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Continuous network of endoplasmic reticulum in cerebellar Purkinje neurons

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ABSTRACT **Purkinje neurons in rat cerebellar slices injected with an oil drop saturated with 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate [DiIC$_{16}$(3) or DiI] to label the endoplasmic reticulum were observed by confocal microscopy. DiI spread throughout the cell body and dendrites and into the axon. DiI spreading is due to diffusion in a continuous bilayer and is not due to membrane trafficking because it also spreads in fixed neurons. DiI stained such features of the endoplasmic reticulum as densities at branch points, reticular networks in the cell body and dendrites, nuclear envelope, spines, and aggregates formed during anoxia in low extracellular Ca^{2+}. In cultured rat hippocampal neurons, where optical conditions provide more detail, DiI labeled a clearly delineated network of endoplasmic reticulum in the cell body. We conclude that there is a continuous compartment of endoplasmic reticulum extending from the cell body throughout the dendrites. This compartment may coordinate and integrate neuronal functions.**

The endoplasmic reticulum (ER) has a variety of intracellular functions, including Ca^{2+} storage, Ca^{2+} release, and protein and lipid synthesis. In the dendrites and cell bodies of neurons, the ER is considered to be an important Ca^{2+}-regulating organelle (1–7). However, the distribution of various functional types of ER is not necessarily coextensive, and it remains an open question to what extent different functional regions of the ER are interconnected. In particular, limitations in the volume of tissue that can be analyzed by thin-section electron microscopy have made it impossible to reconstruct the ER in a large neuron. We approach this problem here by applying a dye labeling method to cerebellar Purkinje neurons in a slice preparation where the ER in soma and dendrites can be examined by confocal microscopy.

This method for labeling ER membranes uses DiI [1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, or DiIC$_{16}$(3)], a fluorescent, lipophilic dye that diffuses only in continuous membrane bilayers. When this dye is introduced into sea urchin eggs as a saturated solution in soybean oil, it spreads throughout a membrane system that contains elements that are generally accepted to be part of the ER (8). Labeling of ER membranes by this method did not damage the egg, as the egg was able to be fertilized and to undergo development. More recently, DiI in oil was injected into the Limulus ventral photoreceptor (9), where it spread in ER membranes and did not affect the physiological responses of the cell to light. We have now used this technique to label the ER in vertebrate central nervous system cells by injecting Purkinje neurons of cerebellar slices and hippocampal neurons in culture.

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MATERIALS AND METHODS

Parasagittal slices of cerebellum (150 μm thick) were prepared from the vermis of isoflurane-anesthetized Sprague–Dawley rats (postnatal days 15–28) by using a vibrating chopper. Slices were perfused at 20–22°C with saline containing 118 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM $NaHCO_3$, and 10 mM D-glucose (gassed with 95% O_2/5% CO_2; pH 7.4). A saturated solution of DiI (Molecular Probes), in soybean oil (Wesson) was pressure-injected [pressure pulses of 20–50 psi (1 psi = 6.89 kPa) for 0.5- to 5-s duration] into Purkinje cell bodies as single oil droplets (2- to 10-μm diameter) by using micropipettes (tip diameter of 1–3 μm). Micropipettes were silanized by treatment with *N,N*-dimethyltrimethylsilylamine (Fluka) at 200°C for 30 min and were front-filled. The perfusion chamber containing the slice was then transferred to the stage of a Zeiss Axioskop upright microscope and imaged by using a Bio-Rad MRC 600 laser scanning confocal microscope with a Zeiss ×40 water immersion objective (n.a. 0.75). Images were analyzed on a MacIntosh II microcomputer with NIH IMAGE (from Wayne Rasband, Research Services Branch, National Institutes of Health, Bethesda, MD) or PHOTOSHOP (Adobe Systems, Mountain View, CA) software and were printed by using a Lasertechnics DIR continuous tone printer (Sandia Imaging, Carrollton, TX). In some instances, living Purkinje neurons were injected, and then the slices were immersed for 2 min in fixative (0.25% glutaraldehyde/0.5% paraformaldehyde/100 mM sodium cacodylate/100 mM sucrose, pH 7.3) within 2 min of the injection. Fixed slices were mounted in buffered medium (100 mM sodium cacodylate/100 mM sucrose, pH 7.3) under coverslips with glass spacers inserted to prevent mechanical distortion of the slice. Plasma membranes of Purkinje neurons were labeled with DiI either by the extracellular placement of a DiI-containing oil droplet or by micropressure ejection of a DiI-containing solution in ethanol. DiI ejected in ethanol readily precipitates from aqueous solution, and the crystalline precipitates settle onto and stain the cell surface.

