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Somatic Ultrastructure May Shape Calcium Dynamics in Purkinje Neurons

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by

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The thesis of Kaitlyn Nicole Robinson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this thesis to my parents, Jack and Karla, and my brother Jack for their endless and unparalleled love and support. Without your faith and guidance, I would not be half as successful as I am today. You are the reason why I graduated college, the reason why I pursue higher learning, and the reason why I am a confident in my career choices. Thank you for believing and trusting in me as I follow my dreams.

EPIGRAPH

I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

-Newton

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ABSTRACT OF THE THESIS

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The spatiotemporal organization of intracellular features is fundamental to understanding the intercommunication of neurons in the brain. Neuronal communication is regulated by receptor activation, which is influenced by signaling messenger availability and internal calcium (Ca^{2+}) concentrations. An excess or deficiency of cytoplasmic Ca^{2+} can lead to changes in secondary messenger diffusion, synaptic plasticity, gene expression, and even cause cell apoptosis. Existing

models of intracellular calcium movement have used image-based recording methods to approximate a uniform distribution within the cytosolic space, but it is still unclear whether or how somatic features shape calcium mobility. A methodic analysis of the relationship between somatic arrangement and the Ca^{2+} signaling pathway does not currently exist. In this study, we used fine-scale electron tomogram reconstructions of cerebellar Purkinje neurons to create a precise model of the interior of the cell. We established structural motifs of endoplasmic reticulum (ER) and noticed a heterogeneous arrangement of organelles within the soma. In addition, we used fluorescent immunohistochemistry to analyze localization patterns of signaling proteins and observed how two receptors that mediate Ca^{2+} efflux from internal ER stores: inositol 1,4,5-trisphosphate receptor 1 (IP_3R1) and ryanodine receptor 1 (RyR1) have distinct angular and radial domains. Overall, we found that ER serves as a diffusion barrier and can cause nonuniform diffusion of Ca^{2+}. This thesis demonstrates how the localization organelles and calcium modulating proteins significantly contribute to intracellular communication and provides an architectural reference for emerging research on somatic neuronal signaling.

I. INTRODUCTION

Intracellular calcium (Ca^{2+}) signaling is central to most neuronal processes in the brain (Ghosh & Greenburg, 1995). An overabundance or absence of internal stores of calcium will influence the activation of cell surface G protein couple receptor (GPCR) kinases, proteins which bind extracellular molecules and cause a cascade of intracellular events (Hoxha *et al.*, 2016). Calcium sensitivity shapes not only cellular phenotypes, but also the overall anatomical structure and function of the brain. Research on internal mechanisms of neurons have shown that motility, memory, and learning pathways are modulated by neuronal calcium sensors (Gomez *et al.*, 2001). Calcium sensors are proteins that bind free floating calcium ions, and these proteins regulate internal calcium levels and the release of neurotransmitters (Burgoyne, 2007). A study on the physiological impacts of disrupting the function of plasma membrane Ca^{2+} ATPases (PMCAs), an important transport protein responsible for removing calcium from the cytoplasm, has revealed that cells with mutant PMCA transporters exhibit abnormal cellular morphology, subcellular signaling degeneration, and cerebellar ataxia (Kozel *et al.*, 1998). Clearly the mechanisms responsible for calcium handling are highly regulated since cellular homeostasis is heavily influenced by the availability of internal and external Ca^{2+} ions (Brini *et al.*, 2014).

Fluctuations in calcium concentrations is driven by interactions between the plasma membrane (PM) and the endoplasmic reticulum (ER), which serve as a calcium source or sink and mediates neuronal functions such as calcium-induced calcium release, gene expression, intracellular spiking, and synaptic plasticity (Berridge 1998). As mentioned, presynaptic neurotransmitters can stimulate the cell surface and cause an internal signal transduction. This stimulus results in secondary messenger propagation and receptor activation on the ER membrane, which releases Ca^{2+} stores from the lumen into the cytosol (Gleichmann & Mattson, 2011). It is widely acknowledged that inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors

(RyRs) on the ER surface are key to regulating the release of calcium from the ER and can produce localized regions of intracellular calcium signaling (Ellisman *et al.*, 1990; Bezprozvanny *et al.*, 1991; Kano *et al.*, 1995). However, the impact of the spatiotemporal organization of somatic features, specifically organelle structures and calcium modulating protein abundance, on subcellular signaling remains uncertain.

The progression of data programming has galvanized scientists to evaluate signaling cascades and neuronal architecture through computational modeling, formerly accomplished in dendritic spines (Martone *et al.*, 1993). This study acknowledged the impact of surface-to-volume ratio of neuronal protrusions on calcium transience as well as document the continuity of the smooth endoplasmic reticulum within the dendrite. Additionally, extensive research on the interplay of voltage-sensitive calcium channels and glutamate receptors been previously conducted on dendritic spine (Bloodgood & Sabatini, 2007). Yet, a comprehensive analysis of calcium handling within the large open space of the cell soma is currently unknown. Early research on Ca^{2+} diffusion has used image-based modeling to identify calcium signaling protein domains within the soma, but these models did not consider the precise intracellular morphological organization of organelles (Fink *et al.*, 2000; Eliers *et al.*, 1995). Evidently, later structural studies determined that internal membranes, specifically the ER, has diverse motifs and pattern arrangements (Banno & Kohno, 1998; Wu *et al.*, 2017).

Subsequent calcium modeling studies which consider cellular anatomy and orientation still assume uniform protein abundance within the cytosol (De Schutter, 1998; Brown *et al.*, 2008; Brown & Loew, 2012). Using more advanced confocal microscopy, data suggests that a highly expressed calcium binding protein in neurons, Calbindin, is heterogeneously distributed radially from the nucleus of the cell which is inconsistent with previous work (Groul *et al.*, 2010; Dio *et*

al., 2005). This finding begs the question whether other Ca^{2+} proteins are spread nonuniformly throughout the cell. Numerous models acknowledge the diverse arrangement of either intracellular features or spatiotemporal dynamics, but there appears to be a disconnect between these two seemingly interdependent concepts. Therefore, our intent in this study is to generate a more accurate model of calcium movement that accounts for realistic three-dimensional membrane ultrastructure and abundance of calcium-signaling proteins within the cell soma.

