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The role of *SERCA* in neural development and brain tumors

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ABSTRACT

Ion channels are essential for neural function, playing a variety of necessary cellular roles including excitability, maintaining ion gradients, and volume control. While the role of ion channels in neurons is well-defined, much less is known about excitability in neural progenitor cells. Recently it has emerged that these neural precursors may be affected by channelopathies, indicating a critical role of ion channels in neural development. Using the model system *Drosophila melanogaster* (fruit fly), I investigated the role of the ion channel SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase). SERCA is a vital calcium ion pump located on the wall of the endoplasmic reticulum, and it is integral in maintenance of cellular homeostasis and signal transduction in larval brain development. The use of RNAi knockdown technology to reduce the expression of SERCA resulted in a significant decrease in larval brain volume. Further experiments found that the *SERCA* RNAi knockdown not only decreased cell numbers in neural stem cell lineages but changed the identity of the lineage, implicating an important role for SERCA in both neural stem cell asymmetric division and cell fate. These results indicated that SERCA channels hold a prominent role in cellular proliferation in normal development. The knockdown of *SERCA* in a brain tumor model additionally showed significantly decreased brain tumor volume, implicating *SERCA* in tumor development and the proliferation of “neural stem cell-like” cancer cells. Together, these data pinpoint *SERCA* as a promising potential treatment target for both neural developmental disorders and cancer.

INTRODUCTION

Neural development is a complicated aspect of early life, consisting of a series of pathways and cell fate choices that eventually form the complex neural circuitry used by animals and humans alike in everyday life. The larger goal of neural development is to form the brain, spinal cord, and nerves that innervate the body. The steps to achieving these endings are affected by the internal and external environments of organs, tissues, and individual cells throughout the developmental stages. Within a single cell, cell fate decisions and cellular identity are influenced by spatial and temporal cues, ion concentration, and the cellular niche (Homem & Knoblich, 2012; Ho et al., 2021). With the plethora of factors essential for healthy development, any source of dysregulation can result in pathway deviations and different individual cell fates— a direct cause of developmental disorders (Ho et al., 2021). Achieving a more mechanistic understanding of neural development provides new avenues of treatment towards developmental diseases and may inform treatment of late-in-life neural degenerative diseases.

Electrolyte balance is a vital component of homeostatic regulation, mediated by ion channels. Found in all cell types, ion channels, pumps, and exchangers control cellular functions, including

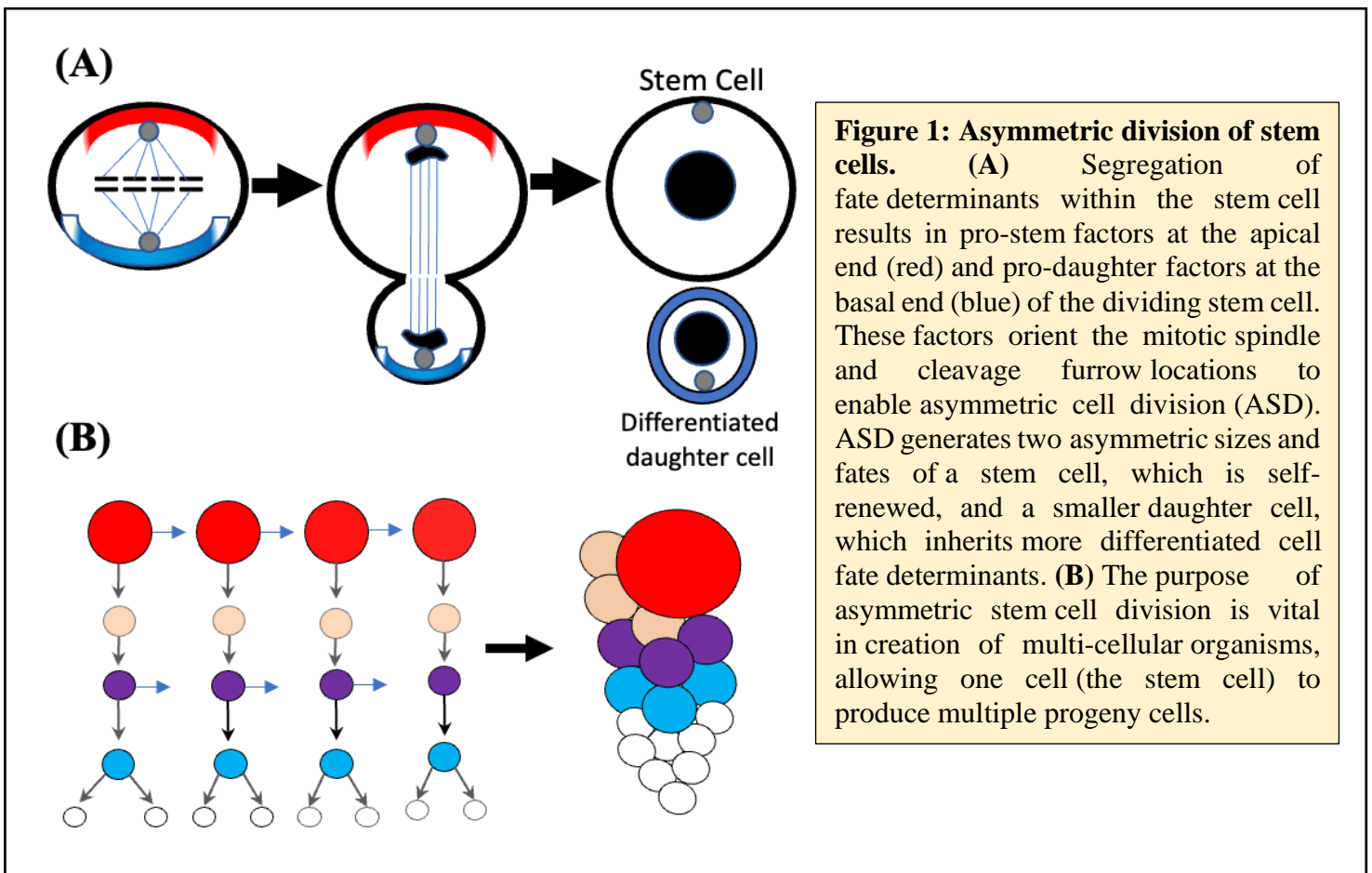
excitability, electrical potential across cell membranes, maintenance of electrochemical gradients, volume control, and the activation of signaling pathways. Ion channels play critical roles in development across all species. In humans, ion channel mutations are directly associated with developmental diseases like seizures and autism (Boczek et al., 2021). Calcium (Ca^{2+}) channels are especially important ion channels, regulating cell activity and inter-/intracellular signaling cascades. Ca^{2+} ions are powerful second messengers, with the capacity to bind to over 200 different cytosolic proteins and cause conformational change and/or further activation of signaling mechanisms (Snoeck, 2020). They exhibit a wide range of roles across tissues, including muscle contraction, neurotransmitter release, metabolic regulation, hormone release, downstream gene activation/deactivation, and cellular migration (Bower et al., 2017; Ho et al., 2021; Horigane et al., 2019; Saghetelyan, 2021; Snoeck, 2020). Studies have found cytoplasmic Ca^{2+} ion concentrations mediate stem cell differentiation of various tissues, such as hematopoietic stem cells and epidermal stem cells, where higher concentrations of Ca^{2+} are associated with cellular differentiation (Snoeck, 2020). Whether the role of Ca^{2+} in differentiation is *conserved* across all tissues, including neurons, remains unresolved. Due to the influential nature of these ions, they are largely sequestered outside of the cell or contained within organelles—like the endoplasmic reticulum (ER)—to maintain low concentrations within the cytoplasm (around $\sim 10^{-7}$ M inside the cell and $\sim 10^{-3}$ M outside the cell, a difference of roughly 10,000 M). Excess calcium concentrations are known to be toxic in cells, so this electrochemical gradient is rigidly maintained by the many Ca^{2+} ion pumps and channels throughout the cell (Boczek et al., 2021).

