Alzheimer’s disease (AD), characterized by a progressive loss of cognitive function, is the most prevalent neurodegenerative disorder of aging. A substantial body of literature has documented evidence of cell cycle re-entry and an increase in DNA ploidy in cases of age-induced neurodegeneration and AD [1,2]. Research on human tissue samples has suggested that hyperploidy in neurons may precede clinical diagnosis of AD [3,4]. Thus, cell cycle re-entry and the resulting hyperploidy could be a proximal cause of age-related neurodegeneration, as well as a useful preclinical marker. Dissecting the precise biological significance of cell cycle re-entry in age-related neural decline will be critical to developing new approaches to combat AD.

Figure 1 (above). An aged adult Drosophila brain, showing glial-specific GFP expression and actin-selective fluorescent staining. This Drosophila brain was dissected from a 50-day-old fly (average lifespan of this strain is 45 days) that expressed nuclear-localized GFP in glial cells (green). The isolated brain was stained with rhodamine phalloidin [red, Cat. No. R415] to label actin and Hoechst™ 33258 (blue, Cat. No. H3369) to label DNA. Image provided by Laura Buttitta and Olga Grushko, University of Michigan.
Developing a model system to study cell cycle re-entry and aging

Extensive literature has reported that cell cycle genes become reactivated in aging brains, and that this reactivation is evolutionarily conserved among diverse species—from flies to humans [5–7]. It has been difficult, however, to observe and quantify cell cycle re-entry of postmitotic cells in the brain due to its rare and potentially transient nature [8,9]. Therefore, its frequency under normal physiological aging conditions has become a matter of debate [3,10–13]. Another barrier to progress in this field has been the difficulty in obtaining aged adult brain samples and monitoring cell cycle re-entry under normal physiological conditions. These challenges, combined with a limited ability to genetically manipulate factors influencing cell cycle re-entry in mammalian models, have hampered investigations.

Progress would be greatly facilitated by the availability of a genetically tractable system that recapitulates the features of age-associated decline in the brain under a rapid lifespan. The simple fruit fly *Drosophila melanogaster* is an attractive model system to study this process because it exhibits age-related neural decline [14], ages on the order of days instead of years, and has well-developed tools for *in vivo* genetic manipulations [15].

Our laboratory is developing genetic tools and fluorescence assays for manipulating cell cycle re-entry in the adult fly brain (Figure 1) and monitoring subsequent effects on DNA ploidy and cell death. Our long-term goal is to understand the contribution of cell cycle misregulation in the brain to age-related neural decline. We want to address several long-standing and important questions in the field, including:

- What causes cell cycle re-entry in the aging brain?
- How do cell cycle re-entry and misregulation affect cell loss and neurodegeneration?
- How does manipulation of cell cycle re-entry impact age-associated neural decline?

Assaying DNA content and cell death with flow cytometry

To begin to investigate these questions, we use a sensitive, high-throughput flow cytometry–based assay to monitor changes in cellular DNA content and cell death in the fly brain (Figure 2). Our method involves dissecting the adult fly brain, dissociating the tissue to a single-cell suspension using gentle trypsinization, and then staining live cells for DNA content with the Vybrant™ DyeCycle™ Violet Stain, a cell-permeant, UV/violet laser–excitable nucleic acid dye.

**Figure 2.** Experimental workflow for examining DNA ploidy and cell death in the aging adult *Drosophila* brain using acoustic focusing cytometry. **(A)** Individual adult fly brains are dissected; in this case, the strain expresses GFP in the nuclei of adult glial cells. **(B)** Cells from the isolated brain are dissociated in 100 µL of a PBS/trypsin solution containing Vybrant™ DyeCycle™ Violet Stain (Cat. No. V35003, 1 µL/mL) and propidium iodide (PI, Cat. No. P3566, 5 µL/mL); tissues are quickly triturated by pipetting in a 1.5 mL microcentrifuge tube cap and then transferred to a microcentrifuge tube containing 400 µL of additional PBS/trypsin/Vybrant™ DyeCycle™ Violet/PI solution and stained for 1 hr. After staining, samples are diluted with 500 µL of PBS and the microcentrifuge tube is placed in the tube holder and run on the Attune™ Acoustic Focusing Cytometer (with blue/violet lasers) in high-sensitivity mode. Doublets and cell clumps or debris are excluded by comparing DNA height and width (see [16] for details) and removed by gating. **(C)** A representative dot plot of cells from an aged adult fly brain showing GFP expression (in glial cells) and DNA content (as measured with Vybrant™ DyeCycle™ Violet Stain), with PI-positive dead and dying cells backgated in pink. Here, GFP-positive glial cells show limited hyperploidy but higher levels of cell death, while the non-glial GFP-negative cells (primarily neurons) show less cell death but slightly increased levels of hyperploidy.
Figure 3. Assessing hyperploidy and cell death in two different cell types of the fly brain. (A) Flies expressing GFP in glial cells were either left untreated (left panel) or induced to re-enter the cell cycle by overexpressing key cell cycle regulators (right panel). Isolated brains were assayed for DNA ploidy and cell death using the workflow described in Figure 2. GFP-expressing glia expressing cell cycle regulators show hyperploidy, including cells exhibiting 4C and 8C DNA content (red arrow). Dead and dying cells incorporate propidium iodide (PI, backgated in pink), and hyperploidy in glia is strongly correlated with PI positivity. (B) Flies expressing GFP in postmitotic neurons were either left untreated (left panels) or induced to re-enter the cell cycle by overexpressing 5-ethynyl-2’-deoxyuridine (EdU) into newly synthesized DNA with the Click-iT™ EdU Alexa Fluor™ 555 Imaging Kit (yellow arrows, yellow signal is due to overlap of GFP and Alexa Fluor™ 555 fluorescence; Cat. No. C10338). (C) Pigment-dispersing factor (PDF)–expressing postmitotic neurons (a subset of neurons influencing circadian rhythms in fly brain) were labeled with a mitochondrial-localized GFP and left untreated (left panel) or induced to re-enter the cell cycle by overexpressing key cell cycle regulators (right panel). Neurons that re-enter the cell cycle exhibit hallmarks associated with neurodegeneration, including loss of mitochondria and aberrant mitochondrial clumping (white arrows).

