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Purkinje Cell Dendrites Grow in Alignment With Bergmann Glia

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ABSTRACT
The pattern of growth of Purkinje cell dendrites has been analyzed and related to their interactions with Bergmann glial radial processes. In cerebellar slice cultures from mice expressing green fluorescent protein (GFP) under the glial fibrillary acidic protein (GFAP) promoter, Purkinje cells were transfected and imaged with two-photon microscopy over 2 days. We report that while the Purkinje cell dendritic tree grows, individual dendrites increase or decrease in length. Importantly, we demonstrate that vertical growth of Purkinje cell dendrites occurs primarily in alignment with radial glial processes. These findings suggest that radial glial processes provide a structural substrate for the directional growth of Purkinje cell dendrites, thus influencing the shape of the dendritic tree.

INTRODUCTION

Neurons have characteristic dendritic morphologies that underlie specific functional properties of the cell. Evidence exists for the regulation of dendritic morphology by both intrinsic cues and extrinsic factors (Voyvodic, 1989; Gao et al., 2000; Polleux et al., 2000; Wong and Ghosh, 2002; Jan and Jan, 2003; Grueber et al., 2003; Niell et al., 2004; Ye et al., 2004). In the cerebellum, mature Purkinje cells have a single primary dendrite that extends toward the pial surface and branches in the molecular layer (Palay and Chan-Palay, 1974). At the molecular layer the Purkinje cell dendrites are contacted by numerous axons of granule cells, which make synapses on dendritic spines. Although the trophic role of granule cell afferents in inducing Purkinje cell dendritic growth has been demonstrated (Rakic, 1975; Sotelo, 1982; Baptista et al., 1994; Morrison and Mason, 1998; Hirai and Launey, 2000), it is unclear whether they are involved in directing the patterned growth of the dendritic tree. Dissociated Purkinje cells in culture form dendrites but lose their polarity and characteristic shape (Baptista et al., 1994), suggesting that other factors might be involved in shaping the Purkinje cell dendritic tree in vivo.

Bergmann glia are cerebellar astrocytes whose radial processes, span the entire molecular layer (Ramon y Cajal, 1911). Early in development, the smooth radial glia fibers play an important role in guiding the migration of granule cells (Rakic, 1971; Gregory et al., 1988; Hatten and Mason, 1990). In the adult brain, numerous Bergmann glial side processes make intimate connections with Purkinje cell dendritic spines and the presynaptic terminals (Altman, 1972; Grosche et al., 2002).

Recently, interactions between Purkinje cells and Bergmann glia were shown to be important for the morphogenesis of Purkinje dendrites; specifically, the transformation from multipolar dendrite to a single primary dendrite morphology is thought to be mediated by the glial glutamate transporter GLAST (Tanaka et al., 2003). Moreover, interactions between the growing tips of Purkinje cell dendrites and Bergmann glial rod-like process have been observed, and the glial processes were postulated to mediate the outgrowth of dendrites toward the pial surface (Yamada et al., 2000).

Using dynamic imaging, we first analyzed how the Purkinje cell dendritic tree expands, and related the growth patterns to the radial glial cells, viewed in the same preparations. We hypothesize that radial processes of the Bergmann glia provide a scaffold for the directed vertical growth of Purkinje cell dendrites. To test this, we performed two-photon time-lapse microscopy of Purkinje cell dendrites in cerebellar slice cultures from transgenic mice expressing GFP in astrocytes. We determined that growth of Purkinje cell dendrites occurs throughout the molecular layer and that most dendrites grow in association with radial glia processes. These data support the proposal that glia provide a substrate which directs dendritic growth and might be involved in shaping the Purkinje cell dendritic morphology.

METHODS AND MATERIALS

Animals

FVB/N-TgN (GFAP–GFP) mice in which all astroglial cells express GFP (Zhuo et al., 1997), were bred in our colony. All animal experiments were approved by the Animal Care and Use Committee of Brown University.

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Cerebellar Organotypic Slices

Sagittal 350-μm cerebellar slices were prepared from postnatal day 11 (P11) mice and incubated in 10% horse serum (HyClone, Logan, UT) BME (Gibco) medium, on Millicell membrane inserts at 5% CO_2, 37°C for 3–4 days. Imaging took place on days 2 and 3 in vitro.

Immunostaining

Slices were fixed in 4% paraformaldehyde and incubated in a rabbit polyclonal antibody against calbindin-D28k (Swant, Bellinzona, Switzerland) overnight at 4°C. After rinsing, slices were incubated in Alexa 595-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR).

Transfection

Slices were transfected by biolistic particle-mediated delivery (Bio-Rad hand-held gene gun) with 1 μm gold particles coated with CMV-DsRed2 vector (Clontech, Palo Alto, CA) immediately after preparation and were kept in culture for 24–48 h before imaging.

