Investigating Cell Cycle Re-entry in the *Drosophila* brain: From the Pupa to the Adult

by

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Dedication

To my mother, who always says, “you should really write more!”

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मीनाक्षीं प्रणतोऽस्मि सन्ततमहं कारुण्यवारांनिधिम् ||

Table Of Contents

List of Tables

List of Figures

Abstract

G_0 associated with terminal differentiation represents the most common cellular state in adult multicellular organisms, yet it is poorly understood. In past years, various tissues of the fruit fly *Drosophila melanogaster* have served as a great model system to understand how cells establish and maintain their non-dividing state. While the *Drosophila* brain has been extensively studied in the context of neurodevelopment, relatively little is known about how the flexibility of cell cycle exit in terminally differentiated neurons and glia. In Chapter 2 of my dissertation, I show that postmitotic neurons and glia in the developing *Drosophila* pupa brain can be forced to re-enter the cell cycle and undergo mitosis after they have exited the cell cycle. Neurons can re-enter the cell cycle up to 24 hours after they have exited the cell cycle whereas glia exhibit greater flexibility and can undergo cell division up to over 48h after they exit the cell cycle. Forcing re-entry in neurons results in cell death, while glial cell division can result in tumor-like growths.

Neurons and glia are some of the longest lived cells in metazoans. How these cells deal with ageing-related damage is poorly understood. My work summarised in Chapter 3 shows that polyploid cells accumulate in the adult fly brain and that polyploidy protects against DNA damage-induced cell death. Multiple types of neurons and glia that are diploid at eclosion, become polyploid in the adult *Drosophila* brain. The optic lobes exhibit the highest levels of polyploidy, associated with an elevated DNA damage response in this brain region. Inducing oxidative stress or exogenous DNA damage leads to an earlier onset of polyploidy, and polyploid

cells in the adult brain are more resistant to DNA damage-induced cell death than diploid cells. Our results suggest polyploidy may serve a protective role for neurons and glia in adult *Drosophila melanogaster* brains.

Chapter 1. Introduction and Review of Literature

The proper development of all multicellular organisms requires coordinated cell proliferation, growth, tissue patterning at critical times and places. Equally critical for an organism's proper development is that cells stop proliferating at the times and places when they should. Not only is stopping proliferation development important for the function of cells, organs, and organ systems, it's compromise can lead to deleterious consequences such as uncontrolled proliferation, cell death, tumour formation etc.

Organisms have evolved conserved, intricate, and elegant systems to control and modulate proliferation and growth spatially and temporally. The process by which cells grow or proliferate is called the cell cycle. Various factors regulating the cell cycle, and its function have been very well studied over the last several decades. However, the mechanisms controlling exit from the cell cycle are less well understood (Buttitta and Edgar, 2007). Moreover, the prolonged maintenance of a non-dividing state is critical for the proper functioning of long lived cells in various tissues throughout the lifespan of an organism. The cells of the nervous system; neurons and glia, are some of the longest lived in many animal. It is known that maintaining a non-dividing state in these cells is critical for brain function (Aranda-Anzaldo and Dent, 2017; Frade and Ovejero-Benito, 2015; Ruggiero, 2012), yet how it is established and maintained in them is poorly understood. The work summarized in this dissertation seeks to obtain a better understanding of this.

My studies use the fruit fly, *Drosophila melanogaster* to study cell cycle exit in the brain. In the course of studying the adult fly brain, we discovered that some cells in adult and ageing fly brains re-enter the cell cycle and become polyploid. Before I introduce the cell cycle, cell cycle exit and re-entry, I will describe why the humble fly is a great model system in which to study this.

1.1. The Merits Of Using *Drosophila*

The reasons to use *Drosophila* to study basic cellular biology and physiology are manifold. Flies have been well studied as a genetic model organism for over one century, and as a result, their development has been catalogued in great detail (Ashburner,1976). Their entire genome is sequenced (Adams et al., 2000), and several tools for facile genetic manipulation have been developed over the years. Genes and proteins involved in important processes such as (but certainly not limited to) the cell cycle are well conserved between flies and humans, and what makes our studies of the cell cycle in flies even more appealing is the relative lack of redundancy. Where humans and mice have several proteins performing overlapping and similar functions, flies generally have only one or two. This makes genetic experiments easier.

Furthermore, sophisticated genetic tools in *Drosophila* developed over several decades allow us to manipulate gene expression in specific cells or tissues to understand their functions at *in vivo*. Flies are also inexpensive and easy to maintain in the laboratory. Of specific interest to my studies is the short lifespan of flies (compared to humans). This makes experiments that require ageing animals last a few weeks, rather than several months it may take to age rats or mice. Flies develop from egg to adult in a matter of approximately ten days and can live up to around two months as adults (Ashburner, 1976). All the above reasons make the fly a very attractive model organism.

The following section of this chapter will introduce the cell cycle, the main factors governing cell cycle control, and the basic cell cycle machinery. While the general structure of the cell cycle is well conserved across eukaryotes from yeast to plants, flies and mammals, this dissertation will focus mostly on the *Drosophila* cell cycle, and where applicable and/or necessary, specify the mammalian orthologues.

1.2. The Cell Cycle

The series of events governing cellular growth, duplication of the genome and cell division are termed the cell cycle, a process well conserved across eukaryotes. Progress through the cell cycle is intricately governed by various regulatory factors, and is orchestrated by phosphorylation events.

The cell cycle has four main phases: A first growth phase termed 'G1' , Replication or synthesis of DNA or 'S' phase, a second growth phase termed 'G2' and mitosis or 'M' phase which marks the division of the nucleus followed by cytokinesis which leads to complete cell division. The cell cycle is termed a 'cycle' because in actively dividing tissues or populations of cells, mitosis and cytokinesis is followed by the subsequent G1, S and so on. The core machinery of the cell cycle are protein complexes comprising cyclins and cyclin-dependent kinases (CDKs) which oscillate in expression as well as activity to ensure progression through the cell cycle. Progression through the cell cycle is robust and is tightly controlled at various regulatory levels.

1.2.1. **Transcriptional Control Of The Cell Cycle**

The synthesis of cell cycle genes at the proper levels and times during the cell cycle is controlled by the E2F family of transcription factor complexes (Duronio and O'Farrell, 1995; Dynlacht et al., 1994; Hiebert et al., 1992; Zheng et al., 1999). E2F factors can be either

transcriptional activators or repressors and are complexed in heterodimers with DP (Dimerization Partner) proteins(van den Heuvel and Dyson, 2008). Drosophila have one activating E2F (encoded by the gene dE2f1) (Ohtani and Nevins, 1994) and one repressive E2F (encoded by the gene dE2f2) as well as one DP (encoded by dDp) in contrast to nine mammalian homologues of E2F (encoded by eight genes) and three of DP (encoded by two genes) (Cayirlioglu et al., 2001; Dynlacht et al., 1994; Lammens et al., 2009; Ohtani and Nevins, 1994; van den Heuvel and Dyson, 2008) .

The E2F1/DP (simply referred to as E2F) complex is particularly important for the transcription of cyclins that regulate progression through G1, the transition from G1 to S phase, as well as key proteins necessary for the initiation of DNA replication (Duronio and O'Farrell, 1995; Dynlacht et al., 1994). The transcriptional activity of E2F complexes is negatively regulated by the retinoblastoma (RB) family of proteins (Rbf1 and Rbf2 in flies, pRb, p130 and p107 in mammals) (Bosco et al., 2001; Cayirlioglu et al., 2003; Dimova et al., 2003; Du and Dyson, 1999; Du et al., 1996; Hiebert et al., 1992). Unphosphorylated RB can bind to E2F1/DP dimers, repressing their transcriptional activity. The phosphorylation of RB in G1 and the G1 to S phase transition allows E2F dependent transcription cell cycle genes (Harbour et al., 1999; Narasimha et al., 2014). The phosphorylation mediated control of cell cycle phase transitions will be further introduced in later sections.

Some critical products of genes transcribed by E2F during G1 and S are Cyclin E, Cyclin A and CDK2, DNA polymerase alpha, the DNA clamp and processivity factor PCNA (Proliferating Cell Nuclear Antigen) (Duronio and O'Farrell, 1995; Herr et al., 2012). Later in the cell cycle, E2F dependent transcription is important for the expression of the kinases Cyclin B, CDK1 and Aurora A, Aurora B, polo and mad2 which are critical for mitosis(Lukas et al.,

1999; Ren et al., 2002). E2F complexes are responsible for the transcription of several hundred cell cycle-related genes at the right times during the cell cycle. The next layer of regulation, thus, is the activity of the proteins encoded by the cell cycle genes, which is controlled at the level of phosphorylation

1.2.2. **Kinases, Phosphatases And The Cell Cycle**

As discussed earlier, the core machinery of the cell cycle is governed by phosphorylation events driven by Cyclin and cyclin dependent kinase activity(Evans et al., 1983). Most cyclins and kinases act in obligate dimers (Morgan and Morgan, 2007). In *Drosophila*, progression through G_1 is marked by accumulation of Cyclin D and its partner CDK4, which are activated by phosphorylation upon external mitogenic cues (Datar et al., 2000). The complex of Cyclin D/CDK4 is responsible for initially phosphorylating RB, partially inactivating it, allowing the release of E2F which in turn transcribes Cyclin E and CDK2 (Harbour et al., 1999; Narasimha et al., 2014). Cyclin E complexes with CDK2 in late G_1 phase to further phosphorylate RB, which in turn promotes more E2F dependent transcription of cell cycle genes that are necessary for DNA synthesis (S phase) (Narasimha et al., 2014). Thus, Cyclin E/CDK2 and E2F form a robust positive feedback loop which promotes the transition to S phase. During S phase, Cyclin D/CDK4 and Cyclin E/CDK2 are necessary for the phosphorylation of several target proteins which are necessary for the progression through the cell cycle (Datar et al., 2000; Duronio and O'Farrell, 1995; Knoblich et al., 1994). Cyclin E/CDK2 phosphorylates and activates various components of the DNA replication machinery as well as proteins responsible for the cell cycle-coupled transcription of histones.

DNA replication begins at genomic regions called origins which are bound by complexed called Origin Recognition complexes (ORCs) These complexes are required for initiation of

DNA replication. The initiation signal is provided by the recruitment of CDC6 (a target of CyclinE/CDK2), which activates Cdt1 to recruit MCM (mini chromosome maintenance) complex proteins, to allow DNA replication. Re-replication of DNA during the cell cycle is prevented by the protein Geminin, which binds to and prevents Cdt1 from reactivating origins (Ayad, 2005; Ballabeni et al., 2013; Lygerou and Nurse, 2000).

In late S and G_2 phases, the levels of Cyclin A/CDK1 and Cyclin B/CDK1 complexes increase in preparation for mitosis (Lukas et al., 1999; Sprenger et al., 1997). Cdk1 is phosphorylated by the kinases Wee and Myt1 and kept inactive (Jin et al., 2005; Liu et al., 2000). The phosphatase string (cdc25c in mammals) counteracts these inhibitory kinases (Edgar et al., 1994). As the levels of Cyclin B increase, the Cyclin B/CDK1 complexes phosphorylate and inactivate Wee and Myt, promoting mitotic entry. During early M phase, Cyclin/CDKs also activate the APC/C complex, activating a feedback loop which will result in their own degradation (Peters, 2002; Vodermaier, 2004; Zachariae and Nasmyth, 1999). The activity of mitotic kinases such as polo, Aurora A and Aurora B promote the progression through the various stages of mitosis and cytokinesi s(Archambault and Carmena, 2012; Barr et al., 2004). At the end of mitosis, the phosphatase complex PP2A-B55 rapidly dephosphorylates the substrates of mitotic kinases prior to the next cell cycle (Pereira and Schiebel, 2016).

1.2.3. **Proteolytic Control of the Cell Cycle**

Timely degradation of cell cycle proteins is another feature of the cell cycle which ensure the robust and switch-like transitions between phases of the cell cycle. Two main classes of ubiquitin-ligase proteolytic complexes play critical roles in the degradation of cell cycle proteins.

1.2.3.1. *SCF Ubiquitin Ligases*

The SCF (Skp1/Cullin/F-box) family of E2-ubiquitin ligase complexes is responsible for degradation of key proteins to promote the G_1 to S transition. The F-box subunit of this complex renders the substrate specificity (Vodermaier, 2004). The substrates of the SCF complexes will be marked for degradation only when phosphorylates, which is another factor which contributes to the specificity of these ligases (Cardozo and Pagano, 2004; Hershko and Ciechanover, 1998; Pickart, 2001). Important targets of SCF complexes include Cyclin E, E2F1 and the cell cycle inhibitor Dacapo (dap) which will be discussed in the following section (Davidson and Duronio, 2012; de Nooij et al., 1996; Dui et al., 2013; Lane et al., 1996; Moberg et al., 2001; Zielke et al., 2011).

1.2.3.2. *APC/C^{Fzy}*

The APC/C (Anaphase Promoting Complex/Cyclosome) is a family of E3-ubiquitin ligases that promote the destruction of substrates during various phases of the cell cycle. The degradation activity of this family of complexes is not dependent on the phosphorylation state of the substrate. Rather, the activity is regulated by the binding of activator subunits, which bind to the complex differentially depending on the phase of the cell cycle, as well as confer specificity (Harper et al., 2002; Peters, 2002).

The APC/C^{Fzy} (APC/C^{cdc20} in mammals) is critical for the metaphase to anaphase transition during mitosis. The activating factor Fizzy (cdc20 in mammals) is responsible for the specificity in this complex (Dawson et al., 1995). The APC/C^{Fzy} complex which is activated by phosphorylation of Fzy by mitotic cyclin/CDK complexes, in turn, mark Cyclin B and other important mitotic kinases for degradation at anaphase. By the end of mitosis, Cyclin A, B and CDK1 are rapidly degraded (Dawson et al., 1995).

1.2.3.3. *APC/C^{Fzr}*

After mitosis, the reactivation of mitotic cyclins is prevented by the APC/C^{Fzr} (APC/C^{cdh1} in mammals) complex which is complexed with the activator subunit Fizzy Related (Fzr). Fzr/Cdh1 specifically targets mitotic cyclins and not the cyclin involved in G_1-S transition (Meghini et al., 2016; Raff et al., 2002), allowing the progression of the cell cycle. Cdh1 is inactivated later in the cell cycle by phosphorylation by G_1-S cyclin/CDK complexes when their levels increase(Peters, 2002; Raff et al., 2002).

Thus, the interplay of degradation complexes acts as a negative feedback loop, promoting the oscillation of cell cycle proteins (Pomerening et al., 2005).

1.2.4. **Cell Cycle Inhibitors**

The action of cyclins and CDKs can also be inhibited by Cyclin-dependent Kinase Inhibitors (CKIs). These can be classified into two groups: the INK (Inhibitor of CDK4) family (Cánepa et al., 2007; Roussel, 1999) of CKIs and the Cip/Kip family which inhibit the activity of CDK2 complexes (Cerqueira et al., 2014; Harper and Elledge, 1996; Xiong et al., 1993). These are referred to as 'families' of proteins only because they are found in quite some diversity in mammals. Mammals possess four INKs (p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}), and three Cip/Kip CKIs (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}). In *Drosophila*, Dacapo (dap) is the predominant CKI, and its function is to antagonize Cyclin E/CDK2 activity (Lane et al., 1996). Flies also have another CKI roughex (rux) which functions as a CyclinA/CDK1 inhibitor during mitosis (Foley and Sprenger, 2001).

1.2.5. **DNA Damage And Cell Cycle Checkpoints**

To ensure fidelity of DNA replication and appropriate segregation of chromosomes, the progression of the cell cycle can be halted by regulatory checkpoints when a cell senses DNA damage. Various checkpoint cascades can be activated depending on when in the cell cycle the damage occurs. These checkpoints serve to halt or slow the progression of the cell cycle, allowing the cell to repair the DNA damage before proceeding further through the cell cycle. Usually, checkpoint proteins are kinases which act rapidly to effect the inhibition of Cyclin/CDKs to slow down the cell cycle. If the damage is too severe, checkpoints can also initiate apoptosis. DNA damage is sensed at two main stages: during replication and during chromosome segregation. A cell actively undergoing DNA synthesis is very susceptible to DNA damage. When DNA damage occurs during S phase, the intra-S checkpoint is activated by the kinase ATM which is recruited to double strand breaks (DSBs) on DNA. ATM can phosphorylate and activate downstream kinases that will ultimately result in the inactivation of Cyclin E/CDK2, slowing down S-phase to allow for DNA repair (Blackford and Jackson, 2017).

One critical target of ATM is the conserved tumor suppressor p53 (Agarwal et al., 1998)which plays important roles in regulating genome stability, progression through the cell cycle and apoptosis at various stages in the cell cycle. In mammals, p53 is known to directly induce the transcription of the CKI $p21^{Cip1}$ which in turn slows down the cell cycle by inhibiting CDK2 activity (Agarwal et al., 1995). In flies, p53 does not play a direct role in slowing down the progression of the cell cycle. However, *Drosophila* p53 functions as a tumor suppressor primarily by inducing the expression of pro-apoptotic genes (Fan et al., 2010; Ollmann et al., 2000; Vousden and Prives, 2009).

During mitosis, DNA damage or improper attachment of chromosomes to microtubules in the mitotic spindle can trigger the spindle checkpoint. The spindle checkpoint cascade results in the inactivation of APC/$C^{Fry/cdc20}$, preventing metaphase to anaphase transition the until chromosome attachments are restored, allowing chromosome segregation to proceed (May and Hardwick, 2006).

Another critical

1.3. G_0: Exit From The Cell Cycle

Most studies of the cell cycle have been performed in actively dividing or cycling cells. Decades of research have dissected the specifics and details of processes underlying cell division. However, most cells in adult organisms are non dividing or 'post-mitotic'. Relatively little is known about how cells acquire this non dividing state, and even less about how they maintain it (Buttitta and Edgar, 2007).

1.3.1. **The Types Of G_0**

The term 'G_0' has been coined as a blanket term to refer to the state or 'phase' in which non-dividing cells reside. Several differing non dividing states are all refereed to as G_0. To better understand $_{G0}$, it important to distinguish the different 'types' of G_0 as they vary in regulation, context, and more importantly: the how permanent they are.

1.3.1.1. ***Quiescence***

Quiescence is a temporary and reversible state of G_0, exemplified in vivo by stem cells and in vitro by cells in culture which are starved of serum or nutrients. Cells in culture that are deprived of nutrients enter a quiescent state which is easily reversible upon re-addition of nutrients (Coller, 2007; Coller et al., 2006). Stem cells can stay dormant (i.e.,

non-dividing) for extended period of time, and be induced to re-enter the cell cycle to divide and produce daughter cells and regenerate in response to damage such as wounding or cell loss (Cho et al., 2019). The maintenance of G_0 in stem cells is important, as compromised G_0 can lead to uncontrolled proliferation and the formation of tumours (Wikenheiser-Brokamp, 2006). Several developmental cancers such as glioblastomas and retinoblastomas are caused by mutations in key cell cycle inhibitor genes (tumor suppressors) that result in uncontrolled proliferation of stem cells resulting in tumor formation (Wikenheiser-Brokamp, 2006). Quiescent cells almost always thought to exit the cell cycle after a mitosis, and their DNA content is diploid (2C) (Coller, 2007). Some stem cells such as those in the developing fly ventral nerve cord (VNC) are also known to "pause" the cell cycle in G2 – these cells have a (4C) DNA content (Contreras et al., 2018; Otsuki and Brand, 2018, 2019). Stem cells can rapidly reenter the cell cycle upon receiving cues, and this feature distinguishes this state of G_0 from the others.

1.3.1.2. *Cellular Senescence*

Cellular Senescence associated G_0 is a permanent arrest from the cell cycle, associated with high levels of DNA damage and cellular ageing (Campisi, 2013; López-Otín et al., 2013). First identified in vitro, cells subjected to repeated passages lose their ability to keep dividing in culture as their telomeres shorten. Senescent cells have since been identified in vivo as well and display similar cellular features (Di Micco et al., 2006; Sapieha and Mallette, 2018). Senescent cells are inert to mitogens and do not respond to growth factors. This state of G_0 is considered irreversible and senescent cells can exit the cell cycle with 2C or 4C DNA content (Gire and Dulic, 2015).

1.3.1.3. *Terminal Differentiation*

Terminal differentiation is the process by which cells acquire their final fate. The most prevalent form of G_0 *in* vivo is associated with terminal differentiation. Cells in developing organisms exhibit an exit from the cell cycle which is coordinated and coincident with terminal differentiation (Buttitta and Edgar, 2007). In a majority of cells, the G_0 associated with terminal differentiation is permanent, examples include muscle cells, neurons and most epithelial cells(Buttitta et al., 2007, 2010; Cunningham et al., 2002; Huh et al., 2004; Zacksenhaus et al., 1996). These cells exit the cell cycle with a diploid (2C) DNA content. A vast majority of cells composing adult organisms reside in this state of G_0.

How exactly do cell-intrinsic and cell-extrinsic developmental signals, terminal differentiation signals and the cell cycle machinery coordinate to make functional cells and tissues? Studies over the past few years has suggested that there are several overlapping and redundant biological pathways that influence this (Flegel et al., 2016; Ma et al., 2015, 2019; Sun and Buttitta, 2015). The sheer ubiquity of this type of G_0, coupled with just how little is known about this state pave an inevitable path of inquiry. For the rest of this chapter, 'G_0' will be used to refer to this third type of cell cycle exit.

1.3.2. **How Is G_0 Established And Maintained?**

G_0 is often termed 'arrest' from the cell cycle and the inherent implication of the noun 'arrest' is that an active process ceases to occur. This presents a somewhat incomplete notion: while it is surely true that the active progression through the cell cycle stops, it does not account for the high metabolic activity of postmitotic cells, nor the factors necessary to ensure that another cell cycle does not occur.

Just as the control of processes within the cell cycle controlled at various levels, exit from the cell cycle is also tightly controlled. I will very briefly attempt to summarise in the following few paragraphs what is known about the factors controlling $_{G0}$ establishment and maintenance.

1.3.2.1. *CKI Mediated Inhibition Of Cyclins*

In mammals, the INK family of CKIs play important, tissue-specific roles in ensuring that exit from the cell cycle is maintained by inhibiting CDK4 activity. The expression of the Cip/Kip family of CKIs are also upregulated, sometimes in a manner induced by factors influencing terminal differentiation to initiate cell cycle exit (Cerqueira et al., 2014; Cunningham et al., 2002; Guo et al., 1995; Parker et al., 1995; Thomas et al., 2004). In *Drosophila*, Dacapo plays a similar role, and is upregulated in postmitotic cells undergoing their final cell cycle and newly differentiated cells (Colonques et al., 2011; de Nooij et al., 1996; Lane et al., 1996; Sukhanova et al., 2007). In neurons, the expression of dap is transcriptionally induced by specific transcription factors upon differentiation. Cells in the eye mutant for dap fail to exit the cell cycle in a timely fashion, and undergo a few extra rounds of cell division prior to exit. However, dap is not necessary to induce cell cycle exit in the wing, suggesting that multiple redundant and tissue specific factors are needed to ensure proper cell cycle exit (Buttitta et al., 2007).

1.3.2.2. *Increased Cyclosome Activity*

Increased activity of the activator of APC/C, Fzr is observed in postmitotic tissues upon cell cycle exit, suggesting a role for the APC/C^{Fzr} complex in marking mitotic cyclins for degradation (Buttitta et al., 2010). Increased levels of various components of the APC/C complex could also play a role in raising the threshold for re-entry into the cell cycle by increasing the amount of G_1-S cyclin/CDKs needed to inactivate the cyclosome. Studies in the fly eye and wing where the

APC/C^{Fzr} is inhibited upon cell cycle exit show that cell cycle exit can be delayed by inhibition of this complex alone (Buttitta et al., 2010).

1.3.2.3. *Transcriptional Repression*

Exit from the cell cycle is often coincident with the silencing of cell cycle gene expression. This is in part effected after by a transcriptional repressor complex comprising Rbf, the repressive E2F, E2F2, Myb and MuvB termed the dREAM/MMB complex (Georlette et al., 2007; Lewis et al., 2004, 2012; Wen et al., 2008).

1.3.2.4. *Changes In Chromatin Accessibility*

In addition to transcriptional repression, the silencing of cell cycle genes can be further reinforced by the DREAM complex by its ability to recruit co-repressors as well as chromatin modifiers (DeBruhl et al., 2013; Fischer and DeCaprio, 2015; Georlette et al., 2007; Lee et al., 2012; Sadasivam and DeCaprio, 2013; Uxa et al., 2019; Wen et al., 2008). Considerable work on this has been performed by my colleagues to investigate how changes at the level of nucleosome remodelling and chromatin accessibility can affect cell cycle exit in the fly eye and wing (Flegel et al., 2016; Ma et al., 2015, 2019; Schreader et al., 2010).

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It is common wisdom in the cell proliferation field that the longer a cell has been in a non-dividing state, the more encouragement it needs to be forced to re-enter the cell cycle. This has been well studied in the fly wing and eye imaginal tissues during metamorphosis (Buttitta and Edgar, 2007; Buttitta et al., 2007; Flegel et al., 2016; Ma et al., 2015, 2019; Sun and Buttitta, 2015). In both these tissues, cell cycle exit is fairly synchronous and occurs 24 hours after the onset of metamorphosis. After the 24h into metamorphosis, cells in the wing and eye display a ‘flexible’ cell cycle exit for a short duration, during which ectopic expression of certain positive

regulators of the cell cycle can induce prolonged cycling or delayed cell cycle exit (Buttitta and Edgar, 2007). After this flexible period, cells in the wing and the eye become 'robustly' postmitotic and increasingly refractory to such signals (Buttitta et al., 2010). This sets up many interesting questions:

Is the duration of flexibility the same in all fly tissues? Do various types of cells in the same tissue display the same properties? If so, can we identify a principle for cell cycle exit maintenance? If not, what underlies the differences? Is it determined by cell fate, structure, morphology, function? Is the underlying chromatin state different between these cells? The work described in Chapter 2 begins to address some of these questions by manipulating cell cycle exit in different cell types of the developing *Drosophila* pupal brain during metamorphosis.

How is G_0 maintained in long-lived cells? Using the ageing adult fly brain, we sought to address this question. Our initial experiments led to a surprising finding which is described in detail in Chapter 3. We discovered that in the adult fly brain, a proportion of postmitotic neurons and glia re-enter the cell cycle to become polyploid. The remainder of this chapter will introduce the concepts of variant cell cycles, polyploidy, its functions and how it plays a role in response to damage and repair.

1.4. Variant Cell Cycles

The canonical cell cycle starts with a diploid cell containing two copies of each chromosome, and at the end of one cycle, results in two daughter cells, each diploid with two copies of each chromosome. Exceptions to this can be seen in several cell types and organisms across the animal and plant kingdoms (Edgar and Orr-Weaver, 2001a; Orr-Weaver, 2015). Variant cell cycles which give rise to a cell that contains more than two copies of the genome are classified as endoreduplication or endoreplication cycles. The resulting cell is polyploid in DNA

content. There are different types of endoreplication cycles, and different contexts in which cells employ them to become polyploid.

Endoreplication cycles utilise parts of the cell cycle machinery to replicate DNA, but these cycles are curtailed and result in one cell with increased DNA content instead of two cells. Endoreplication cycles can involve only cycles of DNA replication and growth (termed endocycles) resulting in one nucleus with increased DNA content, or a cycle of with replication mitosis without an ensuing cytokinesis (termed endomitosis), resulting in two or more nuclei in one cell.

1.4.1. **Endocycles**

Endocycles are variant cell cycles characterised by alternating Gap and DNA synthesis phases (Edgar and Orr-Weaver, 2001a). In flies, endocycling is thought to be driven predominantly by an oscillation of Cyclin E/CDK2 and controlled by the transcriptional activity of E2F (Duronio and O'Farrell, 1995; Edgar and Orr-Weaver, 2001a; Moon and Kim, 2019; Zielke et al., 2011).Another important factor that plays a role in endocycle progression is the $APC/C^{Frz/cdh1}$ which ensures not only the degradation of mitotic CDKs, but also the timely degradation of geminin at the at S phase to prevent re-replication(Edgar et al., 2014). In mammals, variant or non-canonical E2Fs are employed specifically during endocycles implying a specialised role for these regulatory factors (Matondo et al., 2018; Pandit et al., 2012).

Several types of cells in various organisms employ endocycles during development or in contexts of cellular damage. Developmentally regulated endocycles occurs in some cells during development to aid the growth of the organism – cells generated by these endocycles usually possess several to several hundred copies of the genome, and often grow very large in size It is interesting to note that developmentally regulated endocycles can generate cells of vastly varying

ploidies depending on the tissue and context. While the enterocytes of the fly intestinal epithelium show an average ploidy of 8C, nurse cells of the ovaries and cells of the salivary gland can be up to 1024C!(Orr-Weaver, 2015).

Some examples of developmentally regulated endocycles in flies include the larval epidermis, salivary gland, fat body and some Sub-perineurial glia of the blood brain barrier (Britton and Edgar, 1998; Hammond and Laird, 1985; Lee et al., 2009, 2020; Von Stetina et al., 2018; Unhavaithaya and Orr-Weaver, 2012). In the adult fly, the enterocytes in the gut, the nurse and follicle cells of the ovary in adult females (Fox and Duronio, 2013; Royzman et al., 2002). These are all very large cells which either serve a biosynthetic demand or crucial barrier function. The cells resulting from these endocycles are usually constitutively polyploid.

While developmental endocycles have been well studied in *Drosophila*, they are also present and in other organisms. Several tissues in plants such as leaves, roots and trichomes have cells that endocycle after terminal differentiation to support growth(Lang and Schnittger, 2020; Orr-Weaver, 2015; De Veylder et al., 2011). In mammals, the most studied example of endocycling is hepatocytes in the liver, and the trophoblast giant cells of the placenta. Just like in the fly, the different polyploid cells in mammals can exhibit varied levels of polyploidy. Polyploid hepatocytes contain 4C to 8C DNA content, however trophoblast giant cells can have over 1000 copies of the genome (Celton-Morizur and Desdouets, 2010; Jensen et al., 1989; Klisch et al., 1999; Melchiorri et al., 1993; Roszell et al., 1978; Severin et al., 1984; Zuckermann and Head, 1986; Zybina and Zybina, 1996). It is interesting to note here that highly polyploid cells such as nurse cells and trophoblast giant cells which provide critical trophic support are short lived.

