FATED TO FORGET: AN AGE-DEPENDENT DECREASE IN SYD-1 CAUSES AGE-DEPENDENT MEMORY DECLINE

by

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Title: Fated to Forget: An Age-Dependent Decrease in Syd-1 Causes Age-Dependent Memory Decline

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Most organisms experience an age-dependent decline in memory that is caused by a failure to maintain synapses, the connections between neurons. Despite the importance of maintaining synapses during adulthood, the molecular mechanisms responsible are unknown. In aging fruit flies, memory decline occurs because pre-synaptic sites called active zones (AZs) accumulate an excess of the essential structural component Bruchpilot (Brp), but why this happens is unclear. During development, Brp is recruited to AZs by the conserved AZ protein Syd-1. I tested the hypothesis that age-dependent changes in Syd-1 cause the age-dependent accumulation of Brp. I first used an enhancer trap to monitor Syd-1 expression during adulthood. I found that Syd-1 levels decrease with age, suggesting a model in which this decrease might be responsible for the age-dependent increase in Brp. To test this possibility, I asked whether prematurely reducing Syd-1 levels prematurely increases Brp. I found, indeed, that young flies who expressed less Syd-1 from the onset of adulthood onward had higher Brp levels than wild-

type flies of the same age. From this, I can conclude that Syd-1 is required to prevent the accumulation of Brp. I next wanted to test whether preventing the age-dependent decrease in Syd-1 could delay or prevent the age-dependent increase in Brp. I found that overexpressing Syd-1 during adulthood is not sufficient to reduce Brp levels in younger or older adults, suggesting that additional genes contribute to this process. These results deepen our understanding of the components involved in aging that could be manipulated to delay this process.

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Introduction

The aging process is generally accompanied by a decline in physical functions. One such function is the ability to recall memories (Nyberg et al., 2012). In the brain, memories are formed through molecular changes that strengthen synaptic connections (Bruel-Jungerman et al., 2007). The molecular mechanism of forming memories involves neurotransmission, the transfer of information through chemical messengers called neurotransmitters. Neurotransmitters are released from synaptic vesicles (SVs) on the presynaptic side of one neuron into the synaptic gap, where they travel to receptors on the postsynaptic neuron, allowing the latter to receive the signal (Banerjee et al., 2021). Aging is accompanied by an overall decrease in neurotransmission (Barnes et al., 2000), and structural changes within neuronal synapses are associated with the functional decline in memory recall (Koch et al., 2021). In the aging mammalian brain, synapses have been observed to increase in size and decrease in number (Calì et al., 2018). However, the underlying mechanisms that cause these structural alterations have yet to be fully understood.

Aging fruit flies (*Drosophila*) experience similar structural changes in their brains, but at a much faster rate due to their shorter lifespans (Koch et al., 2021). Their rapid life cycles and easy maintenance makes *Drosophila* a well-suited model organism to study the mechanism of age-dependent memory decline (Koch et al., 2021). In *Drosophila*, synapses can be visualized and quantified by monitoring the major presynaptic component, Bruchpilot (Brp) (Chou et al., 2020). Brp[1] localizes in the presynaptic sites that release SVs, known as active zones (AZs), and functions as a docking platform for regulating the release of SVs into the synapse (Chou et al., 2020). Brp accumulates with age, and prior research has found that the age-dependent accumulation of Brp causes age-dependent memory decline: doubling the copies of the

[1] Proteins are referred to by capitalizing the first letter of their name and no italicization.

