Mutations in Reelin pathway are associated with abnormal expression of microglial IgG FC receptors in the cerebellar cortex

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Abstract

Introduction: Microglia are the immune cells of the central nervous system that are involved in a variety of developmental processes such as regulation of cell death and survival, spatial patterning, and contribute to the development of Purkinje cells during migration. Microglia express immunoglobulin G Fc receptors (FcγRs).

Hypothesis: In this report, we describe microglial FcγR expression in the cerebellum during development related to abnormal Purkinje cell migration.

Method: To detect microglial FcγR, the direct anti-IgG (secondary antisera) and high concentrations of Triton X-100 were applied as a method for labeling microglial cells without the use of any specific primary antiserum. By using the *Acp2*^{-/-} mice, which show an excessive Purkinje cell migration into the molecular layer combined with the different knockout mice altering the Reelin pathway (Reeler, Scrambler, and *Apoer2*^{-/-} mice), that Purkinje cells are ectopically located in white matter.

Result: We show the expression of microglial FcγRs in the cerebellum of these mice is absent, but not in the *Acp2*^{-/-} mice .

Discussion: These results suggest a role for FcγRs in reelin signaling pathway, not related to Purkinje cell migration, but may be an adaptation to the environment with the large number of ectopic Purkinje cells.

Conclusion: However, the exact correlation between the ectopic Purkinje cells presence and FcγRs absence in reeler, Scrambler, *Apoer2*^{-/-} mice and the presence of FcγRs in *Acp2*^{-/-} mutant are yet to be determined.

Introduction

Purkinje cells (PCs) are the main output of cerebellar cortex and contribute to a variety of cerebellar functions such as coordination and motor learning (1). Following mitotic division at embryonic day (E) 10.5-12.5, PCs start the migration and form the PC plate, which is a multilayer structure made of PCs in the mantle zone (2). The migration of PCs during cerebellar development happens under tightly controlled conditions and the Reelin pathway plays an important role during this process (3). Reelin is a large protein secreted by the granule cell precursors and cerebellar nuclei neurons in the external germinal zone and cerebellar nuclei, respectively (4). Reelin binds to VLDLR (very low-density lipoprotein receptor) and ApoER2 (apolipoprotein-E receptor 2), and activates a protein known as Dab1 (Disabled 1). Dab1 is an adaptor protein essential for the intracellular transduction of Reelin signaling and regulates the migration and differentiation of post-mitotic neurons during brain development (3, 5). Evidence showed that mutations in *Reelin*, *Dab1*, *Apoer2*, and *Vldlr* genes result in the lack of dispersal of PCs from the cluster and hindered migration of PCs during cerebellar development (6-8). On the other hand, spontaneous mutation in the lysosomal acid phosphatase 2 (*Acp2*) mouse (*nax*-naked-ataxia mutant) are associated with excessive migration of PCs to the molecular layer and lack of PCs monolayer formation (9, 10).

Microglia are myeloid-derived cells in the central nervous system (CNS) involved in a variety of developmental processes such as regulation of cell death and survival, spatial patterning, formation and refinement of neural circuits, and synaptic wiring in the CNS (11, 12). Evidence indicates that microglia contribute to the development of PCs during migration by controlling the survival and synaptogenesis of these cells (13, 14). Microglia express immunoglobulin G Fc receptors (FcγR), which are involved in phagocytic activity, oxidative burst, and inflammatory responses of microglia (15, 16). Although recent evidence shed light on the role of mechanisms involved in PCs migration, it is not clear whether abnormal migration is associated with microglial FcγR during cerebellar development.

In this study, we used different knockout mouse models for Reelin pathway (Reeler, Scrambler, and *Apoer2*^{-/-} mice) and *nax* mouse (*Acp2*^{-/-}), in which PCs migration is decreased and increased, respectively to investigate the expression of microglial FcγRs in the cerebellar cortex of animals during PCs migration.