One such Ca^{2+} ion pump is the sarco/endoplasmic Ca^{2+} -ATPase, or SERCA. SERCA pumps are located on the ER membrane and play a role in ER calcium homeostasis. These P-type ATPases function to transport excess Ca^{2+} ions from the cytoplasm back into the ER lumen, effectively maintaining low cytosolic Ca^{2+} ion concentrations (Guerrero- Hernández et al., 2020). *SERCA* is integral in the halting and removal of calcium microdomains—small pockets of released Ca^{2+} ions that activate cell signaling pathways (Bower et al., 2017). A secondary function of SERCA pumps is to return the internal ER environment to its resting state to ensure proper protein folding and modifications. SERCA proteins are conserved from invertebrates to humans. Humans have three distinct *SERCA* genes, located on different chromosomes. Each gene encodes a different type of SERCA channel (identified primarily by location) resulting in a total of 12 known splicing isoforms. While *SERCA1* and *SERCA3* genes form pumps in specific regions (*SERCA1* in fast-twitch skeletal muscle and *SERCA3* in blood components), *SERCA2b* gene isoform encodes a “housekeeping” pump that is found in all tissues for cellular homeostasis (Britzolaki et al., 2020; Periasamy & Kalyanasundaram, 2007; Stammers et al., 2015). Mutations in *SERCA2b* cause the skin disease known as Darier disease or *follicular keratosis*, resulting from ER stress and incomplete production of vital epidermal proteins (Schmieder & Rosario-Collazo, 2022).

The involvement of *SERCA* in tissue development remains unclear. Deng et al. (2015) found that blocking *SERCA* channels in intestinal stem cells (ISC) of *Drosophila melanogaster* resulted in increased proliferation of the stem cell lineages and increased ER stress. However, different body systems have been observed to differ in calcium-based activities, for example branching and migration of *Drosophila* tracheal cells is directed by low levels of Ca^{2+} ions, a function that is stunted with loss of *SERCA* channels (Bower et al., 2017). These demonstrations of the critical role *SERCA* genes play in cellular homeostasis and signaling of different organ systems underlies the necessity for understanding its functions during brain development.

The model system *Drosophila melanogaster*—also known as the fruit fly—has been used to identify key molecular regulators of neural development. The well-defined anatomy, development, and genetics of *Drosophila* make it a prime resource for understanding complex processes like neurogenesis. Additionally, fruit flies have limited genetic redundancy; for instance, while humans have three *SERCA* genes, flies only have one (an ortholog of the human *SERCA2b* gene) (Britzolaki et al., 2020). This provides a more straightforward system to investigate *SERCA* function without the added complexity from multiple isoforms. Moreover, many aspects of development are conserved from flies to humans, including stem cell asymmetric division.

The neural stem cells of the *Drosophila* central nervous system are known as neuroblasts (NB). They divide asymmetrically via segregation of internal fate determinants, resulting in renewal of the NB and the production of a differentiated daughter cell (**FIGURE 1**). Dysregulation of intracellular fate determinants can cause changes in proliferation, potentially resulting in excess cells and tumor formation or a limited number of cells and reduced neural function. During larval development, these neuroblasts are located throughout the brain lobes and ventral nerve cord (VNC)—the components of the larval *Drosophila* central nervous system (**FIGURE 2 (A-B)**). There are three types of neuroblast lineages that develop in specific regions within these structures, and each has distinct genetic markers and positions, allowing for identification. Type 0 neuroblasts are active only at the embryonic stages, directly producing neurons; however, by the time the embryo reaches larval stages, most Type 0 lineages are completely terminally divided and should not be found (Chen et al., 2022). Type I neuroblasts are located in the brain lobes and VNC and are identified by the expression of Deadpan (Dpn) and Asense (Ase) genetic markers (**FIGURE 2 (C)**). These cells asymmetrically divide to produce two daughter cells—a renewed neuroblast and a more differentiated Ase^+ ganglion mother cell (GMC). The GMC will then divide symmetrically into two neurons. Type I NB lineages produce ~100 neurons. Type II NBs lineages, however, generate the highest numbers of progeny compared to the other NB subtypes (around 500 neurons), and yet have the smallest numbers. Type II neuroblasts are located only in the larval brain lobes, with 8 distinct lineages per lobe (as opposed to the ~90 Type I neuroblasts in the central nervous



system) (Homem & Knoblich, 2012). Type II NBs express only Dpn (are Ase⁻) and asymmetrically divide to produce an immature intermediate neural progenitor (imINP) (**FIGURE 2 (D)**). Upon maturation, these intermediate neural progenitor (INP) cells begin expressing their own Dpn⁺ and Ase⁺ markers and asymmetrically divide as well, producing a GMC while self-renewing the INP. The GMCs then symmetrically divide into neurons. Because of the amplifying step of the INP, Type II neuroblast lineages produce roughly five times as many neurons as Type I (Bello et al. 2008; Boone & Doe, 2008; Bowman et al., 2008; Piggott et al., 2019). The well-established understanding of the *Drosophila* neuroblast provides a platform to investigate novel roles for bioelectricity and ion channel influences on differentiation throughout neurogenesis.

This study was focused on understanding the role of *SERCA* in neurodevelopment and its effect on cell proliferation within the *Drosophila* larval brain. It has been observed that cancer shares physiological properties with stem cells. For instance, many cancers take control of and manipulate natural developmental pathways to increase proliferation. Previous studies have shed light on the potential role of *SERCA* channels in the protection of cancer cells. Park et al. (2018), for example, found in their experiments that human cancer cells upregulate *SERCA* activity to prevent ER stress and apoptosis, circumventing the Unfolded Protein Response (UPR) pathway that is often triggered as an anti-cancer

mechanism (Sehgal et al., 2017). Other groups working with *SERCA* in human cells have corroborated these findings, discovering that inhibition of *SERCA* channels with chemicals, like Thapsigargin, results in decreased tumor volume (Chemaly et al., 2018; Denmead & John, 2004; Sehgal et al., 2017). These findings not only emphasize the importance of cytoplasmic calcium in development and survival, but also present *SERCA* as a potential mechanism for controlling that development, especially in treatment of cancer. Therefore, this study examined the function of *SERCA* pumps in normal brain development, as well as within brain tumor development to elucidate its role more thoroughly in progenitor proliferation and overall neurogenesis patterns.