In addition to cells that undergo developmentally regulated endocycles to become polyploid, some cells show a capacity to enter an endocycle in contexts of wounding and damage. These will be discussed in the following sections.

1.4.2. **Endomitosis**

Endomitosis is another variant cell cycle which differs from endocycles in that it produces a cell with two or more nuclei. Endomitoses comprise a G_1, S, G_2 and a mitosis but no cytokinesis (Edgar and Orr-Weaver, 2001a; Orr-Weaver, 2015). Thus, the regulation of endomitoses is different from that of an endocycle. Endomitotic cell cycles are characterized by a failure to undergo cytokinesis which results in binucleate or multinucleate cells. Endomitotic cells are less common than endocycling cells.

Endomitoses are best studied in the platelet-producing megakaryocyte cells in mammals (Bluteau et al., 2009; Ravid et al., 2002; Zhang et al., 1996; Zimmet and Ravid, 2000). Some SPGs in the fly blood brain barrier are known to become multinucleate by endomitosis (Eliades et al., 2010; Von Stetina et al., 2018; Unhavaithaya and Orr-Weaver, 2012). Examples of endomitosis giving rise to binucleate cells are cardiomyocytes in mouse and human hearts, lactating mammary epithelial cells and the binucleate cells of the *Drosophila* accessory gland (Box et al., 2019; Pandit et al., 2013; Paradis et al., 2014; Rios et al., 2016; Stephen et al., 2009; Taniguchi et al., 2014, 2018).

1.4.3. **Why Become Polyploid?**

Why do some cells become polyploid? What are the benefits of entering a variant cell cycle rather than undergoing cell division? Constitutively polyploid cells, as mentioned before, mainly perform two important functions: they usually have increased biosynthetic capacity, and

they maintain barrier function (Edgar and Orr-Weaver, 2001a; Lee et al., 2009; Øvrebø and Edgar, 2018). Polyploid cells with more copies of the genome can increase cell size and metabolic functions efficiently. Undergoing cell division involves cell rounding, cytoskeletal rearrangements and potential loss of cell-cell contacts (Erenpreisa and Cragg, 2001; Lancaster et al., 2013; Orr-Weaver, 2015; Sauer, 1935). This can be problematic in cells performing important barrier functions. Endocycling can therefore be a way for these cells to grow in size and genome copy number without increasing in cell number (Edgar and Orr-Weaver, 2001a).

One additional benefit of polyploidy is resistance to DNA damage conferred by the number of copies of the genome – somatic mutations in one copy of an gene will not greatly impact the capacity of the cell to function since will have many other copies of the genome. In endomitotic cells: the prevalence of binucleation in contractile tissues could mean that binucleate cells are more resistant to stretch and pull stresses (Edgar and Orr-Weaver, 2001a; Taniguchi et al., 2018; Windmueller et al., 2020).

Thus, given the several benefits of polyploidisation, it is no surprise that these benefits have been adopted by otherwise diploid cells under conditions of stress or damage. Considerable work has been done in *Drosophila* and some mammalian systems to understand the role of polyploidisation in response to wounding and induced cell loss as well as how polyploidy protects cells from DNA damage. This body of work has furthered our understanding of how polyploidy plays a role in acute damage. The next section will first describe how some cells cope with various kinds of damage to become facultatively polyploid. I will then revisit older literature in other (mostly vertebrate) model organisms which has recorded differing levels of polyploidy in tissues which show facultative polyploidy or changes in levels of ploidy in contexts of ageing, as well as with chronic disease. The goal is to appreciate these early

observations with a modern perspective which may help us develop a better understanding of the phenomenon described in Chapter 3.

1.4.3.1. *DNA Damage Resistance*

For over 80 years, scientists have observed that polyploid cells are able to endure and survive DNA damage better than diploid cells (Muntzing and Prakken, 1941). The resistance to DNA damage is attributed, in most part, to the number of copies of a gene that a polyploid cell has. If a cell has several 'spares', DNA damage caused by random somatic mutation to one copy of a crucial gene will not impede the cell's ability to function or survive, as it will have more copies of the gene (Edgar and Orr-Weaver, 2001b; Edgar et al., 2014; Øvrebø and Edgar, 2018). The earliest studies on the resistance polyploid cells show to DNA damage were performed in the 1940s. (Muntzing and Prakken, 1941). These studies compared the response of whole organism tetraploids to diploid rye plants and linked the resistance to radiation damage to ploidy variations.

Functional studies in genetic model organisms have since furthered our understanding of how some polyploid cells may resist DNA damage. The most prominent model used to understand the relationship between polyploidy and DNA damage resistance has been the various polyploid tissues in *Drosophila.* Studies in the follicle cells, fat body as well as salivary glands in the fly have shown that endocycling cells do not undergo apoptosis as a result of induced genome instability(Mehrotra et al., 2008). These polyploid cells can tolerate high levels of DNA damage, and harbour double strand breaks to their DNA, but do not undergo apoptosis. Further studies have shown that low levels of the tumour suppressor protein p53 in these endocycling cells is responsible for conferring their resistance to cell death(Mehrotra et al., 2008; Zhang et al., 2014). The tumour suppressor p53 is responsible for activating the expression of

proapoptotic genes hid, reaper and grim in *Drosophila*, and these proteins are in turn upstream of the caspase cascade. Low levels of p53 in *Drosophila* polyploid cells, combined with chromatin-level silencing of the pro-apoptotic genes confer high levels of resistance to DNA damage-induced cell death in these polyploid cells (Mehrotra et al., 2008; Park et al., 2019; Zhang et al., 2014).

Cells of the *Arabidopsis thaliana* root will increase the proportion of cells that become polyploid as well as increase the degree of polyploidy in these cells in response to various kinds of radiation and chemically induced DNA damage.(Adachi et al., 2011).

Studies of cancer cells show that polyploidy can be induced by DNA damage. This is frequently observed in cancer cells which lack cell cycle checkpoints. Failure of cytokinesis or premature exit from the cell cycle without undergoing mitosis often results in tetraploid cancer cells. Several types of carcinomas with inactivated p53 or Rb have cells with hyperploid DNA content. Severe telomere attrition has been implicated in these cases as the source of DNA damage. Chemotherapy has been shown to give rise to polyploid giant cancer cells (PCGCs) (Davoli and de Lange, 2011; Lazzerini Denchi et al., 2006).

Polyploid cells are protected from DNA damage, and polyploidy can be induced by DNA damage. This suggests that polyploidy has been employed in several types of tissues and organisms as a robust adaptation to DNA damage. In addition to DNA damage, polyploidy has also been employed in other contexts of acute damage, particularly in tissues where very few or no stem cells exist.

1.4.3.2. ***Wound Healing And Compensatory Growth***

Cells in the *Drosophila* adult abdominal epithelium respond to wounding by re-entering the cell cycle as well as undergoing cell fusion to become polyploid close the wound. Induction

of the endocycle in these cells is dependent on the upregulation of E2F by the Hippo/Yorkie pathway as well as the degradation of mitotic cyclins by APC/C^{Fzr}. Polyploidisation is also known to play a role in wound healing in the mammalian corneal endothelium, heart and keratinocytes (Gandarillas et al., 2019; Grendler et al., 2019; Losick, 2016; Losick et al., 2013; Trakala and Malumbres, 2014; Werner et al., 2007).

Endoreplication has also been implicated in alternate modes of regeneration and response to cell loss. The liver remains best studied in this context as well in mammals, but recent studies have shown that polyploidisation occurs in renal tubular epithelial cells in response to ischemic damage (Lazzeri et al., 2018; Matsumoto et al., 2020; Melchiorri et al., 1993). Other examples of endocycling in response to cell loss include the epicardium of the zebrafish heart (Uroz et al., 2019). In *Drosophila,* the enterocytes of the intestinal epithelium, the follicle cells of the ovary and the main cells of the accessory gland can cope with induced cell death by engaging a compensatory cellular hypertrophy or endocycle program to maintain tissue size and homeostasis (Box et al., 2019; Edgar et al., 2014; Øvrebø and Edgar, 2018; Shu et al., 2018; Tamori and Deng, 2013).

Given the benefits of polyploidisation in non-dividing tissues, and relative prevalence of facultative polyploidisation occurring in response to myriad acute sources of damage, one is tempted to ask the following: does polyploidy also play a role in chronic damage? Several long-lived tissues endure constant assaults as well as deteriorating cellular functions such as accumulation of DNA damage, loss of proteostasis, changes in metabolism and increased oxidative stress (Hunt et al., 2019; López-Otín et al., 2013; Rodriguez-Fernandez et al., 2020).

Could polyploidisation serve as an adaptation to cells in ageing tissues with very few or no stem cells? Cytological observations in various murine and other invertebrate and vertebrate

tissues suggest that accumulation of polyploidy in ageing tissues may be more common than is appreciated.

1.4.4. **Age-Dependent Accumulation Of Polyploidy?**

The murine liver and the heart have been extensively studied in the context of polyploidy. In these tissues, most cells are diploid at birth, and the onset of polyploidy occurs at the onset of weaning and acquisition of sexual maturity. A similar pattern of onset of polyploidisation is also observed in the pancreas of mice and rats, and the lacrimal glands of male rats . In addition, an increase in the proportion of polyploid cells with age has been observed and reported in the adrenal and thyroid glands (Bohman et al., 1985; Carriere and Patterson, 1962; Gahan, 1977; Geschwind et al., 1958; Gilbert and Pfitzer; Nguyen and Ravid, 2010; Paulini and Mohr, 1975; Roszell et al., 1978; Teir, 1949). In all of these cases, the proportion of polyploid cells increases rapidly at first (upon weaning), and then gradually over age.

The liver and lacrimal glands exhibit endocrine dependent onset of polyploidy, with the liver being dependent on thyroid and thymus function, and the lacrimal glands, on male gonads. The liver shows diet-dependent increase in polyploidy levels: rats on a restricted diet showed lower levels of accumulated polyploidy whereas rats feeding *ad libitum* showed higher levels of polyploidy accumulation with age, suggesting that the polyploidisation of the liver is dependent on metabolic need and adaptive in nature (Enesco et al., 1991; Paulini and Mohr, 1975).

Similarly, observations of polyploidy and binucleation in cardiomyocytes have been made in several organisms (Brodsky VYa et al., 1994; Derks and Bergmann, 2020; Gan et al., 2019; Hirose et al., 2019). Recent work has linked the onset of polyploidy to endocrine cues and show that the polyploidy is also marked by a metabolic shift from glycolysis to oxidative phosphorylation upon polyploidisation.(Hirose et al., 2019). Induced polyploidy in zebrafish

hearts results in reduced regenerative capacity (González-Rosa et al., 2018). Further, binucleate cells and polyploidy increase with age as well as in diseased hearts (Brodsky VYa et al., 1994; Clubb et al., 1987; Derks and Bergmann, 2020; Dzau and Gibbons, 1988; Lombardi et al., 1989). This has led to the prevailing notion that polyploidisation in the heart is generally not beneficial. The current opinion in the cardioliology field that binucleation directly hampers cardiac regeneration potential linking the of lack of binucleation or polyploidisation with regenerative capacity may be incomplete. Adult mammals and birds(endotherms) show cardiac polyploidy while amphibians and teleosts (ectotherms) do not (Derks and Bergmann, 2020). While most studies view polyploidisation in the heart simply as a loss of regenerative potential, the idea that perhaps the acquisition of polyploidy, instead, is an adaptation to endothermic conditions and oxidative stress warrants further inquiry. Cardiomyocytes are among the longest lived cells in a mammalian body, perhaps polyploidisation (in part) underlies their longevity?

Other observations of polyploidy include the human urothelium, mesothelium, endometrium (during pregnancy), seminal vesicles and squamous epithelium of the mouth under non-cancer conditions in humans. (Biesterfeld et al., 1994). While the potential roles of polyploidy in these cells can be speculated (Gahan, 1977), we do not yet have an understanding of how these may change with age, nor whether it may be a beneficial adaptation.

What about the nervous system? While there have been few reports of polyploidisation in neurons in mammals and other vertebrates, the an understanding of its underlying causes and consequences, for the most part, remains elusive. So I ask, what *is* known about polyploidisation in the nervous system?

1.4.5. **Polyploidy In The Nervous System:From Mollusk To Man.**

1.4.5.1. ***Slugs do it best.***

Sea slugs of the *Aplysia* species have long been used in studies of olfaction and memory formation (Coggeshall et al., 1970; Kukushkin et al., 2019; Moroz, 2011; Nagle et al., 1993; Sattelle and Buckingham, 2006; Yamagishi et al., 2012). These slugs possess giant neurons (roughly the size of one fly brain) which are perhaps the most extreme example of somatic polyploidy, possessing up to 600,000 copies of the genome(600,000C)! While we still do not know exactly why these neurons are so large, it is speculated that in 'simpler' animals, one large cell can perform the functions of several smaller cells, trading off 'complexity' for capacity (López-Sánchez et al., 2011; Mandrioli et al., 2010).

1.4.5.2. ***Fly Peripheral Nervous System***

Endocycling has been observed in the *Drosophila* peripheral nervous system in the bristle cell lineage. Bristle cells are mechanoreceptive cells in the fly thorax. While it has been known for over thirty years that these cells become polyploid (up to 8C) during development (Hartenstein and Posakony, 1989), recent work has provided mechanistic insight into how these cells become polyploid. The bristle lineage consists of a neuron, a glial cell, a sheath cell, and one socket and one shaft cell. The shaft and socket cells become polyploid in a Cyclin A/CDK2 dependent manner, unlike most other tissues in fly which employ CyclinE/CDK2 ocsillations to become polyploid.(Audibert et al., 2005; Furman and Bukharina, 2008; Sallé et al., 2012) However, the reason these cells become polyploid remains unclear.

1.4.5.3. *Teleost Supramedullary Neurons*

Several species of teleosts are also known to possess a small number of highly polyploid neurons called supramedullary neurons on the dorsal surface of the spinal cord or the rostral spinal cord.(Bennett and Nakajima, 1967; Bennett et al., 1959; Dampney et al., 2003; Nakajima and Pappas, 1965). Depending on the species of fish, these neurons can have anywhere between 100 to over 5000 copies of the genome. These neurons are very small in number, and have been proposed as a good *in vivo* model for electrophysiology studies due to their prominent size and convenient location. These large cells are thought to have a neuro-endocrine function as some species of puffer fish produce noradrenalin (Mola and Cuoghi, 2004; Mola et al., 2002).The need for biosynthesis of large amounts of adrenalin may underlie the polyploidy in these cells, however this has not been functionally tested.

1.4.5.4. *Other vertebrates*

Early observations of polyploidy in vertebrate brains involved the study of neurons and glia in the cerebellum by three groups in the 1960's and '70s (Herman and Lapham, 1969, 1973; Lapham, 1963, 1968; Lapham et al., 1971; Lentz and Lapham, 1969, 1970; Mann et al., 1976; Mann and Yates, 1973a, 1973b, 1979; Swartz and Bhatnagar, 1981; Yates and Mann, 1973). While these studies report differing numbers, they both conclude that the cerebellum does indeed possess polyploid cells based on imaging and cytochemical measurements of nuclear size and DNA content. One study measured the proportion of polyploid cells at different ages in the human cerebellum and found that there was no appreciation in the proportion of polyploidy of neurons or glia between ages 8 and 72, suggesting that unlike the liver and heart, the proportion of polyploidy remains constant in the human brain with age . These early studies speculated that

the polyploidisation may contribute to cerebellar memory and specialized function due to their increased transcriptional output.(Mann and Yates, 1973a).

Following a long dormancy in the field that lasted nearly three decades, modern genetic approaches investigating potential cell cycle re-entry in vertebrate brains began taking shape in the early 2000's. In recent years, observations of *bona fide* polyploidy in neurons of the retinal ganglion of the chicken and mouse, cerebral cortex of the rat and neocortex of the mouse have been made using modern flow cytometry and high resolution imaging techniques (Jungas et al., 2020; López-Sánchez and Frade, 2013; Martin et al., 2017; Morillo et al., 2010; Ovejero-Benito and Frade, 2015). Work from the Frade lab has shown that the neurons of the retinal ganglia tetraploidise in an E2F dependent manner. However, this endoreplication program is differentially regulated in chick and mouse brain, as $p27^{kip1}$ is necessary for tetraploidization in the chick, but not the mouse RGCs (López-Sánchez and Frade, 2013; Ovejero-Benito and Frade, 2015). Further advances in imaging and flow cytometry techniques have identified polyploid pyramidal neurons in the cerebrum of the rat, and the neocortex of the mouse, but the function and underlying cause for their polyploidy remain elusive (Jungas et al., 2020; Sigl-Glöckner and Brecht, 2017). These studies show sufficient evidence that polyploidisation does indeed occur in 'higher' vertebrates, suggesting that neuronal polyploidisation may been a well conserved phenomenon. However, while these studies have made detailed observations of polyploidy in neurons, but do not yet answer some important questions:

What causes polyploidisation in neurons?

How is neuronal function impacted by polyploidisation?

How does this relate to neurodegeneration associated cell cycle re-entry ?

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Chapter 2. Glia Establish A More Flexible G_0 Than Neurons In The *Drosophila* Pupa Brain

2.1. Abstract

G_0 associated with terminal differentiation represents the most common cellular state in adult multicellular organisms, yet it is poorly understood. In past years, various tissues of the fruit fly *Drosophila melanogaster* have served as a great model systems to understand how cells establish and maintain their non-dividing state. While the *Drosophila* brain has been extensively studied in the context of neurodevelopment, relatively little is known about how the flexibility of cell cycle exit in terminally differentiated neurons and glia. We sought to understand whether these cells show a capacity to undergo cell division after terminal differentiation.

Here we show that postmitotic neurons and glia in the developing *Drosophila* pupa brain can be forced to re-enter the cell cycle after they have exited the cell cycle. Neurons can re-enter the cell cycle up to 24 hours after they have exited the cell cycle whereas glia exhibit greater flexibility and can undergo cell division up to over 48h after they exit the cell cycle. We show that forcing re-entry in neurons results in cell death, while glial cell division can result in tumor-like growths. Further, we show that most of the glia that display flexibility in cell cycle exit are cortex glia.

2.2. Introduction

The G_0 state of the cell cycle has been extensively studied in various tissues in *Drosophila melanogaster*, such as the wing, eye, (Buttitta and Edgar, 2007; Buttitta et al., 2010; Flegel et al., 2016; Ma et al., 2019; Sun and Buttitta, 2015),testes and intestine (Hétié et al., 2014; Petkau et al., 2014). The fly brain is much more complex, and comprises a heterogeneous population of neuronal and glial cells that perform distinct and highly specialised functions. While the *Drosophila* brain has been thoroughly studied as a model for neural development, very little is known about the regulation of G_0 or cell cycle exit in neurons and glia associated with terminal differentiation. Exit from proliferation has been studied in the context of the neural stem cells which give rise to neurons and glia, but not the differentiated daughters themselves. Our goal is to achieve a better understanding of how post-mitotic cells in the developing brain establish and maintain their non-dividing state to stay postmitotic upon terminal differentiation as well as to understand the 'flexible' and 'robust' nature of their G_0

The adult central nervous system comprises ~150,000 cells, most of which are generated in the larval stages of development from various progenitor cell types (reviewed in (Homem et al., 2015). Type I neuroblasts, Type II neuroblasts (Bayraktar et al., 2010; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009) and mushroom body neuroblasts (Kunz et al., 2012) generate structures in the central brain and ventral nerve cord while the neuroblasts of the inner and outer optic placode (IPC and OPCs) give rise to the cells of the optic lobes(Apitz and Salecker, 2014). Type II neuroblasts undergo self-renewal and give rise to NBs and transit-amplifying cells known as intermediate progenitor cell which undergoes a limited number (3-5) of divisions to generate ganglion mother cells (GMCs) (Walsh and Doe, 2017). OPC, IPC (also known as type III) (Apitz and Salecker, 2014, 2015, 2016), Type I and Mushroom body

neuroblasts divide asymmetrically to generate one NB and one GMC. GMCs subsequently undergo one cell division each to generate terminally differentiated and postmitotic neurons and glia (Homem et al., 2015). T

The type I neuroblasts in the thoracic VNC exit the cell cycle in a prospero-dependent manner in late larval stages (Maurange et al., 2008), while those in the abdominal VNC undergo apoptosis (Cenci and Gould, 2005; Maurange and Gould, 2005). In the central brain, the remaining type I and type II neuroblasts stop proliferation in early pupal stages by undergoing terminal symmetric divisions that lead to depletion of the NBs (Weng et al., 2010). OPC and IPC neuroblasts stop proliferation by undergoing symmetric terminal divisions in the early pupal stages (Apitz and Salecker, 2015, 2016). The mushroom body neuroblasts keep proliferating until late metamorphosis, when they undergo apoptosis (Pahl et al., 2019; Siegrist et al., 2010).

Very little is known about the flexibility of the cell cycle exit in the differentiated progeny from these precursor cells. Do these cells retain a capacity to undergo further cell divisions after differentiation? We examine this question here.

2.3. Results

2.3.1. Cell Cycle Exit In The *Drosophila* Brain Occurs During Early Metamorphosis

Work from several groups has established that proliferation slows down at the larval to pupal transition, and nearly all proliferation of neuroblasts except the mushroom body neuroblasts ceases after 24h APF (Truman and Bate, 1988). The mushroom body NBs continue to divide slowly until late into metamorphosis (Ito and Hotta, 1992; Prokop and Technau, 1994; Stocker et al., 1995). We confirmed that cells do indeed stop proliferation and exit the cell cycle

with a 2C DNA content by performing a flow cytometry-based time course. We measured the DNA content in individual cells from 0hAPF to 96h APF at 24h intervals. We, like others, find that by 24h most of the proliferation has ceased, and by 48h APF, most (>99%) of the cells in the pupal brain have diploid DNA content(Figure 2.1) A small proportion (<3% measured by flow cytometry) of glia exhibit polyploid DNA, consistent with the small population of polyploid endocycling and endomitotic sub-perineurial glia (SPG) which form the *Drosophila* blood-brain-barrier (Von Stetina et al., 2018; Unhavaithaya and Orr-Weaver, 2012).

Previous work from our lab has shown that cells in the *Drosophila* wing and eye imaginal discs exit from the cell cycle during metamorphosis (Buttitta et al., 2007). Cell cycle exit occurs 24 hours after puparium formation (APF) in a relatively synchronous fashion in the eye and the wing. It is known that in the wing and eye, re-activation of Cyclin/Cdks or ectopic expression of the transcription factor E2F and its dimerization partner DP can delay cell cycle exit for a short duration after when cells normally exit the cell cycle (Buttitta et al., 2007). This period has been termed 'flexible' cell cycle exit since the cells retain the capacity to undergo additional divisions. However in both tissues, 12-24 hours later cells become refractory to the activation of Cyclin/Cdks or E2F/DP alone, and require the combination of both simultaneously to continue dividing (Buttitta et al., 2007). This is termed 'robust' cell cycle exit, as cells in this state of G_0 require additional manipulation to force continued cycling or re-entry into the cell cycle (Buttitta et al., 2007, 2010).

In the wing, the timing of cell cycle exit and terminal differentiation are temporally coordinated during metamorphosis by hormonal signals(Guo et al., 2016). In the central nervous system of *Drosophila*, neurons and glia are fated products of terminal cell divisions and are born

as postmitotic cells throughout larval and early pupal development. We sought to ask: how 'flexible' or 'robust' are the states of G_0 in neurons and glia?.

Work comparing the eye and wing has revealed tissue-specific requirement of negative regulators of the cell cycle upon cell cycle exit (Buttitta et al., 2007). Moreover, it is widely believed that neurons are some of the most robustly postmitotic cells in an organism's body. Understanding how a neuron establishes and maintains a non-dividing state can have important ramifications and therapeutic applications in controlling growth. Further, comparing how different cells may behave when establishing G_0 or how the 'flexibility' of G_0 can be modulated in a cell-type specific manner will inform our understanding of the various factors that may regulate it.

2.3.2. **Strategy To Force Cell Cycle Re-Entry In Neurons And Glia**

We previously showed that reversing G0 in differentiated cells required simultaneous reactivation of Cyclin/Cdk and E2F/DP activities (Buttitta et al., 2007). This can be through direct overexpression of Cyclins and Cdks along with the E2F and DP subunits, or via inhibition of the APC/C complex, which degrades Cyclin/Cdk complexes, in combination with overexpression of E2F and DP subunits (Buttitta et al., 2010). We therefore co-overexpressed the E2F/DP transcription factor complex with the G1 Cyclin/Cdk, CycD/CDK4 or we co-overexpressed E2F/DP with Rca1 (Regulator of CyclinA; Emi1 in mammals) (Reimann et al., 2001), an inhibitor of the APC/C Figure 2.1. To ensure that our genetic manipulations of cell cycle regulators do not impair the development of the animal, we used a temperature sensitive $GAL80^{TS}$ in combination with our cell type specific Gal4 drivers to repress transgene expression until metamorphosis (0h APF) (Suster et al., 2004). Animals were collected as white pre pupae

and shifted to higher temperatures which are non-permissive for the $GAL80^{TS}$ and therefore allow GAL4 mediated expression of transgenes under the control of a UAS promoter. We used the pan-glial driver Repo-GAL4 to in our experiments and the neuronal driver OK371-GAL4 for expression of transgenes in a subset of neurons. OK371 is known to express in vesicular glutamatergic neurons (vGlut neurons). We chose this driver instead of the pan-neuronal GAL4 ELAV-GAL4 because ELAV-GAL4 is known to have leaky expression in neuroblasts and glia (Berger et al., 2007; Foo et al., 2017), and is therefore not strictly driving gene expression in postmitotic cells.

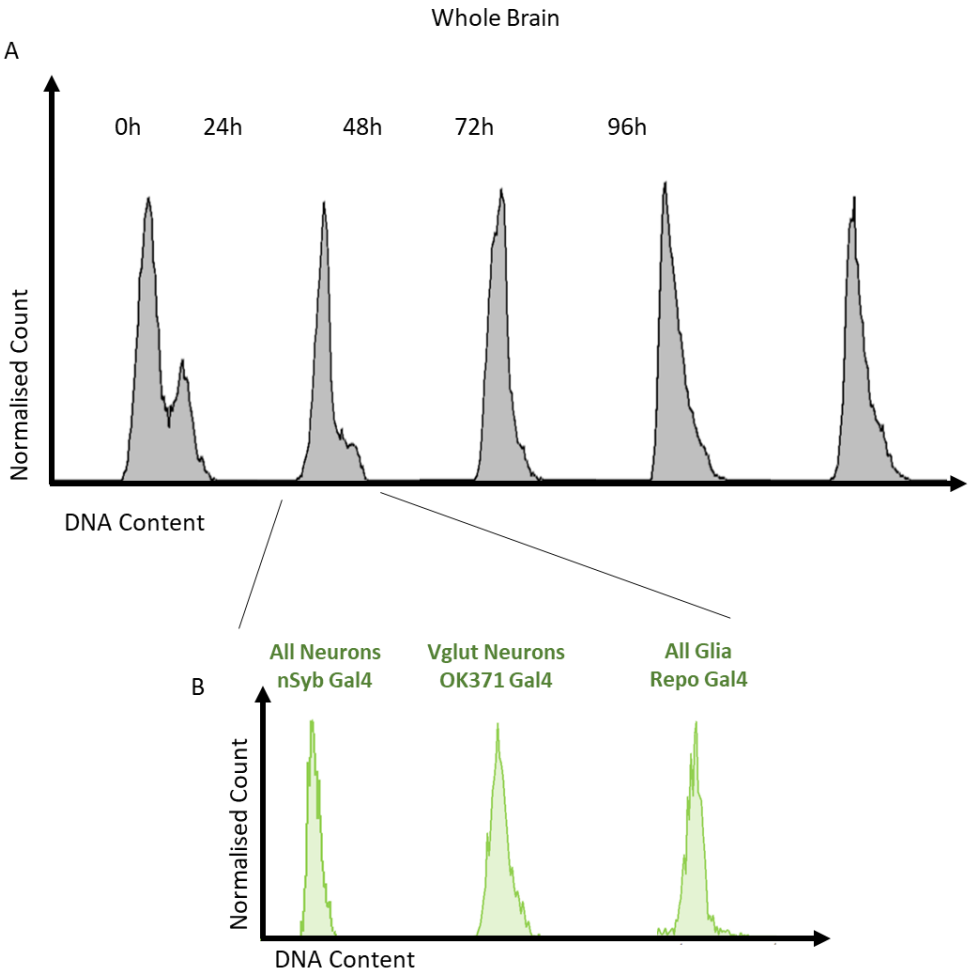


Figure 2.1 Cell Cycle Exit in the Drosophila brain occurs during early metamorphosis

(A) DNA content flow cytometry indicates that most cell proliferation in the pupa brain is complete by 48h APF, most cells exit the cell cycle with a Diploid DNA content. (B) All neurons exit the cell cycle with 2C DNA content. DNA content in all neurons was measured at 24h APF using nSyb-GAL4, UAS nGFP. DNA content of 100% of GFP+ve cells is 2C. Similarly, all neurons expressing OK371 gal4 exit the cell cycle with 2C DNA content. Most glia (97%) show 2C DNA content. The remaining glia showing >2C DNA content are presumed Sub-perineurial glia which are known to be polyploid.