Purkinje neurons are an ideal model for our study on calcium signaling dynamics. Purkinje cells (PCs) have one of the largest neuron somas in the central nervous system (CNS), which enables us to take high-resolution microscopy images of intracellular structures and document interesting membrane distributions (Ross 2012). They facilitate long term depression and tonic firing, which regulates calcium wave concentrations, modifies nerve impulses, and coordinates motor functions (Hernjak *et al.*, 2005; Hoxha *et al.*, 2016). Moreover, due to their large cell bodies PCs have an extensive ER matrix that manages calcium efflux throughout the cytosol and creates sharp calcium gradients through IP_3R and RyR activation (Goto & Mikoshiba, 2011; Kuwajima *et al.*, 1992). Original research on the Purkinje cell dendritic system has shown there is intense interconnectivity of ER but hints at heterogeneity of receptors the ER surface, in particular IP_3R seem to be associated with ER stacks while RyR are absent from the dendritic spines (Ellisman *et al.*, 1990; Walton *et al.*, 1991; Martone *et al.*, 1993). Therefore, having this preconceived idea that Purkinje cells are diversified in their endomembrane areas makes these neurons a more interesting illustration of calcium signaling.

Mitochondrial matrices are also densely packed into the cytosolic space and are a source of adenosine triphosphate (ATP) which is the fuel the drives many biochemical reactions. Mitochondria control mitochondrial Ca^{2+} uptake, mediate ER and plasma membrane Ca^{2+} channels

through membrane contact points and can sense high concentrations of calcium microdomains (Lin *et al.*, 2019; Rizzuto *et al.*, 2012; Wu *et al.*, 2017). Therefore, the location and abundance of a large ultrastructure that includes the mitochondria and the Golgi apparatus in relation to ER membrane may affect passive diffusion of calcium in the soma. Not to mention, the Purkinje somata has an extensive network of calcium regulating proteins including G-coupled protein receptors (GPCRs), calcium buffers (Calbindin and Parvalbumin), cell membrane Ca^{2+} pumps (PMCA), and ER calcium transporters (SERCA, IP_3R, and RyR) that are continuously shaping calcium transience and are important constituents of our model (Fiala *et al.*, 1996; Fierro *et al.*, 1998; Matthews & Dietrich, 2015; Kozel *et al.*, 1998; Cooling *et al.*, 2007).

One subcellular pathway that is a suitable narrative for calcium regulation in PCs is the phospholipase C (PLC) pathway. In the PLC pathway, an extracellular signal is received on the cell surface by a GPCR, activating phospholipase C, an enzyme that effectively digests the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and IP_3 (Berridge 2016). DAG diffuses into the membrane and creates phosphorylation cascades while IP_3 travels to the endoplasmic reticulum and binds to IP_3Rs, along with available cytosolic calcium, to releases bulk stores of Ca^{2+} into the soma. As calcium concentrations rise in within the cytoplasm, additional downstream intracellular responses are triggered, increasing calcium concentrations in neighboring organelles and initiating a wave of calcium efflux into the cytosol and creating a positive feedback system (Berridge 2016). In addition to IP_3-induced calcium release, RyR activation occurs but releases smaller amounts of calcium (Kuwajima *et al.*, 1992). Since modeling has shown that the ER is mobile and can have distinct configurations, the relationship between the PLC pathway and ultrastructure geometries are critical to understanding PC calcium dynamics (Goto & Mikoshiba, 2011; Nixon-Abell *et al.*, 2016).

Although there is a wealth of knowledge available on the significance of cytosolic calcium movement in neurons, a standardized investigation on the importance of somatic compartmentalization is absent. To improve our understanding of internal positioning, morphology, and orientation of organelles in volumetric space we built a three-dimensional reconstruction of the Purkinje cell soma using high resolution electron microscopy. The electron tomograms created allowed us to characterize ER subtypes, identify density domains, and analyze membrane orientation with respect to the plasma membrane boundary. Using this spatial construct, we created a calcium dynamics model to simulate calcium movement through membrane barriers. We also used fluorescent immunohistochemistry to determine calcium signaling protein localization patterns across multiple PCs. To bridge the gap between the structural geometry of the cell and the electrophysiological kinetics of protein interactions, the mitochondrial importer receptor TOM20 stained to compare our analysis methods. This thesis exhibits the impressive influence of internal compartmentalization on somatic calcium signaling and demonstrates an intense need for accurate intracellular simulation models.

II. MATERIALS AND METHODS

Animals

All animal procedures were used in accordance with the Institutional Animal Care and Use Committee (IACUC, protocol number S12254) of University of California, San Diego. For electron microscopy experiments a male C57BL/6NHsd mouse was used from postnatal day 40. For immunohistochemistry experiments, wild-type C57BL/6J (Jackson Laboratories) male and female mice were used from postnatal days 45 to 50.

Tissue preparation for serial block-face scanning electron tomography (ET) microscopy

Tissue preparation was as described in Haberl *et al.,* 2018. A male C57BL/6NHsd mouse postnatal day 40 was anesthetized with ketamine and transcardially perfused for 15 minutes. Tissue sections 100µm thick were cut sagittal from the cerebellum, dehydrated in progressively higher concentrations of ethanol, then washed in acetone overnight. A Durcupan ACM resin was used to embed the tissue for electron microscopy. An electron tomography microscope was used to create high resolution images of mouse cerebellar cortex.

Electron Tomography

Mouse cerebellum was reconstructed using trained prediction algorithms and implemented on the IMOD software system, a software used to create 3D reconstructions of electron microscopy (EM) images. Tomographic reconstruction techniques were used to outline the endoplasmic reticulum (ER). Small training data for the ER were generated from EM datasets and the algorithm was trained to perform more accurate predictions. Mistakes in the algorithm for the ER were hand corrected on IMOD for two out of five datasets. The mitochondria, plasma membrane, nucleus, and Golgi apparatus were hand drawn for the first two datasets. The three remaining datasets used

the re-trained ER algorithm from the first two datasets post-hand corrections. Minimal hand corrections were performed on the organelle structures in the last three datasets.