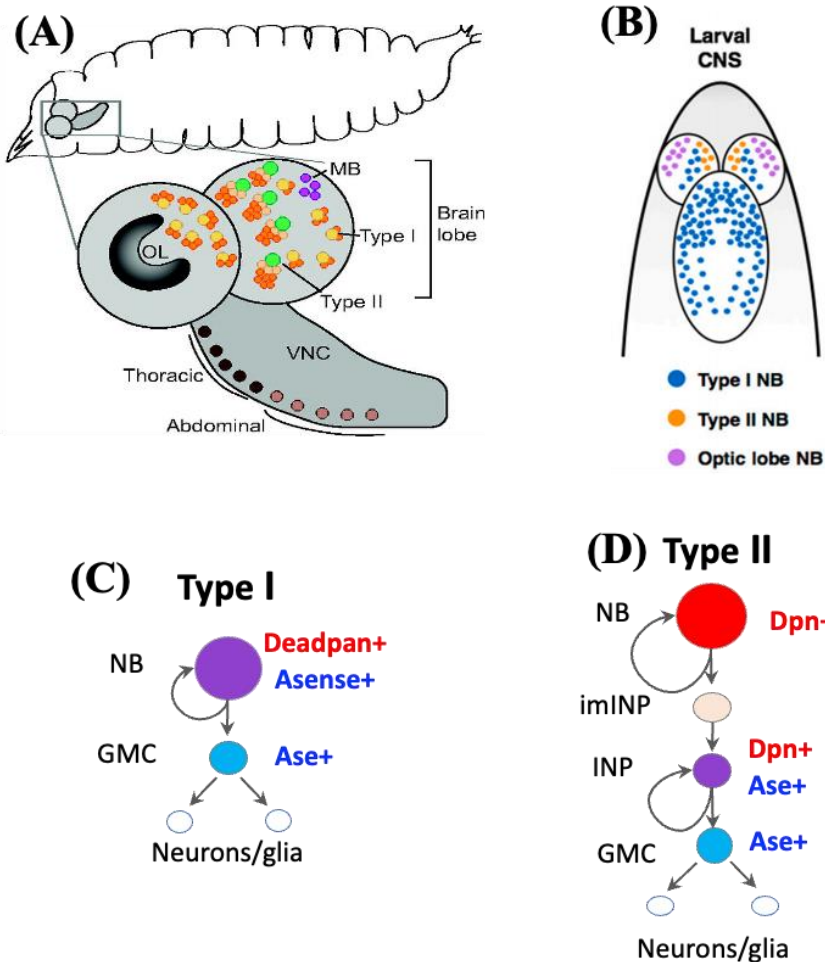


Figure 2: *Drosophila melanogaster* larval brain. (A) Modified from Homem & Knoblich (2012). The larval *Drosophila* brain contains two brain lobes and a ventral nerve cord (VNC). (B) Modified from Harding and White (2018). The different types of *Drosophila* neuroblast (NB) lineages found in the developing fly brain-- Type I, Type II, Optic lobe (OL) and mushroom bodies (MB)-- are located in distinct regions throughout the larval CNS. (C) Representative cartoon of a Type I NB lineage. The neuroblast is marked by Deadpan (**Dpn**) and Ase (Ase) proteins. The NB produces a ganglion mother cell (GMC) that expresses Ase and terminally divides into neurons or glial cells. (D) Representative cartoon of a Type II NB lineages. The Type II NB is marked only by Dpn. It divides to self-renew and generate an immature intermediate neural progenitor (**imINP**), which once matured (**INP**), are marked by both Dpn and Ase and retain the ability to self-renew. This amplification step allows the Type II NB lineage to produce five times the number of cells than Type I NB lineages.

RESULTS

Whole Brain Knockdown of *SERCA* results in decreased overall brain volume.

To uncover the role *SERCA* pumps in neural development, the GAL4-UAS system of *Drosophila* gene expression was utilized. This binary expression system was used to drive RNA interference molecules (RNAi) to effectively degrade *SERCA* mRNA, resulting in reduced protein expression. Specific knockdown of *SERCA* within Type I and Type II neuroblast lineages was accomplished using the *inscuteable-GAL4* (*insc-GAL4*) driver. It was observed that RNAi knockdown of *SERCA* within these lineages resulted in reduced volume throughout the larval brain (FIGURE 3 (A-E)). Reduced proliferation could be one explanation for the decrease in brain size. To test this possibility, brains were stained with the proliferation marker phosphohistone 3 (pH3), which labels histones during mitosis. The

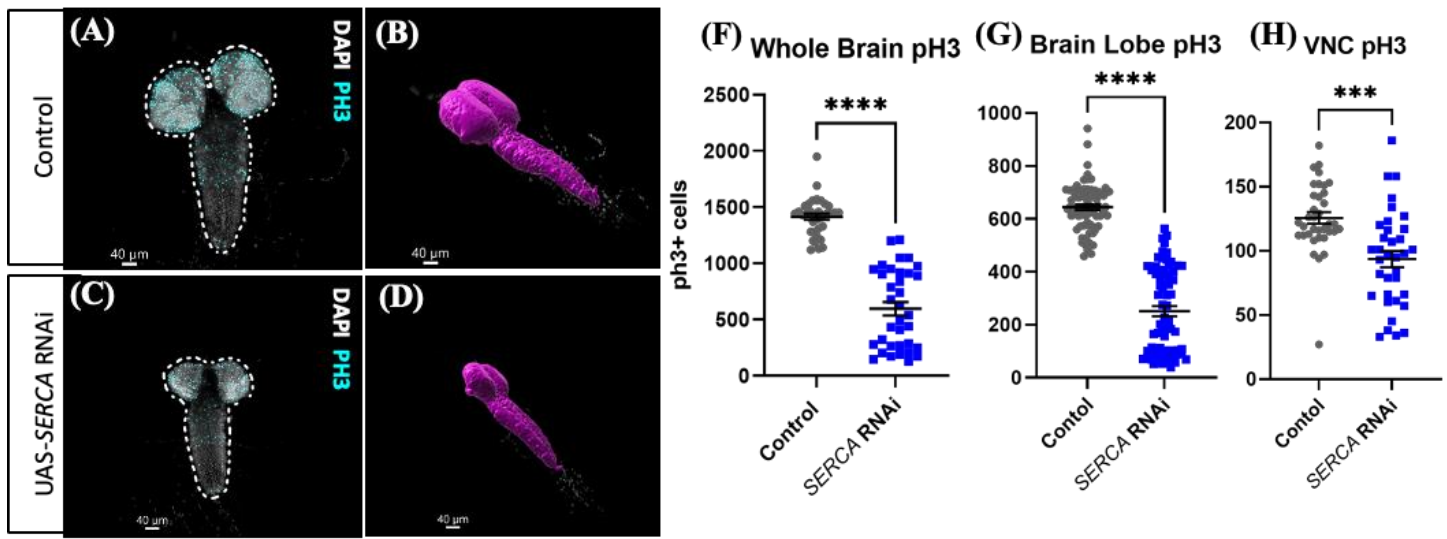


Figure 3: Whole brain *SERCA* RNAi knockdowns showed a decrease in volume and proliferation. Representative images of control (A-B) and *SERCA* RNAi knockdowns (C-D) of third instar larval brains (labelled by DAPI and phosphohistone 3 (pH3) (A, C) and volume rendering of DAPI (B, D)). (E) Comparison of volumes between the control and *SERCA* RNAi knockdown brains displayed significant reduction in volume. (F-H) Quantification of total pH3+ cells throughout the whole brain (F), the brain lobes (G), and the VNC (H) showed decreased proliferation with the *SERCA* RNAi knockdown. Analyzed with parametric t tests. Error bars denote S.E.M. Control n \leq 36. *SERCA* RNAi n \leq 35. *** equals p \leq 0.001, **** equals p \leq 0.0001.

SERCA knockdown brains did display a significant decrease in proliferation. Interestingly, while there is a distinct and visible reduction in the volume and proliferation of the brain lobes of the knockdown, the VNC—while still experiencing decreases in size and dividing cell numbers—does not appear to be as affected as the lobes (**FIGURE 3 (F-H)**). This implicates that *SERCA* channels may have a larger role within the Type II neuroblast lineages (found only in the brain lobes), being localized more specifically within the central cortex of the brain, or alternatively, it may result from the fact that there are more mitotically active cells in the brain lobes compared to the VNC.

***SERCA* knockdown in Type II Neuroblasts caused decreased proliferation and neuroblast identity change.**

To obtain a finer cellular resolution of *SERCA*'s influence on neurogenesis, I restricted *SERCA* RNAi knockdown to the Type II NB lineages by utilization of *Asense*-GAL80 (*Ase-GAL80*). The GAL80 system blocks the GAL4 driver in the specified tissues. In this case, it prevents the knockdown of *SERCA* in neuroblasts (NBs) expressing *Asense*, which would be Type I. Thus, the knockdown occurs only in Type II neuroblast lineages. The purpose of looking only at Type II neuroblasts is three-fold: 1) there are only eight Type II neuroblast lineages in defined and distinct areas, 2) each lineage is easily located, 3) the cell amplifying step of the INPs within the Type II neuroblast correlates closest to the development of the human brain cortex, which also utilizes amplifying progenitor steps.

The knockdown of *SERCA* pumps within the Type II NB lineage resulted in a marked decrease in cell number per lineage (**FIGURE 4 (A-I)**). In addition, all progenitor cell types within the lineage were reduced (**Figure 4 (K)**). The proportional makeup of each cell type compared between control and *SERCA* knockdown lineages is displayed in **FIGURE 4 (L)**. Consistent with whole brain data, a reduction in proliferating cells was also observed when *SERCA* was knocked down in the Type II NB lineage (**FIGURE 4 (J)**).