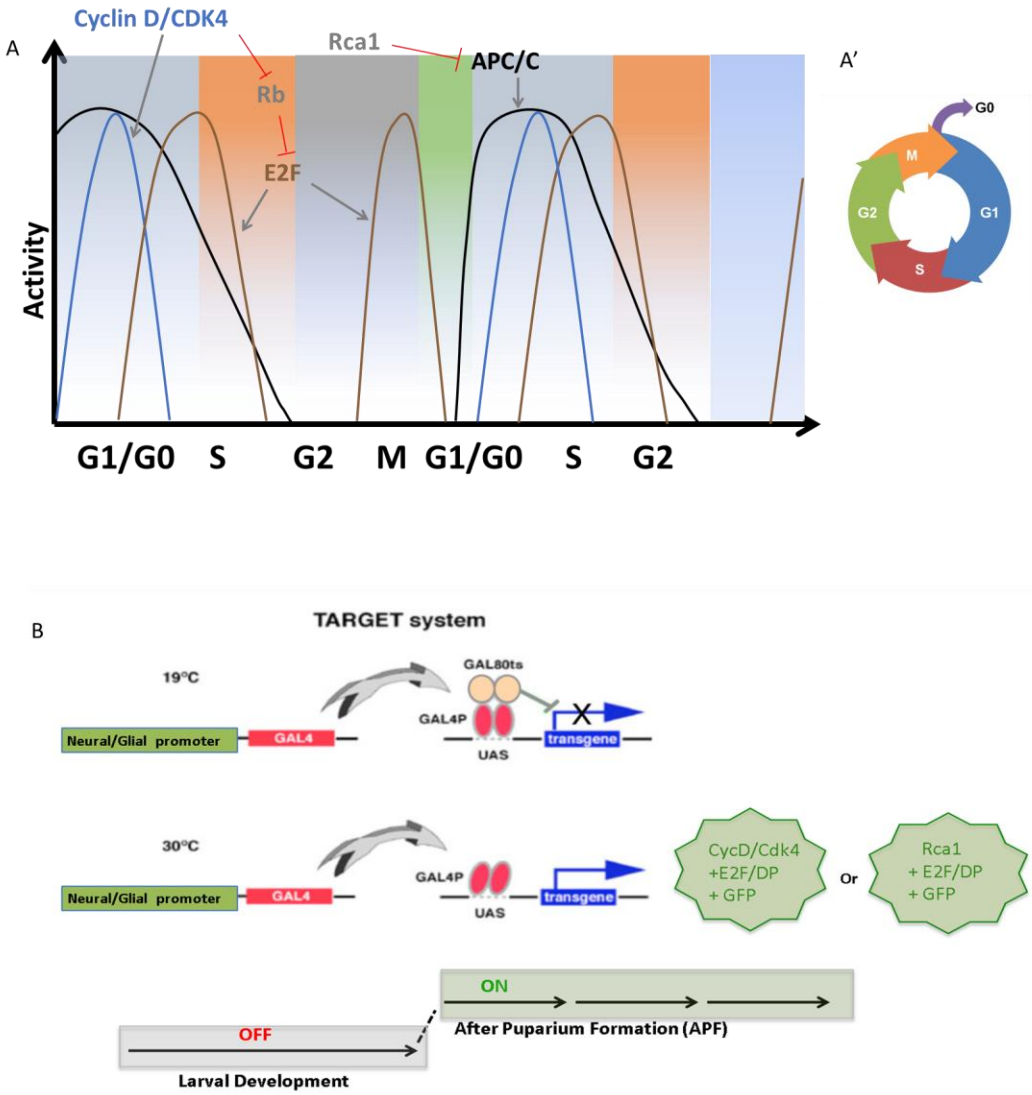


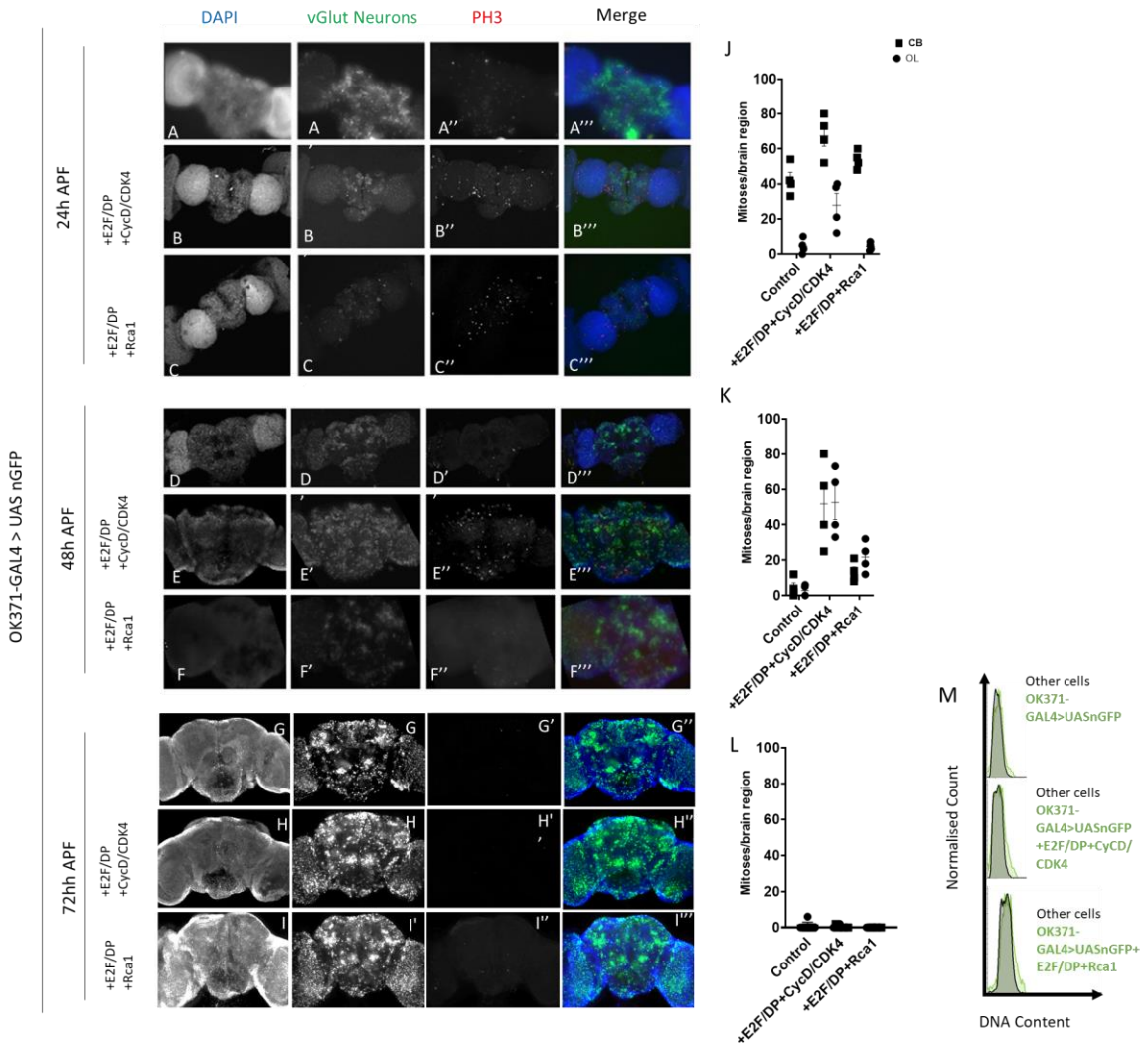
Figure 2.2 Strategy used to force re-entry in postmitotic neurons and glia

(A,A') Cartoon showing the oscillations of cell cycle regulators through the cell cycle (A'). (B) Cartoon showing how the TARGET system will be used to overexpress positive regulators of the cell cycle in transgenic flies to push postmitotic neurons and glia to re-enter the cell cycle.

2.3.3. **Neurons Exhibit A Short Window Of Flexible G_0 Before Becoming Robustly Postmitotic**

After induction of transgene expression in vGlut neurons for 24, 48 or 72 APF, pupae were dissected, fixed, stained for mitoses (anti-phospho Histone H3) and nuclei (DAPI) and mounted on slides for immunofluorescence imaging. Consistent with flow cytometric measurements of DNA content (Figure 2.1) , very few cells in 24h APF brains are normally undergoing mitoses (Figure 2.3), and even fewer (0-4) are observed in control 48 and 72h APF brains. At 24h APF, overexpression of E2F/DP+CycD/CDK4 results in an increase in the number of mitoses in the central brain and optic lobe regions (Figure 2.3B-C’’’, J) , however the overexpression of E2F/DP+Rca1 only results in an increase in mitoses in the central brain, suggesting a different regulation of cell cycle exit at this time point in different brain regions. For comparisons, we quantified the mitotic index of different brain regions with these manipulations at different times (Figure 2.3) . To avoid potentially counting normal mitotic divisions of the MB neuroblasts, our mitotic counts excluded the mushroom body region Interestingly, at 48hAPF, both E2F/DP+CycD/CDK4 and E2F/DP+Rca1overexpression result in increased mitoses in the OL and CB while control brains show nearly no mitoses. This suggests that differentiated V-Glut neurons are capable of re-entering the cell cycle and undergoing mitosis during mid-pupal stages (Figure 2.3D-F’’’, K). This represents a ‘flexible’ G_0 in neurons during development.

By 72h APF, vGlut neurons appear completely refractory to cell cycle re-entry and neither the over expression of E2F/DP+CycD/CDK4 , nor E2F/DP+Rca1 is sufficient to force continued neuronal division (Figure 2.3G-I’’’, L). Flow cytometry analysis of DNA content shows that


OK371-GAL4 > UAS nGFP
24h APF
48h APF
72hh APF
+E2F/DP +CycD/CDK4
+E2F/DP +Rca1
DAPI
vGlut Neurons
PH3
Merge
A
A'
A''
A'''
B
B'
B''
B'''
C
C'
C''
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D
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I
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I''
I'''
J
CB
OL
Mitoses/brain region
Control
+E2F/DP+CycD/CDK4
+E2F/DP+Rca1
K
L
M
Normalised Count
DNA Content
Other cells OK371-GAL4>UASnGFP
Other cells OK371-GAL4>UASnGFP +E2F/DP+CyCD/CDK4
Other cells OK371-GAL4>UASnGFP+E2F/DP+Rca1

Figure 2.3 Postmitotic glutamatergic neurons can be forced to reenter the cell cycle up to 48h APF

Brains from pupae expressing OK371-GAL4, UASnGFP, Tub-GAL80TS only (A-A’’’, D-D’’’), (G-G’’’) or OK371-GAL4, UASnGFP, Tub-GAL80TS+ UASE2F, UAS-DP, UASCyClinD, UAS-CDK4 (B-B’’’) (E-E’’’) (H-H’’’) or OK371-GAL4, UASnGFP, Tub-GAL80TS+ UASE2F, UAS-DP, UAS-Rca1 (C-C’’’) (D-D’’’)(H-H’’’) were shifted up to 29ºC at 0h after puparium formation to induce expression of transgenes. Pupal brains were dissected at indicated timepoints, and stained for mitoses (anti-phospho histone H3) and DAPI. Number of mitoses per brain region were counted at 24, 48 and 72h APF (J, K, L). (G-I’’’, L, M) vGlut neurons are robustly postmitotic at 72hAPF and exit the cell cycle with 2C DNA content even in the presence of cell cycle regulators.

control, E2F/DP+CycD/CDK4 and E2F/DP+Rca1 overexpressing neurons exit the cell cycle with a diploid (2C) DNA content. This suggests that at this stage, vGlut neurons are robustly postmitotic and become refractory cell cycle cues by 72h APF.

2.3.4. Forcing Cell Cycle Re-Entry In Neurons Results In Abnormal Mitoses And Cell Death

High magnification images of vGlut neurons overexpressing E2F/DP+CycD/CDK4 at 48h APF reveals abnormal nuclear morphology Neurons forced to re-enter the cell cycle by overexpression of E2F/DP +CyCD/CDK4 show abnormal mitotic structures such as (Figure 2.4A-A'''') micronucleus formation and (Figure 2.4B-B'''') lagging chromosomes and pyknotic nuclei (not shown). We wondered whether forcing re-entry in neurons may be resulting in cell lethality. We measured cell death by using a propidium iodide incorporation assay with flow cytometry. Brains overexpressing E2F/DP+CycD/CDK4 or E2F/DP+Rca1 in vGlut neurons exhibit greater levels of cell death than control brains at 48h APF, suggesting that forced cell cycle re-entry and mitosis is lethal in developing neurons. Scale bars = 6.8μm.

2.3.5. Glia Exhibit A More Flexible Cell Cycle Exit

We next asked, how flexible is cell cycle exit in glial cells compared to neurons? To ask this, we forced re-entry in glia using Repo-GAL4 to drive the same combinations of transgenes to force re-entry as in neurons. Pupae were collected at the 0hWPP stage and were shifted up to 29ºC to induce expression of respective transgenes. At 24h APF, the overexpression of E2F/DP+CycD/CDK4 and E2F/DP+Rca1 increases the proportion of cells in mitosis in the OL and the CB regions of the brain (Figure 2.5A-C'', J). Glial expression of E2F/DP+Rca1 shows a

greater number of glial mitoses in the CB and OL at 24h APF, and a more mitoses in the CB than overexpression of E2F/DP+CycD/CDK4 at 48h APF (Figure 2.5D-F’’’, K).

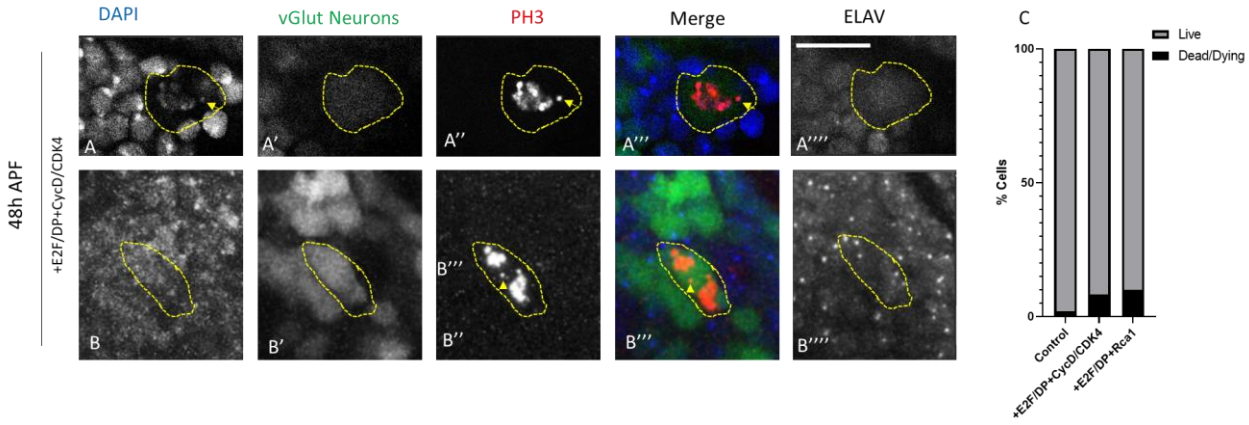

Figure 2.4 Forcing cell cycle re-entry in neurons results in abnormal mitoses and cell death

Neurons forced to re-enter the cell cycle by overexpression of E2F/DP +CyCD/CDK4 exhibit show abnormal mitoses such as (A-A’’’’) micronucleus formation and (B-B’’’’) lagging chromosomes. (C) Overexpression of cell cycle regulators results in cell death measured by propidium iodide incorporation by flow cytometry. Brains overexpressing E2F/DP+CycD/CDK4 or E2F/DP+Rca1 in vGlut neurons exhibit greater levels of cell death than control brains at 48h APF. Scale bars = 6.8µm.

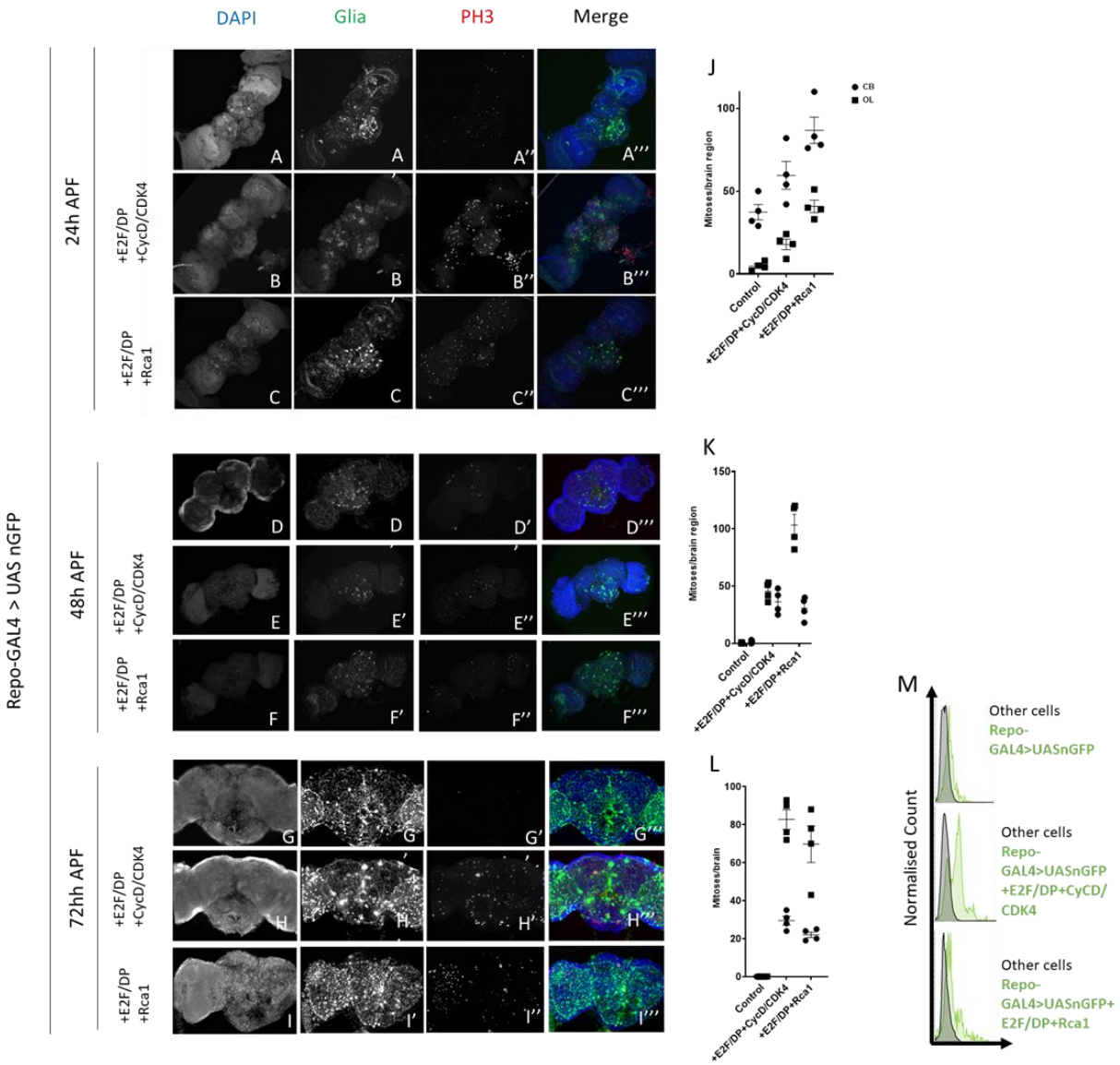
DAPI
Glia
PH3
Merge
Repo-GAL4 > UAS nGFP
24h APF
48h APF
72hh APF
+E2F/DP
+CycD/CDK4
+E2F/DP
+Rca1
J
K
L
M
CB
OL
Mitoses/brain region
Control
+E2F/DP+CycD/CDK4
+E2F/DP+Rca1
Mitoses/brain
Normalised Count
Other cells
Repo-GAL4>UASnGFP
Other cells
Repo-GAL4>UASnGFP +E2F/DP+CyCD/CDK4
Other cells
Repo-GAL4>UASnGFP+ E2F/DP+Rca1

Figure 2.5 Glia exhibit a more flexible cell cycle exit

Brains from pupae expressing Repo-GAL4, UASnGFP, Tub-GAL80TS only (A-A’’’, D-D’’’), (G-G’’’) or Repo-GAL4, UASnGFP, Tub-GAL80TS+ UAS-E2F, UAS-DP, UASCyClinD, UAS-CDK4 (B-B’’’) (E-E’’’) (H-H’’’) or Repo-GAL4, UASnGFP, Tub-GAL80TS+ UASE2F, UAS-DP, UAS-Rca1 (C-C’’’) (D-D’’’)(H-H’’’) were shifted up to 29ºC at 0h after puparium formation to induce expression of respective transgenes. Pupal brains were dissected at indicated timepoints, and stained for mitoses (anti-phospho histone H3) and DAPI. Number of mitoses per brain region were counted at 24, 48 and 72h APF (J, K, L). (G-I’’’, L, M) Glia show greater level of flexibility and continue to undergo mitosis at 72hAPF in the presence of cell cycle regulators. Overexpression of E2F/DP+CycD/CDK4 and E2F/DP+Rca1 increases the proportion of cells with >2C DNA content from <3% in control animals to >6-7% (M).

Unlike neurons, glia continue to undergo mitosis at 72h APF when overexpressing E2F/DP+CycD/CDK4 or E2F/DP+Rca1, suggesting that the flexible period of G_0 in glia lasts a longer duration than in neurons. Interestingly, at 72h APF, both E2F/DP+CycD/CDK4 and E2F/DP+Rca1 drive mitosis in glia at the same levels in both the OL and CB (Figure 2.5G-I'''). Flow cytometry analysis of DNA content (M) in GFP expressing glial cells under different conditions at 72h APF shows that a greater proportion of cells Repo-GAL4 cells driving E2F/DP+CycD/CDK4 (6.5%) or E2F/DP+Rca1 (7.9%) have >4C DNA content than control (<3%). This suggests that forcing cell cycle re-entry can result in polyploid glia in the pupal brain.

2.3.6. **Forcing Re-entry In Glia Can Result In Glial Tumor-like Growth.**

Consistent with the observation that forcing glial cell cycle re-entry results in prolonged cycling in these cells, we also observe tumor-like masses in pupa brains. In 72h APF brains with glial overexpression of E2F/DP+CycD/CDK4 , we observe clones of GFP expressing cells with multiple nuclei (Figure 2.6, yellow dashed outline) as well as cells with large nuclei (Figure 2.6,yellow arrowhead) which are presumably polyploid glial cells. The depth of image section suggests that the large glial cells are not SPG (which are on the surface of the brain), and instead polyploid mitotic nuclei resulting from forced cell cycle re-entry.

2.3.7. **Most Of The Glia Re-Entering The Cell Cycle To Undergo Mitosis Are Cortex Glia**

In previous experiments we observed maximum intensity projections of the brain to visualize mitoses in the whole brain. Closer examination of individual Z-sections reveals that most of the glia which re-enter the cell cycle to undergo mitosis are a specialized subtype of glia termed cortex glial cells. Representative single Z slice images showing mitotic cortex glia in the

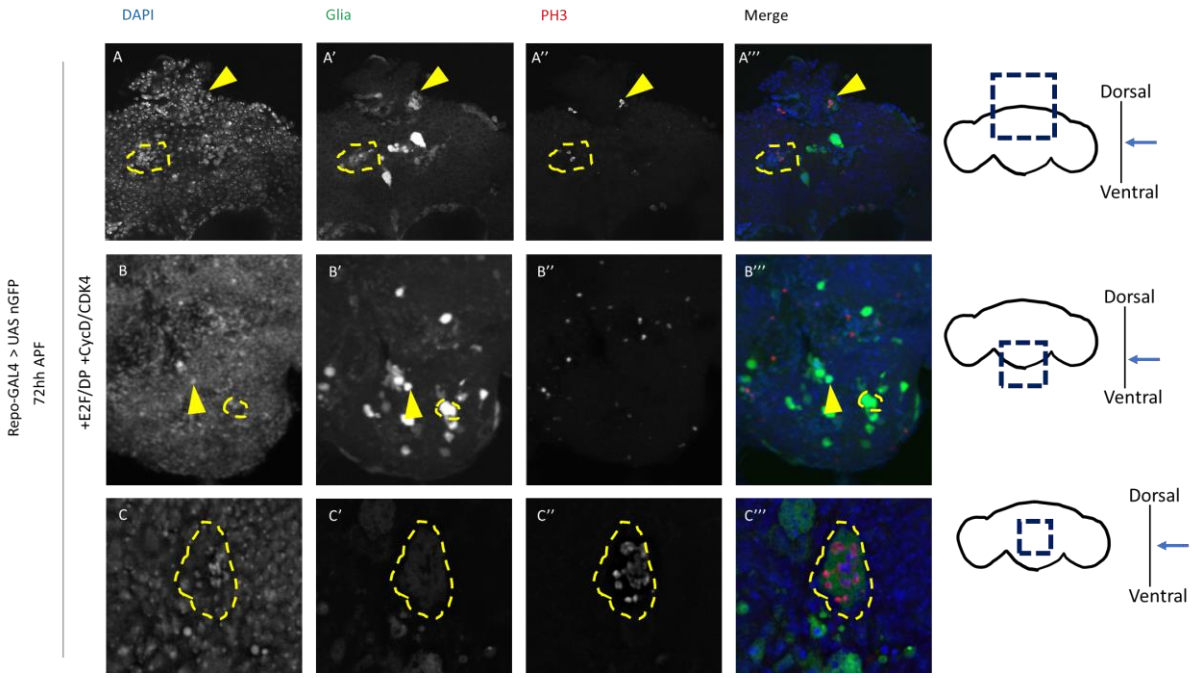

Figure 2.6 Forcing re-entry in glia can result in glial tumors-like growth .

(A-C’’’) Prolonged overexpression of cell cycle regulators in glia can result in glial tumors with multiple cells (yellow dashed outline) and large mononucleate, presumably polyploid glial cells (yellow arrowhead). Depth of image section suggests that the large glial cells are not SPG.

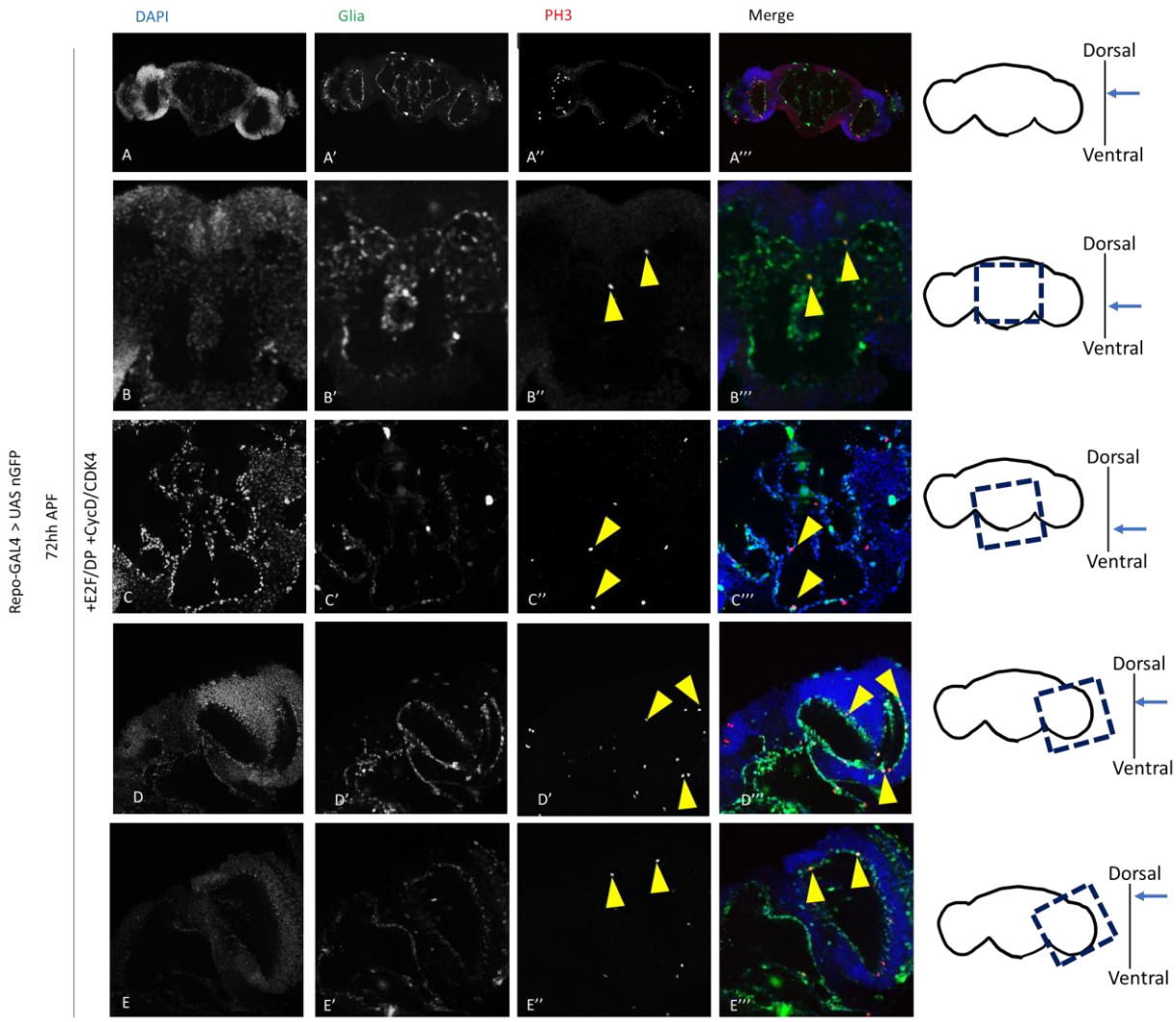

Figure 2.7 Most of the glia re-entering the cell cycle to undergo mitosis are cortex glia

(A-E’’’) Representative single Z slice images showing mitotic cortex glia in the central brain (A, B,C) and in the optic lobe (A,D,E). Cartoons on the right of each image shows the depth of Z section. Most mitotic glia (yellow arrowheads) appear to be cortex glial cells based on position and morphology.

central brain (Most of the glia re-entering the cell cycle to undergo mitosis are cortex glia A, B,C) and in the optic lobe (Most of the glia re-entering the cell cycle to undergo mitosis are cortex glia A,D,E). Cartoons on the right of each image shows the depth of Z section. Most mitotic glia (yellow arrowheads) appear to be cortex glial cells based on position and morphology and location.

