Regeneration in the adult *Drosophila* brain

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Abstract

Understanding the molecular and cellular mechanisms underlying neurogenesis after injury is crucial for developing tools for brain repair. We have established an adult *Drosophila melanogaster* model for investigating regeneration after central brain injury. Within 24 hours after Penetrating Traumatic Brain Injury (PTBI) to the central brain, we observe a significant increase in the number of proliferating cells. Between one- and two-weeks post-injury, we detect the generation of new neurons and glia and the formation of new axon tracts that target appropriate brain regions, suggesting there could be functional regeneration. Consistent with functional regeneration, locomotion abnormalities observed shortly after PTBI are largely reversed within 2 weeks of injury. Further, we find that cells surrounding the injury site upregulate neuroblast genes, such as *asense* and *deadpan*, and demonstrate that these cells give rise to the new neurons and glia. Taken together, our data support the hypothesis that young, adult *Drosophila* brains are capable of neuronal repair after central brain injury. We anticipate that our model will facilitate the dissection of the mechanisms of neural regeneration and anticipate that these processes will have relevance to humans.

Introduction

Different species vary in their ability to regenerate, and within a species, there is tissue to tissue variation in regenerative capacity. (Birnbaum and Sanchez Alvarado, 2008, Bollini et al., 2018, Michalopoulos, 2007, Stanger, 2015). One of the most arguably important organs, the human brain, has become the center of intense investigation regarding the extent to which it can produce new cells in adulthood. Classical studies reported that the brain stopped making new neurons shortly after birth (Ramon y Cajal, 1913, Ramon y Cajal, 1914). However, in 1998, a landmark study concluded that adult humans are able to create new neurons in the hippocampus (Eriksson et al., 1998). Furthermore, this ability is retained throughout later stages of life. These findings have been corroborated (Reif et al., 2006, Manganas et al., 2007), although the exact number varies among studies (Spalding et al., 2013, Dennis et al., 2016, Eriksson et al., 1998, Knoth et al., 2010). Populations of dividing progenitor cells now have been observed in two major regions of the rodent brain: the subventricular zone (SVZ) of the forebrain and the dentate gyrus (DG) of the hippocampus (Altman and Das, 1965) (Altman, 1969) (Kaplan and Hinds, 1977). The progenitors in both brain regions generate multiple cell types, including neurons, astrocytes and oligodendrocytes, over the course of the animal's lifetime (Kuhn et al., 1996).

Identification of the cell types capable of regenerating brain structures and understanding the underlying molecular mechanisms that regulate neuroregeneration is central to developing novel therapeutics for neural degenerative diseases and treatment of brain injuries. Analysis of SVZ and DG neural stem cells continues to provide critical insights into neural regeneration. However, these cells are unlikely to provide progenitors for other brain regions. Therefore, emphasis in the neuroregeneration field has been on transplanting embryonic or induced pluripotent stem cells (Vishwakarma et al., 2014). However, stem cell transplants often are accompanied by tumor formation (Amariglio et al., 2009) and obtaining functional integration of transplanted neural cells remains a major challenge. A more recent

93 focus for the field therefore has been on coaxing resident cells in the brain to undertake
94 regeneration (Gao et al., 2016). This avenue of investigation would be facilitated by the
95 availability of a model system that mimics key aspects of mammalian neurodegeneration
96 phenotypes but is more experimentally accessible.

97 For over a century, the fruitfly *Drosophila melanogaster* has been used as a model
98 organism, furthering many fields, including developmental genetics and neuroscience. Although
99 *Drosophila* are invertebrates, there are many parallels between flies and mammals, and
100 discoveries from *Drosophila* repeatedly have proven relevant to human biology. A *Drosophila*
101 brain is composed of approximately 90,000 neurons (Chiang et al., 2011). While this is a million-
102 fold fewer than the average human brain (Meinertzhagen, 2010), both human and *Drosophila*
103 brains are complex and have analogous neural cell types (Lessing and Bonini, 2009). Common
104 neurotransmitters (GABA, glutamate, and acetylcholine) and biogenic amines (dopamine and
105 serotonin) are utilized in both humans and *Drosophila* (Bellen et al., 2010). In addition, human
106 and *Drosophila* neurons function in similar ways, with neurotransmission occurring at synapses.
107 Even the architecture of synapses is conserved between humans and *Drosophila* (Lessing and
108 Bonini, 2009). Humans and *Drosophila* also have similar physiologies, including homologous
109 sodium and calcium channels that are capable of producing action potentials and regulating
110 membrane potential (Bellen et al., 2010).

111 *Drosophila* have been utilized extensively to study neural development (Spindler and
112 Hartenstein, 2010, Sasse et al., 2015). Most of this research has focused on larval and pupal
113 stages and includes the identification of neural progenitor cells in the brain. During
114 neurogenesis in the embryonic and larval stages of *Drosophila*, neuroblasts (NBs) undergo
115 multiple rounds of asymmetric division to generate one larger and one smaller daughter cell
116 (Homem et al., 2015). At each division, cell-fate determinants are distributed from the NB to the
117 daughter cells. Numb, Prospero (Pros), and Brain tumor (Brat) are partitioned to the basal cell
118 cortex, and atypical kinase C (aPKC) to the apical cell cortex. Therefore, when mitosis is

119 complete, each daughter cell inherits a different set of determinants. As a result, the apical
120 daughter cell remains an NB and continues to proliferate while the basal daughter cell becomes
121 committed to differentiation. Central brain neurons and glia derive from two types of NBs (Boone
122 and Doe, 2008, Egger et al., 2008, Homem and Knoblich, 2012, Weng and Lee, 2011, Homem
123 et al., 2015). Type I NBs express the transcription factors Deadpan (Dpn) and Asense (Ase)
124 and undergo asymmetric cell division to self-renew and produce ganglion mother cells (GMCs)
125 (Knoblich, 2010). Each GMC then divides symmetrically to produce two neurons or glial cells.
126 Type II neuroblasts express Dpn, but not Ase and also self-renew, but, unlike type I NBs, give
127 rise to intermediate neural progenitors (INPs) (Boone and Doe, 2008, Bello et al., 2008,
128 Bowman et al., 2008). INPs undergo a maturation process, during which they do not divide but
129 sequentially initiate expression of the transcription factors Ase and Dpn. After this initial stage,
130 each mature INP undergoes three to five additional rounds of asymmetric division, with each
131 round generating another INP and a GMC that divides terminally into two neurons or glial cells.
132 Other transcription factors required during neurogenesis in the Type I and/or Type II lineages
133 include Prospero (Pros), Inscuteable (Insc), and Earmuff (Erm) which are required for
134 asymmetric cell division (Chia et al., 2008).

135 While neurogenesis is very well studied during development, there remain gaps in our
136 knowledge about homeostasis and response to damage, particularly in the adult brain. This can
137 be partly attributed to previous studies indicating that the adult *Drosophila* brain has limited
138 mitotic activity (~1 dividing cell/brain) (von Trotha et al., 2009) and the fact that known neural
139 progenitors undergo terminal differentiation or apoptosis during metamorphosis (Siegrist et al.,
140 2010). Both of these characteristics are very similar to mammalian brains (Ming and Song,
141 2011, Stiles and Jernigan, 2010). The combination of rare cell proliferation and NB death or
142 terminal differentiation has made the adult *Drosophila* brain an unlikely candidate model in
143 which to investigate neuroregeneration. Nonetheless, other studies have demonstrated that the
144 adult *Drosophila* is capable of neurogenesis after injury (Fernandez-Hernandez et al., 2013,

Moreno et al., 2015). In fact, slowly cycling neural progenitor cells were discovered in the medulla cortex of the optic lobes. These progenitors are activated upon injury (Fernandez-Hernandez et al., 2013) and create new neurons in response to damage. These data support the idea that if there are slowly cycling cells in the central brain, they also may be able to proliferate post-injury and function in regeneration.

The limited information regarding not only the regeneration potential of the adult *Drosophila* brain, led us to examine the regenerative potential of the adult *Drosophila* central brain after injury. We have developed a novel model of brain injury that we call Penetrating Traumatic Brain Injury (PTBI). To inflict PTBI, we use a sterilized metal needle to penetrate the adult head cuticle and puncture the brain. Our goal is to fully exploit the adult *Drosophila* brain to characterize its neurogenic potential post-injury and discover mechanisms that control neuroregeneration. This will further our knowledge of development and homeostasis of the nervous system and may lead to novel neurodegenerative disease therapies.

Results

PTBI as a new model for neuroregeneration

To investigate the neuroregenerative capacity of the adult *Drosophila melanogaster* central brain, we first needed to develop a reproducible injury method. The model we devised is called penetrating traumatic brain injury (PTBI). To induce PTBI, we employed a method of injury termed the penetrating *s*mall *t*argeted *a*rea of the *b*rain (STAB) injury. This was accomplished using a thin metal insect needle (~12.5μm diameter tip, 100 μm diameter rod) to penetrate the head cuticle and the brain, specifically near the mushroom body (MB; **Fig. 1A, B).** We focused on the mushroom body (MB), a region of the central brain critical for learning and memory (Aso et al., 2014), because MB neuroblasts are the last to stop proliferating during development (Siegrist et al., 2010, Ito and Hotta, 1992, Ito et al., 1997). We therefore reasoned that the MB may have the most mitotic potential. Located dorsally in the central brain, the MB is made up of

complex dendrite and axon arbors in large and highly stereotyped arrays. This architecture makes it useful for assaying the regeneration of neurites (Aso et al., 2014). Another factor that we took advantage of is that the cell bodies of the MB lie at the surface of the brain and can be visualized through the head cuticle using the expression of green fluorescent protein (GFP), facilitating for reproducible damage to the MB.

Surprisingly, when we injured 0-6-hour post-eclosion *OK107/yw* adult males and allowed them to recover for 24 hours, we saw no significant decrease in survival of injured males compared to uninjured age-matched controls, with each having an approximate 99% survival rate (**Fig. 1G**). Yet, when adult *OK107/yw* males were aged to 14 days post-eclosion, and then injured, there was a significant increase in mortality to ~13% following PTBI (p-value<0.0001) (**Fig. 1G**). A similar trend was seen when flies were aged to 28 days then injured, with mortality increasing further to ~25% (p-value<0.006). Thus, as flies grow older, their ability to survive a PTBI decreases. This trend showed that resilience in response to injury is specific to younger flies. In subsequent experiments, we therefore focused on injuring flies that were 0-6 hours old.

To quantify the neurodegeneration caused by PTBI, we used a standardized index (Cao et al., 2013) to analyze histological samples from brains 25 days after injury. We observed that there was some increase neurodegeneration following PTBI compared to age-matched uninjured controls (**Fig. 1C-E**). While younger flies were able to survive PTBI for at least 24 hours, we wanted to see how this would impact their lifespan. We therefore examined the lifespan of *OK107/yw* injured males and observed a substantially reduced lifespan compared to uninjured controls, with only 50% survival at around 48 days and no survivors at approximately 74 days post-injury (**Fig. 1F**).

PTBI stimulates cell proliferation

Because one of the first signs of regeneration is cell proliferation, we assayed cell division post-injury using a mitotic marker, phospho-histone H3 (PH3). Histone H3 is transiently

197 phosphorylated during M phase of the cell cycle (Hans and Dimitrov, 2001). Brains from
198 *OK107/yw* adult males that were assayed 24 hours post-PTBI using anti-PH3 had a significant
199 increase of PH3-positive cells, around 11 cells, compared to uninjured controls of the same sex,
200 age, and genotype, in which we observed about 2 cells per brain (p-value<0.0001)(**Fig. 2A-D,**
201 **I**). These PH3-positive cells primarily were located near the area of injury. Similar numbers of
202 proliferating cells were seen at 24 hours using the mitotic marker 5-ethynyl-2'-deoxyuridine
203 (EdU) (**Fig. 3-S1**). To quantify this, we mapped each PH3-positive cell to a hemisphere within
204 the central brain: left or right, with the right hemisphere the location of injury (**Fig. 3-S2A,B**). 24
205 hours post-injury, there is an increased number of PH3-positive cells in the right hemisphere,
206 where the penetrating STAB injury occurred (**Fig. 3-S2C**). Although not statistically significant,
207 we also detect a slight increase in cell proliferation in the left hemisphere at 24 hours post-PTBI
208 (**Fig. 3-S2C**). These data demonstrate that a penetrating STAB injury stimulates cell division in
209 young flies and that the mitotically active cells primarily are located near the area of injury. The
210 new cells could be created to replace the cells damaged by the injury and/or to remove debris
211 caused by the injury.

212 We next wanted to determine whether these newly created cells were maintained or
213 were eliminated soon after injury. Because PH3 only transiently labels dividing cells, we used a
214 different assay for these experiments. Specifically, we utilized EdU which is incorporated into
215 newly synthesized DNA of dividing cells and therefore permanently labels dividing cells and
216 their progeny. We used both pulse-chase and continuous feeding experimental designs and
217 assayed for EdU incorporation using fluorescent 'click chemistry' (InVitrogen®). The pulse-
218 chase experiments allowed us to ask whether the cells incorporating EdU were maintained. If a
219 cell died after synthesizing DNA, the incorporated EdU would either be undetectable or punctate
220 instead of being uniformly allocated within the nuclei following the chase. We saw similar
221 numbers of evenly labeled nuclei in both pulse-chase labeled brains and in brains from animals
222 continuously fed EdU (**Fig. 3-S3**). This indicates that the EdU labeled cells were indeed

proliferating and not dying. Consistent with the PH3 labeling at 24 hours, we observed more EdU-positive cells 7- and 14-days post-injury in PTBI samples compared to uninjured controls (p-value<0.0001 and p-value<0.0002) (**Fig. 3-2E-I**). This indicates that cell proliferation continues between 7 and 14 days post-PTBI. Interestingly, at the later timepoints we saw a statistically significant increase in the number of EdU-positive cells not only in the right hemisphere (region near the injury), but also in the left hemisphere (**Fig. 3-S2D**). This suggests that the penetrating STAB injury may induce both local and widespread proliferation. However, we have not ruled out the possibility that some of the EdU+ cells distant to the injury migrated from areas closer to the wound. Similar effects at a distance from an injury have been observed in rodent models of TBI (Urrea et al., 2007, Ngwenya and Danzer, 2018).

Introducing even a mild injury may also induce cell death, and our histology experiments suggested that PTBI did induce some cell death. To assay cell death, we therefore used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (**Figs. 3-S4, 3-S5**). TUNEL marks the terminal stages of death, both apoptotic and necrotic, where nuclear DNA has been cleaved and degraded by DNases (Grasl-Kraupp et al., 1995). 0-6-hour old *OK107/yw* adult males were injured and allowed to recover for 4 hours before dissecting the brains and TUNEL staining. We classified the brains into groups based on the number of TUNEL-positive cells/brain, with Group 1 having 0 to 9, Group 2 having 10-19, and Group 3 having 20-29 TUNEL-positive cells, respectively. The uninjured controls fell into Group 1, indicating that there is not much cell death occurring in uninjured young adult brains (**Fig. 3-S4A-A',C**). Following PTBI, the brains were classified as either Group 2 or Group 3 (**Fig. 3-S4B-B',C**). This indicates that our standard injury method causes cell death in the brain within 4 hours-post-injury. Next, we wanted to examine the temporal dynamics of the cell death observed after injury. When we analyzed cell death later, at 24 hours post-injury, there were more TUNEL-positive cells in injured brains compared to controls, with controls falling into Group 2 and injured brains falling in the Group 3 category (**Fig 3-S5**). However, when we examined cell death at 10 days post-

injury, there was no significant difference in the numbers of TUNEL-positive cells, with both control and injured brains falling into Group 3 (**Fig 3-S5**). These data indicate two things: 1) PTBI-induced cell death is transient and peaks at earlier timepoints after injury; and 2) that cell death in uninjured adult brains gradually increases following eclosion.

Proliferative ability is age-dependent

Because PTBI in older adult flies results in higher mortality than PTBI in young adult flies (**Fig. 3-1G**), we hypothesized that age might be an important factor in the proliferative ability post-injury. To test this, we aged *OK107/yw* adult males out to 7, 14, and 28 days, then injured them using our standard penetrating STAB injury, and assayed for cell proliferation 24 hours later. For this experiment, we used anti-PH3 to identify cells that were actively dividing. *OK107/yw* adult male brains injured at 7 days had significantly fewer PH3-positive cells 24 hours after injury compared to flies that had been injured 0-6 hours post-eclosion (p-value<0.004) (**Fig. 3-2J**). Although flies injured at 7 days had more PH3-positive cells after injury than the age-matched uninjured controls, this difference was not statistically significant (**Fig. 3-2J**). However, flies injured at either 14- or 28-days exhibited little cell proliferation post-injury, and this was not significantly different compared to age-matched uninjured controls (**Fig. 3-2J**). Taken together, these data support the idea that there is a specific window of time when adult *Drosophila* brains possess the ability for cells to initiate division, but this timeframe is limited to very early adulthood, from eclosion until ~7 days of age. At 7 days of age and further, we see a decrease in the ability of adult Drosophila's ability to respond to a central brain injury. Nonetheless, uninjured flies at 24 hours and 7 days do not have significantly different baseline cell proliferation, despite the fact that response to injury is significantly different (**Fig. 3-2J**). This suggests that the presence of PH3-positive cells does not reflect the ability of the fly brain to regenerate after injury. At 14 and 28 days of age, uninjured flies exhibit a noticeable decrease in baseline cell division compared to 24 hour and 7-day uninjured controls, and injured flies no

longer mount a proliferative response to injury (**Fig. 3-2J**). Taken together, these data indicate that the the climate/microenvironment of young brains may be more permissive of/conducive to cell proliferation.