To better understand the nature of the proliferation deficit, pH3 staining was used to investigate Type II NBs throughout the mitotic stages. In both genotypes, the majority of NBs were in interphase, and the relative proportion of neuroblasts seen in the different mitotic stages (prophase, metaphase, anaphase, and telophase/cytokinesis) was similar between the control and the *SERCA* RNAi knockdown. There was a difference, however, in NB volumes within the stages. It was seen that in the *SERCA* knockdown brains, NBs going through prophase were smaller than the average control prophase volume (**Figure 5 (B-C)**) and NBs in anaphase were larger than the average control anaphase stage (**FIGURE 5 (D-E)**). The reason for alterations in *SERCA* mutant mitotic volumes is unclear. It's known that during mitosis, cellular swelling occurs and can be driven by Na^+/H^+ exchangers (Son et al., 2015; Zlotek-Zlotkiewicz et al., 2015). Our results indicate that calcium-dependent processes may also mediate

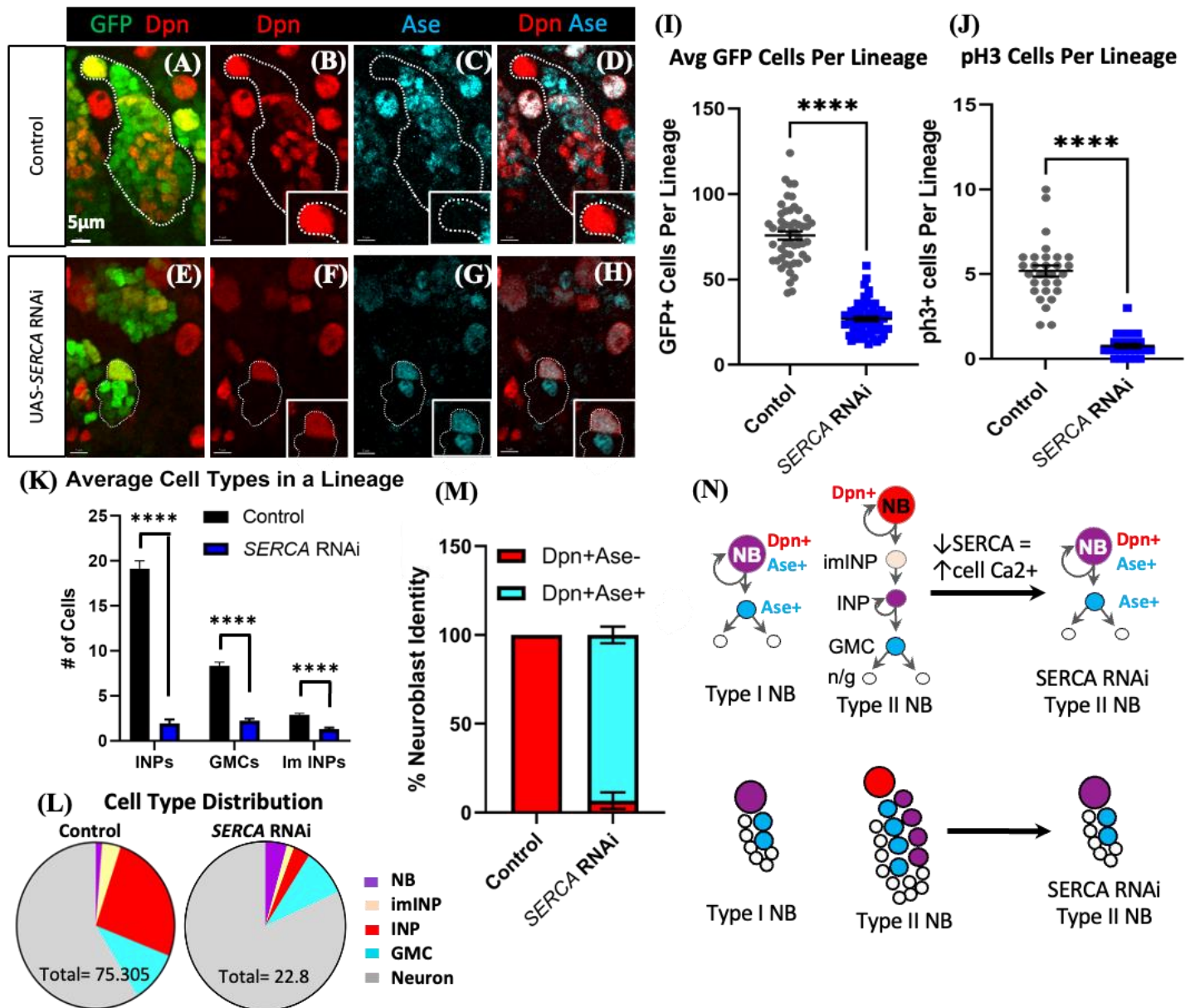


Figure 4: Knockdown of *SERCA* in Type II Neuroblast lineages reduced proliferation and induced cell fate changes. Representative images of control *Drosophila* Type II Neuroblast (NB) lineages (**A-D**), and *SERCA* RNAi knockdown within Type II NB lineages (**E-H**), each stained with GFP (green), Deadpan (Dpn, red), and Asense (Ase, blue). A visible reduction in lineage size and cell number between the control and knockdown images was observed. There was a notable change in Type II NB marker expression, with only Dpn expressed in the control (**D**) while Dpn and Ase were expressed in the *SERCA* knockdown Type II NB (**H**), suggesting a shift in Type II NB identity to a “Type I-like” NB. **(I)** The average GFP cells per lineage was significantly decreased upon *SERCA* knockdown compared to control. **(J)** Quantification of proliferation using phosphohistone 3 (pH3) depicts reduced cell division in the *SERCA* knockdown Type II NB lineage. **(K)** Quantification of average cell types in a lineage demonstrated a reduction in progenitor cell numbers in the *SERCA* RNAi knockdown Type II NB lineages. **(L)** Cell type distribution graphs show that the cellular makeup of a control Type II NB lineage is half neuron/glia cells, with the next most abundant cell type being intermediate neural progenitors (INPs). The cellular makeup of the *SERCA* RNAi knockdown Type II NB lineage is over 75% neuron/glia cells, with the next most abundant cell type being ganglion mother cells (GMC). Overall, **(K-L)** show that there is decreased numbers of each cell type in the lineage in the *SERCA* RNAi knockdown. **(M)** Quantification of neuroblast identity between the control and the knockdown depict a distinct difference in neuroblast staining and identity. **(N)** Model for *SERCA* knockdown, including cartoon representation of NB lineages. Analyzed with parametric t tests. Error bars denote S.E.M. Control $n \leq 52$. *SERCA* RNAi $n \leq 59$. *** equals $p \leq 0.001$, **** equals $p \leq 0.0001$.

cellular swelling. Other possibilities for the altered size could include incomplete mitosis, resulting in aneuploidy.

One of the most profound and unexpected findings was the observed change in neuroblast identity. Type II neuroblasts are Dpn^+ and Ase^- ; yet, upon staining with Ase , it is seen that a majority of NBs within the *SERCA* knockdown brains are Dpn^+ and Ase^+ , as seen in Type I neuroblasts (**FIGURE 4 (M)**). Additionally, the reduction in overall cell numbers and the distinct decrease of INP cells within the lineage correspond visually to a Type I lineage, supporting the idea that these cell lineages have changed identity (**Figure 4 (N)**). *SERCA* pumps function normally to reduce cytoplasmic Ca^{2+} concentrations; thus, we would anticipate that reduction of *SERCA* would increase baseline cytoplasmic Ca^{2+} levels. In other body systems, Ca^{2+} signaling can drive transcription, leading to fate changes (Snoeck, 2020). Therefore, one plausible mechanism could be that elevated Ca^{2+} concentrations from the *SERCA* knockdown activated transcriptional changes, driving the shift in identity from Type II NB to Type I NB lineage. To specifically investigate the potential role of Ca^{2+} concentrations in neuroblast lineage differentiation, we designed experiments to examine baseline Ca^{2+} dynamics.