endogenous *brp*[2] gene has been shown to prematurely accelerate both short-term and middle-term memory (MTM) impairment in younger flies (Gupta et al., 2016). In a subset of neurons in the brain known as the mushroom body (MB), a transient increase in Brp occurs during memory formation, suggesting that Brp levels normally increase temporarily before returning to baseline levels once the memory is formed (Turrel et al., 2022). While the age-dependent increase in Brp causes memory decline, decreasing Brp levels during adulthood also impairs MTM in flies, indicating that a "Goldilocks" level of Brp is required to form new memories (Turrel et al., 2022). Gupta et al. (2016) propose the following model: the age-dependent accumulation of Brp elicits heightened SV release from the presynaptic neuron; while synapses are usually strengthened by increasing the SV release of neurotransmitter signal, synapses may have a "fixed operating range"—that is, their ability to be strengthened may eventually reach an upper limit; at that point, new memories would not be able to form. This model provides a reasonable framework for thinking about how Brp accumulation might impair memory. However, the cause of this Brp accumulation remain unknown.

The components required for assembling synapses prior to adulthood (that is, during development) are well known. I hypothesized that some of the components involved in synaptic assembly may also be involved in synapse maintenance as adults age. My research focused on the presynaptic component, Syd-1, which is one of the first proteins to arrive to AZs during assembly (Chou et al., 2020).

During development, Brp is recruited to the axonal terminal's AZs to remain as an essential structural component (Chou et al., 2020). However, Syd-1 arrives at the AZ very early on in the process of synapse assembly prior to recruiting Brp (Owald et al., 2012). Syd-1 must

[2] Genes and genotypes are referred to by italicizing their entire name and don't require the capitalization of the first letter.

recruit multiple synaptic components to AZs, including the scaffold protein Liprin-α and the cell-surface adhesion molecule Neurexin-1 (Nrx-1), and works together with Liprin-α to recruit the SV release factor Unc13A (Chou et al., 2020). Following their recruitment are the arrival of the downstream components Unc13B, RIM/Unc-10, and Brp (Chou et al., 2020). The interaction between Syd-1 and Nrx-1 has been shown to directly bind and cluster Brp (Owald et al., 2012; Spinner et al., 2018).

Research also shows that when Syd-1 is absent during development, Brp irregularly localizes and becomes misshapen within AZs, demonstrating that Syd-1 is required during synapse assembly to recruit Brp and hold it in place (Owald et al., 2010). It is possible that Syd-1 plays a similar role during adulthood to aid the process of age-dependent memory decline. Syd-1 has been shown to be present at AZs in 4- to 7-day-old adult flies (Fulterer et al., 2018). Using RNA interference (RNAi) to reduce Syd-1 expression in the adult MB disrupts MTM, indicating that Syd-1 is required in adulthood to preserve memory (Ramesh et al., 2023). In my thesis work, I tested the hypothesis that age-dependent changes in Syd-1, a presynaptic protein that recruits Brp during development by direct interaction, cause the age-dependent accumulation of Brp.

Here I show that Syd-1 decreases with age. I found that prematurely decreasing Syd-1 in young adults is sufficient to cause premature accumulation of Brp in their brains, indicating that Syd-1 is normally required to prevent this accumulation and thereby preserve memory. I tested the possibility that preventing the decrease in Syd-1, by overexpressing it in adulthood, might be sufficient to reduce Brp in older animals but saw no effect, indicating that additional genes are involved. These findings support a model in which Syd-1 functions to anchor Brp at AZs during adulthood and the normal decline in Syd-1 with age causes Brp to accumulate. Because Syd-1 and Brp both have mammalian homologs (MSYD1A and ELKS/CAST, respectively) (Chou et

al., 2020), Syd-1's role in maintaining synapses in aging *Drosophila* could theoretically be conserved in mammals. This information may then be used to help delay the process of age-dependent memory decline in humans.