Importantly, while age is an important factor in proliferation rates post-PTBI, once proliferation is initiated, it can persist for more than 7 days. This is supported by the EdU data (**Fig. 3-2I**), in which there is a clear increase in the total number of EdU+ cells between 7 and 14 days. This raised the question of whether EdU+ cells observed at 14 days are continuing to proliferate. The EdU labeling is cumulative, and we needed a real-time assay to address this question. We therefore used anti-PH3 staining to label actively dividing cells. Newly eclosed *OK107/yw* adult males were subjected to PTBI and aged to 24 hours, 7 days, or 14 days post-injury prior to dissecting and fixing their brains and staining them with anti-PH3. At 7 days post-injury, the numbers of PH3+ cells were similar to those at 24 hours post-injury (**Fig. 3-S6**). However, when flies were allowed to recover for 14 days, we observed fewer PH3-positive cells post-injury compared to injured flies aged to 24 hours and 7 days. Additionally, while the number of PH3+ cells in 14-day post-PTBI brains appears to be slightly higher than controls, this difference is not statistically significant (**Fig. 3-S6**). This is consistent with the EdU data in **Fig. 3-2I**, and supports the idea that there is a specific window of time between eclosion and 7 days of age when adult fly brains have a relatively high capacity for (to initiate?) cell division.

<u>Characterizing the cell populations of dividing cells post-injury</u>

Next, we set out to determine the identities of the mitotically active cells. This was accomplished by feeding *OK107/yw* adult males with EdU, then and assaying their brains for EdU, the glial protein Reversed polarity (Repo) and the neuronal protein Embryonic lethal, abnormal vision (Elav). At 7 days post-injury, we observed 4 classes of cells (**Fig. 3-3A-D'''**). Class I consists of cells that were EdU+ but did not express Elav or Repo and therefore had an unknown identity (arrowheads in **Fig. 3-3A-A'''**). Out of 172 EdU+ cells counted, approximately 44% were Class I

(**Fig. 3-3E**). Cells that were EdU+ and Repo+ were defined as Class II (arrowheads in **Fig. 3-3B-B'''**). At 7 days post-injury, approximately 38% of EdU+ cells expressed a glial identity (**Fig. 3-3E**). Class III cells were EdU+ and expressed Elav (arrowheads in **Fig. 3-3C-C'''**). Approximately 13% of cells that had divided by 7 days were EdU+ and Elav+ (**Fig. 3-3E**). These data show that cells actively divide after PTBI, and that the dividing cells either are or become glia and neurons. While most of the new cells that expressed mature cell type markers generated after injury are glia, there were some new neurons. Interestingly, we also observed a fourth class, cells that were EdU+, Repo+, and Elav+ (arrowheads in **Fig. 3-3D-D'''**). Approximately 4% of EdU+ cells were classified as Class IV (**Fig. 3-3E**). These cells that expressed both glial and neuronal identity were not detected in uninjured adult brains. At seven days post-eclosion, approximately 46% of EdU+ cells in control brains are Class I and 53% of of EdU+ cells are Class II (**Fig. 3-3F**). These numbers are similar to what has been seen in previous studies of wildtype brains using a different nucleotide analog, bromodeoxyuridine (BrDU) (von Trotha et al., 2009). In control brains, not only were there many fewer EdU+ cells, we also did not observe either Class III or Class IV cells (**Fig. 3-3F**). This supports the idea that injury stimulates neurogenesis.

Because age plays an important role in the proliferative ability of brain cells, we asked whether the amount of time after injury impacts the types of cells observed. Using the same four classes described in the 7-day post-PTBI experiment, we also looked at the cell types generated at 24 hours and 14 days post-injury (**Fig. 3-3E**) . The proliferation marker we used in the 24-hour assay was anti-PH3 and the marker used in the 14-day assay was EdU incorporation. At 24 hours post-injury, we observed the following distribution of classes: Class I, 56%; Class II, 38%; Class III, 0%; and Class IV, 5% (**Fig. 3-3E**). While there is a slightly lower proportion of Class I (EdU+) cells at 7 days than at 24 hours, the proportions of Class II (EdU+/Repo+) and IV (EdU+/Repo+/Elav+) cells are approximately the same. However, at 24 hours post-PTBI, there are no Class III (EdU+/Elav+) cells (**Fig. 3-3F**). Control uninjured flies at

24 hours had 39% Class I cells and 60% Class II cells (**Fig. 3-3F**). In comparing 7-day to 24-hour control flies, there appears to be a slight trend of increasing Class I cell types and decreasing Class II cells (**Fig. 3-3F**). Together these data demonstrate that new neurons are created later than new glial cells and support the hypothesis that some of the Class I cells (EdU+) are giving rise to Class III cells (EdU+/Elav+).

If we look later, 14 days post-PTBI, there is another striking change in cell type distribution, with only 25% of cells falling into Class I, 45% in Class II, and 28% of cells in Class III (**Fig. 3-3E**). Intriguingly, we did not observe any Class IV cell types 14 days post-PTBI. Class IV (EdU+/Repo+/Elav+) cells express a hybrid glial and neuronal identity that is not observed in control uninjured brains. We speculated that this could represent an abnormal transitional status enroute to differentiation of new glia and neurons. By 14 days post-PTBI, these cells are no longer detected, suggesting that they have committee to either neuronal or glial fate. Control uninjured flies at 14 days had similar patterns as earlier timepoints, with approximately 66% of cells in Class I, 33% of cells in Class II, and no cells in Classes III or IV (**Fig. 3-3F**).

Presence of neuroblast-like cells post-PTBI

To test whether the generation of new neurons post-PTBI follows a normal developmental progression from NBs, we probed PTBI and control brains from *ase-Gal4, UAS-Stinger; UAS-Gal80^{ts}* adult males with anti-Dpn and anti-PH3 antibodies to test whether NB genes were upregulated post-injury. These flies were reared at 18°C until eclosion. At 18°C, the Gal80^{ts} is functional and prevents expression of the fluorescent Stinger protein in *ase*-expressing cells. 0-6 hours after eclosion, adult male flies were subjected to PTBI and placed at 30°C for 24 hours prior to dissection and immunostaining. At 30°C, the Gal80^{ts} is not functional and fluorescent Stinger protein is expressed in *ase*-expressing cells. 24 hours post-injury, we observe both GFP+ cells and cells that are Dpn+/PH3+ in PTBI, but not control, brains (**Fig. 3-4A-D'''**). This indicates that proliferating cells have key features of neuroblast identity, including *ase* and *dpn*

353 expression. Interestingly, we did not observe cells that were GFP+/Dpn+/PH3+. However, cells
354 that were GFP+ and cells that were Dpn+/PH3+ were clustered together and found adjacent to
355 each other, suggesting the two types of cells might share a lineage.

356 Upon cell division, Pros is asymmetrically segregated to the non-NB daughter cell where
357 it migrates from the cytoplasm into the nucleus. In the nucleus, Pros assumes the role of
358 transcription factor, which establishes the neural fate of the daughter cell. Indeed, using *ase-*
359 *Gal4, UAS-mCD8-GFP* flies, we observe large GFP+ cells near the wound at 24 hours post-
360 injury (arrowheads and asterisks in **Fig. 3-4E-F'**). A subset of the GFP+ cells also express
361 cytoplasmic Pros (asterisks in **Fig. 3-4F-F'**). These GFP+/cytoplasmic Pros+ cells are
362 consistent with the presence of NB-like cells post-PTBI. The GFP+/cytoplasmic Pros+ cells are
363 not seen in uninjured controls, which indicates that PTBI is somehow stimulating the generation
364 of these NB-like cell types.

365 To quantify levels of neural progenitor gene expression, we collected and injured
366 *OK107/yw* adult males at 0-6 hours post-eclosion. We then extracted total mRNA at 5 different
367 time points (4 hours, 24 hours, 3 days, 7 days, and 14 days) from post-PTBI and age-matched,
368 uninjured control heads. Relative transcript levels of neural progenitor genes were measured
369 using quantitative real-time PCR (qRT-PCR). At 4 and 24 hours of age, *ase* expression is
370 increased more than 2-fold in injured flies compared to controls (**Fig. 3-4G**). This difference
371 decreases by 3 days and is not significant at later timepoints (**Fig. 3-4G**). Although we observed
372 Dpn+ cells using immunohistochemistry near the area of injury at 24 hours post-PTBI, *dpn*
373 mRNA levels were not detectably increased at any timepoints (**Fig. 3-4H**). This could be
374 because we isolated RNA from whole heads and relatively few cells activate *dpn* following PTBI.

375 Consistent with the idea that NB-like cells are generated following PTBI, transcript levels
376 of *insc* were significantly increased at 4 hours, 24 hours, and 3 days, while *erm* transcript levels
377 also were increased at 4 hours (**Fig. 3-4I,J**). Together these data support the hypothesis that
378 there are NB-like precursor cells in the adult brain that are either generated following PTBI or

activated by PTBI. These cells are the potential precursor cells of adult-born neurons and glia and represent a previously unknown cell type within the adult *Drosophila* brain.

Lineage-tracing the origins of newly created cells

As mentioned previously, in the uninjured wildtype adult central *Drosophila* brain, there is no evidence of NBs. However, post-PTBI, we observe NB-like cells and increases in NB-related transcript levels. The presence of these NB-like cells could be explained in two ways: 1) differentiated cells such as glia or neurons are giving rise to new glia and neurons after injury, via dedifferentiation to NB-like fates; or 2) a quiescent population of adult neural stem cells exists and is activated by damage, similar to what is described in the optic lobe (Fernandez-Hernandez et al., 2013). To test whether glial cells can give rise to neuronal stem cells, we carried out lineage-tracing of glial-derived cells and asked whether they could become neurons. To do this, we used *repo-Gal4* in conjunction with a *flipout-GFP* construct to permanently mark glial lineages. F1 males that were *w[*]; repo-Gal4/P{w[+mC]=Ubi-p63E(FRT.STOP)Stinger}15F2* were injured at 0-6 hours post-eclosion and aged for 14 days prior to dissection and immunostaining. We observed no GFP+ cells that were also Elav+. This indicates that glia do not generate neurons through either trans-or dedifferentiation. We note that ~50% of PH3+ and EdU+ cells post-PTBI are glia (**Fig. 3-3E**). Thus, although we found no evidence that glia give rise to neurons, glia are nonetheless proliferating, especially very early post-PTBI. To test whether neurons can give rise to glia, we used a similar lineage-labeling technique with an *Nsyb-Gal4* driver in a system called Gal4 technique for real-time and clonal expression (G-TRACE) (Evans et al., 2009). F1 males that were *w[*];Nsyb-Gal4/P{w[+mC]=UAS-RedStinger}6,P{w[+mC]=UAS-FLP.Exel}3,P{w[+mC]=Ubi-p63E(FRT.STOP)Stinger}15F* were injured at 0-6 hours post-eclosion and aged for 14 days prior to dissection and immunostaining. We observed no GFP+/Repo+ cells. This indicates that neurons do not give rise to new glia post-injury.

To address the second possibility, that new cells are created by a quiescent NB-like population, we used a similar lineage labeling technique, this time in combination with a neuroblast driver, *dpn-Gal4*. Although we did not detect GFP+/Dpn+ cells using Dpn antibody in the *ase-Gal4* lineage experiments, we did observe GFP+ (i.e. *ase*-expressing) cells near Dpn+ cells, suggesting there might be a clonal relationship. Indeed, in Type II NB lineages during normal development, *dpn* expression in NBs is followed by *ase* expression in the INPs to which those NBs give rise. Also, *dpn* is expressed in both Type I and Type II NBs. We therefore decided to use *dpn-Gal4* to search for quiescent NBs and other NB-like cells. To ensure that neuroblast cells were not labeled during development, we added a temperature sensitive Gal80 and reared the crosses at 18°C. Under these conditions, the Gal80 prevents transcriptional activation by Gal4, thus keeping the lineage tracing system off. F1 males that were *w[*]; dpn-Gal4/P{w[+mC]=tubP-GAL80[ts]}20; P{w[+mC]=UAS-RedStinger}6,P{w[+mC]=UAS-FLP.Exel}3,P{w[+mC]=Ubi-p63E(FRT.STOP)Stinger}15F* were collected or injured at 0-6 hours post-eclosion and aged for 14 days at 30°C prior to dissection and immunostaining. At 30°C, the temperature sensitive Gal80 protein is inactivated and Gal4 can activate transcription. Indeed, 14 days post-injury, we observed GFP+/Elav+ cells in injured brains (arrowheads in **Fig. 3-5A-A''**), but not in uninjured age-matched controls. These results are consistent with the existence of a quiescent stem cell-like population in the adult *Drosophila* brain that is activated by injury to create new neurons and new glia. Several lines of evidence strongly support this view, including the presence of Dpn+ and Ase+ cells near the area of injury 24 hours post-PTBI (**Figs. 3-4A-D''' and 3-5A-A''**) and the elevated expression levels *ase, erm,* and *insc* post-PTBI. However, because no Dpn+ cells are observed in our control uninjured central brains, these putative NB-like cells differ from the NBs present during development because they lack detectable *dpn* expression until stimulated by PTBI.

Structural and Functional Regeneration post-PTBI

To test the extent new neurons were generated following PTBI, we utilized a derivative of the mosaic analysis with a repressible cell marker (MARCM) method (Lee and Luo, 1999). This technique, the Perma-twin system, permanently labels dividing cells and their progeny with either green fluorescent protein (GFP) or red fluorescent protein (RFP). We used adult F1 male flies of the genotype: *w; FRT40A, UAS-CD8-GFP, UAS- CD2-Mir/ FRT40A, UAS-CD2-RFP, UAS-GFP-Mir; act-Gal4 UAS-flp/tub-Gal80^{ts}* that had been reared at 17^{o}C during development to keep the system off. These flies were subjected to PTBI within 24 hours of eclosion, and allowed to recover at 30^{o}C for either 2, 7, or 14 days post-injury before dissecting and analyzing their brains. As expected, based on our prior finding that PTBI stimulates cell proliferation, we observed more clones in injured samples than controls at all timepoints (**Fig. 3-6A-E**). We also found that injured flies had significantly more clones at later timepoints compared to earlier ones, suggesting that regeneration and proliferation were progressive and did not only occur immediately following the initial injury (**Fig. 3-6A-G, N**). Interestingly, we also observed large clones at later timepoints that produced new MB neurons (**Fig. 3-6A-G, N**). These new neurons project dendrites correctly to the MB calyx and axons that correctly project to the MB lobes. This robust regeneration is fairly common in PTBI flies at 14 days post-injury and was observed in approximately 50% of the assayed brains to varying degrees and with varying clone sizes (**Fig. 3N**). Other areas of the brain were also found to regenerate using the Perma-twin system. These include the antennal lobes (AL), the ellipsoid body (EB), and the lateral horn (LH) (**Fig. 3-6H-M**). We observed large clones in each of these regions approximately 26%, 26%, and 20% of the time, respectively (**Fig. 3-6N**). These data suggest that there is structural repair of the damaged MB region.

In order to assay for functional recovery post-PTBI, we assayed locomotor function. Due to their relatively simple nervous system and compact behavioral repertoire, we can ask whether *Drosophila* locomotor circuit function is damaged by PTBI and, if so, whether function is

457 restored at later timepoints. 2-day control uninjured flies displayed stereotypic locomotory
458 patterns throughout a 24-hour period (**Fig. 3-6O**). However, 2-day post-PTBI flies, exhibited
459 significantly different locomotor profiles (p-value<0.001) (**Fig. 3-6O**). Nonetheless, by 14 days,
460 by which time we observe increased cell proliferation and generation of new neurons,
461 PTBI flies display comparable locomotor profiles to age-matched controls (**Fig. 3-6P**). These
462 data indicate that PTBI significantly impacts motor function, and that this damage is largely
463 repaired by 14 days.
464
465 **Discussion**
466 Using a novel penetrating STAB injury, we have shown that the adult *Drosophila* central brain
467 has regenerative potential (**Fig. 3-7**). We demonstrate that PTBI stimulates cell proliferation
468 within 24 hours post-injury. However, the robust proliferative response is primarily in younger
469 flies and as flies age, this ability decreases. These data indicate that age plays an important role
470 in the adult *Drosophila*'s ability to survive a traumatic injury and warrants further investigation to
471 understand what cellular and molecular events transpire that allow for survival. While there is
472 some cell death post-PTBI, this is limited to early timepoints after injury; by 10 days post-PTBI,
473 the amount of cell death is not significantly different from non-injured age-matched controls. At
474 early timepoints post-PTBI, but not in control brains, we observe dividing neuroblast-like cells
475 that are Ase+ or Dpn+. Other neural progenitor genes such as *insc,* and *erm* exhibit elevated
476 transcript levels at early timepoints post-injury. By 7 days post-PTBI, new glia and new neurons
477 have been created. Using cell lineage-tracing techniques, we found that new neurons are
478 generated by cells that had once expressed *dpn.* These *dpn*-expressing cells were not found in
479 uninjured controls. Taken together, our data support the idea that there is a quiescent NB-like
480 population of cells that is activated upon injury, specifically in young adult flies. The newly
481 created cells are able to contribute to the overall regeneration of damaged brain tissue,
482 particularly near the mushroom body.