2.4. Discussion

This study shows that neurons and glia exhibit different levels of flexibility in their respective postmitotic states. We show that both neurons and glia can be forced to re-enter the cell cycle after they have exited the cell cycle, and that neurons have a shorter window of 'flexibility' during which forced re-entry can drive neuronal mitoses. Glia can be forced to cycle for a longer duration, and glial cells undergoing mitoses can even form tumor like masses.

2.4.1. What Regulates The Difference In Flexibility Between Neurons And Glia?

. This study shows that in the Drosophila brain, different types of cells as well as different brain regions show different levels of flexibility in G_0. Glia in the CNS exhibit the most flexibility. They can be pushed to re-enter the cell cycle with either direct or indirect activation of Cyclin/Cdks and the E2F/DP complex for up to 48-72h past their normal terminal cell division. When they eventually exit from the prolonged proliferation they exhibit an increased rate of polyploidy, indicating a possible entry into variant cell cycles, and exhibit features of cellular hypertrophy and multinucleation.

By contrast, neurons exhibit less flexibility in their G_0 state. vGlut neurons of the optic lobes and VNC can only be pushed to divide with direct activation of Cyclin/Cdks and the E2F/DP complex, within about 24 hours of their normal terminal division. Interestingly, vGlut

neurons of the central brain can be pushed to re-enter the cell cycle for a limited window of division simply by inhibition of the APC/C together with overexpression of E2F/DP. This suggests that vGlut neurons in the pupal central brain retain higher levels of endogenous Cyclin/Cdk complexes than those of other regions, and that the higher Cyclin/Cdk levels of the central brain are normally kept in check by APC/C -dependent degradation. Whether these different levels of Cyclin/Cdk complexes are correlated with the function of these neurons and the normal timing of their terminal division is an important issue to address in future studies.

Recent work has shown that changes at the level of chromatin could underlie the flexibility of the postmitotic state (Ma and Buttitta, 2017; Ma et al., 2015, 2019). Studies of chromatin accessibility at cell cycle genes during terminal differentiation with and without compromised cell cycle exit suggest that a subset of key cell cycle genes which play rate-limiting roles is selectively changed. These genes: Cyclin E which plays a rate limiting role at the G1-S transition and the cdc25c phosphatase String, which regulates the G2-M transition have complex enhancers, and their accessibility is selectively modified robust cell cycle exit by developmental signals. This study was performed in the fly wing. Does the same rubric apply to other cells and/or tissues? How do systemic developmental timers impinge upon the cell cycle machinery in a context specific manner? The difference in flexibility between neurons and glia suggests that other factors may mediate the way developmental signals affect chromatin at cell cycle genes. Could these be cell type-specific chromatin remodeling and/or cell fate factors driving changes in transcription of cell cycle genes? These are exciting open avenues for future studies.

2.4.2. **What Underlies The Flexibility Of Cortex Glia?**

Most of the glia exhibiting additional divisions in response to cell cycle re-activation are cortex glia. The functions of cortex glia are well understood in the context of early larval

development (Hartenstein, 2011), however their functions during and after metamorphosis remain relatively unknown. The cortex glial cells are born during early larval stages and ensheath individual neuronal soma in the larval brain to provide trophic support (Ito et al., 1995). In later stages of development, they are known to interact closely with the sub-perineurial glia which form the blood-brain barrier (Kremer et al., 2017). Some cortex glia also ensheath the mushroom body neuroblasts and serve a niche function (Doyle et al., 2017). Recent evidence also suggests an important role for cortex glia in maintaining neuronal firing properties (Melom and Littleton, 2013). Adult mutant flies lacking cortex glia exhibited seizure-like phenotypes (Weiss et al., 2019). It would be interesting to explore how compromising cell cycle exit in these glia during development affects adult physiology.

2.4.3. **Why Are Neuronal Mitoses Often Catastrophic?**

Forced cell cycle re-entry in neurons often leads to abnormal mitoses and cell death, suggesting that maintaining a non-dividing state is critical for neuronal survival. The mid-pupal stage is known to be critical for development of the adult fly brain and is marked by gross morphological changes as well as cellular-level changes such as axon pruning. This period of development may be critical for immature neurons to find their respective pre and post-synaptic partners and form networks of synaptic connections which are potentially critical for future brain function.

It is thought that mitosis and the neuronal state is incompatible as mitosis involved cellular rounding and the potential loss of synaptic connections. But what exactly makes a neuron refractory to cell division cues remains unknown! Do structural changes that a neuron undergoes in the process of maturation underlie its robustness? Are key factors necessary for successful mitosis sequestered away in neurons, or are neurons somehow programmed to

undergo cell death if forced to divide after a certain developmental stage? The implications of understanding exactly how neurons achieve a robust G_0 extend beyond developmental biology to potential therapeutic applications.

The next chapter will explore G_0 in adult fly brains, and how it changes with age.

2.5. Materials and Methods

Reagent	Comment	Source
w;OK-371-GAL4, UAS-nGFP/Cyo-GFP; Tub-GAL80$^{TS/TM6B}$	vGlut Driver	Buttitta Lab Stocks
W;UAS-nGFP/Cyo-GFP;Repo-GAL4,Tub-GAL80TS/TM6B	Pan-glial driver	Buttitta Lab Stocks
y,w,hs-FLP;+;+	'Control'	Buttitta Lab Stocks
y,w,hs-FLP;UAS-E2F,UAS-DP/CyO-GFP;UAS-CycD,UAS-CDK4/TM6B	E2F/DP+CycD/CDK4 overexpression	Buttitta Lab Stocks
y,w,hs-FLP;UAS-E2F,UAS-DP/CyO-GFP;UAS-Rca1/TM6B	E2F/DP+Rca1 overexpression	Buttitta Lab Stocks
w;;nSyb-GAL4	Pan-neuronal driver	Dus Lab
Anti-PH3	1:500	Millipore sigma
Anti-ELAV	1:100	DHSB
DAPI	1:1000	Sigma Aldrich
DyeCycle Violet	1:500	ThermoFisher
Propidium Iodide	2.25:1000	Sigma-Aldrich
Alexa Fluor 633 goat anti-mouse	1:1000	ThermoFisher
Alexa Fluor 568 goat anti-rabbit	1:1000	ThermoFisher

Table 2.1 Key stocks and Reagents

2.5.1. **Fixation, Immunostaining and Imaging**

Drosophila brains were dissected in 1X Phosphate buffered saline (PBS) and fixed in 4% Paraformaldehyde (PFA) in 1X PBS for 25 minutes. Tissues were permeabilised in 1X PBS+0.1% Triton-X, blocked in 1X PBS, 1% BSA 0.1% Triton-X. (PAT) Antibody staining was performed at specified concentrations in PAT (Supplementary File 1) overnight at 4°C, washed, blocked in PBT-X (1X PBS, 2% Goat serum 0.1% Triton-X) prior to incubation with secondary antibody either for 4h at RT or overnight at 4°C. DAPI staining was performed after washes, brains were wet-mounted in vectashield H1000. All imaging was performed on either a Leica SP5 scanning confocal or DMI6000 microscopes..

2.5.2. **Fly Husbandry**

Crosses were set up at room temperature and flies were flipped onto fresh food once every day to ensure that vials were not overcrowded. Progeny were collected as WPP and screened for balancers, then shifted to 29ºC and dissected at the specified timepoints. Since pupal development occurs 1.2 times faster at 29ºC, therefore 24h APF corresponds to 20h, 48APF to 40h and so on..

2.5.3. **Image Quantification**

Number of mitoses were quantified manually from maximum intensity projections of whole brains imaged at 10 or 20x magnifications. Images obtained on DMI6000 fluoresecence microscopes were deconvolved prior to quantification to remove background.

2.5.4. **Flow Cytometry**

Fly brains were dissected in PBS and transferred to 1.5mL microcentrifuge tubes containing 1000uL of a 9:1 Trypsin-EDTA :10XPBS with 1µL Dyecycle Violet and/or 1.12µL PI solution.. Brains were incubated with shaking at 800rpm on a benchtop thermomixer at 25ºC for 45 minutes and vortexed gently every 20 minutes, prior to analysis on the Attune Flow cytometer. The Attune had a laser configuration of a violet laser (VL,405nm) with 6 bandpass (BP) filters and a blue laser (BL,488nm) with 3 bandpass filters. Detection of DyeCycle Violet was performed using VL1 (Emission filter 450/40), GFP using BL1 (Emission filter 530/30), and PI using BL2 (Emission filter 574/24) . A flow rate of 100 to 500 µl/second was used for sample acquisition and a minimum of 20,0000 events gated as 'non doublets' (Figure 1-figure supplement 1) were acquired per sample. Gating Strategy is graphed in Figure 3.2Figure 3.2. Briefly, all cells were plotted on forward vs side scatter (FSC vs. SSC), gated to eliminate debris. Subsequently, 'non-debris' were plotted on VL1(DNA) vs FSC and gated to eliminate unstained events. A third gate was applied plotting VL1(DNA)-H vs VL1(DNA)-A (voltage pulse area vs.height) to eliminate doublets. All events in gate 3 were further subjected to GFP/DNA/PI content analysis.

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Chapter 3. Polyploidy In The Adult *Drosophila* Brain

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3.1. Abstract

Long-lived cells such as terminally differentiated postmitotic neurons and glia must cope with the accumulation of damage over the course of an animal's lifespan. How long-lived cells deal with ageing-related damage is poorly understood. Here we show that polyploid cells accumulate in the adult fly brain and that polyploidy protects against DNA damage-induced cell death. Multiple types of neurons and glia that are diploid at eclosion, become polyploid in the adult *Drosophila* brain. The optic lobes exhibit the highest levels of polyploidy, associated with an elevated DNA damage response in this brain region. Inducing oxidative stress or exogenous DNA damage leads to an earlier onset of polyploidy, and polyploid cells in the adult brain are more resistant to DNA damage-induced cell death than diploid cells. Our results suggest polyploidy may serve a protective role for neurons and glia in adult *Drosophila melanogaster* brains.

3.2. Introduction

Terminally differentiated postmitotic cells such as mature neurons and glia are long-lived and must cope with the accumulation of damage over the course of an animal's lifespan. The mechanisms used by such long-lived cells to deal with aging-related damage are poorly understood. The brain of the fruit fly *Drosophila melanogaster* is an ideal context to examine this since the fly has a relatively short lifespan and the adult fly brain is nearly entirely postmitotic with well understood development and excellent tools for genetic manipulations.

The adult central nervous system of *Drosophila melanogaster* comprises ~110,000 cells, most of which are generated in the larval and early pupal stages of development from various progenitor cell types (Truman and Bate, 1988; White and Kankel, 1978). By late metamorphosis, the *Drosophila* pupal brain is normally completely non-cycling and negative for markers of proliferation such as thymidine analog incorporation and mitotic markers (Awasaki et al., 2008; Pahl et al., 2019; Siegrist et al., 2010)

In the adult, very little neurogenesis and gliogenesis are normally observed (Awasaki et al., 2008; Ito and Hotta, 1992; von Trotha et al., 2009). A population of about 40 adult neural progenitors has been reported in the optic lobe and a population of glial progenitors has been reported in the central brain (Fernández-Hernández et al., 2013; Foo et al., 2017). Upon damage or cell loss, hallmarks of cycling have been shown to be activated, although the overall level of proliferation in the adult brain remains very low (Crocker et al., 2020; Fernandez-Hernandez et al., 2019; Fernández-Hernández et al., 2013; Foo et al., 2017; Li et al., 2020). Thus, the brain of the adult fly is thought to be almost entirely postmitotic with most cells in G0 with a diploid (2C) DNA content. One known exception to this are the cells that constitute the "blood-brain barrier" of *Drosophila.*

The “blood-brain barrier” in *Drosophila* is made up of specialised cells called the Sub-perineurial glia (SPGs). These cells are very few in number and achieve growth without cell division by employing variant cell cycles termed endocycles, that involve DNA replication without karyokinesis or cytokinesis, as well as endomitotic cycles that involve DNA replication and karyokinesis without cytokinesis (Von Stetina et al., 2018; Unhavaithaya and Orr-Weaver, 2012). The SPGs undergo these variant cell cycles to increase their size rapidly to sustain the growth of the underlying brain during larval development. The polyploidisation of these cells plays an important role in maintaining their epithelial barrier function, although it remains unclear whether these cells continue to endocycle or endomitose in the adult.

Polyploidy can also confer an increased biosynthetic capacity to cells and resistance to DNA damage induced cell death (Edgar and Orr-Weaver, 2001; Lee et al., 2009; Mehrotra et al., 2008; Zhang et al., 2014). Several studies have noted neurons and glia in the adult fly brain with large nuclei (Robinow and White, 1991; Winberg et al., 1992) and in some cases neurons and glia of other insect species in the adult CNS are known to be polyploid (Nordlander and Edwards, 1969). Rare instances of neuronal polyploidy have been reported in vertebrates under normal conditions (Morillo et al., 2010) and even in the CNS of mammals (López-Sánchez and Frade, 2013; Shai et al., 2015).

Polyploidisation is employed in response to tissue damage and helps maintain organ size (Cohen et al., 2018; Tamori and Deng, 2013) (Losick et al., 2013, 2016). Therefore, polyploidy may be a strategy to deal with damage accumulated with age in the brain, a tissue with very limited cell division potential. Here we show that polyploid cells accumulate in the adult fly brain and that this proportion of polyploidy increases as the animals approach middle-age. We show that multiple types of neurons and glia which are diploid at eclosion which become

polyploid specifically in the adult brain. We have found that the optic lobes of the brain contribute to most of the observed polyploidy. We also observe increased DNA damage with age, and show that inducing oxidative stress and exogenous DNA damage can lead to increased levels of polyploidy. We find that polyploid cells in the adult brain are resistant to DNA damage-induced cell death and propose a potentially protective role for polyploidy in neurons and glia in adult brains.

3.3. Results

Cell ploidy often scales with cell size and biosynthetic capacity (Edgar and Orr-Weaver, 2001; Orr-Weaver, 2015). The brain is thought to be a notable exception to this rule, where the size of postmitotic diploid neurons and glia can be highly variable. We wondered whether alterations in ploidy during late development or early adulthood may contribute to the variability in neuronal and glial cell size in the mature *Drosophila* brain. We therefore developed a sensitive flow cytometry assay to measure DNA content in *Drosophila* pupal and adult brains. Briefly, this assay involves dissociating brains with a trypsin or collagenase based solution followed by quenching the dissociation and labeling DNA with DyeCycle Violet (Grushko and Buttitta, 2015) in the same tube, to avoid cell loss from washes. Samples are then immediately run live on a flow cytometer for analysis. We employed strict gating parameters to eliminate doublets (Figure 3.2) (López-Sánchez et al., 2017a). This assay is sensitive enough to measure DNA content from small subsets of cells (e.g. *Mz19-GFP* expressing neurons) from individual pupal or adult brains (Figure 3.2D). Using this approach we confirmed that under normal culturing conditions, cell proliferation and DNA replication ceases in the pupal brain after 24h into metamorphosis (24h APF) (Figure 3.2E), and that only the previously described mushroom body neuroblasts continue to replicate their DNA and divide in late pupa (Siegrist et al., 2010). The

brains of newly eclosed adult flies are 98-99% diploid and we, like others, only rarely observe EdU incorporation during the first week of adulthood in wild-type flies under normal conditions but not later in adulthood (Figure 3.2F.) (Fernández-Hernández et al., 2013; Foo et al., 2017; Kato et al., 2009; Siegrist et al., 2010; von Trotha et al., 2009). We were therefore surprised to find a distinct population of cells with DNA content of 4C and up to >16C appearing in brains of aged animals of various genotypes under normal culture conditions (Figure 3.2G).

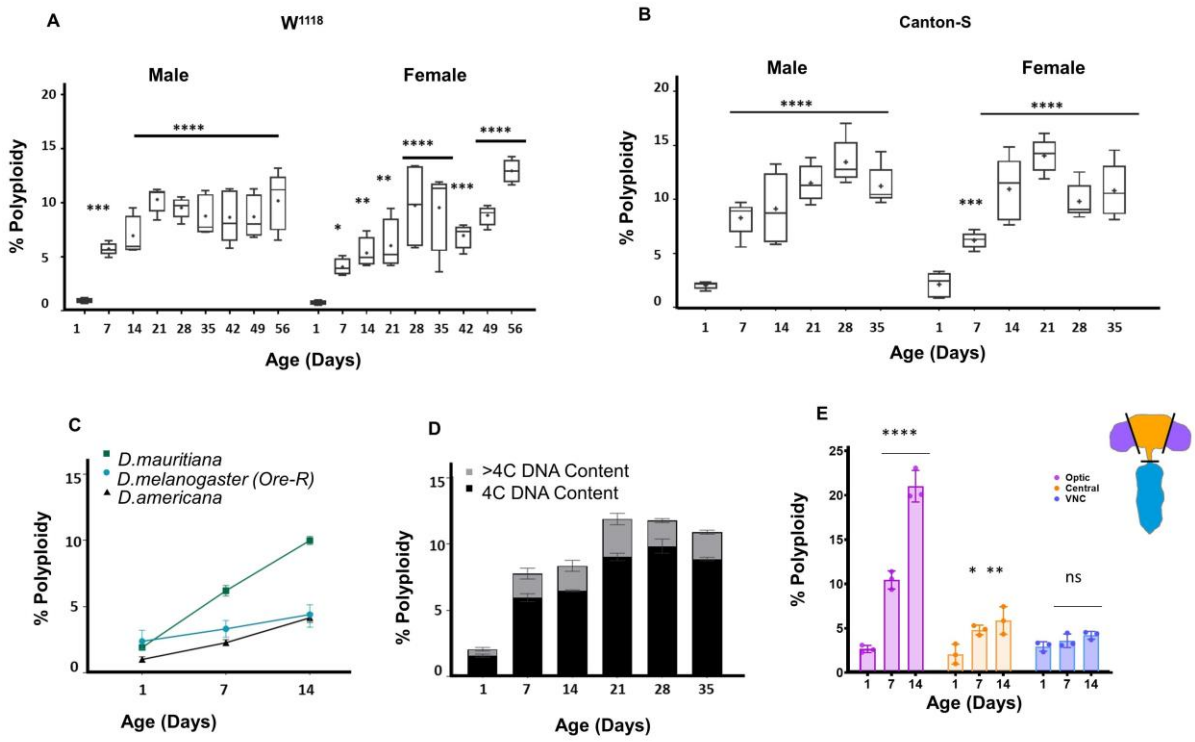


Figure 3.1 Polyploid cells accumulate in the adult Drosophila brain

(A,B) Percentage of cells in individual brains exhibiting polyploidy in w^{1118} (A) and *Canton-S* (B) male and female whole brains. Age in days indicates days post-eclosion. Box plots showing range, dot indicates mean (n=10).(Two-way ANOVA with greenhouse-geisser correction for unequal SDs followed by Holm-Sidak's multiple comparisons test. P values: ns > 0.1234; <0.0332 *; <0.0021 **; <0.0002 ***; **** <0.0001) (C) Accumulation of polyploidy is also observed in other *Drosophila* species. *D.mauritiana and D.americana* shown respectively in green and black compared to *Oregon-R* (*D.melanogaster)* shown in teal at different time points post-eclosion. Shapes indicate mean polyploidy observed, bars show range. 3 brains each per sample, n=2 per time point. (D) Stacked bar plot showing proportion of polyploid cells with tetraploid or 4C DNA content (black) and greater than tetraploid or >4C DNA content (grey) in *Canton-S* males at different ages. (E). Percentage of polyploidy in Optic lobes (OL shown in purple), central brain (CB, shown in orange) and ventral nerve cord (VNC shown in blue) at different ages, w^{1118} (Error bars show mean±SEM n=3).

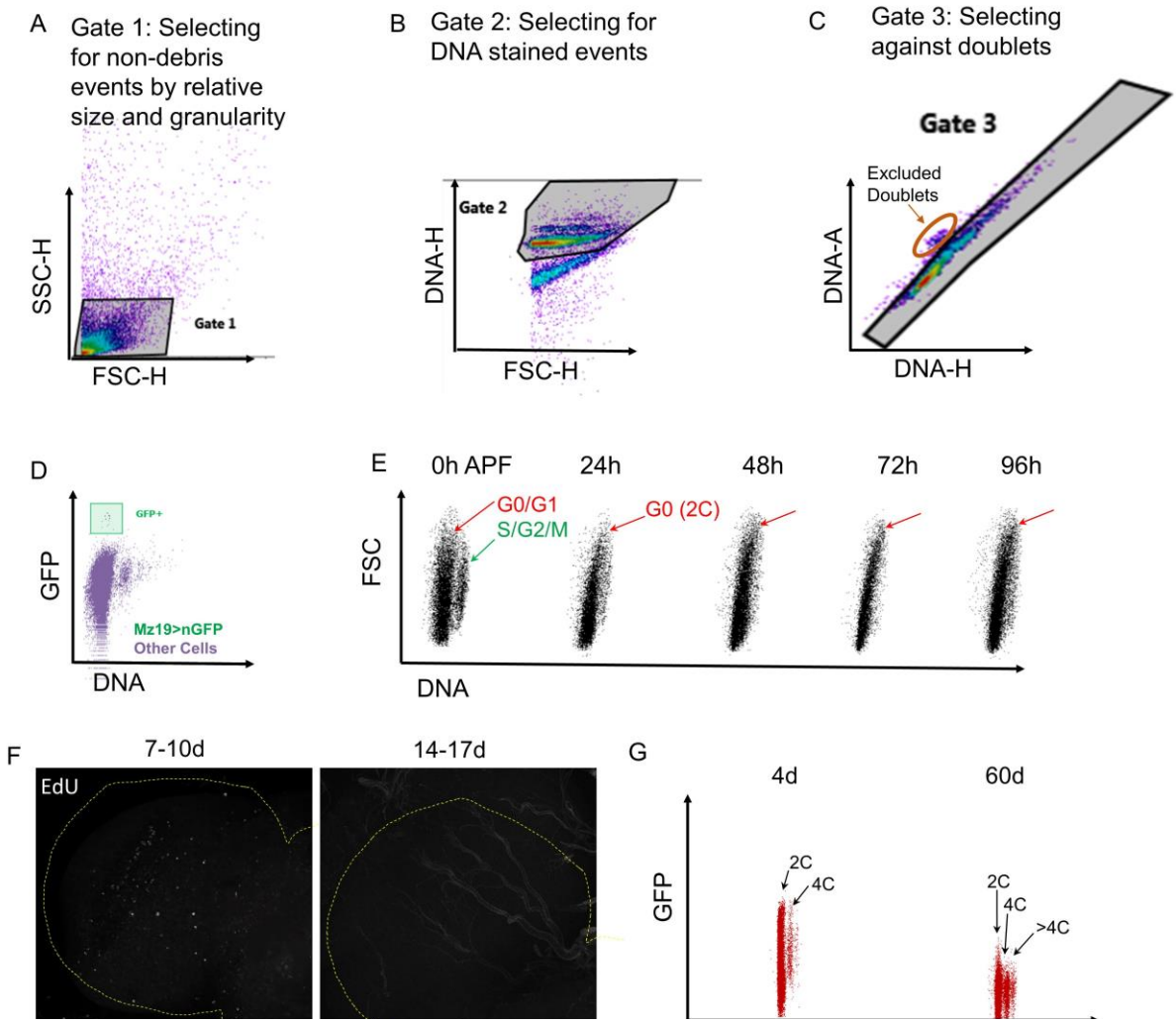


Figure 3.2 Gating Strategy

(A-C) Flow Cytometry gating strategy to ensure doublet discrimination. (D) The small population of *mz19-GAL4, UAS nGFP* labelled neurons are detectable by flow cytometry assay. (E) Dot plots showing DNA content during metamorphosis in *Drosophila melanogaster* w^{1118} brains. APF = after puparium formation. Cell cycle exit in most cells occurs by 24h APF. (F) Sparse EdU labelling observed in OL (outlined with yellow dotted line) before 10d but not after 2 weeks in the adult brain. (G) Increased DNA content observed in 60d old brains compared to 4d. Dot plots showing all cells in *Elav-GAL4, UAS-nGFP* brains at each time point Polyploid cells indicated as 4C and >4C. *Elav-GAL4* driver shows weak expression in older brains.

3.3.1. **Polyploid Cells Accumulate In The Adult *Drosophila* Brain**

We performed a systematic time-course to measure accumulation of polyploid cells in the adult brain in isogenic w^{1118} male and female flies cultured under standard conditions (Linford et al., 2013). We measured the percentage of polyploid cells in individual brains from the day of eclosion until 56 days (8 weeks) at weekly intervals. Polyploid cells appear as early as 7 days into adulthood, and the proportion of polyploidy continues to rise until animals are 21 days old (Figure 3.1A). This increase in polyploidy is only observed until week 3, after which the proportion of polyploid cells observed remains variable from animal to animal, but on average, does not increase. We observe similar patterns of polyploidy accumulation in males and females (Figure 3.1A). To ensure that the polyploidy we observe is not an artefact of one particular strain, we performed similar measurements across the lifespan in other commonly used lab 'wild-type' strains *Canton-S* (Figure 3.1B) and *Oregon-R* (Figure 3.1Figure 3.2C). Interestingly, *Oregon-R* flies show lower levels of polyploidy in the first two weeks than w^{1118} and *Canton-S* suggesting that different genetic backgrounds may influence polyploidy in the brain. We also performed DNA content measurements of brains from the distantly related *D.americana* which diverged ~50 million years ago and a more closely related species, *D.mauritiana*, which diverged ~2 million years ago (Figure 3.1C). While both species show accumulation of polyploidy, it is interesting to note that they show differences in levels of polyploidy.

We next measured changes in ploidy in the *D.melanogaster* adult brain over time. We pooled data from multiple animals and binned polyploid cells from w^{1118} brains into two categories: cells with 4C (tetraploid) DNA content measured by flow cytometry and cells with >4C DNA content - this includes 8C, 16C and even some 32C cells (D). The majority of the

polyploid cells appear to be tetraploid, and the fraction of cells exhibiting >4C DNA content increases during the first week of adulthood, but remains relatively consistent with age.

We next asked whether polyploid cells are located in a specific region of the brain. We dissected the *Drosophila* central nervous system (CNS) into the central brain, optic lobes, and ventral nerve cord (VNC) and measured levels of polyploidy in each region from day of eclosion to 2 weeks into adulthood (Fig 1E). We found that while there is a low level of polyploidy in the central brain and VNC that increases with age, most of the polyploidy comes from the optic lobes. Strikingly, by 3 weeks, over 20% of the cells in the optic lobes can exhibit polyploidy.

Since the optic lobes contribute to most of the polyploidy observed, we wondered if this phenomenon may be dependent on light. *Canton-S* animals reared in complete darkness did not show difference in polyploidy compared to age-matched controls raised in regular 12-hour light / 12-hour dark cycles (Figure 3.3A). Next, we hypothesized that polyploidy accumulation may depend on proper photoreceptor function. However, $glass^{60j}$ flies devoid of photoreceptors and pigment cells in compound eyes (Helfrich-Förster et al., 2001) still show polyploidy Figure 3.3B).

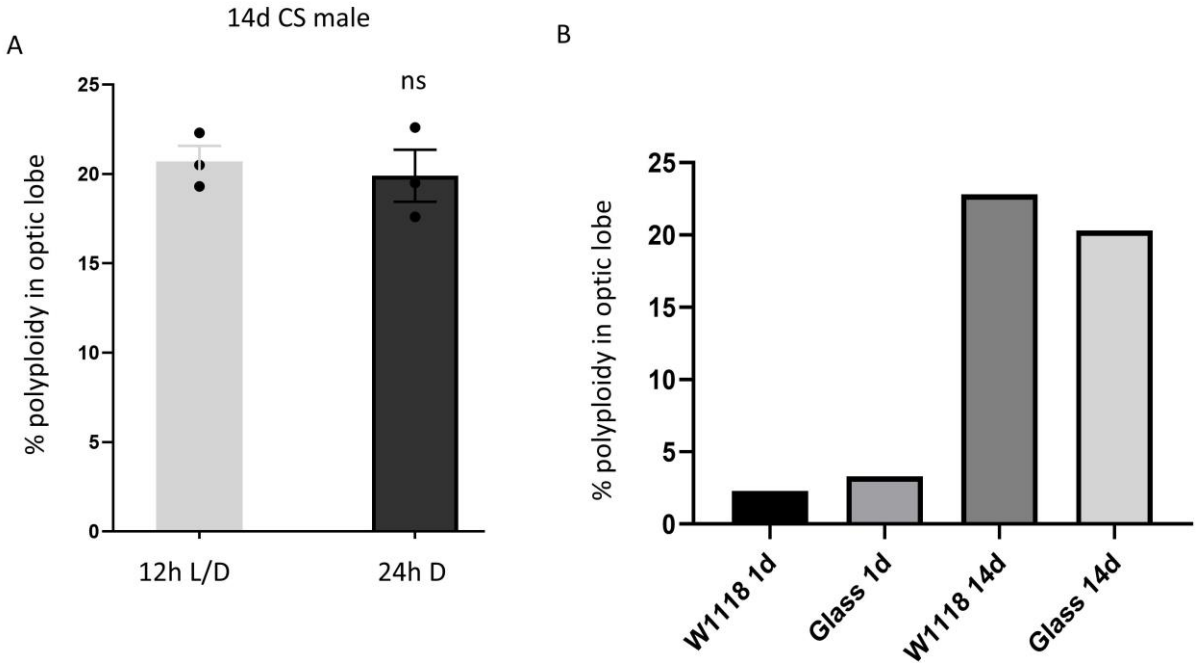


Figure 3.3 Polyploidy in the optic lobes is not light or photoreceptor-dependent

(A) 14d *Canton-S* (CS) males reared in 12h L/D cycles do not show significantly different polyploidy compared to age-matched CS males reared in 24h darkness from eclosion. Error bars = mean±SEM; unpaired t-test with Welch's correction. (B) Percentage of polyploidy observed in *glass*^{60j} mutants lacking photoreceptors and age-matched *w*^{1118} controls at 1 and 14 days. Bars show mean % polyploidy in samples containing 3 pooled brains.