Time-Lapse Imaging

Labeled slices were transferred to a microscope stage (35–37°C) and were perfused with oxygenated artificial corticospinal fluid. Dendrites and glial shafts were imaged with a two-photon microscope (Radiance 2000, Bio-Rad, coupled to Nikon E-600-FN microscope, and a Ti:S laser providing 100-fs pulses at 80 MHz at a wavelength of 920 nm (Mira 900; Coherent, Santa Clara, CA) pumped by a 10-W solid-state source (Verdi, Coherent). Fluorescence was detected by using two external detectors. Images were collected with a 60× objective, NA 1, at a digital zoom of 1–3. Stacks of 5–6 focal planes, 0.5 μm apart, were scanned simultaneously for the two fluorophores. After imaging, the filters carrying the imaged slices were placed on new inserts and returned to the incubator for 24 h.

Image Processing and Analysis

Images were processed and analyzed by NIH-Image and ImageJ using custom-written macros. Measurements of dendritic length were made on maximal intensity projections of the stacked images. The length between the tip of a dendrite and the next branch point was measured. Dendrites whose length changed for ≥0.2 μm were considered to have grown or retracted. To view interactions between dendrites and glial shaft processes, the images from the two channels were superimposed. To determine whether dendrites grew while in contact with glial processes, either the tip of the dendrite or at least three spines on the tip had to be in close apposition to the glial shaft. The determination of dendrite apposition with glial process was performed without prior knowledge of the dendritic growth history.

To estimate the area of a slice covered by glial radial processes, stacks of 3–4 images were projected, thresholded to highlight the radial glia processes, and then binarized. For each image we determined the ratio of number of pixels representing the glial radial processes to number of pixels representing the rest of the slice. An average of these ratios was then computed.

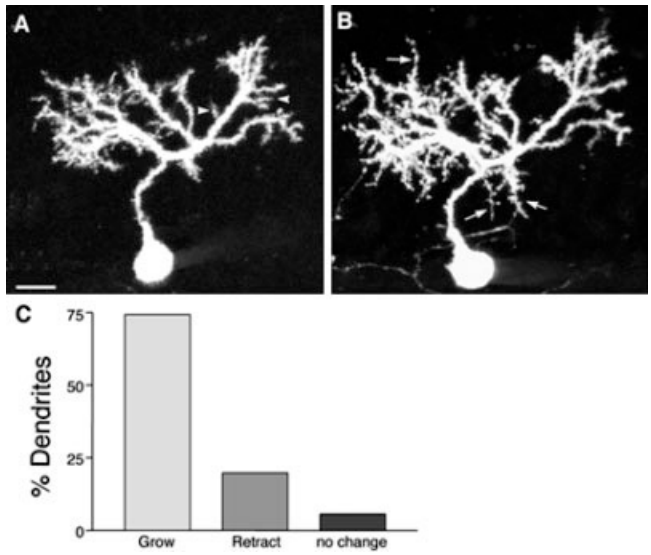

Fig. 1. Growth of Purkinje cell dendrites in cerebellar slice cultures. DsRed2-labeled Purkinje cell imaged from postnatal day 11 is imaged at 2 (**A**) and 3 (**B**) days in vitro. Note growth (arrows in B) and retraction (arrowheads in A) of individual dendrites. **C:** Percentage of dendrites that grew, retracted or did not change in length over a period of 24 h. Scale bar = 15 μm in A.

RESULTS

Purkinje Cell Dendritic Development in Organotypic Slice Cultures

To determine how Purkinje cell dendrites develop, we first analyzed the pattern of growth of Purkinje cell dendrites in cerebellar slice cultures. DsRed2-expressing Purkinje cells in slices from postnatal day11 (P11) cerebellum were imaged with two-photon microscopy. The same cells were easily identified again 24 h later (Fig. 1), and the dendrites (3–12 per cell) were re-imaged.

Quantitative analysis of dendritic length demonstrated that 74.4% of dendrites grew while 19.9% of dendrites retracted during 1 day in culture (156 dendrites, 18 cells). The average rate of dendritic elongation and retraction was 2.1 ± 0.2 and 1.8 ± 0.3 μm/day, respectively (mean ± SEM). The average net change of individual dendrites was an increase of 1.2 ± 0.2 μm/day. Thus, although individual dendrites can either grow or