Although glia do not give rise to new neurons, and neurons do not give rise to new glia, we have not ruled out the possibility that a process of dedifferentiation is occurring to give rise to neural progenitors. For instance, it could be that mature cell types, such as neurons which are not known to have proliferative abilities, are able to adopt more plastic fates upon injury and create more neurons. Although we detect new neurons and new glia at 7 days post-injury, these cell types are not generated in equal proportions. This trend continues even out to 14 days post-PTBI. There appears to be an initial wave of gliogenesis, followed by a delayed wave of neurogenesis. We originally thought that this was because glia were the precursors of both new glia and new neurons, and that glial cells dedifferentiated to a more primitive state before giving rise to new neurons. However, our lineage studies do not support this hypothesis. While glia proliferate following PTBI, they do not give rise to new neurons.

Taking the 24 hour, 7 day, and 14 day timepoints together, we can identify noticeable trends. At the earliest timepoint post-injury, we see the greatest proportion of Class I cells which have incorporated EdU but are neither neurons nor glia. As time progresses post-PTBI, the proportion of Class I cells decreases. This is consistent with progressive differentiation of the newly born cells. Also consistent is that at earlier timepoints, there are no Class III cells (EdU+/Elav+, i.e. new neurons). However, the further we assay after injury, the higher the proportion of Class III cells. At 14 days post-injury the proportion of Class III cells is more than double what was observed at 7 days (**Fig. 3-3E**).

Not only is cell proliferation quite low in control brains, but the identities of the new cells exhibit different trends. Specifically, there are no Class III (proliferation+/Elav+) or IV (proliferation+/Elav+/Repo+) cells at any of the three timepoints. There also is a slight increase in the proportion of Class I (proliferation+/) cells over time in the controls, in direct proportion to the decrease in the proportion of Class II (proliferation+/Repo+) cells (**Fig. 3-3F**). This suggests that the cells that are dividing in control brains are slowly losing their glial identity over time. This also corresponds with the decrease in the adult Drosophila brain cells ability to proliferate in

response to injury as they age and could be evidence of a progenitor cell type, specifically found in younger adult flies, that is lost with age. Most important however, is that Class III (proliferation+/Elav+) cells are not observed in controls at any timepoint, indicating that PTBI brains make new neurons, but control brains do not (**Fig. 3-3F**).

Nonetheless, our studies do not rule out the possibility that glia undergo a process of partial deregulation before making more glial cells. This is supported by the observation of Class IV cells (proliferation+/Repo+/Elav+/) cells seen at 24 hours and 7 days post-PTBI. Repo+/Elav+ cells are not found in uninjured control adult brains, but have been reported during larval development (Berger et al., 2007) and in certain brain tumors (Beaucher et al., 2007). Elav also is known to be transiently expressed in some NB-like cells during development (Beaucher et al., 2007). Thus, the presence of dividing cells that are Repo+/Elav+ is consistent with a less differentiated state. Further investigation is needed to conclusively identify the origins of the glia created post-injury.

Previous studies have indicated a higher occurrence of certain brain tumors, such as glioblastomas, in people that have previously experienced TBI (Tyagi et al., 2016). In our model of PTBI, we do not find evidence of unregulated growth. Although there is an increase in new cells over time post-injury as assayed EdU labeling, this is a cumulative view of all the cells that proliferated and does not represent how many cells are still mitotically active. To measure the number of cells that are actively proliferating at later timepoints, we injured flies at 0-6 hours post-eclosion, allowed them to recover for 14 days and then assayed brains using anti-PH3, which specifically marks cells in mitosis. At 14 days post-PTBI, although injured flies have slightly more PH3+ cells than age-matched uninjured controls, the number is significantly lower than at 7 days post-PTBI (**Fig. 3-S6**). Additionally, we have observed that the proportion of Class I (prolif+ only) cells decreases over time, with 56% of cell in Class I at 24 hours, 44% of cells in Class I at 7 days, and 26% of cells in Class I at 14 days post-PTBI. We also observed a unique cell type, that was dividing and expressed Repo and Elav (Class IV cell). The timing of

these shifts in proportions suggest that a process of deregulation is occurring, and that a mature cell type may be in the process of adopting a more plastic fate. While there are ~5% Class IV cells at both 24 hours and 7 days post-PTBI, we did not observe any Class IV cells in 14-day injured brains. Together, these data support the idea that PTBI does not stimulate uncontrolled cell division. The mechanisms behind this need to be further analyzed to understand how *Drosophila* are able to give a measured proliferative cell response to regenerate damaged tissue.

In addition to proliferating glial cells, we also have identified a novel population of cells that upregulate the expression of *dpn* post-PTBI and give rise to new neurons. Using Dpn and PH3 antibodies, we found that uninjured adult central brains lack Dpn and have few mitotic cells, while injured brains have Dpn+ cells that are mitotically active. Our lineage analysis is consistent with this and indicates that *dpn*-expressing cells can generate neurons. We propose that these cells represent a previously unknown population of NB-like cells that are quiescent in uninjured brains. This is significant for several reasons. First, uninjured brains have cells that cannot be identified with standard neural progenitor markers, but that nonetheless have regenerative potential. If such cells exist in *Drosophila*, perhaps they also exist in humans. Second, regeneration can be stimulated by mild injury. This provides us with an avenue for identifying these cells for future study. It also provides us with a novel model system for screening pharmacologic agents for those that activate the regenerative program. This could lead to novel therapeutic approaches for both neurodegenerative diseases and brain injuries.

Our results in the central brain are somewhat reminiscent of optic lobe regeneration after injury. However, while we have identified 2 distinct populations of new cells: glia and neurons, the Fernandez-Hernandez *et al.* study identified one, neurons. Another intriguing difference is that the proliferating optic lobe cells express the neural stem cell gene *deadpan* (*dpn*), but not the proneural gene *asense* (*ase*). In the central brain after PTBI, there is *ase* upregulation as assayed by both qRT-PCR and immunohistochemistry. Interestingly, we did not observe cells

561 expressing Ase that were Dpn+/PH3+. Instead, the *ase*→GFP cells were located adjacent to
562 Dpn+/PH3+ cells. This could indicate a lineage relationship between the Dpn+ and Ase+ cells.
563 For example, Dpn+/PH3+ cells could indicate a type II NB cell type while cells expressing Ase
564 might be progenitors, similar to INPs or GMCs. Two different populations of progenitor cells
565 could also explain the differences seen in the creation of glia versus neurons. Perhaps glia are
566 able to adopt more plastic like fates, akin to GMCs, and create more glia post-injury. It could
567 also be the case that the proliferating cells seen in uninjured control brains that express Repo
568 represent an unknown glial progenitor cell type that respond by creating more glia post-PTBI.
569 Further investigation is needed to understand how these cells contribute to proliferation post-
570 PTBI and what mechanisms allow this to occur. Together, these results indicate that
571 neurogenesis in adult *Drosophila* is different in the central brain than it is in the optic lobe and
572 that the neural progenitors in these brain regions have distinct developmental potentials.
573 Using our novel injury paradigm, we have been able to establish that young adult
574 *Drosophila* are capable of robust regeneration, with the creation of new neurons and glia and
575 functional recovery from locomotor defects by 14 days post-PTBI. We have demonstrated that
576 regeneration is accomplished via proliferation and by a neuroblast-like population that is
577 activated by injury. Further questions remain about the origin and properties of the NB-like cells.
578 Additionally, the molecular mechanisms that trigger regeneration and the activation of the
579 neuroblast-like population(s) have yet to be identified. In order to translate promise into
580 progress, we began at a basic level to elucidate the complex process of regeneration. By
581 utilizing *Drosophila melanogaster,* we can reap the benefits of a low-cost model and powerful
582 genetic and molecular tools. Once the myriad of components involved in neuroregeneration are
583 dissected and teased apart, we can work our way towards converting our knowledge of
584 *Drosophila* neurogenesis to humans.
585
586

Materials & Methods

Fly Stocks and Rearing

Unless otherwise specified, flies were reared at 25°C on a standard cornmeal-sugar medium. The following stocks were obtained from the Bloomington Drosophila Stock Center (BDSC): **#854** (*w[*]; P{w[+mW.hs]=GawB}OK107 ey[OK107]/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]*; **#1495** (*y[1] w[1]*); **#4539** *y[1] w[*]; P{w[+mC]=UAS-FLP.D}JD1*; **#5130** (*y[1] w[1]; Pin[Yt]/CyO; P{w[+mC]=UAS-mCD8::GFP.L}LL6)*; **#7018** (*w[*]; sna[Sco]/CyO;P{w[+mC]=tubP-GAL80[ts]}ncd[GAL80ts-7]*); **#7019** (*w[*];P{w[+mC]=tubP-GAL80[ts]}20;TM2/TM6B, Tb[1]*; **#7415** (*w^{1118}; P{w[+m*]=GAL4}repo/TM3, Sb^{1}*); **#28281** (*w[*];P{w[+mC]=UAS-RedStinger}6,P{w[+mC]=UAS-FLP.Exel}3,P{w[+mC]=Ubi-p63E(FRT.STOP)Stinger}15F*); **#32251** (*w[*]; P{w[+mC]=Ubi-p63E(FRT.STOP)Stinger}15F2*); **#47859** *w[1118]; P{y[+t7.7] w[+mC]=GMR13CO2-GAL4}attP2*; **#51635** (*y^{1} w^{*}; P{w[+m*]=nSyb-GAL4S}3*): **#65408** (*P{w[+mC]=UAS-Stinger}2, P{w[+mC]=UAS-hid.Z}2/CyO*). Other lines used were *ase-Gal4/CyO; Dr/TM6B* (a gift of Dr. Cheng-Yu Lee); *w; FRT40A, UAS-CD8-GFP, UAS- CD2-Mir; act-Gal4 UAS-flp/TM6B*; and *w; FRT40A, UAS-CD2-RFP, UAS-GFP-Mir; tub-Gal80ts/TM6B* (both gifts of Dr. Eduardo Moreno).

Standard cross

To minimize variations due to genetic background and sex, we routinely analyzed F1 males from the following cross: *w[*]; UAS-mCD8-GFP;; OK107-Gal4* X *y[1] w[1]*. This cross was maintained at 25ºC. PTBI flies were kept at 25ºC until their brains were dissected and fixed for analysis. For simplicity, this genotype is referred to as "*OK107/yw*" hereafter.

Perma-twin flies

Perma-twin flies were generated by crossing *w; FRT40A, UAS-CD2-RFP, UAS-GFP-Mir; tub-$Gal80^{ts}$/TM6B* virgin females to *w; FRT40A, UAS-CD8-GFP, UAS- CD2-Mir; act-Gal4 UAS-*

613 *flp/TM6B* males (Fernandez-Hernandez et al., 2013). These crosses were maintained at 17ºC.
614 F1 progeny of the genotype: *w; FRT40A, UAS-CD8-GFP, UAS- CD2-Mir/ FRT40A, UAS-CD2-*
615 *RFP, UAS-GFP-Mir; act-Gal4 UAS-flp/tub-Gal80^{ts}* were collected at eclosion, subjected to PTBI
616 or kept as uninjured controls and maintained at 30ºC until their brains were dissected and fixed
617 for analysis.
618
619 G-TRACE crosses
620 Lineage-labeling was accomplished using a G-TRACE line (**#28281** (*w[*];P{w[+mC]=UAS-*
621 *RedStinger}6,P{w[+mC]=UAS-FLP.Exel}3,P{w[+mC]=Ubi-p63E(FRT.STOP)Stinger}15F*)
622 crossed to various Gal4 driver strains listed above. These crosses were maintained at 17ºC. F1
623 progeny of the desired genotyped were selected at eclosion, subjected to PTBI or kept as
624 uninjured controls and maintained at 30ºC for 14 days when their brains were dissected and
625 fixed for analysis.
626
627 Penetrating Traumatic Brain Injury
628 To induce PTBI, we used thin metal needles (~12.5 µm diameter tip, 100 µm diameter rod; Fine
629 Science Tools) sterilized in 70% ethanol to penetrate the head capsule of CO_2-anesthetized
630 adult flies. Injured flies were transferred back to our standard sugar food for recovery and aging.
631
632 Immunohistochemistry
633 Brains were dissected in PBS (phosphate-buffered saline; 100 mM K_2HPO4, 140 mM NaCl pH
634 7.0) and fixed in a 3.7% formaldehyde in a PEM (100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$)
635 solution for 20 minutes at 25°C. Fixed brain samples were washed in PT (PBS and 0.1% Triton
636 X-100), blocked with 2% BSA in PT solution (PBT), and then incubated with primary antibodies
637 overnight at 4°C in PBT. Following primary antibody incubation, the samples were washed with
638 PT (5 times over the course of an hour) and incubated overnight in secondary antibody at 4°C.

639 The next day, samples were washed in PT, stained with DAPI (1:10,000, ThermoFisher) for 8
640 minutes, and mounted in Vectashield anti-fade mountant (Vector Labs) and imaged using a
641 Nikon A1RS system and analyzed using the Nikon NIS Elements software. Cell counting was
642 done both manually and using the Nikon NIS-Elements software to analyze regions of interest
643 (ROIs) with a threshold of over 1000 and an area of at least 10μm.
644 The primary antibodies used in this study were: rabbit anti-PH3 (1:500, Santa Cruz
645 Biotechnology, Inc); mouse anti-FasII (1:20, Developmental Studies Hybridoma Bank; DSHB);
646 mouse anti-Repo (1:20, DSHB); rat anti-Elav (1:20, DSHB); mouse anti-Pros (1:20, DSHB); and
647 rat anti-Dpn (1:50, AbCam). Secondary antibodies used were: anti-rabbit Alexa Fluor 568
648 (1:200, ThermoFisher); anti-rabbit Cy5 (1:400, Jackson ImmunoResearch, Inc.); anti-mouse
649 Cy5 (1:100, Jackson ImmunoResearch, Inc.); anti-rat Alexa Fluor 488 (1:400, ThermoFisher);
650 anti-rat Alexa Fluor 568 (1:400, ThermoFisher); and anti-rat Cy5 (1:200, Jackson
651 ImmunoResearch, Inc.).
652
653 EdU labeling
654 The standard injury method was used on flies for 5-ethynyl-2'-deoxyuridine (EdU) labeling,
655 except flies were fed 50 mM EdU in 10% sucrose solution on a size 3 Whatman filter for six
656 hours prior to PTBI and allowed to recover on the same solution for the desired amount of time.
657 The EdU solution was replaced every 24 hours. Brains were dissected, processed, and antibody
658 stained as described above with the exception of using buffers without azide. To detect EdU
659 incorporation, Click-IT® reagents from InVitrogen were used according to the manufacturer's
660 instructions. The brains then were antibody stained mounted and imaged as described above.
661
662 Quantitative Real-Time PCR
663 Transcript levels of target genes were measured by quantitative real-time PCR (qRT-PCR)
664 using methods described in (Ihry et al., 2012). RNA was isolated from appropriately staged

665 animals using TRIzol Reagent used according to the manufacturer's instructions (Thermo Fisher
666 Scientific). cDNA was synthesized from 40 to 400 ng of total RNA using the SuperScript III First-
667 Strand Synthesis System (Invitrogen). qPCR was performed on a Roche 480 LightCycler using
668 the LightCycler 480 DNA SYBR Green I Master kit (Roche). In all cases, samples were run
669 simultaneously with three independent biological replicates for each target gene, and *rp49* was
670 used as the reference gene. To calculate changes in relative expression, the Relative
671 Expression Software Tool was used (Pfaffl et al., 2002). We used the following primers to detect
672 transcript levels: *ase* Forward: 5'-CAGTGATCTCCTGCCTAGTTTG-3' & Reverse: 5'-
673 GTGTTGGTTCCTGGTATTCTGATG-3' (gift from Stanislava Chtarbanova); *dpn* Forward: 5'-
674 CGCTATGTAAGCCAAATGGATGG-3' & Reverse: 5'-CTATTGGCACACTGGTTAAGATGG-3'
675 (gift from Stanislava Chtarbanova); *elav* Forward: 5'-CGCAGCCCAATACGAATGG-3' &
676 Reverse: 5'-CATTGTTTGCGGCAA GTAGTTG-3' (Fly Primer Bank); *erm* Forward: 5'-
677 GTCCCCTAAAGTTTTCGATAGCC-3' & Reverse: 5'- GAGTCATAGTTGACAGTGGATGG-3'
678 (Fly Primer Bank); *insc* Forward: 5'- CCCTGGGCAATCTGTCCTG-3' & Reverse: 5'-
679 GAGAAGCCCGAATCCTGACT-3' (Fly Primer Bank); *myc* Forward: 5'-
680 AGCCAGAGATCCGCAACATC-3' & Reverse: 5'- CGCGCTGTAGAGATTCGTAGAG-3' (Fly
681 Primer Bank); *repo* Forward: 5'-TCGCCCAACTATGTGACCAAG-3' & Reverse: 5'-
682 CGGCGCACTAATGTACTCG-3' (Fly Primer Bank;; *Rp49* Forward: 5'-
683 CCAGTCGGATCGATATGCTAA-3' & Reverse: 5'-ACGTTGTGCACCAGGAACTT-3' (Denton et
684 al., 2009).
685
686 Locomotor assays
687 0-6-hour post-eclosion *OK107/yw* males were collected, subjected to PTBI, and aged to 2 days
688 and 14 days, respectively. The 2 and 14 day injured and age-matched uninjured controls were
689 placed in the *Drosophila* Activity Monitor (DAM) system (TriKinetics, Waltham, MA) to record