Immunohistochemistry

Mice were anesthetized with 90 µl of ketamine and transcardially perfused at 37˚C by first flushing with a 1X phosphate buffer saline (PBS) and heparin solution for 5 minutes, then perfused with 4% paraformaldehyde (PFA) diluted from a 16% solution (Electron Microscopy Sciences 15710) in 1X PBS for 15 minutes. Whole brain extractions were performed and fixed in 4% PFA overnight. Post-fix brains were cryo-protected in 30% sucrose for 48 hours. Cerebellar slices were prepared using a Leica microtome and 50 µm thick sagittal cerebellar tissue slices were placed in cryo-preservative solution for a minimum of 24 hours.

Regions from the Vermis of the cerebellum were selected to undergo immunohistochemistry procedures. Three brain slices from three different mice were stained per protein: biological replicates; n=3. Good quality brain tissue was rinsed in ice cold 1X PBS for 10 minutes, 3 times. Tissue was then permeabilized for 10 minutes at 37°C. Permeabilization buffer consists of 8 ml ddH_2O, 1 ml 10X PBS, 4 ml 10% triton X-100. Tissue slices were incubated at 4°C overnight in blocking buffer: 1 ml 10X PBS, 1 ml 10% bovine serum albumin (BSA), 1 ml 10% fish gelatin, 300 µl Normal Donkey Serum (Sigma Aldrich D9663), 100 µl 10% triton-X 100 (Sigma Aldrich 10789704001).

Prior to primary antibodies incubation, if the protein receptor primary antibody was raised in mouse the tissue slices were incubated for 2 hours at 37°C with 0.1mg/mL anti-mouse IgG (H+L) polyclonal antibody and washed 3 times in working buffer for 10 minutes.

Slices were washed at 4°C with working buffer, 3 times: 15.8 ml ddH_2O, 2 ml 10X PBS, 2 ml blocking buffer mentioned previously, 200 µl 10% triton-X 100. After, tissue was incubated with the primary antibody cocktail. The following primary antibodies were used: 1/800 guinea pig anti-calbindin polyclonal (Synaptic Systems 214004), 0.1mg/mL anti-mouse IgG (H+L) polyclonal (Jackson 2338476), 1/100 rabbit anti-IP3R1 polyclonal (Invitrogen PA1-901), 1/250 Rabbit anti-PMCA2 polyclonal (Abcam ab3529), 1/100 Mouse anti-RyR1 monoclonal (Invitrogen MA3-925), 1/100 Rabbit anti-TOM20 polyclonal (Proteintech 11802-1-AP). Cerebellar tissue was incubated in 4°C for 72 hours on a shaking plate.

Incubated primary antibody tissue was washed 3 times in the working buffer for 5 minutes before adding the secondary antibody cocktail. All secondary antibodies were raised in donkeys. Anti-donkey secondary antibody conjugates were Alexa 488, 594, or 647 fluorophores. Tissue was incubated for 24 hours at 4°C on a shaking plate.

Tissue Mounting

Tissue was rinsed 3 times with 1X PBS for 5 minutes and mounted on a microscope slide with 4′,6-diamidino-2-phenylindole (DAPI) Fluoromount-G mounting media (Southern Biotech 0100-20). DAPI was used to stain the dsDNA within the tissue, therefore the nuclei of the Purkinje cells and granule cell layer.

Confocal microscopy

High resolution images of neurons were recorded from Cerebellar Vermis lobes IV/V or VI using a Nikon A1 confocal microscope. Image sizes were 1024 X 1024 pixels and deconvolved with the Landweber algorithm by NIS-Elements software. Only Purkinje cells with nuclei and

dendrite on the same z-plane for a minimum of five z-stacks were reconstructed and used for qualitative and quantitative analysis of the radial and angular fluorescence distribution in this study.

Data Analysis

Raw high resolution deconvolved z stack images taken on a confocal microscope were processed on FIJI software and pixel saturation was normalized to 0.01% contrast for all channels: DAPI enhanced contrast at 10%. Processed images were saved as composite .tif images to be further processed in MATLAB for radial and angular distribution. Calbindin and DAPI channels were saved individually under grayscale for IMOD mask creation.

Tissue images were rotated so that the dendritic pole is at 270°, axonal pole is at 90°, and the nucleus centroid is at (0,0). Six technical replicates were collected from each of the three brains for radial and angular fluorescence analysis: total n=18. Grayscale .tif files were converted to .mrc files and used to create automated positioning for the soma, dendrite, and nucleus outlines hand corrected in IMOD computer software system. The Calbindin channel of the immunostained images were used to outline the soma and dendrite of the cells per z-stack while the DAPI channel was used to outline the nucleus. Masks were used to isolate fluorescence from the boundary between the nucleus and plasma membrane (the soma) of a single neuron on a single z-plane image. Angular fluorescent intensity for each z stack was extracted into twelve 30° angular bins with nucleus centroid at (0,0). The average fluorescence intensity was calculated for each cell, as well as an average fluorescent intensity for the entire dataset; n=18. Radial fluorescence was measured by normalizing vector lengths from the nucleus centroid to the plasma membrane across

the same twelve 30° angular bins. The total average radial fluorescence was measured by taking the 0° to 360° lateral distribution from each cell.

Computer model simulation of calcium movement

A partial differential equation (PDE) computational model has been made to analyze the spatiotemporal calcium dynamics in Purkinje cell somas using EM datasets to create a mesh geometry for proper organelle characterizations and orientation, as well as fluorescent data for protein abundance.

III. RESULTS

1. Electron tomography reveals non-uniform organelle orientation

A variety of calcium signaling models currently exist to hypothesize internal space and the biochemistry behind cellular communication (Fink *et al.*, 2000; Cooling *et al.*, 2007; Fiala *et al.*, 1996). In this study, fine-scale reconstructions of organelle features were generated from high-resolution electron tomography (ET) microscopy images of mouse cerebellar tissue to determine if spatial localization can affect calcium movement (Figure 1.1). Two-dimensional reconstructions were created to outline the boundaries of five major cellular components: PM, ER, mitochondria, Golgi apparatus, and nucleus. A 3D mesh geometry of these organelles was built to help discern the structural organization of organelles in Purkinje cell somatas (Figure 1.1b-1.1c). The maps of segmented intracellular features were created from five separate electron tomogram volumes (two from the axonal pole and three from the dendritic pole) to consider whole-cell spatial variance and characterizations of the ultrastructure within the soma.