3.3.2. **Multiple Cell Types Exhibit Adult-Onset Polyploidy In The Brain**

To identify which cell types in the brain are becoming polyploid, we used the binary GAL4/UAS system to drive the expression of a nuclear-localised green or red fluorescent protein (nGRP or nRFP) with cell type-specific drivers. We then measured DNA content using Dye-Cycle Violet in the GFP or RFP-positive populations.

We first examined the SPGs, as previous work from the Orr-Weaver lab identified these to be highly polyploid (Von Stetina et al., 2018; Unhavaithaya and Orr-Weaver, 2012). When we used the SPG driver *moody-GAL4,* we found that the SPGs are highly polyploid (Unhavaithaya and Orr-Weaver, 2012), but contributed to less than 5% the polyploid cells observed in mature adult brains (Figure 3.4A,C). Another class of cells previously shown to be polyploid in some contexts are tracheal cells that carry oxygen to tissues (Zhou et al., 2016). Using the tracheal driver *breathless-GAL4*, we found that in 10 day old adult brains, tracheal cells comprise less than 3% of all polyploid cells (Figure 3.4B,C). Thus, 90% of the polyploid cells we observe in the brain arise from cell types not previously known to become polyploid.

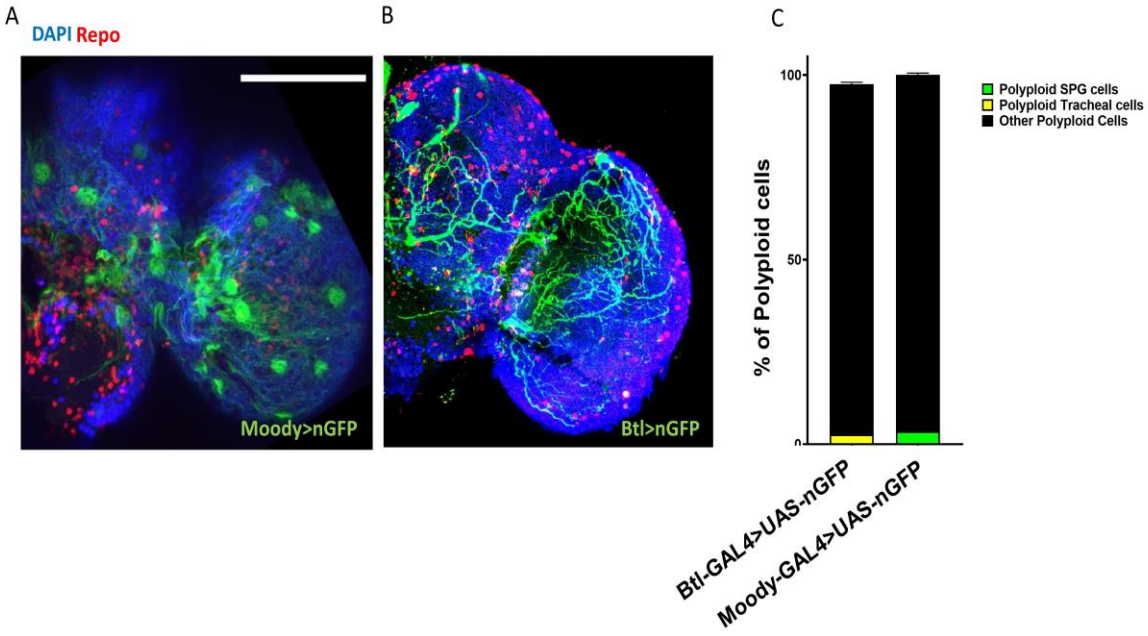


Figure 3.4 Trachea and Sub-perineurial glia comprise less than 5% of all polyploid cells

(A,B) Micrographs showing expression pattern of *moody-GAL4* (A) and (Breathless) *btl-GAL4* (B) in the adult brain to label SPGs and tracheal cells respectively. (C) Flow cytometry based quantification showing the contribution of Btl-GAL4 (yellow) and *moody-GAL4* (green) driving cells to total polyploidy observed in 10d adult brains.

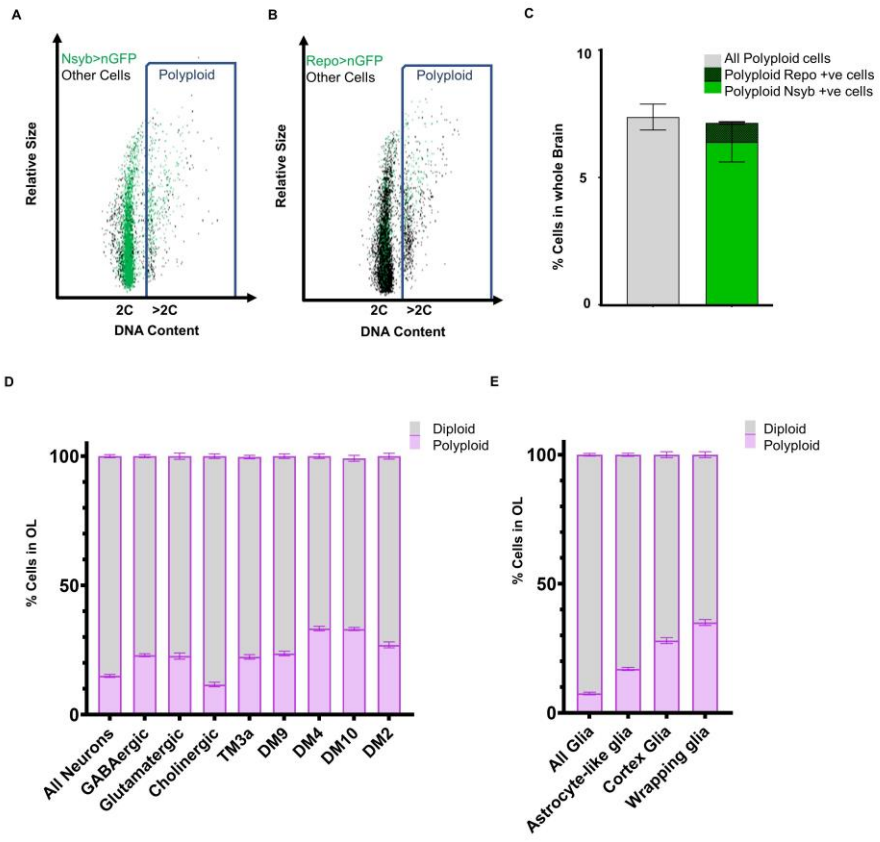


Figure 3.5 Identification of various neuronal and glial cell types that become polyploid in the adult brain

Representative flow cytometry dot plots showing polyploid neuronal (A) and glial (B) cells in 2 week old male brain (A) Neuronal nuclei are labelled using *nsyb-GAL4*, *UAS-nGFP*, neuronal cells are shown in the dot plot as green dots and 'other' cells unlabelled by *nsyb-GAL4* are shown in black. Blue rectangle highlights cells with polyploid or >2C DNA content (B) Glial nuclei are labelled using *Repo-GAL4*, *UAS-nGFP*, glial cells are shown in the dot plot as green dots and 'other' cells unlabelled by Repo-GAL4 are shown in black. Blue rectangle highlights cells with polyploid or >2C DNA content. (C) Plot showing proportion of polyploid neurons (bold green) and polyploid glia (checked green) at 2 weeks compared to total polyploidy in the brain in w^{1118} control (grey) (error bars show mean ± SEM, n=3). (D) Proportion of polyploidy observed at 7 days in the optic lobes in various classes of neurons (D) and glia (E). Stacked bar plot showing mean ± SEM; percentage of polyploidy (purple) and diploidy (grey) per sample,

each sample contains pooled OLs from 3 or more brains; n=3. Proportions of cells also represented as tables in Table 3.1 and Table 3.2.

Cell Type	**% of total cells**	**% polyploid**	**% of total polyploidy**
All Neurons	91%	5.8%	84.7%
All Glia	8%	6.3%	13.2%
Tracheal cells	0.3%	100%	2.8%
Sub Perineurial Glia	1%	100%	4.5%

Table 3.1 Proportions of cell types polyploid in the whole brain

Table 3.2 Types of neurons and glia polyploid in the OL

Cell Type	% of total cells	% polyploid	% of total polyploidy
All neurons	86%	15%%	85%
GABAergic neurons	58%	23%	54%
Glutamatergic neurons	35%	22.6%	33%
Cholinergic neurons	25%	11.7%	15%
TM3a	3.2%	22.3%	3.7%
DM9	1.3%	23.6%	2.2%
DM4	1.3%	33.33%	0.7%
DM10	5%	33.1%	2.8%
DM2	1.9%	27%	1.6%
All glia	7%	7.5%	13%
Astrocyte-like glia	2%	17%	1.1%
Cortex Glia	5.6%	28%	7%
Wrapping glia	3%	35%	5%

The adult fly brain is thought to be composed almost entirely of neurons (90% of total population) and glia (10% of total population). First, we asked if neurons become polyploid by using a pan-neuronal driver, *nSyb-GAL4* to drive *UAS-nGFP* (Figure 3.5Figure 3.5 Identification of various neuronal and glial cell types that become polyploid in the adult brainA). We found that indeed, by two weeks ~5-6% of cells expressing nSyb-GAL4 show >2C DNA content. Similarly, we used the pan-glial driver *Repo-GAL4* and found that by 2 weeks ~6-7% of glia also become polyploid in the adult brain (Figure 3.5B). Neurons outnumber glial cells in the fly brain, and we find that the relative proportions of polyploid cells reflect the total ratio of neurons vs. glia in the adult brain (Figure 3.5C). We next asked whether specific types of neurons or glia show higher levels of polyploidy. We measured the proportion of polyploid vs diploid cells in various classes of neurons (Figure 3.5D) and glia (Figure 3.5E) in 7 day old optic lobes. Interestingly, we found that most differentiated cell types we assayed in the optic lobes show some level of polyploidy by one week of age. We conclude that polyploidy arises in multiple neuronal and glial types that are initially diploid upon eclosion and become polyploid after terminal differentiation and specifically during adulthood.

3.3.3. **Most Of The Polyploidy Is Not A Result Of Cell Fusion**

We reasoned that cells in the brain could become polyploid either by re-entering the cell cycle or by undergoing cell fusion (Alvarez-Dolado and Martínez-Losa, 2011; Giordano-Santini et al., 2016; Grendler et al., 2019; Losick et al., 2013; Schoenfelder et al., 2014; Shu et al., 2018; Starnes et al., 2016). To examine whether cell fusion occurs, we used a genetic labelling tool called CoinFLP (Bosch et al., 2015). The CoinFLP genetic cassette contains two overlapping but

exclusive Flippase Recombination Target (FRT) sites flanking a stop cassette that can be 'flipped -out' using FRT mediated recombination to give rise to cells expressing either a LexGAD driver or a GAL4 driver, which can be used to drive expression of *lexA$_{op}$-GFP* (green) and *UAS-RFP* (red). In animals heterozygous for CoinFLP, a diploid cell has only one copy of the transgenic cassette which can only be 'flipped' to give rise to a cell permanently labelled with either red or green fluorescent proteins, hence the name CoinFLP. If labeling is induced in the brain early during development before eclosion, cells become stochastically and permanently labeled with either red or green fluorescent proteins. If cells fuse in the ageing brain, up to ⅓ of cells undergoing fusion could fuse a red-labeled cell with a green cell and appear yellow. We used a FLP recombinase (flippase) under the control of the *eyeless* promoter (ey-FLP) to label most of the cells red or green in the optic lobes early in development (Figure 3.6A-B') and did not observe any double labelled (yellow) cells in young adult brains or older brains. We also expressed flippase enzyme more broadly under the control of a heat-shock promoter (hs-FLP) and labelled cells using a nuclear GFP or RFP at larval L2-L3 stages (Figure 3.6C) and measured the number of double-labelled cells in the optic lobes at on the day of eclosion or after aging at 14 days post-eclosion. We never observed more than 10-12 cells per optic lobes exhibiting double-labelling under these 'early-FLP' conditions. These double-labelled cells under the larval hs-FLP conditions likely include the SPG cells which become polyploid early in larval development and do not express ey-FLP. We conclude that very little cell fusion occurs in the adult OL, even with age.

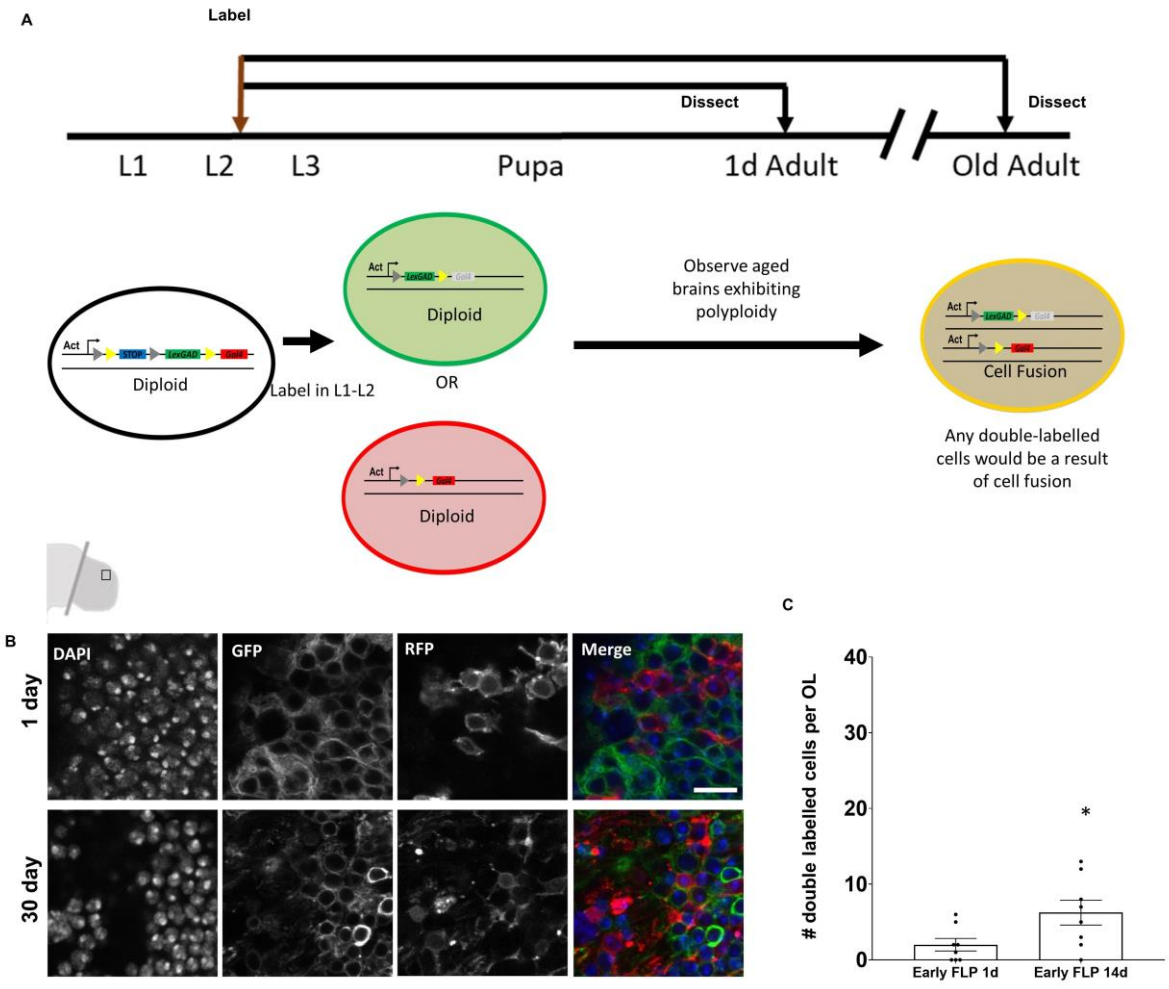


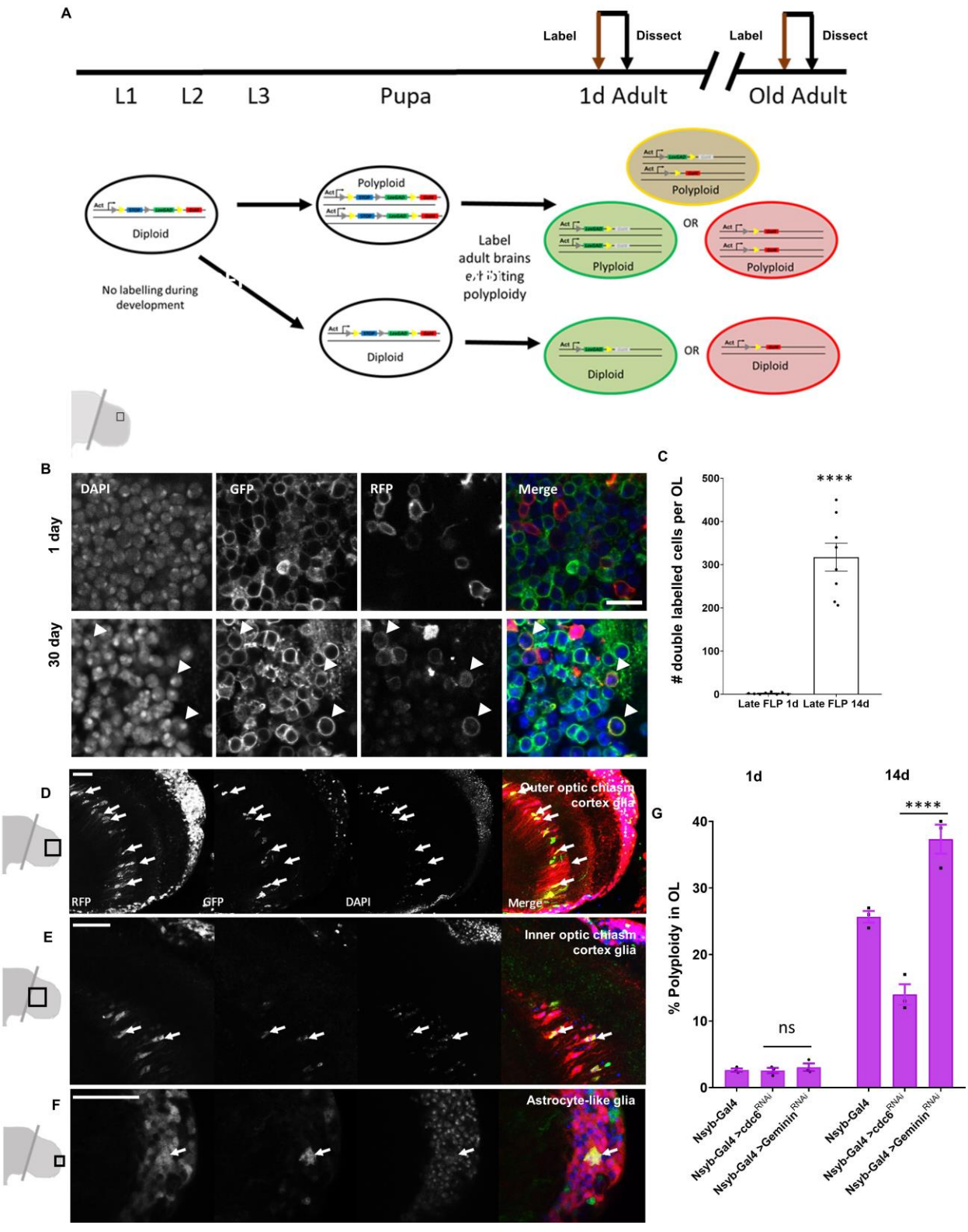
Figure 3.6 Very few polyploid cells arise from cell fusion in the adult brain

(A) Schematic of 'early labeling' using CoinFLP to identify potential cell fusion events. Early CoinFLP labeling will label diploid cells either with GFP or RFP. Any double-labelled cells in an older, polyploid brain will be a result of cell fusion. Representative images of 0 day (B) and 30 day polyploid optic lobes showing no double-labelled cells under 'Early-FLP' conditions when labelled using *ey-FLP* and membrane GFP and RFP. (C) Quantification of double labelled cells using nuclear GFP and RFP observed per brain lobe in early 'FLP' condition at 14 days. p value=0.0428 significance calculated using unpaired t-test with Welch's correction. Early 'FLP' in (C) was induced at L2-early L3 stages using *hs-FLP*. Scale bars = 8.3µm.

3.3.4. **‘Late-Flp’ Can Label Polyploid Cells That Arise From Cell Cycle Re-Entry**

By using a modified labelling paradigm, we can also use CoinFLP to label polyploid cells *in situ* (Figure 3.7A). Previous work with CoinFLP has shown that inducing ‘flipping’ in cells that are already polyploid results in a fraction of double labelled yellow cells (Bosch et al., 2015). We therefore reasoned that heterozygous CoinFLP brain cells that become polyploid by replicating their genome during adulthood will contain 2 or more copies of the CoinFLP transgene cassette. If we label cells by activating *hs-FLP* late in adulthood after polyploidy appears, some polyploid cells may ‘flip’ one copy green and one copy red, appearing yellow. When we induce an adult FLP at one day, before polyploidy occurs, we do not observe any double-labeled cells in the optic lobe (Figure 3.7B) but when we induce an adult FLP at 30 days post-eclosion, we observe several double labeled cells (Figure 3.7B’) indicating that these cells have undergone genome replication and contain at least two heterozygous copies of the CoinFLP transgenic cassette. To quantify this, we used an adult ‘late-FLP’ to drive nuclear GFP and RFP, and we observe around 300 double-labelled cells per optic lobes in 14 day old brains (Figure 3.7C). The presence of double-labelled nuclei in aged optic lobes suggest cells become polyploid by cell cycle re-entry and endoreduplicating DNA.

We further confirmed that the double-labelled cells visualised by microscopy are polyploid by performing DAPI intensity quantification in high-magnification images (Figure 3.8) and flow cytometry. (Figure 3.9). All CoinFLP double-labelled cells were confirmed to be polyploid by DAPI integrated intensity measurements and 97% of all double-labelled cells show >2C DNA content by FACS (Figure 3.9) CoinFLP double-labelling confirmed several types of glia identifiable by location and shape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Old Adult
Diploid
Polyploid
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Polyploid
OR
Plyploid
Polyploid
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OR
Diploid
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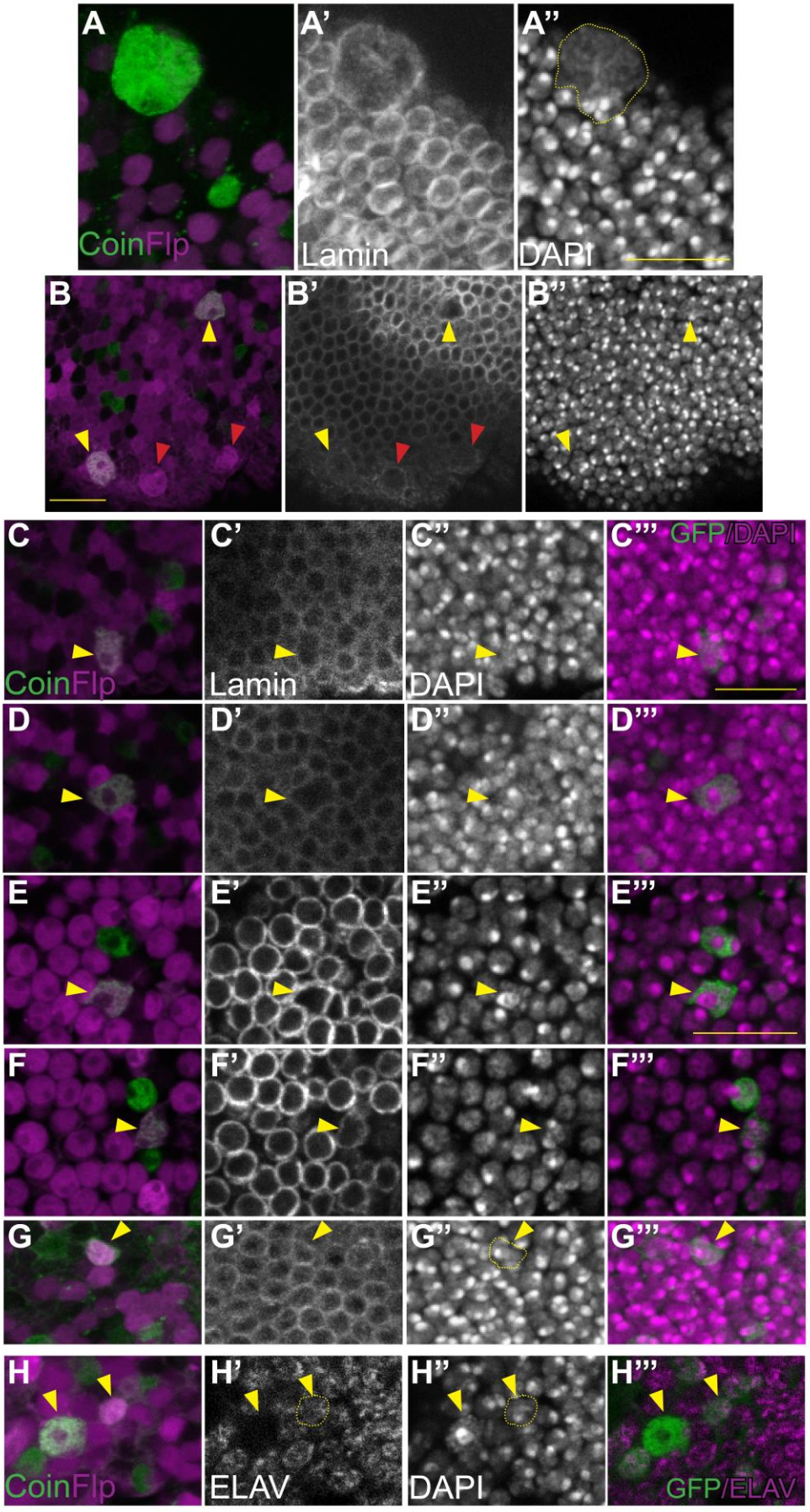
Nsyb-Gal4 >GeminiRNAi
Nsyb-Gal4
Nsyb-Gal4 >cdc6RNAi
Nsyb-Gal4 >GeminiRNAi

Figure 3.7 Cells in the OL undergo cell cycle re-entry to become polyploid

(A) Schematic showing labelling protocol for inducing a 'late-FLP' in brains where polyploidy is expected to identify polyploid cells *in situ*. A proportion of cells with multiple copies of the genome will be double-labelled. Representative images of 1 day optic lobe heat shocked soon after eclosion and a 30 day old optic lobe heat shocked at 29 days to induce labelling (B). Older optic lobes have double-labelled cells marked with membrane GFP and RFP. Scale bar =8.3µm. (C) Quantification of double labelled cells using nuclear GFP and RFP observed per brain lobe in 'late-FLP' condition. Labelling was induced 24h prior to dissection for both 1d and 14d using *hs-FLP*. P value <0.0001 significance calculated using unpaired t-test with Welch's correction.(D-F) Representative images showing cortex glia of the outer (D) and inner (E) optic chiasm as well as astrocyte-like (F) glial nuclei that can be identified as polyploid based on position and morphology using CoinFLP 'late-FLP' labelling method. Polyploid, double-labelled glia of each type are indicated with white arrows (G) Inhibition of DNA replication licensing factor *cdc6* by RNAi in neurons using the driver *nsyb-GAL4* results in lower levels of polyploidy (measured by flow cytometry) in male optic lobes compared to control (GAL4 driver alone). Knockdown of replication inhibitor *geminin* increases levels of polyploidy in 14 day old male optic lobes. Error bars show mean ± SEM, n=3. (Two way anova with greenhouse geisser correction for unequal SDs followed by Holm-Sidak's multiple comparisons test p values: 0.1234=ns; <0.0332 *; <0.0021 **; <0.0002 ***; **** <0.0001) Scale bars for D-F = 20µm.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ELAV
DAPI
GFP/ELAV

Figure 3.8 CoinFLP douoble positive cells are polyploid

Heat-shock induced CoinFLP was used to stochastically label cells in the adult brain with *UAS-GFP$_{NLS}$* (magenta), *LexA$_{op}$-RFP$_{NLS}$* (green), or both 24h prior to dissection. Optic lobes at 14 days (A-G) or 7days (H) are shown. Projections of three one micron z-sections (A,B) or single z-sections (C-H) with anti-lamin and DAPI staining were used to delineate nuclear boundaries and quantify DNA content (Figure 4-figure supplement 2A). (A) Very large polyploid cells likely to be sub-perineurial glia, are easily discernible on the surface of optic lobes. (B) Visibly larger polyploid cells scattered throughout optic lobes are double-labeled with CoinFLP (yellow arrowheads) but can also be single-labeled (e.g. expressing *UAS-RFP$_{NLS}$/ UAS-RFP$_{NLS}$*, magenta arrowhead) due to the stochastic nature of CoinFLP labeling. (C-G) Arrowheads indicate examples of double-labeled CoinFLP cells. All were confirmed to be polyploid by Dapi quantifications (Figure 4- figure supplement 2A). Polyploid nuclei can exhibit a visibly dispersed chromocenter (C), intact chromocenters with decondensed peripheral chromatin (D,E) multiple chromocenters (F) or a normal nuclear appearance (G). (H) An example of a double-labeled CoinFLP, ELAV positive cell, confirmed to be tetraploid by Dapi quantification (yellow arrowhead) with a neighboring double-labeled CoinFLP, ELAV negative polyploid cell (white arrowhead). Scale bar =10µm.