Materials And Methods

Animal maintenance and tissue processing

All animal procedures were performed in accordance with institutional regulations and the Guide to the Care and Use of Experimental Animals from the Canadian Council for Animal Care and has been approved by local authorities “the Bannatyne Campus Animal Care Committee”, University of Manitoba Animal Care Committee (ACC), and University of Calgary Animal Care Committee (ACC). Animals were maintained at room temperature and in controlled humidity (50– 60%) with a 12-h light dark cycle. All efforts were made to minimize the number of animals used, and the animals were treated in a humane manner. In this study, we used *nax* (*Acp2*^{-/-}), Reeler (reelin mutant), SCM (*Dab1* mutant), and *Apoer* (*apoE* receptor mutant) mice. An *Acp2*^{-/-} colony was established in the Genetic Modeling Center at the Faculty of Health Sciences, University of Manitoba, by breeding mice (C57BL/6) heterozygous for the *Acp2*^{-/-} mutation (17, 18). Mice null for Reelin (B6C3Fe a/a-*Reln*l/J; (19, 20)), *Dab1* (CBy.129S4-*Dab1*tm1Cpr/J; (21–23)), and *Apoer* (B6;129S6-Lrp8tm1Her/J; (4, 8)) were the generous gift of Dr. Richard Hawkes (University of Calgary), which were purchased from The Jackson Laboratory. Wild type littermates were used as controls in all studies. The animals were perfused and post-fixed with 4% paraformaldehyde and then cryosectioned at 20 μm and processed for immunohistochemistry according to methods described in Bailey et al. (18).

Antisera

We used rabbit polyclonal Anti-Iba1 antibody (Millipore #MABN92). Iba1 is a 17-kDa EF hand protein that is specifically expressed in macrophages/microglia (24). Biotinylated anti- FcγRs (α-IgGs; secondary antibodies or antisera) can be detected with the ABC method and avidin-bound fluorochromes (Vectastain, Vector Laboratories Inc., Burlingame CA., USA), as we used this method to detect microglia (25). Rabbit anti-GFAP (1:2000 dilution, Chemicon Inc. Temecula U.S.A.) specifically recognizes

astrocytes. We also used Rabbit polyclonal anti-MBP (Santa Cruz #sc-13914) and mouse monoclonal anti-Calb1 (26).

Immunohistochemistry

Peroxidase immunohistochemistry was carried out on cerebellar sections as described previously (17, 27). Briefly, tissue sections were washed thoroughly blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and incubated in 0.1M PBS buffer containing 0.1% Triton-X and the primary antibody for 16–18 hours at 4 °C. Secondary incubation in horseradish peroxidase (HRP) conjugated goat anti-rabbit or HRP-conjugated goat anti-mouse antibody (diluted 1:200 in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA) lasted 2 hrs at room temperature. Diaminobenzidine (DAB, 0.5 mg/ml) was used to visualize the reaction product. Cerebellar sections for double-label fluorescent immunohistochemistry were processed as described previously (27). To detect microglial immunoglobulin G Fc receptors (FcγR), the direct anti-IgG (secondary antisera) and high concentrations of Triton X-100 (1%) was applied as a method for labeling microglial cells without the use of any specific primary antiserum (25). Biotinylated anti- FcγRs (α-IgGs; secondary antibodies or antisera) can be detected with the avidin-biotin-immunoperoxidase (ABC) method and with avidin-bound fluorochromes. To reveal the secondary antisera binding, the ABC method was used. Biotinylated secondary antisera diluted at 1:200 in PBS and incubated for 2 hr. The Vectastain Elite ABCkit (Vector, Burlingame, CA; #PK-61000) was diluted at 1:200 as well and incubated for 3 hr. Tissue-bound peroxidase was revealed by incubating the sections in a solution containing 0.002% (w/v) 3,3'-diaminobenzidine(DAB; Sigma, St Louis, MO) and 0.003% (v/v) H₂O₂ in 0.05 M Tris-HCl buffer, pH 7.6. The sections were mounted, dehydrated, and cover slipped with Entellan (Merck; Darmstadt, Germany).

Figure Preparation

For bright field microscopy, an Olympus BH-2 microscope was used and images were captured using Image-Pro Expression software. For fluorescence microscopy, a Ziess Lumar.V12 stereomicroscope was used to capture images of entire cerebellar sections using AxioVision 4 software. For high magnification fluorescence microscopy a Ziess Z1.Imager with AxioVision 4 software and a Zeiss LSM 700 confocal microscope with Zen software were used to obtain images. Images were cropped, corrected for brightness and contrast, and assembled into montages using Adobe Photoshop CS5 Version 12.

Results

To visualize the microglia, we utilized biotinylated anti-mouse FcγR secondary antibody by addition of high concentrations of Triton X-100 (1%) and revealed secondary antibody binding by the avidin-biotin-immunoperoxidase (ABC) method (25, 28). Application of this method revealed the non-neuronal cells with branches similar to the microglia in the cerebellar cortex and raises the speculation that FcγRs are localized on microglia (Fig. 1A, D). To confirm that, the ionized calcium binding adaptor molecule 1 (Iba1)

marker was used to demonstrate the morphological features of microglial cells (29–31). A transverse section of the cerebellum immunostained with Iba1 shows that microglia are the only immuno-reactive cells with this marker and the morphology closely resemble the FcγRs⁺ cells. (Fig. 1B, C). Double immunostaining confirmed that FcγRs are co-labeled with the Iba1 (Fig. 1F). The detail of co-localization of FcγR and Iba1 immunostaining is demonstrated in higher magnification (Fig. 1D-F). Positive microglial cell appeared to contain FcγR-like immunoreactivity.