In some experiments, cerebellar slices were perfused on the stage of the confocal microscope with saline of the following composition: 118 mM NaCl/3 mM KCl/1.5 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes·NaOH/11 mM D-glucose (gassed with 100% O2; pH 7.4). The perfusate was changed to an anoxic (N_2-gassed) solution containing increased $MgCl_2$ (10 mM), and $CaCl_2$ was omitted. Electron microscopy was performed on anoxic or recovered slices by treating with concentrated aldehyde followed by fixing in 1% osmium tetroxide and embedding in Araldite.

Cultured rat hippocampal neurons were prepared by the method of Goslin and Banker (10). Micropipettes of borosilicate glass with an internal fiber (tip diameter of ≈1 μm) were

Abbreviations: DiI, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate [DiIC$_{16}$(3)]; ER, endoplasmic reticulum.

back-filled with DiI-saturated oil by using an Eppendorf Microloader pipette tip. Neurons were microinjected in conditioned serum-free minimal essential medium with N2 supplements (GIBCO/BRL) on the stage of a Zeiss Axiovert 135 inverted microscope. Single DiI-containing oil droplets of 5- to 12-μm diameter were delivered into cell somas by air pressure pulses controlled by a pressure injector [Narishige model IM-200 (Tokyo)]. The cells were out of the incubator for no longer than 5 min. Coverslips containing the injected cells were mounted in a Sykes–Moore chamber with warm, conditioned N2 medium and observed on the heated (35–37°C) stage of an Olympus (New Hyde Park, NY) LSM GB200 laser scanning confocal microscope coupled with an Olympus IMT-2 inverted microscope. Fluorescence was imaged with a ×60 SPlanapo oil-immersion lens (n.a. 1.4).

RESULTS

All of the DiI in oil drops injected into Purkinje neurons left the oil drop and spread into the dendrites and axon to its cut end by 4–5 hr after injection (Fig. 1). DiI spread approximately to the edge of the slice, which is the edge of the dendritic tree, indicating that the dye spreads throughout the entire Purkinje cell dendritic tree.

DiI Spreads by Diffusion in a Continuous Membrane. It is now generally believed that DiI diffuses only within a continuous lipid bilayer (11). One way that DiI could spread in Purkinje neurons is by diffusion in a continuous bilayer such as the plasma membrane or some intracellular membrane compartment. The other way is by membrane traffic, where a vesicle buds from labeled membranes and then fuses with unlabeled membranes. To investigate how DiI actually spreads in Purkinje neurons, we first observed the time course of spreading in living injected neurons (Fig. 2 *a* and *b*). Over 1.5 hr, the dye front moved at ≈0.25 μm/min (measured directly on the video monitor at multiple time points), a rate consistent with diffusion in a membrane (12) but slower than the 2–130 μm/min rate of fast axonal transport (13).

We also observed DiI in cells that were injected and then fixed 2 min later. DiI also spread in these cells, resulting in a distribution of label indistinguishable from that seen in living cells (Fig. 2 *c* and *d*), though the rate of spreading was somewhat slower. DiI also diffuses more slowly in other fixed membranes (14, 15). Thus, DiI spreading in a fixed Purkinje cell is very likely to be in a continuous membrane in which DiI diffusion has been slowed by fixation.