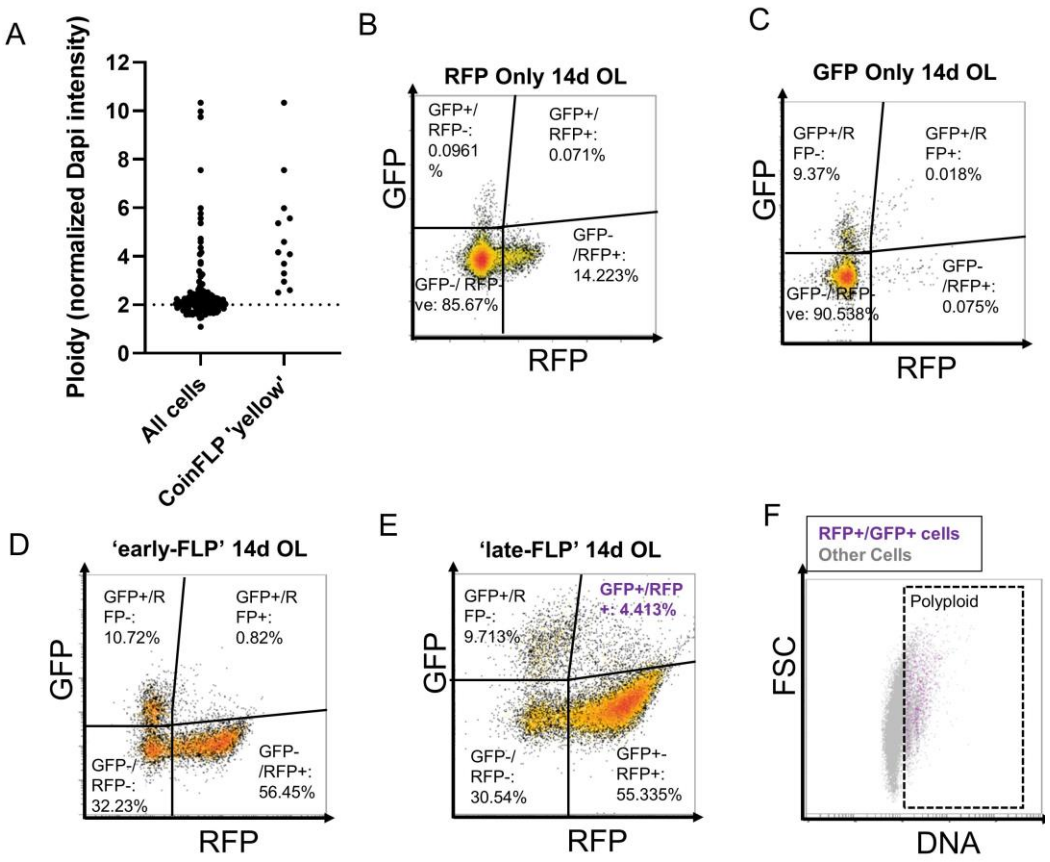


Figure 3.9 CoinFLP double positive cells have polyploid DNA content

(A) DAPI integrated intensity was quantified for 132 nuclei from CoinFLP- labeled 14 day optic lobes using FIJI. Nuclear boundaries were guided by lamin staining for Dapi quantifications from at least 10 randomly chosen cells from 11 different representative images, from 8 different brains. (B,C) Single colour controls used for gating.(B) shows RFP only and (C) shows GFP only controls. (D) CoinFLP animals were heat shocked at day of eclosion to induce labelling and brains were harvested at 14 days post eclosion. Inducing labelling at day 1 shows very few (0.82%) of cells in the OL that are double labelled. (E) CoinFLP labelling was performed 24h before harvesting. 4.413% of the OL are double labelled. (F) DNA content dot plot showing all cells in the brain in grey and the double labelled cells from (E) in purple. 97% of double labelled (GFP+/RFP+) cells from 'late-FLP' OL show polyploid DNA content. Single colour controls in (B) and (C) are siblings from the cross *y,w,hsflp;lexAop-nlsRFP/Cyo;UAS-nlsGFP/TM6B* x *y,w; CoinFLP*. 'RFP only' and 'GFP only' are progeny that respectively, did not inherit the *UAS-GFP$_{nls}$* and *LexA$_{op}$-RFP$_{nls}$*. cassettes and therefore contain

to become polyploid, such as a subset of cortex glia of the outer (Figure 3.74D) and inner (Figure 3.7E) optic chiasm and astrocyte-like glia (Figure 3.7F) in the medulla of the OL.

To test whether polyploidy in the adult optic lobes is driven by cell cycle re-entry, we used cell-type specific RNA-interference (RNAi) to modulate the DNA replication licensing factors cdc6 and Geminin in postmitotic neurons. Cdc6 is an essential factor for DNA replication licensing that promotes the recruitment of the MCM complex to load the DNA replication complex (Kang et al., 2014), while Geminin is a replication licensing inhibitor that sequesters DNA replication licensing factors to inhibit DNA re-replication (Lutzmann et al., 2006). Using *nSyb-GAL4*, we expressed *UAS-cdc6*^{RNAi} in differentiated neurons which significantly reduced levels of polyploidy by 14d (Figure 3.7G) from ~25% in control optic lobes to ~14% on optic lobes expressing the RNAi. We next knocked down geminin and found that we increase levels of polyploidy in the optic lobes (Figure 3.7G). This suggests that a fraction of post-mitotic neurons reactivate DNA replication to become polyploid in the adult fly brain.

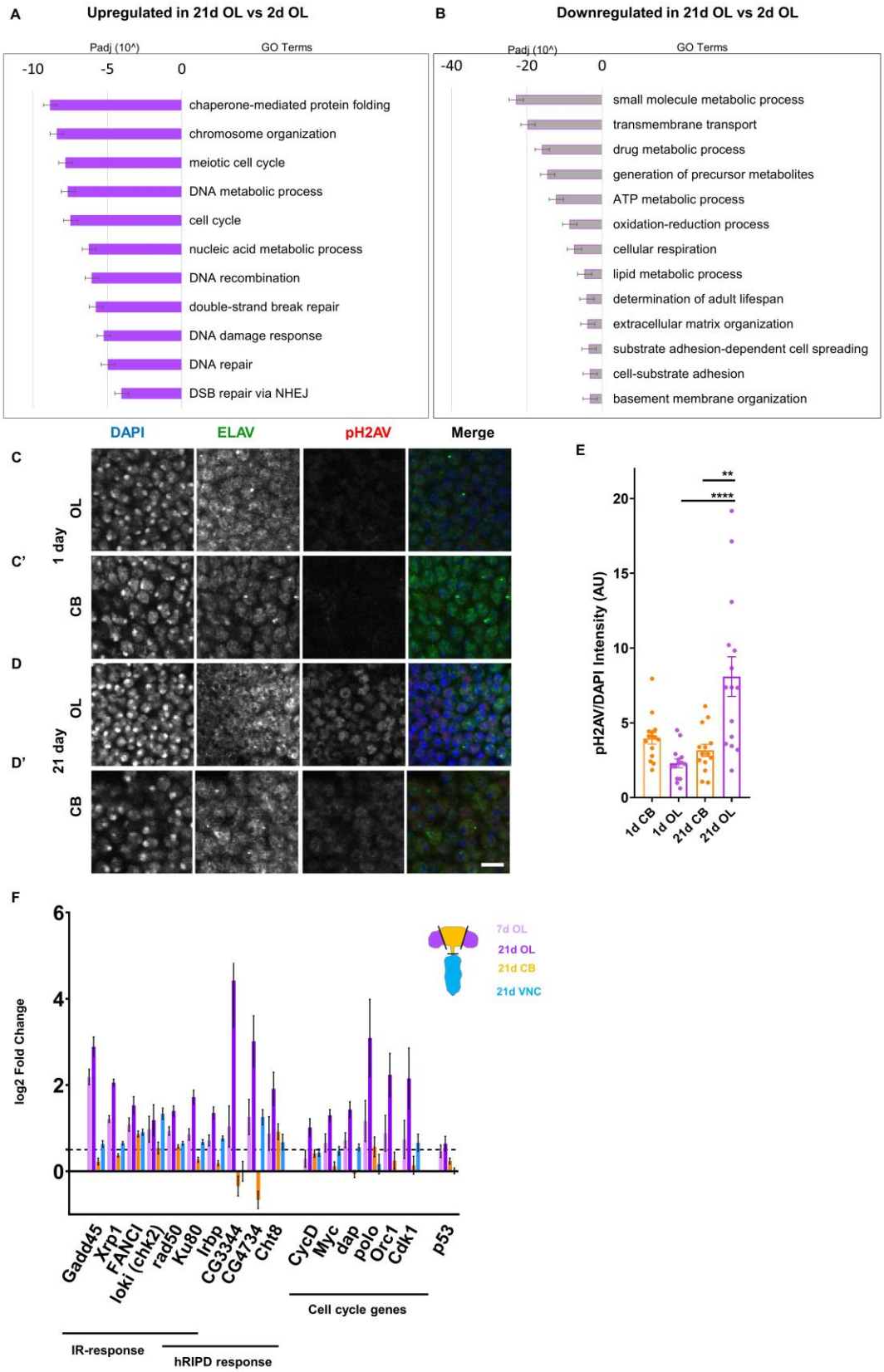
3.3.5. **DNA Damage Accumulates In The Adult Optic Lobes**

To investigate whether transcriptional changes that occur with age may be associated with cell cycle reactivation and polyploidy in the brain, we performed RNA sequencing on three parts of the CNS: optic lobes, central brain and VNC from male and female *Canton-S* animals at different time points: 1 day, 2 days, 7 days and 21 days post-eclosion. To infer biological processes that are affected with age, gene ontology analysis was performed using GOrilla and redundant terms were filtered using reviGO. The most significant changes observed in the optic lobes at 21d compared to 2d are shown in Figure 3.10 A and B. Among the most significantly upregulated groups of genes are those associated with the cell cycle, DNA damage response and DNA damage repair. The enrichment of up-regulated genes associated with the DNA damage

response was also observed in the optic lobes at 7 days (Figure 3.11A), but the enrichment and fold-induction of specific genes is stronger at day 21 (Figure 3.10A). A gene expression signature associated with DNA damage is specific to the optic lobes. However, the most significantly downregulated GO terms in the optic lobes at 21d are shared with the central brain and VNC and include metabolism, transmembrane transport and cellular respiration-associated processes (Figure 3.11).

To examine whether DNA damage is higher in the OL, we performed immunostaining against the phosphorylated histone 2A variant (pH2AV) in 1d optic lobes and central brain and 21d optic lobes and central brain (Figure 3.10C-E) from *Canton-S* male brains. Young brains show very low levels of pH2AV in both the optic lobes and central brain (Figure 3.10C,C',E) but older brains show higher levels of pH2AV in the optic lobes compared to the central brain (Figure 3.10D,D',E).

To further understand the DNA damage and cell cycle signatures observed with age, we looked at the change in expression of specific genes involved in the DNA damage response and the cell cycle (Figure 3.10F). Recent work has identified a specific transcriptional response to induced DNA damage in the head that involved a non-canonical role for tumor suppressor protein p53 (Kurtz et al., 2019). This signature was termed head Radiation Induced p53-Dependent or hRIPD. In addition to genes such as *FANCI, loki, rad50* and *xrp1* which are involved in a canonical, ionising radiation-induced DNA damage response, we also find robust upregulation of hRIPD genes *Ku80, Irbp, Cht8, CG3344 and CG4734* in our RNAseq data set at 7d and 21d in optic lobes. However, these genes are not as strongly upregulated in the aged central brain or VNC and *p53* itself shows only a small increase in the optic lobes at 21d (Figure 3.10F).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nucleic acid metabolic process
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DNA damage response
DNA repair
DSB repair via NHEJ
B
Downregulated in 21d OL vs 2d OL
Padj (10^)
GO Terms
-40
-20
0
small molecule metabolic process
transmembrane transport
drug metabolic process
generation of precursor metabolites
ATP metabolic process
oxidation-reduction process
cellular respiration
lipid metabolic process
determination of adult lifespan
extracellular matrix organization
substrate adhesion-dependent cell spreading
cell-substrate adhesion
basement membrane organization
DAPI
ELAV
pH2AV
Merge
C
C'
D
D'
1 day
21 day
OL
CB
E
pH2AV/DAPI Intensity (AU)
**
****
1d CB
1d OL
21d CB
21d OL
F
log2 Fold Change
7d OL
21d OL
21d CB
21d VNC
Gadd45
Xrp1
FANCI
loki (chk2)
rad50
Ku80
Irbp
CG33344
CG4734
Cht8
CycD
Myc
dap
polo
Orc1
Cdk1
p53
Cell cycle genes
IR-response
hRIPD response

Figure 3.10 DNA Damage accumulates in the Optic Lobes

Changes in gene expression in 21d OL compared to 2d OL shown by GO term analysis. Padj= adjusted P value. Upregulated GO terms shown in solid purple (A), downregulated GO terms shown in grey bars outlined with purple (B'). Representative images showing pH2AV foci in 1 (C,C') and 21 day (D,D') Central Brain (CB) and Optic Lobes (OL) Neurons are labelled in green (ELAV), phosphorylated histone 2A variant (pH2AV) in red nuclei are labelled in blue (DAPI). (E) Accumulation of DNA damage is quantified by measuring pH2AV intensity/DAPI intensity per frame in 5 brains per sample. Significance determined by performing unpaired t-test with Welch's correction for unequal SD. Scale bars = 20μm. (F) Genes involved in canonical Ionising Radiation (IR) response, head radiation induced p53 dependent (hRIPD) and cell cycle genes showing changes in expression compared to 2day. Dotted line indicates threshold for significance. Genes showing changes in 7d OL are shown in light purple, 21d OL are shown in dark purple, 21D CB in yellow and 21d VNC in blue.

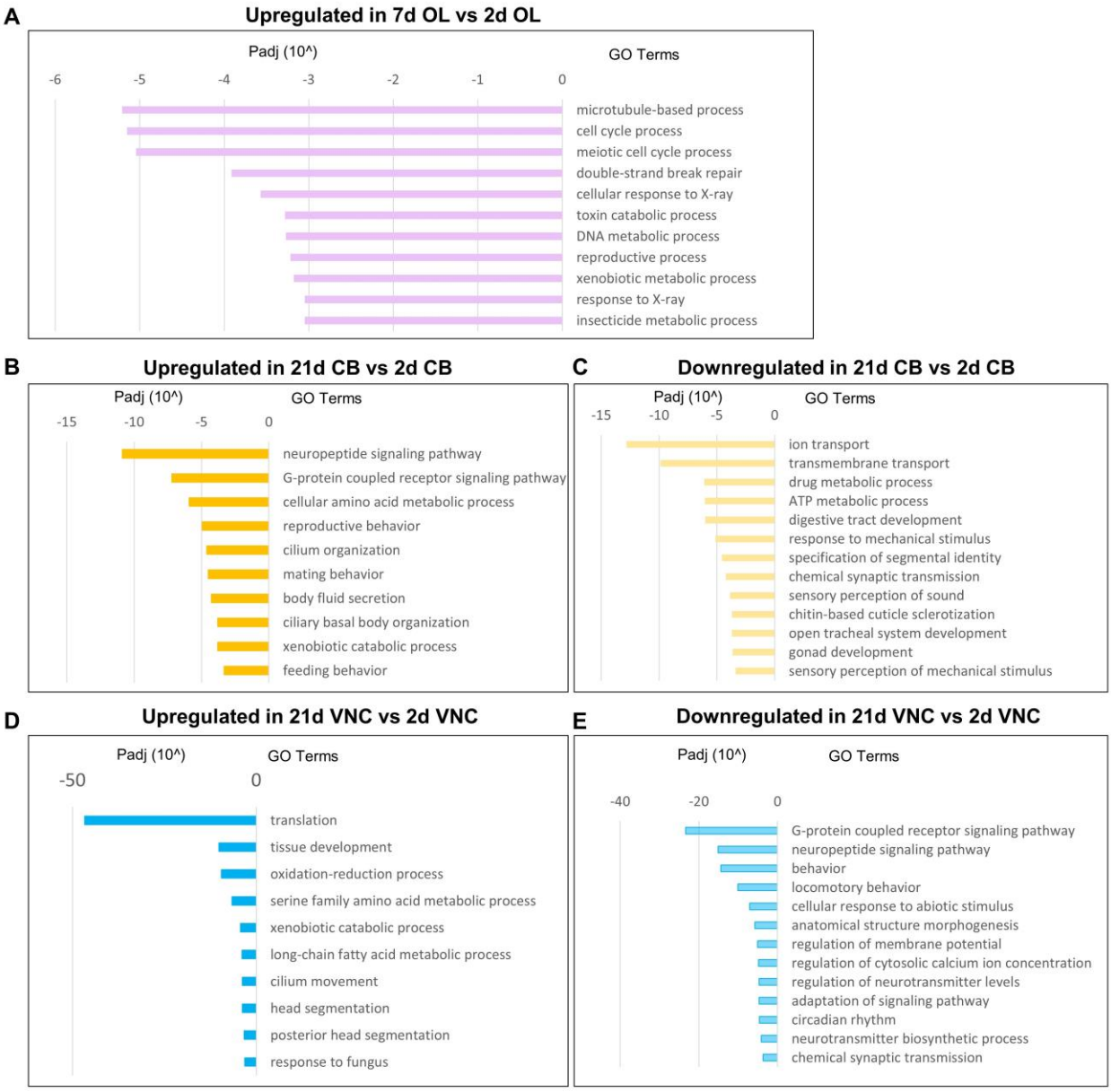

Figure 3.11 Supplemental RNAseq GO analysis

(A) Most significantly upregulated GO terms in 7d OL compared to 2d OL. Most significantly upregulated (B) and downregulated (C) GO terms in 21d CB compared to 2d CB. Most significantly upregulated (D) and downregulated (E) GO terms in 21d VNC compared to 2d VNC.

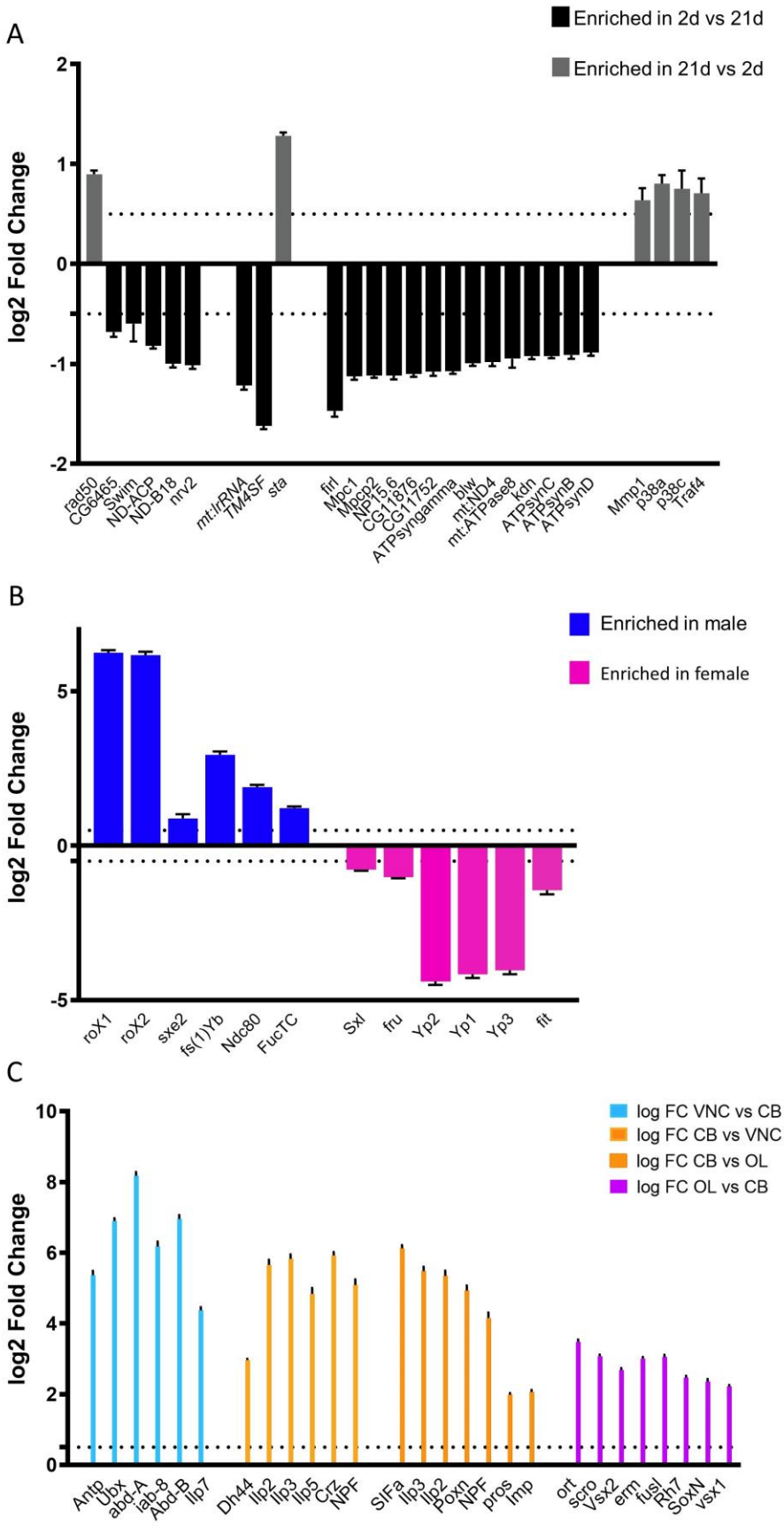
A
Enriched in 2d vs 21d
Enriched in 21d vs 2d
log2 Fold Change
2
1
0
-1
-2
rad50
CG6465
Swim
ND-ACP
ND-B18
nrv2
mt:lrRNA
TM4SF
sta
firl
Mpc1
Mpcp2
NP1516
CG11876
CG11752
ATPsyngamma
blw
mt:ND4
mt:ATPase8
kdn
ATPsynC
ATPsynB
ATPsynD
Mmp1
p38a
p38c
Traf4
B
Enriched in male
Enriched in female
log2 Fold Change
5
0
-5
roX1
roX2
sxe2
fs(1)Yb
Ndc80
FucTC
Sxl
fru
Yp2
Yp1
Yp3
flt
C
log FC VNC vs CB
log FC CB vs VNC
log FC CB vs OL
log FC OL vs CB
log2 Fold Change
10
8
6
4
2
0
Antp
Ubx
abd-A
iab-8
Abd-B
Ilp7
Dh44
Ilp2
Ilp3
Ilp5
Crz
NPF
SIFa
Ilp3
Ilp2
Poxn
NPF
pros
Imp
ort
scro
Vsx2
erm
fusl
Rh7
SoxN
vsx1

Figure 3.12 Validation of RNAseq data

Validation of our RNA sequencing dataset was performed by comparing our dataset to other published datasets. Aggregated data based on (A) age (2d vs 21d, n=6 replicates), sex (B, n=9 replicates) or brain region (C n=9 replicates) was compared to previously published datasets. Bar graphs show Mean log 2 fold change +/- SEM of select genes. Further details on references used are provided as a table in Supplemental File 3.

Table 3.3 - The list of comparisons and references for validation of RNAseq dataset

Gene(s)	Comparison	Reference(s)
ubx , abd-a, abd-b	Enriched in VNC vs CB	(Estacio-Gómez et al., 2013)
antp	Enriched in VNC vs CB	(Kuert et al., 2014)
dilp7	Enriched in VNC vs CB	(Nässel et al., 2008)
Ilp2, ilp3, ilp5	Enriched in CB vs VNC	(Cao et al., 2014)
dh44	Enriched in CB vs VNC	(Dus et al., 2015)
NPF	Enriched in CB vs VNC	(Shao et al., 2017)
crz	Enriched in CB vs VNC	(Lee et al., 2008)
NPF, poxn, pros, imp	Enriched in CB vs OL	(Davie et al., 2018)
ilp2, ilp3	Enriched in CB vs OL	(Cao et al., 2014)
ort	Enriched in OL vs CB	(Hong et al., 2006)
scro	Enriched in OL vs CB	(Davie et al., 2018)
vsx2	Enriched in OL vs CB	(Erclik et al., 2008)
fusl	Enriched in OL vs CB	(Long et al., 2008)
erm	Enriched in OL vs CB	(Peng et al., 2018)
rh7	Enriched in OL vs CB	(Kistenpfennig et al., 2017)
soxN	Enriched in OL vs CB	(Schilling et al., 2019)
vsx1	Enriched in OL vs CB	(Davie et al., 2018)
rad50,CG6465,swim, ND-ACP,ND-B18, nrv2	Differential expression in 21d vs 2d	(McCarroll et al., 2004)
mt:lrRNA, TM4SF, sta	Differential expression in 21d vs 2d	(Davie et al., 2018)

Firl, Mpc1, Mpcp2, NP15.6, CG11876, CG11752, ATPsyngamma, blw, mt:ND4, mt:ATPase8, kdn, ATPsynC, ATPsynB, ATPsynD	Genes involved in oxidative phosphorylation that decline with age (Downregulated at 21d compared to 2d)	(Davie et al., 2018)
Mmp1, p38a, p38c, Traf4	Injury/Stress response (Enriched in 21d vs 2d)	(Purice et al., 2017)
roX1, roX2, sxe2, fs(1)Yb, Ndc80, FucTC,	Enriched in male vs female	(Catalán et al., 2012; Chang et al., 2011)
Sxl, fru, Yp2, Yp1, Yp3, fit	Enriched in female vs male	(Chang et al., 2011)

Consistent with cell cycle re-entry in a fraction of cells in the OL, upregulation of cell cycle genes such as *myc, cyclin D, orc1* is observed specifically in the optic lobes and increases with age.

3.3.6. **Polyploidy accumulation in neurons is p53-independent**

Work in other polyploid tissues in Drosophila has shown that polyploid cells in the salivary gland can tolerate high levels of DNA double-strand breaks and resist apoptosis caused by DNA damage (Hassel et al., 2014; Mehrotra et al., 2008; Qi and Calvi, 2016; Zhang et al., 2014). This is possible because polyploid cells in tissues such as the salivary gland have intrinsically low levels of p53 protein and also suppress the expression of pro-apoptotic genes (Zhang et al., 2014). It has also been shown in various tissues and organisms that DNA damage can induce polyploidisation (Bretscher and Fox, 2016; Donovan and Corbo, 2012; Grendler et al., 2019). Since we observe a modest upregulation of p53 as well as a p53-dependent gene expression signature in older optic lobes, we asked if the induction of polyploidy in neurons is p53 dependent. To address this, we overexpressed wildtype (p53WT) or a dominant-negative allele of p53 (p53DN) that is unable to bind to DNA and evoke a transcriptional response in neurons using the nSyb-GAL4 driver (Figure 3.13A). We did not see a significant difference in levels of polyploidy in 7day old brains with overexpression of either WT or mutant p53, suggesting that accumulation of polyploidy in neurons is p53-independent. We also performed cell death measurement in the brain using flow cytometry. We calculated cell death by measuring proportions of cells incorporating either Propidium Iodide (PI) or Sytox-Green (Figure 3.14A). We did not see a significant difference in the proportion of dead cells in

overexpression of p53WT or p53DN conditions compared to control (Figure 3.14B) consistent with recent work suggesting a non-canonical, non-apoptotic role for p53 in the adult Drosophila brain (Kurtz et al., 2019).

3.3.7. **Exogeneous DNA damage leads to increased polyploidy**

We next asked if exogenous stress can impact levels of polyploidy in the brain. Increased oxidative stress is commonly associated with ageing (Haddadi et al., 2014; Hussain et al., 2018; Pinto and Moraes, 2015). We first treated flies with a low dose of paraquat (PQ) to mimic oxidative stress (Bonilla et al., 2006; Dudas and Arking, 1995; Hosamani and Muralidhara, 2013; Zou et al., 2000). w^{1118} flies treated with low dose of 2mM PQ from eclosion show increased DNA damage as well as increased polyploidy at 7d -14d (Figure 3.13B,D) but not increased cell death (Figure 3.14).

We next tested whether inducing DNA damage directly affects polyploidy. We treated flies with 900mJ of UV radiation by placing flies in a UV Stratalinker at 2 days post-eclosion (Grendler et al., 2019; Kang and Bashirullah, 2014) and observed significantly increased levels of polyploidy at day 7 in UV-treated flies compared to mock-treated controls (Figure 3.13C). We measured cell death using propidium-iodide (PI) incorporation (Grushko and Buttitta, 2015) and observed an acute increase in cell death 16h post-exposure to UV (Figure 3.13E), but no difference in cell death 5 days post-exposure. This suggests that cell death precedes accumulation of polyploidy upon induction of exogenous DNA damage.