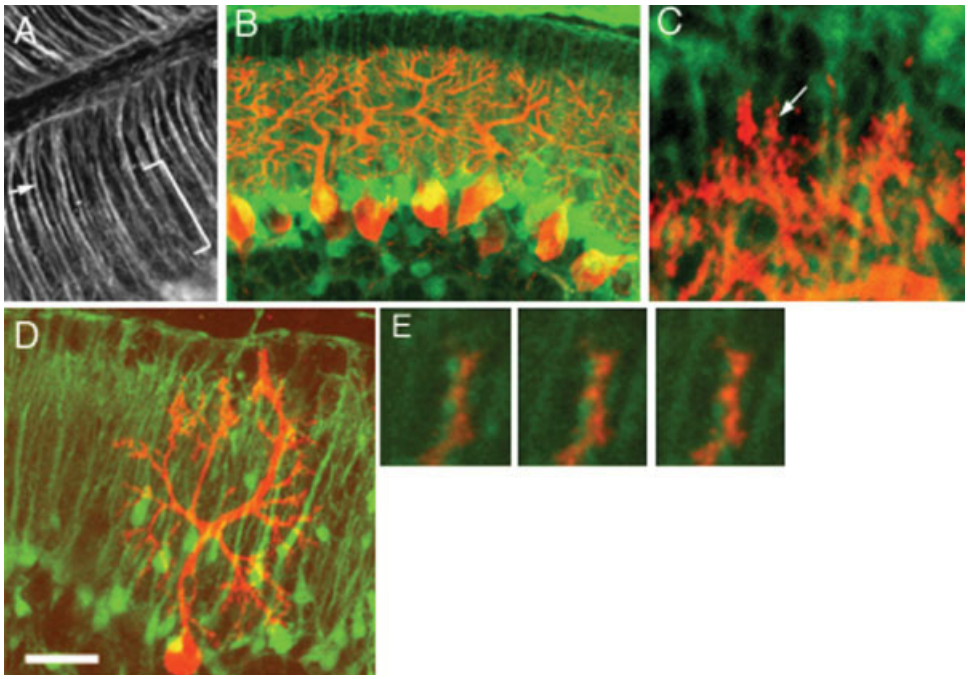

Fig. 2. Interactions between Purkinje cell dendrites and glial processes. **A:** Cerebellar slice from GFAP-GFP mouse visualizing the glial radial processes (arrow) as well as the glial side processes (bracket). **B:** Calbindin immunostaining (red) demonstrating the coextension of Purkinje cell dendrites and developed GFP-expressing Bergmann glial processes at the molecular layer. **C:** High magnification of Calbindin-immunostained Purkinje cell dendrite in close contact with a radial glial process at the pial surface (arrow). **D:** DsRed2-expressing Purkinje cells in cerebellar slice culture from GFAP-GFP mouse. Note close contacts between some dendrite and radial glia all throughout the molecular layer. **E:** Apparent contact between glial process and a dendrite is visible in multiple individual focal planes (not projections). Scale bars = 23 μm in A; 21 μm in B; 10 μm in C,E; 30 μm in D.

retract, the total dendritic tree grows with time. We analyzed the change in dendritic length of distal dendrites throughout the molecular layer and found that dendrites grow both vertically, either up toward the pia or away from the pia (2.2 μm/day) or horizontally/obliquely (1.8 μm /day, $P = 0.1$, t-test).

Purkinje Cell Dendritic Tips Interact With Bergmann Glia Shaft Processes

We next examined the relationship between Purkinje cell dendrites and Bergmann glia shaft processes in mice that expresses GFP under the control of the GFAP promoter, thus labeling all astrocytes in the brain, including the cerebellar Bergmann glia (Zhuo et al., 1997) (Fig. 2A).

To determine the relationship between Purkinje cell dendrites and glial radial processes, we immunostained cerebellar slices from GFAP-GFP mice with a Purkinje cell marker Calbindin D_{28}. We demonstrate that the development of the glial cells occurs in concert with the development of the Purkinje cell dendrites; the dendritic tree and the portion of the glial fibers that have developed side processes are coextensive at the molecular layer (Fig. 2B). We have also observed dendritic tips protruding into the external granular layer while parallel to and in close association with the Bergmann glia fibers, as has been previously reported (Yamada et al., 2000) (Fig. 2C).

To determine whether dendrites and glial radial processes associate with each other, and whether these associations are limited to the tips in the external granular layer, we transfected cerebellar slices from GFAP-GFP with a DsRed2 construct. Visualizing individual Purkinje cells allowed a better appreciation of the interaction of the dendrites with the glial processes at the molecular layer. We report that the close interactions (putative contacts) between dendritic tips and glial processes were found throughout the molecular layer (Fig. 2D,E), and were not confined to the pial surface, as previously suggested (Yamada et al., 2000).

Purkinje Cell Dendrites Grow Vertically While in Putative Contact With Bergmann Glia Radial Processes

The close association between dendritic tips and Bergmann fibers suggests that the Bergmann glia processes could provide structural substrates for the directional growth of the Purkinje cell dendrites. To test this, we measured the growth of dendrites over 24 h in culture and analyzed the relationship of the dendrites with the glial processes. We found several different interactions between dendritic segments and glial processes. In some cases dendritic segments appeared to be in close contact with a glial process throughout their length (from branch point to tip; Fig. 3C,D), while in others such contact was not continuous (Fig. 3A,D). Out of 79 vertically arrayed imaged dendrites, 58 were scored to be in putative contact with a glial radial process. For example the dendrites in Figure 3B marked with an arrowhead and an arrow were considered to be in contact and not in contact with a glial process, respectively. In most cases in which a dendrite was scored to be in contact with a glial process the contact was evident in multiple focal planes (Fig. 2E).