To determine whether FcγR is expressed in other non-neuronal cells, the wt cerebellar sections were stained by double-fluorescence immunohistochemistry, FcγRs -like immunoreactivity was not colocalized in GFAP-positive astrocytes (Fig. 2A–C) and in MBP-positive oligodendrocytes (Fig. 2D–F).

To understand the expression of FcγRs during the development of the microglia, the cerebellar sections were used during perinatal development. FcγRs immunoreactivity is distinguishable at prenatal stages of development and by E17, FcγR immunoreactivity is weak at the core of the cerebellar sections in developing white matter (Fig. 3A, B). The FcγR immunoreactivity is strong in microglial precursor cells which are located in developing white matter at around P1 (Fig. 3C) and P3 (Fig. 3D). By P10, developing microglial cells with their extended process are clearly detectable by FcγRs which are highly populated in the white matter and indicated stream migratory pathway to cerebellar cortex (Fig. 3E, F).

To determine whether FcγRs expression profile is altered in the cerebellum with PCs migration disorders, we used nax mutant cerebellum which shows an excessive PC migration and compared it with other Reelin mutant mice cerebellum with prominent ectopic PCs in white matter and lack of PCs cluster dispersal.

The cerebellar cortex of nax mouse is abnormal and three layer of the cortex is indistinguishable with PCs invading the molecular layer and significantly reduce in the number of granule cells ((18) and Fig. 4). In a P17 wt mouse, the PCs bodies almost form a monolayer while their dendrite extension is apparent in developing molecular layer and directed toward the pial surface (Fig. 4A) while, in nax cerebellum PC bodies with less dendritic arborization and form a multilayer of the cells instead of a monolayer in cortex (Fig. 4D). Multilayer PCs occupy all molecular layer and their dendrites are branched in multidirectional pattern in nax cerebellum (Fig. 4D) in comparison to the wt (Fig. 4A). Recently we have shown the distribution of both ramified and activated microglial cells in nax cerebellum compared to the wt siblings cerebellum (32). In spite of the activated microglia, the FcγR expression in microglia of the nax cerebellum with excessive PCs migration which were detected by Iba1 at P17 were colocalized, which is similar to the wt cerebellum (Fig. 4B, E; higher magnification in Fig. 4C,F). To determine the FcγR expression profile in cerebellar with ectopic PCs in white matter, FcγR and Iba1 immunoperoxidase staining performed using the scm cerebellum (Fig. 5). Surprisingly, no FcγR immunoreactivity was observed in scm cerebellum (Fig. 5D, E) compare to scm wt cerebellum (Fig. 5A, B, C). Iba1 immunostaining was utilized to determine whether the lack of immunoreactivity in FcγRs is due to the lack of microglia in cerebellum. Results revealed the existence of ramified microglia and they are not activated in respond to ectopic neuronal environment (Fig. 5F, G). To test the hypotheis that FcγR expression is

absent in scm pathway, we have pursued in *Reeler* and *Apoer*^{-/-} mutants. Double immunofluorescent staining using anti- FcγR and Iba1 antibodies (Fig. 6) in *reeler* wt confirmed the colocalization of the FcγR and Iba1 (Fig. 6A-C). However, double immunofluorescent staining using anti- FcγR and Iba1 antibodies in *reeler* (Fig. 6D-F), *Apoer*^{-/-} (Fig. 6G-I), and *scm* (Fig. 6J-L) mutants revealed lack of FcγR expression on their microglia despite their well-organized Iba1 immunopositive microglia when compared with control wt (Fig. 6A-C).