3.3.8. **Polyploid cells are protected from cell death**

Polyploid cells in other tissues are known to sustain high levels of DNA damage as well as resist cell death (Zhang et al., 2014). We and others do not observe reproducible caspase-

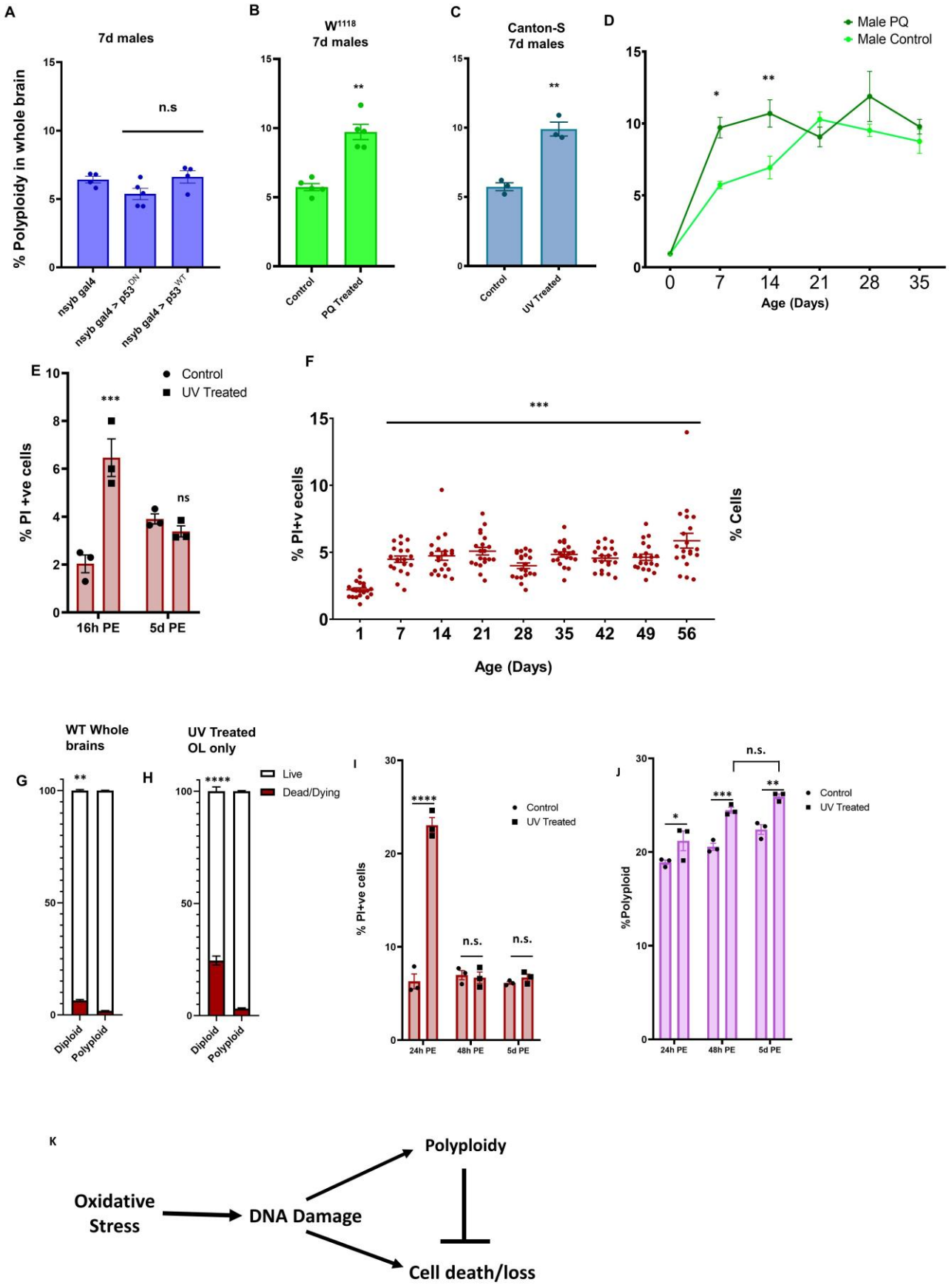
A
7d males
% Polyploidy in whole brain
n.s
nsyb gal4
nsyb gal4 > p53DN
nsyb gal4 > p53WT
B
W1118
7d males
Control
PQ Treated
C
Canton-S
7d males
Control
UV Treated
D
Male PQ
Male Control
Age (Days)
E
Control
UV Treated
% PI +ve cells
16h PE
5d PE
ns
F
% PI+v ecells
% Cells
Age (Days)
G
WT Whole brains
Diploid
Polyploid
H
UV Treated OL only
Live
Dead/Dying
I
% PI+ve cells
24h PE
48h PE
5d PE
J
%Polyploid
K
Oxidative Stress
DNA Damage
Polyploidy
Cell death/loss

Figure 3.13 Oxidative stress and DNA damage results in increased polyploidy during early adulthood and polyploid cells are protected from cell death

(A) Polyploidy in neurons is not p53 dependent. Percentage of polyploidy under each condition was measured in individual 7d male brains (n=5). (B) w^{1118} males treated with 2mM paraquat (PQ) from day of eclosion exhibit higher levels of polyploidy at 7 days compared to control w^{1118} males (n=5). (C) UV treated (960mJ exposure 5 days prior to dissection and flow cytometry) *Canton-S* flies show greater levels of polyploidy at 7 days compared to control (n=3). Error bars are mean±SEM, significance was calculated by performing unpaired t-test with Welch's correction for unequal SD. (D) Accumulation of polyploidy over a time course in w^{1118} males on 2mM PQ (dark green) compared to control w^{1118} males (light green). Shapes show mean, bars show SEM. Significance was calculated using 2 way ANOVA with Greenhouse-geisser correction for unequal SDs, multiple comparisons with Holm-Sidak's test; 0.1234=ns; <0.0332 *; <0.0021 **; <0.0002 ***. (E) Cell death measured by Propidium Iodide incorporation in animals treated with 960mJ UV at 16h post-exposure and 5 days post-exposure. Cell death precedes accumulation of polyploidy upon induced DNA damage. Significance was calculated using 2 way ANOVA with Greenhouse-geisser correction for unequal SDs, multiple comparisons with Holm-Sidak's test; 0.1234=ns; <0.0332 *; <0.0021 **; <0.0002 ***; **** <0.0001. (F) PI incorporation shows percentage of dead/dying cells in individual brains, male and female, w^{1118} at different ages post-eclosion. Significance was calculated using 2 way ANOVA with Greenhouse-geisser correction for unequal SDs, multiple comparisons with Holm-Sidak's test; 0.1234=ns; <0.0332 *; <0.0021 **; <0.0002 ***; **** <0.0001 (G). Proportion of PI+ cells that are diploid (2C) and polyploid (>2C) in pooled 14 day old *Canton-S* male brains. (H-J) Animals were exposed to 480 mJ UV at 21days and dissected 24h, 48h or 5d post exposure and cell death (H,I) and polyploidy (J) was measured by flow cytometry. (H) Proportion of PI+ cells that are diploid (2C) and polyploid (>2C) in pooled 21d w^{1118} male OL 24h post-exposure to 480mJ UV. (K) Proposed Model. For (G-I) n=3, Error bars are mean±SEM, significance was calculated by performing Two way ANOVA, multiple comparisons with Holm-Sidak's test; 0.1234=ns; <0.0332 *; <0.0021 **; <0.0002 ***; **** <0.0001.

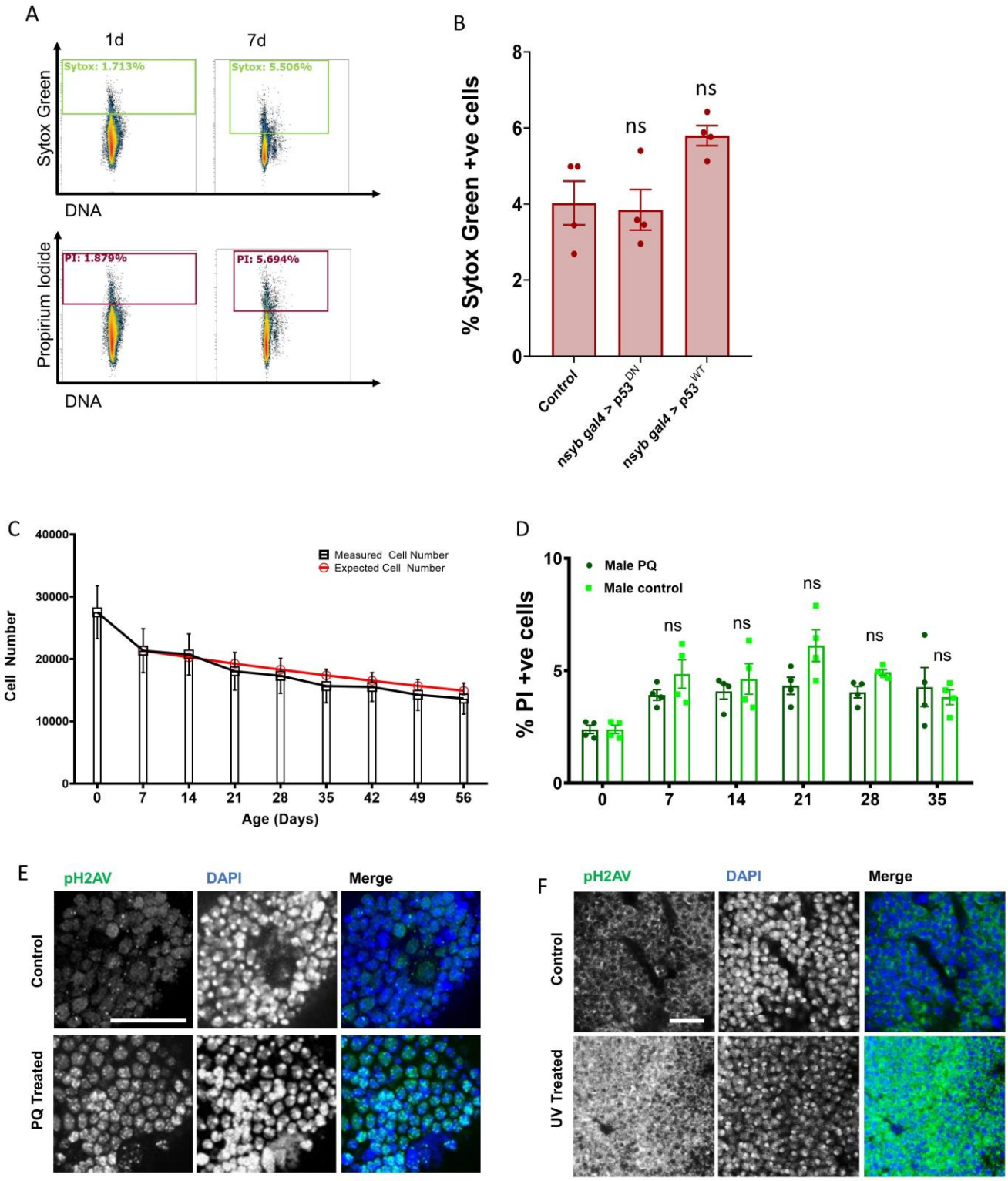
A
1d
7d
Sytox: 1.713%
Sytox: 5.506%
Sytox Green
DNA
PI: 1.879%
PI: 5.694%
Propirium Iodide
DNA
B
% Sytox Green +ve cells
ns
ns
Control
nsyb gal4 > p53DN
nsyb gal4 > p53WT
C
Measured Cell Number
Expected Cell Number
Cell Number
Age (Days)
D
Male PQ
Male control
% PI +ve cells
ns
E
pH2AV
DAPI
Merge
Control
PQ Treated
F
pH2AV
DAPI
Merge
Control
UV Treated

Figure 3.14 Supplemental Cell death and DNA damage data

(A) One day and 7d *Canton-S* brains stained with both Sytox-Green (green) and Propidium Iodide (red) show similar labelling with both cell death markers. (B) Percentage of cells incorporating Sytox-green under each condition was measured in individual 7d male brains (n=5) Error bars are mean±SEM, significance was calculated by performing unpaired t-test with Welch's correction for unequal SD. (C) Total cell counts from individual brains measured by flow cytometry in w^{1118} males. (black box = mean, bars = SEM) Red circles indicate the expected cell number, estimated by plotting 5% cell loss every week to model the death rate we observe inFigure 3.13. (D) PI incorporation with 2mM pQ treatment measured over a time course in w^{1118} males. 2mM PQ (dark green) compared to control w^{1118} males (light green). Error bars show SEM. Significance was calculated using 2 way ANOVA with Greenhouse-geisser correction for unequal SDs, multiple comparisons with Holm-Sidak's test. (E) 30d w^{1118} Control vs PQ-Treated brains stained with anti-pH2AV showing increased pH2AV immunofluorescence in PQ treated brains. Scale bar=20µm. (F) 21d w^{1118} Control vs UV-treated (480mJ) OL stained with anti-pH2AV showing increased pH2AV immunofluorescence in UV treated OL. Scale bar = 8µm.

dependent cell death in the adult brain beyond the first 5 days after eclosion (Foo et al., 2017). We measured cell death and necrosis in individual w^{1118} adult brains over a time-course using PI incorporation from day 1 post-eclosion until day 56 (Grushko and Buttitta, 2015). We found that newly eclosed flies exhibit a low level of dead or dying cells but from day 7 to day 56, the brain shows a relatively steady level (~5%) of dead or dying cells (Figure 3.13F) although there is variability from animal to animal. Since dead cells are cleared in the brain (Kurant, 2011), we expect this steady rate of cell death to result in a predictable rate of cell loss in the brain with age, which closely agrees with our total cell counts performed using flow cytometry (Figure 3.14).

We next examined whether the polyploid cells in aged brains are protected from cell death. Since the numbers of dead or dying cells measured in individual brains was very small, we pooled brains from 2 week old *Canton-S* males to obtain a measurement of ploidy in the PI positive cells by co-staining with the DNA content dye DyeCycle Violet. We found that while ~7% of the diploid cells incorporate PI, less than ~2.5% of polyploid cells incorporate PI (Figure 3.13G), suggesting that polyploid cells are more resistant to cell death.

To examine whether polyploid cells are resistant to cell death upon external DNA damage, we aged animals to 21 days, a time point where the OL exhibits high levels of polyploidy. We then exposed these flies to 480mJ UV to induce DNA damage and measured the levels of cell death and polyploidy from 24 hours – 5 days post exposure to UV. We observe high levels of PI incorporation (Figure 3.13H,I) at 24h post exposure, but normal levels by 48h, indicating an acute response of DNA damage induced cell death in the brain. Many diploid cells die in response to this dose of UV (~24.5%). In contrast, the polyploid cells show very low levels of PI incorporation (~3.1%, Figure 3.13H), suggesting that the polyploid cells in older adult

brains are also resistant to DNA damage induced cell death. We next examined whether the exposure to DNA damage also altered polyploidy, as we had observed in younger animals (Figure 3.13C). At 48h we observed, on average, a 4% increase in polyploidy for UV exposed animals. This early increase can be almost entirely attributed to the loss of the diploid cells that are PI positive at 24 hours post exposure (a loss of 24.5% of diploid cells increases the proportion of polyploid cells from 18.9% to 23%). When comparing 48h to 5 days post exposure, we see no significant increase in polyploidy, suggesting that after 3 weeks of age, animals lose the ability to further increase polyploidy in response to damage. This is in contrast to our experiment in young animals, using a low dose of paraquat to cause oxidative damage (Figure 3.13B, D) where we see an earlier accumulation of polyploid cells without any obvious increase in cell death (Figure 3.14).

The work described in this study supports a model (Figure 3.13) where cells in the early adult fly brain undergo endoreplication and polyploidisation in response to DNA damage and oxidative stress accumulated with age. Our data also suggests that polyploid cells are more resistant to cell death and may serve a beneficial or neuroprotective role in the ageing brain.

3.4. Discussion

3.4.1. Adult -onset polyploidy in neurons and glia

In this study we describe a surprising discovery, that diploid cells in the adult *Drosophila* brain can re-enter the cell cycle and become polyploid. We have identified several classes of neurons as well as glia that exhibit adult-onset polyploidy. We have also characterised which regions of the brain show increased polyploidy, and find that polyploidy is closely correlated with the expression of a DNA damage response signature. Other work has also shown that a

small population of about 40 stem cells in the optic lobes of *Drosophila* respond to acute injury by generating adult-born neurons (Fernández-Hernández et al., 2013). We considered the possibility that a fraction of cells with 4C DNA content may be in G2 and poised to undergo mitosis. We stained for G2 and mitotic cell cycle markers (phospho-histone H3 and Cyclin A) in over 100 adult brains at different ages and never observed a convincing G2 or mitotic event. However, we may have missed rare, transient cell cycle events that are captured by permanent lineage tracing approaches (Crocker et al., 2020; Fernandez-Hernandez et al., 2019). Both our cell number counts and cell death measurements indicate a steady decline in cell number in the adult brain with age (Figure 3.14), and suggest that under normal ageing conditions mitoses are likely rare. Moreover, we observe hundreds to thousands of tetraploid or polyploid cells by FACS or CoinFLP, suggesting that only a very small proportion of tetraploid cells would be expected to be in G2. We suggest that multiple mechanisms are employed in this brain region to ensure proper function and tissue integrity with age.

Polyploidy in neurons has previously been reported in the mouse cerebral cortex (López-Sánchez and Frade, 2013; López-Sánchez et al., 2017b) and chick retinal ganglion cells (Morillo et al., 2010). Whether purkinje cells in the mammalian cerebellum are polyploid has been a matter of considerable debate over the past several decades. (Brodskii et al., 1971; Kemp et al., 2012; Lapham, 1968; Lapham et al., 1971; Mares et al., 1973; Del Monte, 2006; Swartz and Bhatnagar, 1981). Perhaps the most exaggerated examples of polyploidy are from the giant neurons in the terrestrial slug *Limax* (Yamagishi et al., 2011) and the sea slug *Aplysia* (Coggeshall et al., 1970) where giant neurons contain >100,000 copies of the diploid genome. However, in all these cases, polyploid neurons appear during development. Our study describes a

novel phenomenon of adult onset and accumulation of polyploidy in the *Drosophila* brain under normal physiological ageing conditions.

3.4.2. **What is the function of polyploidisation in the brain?**

We have shown that many cell types become polyploid in the adult brain (Figure 3.5). These cell types have distinct physiology and functions. How polyploidisation affects the function of these various cell types is an exciting avenue for future research. Polyploidy can confer cell-type and context specific benefits in various tissues. In *Drosophila*, polyploid intestinal enterocytes (Miguel-Aliaga et al., 2018), SPGs (Von Stetina et al., 2018; Unhavaithaya and Orr-Weaver, 2012) and cells in the wounded epithelium (Losick, 2016; Losick et al., 2013) undergo endoreduplication and do not undergo cytokinesis to maintain the integrity of the blood brain barrier and the cell-cell junctions in the epithelium respectively. One possibility is that polyploidy in neurons or glia may allow cells to compensate for cell loss while maintaining established cell-cell contacts (Unhavaithaya and Orr-Weaver, 2012). The compound eye and optic lobes of *Drosophila* contain ~750-800 ommatidial 'units' that form a highly organised and crystalline structure (Bates et al., 2019; Nériec and Desplan, 2016; Pecot et al., 2014). Numerically and topographically matched cells in the medulla cortex of the optic lobes receive inputs from the lamina which in turn receives inputs from the retina (Bates et al., 2019; Pecot et al., 2014). We observe polyploidisation in multiple neuronal types found in the medulla, yet several cell types in the brain show a decline in number with age (Bates et al., 2019). In neurons, polyploidy could play a role in helping cells increase their soma size and dendritic arbors (Morillo et al., 2010; Szaro and Tompkins, 1987). It is possible that polyploidy allows neurons to form more presynaptic and postsynaptic connections to compensate for lost cells while maintaining the integrity of existing connections in the visual system.

Nurse cells in the egg chamber (Lilly and Spradling, 1996; Wattiaux and Tsien, 1971), cells in the accessory gland (Box et al., 2019; Sitnik et al., 2016), salivary gland (Edgar and Orr-Weaver, 2001) and fat body (Guarner et al., 2017), on the other hand become polyploid to fulfill increased biosynthetic demands. In addition to an upregulation of DNA damage and cell cycle in the optic lobes, our RNAseq data suggests compromised metabolism with age in all parts of the brain. One of the main functions of glial cells is to provide metabolic support to neurons in the brain (Kremer et al., 2017; Schirmeier et al., 2016; Volkenhoff et al., 2015). Polyploidisation in astrocyte and cortex glial cells might also serve to increase their metabolic output and compensate for the reduced metabolic output in the ageing brain.

3.4.3. **DNA damage accumulates in the optic lobes with age**

We observe higher levels of expression of DNA damage-associated genes in the optic lobes than in other parts of the brain (Figure 3.10A). We also see higher levels of DNA damage foci in the optic lobes than the central brain. We observe this signature even at 7 days in the OL, but it becomes stronger by 21 days (Figure 3.11Figure 3.10). This is consistent with other studies that report that signatures of ageing appear gradually over the course of an organism's lifespan and not abruptly at later chronological ages (Ben-Zvi et al., 2009; Labbadia and Morimoto, 2014; Shavlakadze et al., 2019). We do not know whether the increased DNA damage signature we observe in the optic lobes is because the optic lobes intrinsically sustain higher levels of DNA damage or whether other parts of the brain are better equipped to resolve DNA lesions. We also see an upregulation of cell cycle-associated genes specifically in the optic lobes with age. The transcription of cell cycle genes and genes involved in the DNA damage response and repair are intimately coordinated and can be controlled by intersecting pathways. (Chen et al., 2010; Herrup et al., 2013; Uxa et al., 2019). Homology-directed repair of DNA lesions occurs in S and

G2 phases of the cell cycle in actively dividing cells (Herrup and Yang, 2007). In other phases of the cell cycle, and after cell cycle exit, cells have to rely on error-prone non-homologous end joining mediated repair. It is tempting to speculate that re-entering the cell cycle allows postmitotic cells to repair DNA damage better and survive.

3.4.4. **Is polyploidy protective?**

We and others observe a steady decline in the number of cells in the adult *Drosophila* brain with age (Figure 3.14), (Bates et al., 2019; Foo et al., 2017). The continual loss of cells in the ageing brain may be analogous to wounding, which induces polyploidisation or compensatory cellular hypertrophy in other *Drosophila* tissues (Bretscher and Fox, 2016; Cohen et al., 2018; Losick et al., 2013), ((Box et al., 2019; Tamori and Deng, 2013). We suggest neurons and glia in the adult brain may employ a similar strategy, to compensate for cell loss in a non-autonomous fashion. When we induce damage that does not increase cell death in young brains (Figure 3.13B, D) we observe an earlier increase in polyploidy, suggesting that in younger animals polyploidy can be an adaptive response to damage. By contrast in older animals, we find that polyploidy can protect from acute cell loss. However, levels of subsequent polyploidy do not further increase in aged animals, suggesting there is a permissive window for damage-induced polyploidy during adulthood (Figure 3.13). This may explain why levels of polyploidy plateau after 3-4 weeks of age in various strains (Figure 3.1). It will be interesting to further test the nature of this compensation for cell loss in early adulthood by performing genetic experiments to ablate specific cell types or sub-populations of cells.

3.4.5. **How does polyploidy relate to neurodegeneration?**

Over the past two decades, several studies have reported an interesting correlation between neurodegeneration and cell cycle re-entry in neurons (Chen et al., 2010; Frade and Ovejero-Benito, 2015; Herrup, 2012; Moh et al., 2011; Rimkus et al., 2008; Yang and Herrup, 2005). Most of these observations are from post-mortem brains containing neurons expressing cell cycle genes or exhibiting hyperploidy (>2N DNA content). More hyperploidy is observed in brains of patients with preclinical Alzheimer's compared to age-matched controls, which has led to the hypothesis that cell cycle re-entry may precede cell death and neurodegeneration. Whether cell cycle re-entry is a cause or a consequence of neurodegeneration has been difficult to test, since both are associated with age and damage. Our data suggests that re-entry into the cell cycle may be a normal physiological response to the accumulation of damage in early adulthood and that it can serve a beneficial and protective function in neurons and glia. However, we do not know how polyploidy may impact neuronal and glial function and whether it may become detrimental over time. In geriatric animals (beyond 4 weeks) we observe increased variation in the levels of polyploidy and we note that a subset of animals also exhibit extreme levels of cell death (Figure 3.13C). It is possible that these animals represent a fraction of the aged population that exhibit neurodegeneration. Our single-animal assays will be essential to identify these outliers for further study.

3.5. Materials and Methods

Table 3.4List of Key resources and Reagents

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
Genetic reagent (*D. melanogaste*r)	w^{1118}	Bloomington Drosophila Stock Center	BDSC 5905	isogenic
Genetic reagent (*D. melanogaste*r)	*Canton-S*	O. Shafer lab	n/a	WT
Genetic reagent (*D. melanogaste*r)	*Oregon-R*	C. Collins lab	n/a	WT
Genetic reagent (*D. americana*)	*Drosophila americana*	P. Wittkopp lab	n/a	Non melanogaster *Drosophila*
Genetic reagent (*D. mauritiana*)	*Drosophila mauritiana*	P. Wittkopp lab	n/a	Non melanogaster *Drosophila*
Genetic reagent (*D. melanogaste*r)	$glass^{60J}$	O. Shafer lab	n/a	Mutant for glass

Genetic reagent (*D. melanogaste*r)	*w;nSyb-GAL4/Cyo*	M. Dus lab	n/a	pan-neuronal
Genetic reagent (*D. melanogaste*r)	*w;+;nSyb-GAL4*	M. Dus lab	n/a	pan-neuronal
Genetic reagent (*D. melanogaste*r)	*w;UAS-nGFP;Repo-GAL4, tubulin GAL80TS*	Buttitta lab stocks	n/a	pan-glial
Genetic reagent (*D. melanogaste*r)	*w;UAS-nGFP*	Buttitta lab stocks	n/a	UAS nuclear GFP
Genetic reagent (*D. melanogaste*r)	*w;+;UAS-nGFP*	Buttitta lab stocks	n/a	UAS nuclear GFP
Genetic reagent (*D. melanogaste*r)	*w;Moody-GAL4*	C. Collins lab via Klambt Lab	n/a	Sub-perineurial glia
Genetic reagent (*D. melanogaste*r)	*y,w;mz19-mCD8::GFP*	Bloomington Drosophila Stock Center	BDSC 23300	Antennal lobe projection neuron
Genetic reagent (*D. melanogaste*r)	*w^{1118};ELAV-GAL4,UAS-nGFP*	Bloomington Drosophila Stock Center	BDSC 49226	pan-neuronal
Genetic reagent (*D. melanogaste*r)	*y,w;breathless-GAL4*	DGRC Kyoto	105276	Trachea

Genetic reagent (*D. melanogaste*r)	*w-;GAD1-GAL4/SM6*	O. Shafer lab	n/a	GABAergic
Genetic reagent (*D. melanogaste*r)	*w-;OK371-GAL4,UASn-GFP*	Buttitta lab stocks	n/a	Glutamatergic
Genetic reagent (*D. melanogaste*r)	*w;ChaT-GAL4*	O. Shafer lab	n/a	Cholinergic
Genetic reagent (*D. melanogaste*r)	w^{1118}*;+;GMR-12C11-GAL4*	Bloomington Drosophila Stock Center	BDSC 76324	Tm3a
Genetic reagent (*D. melanogaste*r)	w^{1118}*;+;GMR-42H01-GAL4*	Bloomington Drosophila Stock Center	BDSC 48150	Dm9
Genetic reagent (*D. melanogaste*r)	w^{1118}*;+;GMR-23G11-GAL4*	Bloomington Drosophila Stock Center	BDSC 49043	Dm4
Genetic reagent (*D. melanogaste*r)	w^{1118}*;+;GMR-30B06-GAL4*	Bloomington Drosophila Stock Center	BDSC 47529	Dm10
Genetic reagent (*D. melanogaste*r)	w^{1118}*;+;GMR-26H07-GAL4*	Bloomington Drosophila Stock Center	BDSC 49204	Dm2

Genetic reagent (*D. melanogaste*r)	*y;w;NP3233-GAL4/Cyo*	DGRC Kyoto	113173	Astrocyte-like
Genetic reagent (*D. melanogaste*r)	*y;w;NP2222-GAL4/Cyo*	DGRC Kyoto	112830	Cortex glia
Genetic reagent (*D. melanogaste*r)	*w;mz97-GAL4*	C. Collins lab via Klambt Lab	n/a	Wrapping glia
Genetic reagent (*D. melanogaste*r)	*y,w,UAS-mCD8::RFP,LexA$_{op}$2-mCD8::GFP; CoinFLP-LexA::GAD.GAL4*	Bloomington Drosophila Stock Center	BDSC 59270 and 59271	CoinFLP
Genetic reagent (*D. melanogaste*r)	*y,w,hs-FLP;LexA$_{op}$-RFP$_{nls}$; UAS-GFP$_{nls}$*	Buttitta lab stocks	n/a	*hs-FLP* used with with CoinFLP nuclear GFP and RFP
Genetic reagent (*D. melanogaste*r)	*ey-FLP*	Bloomington Drosophila Stock Center	BDSC 5576	ey-FLP
Genetic reagent (*D. melanogaste*r)	*y,sev,w;UAS-cdc6^{RNAi}*	Bloomington Drosophila Stock Center	BDSC 55734	cdc6KD

Genetic reagent (*D. melanogaste*r)	*w; UAS-geminin^{RNAi}*	Bloomington Drosophila Stock Center	BDSC 30929 and 50720	geminin KD
Genetic reagent (*D. melanogaste*r)	*w1118;GUS-p53*	Bloomington Drosophila Stock Center	BDSC 6584	UAS-p53WT
Genetic reagent (*D. melanogaste*r)	*y,w1118; UAS-p53^{259N}*	Bloomington Drosophila Stock Center	BDSC 6582	UAS-p53DN
Antibody	anti-ELAV (rat monoclonal)	Developmental Studies Hybridoma Bank	Rat-ELAV-7E8A10	1 : 100
Antibody	anti-pH2AV (mouse monoclonal)	Developmental Studies Hybridoma Bank	UNC93-5.2.1	1 : 100
Antibody	anti-Repo (mouse monoclonal)	Developmental Studies Hybridoma Bank	8D12	1 : 100
Antibody	anti-Lamin (mouse monoclonal)	Developmental Studies Hybridoma Bank	ADL67.10	1 : 100

Antibody	Alexa Fluor 568 anti-mouse (goat polyclonal)	ThermoFisher	A11031	1 : 1000
Antibody	Alexa Fluor 568 anti-rat (goat polyclonal)	ThermoFisher	A11077	1 : 1000
Antibody	Alexa Fluor 488 anti-mouse (goat polyclonal)	ThermoFisher	A11029	1 : 1000
Antibody	Alexa Fluor 488 anti-rat (donkey polyclonal)	ThermoFisher	A21208	1 : 1000
Other	DAPI	Sigma-Aldrich	D9542	1 : 1000
Other	Dye-cycle violet	ThermoFisher	V35003	2 : 1000
Other	Sytox Green	ThermoFisher	S7020	2 : 1000
Other	Propidium Iodide	Sigma-Aldrich	P4170	2.25 : 1000

3.5.1. **Fixation, Immunostaining and Imaging**

Drosophila brains were dissected in 1X Phosphate buffered saline (PBS) and fixed in 4% Paraformaldehyde (PFA) in 1X PBS for 25 minutes. Tissues were permeabilised in 1X PBS+0.5% Triton-X, blocked in 1X PBS, 1% BSA 0.1% Triton-X. (PAT) Antibody staining was performed at specified concentrations in PAT (Supplementary File 1) overnight at 4°C, washed, blocked in PBT-X (1X PBS, 2% Goat serum 0.3% Triton-X) prior to incubation with secondary antibody either for 4h at RT or overnight at 4°C. DAPI staining was performed after washes, brains were wet-mounted in vectashield H1000. All imaging was performed on either a Leica SP5 or SP8 laser scanning confocal microscopes. For EdU incorporation assays, flies were placed on 10mM EdU containing food with food colouring for 3 days prior to dissection. Only flies with visibly coloured abdomens were dissected. Click-iT Plus^{TM} staining with picolyl Azide was done as per the protocol recommended by ThermoFisher.