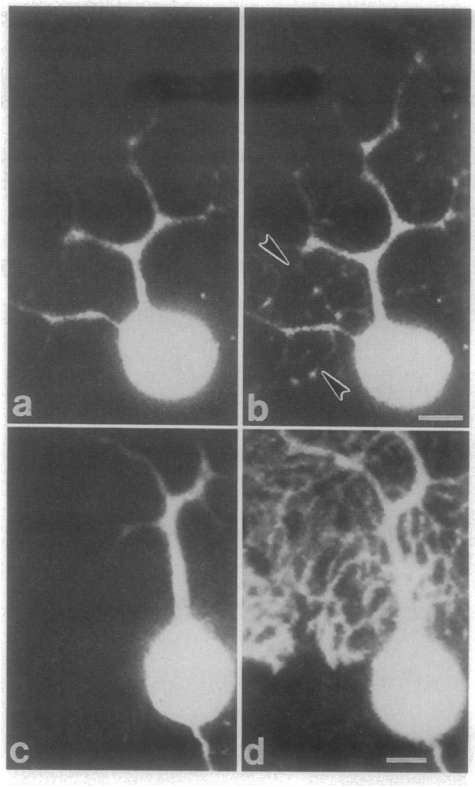

FIG. 2. Spread of DiI from injected oil drop in living (*Upper*) and fixed (*Lower*) Purkinje cells. DiI was injected in the soma of living Purkinje cells in two separate slices. (*Upper*) Images display the spread of the dye at 30 min (*a*) and 2 hr (*b*) after dye injection in a living cell; at 2 hr many fine branches (arrowheads) are apparent. (*Lower*) Images show the spread of the dye in a Purkinje cell fixed 2 min after dye injection and imaged 15 min (*c*) and 14 hr (*d*) after fixation. While the visualization of dendritic structure was impaired to some degree in the fixed tissue because of autofluorescence associated with the fixative, dye spreading throughout the branches of the Purkinje cells in the fixed tissue can nevertheless be clearly observed. (Bars = 10 μm.)

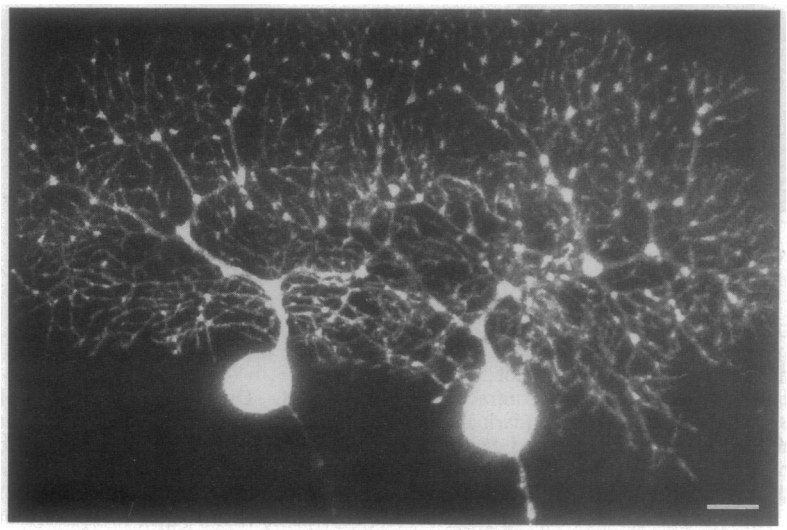

FIG. 1. Continuous internal network of ER in two adjacent living cerebellar Purkinje cells. The long-chain lipophilic dye DiI in an oil drop was injected into the soma of both Purkinje cells in a living cerebellar slice, and the resulting spread of fluorescence was imaged by using a laser scanning confocal microscope 4 hr after dye injection. To obtain the image of the entire cellular network, a Z-series scan was performed, and the images were superimposed. Note the spread of the dye throughout the soma, dendritic tree, and axon of each cell, with concentrations of bright dye at dendritic branch points, extending to the most distal regions of the cell. (Bar = 10 μm.)

DiI Spreads in Membranes of the ER. Several observations indicate that the spread of DiI is in ER membranes. At high magnification, there was a reticular staining pattern in the cell body and main branches (Fig. 3*b*). This pattern was similar to the electron microscopic appearance of the ER in these regions (16). The dye also stained the dendritic branch points more intensely (Fig. 1). This pattern corresponds to the observed higher concentration of ER membranes in these regions by electron microscopy (16) and to higher concentrations of inositol trisphosphate receptor and ryanodine receptor (2, 4). DiI also labeled the nuclear envelope (not shown), which is continuous with the ER.

It remains possible that some DiI molecules spread simultaneously in the plasma membrane. In particular, the staining was not well resolved in the smallest dendritic branches, where it was difficult to distinguish between plasma membrane and internal membrane staining. This issue was addressed by examining preparations in which only the plasma membrane was stained, either by injection of ethanolic DiI into the extracellular space (15) or, in a few instances, by deposition of a DiI-saturated oil drop outside the cell. Plasma membrane staining was characterized by a sharp outline around the cell body and larger dendritic branches (Fig. 3*a*) and by staining of the small dendritic branches (not shown). The sharp outline around the cell body and large dendritic branches was not seen in cells injected with a DiI-saturated oil drop (Fig. 3*a*), showing that the plasma membrane was not stained and therefore that none of the spread of DiI from an injected oil drop is likely to be in the plasma membrane.