Discussion

In the current work, we found that cerebellar microglia express FcγRs during development starting at E17 until postnatal stage at P10. We also found that there is an increase in the expression of microglial FcγRs at the postnatal stage in the cerebellum in comparison with that of the prenatal stage. Previous studies have shown that FcγRs are expressed in a variety of cell types in the brain during adulthood (16), but there are few studies about the role and expression of FcγRs during gestational period and early development (33). Also, we showed that cerebellar astrocytes do not express FcγRs. This report is consistent with a previous study, which showed astrocytes in the cerebellum do not express FcγRs (34). Although there are few evidence on the expression of FcγRs on the cerebellar microglia during gestational period, but our data showed that FcγRs are expressed on cerebellar microglia suggesting their involvement in cerebellar development. It has been shown that the numbers of microglia increase progressively during cerebellar development (E11.5) until postnatal period (35). In addition, evidence indicates that programmed cell death plays an important role in the brain development (36, 37), and microglia are responsible for the phagocytosis of neurons following apoptosis in order to clear pathways for developing cortical afferents and efferents during cerebellar development (14, 35, 38). It is important to note that FcγRs are essential for the phagocytic activity of microglia, and increased expression of FcγRs on cerebellar microglia in our study is associated with the increased number of microglia during development (39, 40).

Further, we showed that manipulation of the Reelin pathway is associated with the lack of dispersal of PCs from the cluster and hindered migration of PCs during cerebellar development. In this context, previous studies have shown that *reeler* mice (mutation in the *Reln* gene), *scrambler* mice (mutation in the *Dab1* gene), and *Apoer2*^{-/-} mice exhibit ectopic PCs and impairments in PCs migration (6–8). On the other hand, *nax* mice (mutation in the *Acp2* gene) showed excessive migration of PCs to the molecular layer and lack of PCs monolayer formation. In this study, our observation in both Reelin pathway manipulated mice and *nax* mice cerebellum suggests that that microglia are present in the cerebellum of all animals and decreased or increased PCs migration has no effect on Iba1 expression. Interestingly, unlike *nax* mice, *reeler*, *scrambler*, and *Apoer2*^{-/-} mice had no expression of FcγRs on their microglia. Our results revealed that manipulations in Reelin pathway affected the expression of microglial FcγRs in the cerebellum. Previous studies demonstrated that manipulation of Reelin signaling directly changes radial glia morphology and biochemical maturation (41–43). Radial glial cells play a crucial role in the radial migration of neurons and are characterized by their astroglial properties and long radial processes (44).

However, the impact of Reelin signaling on the cerebellar microglia is not clear during development. Previous studies revealed that microglia express Reelin receptors VLDL and ApoE2 (45–47). Evidence indicates that activation of ApoE2 and VLDL in microglia is associated with their improved ability to phagocytosis and clearance of apoptotic bodies (47–49). In our study, microglia of reeler, scrambler, and ApoE2^{-/-} mice showed no expression of FcγRs. A research by Bigler and colleagues showed that lipoproteins (namely low density lipoproteins) are the regulators of FcγR-mediated phagocytosis (50). In addition, another study showed that Dab1 is associated with phagocytic activity of macrophages/microglia (51). In our study, lack of LDL receptor family members (such as ApoE2) and their signaling downstream (Dab1) resulted in the lack of microglial FcγRs expression. Since phagocytic activity of microglia is important during cerebellar development, it is possible that manipulations of Reelin signaling affect microglial activity through ablation of FcγRs.

Conclusion

These results suggest a role for FcγRs in reelin signaling pathway, not related to Purkinje cell migration, but may be an adaptation to the environment with the large number of ectopic Purkinje cells.

Declarations

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The article includes all the data used to support the conclusions.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

MRB (conducting experiment and writing manuscript), XJ (assisting in conducting experiment), AD (assisting in conducting experiment), SS (assisting in conducting experiment), SA (writing manuscript), SHC (editing manuscript), JK (editing manuscript), HM (editing manuscript and designing study).

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Figures

Figure 2

The expression of FcγR in the adult mouse cerebellum. A, B, C. Double immunofluorescence staining for FcγRs (A - red) and Iba1 (B - green; C - merged) of a transverse section at adult mouse cerebellum showing the microglia (arrows) that scattered in non-overlapping manner in the molecular layer (ml), Purkinje cell layer (Pcl), and granular layer (gl). D, E, F: A high magnification view of the microglia double immunofluorescence staining for FcγR receptor (D - red) and Iba1 (E - green; F - merged) showing detail of FcγR distribution on microglial cell body (asterisks) and branches (arrowheads) which are co-labeled with Iba1. Scale bars: 100μm (C applies to A, B); 25μm (F applies to D, E) .