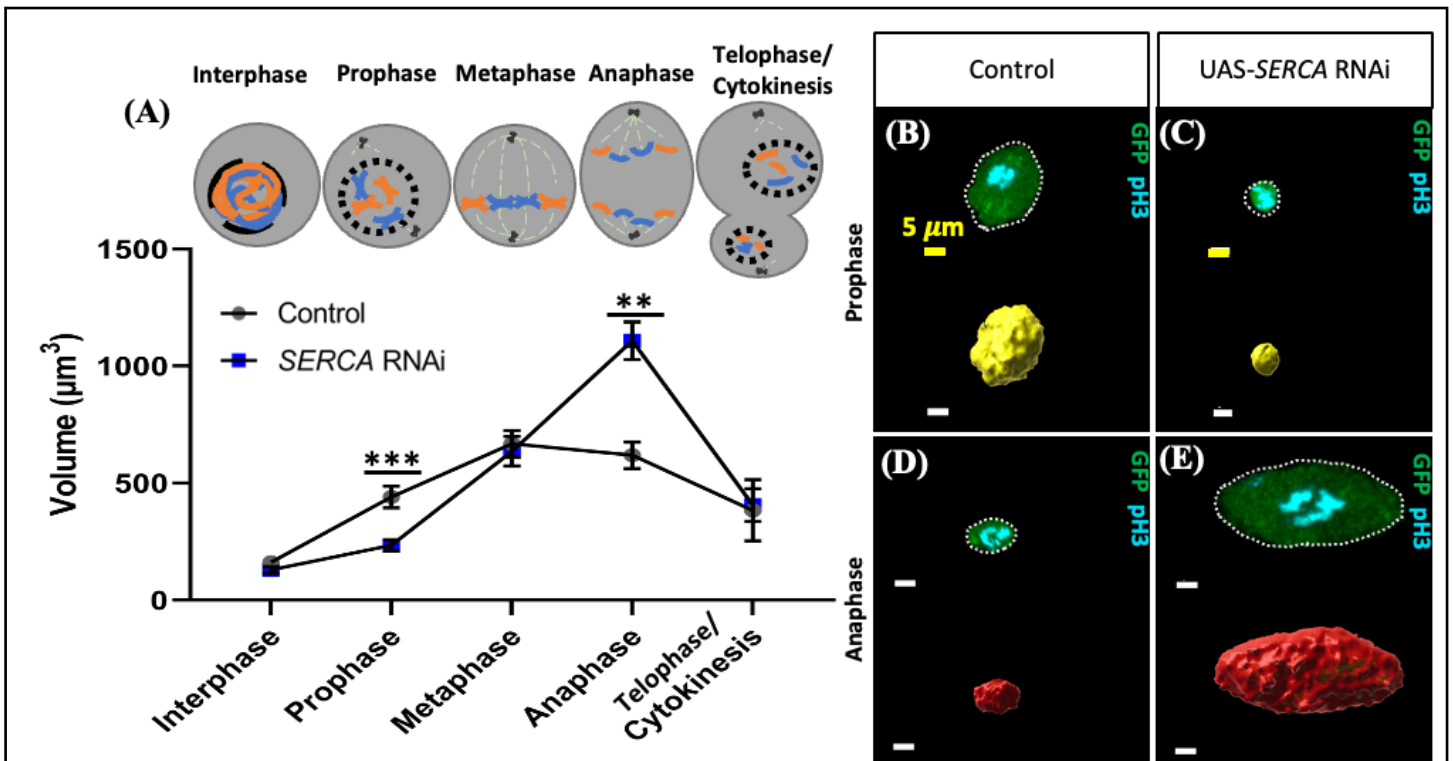


Figure 5: *Drosophila* Neuroblast volumes through the mitotic stages. (A) Neuroblast (NB) volume changes throughout mitosis were seen to differ between Type II NB control and the *SERCA* RNAi knockdown at two points in the cell cycle: prophase and anaphase. Representative images of control (B) and *SERCA* knockdown (C) NBs (stained with GFP and pH3) in prophase show decreased volume upon *SERCA* RNAi knockdown. Representative images of control (D) and *SERCA* knockdown (E) NBs (stained with GFP and pH3) in anaphase depict increased cellular volume in the *SERCA* RNAi knockdown. Analyzed with parametric t tests. Error bars denote S.E.M. All mitotic stages $n \leq 2$. ** equals $p \leq 0.01$. *** equals $p \leq 0.001$.

GCaMP Experiments

The results of our previous experiments implicated intracellular Ca^{2+} ion concentrations as a driver of cell fate; thus, it became pertinent to look for differences in baseline calcium levels between the stem cell and its differentiated cells. To explore this, a genetically encoded calcium sensor, GCaMP, was driven in the Type II NB lineages of the larval brain. GCaMP is a fusion of GFP to calcium-binding protein calmodulin (CaM). Increases in cytoplasmic Ca^{2+} concentrations will cause binding to the CaM domain of GCaMP, resulting in a conformational change that effectively increases GFP fluorescence. The fluorescence intensity generally reflects the concentration of Ca^{2+} ions present.

Comparison of cells within the Type II NB lineage showed a distinct difference in cytoplasmic calcium concentrations, with the NBs displaying much lower fluorescence levels in comparison to the progenitors and the terminally differentiated neural cells (**FIGURE 6 (B-C, E-F)**). We also separately examined fluorescence intensity of GFP driven by the *insc-GAL4* NB promoter. This acted as an expression control, allowing us to determine if the observed trend in fluorescence intensity was specific to Ca^{2+} levels and not merely a reflection of GCaMP expression throughout the NB lineage. Quantification of fluorescence levels in the GFP control showed high GFP activity throughout the lineage, with the highest levels of fluorescence localized in the neuroblast itself and a progressive decline in fluorescence with lineage differentiation and distance from the neuroblast (**Figure 6 (A-B, G)**). This is in direct opposition of the GCaMP control, in which the lowest fluorescence levels are in the neuroblast. Altogether, this indicated that the GCaMP calcium sensor fluoresced appropriately and with specificity to Ca^{2+} ions.

Because SERCA transports intracellular calcium into the ER lumen, we predicted that knockdown of *SERCA* in NB lineages should increase intracellular calcium levels. RNAi knockdown of *SERCA* in this GCaMP experiment indeed resulted in increased GFP fluorescence throughout the lineage; however, it still maintained the proportional “lower” brightness in the NBs and “greater” brightness in the differentiated lineage (**FIGURE 6 (D)**). This experiment confirms that there is increased Ca^{2+} ion levels within the cytoplasm upon knockdown of SERCA channels. It also provides further evidence that calcium plays a key role in developmental processes correlated with cellular identity.

Knockdown of *SERCA* in a tumor model decreased in tumor mass.

Cancer cells frequently hijack developmental pathways to increase proliferation, making it a useful tool for studying “stem-cell like” behavior in a highly proliferating model. Additionally, previous studies have proposed cancer cells upregulate SERCA pumps to combat Ca^{2+} -mediated cellular apoptosis. Therefore, we used a neuroblast-derived brain tumor model to observe the role of *SERCA* in this heightened proliferative tumor state.