Materials and Methods

Drosophila Stocks

The following lines were used: (1) *syd-1GFSTF/TM3*, (2) *w; FRT82w^{+},* (3) *w; FRT82w^{+}, syd-1^{CD}/TM6B* (4) *UAS-Dcr; C7702RNAi*, (5) *UAS-Dcr; sy-1dRNAi/CyO*, (6) *w; UAS-syd-1^{WT}*, (7) *w; 5XUAS-lacZ*, (8) *C155-Gal4/w*, (9) *C155-Gal4;UAS-Dcr*, (10) *C155-Gal4; Gal80^{ts}*, (11) *UAS-syt-GFP*, (12) *Nsyb-Gal4*, (13) *Gal80^{ts}; Nsyb-Gal4/TM6B*, (14) *Bloomington Control* (used to compare to *syd-1RNAi on 3*) (#35788), (15) *syd-1RNAi on 3* (# 32946), (16) *syd-1Dup*, (17) *futschRNAi/CyoGFP* (#40834) (18) *PR7/20C11FLP;UASfutschtomato/TM6B* (19) *w;UASFLP,Gal80^{ts}/CyoGFP;TM2/TM6B* (20) *PR7-Gal4/CyoGFP;brpGFP,UAStomato/TM6B.* All stocks were acquired from the Bloomington *Drosophila* Stock Center or the Vienna *Drosophila* Resource Center except for the stock containing *UAS-syd-1^{WT}*, which was generated by Spinner et al. 2020, and (19), which was generated using stocks from Bloomington.

DNA Constructs

Measuring Syd-1 *levels*

syd-1GFSTF/TM3 parent vials were raised at 18°C and flipped every 3-4 days. Female F1 progeny were collected and maintained at 18°C to be dissected at a variety of ages on the same day.

Assessment of adding Gal80^{ts} *to the* Gal4 *system*

UAS-syt-GFP males were crossed to the following lines: *C155-Gal4/w* (control), *Nsyb-Gal4* (control), *C155-Gal4; Gal80^{ts}* (manipulation), and *Gal80^{ts}; Nsyb-G4/TM6B* (manipulation), and raised at either 18°C, 25°C, or 29°C. Crosses were flipped every 3-4 days. Females of the

correct genotypes were collected and either maintained at the temperature they were raised at, or transferred from 18°C to either 25°C or 29°C at some time during adulthood.

Decreasing syd-1 expression during development and adulthood

C155-Gal4/w was crossed to *Bloomington control* (control) or *syd-1RNAi on 3* (manipulation). Crosses were kept at 29°C and flipped every 3-4 days. Females of the correct genotypes were collected and maintained at 29°C prior to dissection.

Increasing syd-1 expression during development and adulthood

C155-Gal4/w was used as the control or crossed to *w; UAS-syd-1^{WT}* (manipulation). Crosses were kept at 18°C and females were collected and transferred to 25°C upon eclosion. Females were maintained at 25°C prior to dissection.

Temperature-manipulated syd-1 expression

The Gal4/Gal80^{ts} system was employed to temporally control expression of the targeted transgene. *Gal80^{ts};Nsyb-Gal4/TM6B* female virgins were crossed to the following males: *syd-1RNAi on 3* (manipulation), *Bloomington Control* (control), *UAS-Dcr; syd-1RNAi/Cyo* (manipulation), *UAS-Dcr; C7702RNAi* (control), *w; UAS-syd-1^{WT}* (manipulation), *w; 5XUAS-lacZ* (control), and *fustchRNAi/CyoGFP* (manipulation) and raised at 18°C. Crosses were flipped every 3-4 days. Females of the correct genotypes were collected and transferred to 29°C upon eclosion to induce a decrease or increase in gene expression. Females were maintained in 29°C for 10 and 30 days prior to dissection. Some *UAS-syd-1^{WT}* female progeny were collected and maintained in 18°C to be transferred to 29°C at a later timepoint during adulthood.

UASFLP,Gal80^{ts} STaR recombinant candidates

w;UASFLP,Gal80^{ts}/CyoGFP;TM2/TM6B virgins were crossed to *PR7-Gal4/CyoGFP;brpGFP,UAStomato/TM6B* males and kept at 18°C. Females or males of the correct genotypes were collected, kept at 18°C, and dissected between 1-3 days into adulthood.