Figure 3

Double immunofluorescence-stained transverse sections through the adult cerebellum to show the FcγR expression and GFAP and MBP expression in astrocytes and oligodendrocytes. A, B, C. Double immunofluorescence staining of the cerebellum for anti FcγR (A – red) and GFAP (B – red; C – merged) shows that the FcγR+ cell (arrow) in molecular layer and GFAP+ astrocytes (B - arrowhead) and Bergmann Glial fibers (B – arrow) in molecular layer (ml) which are not co-labeled with FcγR+ microglia (C – arrow and arrowhead). D, E, F. Double immunofluorescence staining of the cerebellum for anti FcγR (A – red) and MBP (B – red; C – merged) shows that the FcγR+ cells in cerebellar cortex and indicated by arrow in molecular layer and strong MBP immunoreactivity in granular layer (gl) (E - arrowhead) which are not co-labeled with FcγR+ microglia (F – arrow indicates microglia in the molecular layer (ml)). Scale bars: 50μm (C applies to A, B); 100μm (F applies to D, E).

Figure 6

Peroxidase immunostaining of the cerebellar sections to show the development of the FcγR⁺ cells. A, B, C, D. FcγRs immunoperoxidase staining of a frontal section through the cerebellar at E17 showing weak immunoreactivity at the cerebellar core that is indicated in a higher magnification view of the same section by the arrowhead in "B". FcγR immunoperoxidase reactivity is strongly outlined microglial cell body at P1 (C) and P3 (D). E, F. FcγR immunoperoxidase staining of a sagittal section through the cerebellum at P10 shows strong immunoreactivity in microglia (e.g. arrow) in white matter and scattered

in cerebellar cortex. F) A higher magnification view of the same “E” shows microglial cell body (arrow) and developing branches of the microglial indicated by arrowhead. Scale bars: 1mm (A); 250μm (B); 50μm (D applies to C); 100μm (E); 25μm (F).

Figure 8

Transverse section of Acp2^{-/-} and wt cerebellum, immunostained with Calb1 at P17 and FcgR/Iba1 at P21. A, D. Immunostaining of wild type mouse at P17 showing the Purkinje cell bodies form a monolayer

with their dendritic arborization in molecular layer (ml) that is directed toward pia mater, while in *Acp2*^{-/-} Purkinje cell bodies are invaded in ml and form a multilayer of the cells and dendrites are less developed with multidirectional in ml. B, C, E, F. Frontal section of *Acp2*^{-/-} and wt cerebellum, immunostained with anti- FcγR (green) and Iba1 (red) at P21 shows a co-localization of FcγR and Iba1 in microglia in wt (B, C) and *Acp2*^{-/-} (E, F). A high magnification view of the microglia in C and F from B and E, respectively. Scale bars: 100μm (D applies to A); 20μm (E applies to B); 25μm (F applies to C).

Figure 9

Transverse section of adult scm (Dab1^{-/-}) and wt cerebellum, immunostained for FcgR/Iba1. A, B, C. transverse section of the wt cerebellum immunostained with FcgR shows microglia scattered in cerebellar cortex (A), and higher magnification shows the microglia distribution, cell bodies and branches in “B” and “C”. D, E, F, G. Transverse section through the scm (Dab1^{-/-}) cerebellum and brain stem/medulla oblongata (bs/mo) immunostained with anti FcgR showing no positive immunoreactivity in the cerebellum and mo (D), clearly shows in high magnification (E). Transverse section of scm (Dab1^{-/-}) cerebellum immunostained with Iba1 showing the presence of microglia in cerebellar cortex (G) and higher magnification (F). Scale bars: 250µm (A and G); 50µm (B and E); 25µm (C and F); 1mm (D).

Figure 11

Transverse sections of wild type, scm (*Dab1*^{-/-}), reeler (*rl*^{-/-}), *Apoer*^{-/-} cerebella immunostained with anti-FcgRs and anti-Iba1. A, B, C. Transverse sections of wild type cerebellum immunostained with anti-FcgRs (A-red) and anti-Iba1 (B-green; C - merged) showing FcgR and Iba1 co-localization on microglia. D, E, F. Transverse sections of scm (*Dab1*^{-/-}) cerebellum double immunostained with anti-FcgRs (D - red) and anti-Iba1 (E - green; F - merged) showing that FcgR is not expressed in microglia which are present and

express Iba1. G, H, I. Transverse sections of reeler (rl^{-/-}) cerebellum double immunostained with anti-FcγRs (G - red) and anti-Iba1 (H – green; I - merged) showing that FcγR is not expressed in microglia which are present and express Iba1. J, K, L. Transverse sections of Apoer^{-/-} cerebellum double immunostained with anti-FcγRs (J - red) and anti-Iba1 (K – green; L - merged) showing that FcγR is not expressed in microglia which are present and express Iba1. Scale bars: 40μm (C applies to A, B and L applies to J, K); 25 μm (F applies to D, E); 20μm (I apply to G, H).