Genetically manipulating cell cycle regulators

We use the binary Gal4/UAS system to activate gene expression in specific cell types in the Drosophila brain [17]. To force cell cycle re-entry, we express a combination of cell cycle regulators—cyclin G1, cyclin D, its partner kinase Cdk4, and the cell cycle transcriptional activator E2F—which we have previously reported can reverse cell cycle exit in postmitotic tissues when overexpressed [18]. Thus, we can simultaneously activate cell cycle genes with the Gal4/UAS system, induce GFP or another fluorescent marker in a cell type–specific manner, and monitor the ploidy and death of subpopulations of cells in the adult fly brain.

Figure 3A shows a pair of GFP fluorescence vs. DNA content dot plots comparing control cells expressing GFP under a glial-specific promoter, to GFP-positive glial cells that have been forced to re-enter the cell cycle. The cell population forced to re-enter exhibits increased DNA hyperploidy, including 4C and 8C cells, which is also strongly correlated with PI positivity.

We can also force cell cycle re-entry in postmitotic neurons, such as those shown in Figure 3B, and observe hallmark changes in active cell cycling via incorporation of 5-ethynyl-2’-deoxyuridine (EdU) and subsequent detection using the Click-iT™ EdU Alexa Fluor™ 555 Imaging Kit. Forced cell cycle re-entry in postmitotic neurons leads to phenotypes consistent with neurodegeneration, such as mitochondrial clumping and loss [19] (Figure 3C).
Watch the JoVE video on cell cycle analysis of Drosophila tissues

Follow along with the protocol for “Live cell cycle analysis of Drosophila tissues using the Attune Acoustic Focusing Cytometer and Vybrant DyeCycle Violet DNA Stain” by Kerry Flegel, Dan Sun, Olga Grushko, Yiqin Ma, and Laura Buttitta (Molecular, Cellular, and Developmental Biology, University of Michigan) at jove.com/video/50239/live-cell-cycle-analysis-drosophila-tissues-using-attune-acoustic [a subscription to JoVE is required]. In this 11-minute video, the authors discuss fly dissection, tissue dissection, DNA staining, and subsequent analysis by acoustic focusing cytometry. Their cell cycle analysis protocol provides a method for determining relative cell size, cell number, and DNA content, as well as cell type via lineage tracing or cell type-specific fluorescent protein expression. This video has been viewed thousands of times by universities and research labs worldwide.

Laura Buttitta, PhD, is an assistant professor in the Department of Molecular, Cellular, and Developmental Biology at the University of Michigan. Her lab studies how the cell cycle is shut off in quiescent cells, such as differentiated or stem cells, using both Drosophila and mammalian cell systems. Olga Grushko, PhD, a research specialist at the University of Michigan, is studying the adult Drosophila brain, neuronal populations involved in feeding behaviors, and neurodegeneration. Correspondence can be sent to buttitta@umich.edu.

Future directions

While hyperploidy has been observed in aged human brains and in cases of AD, it remains unclear whether hyperploidy is restricted to neurons or glia, and which cell types are responsible for the neural decline. Glia play a critical support role for maintaining neuronal survival in the brain, and disruption of their quiescence may have huge impacts on the brain. Indeed, cell cycle re-entry in glia may lead to neurodegenerative phenotypes in Drosophila [20,21].

Using the adult fly brain as our model system, we can both assay and manipulate cell cycle re-entry in multiple cell types under physiological aging conditions. Our future investigations aim to establish a new model system for examining the role of cell cycle re-entry in age-related neural decline. If successful, our work will provide information about how aging impacts critical cell cycle controls in the brain, which may suggest novel approaches to combat age-related declines in cognitive function.

References


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