Fly husbandry

Unless stated, flies were raised and maintained at 25°C. All strains were reared in the same conditions in either 18°C, 25°C, or 29°C. All incubators were under the conditions of 50% humidity with a constant 12:12 h light/dark cycle. All strains were fed standard media and progeny vials were flipped every 3-4 days.

Dissections and immunohistochemistry

All dissections were performed at room temperature (21°C). Adult females were beheaded in groups of six and then kept in HL3 (as in Gupta et al 2016). Each brain was dissected in HL3 and then kept submerged in HL3 on ice. Every 2 brains dissected were fixed for 20 minutes in 4% PFA (8% PFA + 2X Phosphate buffered solution (PBS) (Sigma Aldrich 4101737145)). Then brains were washed in 1% PBT (1X PBS + 1% Triton X-100) for 20 minutes, washed in 0.3% PBT (1X PBS + 0.3% Triton X-100) for 20 minutes, and refrigerated overnight in 10% NGS Block (0.6% 2X PBT + 10% NGS + 0.05% sodium azide). Antibodies were diluted in 5% NGS Block (0.6% 2X PBT + 5% NGS + 0.05% sodium azide).

Brain-wide Syd-1 *fluorescence*

In each well, rabbit anti-GFP primary antibody (1:1000; A11122 from Thermo-Fisher) was applied for 2 days at room temperature. Brains were washed three times in 0.3% PBT and sat in 0.3% PBT for 30 minutes in between each wash. On fourth wash, brains were refrigerated

in 0.3% PBT overnight. Goat anti-Rabbit Alexa 488 secondary antibody (1:250; A11034 from Thermo-Fisher) was applied for 1 day at room temperature. Again, brains were washed three times in 0.3% PBT and sat in 0.3% PBT for 30 minutes in between each wash. On fourth wash, brains were refrigerated in 0.3% PBT overnight.

Brain-wide Brp *fluorescence*

In each well, mouse anti-Brp primary antibody (1:100; NC82 from Developmental Studies Hybridoma Bank) was applied for 2 days at room temperature. Brains were washed three times in 0.3% PBT and sat in 0.3% PBT for 30 minutes in between each wash. On fourth wash, brains were refrigerated in 0.3% PBT overnight. Goat anti-mouse Alexa 488 secondary antibody (1:400; A11029 from Thermo-Fisher) was applied for 1 day at room temperature. Again, brains were washed three times in 0.3% PBT and sat in 0.3% PBT for 30 minutes in between each wash. On fourth wash, brains were refrigerated in 0.3% PBT overnight.

Prior to mounting anti-Brp and GFP brains, 2 cover slips were attached with nail polish to each side of each microscope slide for equal compression of each brain on each slide. Brains were washed in 0.3% PBT one more time before mounting. Vectashield mounting medium was used to mount 2 brains per slide. Each brain was oriented anterior-side up and in between the 2 cover slips. A third coverslip was placed in between the 2 coverslips to secure the brains mounted on each slide.

AZ-level labeling of neurons and Brp

The approaches described in Chen et al. (2014) were further developed for the goal of visualizing Brp-containing individual active zones in wild-type, *syd-1RNAi*-expressing, and *UAS-syd-1*^{WT}-overexpressing adults. In each well, chicken anti-GFP primary antibody (1:800; 13970 from Abcam) and rabbit anti-DsRed primary (1:200; 632496 from Takara Bio) were

applied for 2 days at room temperature. Brains were washed three times in 0.3% PBT and sat in 0.3% PBT for 30 minutes in between each wash. On fourth wash, brains were refrigerated in 0.3% PBT overnight. Anti-chicken Alexa 488 secondary antibody (1:500; A32931 from Thermo-Fisher) and Anti-rabbit Alexa 555 secondary antibody (1:500; A21428 from Thermo-Fisher) were both applied for 1 day at room temperature. Again, brains were washed three times in 0.3% PBT and sat in 0.3% PBT for 30 minutes in between each wash. On fourth wash, brains were refrigerated in 0.3% PBT overnight.