690 locomotory behavior. The circadian locomotor activity of flies was assayed and analyzed as
691 previously described (Hamblen et al. 1986; Sehgal et al, 1992).
692
693 Statistical analysis
694 For all cell/clone counting and locomotor assays, counts were expressed as means ± standard
695 deviations. Two-tailed t-tests were performed using Graphpad Prism Version 8.3.0 for Mac
696 (GraphPad Software, La Jolla California USA, www.graphpad.com). An alpha value of 0.05 was
697 considered significant. The following symbols represent significance; * significant at $p \leq 0.05$; **
698 significant at $p \leq 0.01$; *** significant at $p \leq 0.001$; **** significant at $p \leq 0.0001$.
699
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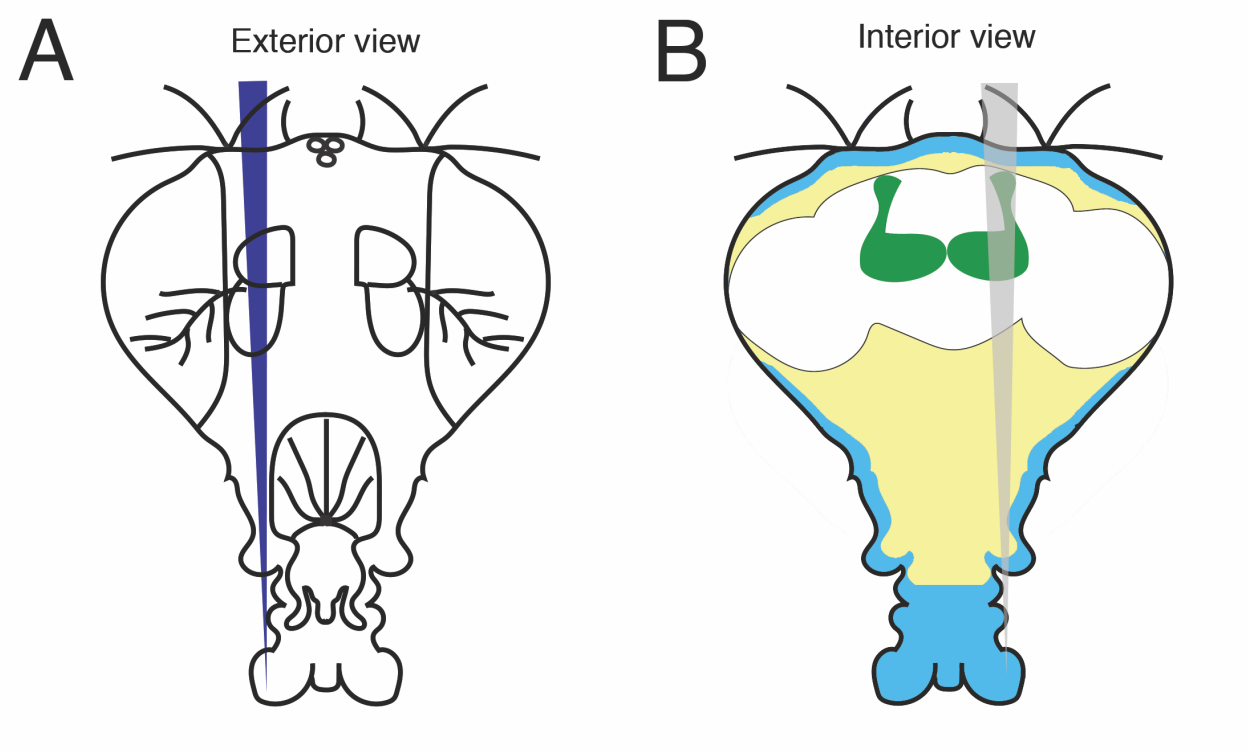

Figure 3-1A,B. Penetrating Traumatic Brain Injury (PTBI) as a new model to study neuroregeneration. In our standard injury protocol, we anesthetize young flies within 0-6 hours of eclosion and unilaterally injure the right central brain. Specifically, we target the right mushroom body (MB) region using a sterilized steel minutien pin (~12.5 um diameter tip, 100 um rod diameter; Fine Science Tools) to penetrate the head cuticle and the brain. **A.** This schematic shows an external frontal view of the *Drosophila* head and the location of injury with the needle trajectory indicated in blue). In this diagram, the right side of the brain is on the left because it is a frontal view. **B.** As described above, our penetrating STAB injury (trajectory indicated in grey) damages multiple cell types, including the mushroom body (MB, green), parts of the brain (white), such as glia and neurons, and cells outside the brain, such as the fat body (blue) and hemocytes (red). CB = central brain region. OL= optic lobe region. In this diagram, the right side of the brain is to the right.

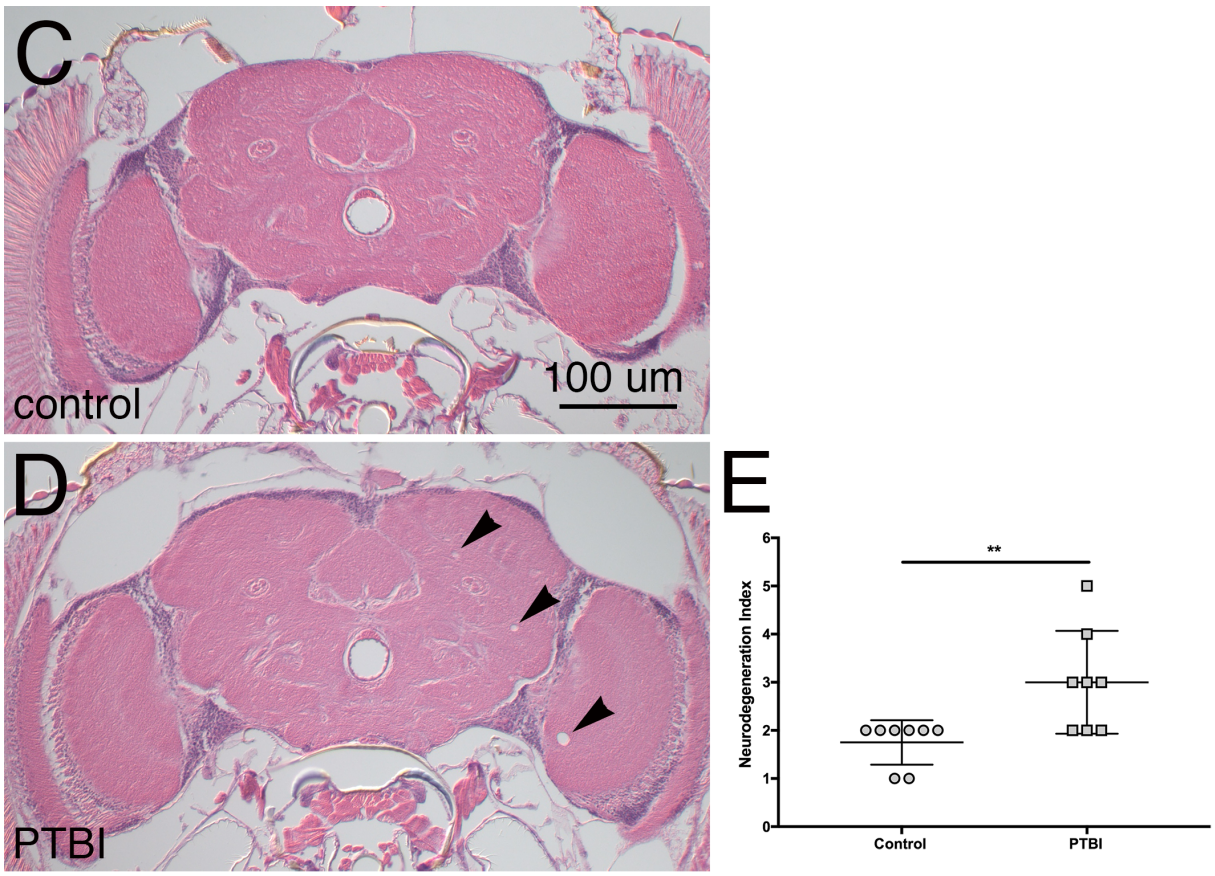

Figure 3-1C-E. PTBI increases neurodegeneration. To understand the long-term consequences of PTBI, we evaluated control and injured brains by histology at 25 days post-eclosion. Controls exhibited little neurodegeneration at 25 days (**C**). In PTBI flies, we observed an increase in the number of lesions (**D**). We used the neurodegeneration index described in Cao *et al.*, 2013 to quantify this neurodegeneration. Controls had an average neurodegeneration index score of 1.7+/-0.2, while PTBI flies had an average neurodegeneration index score of 3.0+/-0.4 (**E**). This represented a statistically significant difference (p-value=0.0089). Error bars reflect the standard deviation (SD).

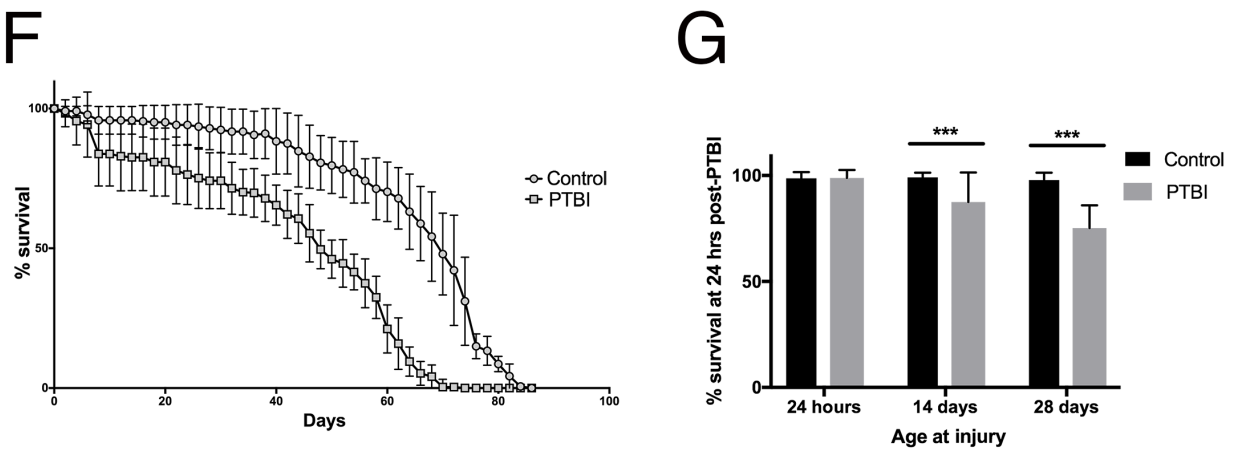

Figure 3-1F,G. PTBI decreases lifespan. F. To assess the impact of PTBI on viability, we performed a lifespan assay with control and PTBI flies. Within the first 12 days, there was no significant difference in survival between control and injured flies. However, beyond 12 days, we saw a significant drop in survival in injured flies. Control flies reached 50% survival at 70 days of age, while injured flies reached 50% survival at 48 days. The maximum lifespan was 84 days for uninjured flies and 74 days for injured flies. This indicates that PTBI does have long-term consequences, similar to what is observed with other forms of TBI. Yet, in the window of 0-12 days, there is no significant difference in survival, suggesting that something is occurring to combat or delay the negative effects of injury. **G.** Because we observed little death within the first 12 days post-injury, we suspected that age might play an important role in this outcome. To test this, we collected, aged, and injured flies that were 0-6 hours post-eclosion, 14 days old, and 28 days old. Survival was then assayed 24 hours post-PTBI. Young flies (0-6 hours post-eclosion), exhibited no significant difference in survival compared to control flies (p-value=0.69), while older flies at 14 days (p-value<0.0001) and 28 days (p<0.0001) of age exhibited decreased survival compared to age-matched uninjured controls. Error bars reflect the standard deviation (SD).

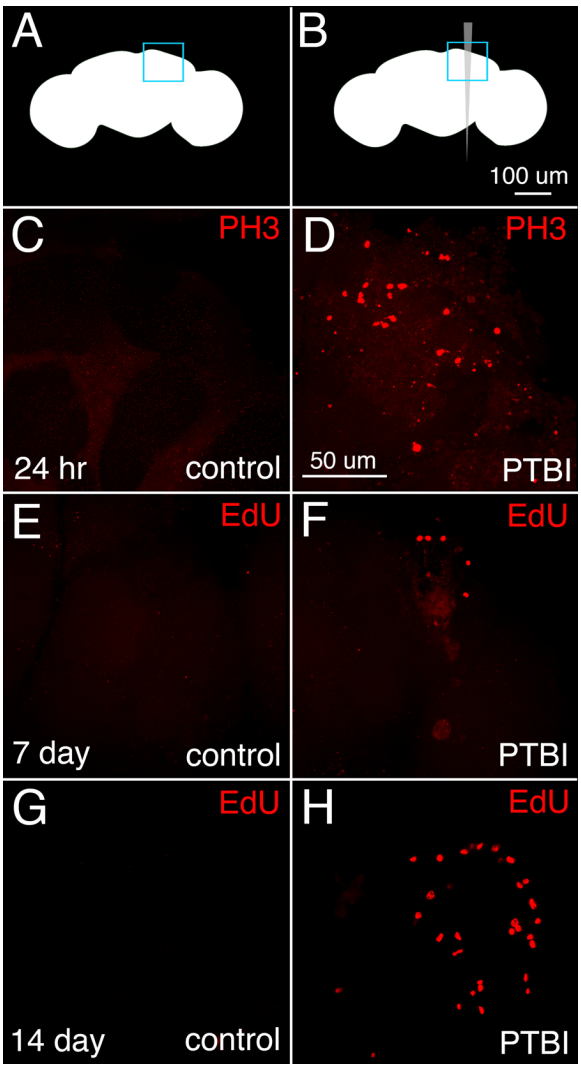

Figure 3-2A-H. PTBI stimulates cell proliferation. A, uninjured, control and **B, PTBI** brain schematics. The blue boxes in the upper right corner of these brains indicate the area near the MB imaged in **C-H. C** and **D.** To assay for cell proliferation, we used an anti-PH3 antibody (red) and stained brains 24 hours after injury. In control brains (**C)** there are few PH3+ cells, and none near the MB. However, in PTBI brains (**D**), there is an increase in the number of PH3+ cells specifically near the MB. **E** and **F.** To test whether newly created cells are surviving or being eliminated, we conducted a pulse-chase EdU experiment. We fed flies EdU (red) for 4 days post-injury (a pulse) and followed this with a 3-day period without EdU (the chase). In the 7-day control brain (**E**), there is little EdU incorporation. In 7-day PTBI brains (**F**), there are more EdU+ cells and many of these cells are near the MB. **G.**In 14-day control brains, there are few EdU+ cells. **H.** However, in 14-day injured brains, there is an increase in EdU+ cells near the MB. All brains are from males of our standard genotype (*OK107/yw*).

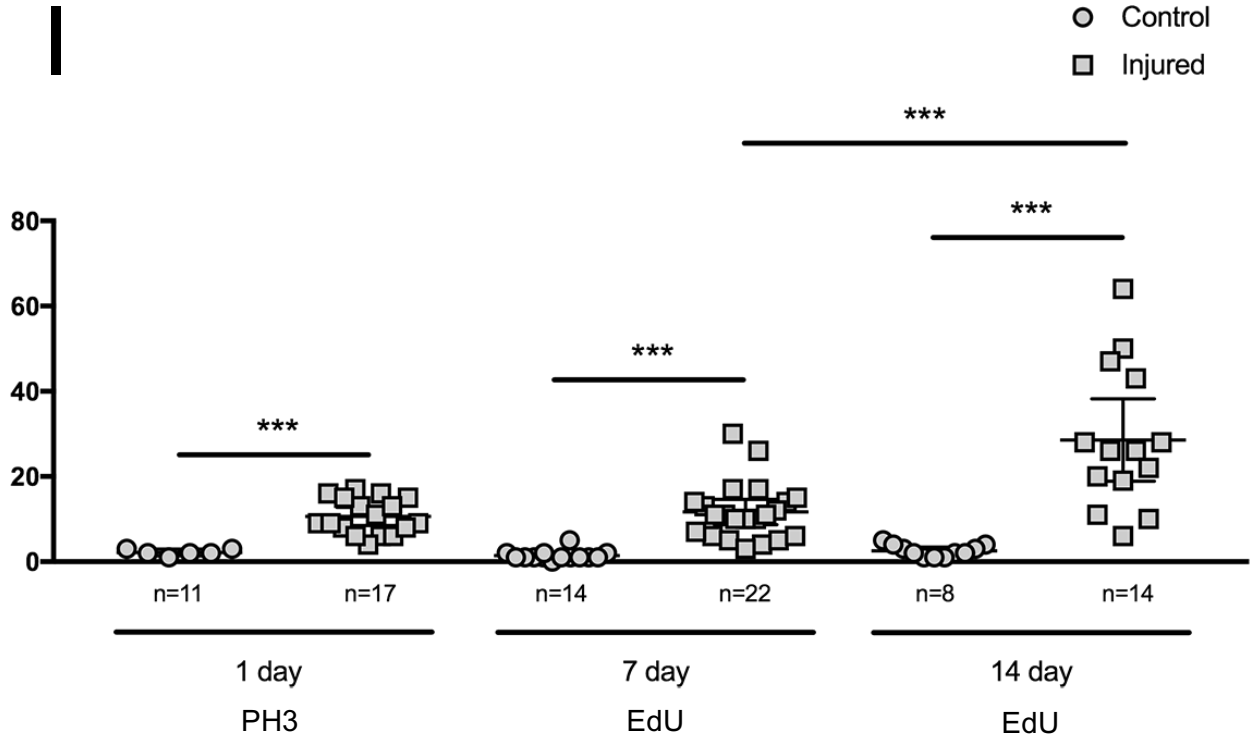

Figure 3-2I. The number of proliferating cells increases over time post-PTBI. We quantified proliferating cells using anti-PH3 at 24 hours and EdU at 7 and 14 days. EdU permanently marks dividing cells and would therefore allow us to measure the cumulative number of cells that had divided since the PTBI. At 24 hours, uninjured control brains had an average of 2.5 PH3+ cells per brain (n=11), while 24-hour post-PTBI brains had an average of approximately 11 PH3+ cells per brain (n=17). This is significantly different (p-value>0.0001). At 7 days, uninjured controls have very few EdU+ cells, with an average of approximately 1 EdU+ cell per brain (n=14), while 7-day post-PTBI brains had approximately 12 EdU+ cells per brain (n=22; p-value<0.0001). At a later timepoint, 14 days post-PTBI, uninjured controls have very few EdU+ cells, with an average of approximately 1 EdU+ per brain (n=8). In 14-day post-PTBI brains, there were an average of 29 EdU+ cells per brain (n=14; p-value=0.0002). Interestingly, although there was no significant difference between the number of proliferating cells between 24 hours and 7 days, by 14 days post-PTBI there was an increase in the number of dividing cells (p-value=0.0002). This indicates that cell proliferation continues between 7 and 14 days. Error bars reflect the standard deviation (SD).