A variety of widely known morphological ER subtypes were established and categorized in this neuronal model: layered ER sheets, subsurface ER, and ER stacks (Figure 1.1e-f). Quantifications from these ETs have revealed that the average diameter of ER in the soma is largely variable, between 20nm and 90nm in length, while the length can be anywhere from less than 1nm to 3000nm (Haberl *et al*., in preparation). These ER motifs are not only different configuratively, varying in length and diameter, but are also distinctly dispersed through the cell. Long segments of layered ER sheets with a parallel orientation to the nuclear envelope are densely compacted near the nucleus of the cell as expected (Figure 1.1f). While shorter ER structural motifs, namely ER cisternae and subsurface ER, are clustered further from the centroid of the cell, closer to the plasma membrane, and seem to be connective structures medial of the cytosol (Figure 1.1f). Local density maps of ER were created to further demonstrate that the identified ER motifs

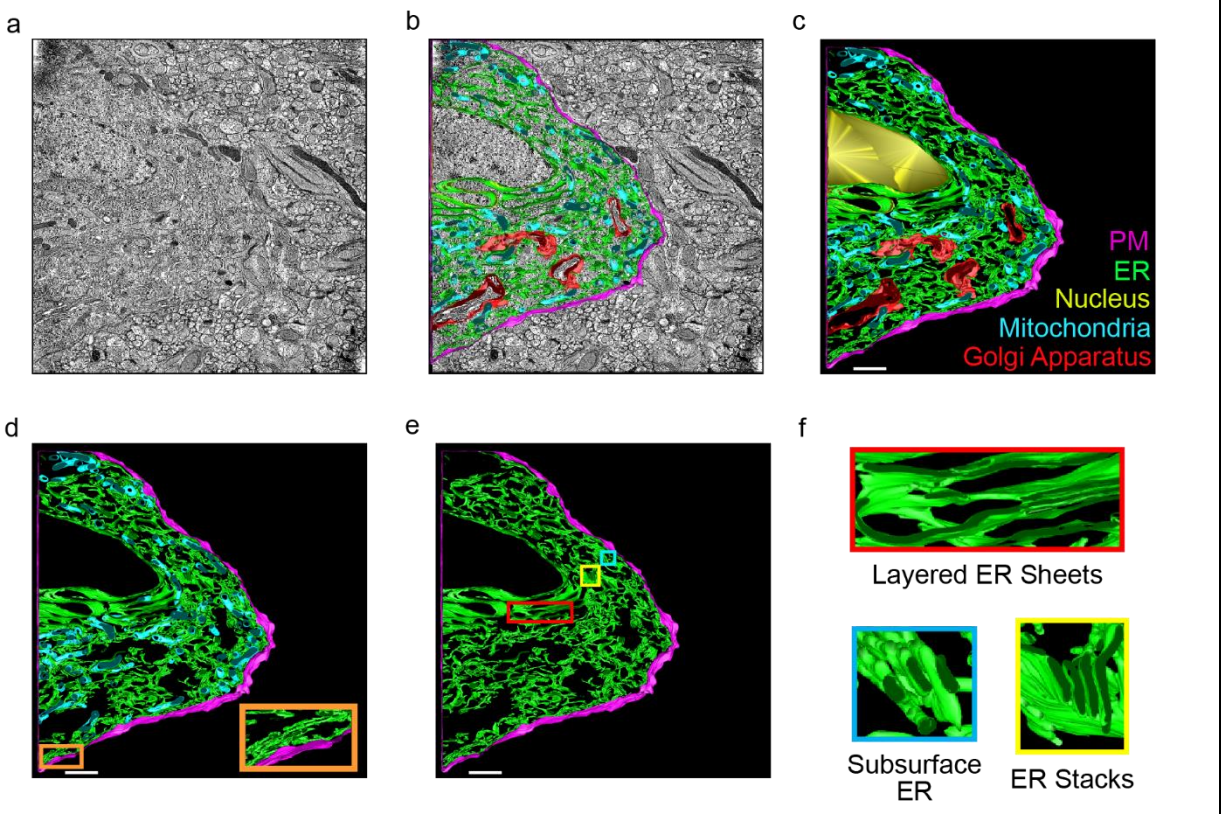

Figure 1.1: Organization of Purkinje soma ultrastructure visualized by electron microscopy. (a) Electron tomogram (ET) dimensions (2,000 x 2,000) of a Purkinje neuron soma portion generated from cryofixation of high-pressure frozen cerebellar tissue. **(b)** Subcellular organelle reconstruction of cell soma overlaid with z-stack images from ET (2,000 x 2,000 x 73 volume). **(c)** 3D model view of organelle structures with labelled cellular features: plasma membrane (PM), endoplasmic reticulum (ER), nucleus, mitochondria, and Golgi apparatus perimeter. Scale bar = 50 pixels**. (d)** ER and PM membrane proximity and orientation. **(e-f)** Endoplasmic reticulum subtypes: layered ER sheets, ER stacks, and subsurface ER.

are in concentrated regions of the reconstruction (Haberl *et al.*, in preparation). These density maps reveal that peaks in ER clusters correspond to the established structural motifs.

Based upon small-scale reconstructions, there is a tight association between ER and the PM which may influence the final structural model (Figure 1.1d, orange panel). Therefore, the relative frequency and orientation of endoplasmic reticulum with respect to the plasma membrane were analyzed. There is a logical trend that ER stacks are closer to the surface of the cell and

preferentially angled parallel to the plasma membrane (Figure 1.1d, orange box). Parallel orientation of ER with relation to the PM is limited to 30° in either direction of the horizontal while perpendicular ER is the interval of 80° to 120°. Any ER that falls in between these angles were not included in the relative frequency data. We found overall, from the nuclear to plasma membrane bound, ER is indeed preferentially parallel to the plasma membrane. Moreover, these strands are predominantly (93%) oriented parallel when less than 100nm from the cell surface (Haberl *et al*., in preparation). Data also suggests that there are significantly more ER strands near the subsurface of the soma, less than 100nm from the plasma membrane. The ER-PM distance varies between nm and μm away, which may drastically influence the calcium dynamics model.