3.5.2. **Fly Husbandry**

Flies were reared and aged in a protocol modified from (Linford et al., 2013). Ageing flies were collected soon after eclosion as virgin males and females and segregated into vials containing no more than 20 flies/vial. Ageing flies were flipped onto fresh Bloomington Cornmeal food every 5-7 days. A list of all fly stocks used in this study is supplied in Table 3.4.

3.5.3. **Image Quantification**

For pH2AV quantification, 5 non-overlapping Regions of Interest (ROIs) were chosen per brain region per brain. Average Intensity of pH2AV and DAPI per ROI were computed on

individual channels using ImageJ. All brains were imaged at the same laser intensity and gain settings at different ages.

CoinFLP double-labelled cell counting was performed manually. Individual optic lobes were imaged at 100x magnification with 0.5 micron Z-sections. Quantification was performed by cropping 2-5 confocal Z-sections at a time, performing maximum intensity projections of each cropped image, and counting cells that showed DAPI, GFP and RFP signal overlap. Lamin staining was used to discern nuclear boundaries for DAPI Integrated Intensity measurements of 132 cells using FIJI. DAPI intensity was normalized to diploid cells (2N) measured on the same slide.

3.5.4. **Heat shock protocol**

CoinFLP labelling was induced with heat shock induction. Flies were placed in plastic vials and completely submerged in 37°C water bath for 15 minutes. For 'early-FLP', flies were moved back to 23°C and dissected at day 1 or day 10. For 'late-FLP', heat shock induction was performed 24 hours prior to dissection. All incubations and culturing except heat shock was performed at 23°C. We noted that the frequency of CoinFLP flipping resulted in a ratio of LexA:GAL4 expressing cells that is between 4:1 and 4.6:1 (Bosch et al 2015). We calculated the expected number of double labeled cells for 'late-FLP' in Fig 4 and Figure 4-figure supplement 2 as follows: If flipping is complete (100% of cells flip), we would expect 80% of diploid cells to label red and ~20% to label green. Only 4% of all polyploid cells will label green/green (probability of green is 0.2 therefore 0.2* 0.2=0.04*100), 64% red/red (probability of red is 0.8 thus, 0.8*0.8=0.64*100) and 32% will label green/red or red/green and appear yellow or 'double-labelled'. If we assume that about 20% of cells in the optic lobes are tetraploid (20% of ~30,000 = 6,000 cells), we can expect 1,920 cells (32% of 6,000) to label yellow per optic lobe

under 100% flipping conditions. If we flip ~50% of cells, we expect about 900 yellow cells per optic lobe. In our measurements the amount of flipping was variable from animal to animal and we estimate that in our samples with the lowest flipping we flip about ⅓ of cells and with our strongest flipping we label about ¾ cells.

3.5.5. **Flow Cytometry**

Fly brains were dissected in PBS and transferred to 1.5mL microcentrifuge tube caps containing 100uL of solution containing 9:1 Trypsin-EDTA :10XPBS with 1µL Dyecycle Violet and/or 1.12µL PI or 1µL Sytox green. Brains were incubated for 20 minutes in the microcentrifuge tube caps, triturated using low retention p200 pipette tips for 60 seconds then transferred into the microcentrifuge tubes containing 400µL of the trypsin-EDTA solution with dyes and capped, and incubated further for 45 minutes at room temperature without agitation. After incubation, each sample was diluted with 500µL 1XPBS and gently vortexed at speed 8 before being loaded onto Attune or Attune NxT flow cytometer for flow cytometry analysis. The Attune had a laser configuration of a violet laser (VL,405nm) with 6 bandpass (BP) filters and a blue laser (BL,488nm) with 3 bandpass filters. The Attune NxT is configured with VL (6 BP filters), BL with 2 BP filters, a yellow laser (YL, 561nm) with 3 BP filters and a red laser (RL, 637nm) with 3 BP filters. The detection of DyeCycle Violet was performed using VL1 (Emission filter 450/40), GFP and Sytox Green using BL1 (Emission filter 530/30), RFP and PI using BL2 (Emission filter 574/24) on the Attune and YL1 (585/16) on the Attune NxT. A flow rate of 100 to 500 µl/second was used for sample acquisition and a minimum of 20,0000 events gated as ‘non doublets’ (Figure 1-figure supplement 1) were acquired per sample. Gating Strategy is graphed in Figure 3.2Figure 3.2. Briefly, all cells were plotted on forward vs side

scatter (FSC vs. SSC), gated to eliminate debris. Subsequently, 'non-debris' were plotted on VL1(DNA) vs FSC and gated to eliminate unstained events. A third gate was applied plotting VL1(DNA)-H vs VL1(DNA)-A (voltage pulse area vs.height) to eliminate doublets. All events in gate 3 were further subjected to GFP/DNA/PI content analysis.

3.5.6. **RNA sequencing**

10 CNSs from Canton-S females and males raised at 25°C on Cornmeal/Dextrose food under normal 12h L/D cycles at ZT = 2, were dissected into optic lobes, VNC and central brain at the indicated ages with 3 biological replicates for each sex, age and region (72 samples total). Tissues were directly dissolved into TRIZOL-LS. (Invitrogen) and RNA was prepared as directed by the manufacturer. Total RNA (2-5µg) was provided to the University of Michigan Sequencing Core for polyA selection and unstranded mRNA library preparation for the Illumina HiSeq4000 platform

3.5.7. **RNAseq data analysis and GO term analysis**

RNAseq analysis was performed at the U.Michigan Bioinformatics Core using the following pipeline:

1. Read files from the Sequencing Core were concatenated into single fastq files for each sample. 2. Quality of the raw reads data for each sample was checked using FastQC(version v0.11.7). 3. Adaptors and poor quality bases were trimmed from reads using bbduk from the BBTools suite (v37.90).4. Quality processed reads were aligned to the Ensembl Dm6 genome using STAR (v2.6.1a) with quantMode GeneCounts flag option set to produce gene level counts. MultiQC (v1.6a0) was run to summarize QC information for raw reads, QC processed reads,

alignment, and gene count information. Differential expression analyses were carried out using DESeq2 (v1.14.1). Data were pre-filtered to remove genes with 0 counts in all samples. Normalization and differential expression was performed with DESeq2, using a negative binomial generalized linear model. Plots were generated using variations or alternative representations of native DESeq2 plotting functions, ggplot2, plotly, and other packages within the R environment.

Genes called as at least 2-fold differentially expressed between day 2 and day 21 were examined for enriched GO terms using target and background unranked lists in GOrilla and redundant GO terms were filtered using ReviGO. GO term Enrichment is presented as the -log10 of the p-value with a cutoff at p-values higher than 10^-3. The full dataset has been uploaded to GEO and can be found using the accession number: **GSE153165**

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Chapter 4. Concluding Remarks and Future Directions

In Chapter 2, I show that postmitotic neurons and glia in the developing *Drosophila* brain can be forced to re-enter the cell cycle after they have terminally differentiated. My studies show that forced re-entry in both neurons and glia can result in mitosis, however, neurons that are forced to divide frequently undergo cell death potentially due to catastrophic mitoses, while glial cells can form tumour-like masses. I also show that the time window of flexible cell cycle exit in neurons is shorter, whereas glia exhibit an intrinsically greater flexibility in their G_0 state.

In Chapter 3, I describe the discovery of polyploid neurons and glia in the adult *Drosophila* brain. This is the first recognition of age-associated, adult specific increase in DNA content in neurons and glia in *Drosophila*. I found that the optic lobes show higher levels of polyploidy than the central brain and the ventral nerve cord. I also show that an increase in polyploidy occurs within the first week after eclosion. In addition, exogenous DNA damage and oxidative stress can induce even higher levels of polyploidy, and the polyploid cells are protected from cell death.

The studies described in chapters 2 and 3 show that the establishment and maintenance of G_0 in non-dividing cells is a complex and dynamic process. Neurons are sensitive to cell cycle activation in early metamorphosis, and can even undergo mitosis. In late metamorphosis, they become completely refractory to the same cues which could drive them into mitosis at earlier stages. Later, in the adult, neurons readily re-enter the cell cycle to become polyploid as flies get older but do not undergo mitosis. Indeed, overexpression of the same factors which could drive

neuronal mitosis at 48h APF do not result in increased polyploidy in older brains suggesting that different factors may be involved in the regulation of adult-onset polyploidy in neurons.

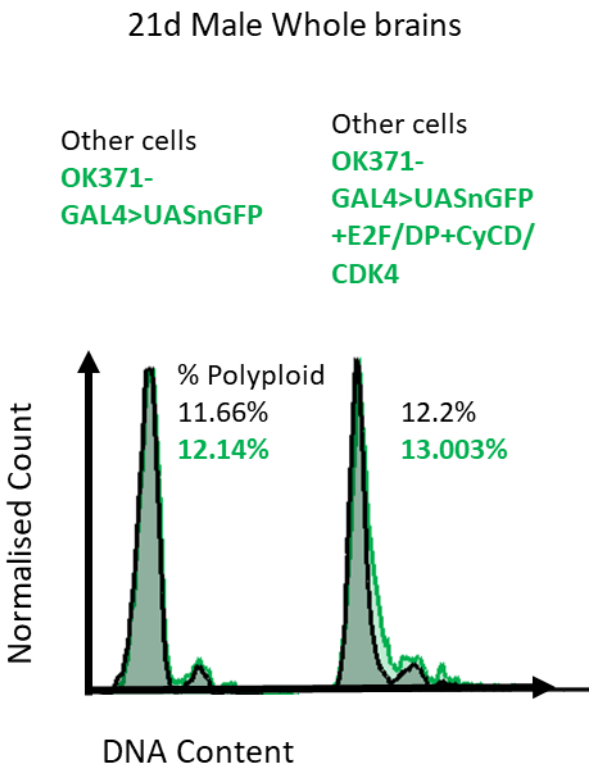

Figure 4.1Overexpression of E2F+CycD/CDK4 does not ult in increased polyploidy in adult neurons

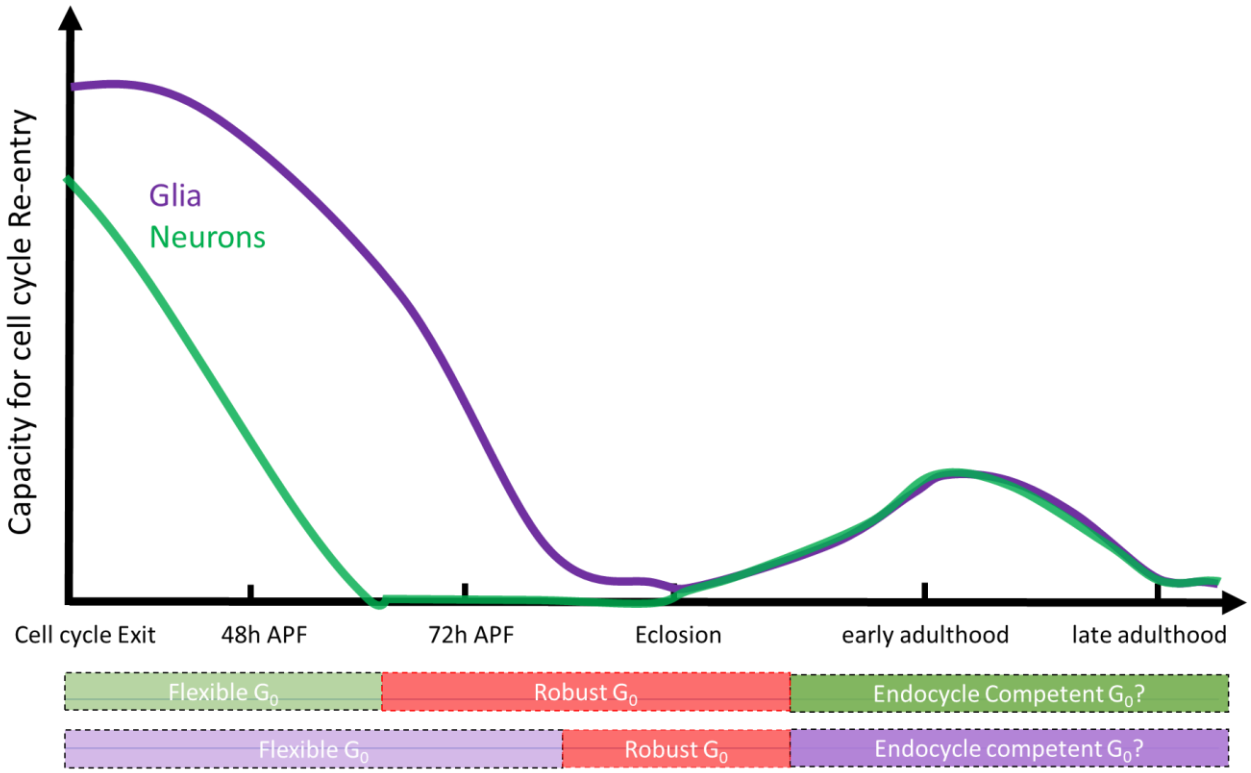

Figure 4.2 Schematic diagram summarising the difference in capacity for cell cycle re-entry in Drosophila neurons and glia

I concluded the first chapter with some questions. In this final chapter, I will attempt to address them with some of the perspective gained from the work described in Chapter 3, and then discuss some potential future avenues of research that can be explored.

4.1. An Endocrine Polyploidy Trigger?

What causes polyploidy in neurons and glia specifically in the adult? Is the onset of polyploidy in the fly brain 'developmentally' regulated in the first week of fly adulthood? Are the endocycling cells in early adulthood responding to endogenous DNA damage or are they endocycling in anticipation of DNA damage? Recent work in the fly prostate (accessory gland)

shows that this tissue also exhibits endocycle-associated growth in gland size after eclosion(Box et al., 2019). Just as cells in the brain are able to increase levels of polyploidy upon oxidative and DNA damage, the accessory gland also shows a capacity to regenerate in response to cell loss, suggesting that various tissues in the display a capacity to respond to and recuperate from damage in early adulthood by engaging an endocycle program. The increase in polyploidy in the brain within the first week of adulthood suggests that this may be developmentally regulated.

The early work on facultative polyploidy in mammalian tissues I described in Chapter 1 suggests that there may be endocrine factors upstream of the cell cycle machinery to modulate the competency of cells in several tissues to endocycle at weaning and acquisition of sexual maturity (Gilbert and Pfitzer). The onset of polyploidy in the murine heart, liver, pancreas, lacrimal glands and some other tissues are all under the control of various endocrine systems, namely the thymus, thyroid or sex hormones (Geschwind et al., 1958; Paulini and Mohr, 1975; Roszell et al., 1978). In mice and rats, weaning regulates and is coincident with sexual maturity and it is still unclear to me which may play a bigger role in regulating polyploidy in these tissues (Fortier et al., 2017). In vertebrates, thyroid hormones are essential for early development in various tissues, and specifically the brain (Bohman et al., 1985; Enesco et al., 1991; Gilbert and Pfitzer). Thyroid hormones are less critical but nevertheless important for sexual maturity.

In the plant model *Arabidopsis thaliana*, the onset of flowering (sexual maturity) is marked by an uptick in endocycling in cells of the leaves and root, this switch to an endocycle is regulated by the developmental hormone auxin. This increase in endoreplication has also been observed in other flowering plants, and has been linked to an increased need for biosynthesis in preparation for generating fruits/seeds (Bhosale et al., 2018; Lammens et al., 2008; Pacey et al., 2019; Del Prete et al., 2019).

What regulates sexual maturity in insects? The Juvenile Hormone (JH) is a good candidate (Ren et al., 2020; Wu et al., 2016, 2018). While JH has been extensively studied in early larval development, its role in the adult are only beginning to be understood. Recent work has shown that JH is important for mating associated behaviour in males and changes in physiology and metabolism in females (Bilen et al., 2013; Lee et al., 2017; Reiff et al., 2015; Wijesekera et al., 2016). Could JH regulate endocycling in adult fly tissues? Work in locusts has shown that the polyploidization of the adult fat body and cells in the ovary is regulated by the action of juvenile hormone (JH) (Guo et al., 2014; Irvine and Brasch, 1981; Nair et al., 1981; Wu et al., 2018). Indeed, studies have also shown that JH binding to its receptor Met and coreceptor Tai can directly activate the transcription of S phase genes. The peak of JH titre in young adults adult coincide with the onset of polyploidy in the brain (Bownes and Rembold, 1987; Yamamoto et al., 2013). (Figure 3.13 Oxidative stress and DNA damage results in increased polyploidy during early adulthood and polyploid cells are protected from cell death)in Chapter 3 shows that younger brains can respond to DNA damage by increasing levels of polyploidy. However older brains do not respond to DNA damage by increasing levels of polyploidy, even as the polyploid cells remain protected from cell death. This suggests a critical period in early adulthood when cells in the brain are able to compensate for cell loss by re-entering the cell cycle. Could JH regulate onset of polyploidy in the adult brain and mediate endocycle competency? If so, then the polyploidy we observe in the brain could be somehow related to sexual maturity.

All of my initial experiments measuring ploidy changes in the brain were performed in unmated (virgin) male and female animals. My subsequent experiments have shown that mating status does not influence levels of polyploidy (not shown). If there is indeed a hormonal

regulation of endocycling in adult tissues, it is perhaps the onset of sexual aptitude and not mating status which plays a role.

I posit that the transition to a reproduction-capable stage in sexually reproducing multicellular organisms could result in a widespread competence for endocycling in various postmitotic tissues. This could represent a late 'developmental' transition with potentially important consequences for health span in adult organisms.

4.2. DNA Damage and Polyploidy

We show in Chapter 3 that exogeneous DNA damage and oxidative stress can increase levels of polyploidy. These experiments were designed to test the hypothesis and demonstrate that exogeneous DNA damage leads to increased polyploidy, and that polyploid cells are protected from DNA damage induced cell death. Exposure to paraquat and UV both elicit a DNA damage response in the brain, and result in increased polyploidy. Work in other endocycling tissues in flies has shown that endocycling cells are protected from DNA damage induced cell death due to low levels of p53 (Mehrotra et al., 2008; Zhang et al., 2014). We show that, in adult neurons, neither the overexpression of wild type p53, nor a dominant negative p53 impact the levels of polyploidy. This suggests that the survival of polyploid neurons is not dependent on p53 inhibition. Future studies should dissect the relationship between polyploidy and DNA damage pathways in the brain.

4.2.1. **What is the source of endogenous DNA damage and how do DNA damage signals influence polyploidy?**

Our RNAseq experiments have generated a rich dataset. From our dataset, we know which genes pertaining to the DNA damage response and repair are upregulated in which parts

of the brain, and when. The optic lobes, which show the highest levels of polyploidy, also show an upregulation of DNA damage related genes as early as 7 days old, and an even higher DNA damage gene expression signature at 21 days. Our interpretation of this is that the OLs begin to cope with DNA damage quite early in adulthood, and one way that they do this is by increasing polyploidy. But what exactly is the source of endogenous DNA damage and what are the signals that induce? We can start systematically testing different DNA damage response pathways to ask which ones impact polyploidy by looking at candidate genes that are most upregulated. It is possible that multiple pathways are upstream of polyploidy induction.

4.2.1.1. *Are Transposons The Source Of DNA Damage In The Adult Brain?*

Recent work in ageing *Drosophila* has shown that transposon silencing becomes compromised with age in the brain and has been linked with conditions of neurodegeneration and decline in brain function (Chang and Dubnau, 2019; Chang et al., 2019; Krug et al., 2017; Li et al., 2013). This has been termed the 'transposon storm' hypothesis of ageing and neurodegeneration. Transposon reactivation has also recently been observed in ageing fly guts, albeit at different levels (Riddiford et al., 2020). Could transposon reactivation represent a portion of the endogenous DNA damage that cells in the brain have to endure and overcome as they age?

4.2.1.2. *Is Transcriptional Activity Causing DNA Damage In The Adult Brain?*

Another potential source of endogenous DNA damage is DNA damage associated with high transcriptional activity (D'Alessandro and d Adda di Fagagna, 2017). Highly transcribed loci in the genome are known to be susceptible to damage as a result of RNA:DNA hybrid formation. Recent work has shown that proteins implicated in neurodegenerative diseases such as TDP-43 are involved in preventing and contributing to repair at sites of transcription

associated DNA damage. Age associated decline in TDP43 (Hill et al., 2016; Langellotti et al., 2016), coupled with high levels of transcription in neurons could contribute to unresolved DNA damage resulting from transcription-associated DNA lesions.

4.2.1.3. *Does Increased Oxidative Stress As A Result Of Reduced Mitochondrial Integrity Lead To DNA Damage In The Adult Brain?*

Our RNAseq dataset suggests that older flies show reduced ATP metabolism, oxidative phosphorylation and cellular respiration. This may indicate compromised mitochondrial function, which is a known hallmark of ageing and a well known source of cellular oxidative stress (López-Otín et al., 2013). Compromised mitochondrial function can lead to increased levels of intracellular peroxide and superoxide radicals which can lead to oxidative DNA damage. Oxidised bases in DNA may evoke the need for base or nucleotide excision repair pathways to repair lesions.

4.2.2. **Do Different Cells Respond To DNA Damage Differently?**

The brain is a glorious mixed bag of different cell types performing different functions. While we know that the neuronal polyploidy is not p53 dependent, it is possible that the same cannot be said of glia. Given the very different roles neurons and glia play in the brain, and their inherent differences in metabolism and transcription, future studies should use cell type specific drivers in different and less numerous populations of cells to start addressing this.

4.3. How Do Cells In The Adult Brain Die?

Work from the Calvi lab has shown that polyploid cells are protected from DNA damage induced cell death as a result of low levels of p53 and chromatin-level silencing of pro-apoptotic genes (hid, reaper and grim – collectively known as the H99 locus) which are upstream of the

caspase cascade that directs apoptosis (Zhang et al., 2014). However, in the brain, at least in neurons, the polyploidy does not appear to be dependent on p53. Our experiments overexpressing wild type and dominant negative versions of p53 in neurons show that the levels of cell death and polyploidy are not altered in the adult brain. This indicates that p53 is not required for neuronal survival or the survival of polyploid neurons. Our data also indicates that the establishment of polyploidy in neurons is p53 independent.

Additionally, recent work from the Abrams lab shows that the drosophila adult head (brain) engages a non-canonical p53 dependent transcriptional program which drives the expression of genes involved in DNA repair, metabolism and proteolysis, but not apoptosis (Kurtz et al., 2019).

Consistent with this, in our studies, we have not been able to successfully detect evidence of apoptotic cell death in the adult brain using antibodies commonly used to detect caspases. However, we do see steady rates of cell loss with age as well as increased cell death upon induced DNA damage by propidium iodide staining and SYTOX green staining. Our observations of cell loss are consistent with recent single cell work from the Aerts lab. This means that cell loss occurs via a yet-unknown mechanism in the brain Work in the developing brain has shown that some neuroblasts are eliminated through necrosis in the absence of Fzy, suggesting that cells in the brain may adopt non apoptotic programs to eliminate cells. Excitotoxicity-mediated cell loss is yet another way cells in the brain may be lost. It is also likely that a completely unknown mechanism of cell death occurs in the adult fly brain.

4.4. How Does Polyploidisation Affect Neuronal And Glial Function?

I’ve described polyploidy in the fly brain (except for SPGs and some tracheal cells (Djabrayan et al., 2014; Von Stetina et al., 2018; Unhavaithaya and Orr-Weaver, 2012)) as an

adult specific phenomenon, and as a potential facultative adaptation to respond to age associated oxidative stress and DNA damage. We still don't know how the polyploid state impacts cellular function. Just as neurons and glia may respond differently to DNA damage, their respective functions may also be affected differently upon polyploidisation.

Speculation about the role that tetraploidy plays in neurons has varied from generation of neuronal diversity to increased capacity for dendritic arborization. One study performed over thirty years ago (Szaro and Tompkins, 1987) compared the dendritic arbors of two *Xenopus* species, one diploid species and another which displays whole organism tetraploidy (where the entire organism has a larger genome). This study showed that while the brains from these two organisms were the same size, but the neurons from the tetraploid species showed longer dendritic segments as well as larger dendritic arbors. This could mean that tetraploid neurons are able to make more synaptic connections and participate in larger neuronal networks, contributing to functional diversity. Polyploid neurons could also, as a virtue of increased biosynthetic capacity, increase production of neurotransmitters, resulting in robust signaling.

Increased biosynthetic capacity in wrapping glia as a result of endocycling could ensure better sheathing of axon bundles and enhanced neuronal conductivity. Similarly, increased biosynthetic capacity could improve phagocytic glial function and aid in better clearance of cellular debris in the adult brain. Glial cells provide the bulk of the glycolytic support to the neurons in the brain, in fact glial glycolysis has been shown to be essential for neuronal survival (Volkenhoff et al., 2015) in the adult brain. Glia are also outnumbered by neurons approximately 9:1. Our RNAseq data shows that oxidative phosphorylation becomes compromised with age in the brain. Could polyploidisation be one way that glial cells enhance their trophic capacity by increasing their biosynthetic ability?

4.5. How Can We Visualize And Manipulate Polyploid Cells? Progress Toward Developing A Polyploidy Sensor And Gene Expression Tool.

Currently the best tool at our disposal to visualize polyploid cells in the brain *in situ* is the CoinFLP tool (Bosch et al., 2015). While it is great for marking and visualizing polyploid cells, it is not without limitations. CoinFLP uses two overlapping and exclusive FRT cassettes to 'flip' out a transgenic 'STOP' cassette to allow expression of either a LexGAD or a GAL4 driver. To label polyploid cells, the CoinFLP containing flies have to be crossed to animals that have three more transgenes, namely an inducible FLP recombinase, a UAS-fluorescent protein, and a $LexA_{op}$-fluorescent protein. This makes combining the genotypes with additional genetic manipulations difficult, and does not allow for manipulation of polyploid cells. Moreover, CoinFLP 'flipping' is unequal – LexGAD expression is 4 times as likely as GAL4 expression.

To overcome some of these problems, we propose the polyploidy sensor – transgenic fly which will reconstitute a split GAL4 only in polyploid cells. The idea is similar to that of CoinFLP, but the difference is that instead of expressing either LexGAD or GAL4 in diploid cells, and both in some of the polyploid cells, we will make a transgenic fly which will, upon induction of FLP recombinase, reconstitute a GAL4 driver whose expression can be used to perform genetic manipulations specifically in polyploid cells. Since this will be a random flip-mediated labelling, just like CoinFLP, it will not label all the polyploid cells. This can be used to our advantage: we can ask interesting questions like, what happens when we only ablate some of the polyploid cells, or how do unmanipulated polyploid cells compensate for cell loss or damage in a non-autonomous way.

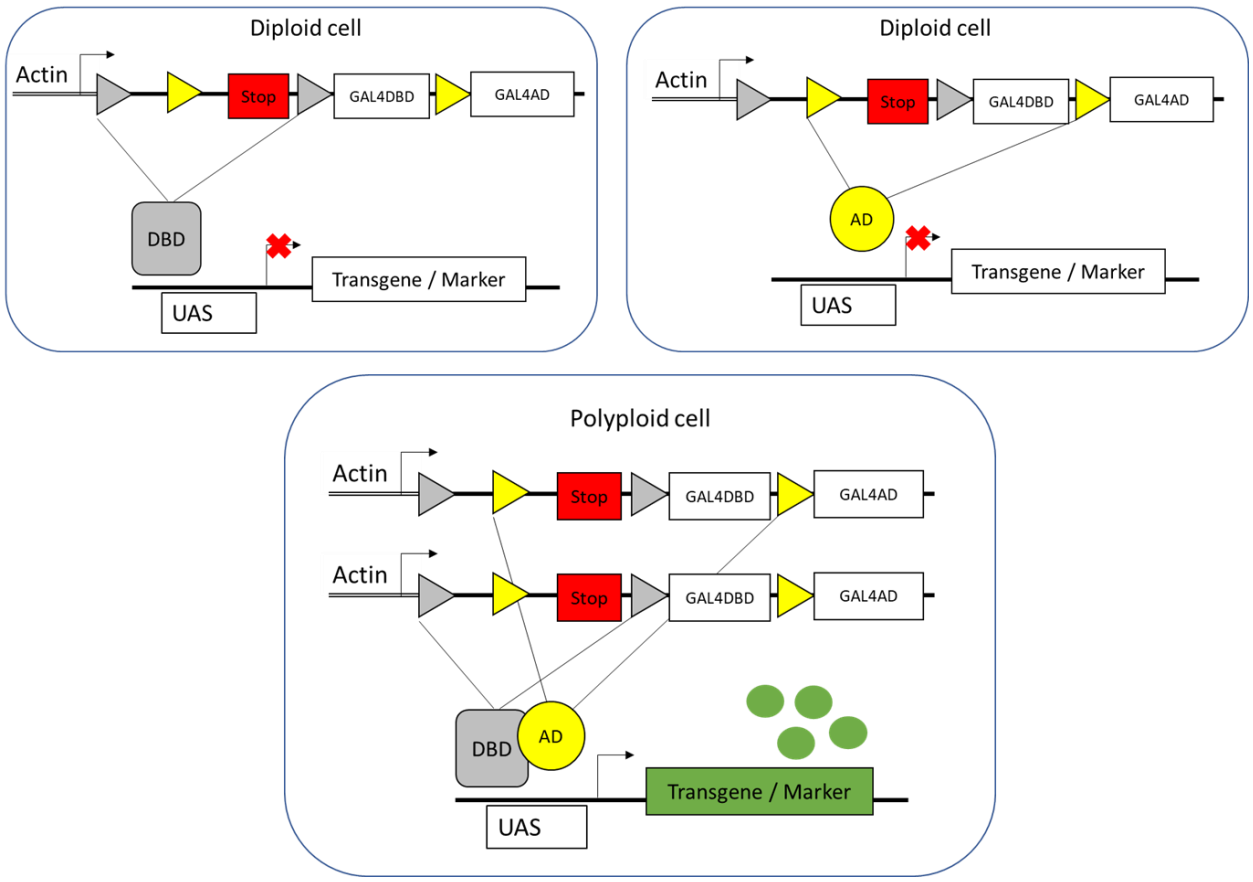

Figure 4.3 Cartoon illustrating the proposed 'polyploidy' sensor tool

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