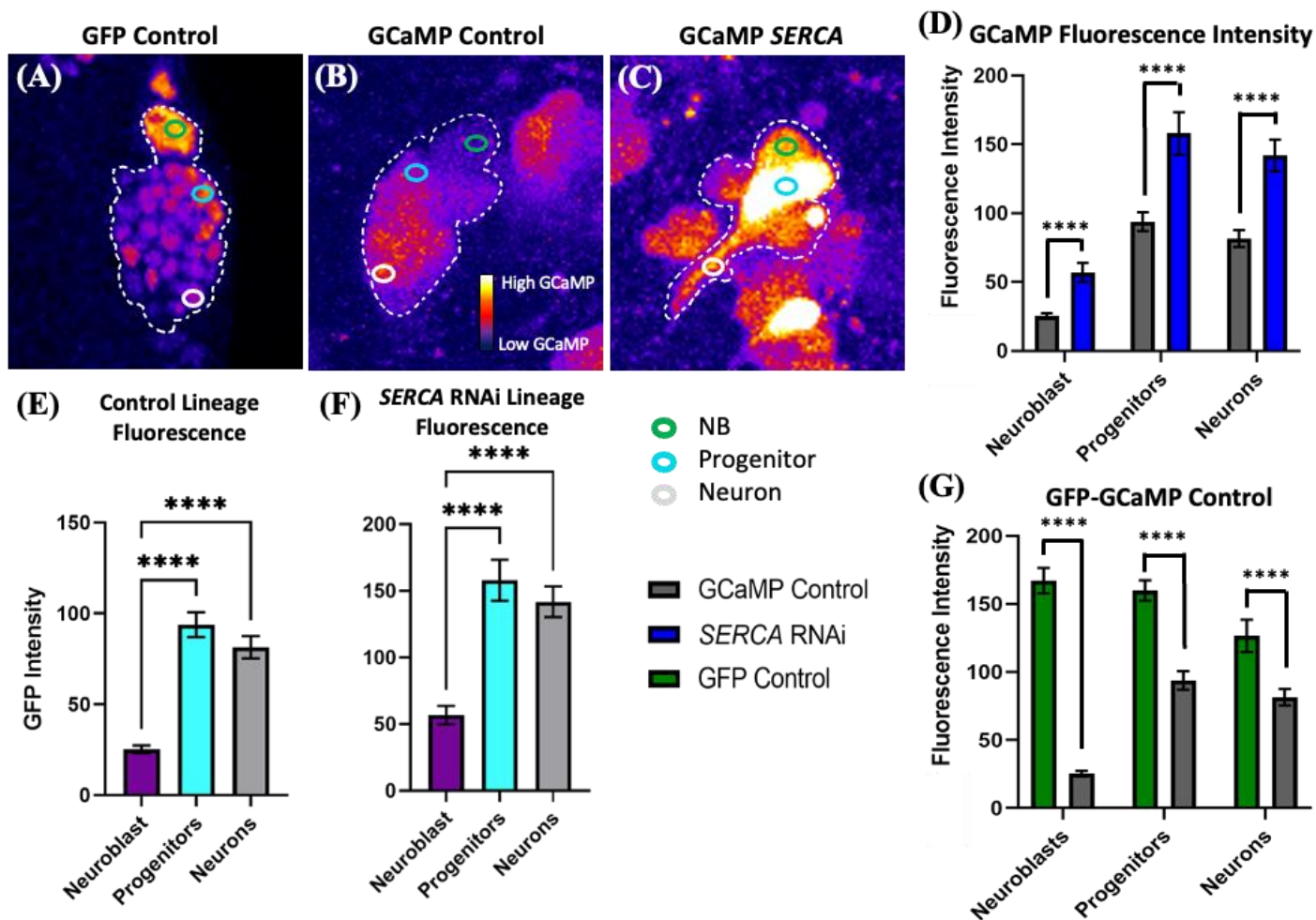


Figure 6: Use of GCaMP Ca^{2+} sensor displayed higher levels of cytoplasmic Ca^{2+} in the Type II Neuroblast when *SERCA* is reduced. (A-C) Representative images of *Drosophila* Type II NB lineages. The black circle denotes the neuroblast (NB), the teal blue circle denotes progenitor cells, and the gray circle denotes neurons. Brighter areas depict greater cytoplasmic Ca^{2+} concentrations. (A) GFP fluorescence intensity control as a readout of promoter driven (*insc-GAL4; ase-GAL80*) expression level throughout Type II NB lineage. (B) GCaMP activity in control NB lineages increased with differentiation, indicating that more differentiated cells have more cytoplasmic Ca^{2+} ions. (C) *SERCA* RNAi knockdown lineages displayed higher Ca^{2+} levels compared to control, quantified in (D). (E-F) Fluorescence was lowest in the neuroblast but increased with differentiation of cells in both the control (E) and the *SERCA* knockdown (F), although there were overall higher intensities in the knockdown lineage. (G) Quantification of the GFP Control versus the GCaMP Control showed that in the GFP Control, the highest GFP expression was in the NB and progressively decreased with differentiation. An opposite trend was observed in the GCaMP control where GCaMP fluorescence was low in the NB and progressively increased with differentiation. This supports the idea that GCaMP activity reflects baseline cytoplasmic calcium levels. Analyzed with parametric t tests. Error bars denote S.E.M. GFP Control $n \leq 19$. GCaMP Control $n \leq 34$. *SERCA* RNAi knockdown $n \leq 25$. **** equals $p \leq 0.0001$.

The tumor model was created via ectopic overexpression (OE) of the bHLH transcriptional repressor Deadpan (Dpn), the protein that enables self-renewal of neuroblasts during asymmetric division (Figure 7 (A)) (San-Juan & Baonza, 2011; Piggott et al., 2019). Ectopic overexpression of Dpn in all neuroblasts and their progeny results in excessive NB proliferation at the expense of neurons. The Dpn^{OE} tumor model, filled with “NB-like cancer stem cells”, increased in size compared to non-tumor larval brains. Knockdown of the *SERCA* gene in the tumor model caused overall reductions in brain volume (FIGURE 7 (B-L)), as well as decreased tumor proliferation. These results show that SERCA channels guide important developmental processes and could also be a therapeutic target for aberrant growth of various cancers.

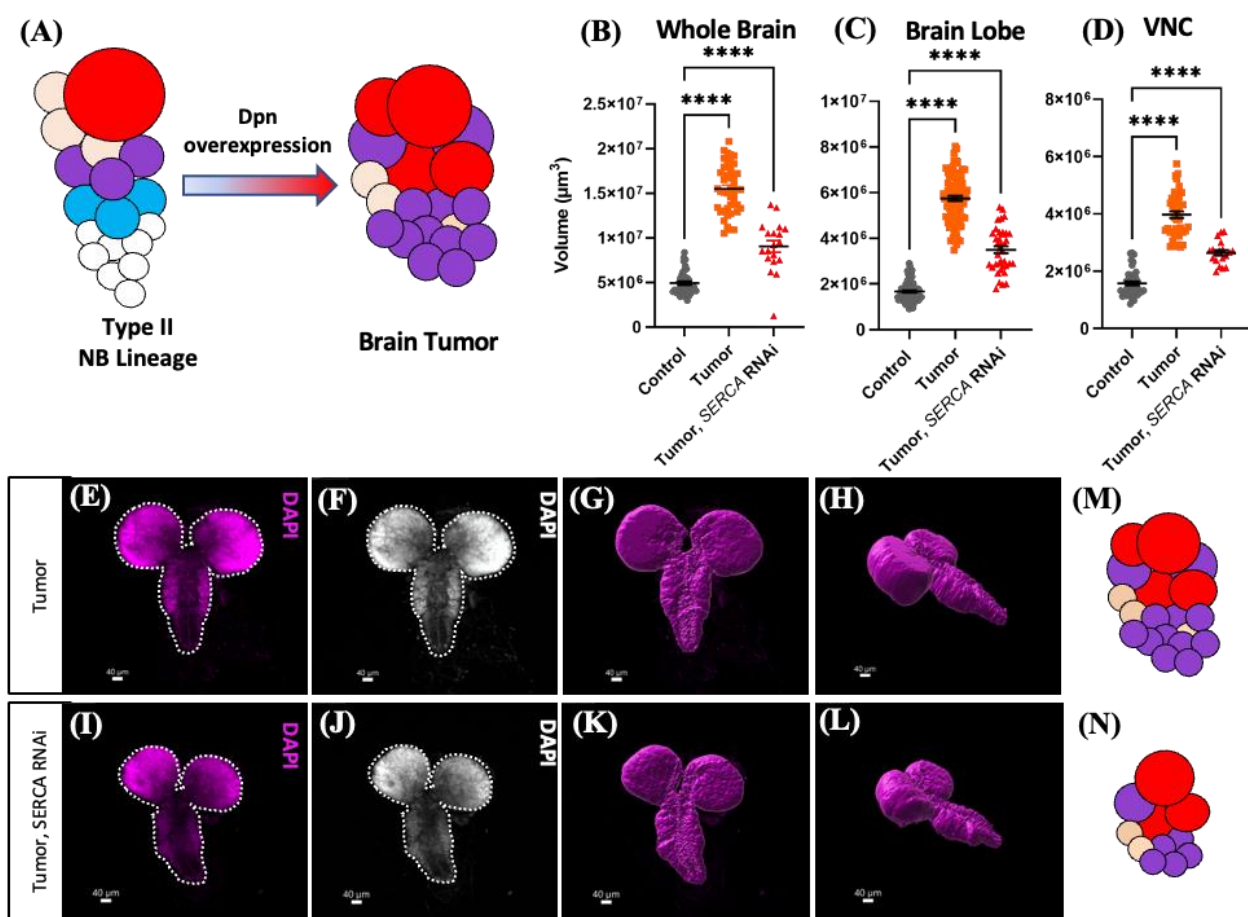


Figure 7: *SERCA* RNAi knockdown in a neuroblast-derived brain tumor model resulted in decreased brain volume. (A) Cartoon depiction of brain tumor creation via ectopic overexpression of Deadpan (Dpn), which normally enables self-renewal of stem cells in asymmetric division but drives uncontrolled proliferation and tumor formation when overexpressed throughout the NB lineage. Knockdown of *SERCA* in the tumor model significantly decreased volume of the (B) whole brain, (C) brain lobes, and (D) ventral nerve cord (VNC). Representative images display a visual size comparison between the tumor model (E-H) and the *SERCA* RNAi knockdown in the tumor model (I-L), stained with DAPI (E, F, I, J). (M) and (N) depict cartoon representations of a Type II NB lineage for both the tumor model and the *SERCA* RNAi knockdown within the tumor model, simulating the decrease in size and cell number resulting from the knockdown. This reflected fewer tumor-like cells in the *SERCA* knockdown. Analyzed with parametric t tests. Error bars denote S.E.M. Control n ≤ 43. *SERCA* RNAi knockdown n ≤ 19. **** equals p ≤ 0.0001.