Intracellular components, such as the distance between mitochondria, and endoplasmic reticulum, may act as barriers to calcium flux. Extending the previous analysis between ER-PM, the radial distribution of mitochondria indicates that mitochondrion are largely absent near the plasma membrane relative to presence within the cell (Figure 1.1d). It has been quantitatively determined from the volumetric ET datasets that mitochondria are predominantly located 200nm from the cell surface while ER structures are between 0-100nm from the cell surface, resulting in mirrored ultrastructure volumes for ER and mitochondria (Haberl *et al.*, in preparation). Thus, calcium may be obstructed by mitochondria and, by extension, the Golgi apparatus in layered ER sheets and ER stacks that are proximal to the nucleus.

2. Diversity of calcium signaling protein abundance in Purkinje cell somas

Presently, our model includes the precise structural geometry and spatial organization of neuronal features, but still presumes homogeneous distribution of the biochemical factors involved in calcium homeostasis. There are already models which exist that consider the unique biochemical and electrophysiological kinetics of calcium handling within the cell (Wu *et al*., 2017; Fink *et al.*,

2000; Eliers *et al*., 1995). To create the most accurate model of Purkinje cell somas, all components which influence calcium dynamics must be investigated. Since our previous findings suggest there is heterogeneity in ultrastructure subtypes and cellular occupancy, next we will observe localization patterns of calcium signaling proteins spanning the neuron soma. We hypothesize that the selected intracellular proteins will be localized to distinct regions of the soma. We want to quantitatively determine protein distributions of Calbindin and PMCA, which bind calcium molecules and remove from the cytosol, as well as IP_3R and RyR which release calcium from internal stores into the soma.

To investigate the localization patterns of calcium-dependent protein receptors in Purkinje cell somas, we used fluorescence immunohistochemistry to identify the somatic boundary of each cell without the nuclei and evaluate the abundance of proteins across multiple cells (Figure 2.1a-e). The arrangement of fluorescence calcium-signaling protein receptors and buffers were recorded qualitatively, and representative images were pulled from each dataset (Figure 2.1f, 2.2a, 2.3a). To quantitatively determine the probability of protein abundance, a standard coordinate system for all cells was created and aligned linearly such that the fluorescent intensity in the cytoplasmic domain could be analyzed around the angular and radial axes for multiple cells (Figure 2.1b). To validate our analysis system, we first considered a well-known and highly expressed protein, Calbindin, (Figure 2.1).

As previously stated, Calbindin is a highly expressed endogenous calcium buffer that moves freely throughout the proximal to distal regions of the soma (Fierro and Llano, 1996). Therefore, we expected Calbindin to be dispersed relatively uniform throughout the cytoplasm. Probabilities of protein localization across angular and radial axes were recorded and as expected, resulted in very little variability throughout the cell soma (Figure 2.1g-h). There was no significant