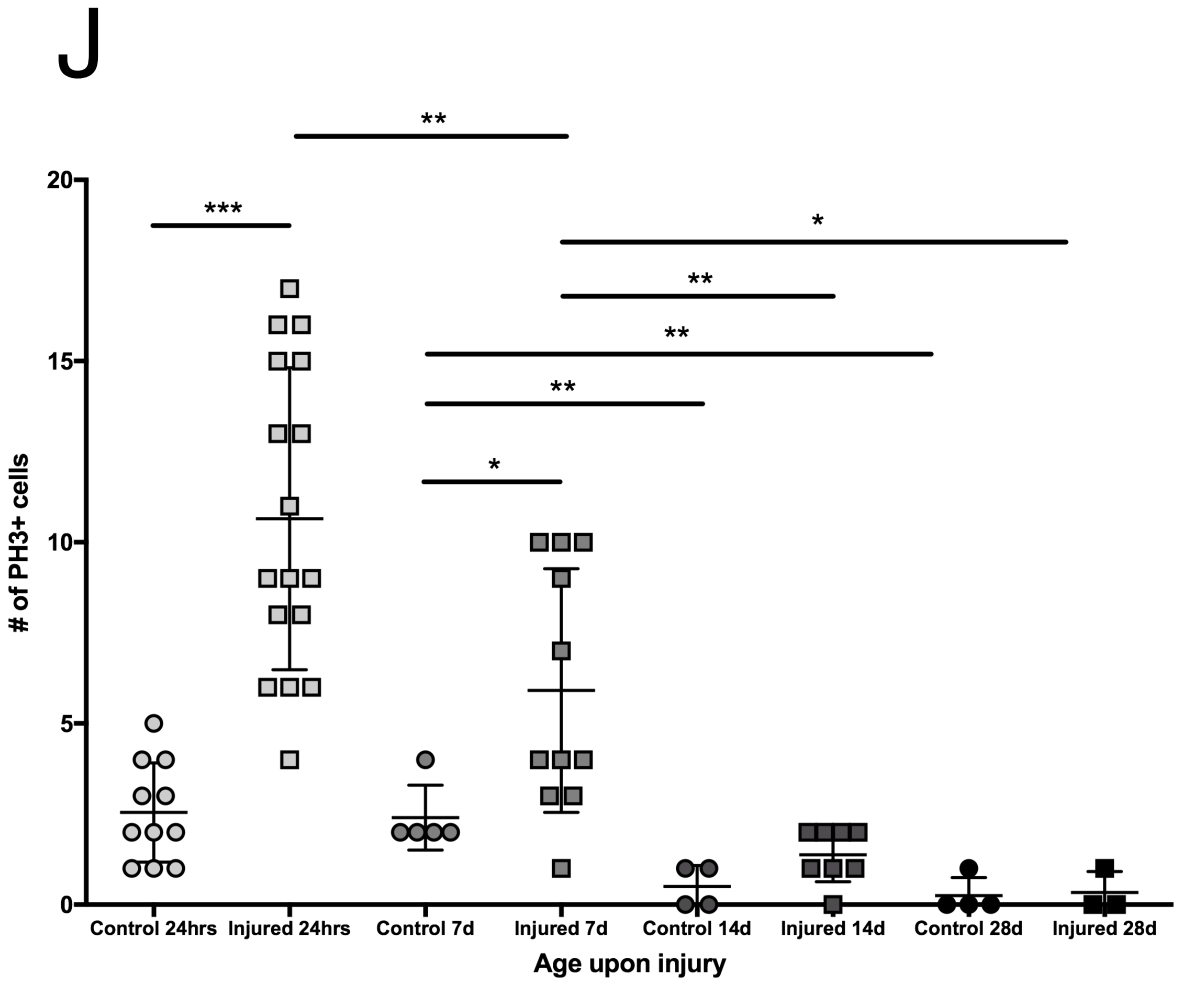

Figure 3-2J. Robust proliferative responses decrease with age. To explore whether age impacts the amount of cell proliferation that occurs post-injury, we aged adult flies to 24 hours, 7 days, 14 days, and 28 days prior to PTBI and used anti-PH3 to assay cell proliferation 24 hours after injury. Flies injured at 24 hours had an average of 11 PH3+ cells/brain compared to an average of 2.5 PH3+ cells/brain in age-matched controls. (p-value<0.0001). Flies that were aged to 7 days, then subjected to PTBI and allowed to recover for 24 hours had an average of 6 PH3+ cells per brain (n=11). While this is a significant increase from uninjured control 7-day brains that had an average number of 2 PH3+ cells per brain (n=5; p-value=0.04), this was significantly fewer PH3+ cells than when flies were injured at 24 hours post-eclosion (p-value=0.004). When flies were aged to 14 days, subjected to PTBI, and assayed 24 hours later, there was an average of 1 PH3+ cell per brain. In 14-day controls, there were also very few PH3+ cells, with an average of 0.5 PH3+ per brain. This was not significantly different from the average number of PH3+ cells in injured brains. In 28-day controls, there were an average of 0.25 PH3+ cells in control brains and an average of 0.33 PH3+ cells in injured brains.

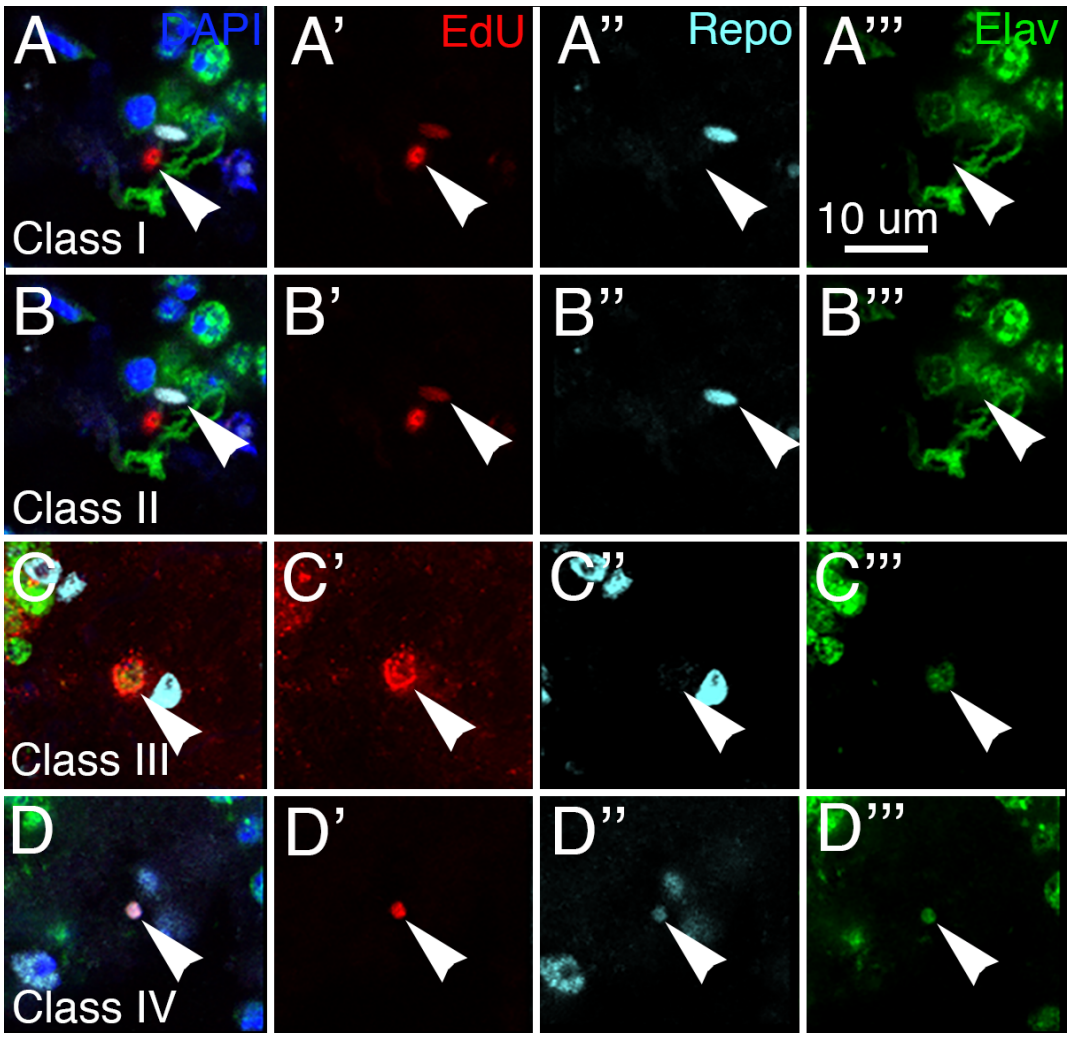

Figure 3-3A-D'''. Analysis of new cell identities 7 days post-PTBI. To determine what types of new cells had been generated in the first 7 days post-PTBI, we used pulse-chase experiments with EdU in combination with the glial marker anti-Repo and the neuronal marker anti-Elav. At 7 days post-PTBI, we found four classes of cells resulting from proliferation: Class I cells were cells that were EdU+ and did not express either Repo or Elav (**A-A'''**); Class II cells were Repo+/EdU+ (**B-B'''**); Class III were Elav+/EdU+ (**C-C'''**); and Class IV cells were Repo+/Elav+/EdU+ (**D-D'''**). Arrowheads indicate representative cells in each class. The nuclear dye DAPI is in blue.

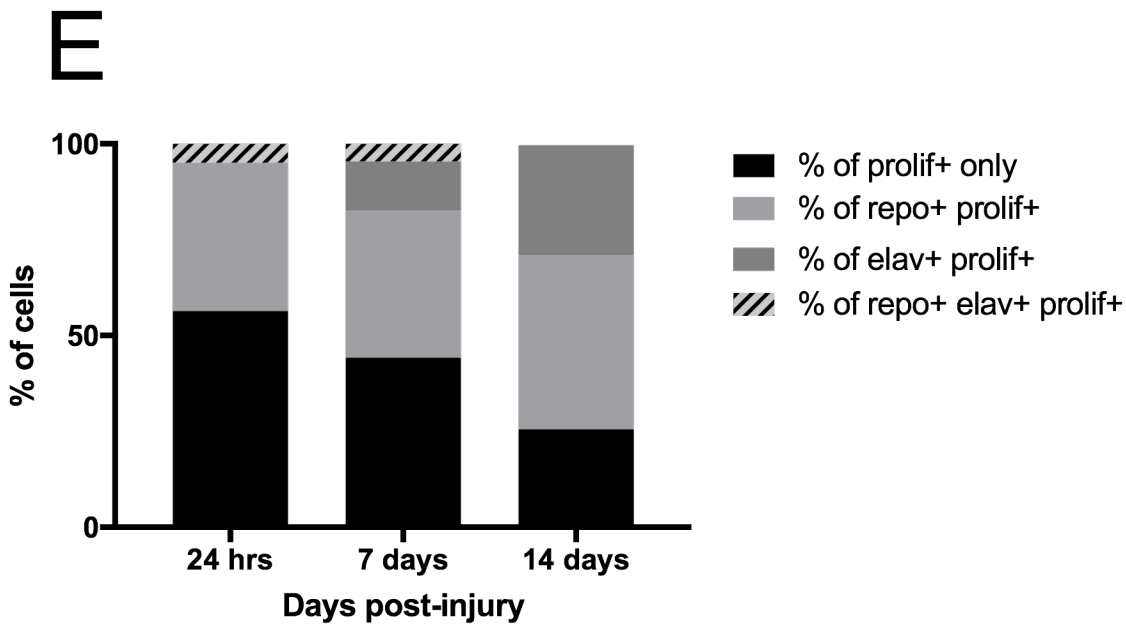

Figure 3-3E. New cells induced by PTBI express different cell markers at different times post injury. We used cell proliferation markers, such as anti-PH3 at 24 hours and EdU at 7 and 14 days in combination with the glial marker anti-Repo and the neuronal marker anti-Elav to quantify the cells in each class. EdU permanently marks dividing cells and would therefore allow us to see the cumulative number of cells that had been generated since the injury. At 24 hours post-injury, we observed the following approximate distribution of cell classes: 56% Class I (proliferation+ only), 39% Class II (Repo+/proliferation+), 0% Class III (Elav+/proliferation+), and 5% Class IV (Repo+/Elav+/ proliferation+). For 24-hour PTBI brains, n=17 and 181 PH3+ cells were counted. At 7 days post-injury, we saw the following distribution of cell classes: 44% Class I (proliferation+ only), 38% Class II (Repo+/proliferation+), 13% Class III (Elav+/proliferation+), and 5% Class IV (Repo+/Elav+/proliferation +). For the 7-day PTBI brains, n=15 and 172 EdU+ cells were counted. At 14 days post-injury, we saw the following distribution of cell classes: 26% Class I (proliferation+ only), 45% Class II (Repo+/proliferation+), 29% Class III (Elav+/proliferation+), and 0% Class IV (Repo+/Elav+/proliferation +). For the 14-day PTBI brain, n=8 and 278 EdU+ cells were counted. These data demonstrate that cell classes change over time. There are several trends that appear. Class I cells (proliferation+ only) are most frequent at 24 hours post-PTBI at 55%. As time goes on, the proportion of Class I cells decreases, with 44% at 7 days and 26% at 14 days. Class II cells (Repo+/proliferation+) are found in approximately the same percentage at 24 hours and 7 days post-injury, 39% and 38%, respectively. However, at 14 days, there are proportionately more Class II cells, with about 45% of cells in this class. Interestingly, at 24 hours post-PTBI, we see no Class III cells (Elav+/proliferation+). However, at 7 days PTBI, there are 13% of Class III cells, and this percentage more than doubles at 14 days PTBI to 29%. The most unusual cell class, Class IV (Repo+/Elav+/proliferation+) composed 5% of proliferating cells at 24 hours post-injury and was similar at 7 days post-PTBI. However, at 14 days PTBI, we observed 0% of proliferating cells Class IV, suggesting that these cells had transitioned into other classes, such as Class II or III.

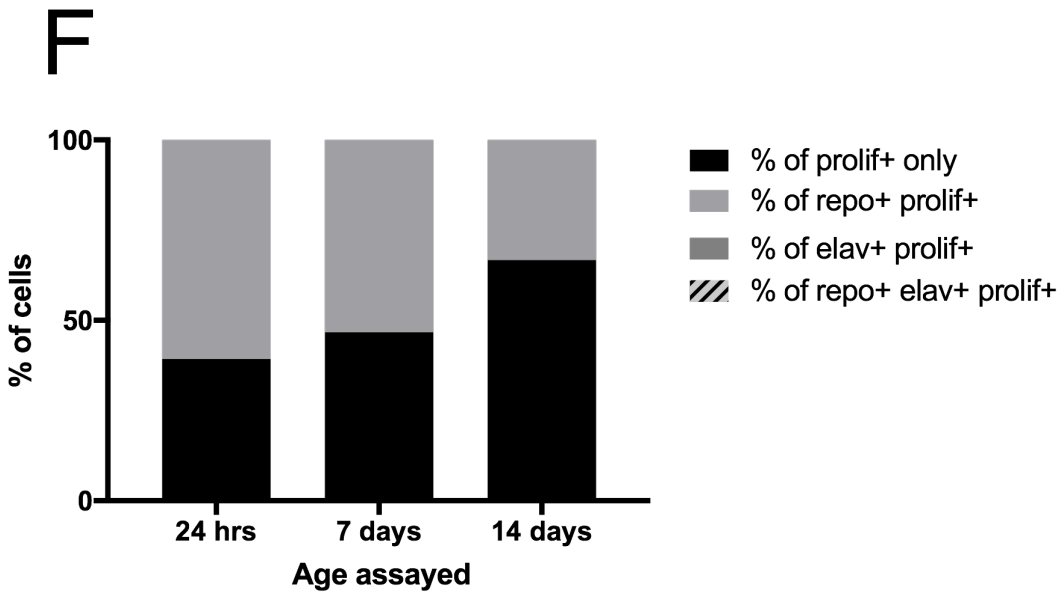

Figure 3-3F. Proliferating cells in uninjured brains express different cell markers at different ages. To quantify the proportions of cell classes in control brains, we used the same cell proliferation markers, i.e. anti-PH3 at 24 hours and EdU at 7 and 14 days. At 24 hours, we saw the following distribution of cell classes: 39% Class I (proliferation+ only) and 61% Class II (Repo+/proliferation+). For the 24-hour control brains, n=11 and 28 PH3+ cells were counted. At 7 days of age, we saw the following distribution of cell classes: 47% Class I (proliferation+ only) and 53% Class II (Repo+/proliferation+). For the 7-day control brains, n=9 and 15 EdU+ cells were counted. At 14 days post-injury, we saw the following distribution of cell classes: 67% Class I (proliferation+ only) and 33% Class II (Repo+/proliferation+). For the 14-day PTBI brain, n=2 and 3 EdU+ cells were counted. These data demonstrate that cell classes change over time in control brains and there are several trends that appear. Class I cells (proliferation+ only) appear to increase in proportion over time, from 39% at 24 hours, 47% at 7 days, and 67% at 14 days. This is the opposite trend in Class II cells (Repo+/proliferation+). Class II cells decrease in proportion over time, with 61% at 24 hours, 53% at 7 days, and 33% at 14 days. Notably, neither Class III (Elav+/proliferation+) or Class IV (Repo+/Elav+/proliferation+) were observed in the control brains.