DISCUSSION

Ca^{2+} ions are powerful second messengers, with vital roles in cell survival, signaling, and function. There is increasing evidence that Ca^{2+} concentrations play a critical part in development, directing major processes such as proliferation, migration, and cell fate (Bower et al., 2017; Deng et al., 2015; Snoek, 2020). While low cytoplasmic Ca^{2+} concentrations are rigidly maintained by ion channels and pumps, issues in proper regulation of cellular Ca^{2+} levels throughout the developmental process have been linked to neurological disorders like autism and seizures (Boczek et al., 2021). Prior studies have examined the role of Ca^{2+} ions in the development of various cell types (i.e., *Drosophila* ISCs (Deng et al., 2015) and tracheal cells (Bower et al, 2017), as well as human hematopoietic (Snoek, 2020) and epidermal cells (Schmieder & Rosario-Collazo, 2022)). However, calcium-based developmental activities may be specialized, as calcium dynamics appear to vary between organ systems. This highlights a necessity for understanding calcium signaling and its function specifically in brain development.

The goal of our research was to better understand the role of Ca^{2+} concentrations in neurogenesis. To complete this, we used the model system *Drosophila melanogaster* to manipulate a prominent Ca^{2+} ion pump—SERCA—that is conserved between invertebrates and humans. Reduction of SERCA in the fly larval brain was accomplished through RNAi knockdown. This genetic manipulation allowed us to observe how developmental outcomes are influenced by dysregulation of Ca^{2+} levels. Our work has revealed that SERCA regulates Ca^{2+} concentrations, enabling control of critical aspects of neural development such as proliferation and cellular identity.

Firstly, we discovered that a reduction in SERCA resulted in decreased total volume of the larval brain (**Figure 3 (A-E)**) and reduced cell numbers in the Type II NB lineage (**Figure 4 (A-I, K-L)**). It is known that dysregulation of *SERCA* causes ER stress and activates the Unfolded Protein Response (UPR), an event that can lead to cell apoptosis (Guerrero-Hernández et al., 2020; Park et al., 2018). One idea is that the knockdown of *SERCA* caused apoptosis, resulting in the observed decreases in brain size and cell numbers. However, further investigation of the reduction of SERCA pumps in the Type II NB lineage revealed another potential source of decreased cell numbers. In this experiment, the knockdown of *SERCA* caused the neuroblasts, which are typically marked by Dpn only, to express both Dpn and Ase proteins (**Figure 4 (G-H, M)**). This indicated a change in cellular identity. Type I NB lineages, which produce five times fewer cells than the Type II NB lineage, due to lack of the transit-amplification step, have neuroblasts that express both Dpn and Ase. The idea that changes in Ca^{2+} concentration (due to the knockdown of *SERCA*) transformed the Type II NB into a “Type I-like” NB, which then resulted in decreased cell numbers, is supported by further analysis. Type I neuroblasts produce GMC cells, which then terminally divide into neuron/glia cells. Type II neuroblasts produce imINP cells, which then mature into self-renewing INPs that produce GMCs. Quantification of progenitor numbers showed that in the

SERCA knockdown lineages, there was a significant reduction of imINPs and INPs, causing the proportion of GMCs in the lineage to be much greater than the proportion of GMCs in the control Type II NB lineage (**Figure 4 (K-L)**). This cell type analysis showed that the Type II NB lineages with reduced *SERCA* expression more closely resembled Type I NB lineages.

Previous work finds that in many cell types, increases in Ca^{2+} concentrations are associated with further differentiation of cells (Ho et al., 2021). Additionally, due to the plethora of calcium-binding partners, Ca^{2+} ions are often co-factors for the transcription of cell-specific proteins. Therefore, in other systems, increased cytoplasmic calcium concentrations were sufficient to drive cellular differentiation. In order to apply this hypothesis to our findings, we investigated baseline calcium levels between stem cells and their progeny. Use of the genetically encoded calcium sensor GCaMP in the Type II NB lineage displayed distinct differences in cytoplasmic Ca^{2+} concentrations. The neuroblasts had significantly lower fluorescence levels compared to more differentiated neural progenitors and the terminally differentiated neurons (**Figure 6 (B-C, E-F)**). This supports the idea that Ca^{2+} levels increase with further differentiation, implying that manipulation of stem cells to have greater cytoplasmic Ca^{2+} concentrations can drive cell changes and differentiation of the stem cell. RNAi knockdown of *SERCA* in this GCaMP experiment resulted in a substantial increase in fluorescence throughout the lineage, meaning there was much greater levels of cytoplasmic Ca^{2+} ions, which correlates to further differentiation (**Figure 6 (B-D)**). Interestingly, the cellular fate transition of the *SERCA* knockdown from Type II NB to a Type I NB fate could represent a type of “neuroblast differentiation.” Type I NBs express both *Ase* and *Dpn* cell markers, as do INP cells in the Type II NB lineage. INPs are more differentiated daughter cells from the Type II NB (**Figure 2 (D)**). In future experiments, it will be fascinating to investigate if Type I NBs display lower Ca^{2+} levels than the Type II NBs, and/or similar levels to the INP cells. The results gathered in these experiments provides evidence that calcium concentrations may be directly linked to differentiation during neurogenesis.

Our experiments additionally revealed a role for *SERCA* pumps in mitosis, as RNAi knockdown decreased proliferation throughout the whole larval brain (**Figure 3 (F-H)**) and within the Type II NB lineage (**Figure 4 (J)**). We anticipate that *SERCA* broadly influences proliferation throughout the NB lineages, although future experiments will need to be performed to determine if this is universally true. Proliferation was measured using the marker phosphohistone 3 (pH3), which labels histones during mitosis. It is known that cells can arrest mitosis at a series of checkpoints should certain cellular parameters not be met—size, nutrition, DNA integrity, molecular signals, etc. Often, “unhealthy” cells do not complete the cell cycle and are arrested at one of the checkpoints indefinitely until improvement or cell death. One potential mechanism of the observed reduced proliferation is that ER stress and the UPR (triggered by the knockdown of *SERCA*) arrested mitosis and are preventing proliferation. Studies show

that these cellular responses do often stop the cell cycle at the G2/M checkpoint of interphase (Lee et al., 2019). Researchers Simon and Moran (2001) also found that total loss of SERCA pump function via inhibition by Thapsigargin (Tg) in human embryonic kidney cells completely arrested cells at the G1/S checkpoint.

To better investigate this, analysis was completed looking at the Type II NB volumes throughout the mitotic stages. The control NBs showed—as expected—a parabolic graph, with natural increases in volume from interphase to anaphase and natural decreases in volume from anaphase to cytokinesis. The NBs in the *SERCA* RNAi knockdown of the Type II lineage were quite distinct, however, at two points: prophase and anaphase. It was observed that in the *SERCA* knockdown brains, the NBs in prophase were significantly smaller than the average control NB prophase volumes (**Figure 5 (A-C)**). Additionally, the NBs in anaphase were significantly larger than the average control NB anaphase volumes (**Figure 5 (A, D-E)**). These changes caused a much more dramatic volume graph—almost sinusoidal. While the differences in mitosis volume between the *SERCA* knockdown and control were striking, particularly during anaphase, the proportion of NBs in the various stages of the cell cycle was similar. If cell cycle arrest was occurring, we would anticipate that a greater proportion of *SERCA* knockdown NB would reside in a stage of proliferation blockage. The fact that this didn't occur suggests that cell cycle arrest in the neural stem cells is not the primary contributor to reduced proliferation. Alternative explanations for reduced proliferation could include reduced cell cycle speed or the susceptibility of daughter cells to apoptosis following NB division. Further experiments will explore these potential possibilities.