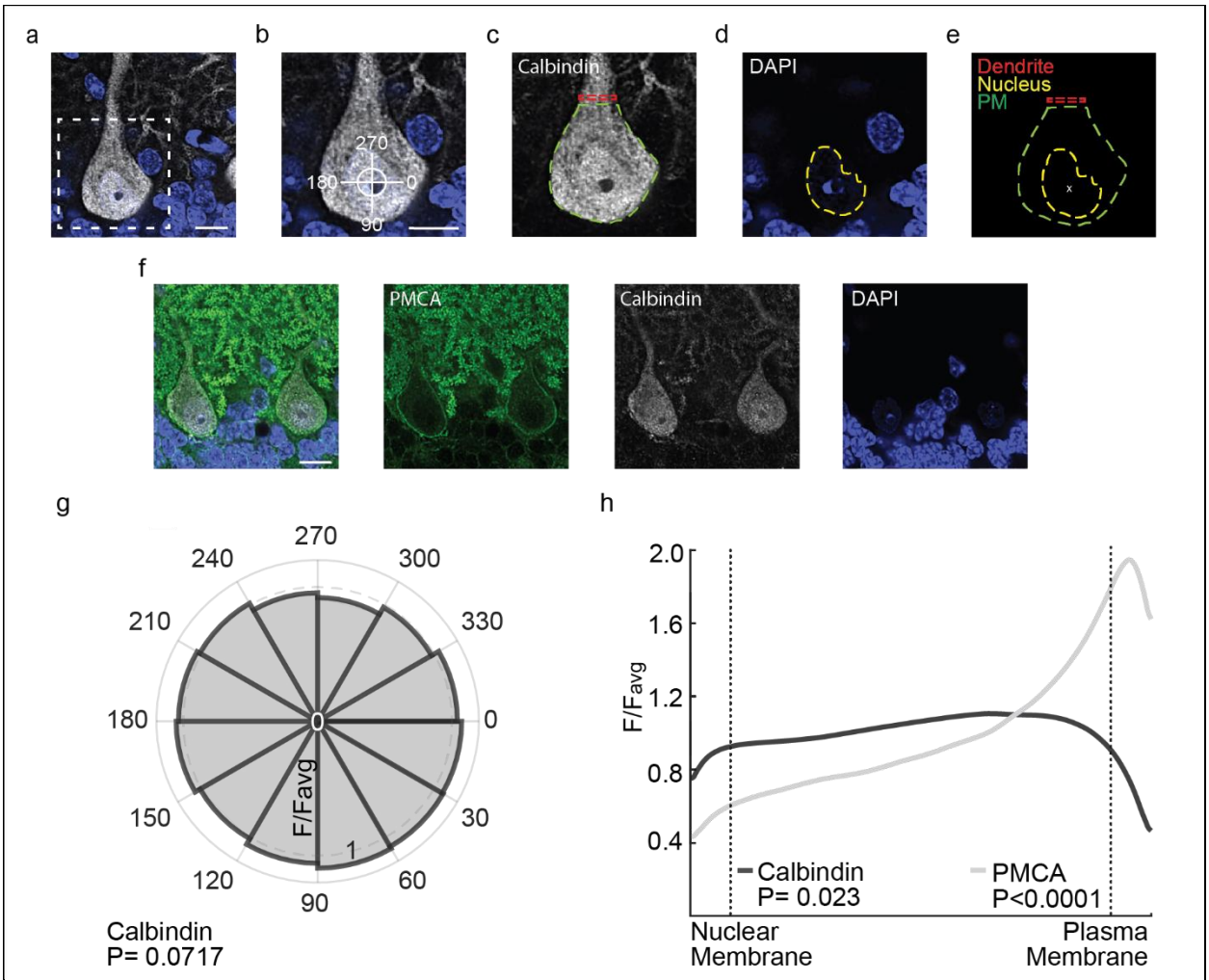

Figure 2.1: Method validation using Calbindin and PMCA protein distributions within Purkinje cell somas. (a-e) Fluorescent immunohistochemistry (IHC) evaluation of calbindin in mouse cerebellum. **(a)** Cell identification for data analysis. Calbindin (grey) used to identify cell soma and dendrite in the same z-plane. DAPI (blue) is used to identify cell nuclei in plane with dendrite. White box indicates the analyzed cell. Scale bar = 10µm. **(b)** Cell orientation; axonal pole at 90°, dendritic pole at 270°, center of nucleus at (0,0). Scale bar = 10µm **(e)** Composite outline of cell boundaries for a single z-stack where X indicates the centroid of the nucleus; **(c)** plasma membrane (green) and dendrite (red) derived from calbindin channel; **(d)** nucleus (yellow) resulting from the DAPI channel. **(f)** Representative multi-channel and single-channel images of PMCA **(g)** Scaled angular distribution of calbindin divided by 12, 30° bins. Significance indicated by $p<0.05$, n=18. Calbindin p=0.0717 across 0-360°. Dotted grey line indicates $F/F_{avg}=1$, the normalized average fluorescent intensity. **(h)** Scaled radial distribution of average fluorescence from nucleus to plasma membrane (PM) across 0-360°; significance indicated by $p<0.05$, n=18. Radial distribution of calbindin (black line); p=0.023, bias towards PM. Radial distribution of PMCA (grey line); $p<0.0001$, bias towards PM.

difference in the angular distribution of Calbindin ($p=0.0717$) in the soma which supports the veracity of our model system (Figure 2.1g). Yet, when calbindin is analyzed radially from 0-360° our data indicates there is a minor, but significant ($p=0.023$), bias towards the plasma membrane (Figure 2.1h). This buffering pattern may be attributed to limitations in geometric space caused by ultrastructure densities pushing Calbindin and other calcium buffers towards the plasma membrane. This theory is due to overlapping evidence from Figure 1.1, where organelle features such as mitochondrion are preferentially located distal from the PM. However, more evidence needs to be gathered before residing on this conclusion.

Calbindin was used to showcase our methodology with well-known homogeneously distributed calcium buffers, but to further validate our model we wanted to determine if our system can detect highly localized proteins. PMCA is a transporter protein that removes calcium from the cytosol and is ubiquitous across the cell membrane surface (Figure 2.1f). Analysis of the radial distribution of PMCA from 0-360° exhibited an intense polarization ($p<0.0001$) strongly preferring the plasma membrane boundary of the cell (distal membrane 1.87 F/F_{avg} and proximal membrane 0.57 F/F_{avg}), as predicted (Figure 2.1g). Jointly, Calbindin and PMCA provide a foundation for our analysis technique to be used with other signaling proteins that have undetermined distributions, such as IP_3R and RyR, since our method can record both homogeneous and heterogeneous fluorescent patterns.

After establishing our technique, our next aim is to analyze the distribution of other calcium modulating proteins that we are including in our final model. IP_3R and RyR localizations are imperative to understand molecular handling within the soma since both receptors regulate the efflux of calcium from internal ER stores and propagate intracellular calcium flux. IP_3R and RyR staining was conducted on the same tissue batch, indicated in figure 2.2, for the purpose of directly