In injected cells where the staining was particularly bright (either living or fixed), we were able to image projections from the dendrites that correspond to the position and density of spines (Fig. 3*c*) and presumably represent DiI labeling of the spine ER. Previous investigators have shown by thin-section electron microscopy that the spine ER is continuous with the dendritic shaft ER (17). DiI labeling of the spine ER

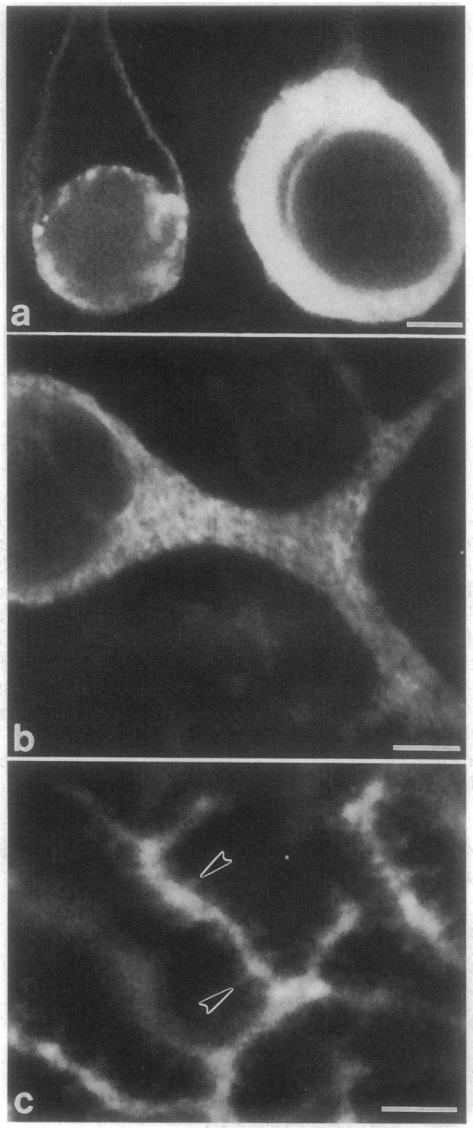

FIG. 3. Structural features of the distribution of DiI in Purkinje cells. (*a*) Two cerebellar Purkinje cell soma have been labeled by DiI and imaged in a single plane through the center of the soma. The left-hand cell was labeled by ejection of the oil drop at the surface of the soma, thereby labeling the plasma membrane but not the ER. The only other staining corresponds to the original position of the oil droplet (out of focus). The right-hand cell was injected intracellularly, and the fluorescence has spread out of the oil drop (dark circle in the center of the soma) and filled the soma interior and dendrite (out of the plane of the section). (*b*) Organization of labeling pattern in the cell soma and proximal dendrite of a living Purkinje cell. A complex reticulum is apparent in the cell soma and proximal dendrite of living Purkinje cells. The unstained region in the soma represents the oil drop, depleted of dye, and cell nucleus. (*c*) High magnification image of fluorescence in the distal dendrite of a Purkinje cell fixed 2 min after dye injection. The fluorescent label can be clearly seen in both the dendrite and spinous processes (arrowheads), indicating the continuity of the ER in dendrite and spine. (Bar = 5 μm.)