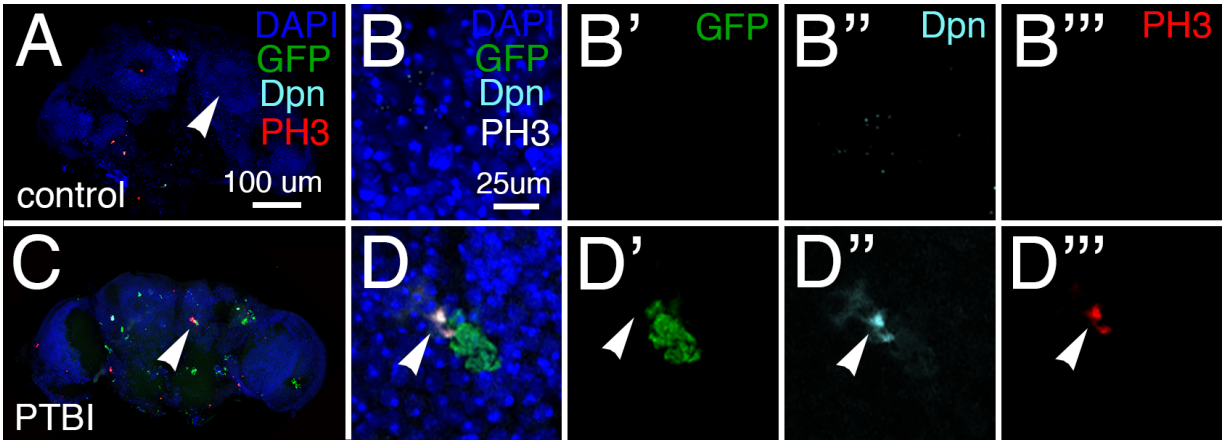

Figure 3-4A-D'''. Neuroblast gene expression is activated by PTBI. (A) and (**C**) are low magnification views of the control and PTBI brains shown at higher resolution in **B-B'''** and **D-D'''**, respectively. **A-B'''.** Images from an uninjured *ase-Gal4, UAS-Stinger; UAS-Gal80^{ts}* probed with anti-PH3 (red) and anti-Dpn (cyan). Stinger expressed under control of the *ase* regulatory sequence is in green. The nuclear dye DAPI is in blue. Arrowheads in (**A**) and (**C**) indicate the regions where higher magnification images were collected. At 24 hours in injured (C-D'''), but not in control (A-B''') brains, we observe Stinger+ cells, indicating the expression of *ase*, which is a neuroblast and neural progenitor gene. (**D-D'**). We observed cells that were Dpn+ (cyan) and PH3+ (red) in injured brains (**D''-D'''**), but not in controls (**B''-B'''**). *dpn* also is a neuroblast and neural progenitor gene. We did not observe cells that were GFP+/Dpn+/PH3+ in either injured or control brains. However, the Dpn+/PH3+ cells were often in close proximity to Stinger+ cells (**D**).

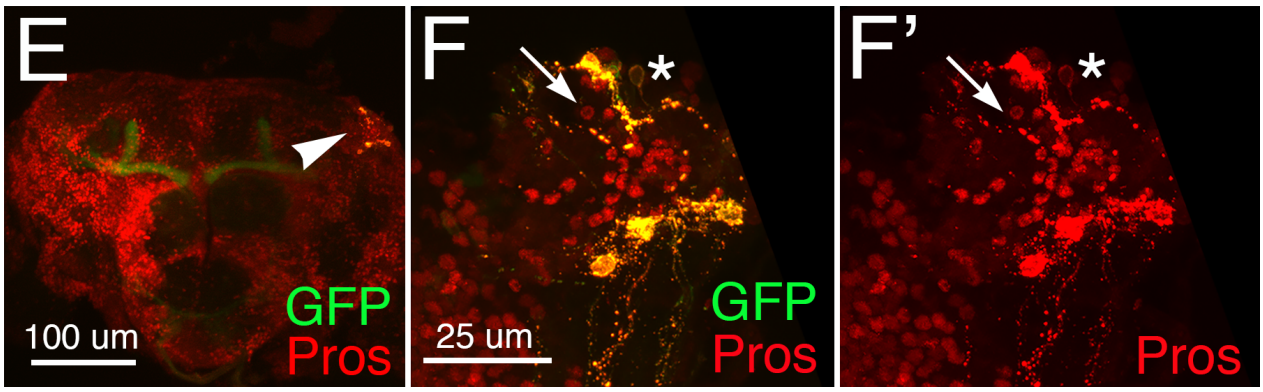

Figure 3-4E-F'. Cytoplasmic Pros seen in cells expressing *ase* at 24 hours post-PTBI. E-F'. An *ase-Gal4>>mCD8-GFP* brain 24 hours post-PTBI immunostained with anti-Pros (red). In this brain, GFP (green) is expressed in the cells that express *ase*. **E.** Low magnification view. Arrowhead indicates region that is seen in higher magnification in **F** and **F'. F** and **F'.** High magnification view of area near arrowhead in **E**. There are several cells in which Pros is cytoplasmic, one of which also expresses *ase* (asterisk) and another which lacks *ase* (arrow). Cytoplasmic Pros is characteristic of neuroblasts and neural progenitors and is not observed in control uninjured brains, while nuclear Pros is a hallmark of differentiated neurons and glia.

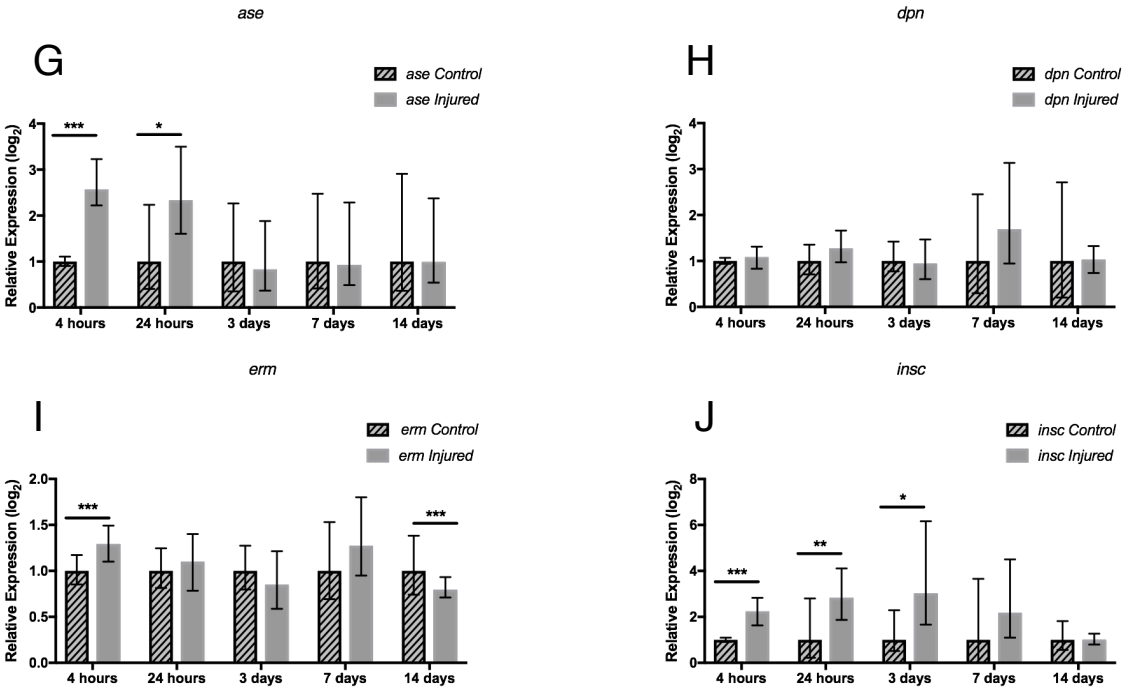

Figure 3-4G-J. qRT-PCR reveals increases in neural progenitor gene expression following PTBI. The mRNA levels of four different neural progenitor genes were assayed at 4 hours, 24 hours, 3 days, 7 days, and 14 days. **G.** The level of *ase* mRNA is increased more than 5-fold by 4 hours and remains elevated at 24 hours. However, at 3, 7, and 14 days, *ase* mRNA levels are no longer higher than in controls. **H.** The level of *dpn* mRNA was not detectably increased at any timepoints. **I.** mRNA levels of *erm* are increased almost 3-fold at 4 hours post-injury. However, by 24 hours, 3 days, and 7 days, *erm* mRNA levels have returned to baseline. **J.** *insc* mRNA levels are increased 6-fold at 4 hours, 24 hours, and 3 days post-injury. At later timepoints, 7 and 14 days, *insc* mRNA levels return to near baseline. The qPCR results reflect triplicate biological samples, represented relative to the levels of *rp49*, and then normalized to the corresponding levels in time-matched controls. Error bars calculated by Relative Expression Software Tool analysis and reflect the standard error of the mean (SEM). Note that scales on Y axes differ among the graphs.

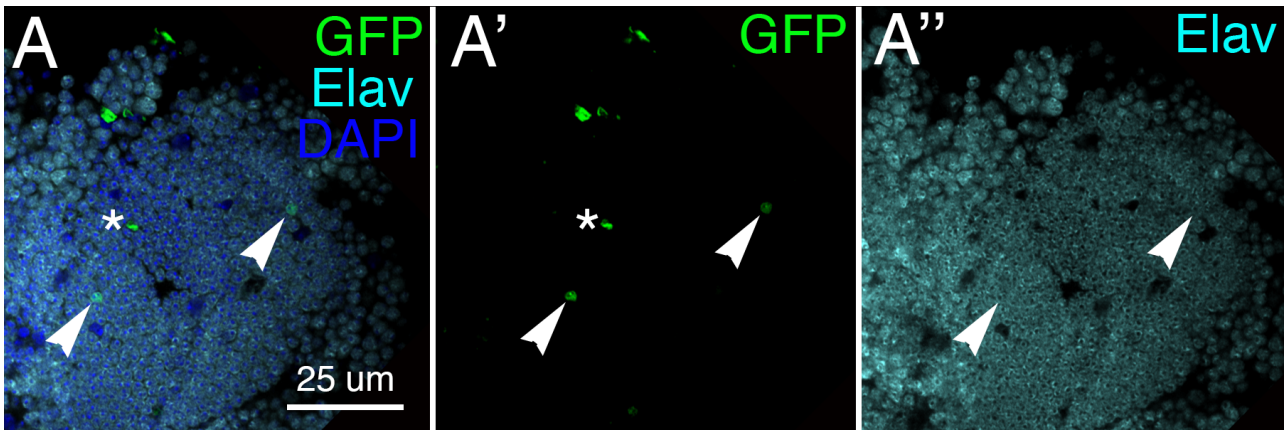

Figure 3-5A-A''. Lineage-tracing shows that neurons are created through Dpn expressing cells 14 days post-PTBI. Using *dpn-Gal4*, the G-TRACE lineage-tracing system, and a temperature sensitive Gal80, we observed cells that were GFP+/Elav+ at 14 days post-PTBI near the mushroom body, indicated with arrowheads. Other GFP+ cells that did not stain with Elav were also observed, indicated with asterisk. No GFP+/Elav+ cells were observed in age-matched uninjured control brains. DAPI is shown in blue and Elav is shown in Cyan.

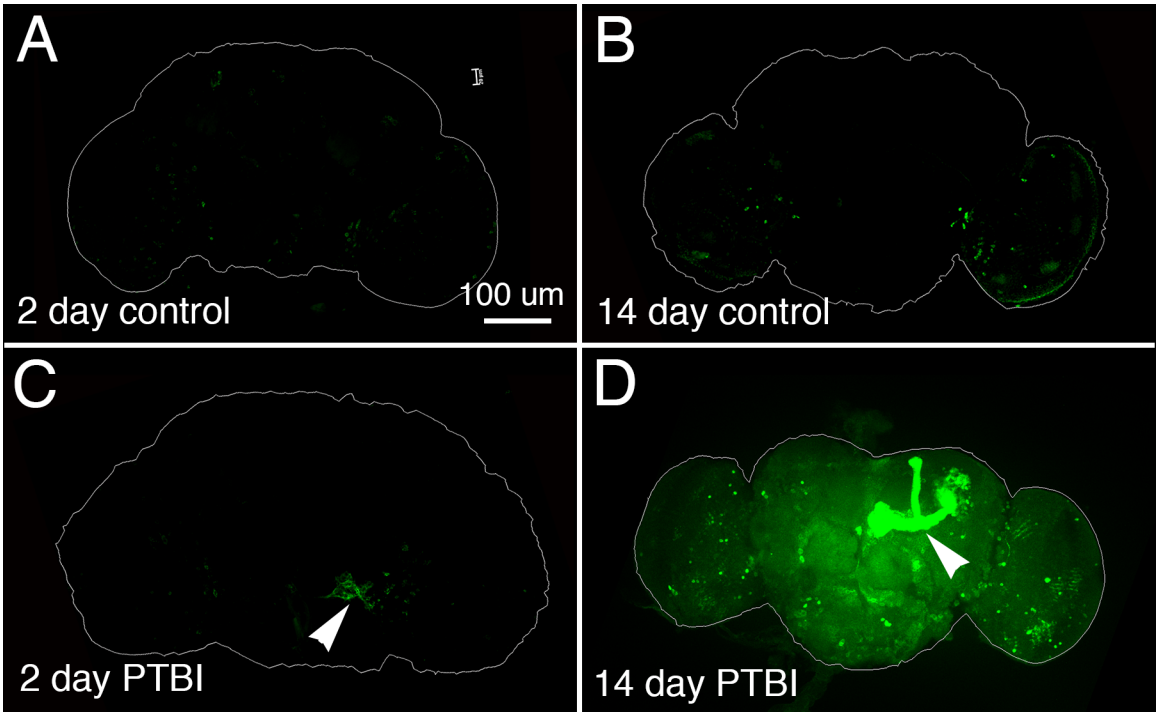

Figure 3-6A-D. Perma-twin lineage tracing demonstrates brain regeneration and appropriate targeting of axons following PTBI. To analyze neurogenesis after PTBI, we utilized a lineage-tracing system, called the Perma-twin system, which permanently labels dividing cells and their progeny with either green fluorescent protein (GFP) or red fluorescent protein (RFP). Flies were reared at 17°C to keep the system off during development. Upon eclosion, F1 males carrying transgenes of the Perma-twin system were collected and injured using our standard protocol. Flies were then placed at 30°C to recover for either 2 days or 14 days. **A.** In 2-day uninjured controls, there are some GFP+ cells, and these cells are scattered throughout the brain. **B.** At 14 days, there are relatively few GFP+ cells present in the control central brain. **C.** In comparison, 2-day injured brains have significantly more GFP+ cells, and these cells appear in a cluster near the injury, indicated with arrowhead. **D.** At 14 days post-injury, there are large clones near the site of injury. These clones also have axons and dendrites that correctly project to target areas that were damaged during PTBI. Only the GFP channel is shown here for simplicity; there were similar RFP+ clones in the PTBI samples.