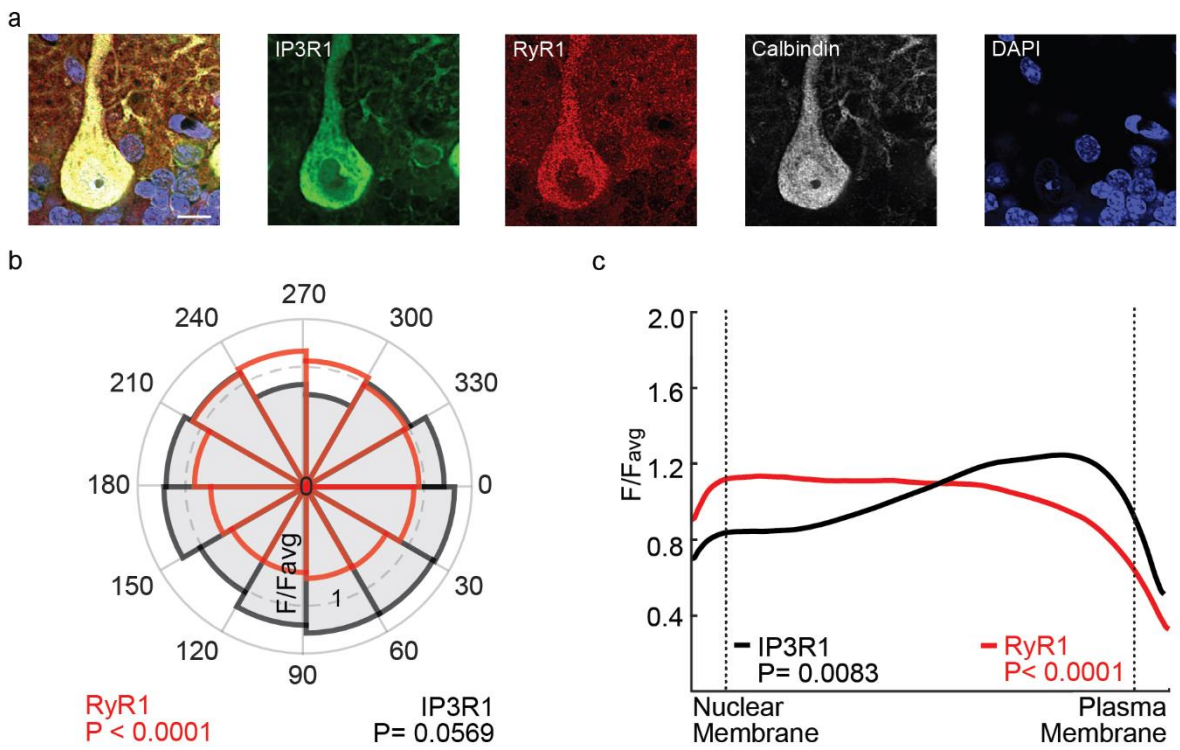

Figure 2.2: Heterogeneous distribution of IP$_3$R1 and RyR1 in Purkinje cell somas. **(a)** Representative multi-channel and single-channel fluorescent immunohistochemical (IHC) evaluation of mouse cerebellar tissue. Green channel, inositol 1,4,5-trisphosphate receptor 1 (IP$_3$R1); red channel, ryanodine receptor 1 (RyR1). Scale bar = 10µm. **(b)** Scaled angular distribution of IP$_3$R1 and RyR1 divided by 12 equal 30° bins across 0-360°; Significance is indicated by $p<0.05$, n=18. Angular distribution of RyR1 (red); $p<0.0001$, bias towards dendritic pole. IP$_3$R1 (black); p=0.0569. Dotted grey line indicates $F/F_{avg}=1$, the normalized average fluorescent intensity. **(c)** Scaled radial distribution of average fluorescence from nucleus to plasma membrane (PM) across 0-360°; significance is indicated by $p<0.05$; n=18. Radial distribution of IP$_3$R1 (black line); p=0.0083, bias towards nuclear membrane. Radial distribution of RyR1 (red line); $p<0.0001$, bias towards PM.

comparing protein abundance regions within the soma. Trends of protein localization patterns are demonstrated in the angular and radial analysis of the cell population. There was no significant difference in the angular fluorescence of IP$_3$R from 0-360° (p=0.0569), however there is notable variation between angular bins (Figure 2.2b). The IP$_3$R signal from bins 240-270° and 270-300° is less than the normalized average fluorescence ($F/F_{avg}=1$), as well as significantly lower than the fluorescent intensity of other bins on the axonal end of the cell.

Although there is not an overall angular bias, the significant polarities between bins may suggest that IP_3Rs are more closely associate with the axonal pole of the cell and could be correlated with the availability of subsurface ER around the cell. As mentioned previously in our ET geometry, endoplasmic reticulum is not evenly distributed throughout the cell and is more densely populated near the distal end of the cell less than 100nm from the cell surface (Figure 1.1d). In conjunction with this data, fluorescent analysis has also shown there is an askew nuclei centroid location the basal side of Purkinje neurons and an absence of plasma membrane at 270° due to the dendrite opening (Figure 2.1e). Thus, analysis of bins from 240-300° may have a reduced abundance of subsurface ER while retaining proximal ER sheets and stacks. Therefore, the decrease in angular fluorescence at the dendritic pole may be associated with the enriched distal polarization ($p<0.0083$) of IP_3R along the radii of the cell from 0-360°, revealing that IP_3R may be more closely associated with ER near the plasma membrane (Figure 2.2c).

In opposition to IP_3R angular analysis, the distribution of RyR across 0-360° reveals not only a significant angular bias towards the apical dendrite ($p<0.0001$), but also a decrease in relative abundance of RyR especially on the axonal side (Figure 2.2b). This is the first indicator that RyRs may be associate with proximal ER. As expected, the radial analysis of RyR localization is enriched ($p<0.0001$) towards the nuclear membrane of the cell from 0-360° and is in stark contrast to IP_3R results (Figure 2.2c). Given these contrasting results, although both IP_3R and RyR reside on the ER surface we observed unique angular and radial patterns. This supports the idea that signaling proteins can be localized to distinct regions in the soma.

Since we have established that signaling proteins can be heterogeneously dispersed throughout the cytoplasmic space and intend to implement this in our model, our next goal is to verify that both of our analysis methods are comparable. To bridge the gap between ET and IHC,