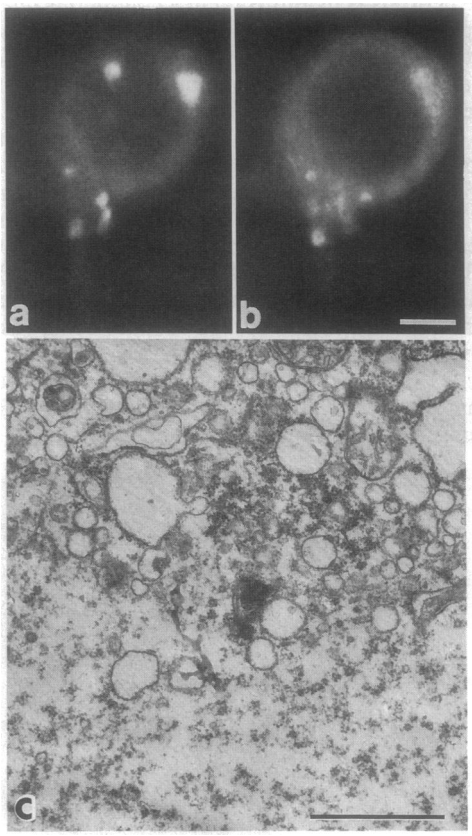

FIG. 4. Dynamic reorganization of the ER after exposure to an anoxic environment with low extracellular Ca^{2+}. (*a*) Fluorescent pattern in a living Purkinje cell exposed to a low-Ca^{2+}, oxygen-deprived medium for 1 hr and 15 min. The label is no longer in a diffuse reticulum (compare with Fig. 3) but is clustered into compact sites of label. (*b*) After reexposure of the slice to a normal-Ca^{2+}, oxygenated medium for 1 hr, clustering partially reversed. (Bar = 5 μm.) (*c*) Transmission electron micrograph obtained from a slice fixed in the presence of a low-Ca^{2+}, oxygen-deprived medium, showing clustering of membrane components (top of micrograph). The remainder of the cytoplasm is devoid of membranous structures in other regions (lower right-hand portion). (Bar = 1 μm.)

in fixed cells provides therefore additional evidence that it spreads in the ER. Furthermore, since DiI seemed to spread into the majority of the spines in fixed cells, much of the spine ER appears to be continuous with the ER of the dendritic shaft.

The identification of DiI-stained material in the cell body was further investigated by comparing its behavior under conditions reported to alter the distribution of ER. Both low extracellular Ca^{2+} (18) and anoxia (19–21) have been reported to cause reorganization of neuronal ER into lamellar aggregates. In our experiments, exposure to Ca^{2+}-free medium or anoxic medium alone did not produce any change in the staining pattern, but a dramatic, reversible change was observed in anoxic Ca^{2+}-free buffer. Bright areas of DiI labeling in the cell body developed (Fig. 4*a*), and this pattern was partially reversed by restoring oxygen (Fig. 4*b*). Electron microscopy showed that there were accumulations of membranes in the anoxic cell that are similar to those previously reported (18) and that seem to correspond with the bright areas of DiI labeling (Fig. 4*c*).

Injection of Neurons in Culture. Cultured neurons growing on cover slips could be examined with high numerical aperture, oil-immersion objectives that produce higher resolution images than the long working distance, water immersion objectives that were necessary for examining cerebellar slices. A more detailed image of the ER network was seen in cultured rat hippocampal neurons injected with a DiI-saturated oil drop (Fig. 5). The cell body ER appeared as a continuous branching reticulum lacking any ends or discontinuities. This ER appeared to be stationary (time lapse images at 10-s intervals at 35°C) in contrast to the ER in sea urchin eggs, which is in continuous motion (8). It was difficult to resolve details of the ER in the neurite, apparently because this ER is more closely spaced than the resolution of the light microscope (≈0.2–0.5 microns). In younger neurons (at 2–4 days after plating, before the axon became too long to trace), DiI spread from the cell body to the ends of the neuritic processes and into growth cones.

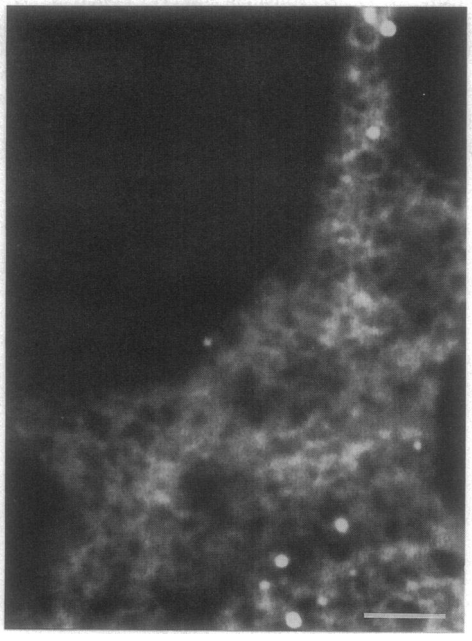

FIG. 5. DiI labeling in the cell body of a cultured hippocampal neuron. A living rat hippocampal neuron, 2 weeks after plating in culture, was injected with DiI in oil. This confocal image was taken in a plane 5 μm above the cell substrate in a single 40-sec scan. A complex reticulum is present in the cell body and appears to be continuous with a reticulum in the neurites. The detail is greater than in the Purkinje neurons because the optical conditions are better for cells growing directly on glass coverslips than for cells embedded in a tissue. (Bar = 5 μm.)