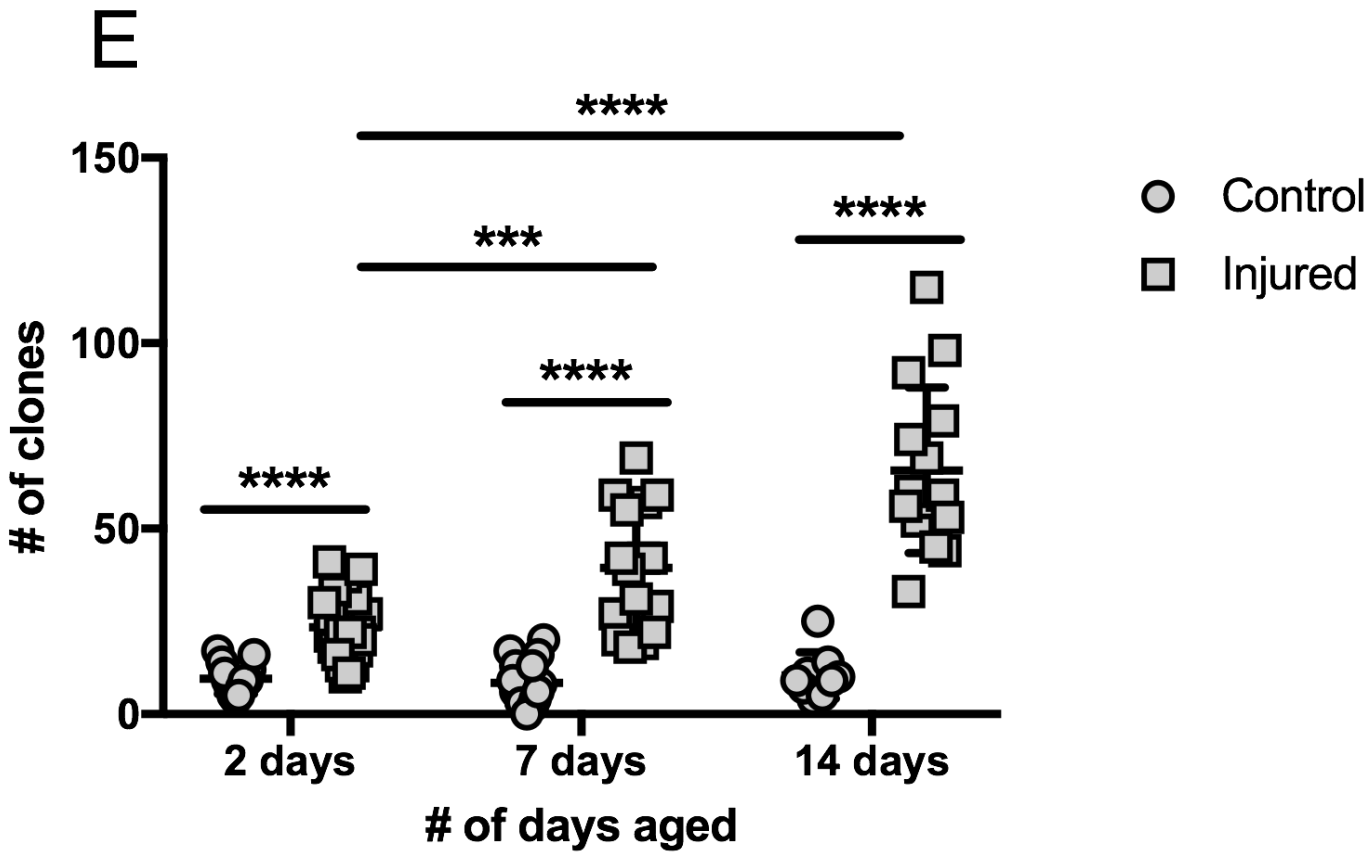

Figure 3-6E. The number of clones increases over time post-PTBI. Control uninjured brains (n=13) have approximately 10 clones at 2 days whereas 2-day PTBI brains (n=20) have a much higher mean of 23 clones ($p<0.00002$). At 7 days, control brains had an average of 9 clones per brain (n=18), while 7-day PTBI brain had substantially more clones, with an average of 39 clones per brain (n=16) ($p\text{-value}<0.00000002$). This is significantly more compared to the number of clones seen at 2 days post-injury ($p\text{-value}>0.0009$). In 14-day uninjured brains, there are ~10 clones per brain, which is not significantly different from the 2-day and 7-day controls. However, at 14 days post-PTBI, there are 66 GFP+ clones, which is significantly more than in age-matched controls ($p<0.0000003$). The number of clones seen at 14-days post-PTBI is significantly increased compared to 2-day post-PTBI brains ($p\text{-value}<0.0001$). Error bars reflect SD.

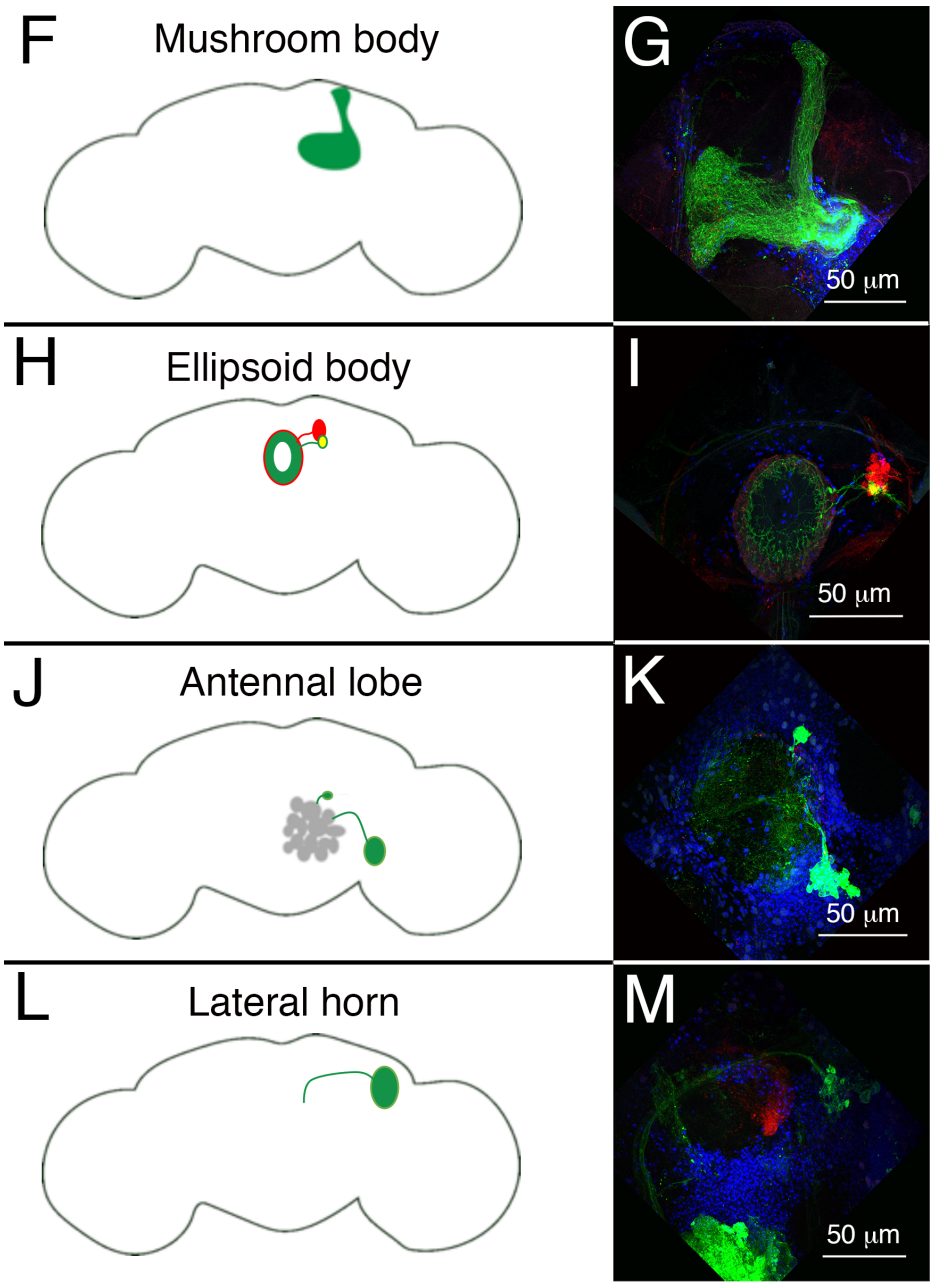

Figure 3-6F-M. PTBI stimulates clone formation in multiple regions in the brain. Panels on the left side show schematics of regions where large clones were found 14 days post-PTBI (**A**, **H**, **J**, **L**). Panels on the left show high magnification of representative images (**G**, **I**, **K**, **M**). Many 14-day brains exhibited clones that had striking large clones that seem to project to correct target areas. There were several structural regions that seem to be able to regenerate post-injury, including the mushroom body (MB) (**F**, **G**), the ellipsoid body (EB) (**H**, **I**), the antennal lobe (AL) (**J**, **K**), and the lateral horn (LH) (**L**, **M**).

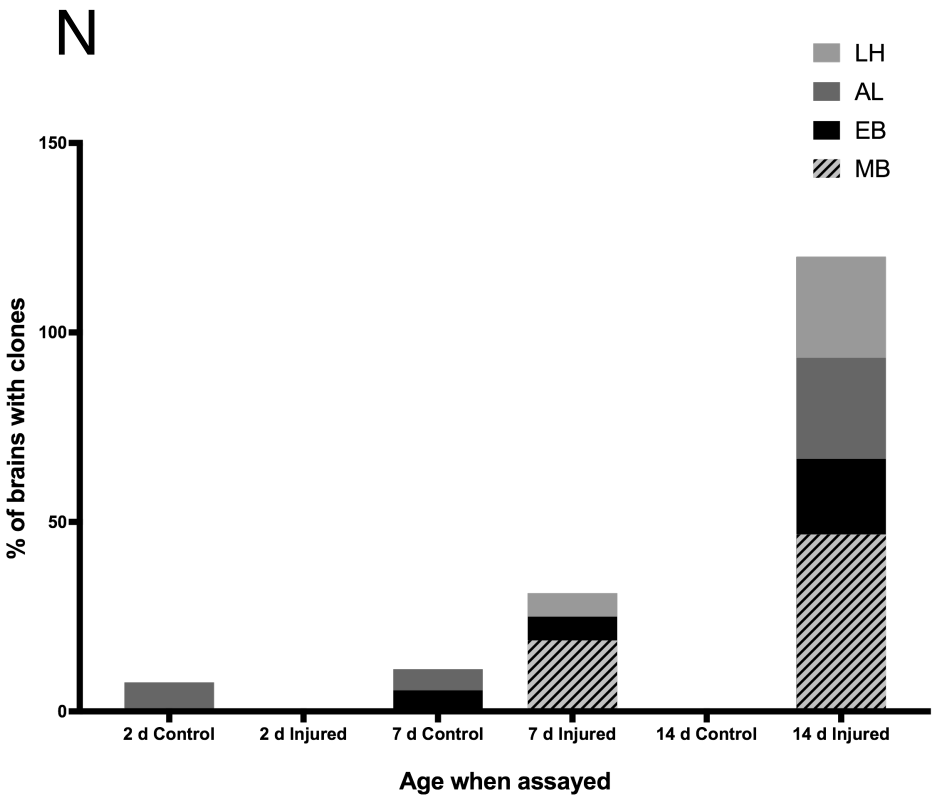

Figure 3-6N. Brain regeneration increases over time post-PTBI. The proportion of anatomical structures that regenerated were calculated at 2, 7, and 14 days in controls and injured brains. At 2 days, approximately 8% of control brains (n=13) showed AL regeneration, while in 2-day injured brains (n=20), there were no areas of regeneration. 7-day control brains also exhibited some regions of regeneration (n=18), with 6% showing AL and 6% showing EB clones. At 7 days post-injury, there are many more areas with large clones (n=16), with 6% of brains showing AL, 6% showing EB, and 19% of brain showing large MB clones. At 14 days, control brains (n=9) did not exhibit any specific areas that had regenerated, but 47% of PTBI brains (n=15) had MB clones, 20% had AL clones, 27% had EB clones, and 27% had LH clones.

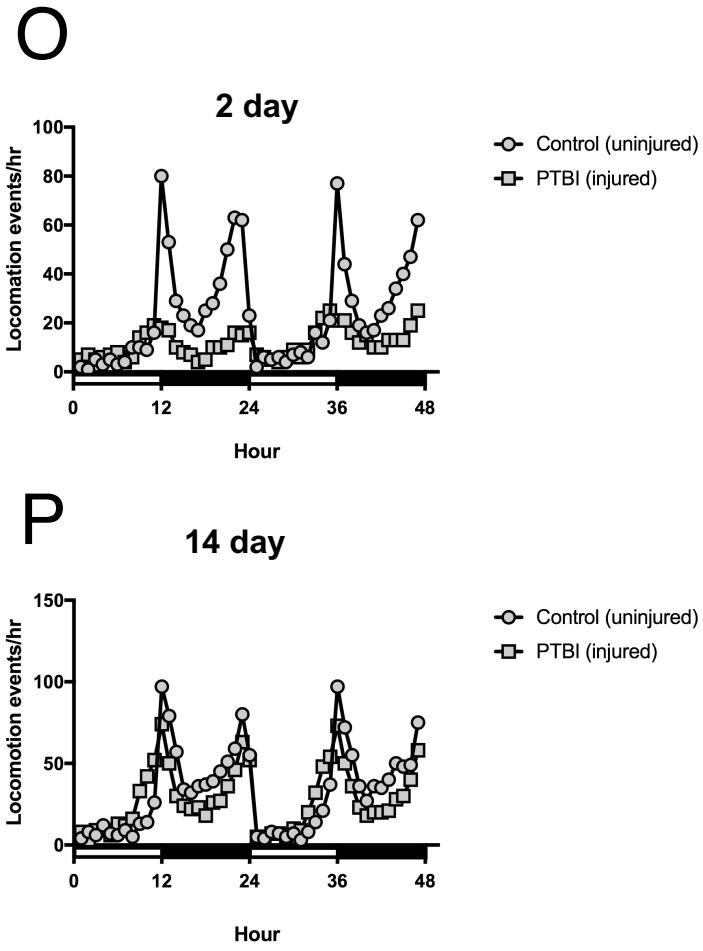

Figure 3-6O,P. Locomotor defects observed at 2 days post-PTBI have recovered by 14 days post-PTBI. In order to assay for functional recovery post-PTBI, we examined locomotor function. The 2 and 14 day injured, and age-matched uninjured controls were placed in the *Drosophila* Activity Monitor (DAM) system (TriKinetics, Waltham, MA) to record locomotory behavior. **O.** 2-day control uninjured flies displayed stereotypic locomotory patterns throughout a 24-hour period. However, 2-day post-PTBI flies, exhibited significantly different locomotor profiles (p-value<0.001). **P.** Nonetheless, by 14 days, PTBI flies display comparable locomotor profiles to age-matched controls.

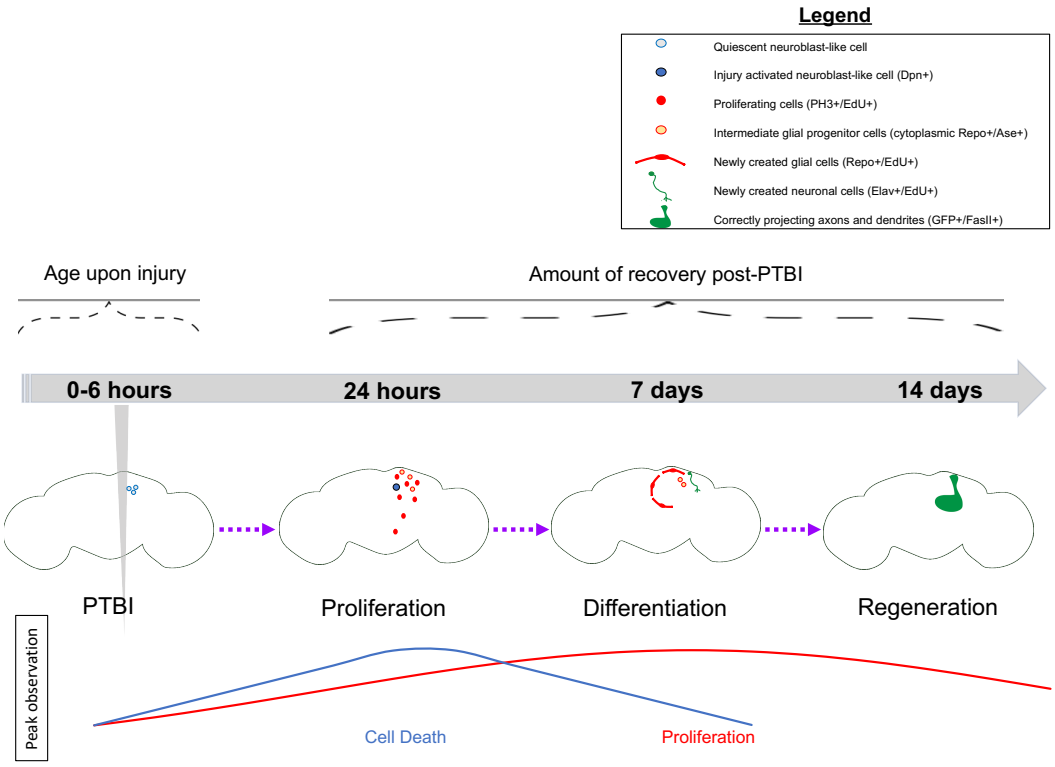

Figure 3-7. Summary model for regeneration following penetrating traumatic brain injury (PTBI). We propose that in young adult *Drosophila* there are quiescent NB-like cells within the central brain that lack expression of canonical NB genes. By 24 hours post-PTBI, the quiescent NB-like cells are activated, express NB genes, and have begun to proliferate. At both 4 hours and 24 hours post-PTBI, there is a wave of cell death as assayed using TUNEL. At 7 days, the proliferation rate is still high, and many of the new cells have adopted mature cell identities, becoming neurons or glia. At 10 days post-PTBI, there is no longer a difference in TUNEL+ cells between uninjured brains and injured brains, indicating that the wave of cell death has ended. Because the peaks of both cell death and proliferation occur at the same time post-injury, this could explain why there is not a significant increase in the number of EdU+ cells seen at 7 days compared to the number of PH3+ cells seen at 24 hours. However, by 10 days, cell death is back to control levels while proliferation has decreased but is still slightly above baseline levels. This could explain why there is an increase in the number of EdU+ by 14 days. At 14 days post-PTBI, there are large clones of new neurons with axons and dendrites correctly projecting to their respective target areas. Locomotor defects are also restored by 14 days, suggesting that adult *Drosophila* are able to regenerate functionally as well as structurally.