These findings draw inquiries into potential changes in ionic volume control due to higher concentrations of Ca^{2+} ions in the cytoplasm. Not much is known about CALX, a *Drosophila* $\text{Na}^+/\text{Ca}^{2+}$ exchanger, but current research is being conducted to explore the possibility of the protein's involvement in cellular volume control. Additional investigation into its function throughout the process of mitosis may reveal more about its potential effects of calcium on cell size during division. Another avenue to consider is the possibility of incomplete mitosis and aneuploidy. Calcium is known to play a role in the spindle apparatus. Calmodulin (CAM), a calcium-binding protein, has been shown to regulate the *Drosophila* protein called abnormal spindle (ASP) in embryonic neuroblasts. Together, these proteins control pole focusing, centrosome attachment, and microtubule crosslinking—important steps in cell orientation and polarity, as well as controlled chromosomal separation (Schoborg et al., 2015). Mutation and dysregulation of ASP is linked to the disease microencephaly, a condition that results in reduced head and brain size (Bond et al., 2002).

Finally, we studied the effect of *SERCA* knockdown in a brain tumor model. Cancer cells can act in a “stem cell-like” manner, repeatedly dividing without differentiation. Because of this, it's not surprising that these cells often use developmental pathways to increase their proliferation. Utilization of

a neuroblast-derived brain tumor model showed us that the RNAi knockdown of *SERCA* throughout the brain tumor model reduced brain volume size and decreased tumor cell proliferation (**Figure 7 (B-L)**). This is remarkably similar to what occurred in the non-tumor whole brain *SERCA* knockdown, showing the conservation of developmental pathways between stem cell division and cancer proliferation. Previous studies have identified *SERCA* pumps as a frequently repurposed or “hijacked” protein that cancer cells use to combat Ca^{2+} -mediated cellular apoptosis, circumventing both intrinsic and extrinsic anti-cancer mechanisms (Park et al., 2018). Studies conducted utilizing chemical inhibition of *SERCA* pumps with Thapsigargin (Tg), have found that blocking *SERCA* activity decreases tumor size as well, corroborating our findings (Chemaly et al., 2018; Denmead & John, 2004; Sehgal et al., 2017). These results emphasized the importance of cytoplasmic calcium regulation in development and survival, as well as implicated *SERCA* as a potential mechanism for controlling development—especially as a therapeutic target for “stem-cell like” tumor growth.

This study identified a role for Ca^{2+} -ATPase *SERCA* in both neural development and cancer. It has also shown the importance of calcium regulation within the cell, uncovering the novel finding that changes in cytoplasmic Ca^{2+} concentrations can drive cell fate during development. Further inquiry into the influence of calcium in the process of mitosis is necessary to fully comprehend the role of electrolytes in regulation of cell cycle dynamics. Examination of other Ca^{2+} ion regulators, such as *CALX*, and downstream Ca^{2+} signaling pathways will provide a more robust grasp of calcium’s influence on neurogenesis. Ion channels, exchangers, and pumps—like *SERCA*—are druggable targets; however, their roles in regulating proliferation and differentiation remains poorly understood. Achieving greater understanding of the bioelectric mechanisms of these developmental processes is knowledge that can be harnessed to treat various diseases. For example, ion channels that promote proliferation could be stimulated with drugs to facilitate regeneration of cells in treatment of developmental disorders. Conversely, blocking these proliferative channels could be instrumental in limiting aberrant cancer growth.

METHODS AND MATERIALS

***Drosophila melanogaster* stocks**

Developing Larval Whole Brain Experiments:

Control group created by crossing females from stock *yw, UAS-mCD8GFP; insc-GAL4, UAS-Dcr2/cyowEEP* with male *y1v1;; UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2) stock, selecting for progeny *yw, UAS-mCD8GFP; insc-GAL4, UAS-Dcr2; UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2).

Experimental group created by crossing females from stock *yw, UAS-mCD8GFP; insc-GAL4, UAS-Dcr2/cyowEEP* with male *yIsc*vIsev2I;; UAS-SERCA RNAi (HMS)* (BL44581 P{TRiP.HMS02878}attP2) stock, selecting for progeny *yw, UAS-mCD8GFP; insc-GAL4, UAS-Dcr2; UAS-SERCA RNAi (HMS)*.

Type II Neuroblast Experiments:

Control group created by crossing females from stock *w-; insc-GAL4, UAS-GFP^{NLS}/cyowEEP*; *Ase-GAL80/Tm6B, tb* with males from stock *yIvI;; UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2), selecting for progeny *w-; insc-GAL4, UAS-GFP^{NLS}; Ase-GAL80/UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2).

Experimental group created by crossing females from stock *w-; insc-GAL4, UAS-GFP^{NLS}/cyowEEP*; *Ase-GAL80/Tm6B, tb* with males from stock *yIsc*vIsev2I;; UAS-SERCA RNAi (HMS)* (BL44581 P{TRiP.HMS02878}attP2), selecting for progeny *w-; insc-GAL4, UAS-GFP^{NLS}; Ase-GAL80/UAS-SERCA RNAi (HMS)* (BL44581 P{TRiP.HMS02878}attP2).

GCaMP Experiments:

Control group created by crossing females from stock *w-; insc-GAL4/cyowEEP*; *Ase-GAL80/Tm6B* with males from stock *w-; UAS-Gcamp6f, attp40/cyowEEP*; *UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2), selecting for progeny *w-; insc-GAL4/UAS-Gcamp6f, attp40⁰; Ase-GAL80/UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2).

Experimental group created by crossing females from stock *w-; insc-GAL4/cyowEEP*; *Ase-GAL80/Tm6B* with males from stock *w-; UAS-Gcamp6f, attp40/cyowEEP*; *UAS-SERCA RNAi (HMS)* (BL44581 P{TRiP.HMS02878}attP2), selecting for progeny *w-; insc-GAL4/UAS-Gcamp6f, attp40; Ase-GAL80/UAS-SERCA RNAi (HMS)* (BL44581 P{TRiP.HMS02878}attP2).

Tumor Whole Brain Experiments

Control group created by crossing females from stock *w-, UAS-mCD8GFP; insc-GAL4/cyo,tb-GAL80; UAS-Dpn,UAS-Dcr2/Tm6B* with males from stock *yIvI;; UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2), selecting for progeny *w-, UAS-mCD8GFP; insc-GAL4/cyo,tb-GAL80; UAS-Dpn,UAS-Dcr2/UAS-Luciferase RNAi* (BL35788 P{UAS-LUC.VALIUM10}attP2).

Experimental group created by crossing females from stock *w-, UAS-mCD8GFP; insc-GAL4/cyo,tb-GAL80; UAS-Dpn,UAS-Dcr2/Tm6B* with males from stock *yIsc*vIsev2I;; UAS-SERCA RNAi (HMS)* (BL44581 P{TRiP.HMS02878}attP2), selecting for progeny *w-, UAS-mCD8GFP; insc-*

GAL4/cyo,tub-GAL80; UAS-Dpn,UAS-Dcr2/UAS-SERCA RNAi (HMS) (BL44581 P{TRiP.HMS02878}attP2).

Tissue Immunohistochemistry and microscopy

Third-instar larval brains were dissected, fixed, and stained similarly to methods described in Hafer & Schedl (2006). Primary antibodies: guinea pig anti-Ase (1:1000), rabbit anti-Dpn (1:500), chicken anti-GFP (1:400), and mouse anti-pH3 (1:200). Secondary antibodies: Alexa Fluor 488 (1:400), Alexa Fluor 565 (1:400), Alexa Fluor 640 (1:200), and DAPI (1:500). After staining, the brains were mounted on slides with vectashield and a coverslip was placed overtop, secured with clear nail polish. Imaging was completed using a Zeiss Confocal. Non-tumor and tumor whole brain images were taken with a 20x objective with 1- μ m stacks. Type II lineages were imaged with an 40x oil-objective with 1- μ m stacks. Analysis was completed using the Imaris program, and statistics were completed on GraphPad Prism.

Live GCaMP Recording

Third-instar larval brains were dissected similarly to methods described in Hafer & Schedl (2006). Brains were placed on slides with PBS solution and a coverslip overtop. Image stacks were taken using a Zeiss Confocal, using a 40x water-objective lens with 0.5- μ m stacks. Timed series videos were taken using the 40x water-objective lens, with 150 cycles of images taken at 1 second intervals (2.5 minutes of exposure). Analysis was completed using the FIJI (Image J) program, and statistics were completed on GraphPad Prism.

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