DISCUSSION

A major class of intracellular membranes, the ER, was originally seen in whole-mount electron micrographs of fibroblasts to branch throughout much of the cell (22). It is now established that the ER supports a variety of functions such as Ca^{2+} regulation and synthesis of proteins and lipids (23). To what extent the ER membranes are continuous through a large complex cell, such as a neuron, remains unclear.

Previous studies to address ER continuity have used special electron-microscopic techniques (24–26). When thick (1 μm) sections of fixed tissue impregnated by heavy metal were observed by high-voltage electron microscopy, extensive regions of interconnected tubular ER membranes were demonstrated, first in the axon and axon hillock and more recently in Purkinje cell dendrites (26). Although it seems a reasonable hypothesis that these membranes are interconnected throughout the cell, no cell has been entirely reconstructed, so the overall continuity of the ER remains unclear.

The fluorescent dicarbocyanine DiI is known to trace the continuous plasma membrane of neurons (11, 12, 15) and recently has also been used to investigate the continuity of the ER. DiI was introduced into the cytoplasm of living and fixed sea urchin eggs by microinjecting a dye-saturated oil drop (8, 14). Study of eggs fixed before injection made it clear that the DiI spread through continuous membranes and that the cortical ER network, the interior membrane lamellae, and the nuclear envelope are continuous. In another study, DiI was injected into fixed, isolated Limulus ventral photoreceptor cells, where it was concluded that the ER is continuous between the phototransducing lobe and the lobe containing the nucleus (9).

We now have used the DiI oil drop method to investigate the overall continuity of the ER in a central nervous system neuron, the cerebellar Purkinje cell. The dye spread and filled the cell body and dendritic tree and also spread into the axon. There are several reasons to believe that this spread is through continuous lipid membranes. The rate of movement, 0.25 μm/min, is more consistent with lipid diffusion rates than with rapid vesicular transport, and the spread occurs in cells fixed soon after injection, thereby excluding the possibility of dye transfer by vesicular membrane traffic.

That DiI is spreading in ER membranes is supported by its reticular staining distribution in the cell body, its concentration at the major branch points of dendrites, and its presence in dendritic spines (16). The reticular pattern is particularly clear in cultured hippocampal neurons where it extends into proximal neurites. Also, this staining pattern is not consistent with staining of the plasma membranes, from which it can be clearly distinguished. The DiI-labeled membranes under conditions of anoxia and low extracellular Ca^{2+} also reversibly clumped into aggregates of membranes, in this respect showing the behavior reported for ER (18–21).

Our observations lead us to conclude that DiI spreads in a continuous compartment of ER in the Purkinje cell; that, for the most part, only ER membranes are stained; and that the major known parts of the ER are stained. These results indicate that there is a continuous compartment of ER that is distributed from the cell body throughout the entire dendritic tree. It remains possible, however, that the ER has undetected discontinuities in the cell body of Purkinje neurons. Because the oil drop takes up a relatively large fraction of the cell body volume (probably 5–10%), DiI could be spreading in two or more interdigitated continuous ER membranes that independently contact the oil drop, so the spread into axons and dendrites could depend on separate membrane systems. However, this appears to be unlikely because the images of

the ER in cultured neurons show a dense membrane system, which appears to be a single interconnected system.

Another limitation is that DiI would not label any ER membranes not in continuity with the main ER—the ER membrane system could include discontinuous compartments in addition to the continuous membranes in which DiI spreads. Thus, it remains unclear whether inositol trisphosphate receptors and ryanodine receptors, which have been localized in different parts of the Purkinje neuron ER (4), are present on the same membrane.

What might be the functional advantage of a continuous compartment of ER in the Purkinje cell? One advantage might be that release or uptake of Ca^{2+} that would deplete or saturate an isolated compartment, like a vesicle, would be able to draw on a large volume of ER. In analogy with ion currents and gradients generated by the plasma membrane (27), regions in the ER of pumping activity distinct from regions of release activity could also establish ionic currents and concentration gradients both within the ER lumen and in the cytoplasm. Local synthesis of proteins and lipids thought to be synthesized in dendritic ER (28) could be influenced by conditions in another part of the neuron by signals, possibly diffusional, through the continuous ER compartment.

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