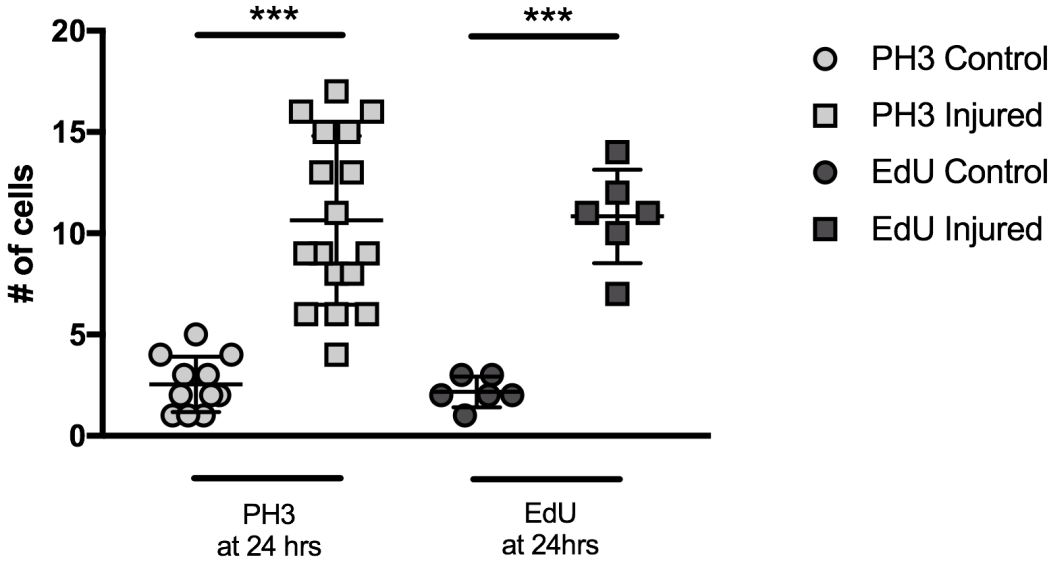

Figure 3-S1. There is no significant difference in the number of PH3+ and EdU+ cells observed 24 hours post-PTBI. We use two methods, anti-PH3 immunochemistry to label mitotic cells and EdU labeling of newly synthesized DNA to assay cell division. To assess the extent to which anti-PH3 and EdU labeling are comparable, we evaluated both control and PTBI brains with both methods at 24 hours. In control brains, there were an average of 2.5 PH3+ (n=11) cells and 2.2 EdU+ cells (n=6). In PTBI brains, there were an average of 10.7 PH3+ cells (n=16) and 10.8 EdU+ cells (n=6). Thus, while control and injured brains displayed significant differences in cell proliferation with both assays (PH3: p-value>0.0001, EdU: p-value>0.0005), the number of proliferating cells detected with the two methods was not significantly different. Error bars reflect the standard deviation (SD).

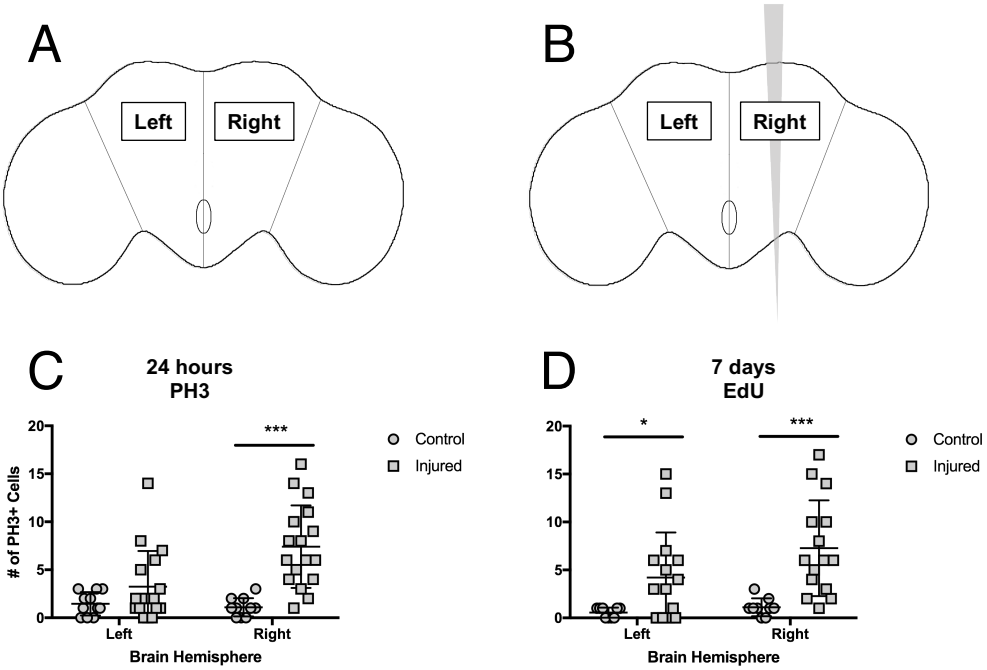

Figure 3-S2A-D. Cell proliferation is concentrated in the damaged right hemisphere. In order to determine where cell proliferation occurs post-PTBI, we counted PH3+ or EdU+ cells in the left and right hemispheres of the central brain. The schematics in **(A)** and **(B)** illustrate the brain regions assayed. **C.** At 24 hours, control brains (n=11) have ~2 PH3+ cells and in PTBI brains (n=17) have ~3 PH3+ cells in the left hemisphere. In the right hemispheres, control brains have ~1 PH3+ cell while PTBI brains have ~7 PH3+ cells (p-value<0.00006). **D.** At 7 days, in the left hemispheres, control brains (n=8) have an average of 0.6 EdU+ cells while PTBI brains have an average of 4 EdU+ cells (p-value=0.04). In the right hemisphere, control brains (n=8) have an average of 1 EdU+ cell, while PTBI brains (n=14) have an average of 7 EdU+ cells (p-value=0.002). Error bars reflect the standard deviation (SD).

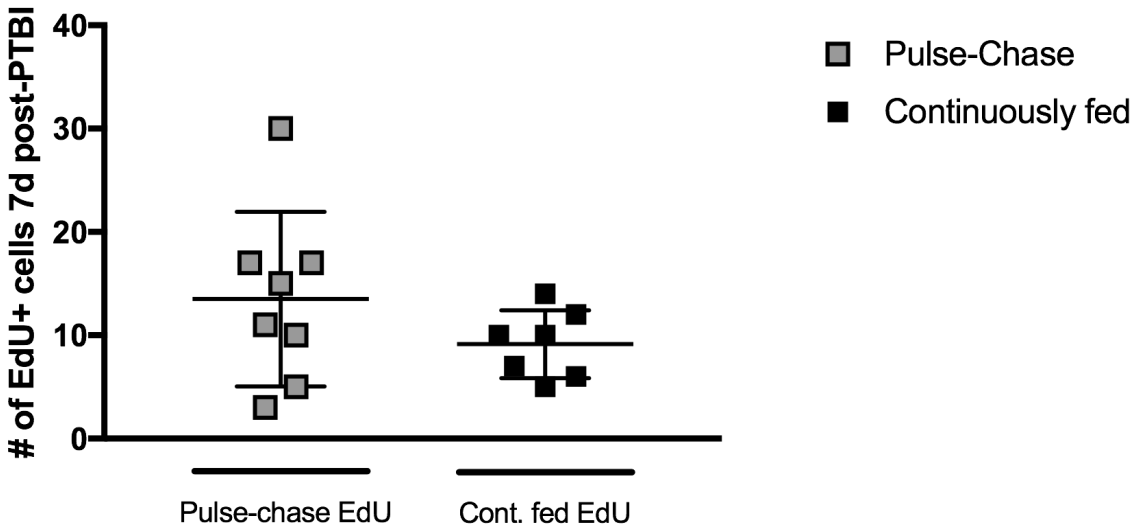

Figure 3-S3. Pulse-chase and continuously fed EdU animals exhibit no difference in the number of EdU+ cells at 7 days post-PTBI. To assess whether mitotically active cells survive post-PTBI, we used two methods of feeding EdU, pulse-chase (flies are fed EdU for 4 days, then placed on standard sugar food for 3 days) and continuously fed (flies are fed EdU every day before being assayed). At 7 days post-PTBI, we find that pulse-chase PTBI brains have an average of 13.5 EdU+ cells (n=8) while continuously fed PTBI brains have an average of 9.1 EdU+ cells (n=7), this is not significantly different. Error bars reflect the standard deviation (SD).

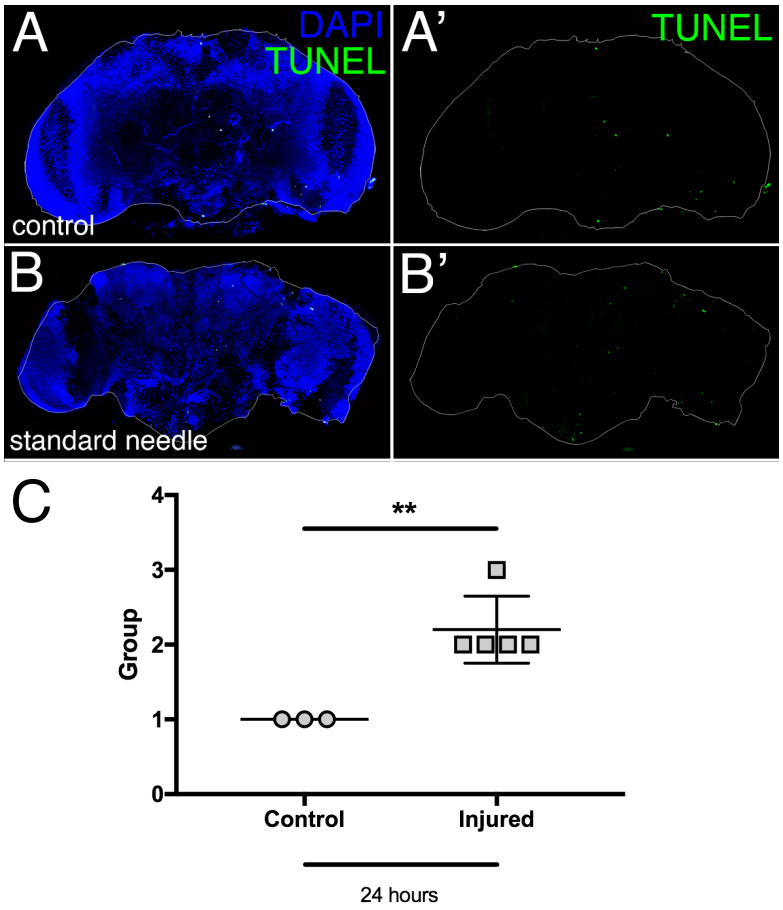

Figure 3-S4A-C. Cell death following PTBI is increased after injury. We assayed cell death using TUNEL staining (in green) in control and PTBI brains. At 24 hours, there is little cell death following our standard penetrating STAB injury (compare **B** and **B'** to **A** and **A'**). Quantification of neurodegeneration is provided in **C**. Groups 1, 2, 3 and 4 have 0-9, 10-19, 20-29, and 30+ TUNEL+ cells/brain respectively. Error bars reflect the standard deviation (SD).

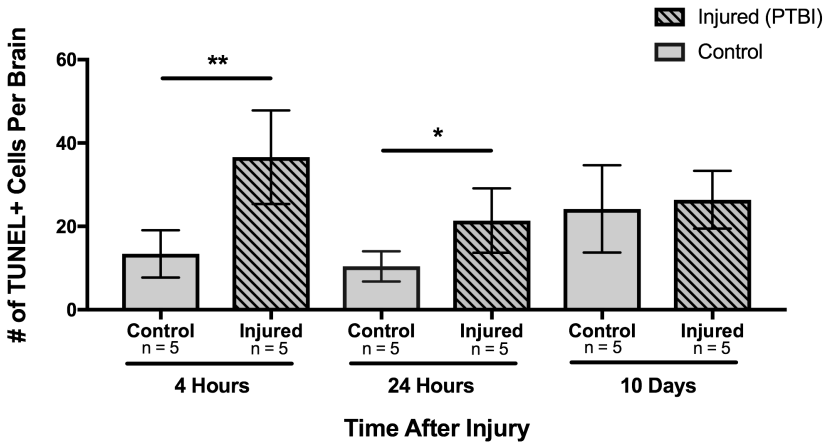

Figure 3-S5. Increase in cell death is inversely correlated with time after injury. In order to understand the temporal dynamics of cell death after PTBI, we used the TUNEL assay to determine how much cell death was present at three time points. We used 4 hours after injury to detect primary cell death resulting from the mechanical injury and 24 hours and 10 days after injury to detect cell death resulting from secondary injury. We found that the both the 4 hour and 24 hour injured samples exhibited significantly higher levels of cell death than the time-matched controls. However, there is no difference between amount of cell death seen in 10-day control and injured brains. Average number of TUNEL+ cells are 13.4±2.5 for 4 hour control and 36.6±5.0 for 4 hour injured samples (p-value=0.0033); 10.4±1.6 for 24 hour controls and 21.4±3.4 for 24 hour injured samples (p-value=0.0203); and 24.2±4.7 for 10 day control and 26.4±3.1 for 10-day injured samples (p-value=0.7053). Error bars reflect the standard deviation (SD).

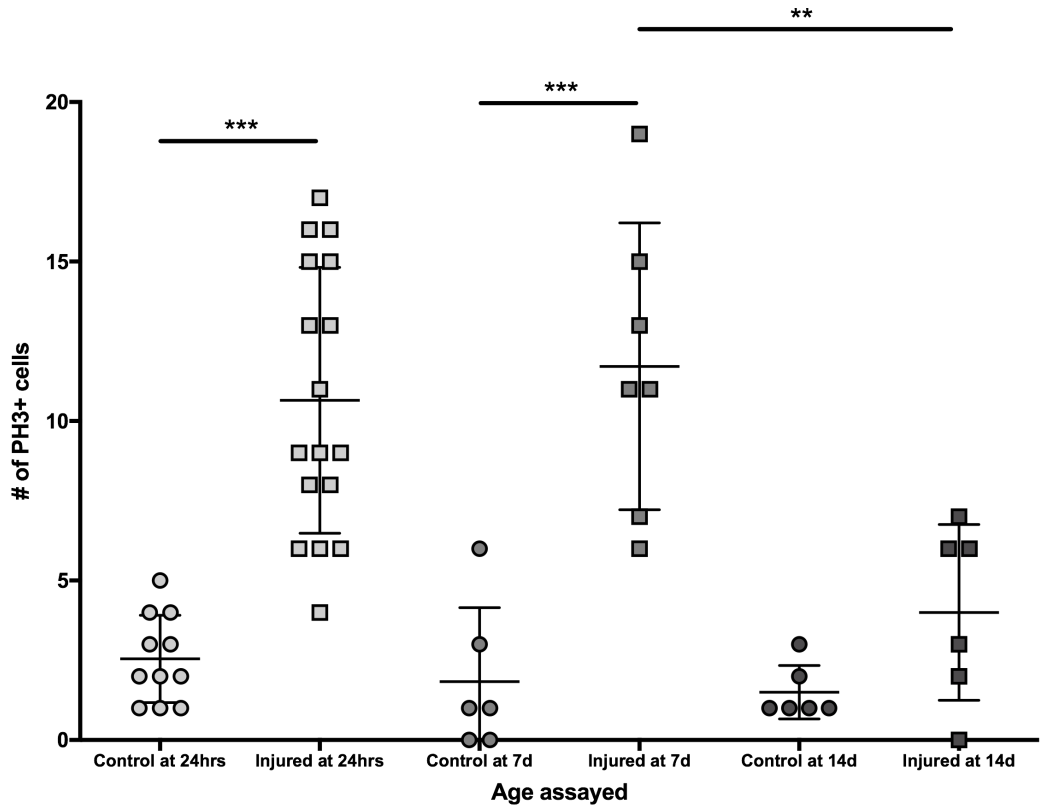

Figure 3-S6. Mitotic activity highest between 24 hours and 7 days. To assay how many cells were actively dividing as time progressed, we used anti-PH3 at three timepoints: 24 hours, 7 days, and 14 days post-injury. At 24 hours, uninjured control brains had an average of 2.5 PH3+ cells per brain (n=11), while 24-hour post-PTBI brains had an average of approximately 11 PH3+ cells per brain (n=17), which is significantly different (p-value<0.0001). At 7 days, control brains had an average of 1.8 PH3+ cells per brain, while 7 days post-PTBI brains had an average of 11.7 PH3+ cells per brain, which is significantly different (p-value=0.0005). However, 24-hour PTBI and 7-day PTBI brains did not have significantly different numbers of PH3+ cells. At 14 days, control brains had an average of 1.5 PH3+ cells and 14-day post-PTBI brains had an average of 4 PH3+. While this is slightly more than controls, it is not quite statistically significant. Error bars reflect the standard deviation (SD).