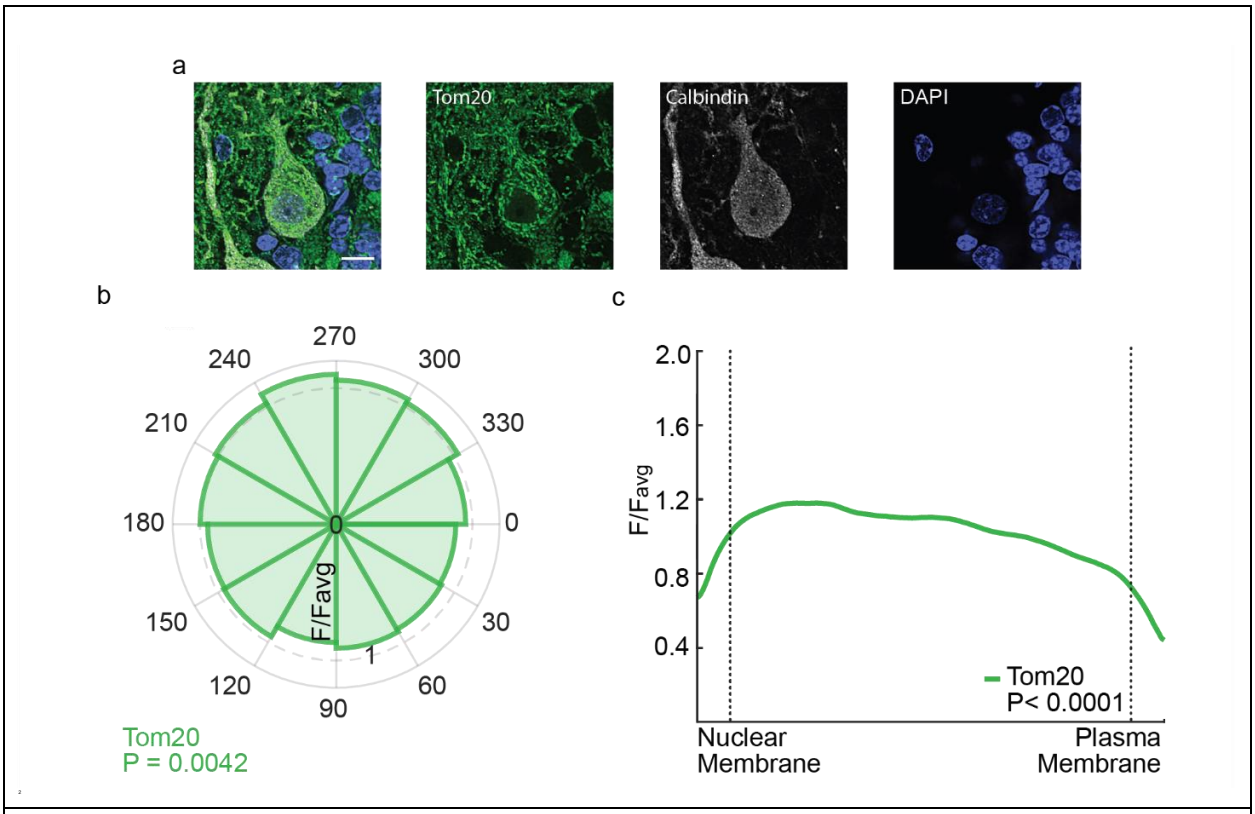

Figure 2.3: Biased distribution of mitochondrial import receptor subunit TOM20 in Purkinje cell somas. (**a**) Representative multi-channel and single-channel fluorescent IHC evaluation of translocase of outer membrane 20 kDa subunit (TOM20) in mouse cerebellar tissue. Scale bar = 10µm. (**b**) Scaled angular distribution of TOM20 divided by 12 equal 30° bins across 0-360°; Significance $p<0.05$, n=18. Angular fluorescence for Tom20; $p<0.0042$, bias towards dendritic pole. Dotted grey line indicates $F/F_{avg}=1$, the normalized average fluorescent intensity. (**c**) Scaled radial distribution of average fluorescence from nucleus to plasma membrane (PM) across 0-360°; significance $p<0.05$; n=18. Radial distribution of TOM20; $p<0.0001$, bias towards nuclear membrane.

we immunostained cortical mitochondrial import receptor subunit TOM20 and compared the localization patterns to the ultrastructure motif of mitochondria (Figure 2.3; Figure 1.1d). To ensure the final dynamics model is being populated faithfully, we expect to see similar results between the structural analysis and fluorescent data. The angular distribution of TOM20 has a slight preference towards the dendritic pole ($p=0.0042$) and is significantly enriched ($p<0.0001$) near the nuclear membrane when analyzed from 0-360° (Figure 2.3b, c). These results correspond

directly with the electron tomography dataset analysis. Therefore, a final PDE model using all the results above is feasible.

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IV. DISCUSSION

The findings in this thesis demonstrates the fundamental relationship between intracellular organelles and biochemical components that shape spatiotemporal dynamics of calcium. Firstly, we were able to validate our model by comparing structural motifs and known polarized and non-polarized calcium protein placements to other works (Wu *et al.*, 2017; Banno & Kohno, 1998; Dio *et al.*, 2005; Kozel *et al.*, 1998). Using electron tomography, we categorized ER subtypes and quantified calcium diffusion rates with respected organelle orientations. This sheds new light on the possibility of the somatic ultrastructure passively and actively altering calcium flow in the open space of the cell body. Ultimately, our assumption that the ER plays an important role in maintaining calcium homeostasis was confirmed and contextualized in this thesis by using calcium simulation models which accounted for ER preferential orientation and distance between ER strands (Figure 1.1d; Haberl *et al.*, in preparation). This is reflected in the neuronal cell body diagram created in Wu *et al.*, which showed a higher concentration of parallel strands of endoplasmic reticulum near the plasma membrane (Wu *et al.*, 2017).

Another point that may want to be explored in subsequent studies on the structural geometry is the interactions between ER and mitochondria. The same study as above suggests that ER-PM membrane contact sites (MCS) may serve as store-operated Ca^{2+} entry (SOCE) points or mediate calcium release from the ER. But additional research has shown MCS between ER and mitochondria as well, which may be correlated with gated calcium movement between organelles (Wu *et al.*, 2017; Rizzuto *et al.*, 2012). Therefore, if more parameters were added to the final PDE model it may be interesting to document the nm-separation between ER and mitochondria to see if mitochondria are more than just passive diffusion (Figure 1.1d; Figure 2.3).

Given the conclusions from our IHC experiments, there seems to be a heterogeneous distribution of IP_3R and RyR within the soma and each receptor-type heavily governs a particular

region of the cytosol (Figure 2.2). In the greater context of calcium modulation within neurobiology, recent studies have begun to unearth how endogenous IP3Rs on the surface of the ER are arranged in small clusters and may also transform calcium dynamics (Thillaiappan *et al.,* 2017). Therefore, evidence suggests that the internal cavity of the cell is more complex that previously though (Figure 2.2). Furthermore, through repeated analysis on the clusters of IP_3R they noticed mobile and immobile IP_3R that were embedded in the membrane. Unexpectedly, transient IP_3Rs were not associated with calcium efflux from the ER, but immobile receptors were essential to Ca^{2+} release, suggesting that there is diversity even within IP_3Rs (Thillaiappan *et al.*, 2017). Thus, our fluorescent analysis of IP_3R protein abundance may not be completely accurate since our method did not distinguish between mobile and immobile receptor types. This finding, coupled with the idea that organelles are heterogeneously organized throughout the cytosol, may affect further affect the signaling dynamics model and the neuronal pathology of the cerebellar cortex.

Overall, our results adequately build upon a narrative that connected existing model studies on cellular morphology, structural orientation, and calcium diffusion patterns and may continue to add to the greater knowledge of calcium handling within neuron somas (Eliers *et al.*, 1995; Fierro & Llano, 1996; De Schutter 1998).

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