Image acquisition and analysis

Brain-wide intensity analyses

All NC82 and GFP brains were imaged with the Zeiss LSM 880 confocal microscope. The following settings were used to scan anti-Brp and GFP brains: 20X lens, 488 laser, 16-bit depth. Images were taken in Z-stacks in 1.5um slices. Images were processed using Fiji. The sum image of every Z-stack was created. The right side of each brain was outlined manually and subsequently used to quantify the average intensity of each brain.

Individually labeled AZ analyses

All STaR-labeled brains were imaged with the Zeiss LSM 880 confocal microscope. The best-mounted optic lobe from each brain was scanned under the following settings: 20X lens, 488 and 561 lasers, 16-bit depth. Images were taken in Z-stacks in 1.0um slices, starting from the first row of axon terminals and ending 4-5 rows in. Images were processed using Huygens deconvolution software and then analyzed using Fiji. In Fiji, 5 axon terminals located toward the middle of each optic lobe were chosen, where the brightest slice for each axon terminal was then selected for further analysis. With a pre-made circular shape, each axon terminal head was

outlined and its tomato intensity was measured. Each of the 5 axon terminals' tomato intensities were used to quantify the average intensity of each brain.

Statistical analysis

Statistical analyses were performed in Excel and Prism. Statistical significance was determined in Excel using the two-tailed or one-tailed unpaired t-test between each manipulated and its control genotype. T-tests were only applied to analyze data sets dissected on the same day. I used Graphpad Prism to create box-and-whisker plots for each data set where each data point showed an individual brain's normalized average Brp intensity in reference to each control group's average. For the trendline analysis of the age-dependent decrease in Syd-1, Graphpad Prism was used to perform an extra-sum-of-squares F test to determine whether one curve adequately fit all data sets. For analysis of the *UASFLP,Gal80^{ts}* STaR recombinant candidates at 18°C, I performed a one-way ANOVA followed by the Dunnett post-hoc test to determine significance between each candidate and the control group.

Results

Syd-1 levels decrease with age

Syd-1 is present at AZs in 4- to 7-day-old adult flies (Fulterer et al., 2018). However, whether Syd-1 levels change as flies age is unknown. Because Syd-1's role during synapse assembly is to recruit Brp to AZs, I hypothesized that the age-dependent accumulation of Brp could theoretically result from an age-dependent increase in Syd-1. To test this, I used an existing protein trap that causes the endogenous Syd-1 protein to be fused with GFP (*sydGFSTF/TM3*). I measured Syd-1-GFP intensity in the central brains of young (3-day-old) and middle-aged (32-day-old) adult flies grown at 18°C. Contrary to my prediction, I found that Syd-1-GFP intensity significantly[3] decreased by 21.29% between these two timepoints (Figure 1A, B).

I reasoned that, despite this observed decrease in middle age, Syd-1 might later increase as adults get older. To rule out this possibility, I needed to follow the time course of Syd-1 expression throughout adulthood. To minimize variation, I performed a series of experiments, spanning 1-60 days of adulthood. In each experiment, I compared flies of different ages, all dissected on the same day to ensure that any differences observed were due to age and not other factors. Flies age approximately twice as fast at the standard growth temperature of 25°C than at 18°C (Bloomington *Drosophila* Stock Center). To expedite these experiments, I therefore grew all flies at 25°C. Note that the 32-day-old flies that I grew in the initial 18°C experiment were roughly equivalent to 16-day-old flies grown at 25°; I therefore expected to see a decrease in

[3] The use of "significant" and "significantly" describes the statistical significance of quantitative data at the threshold of $p<0.05$.

Syd-1 between 1- and 16-day-old flies at 25°C. Consistent with my prediction, I observed that this was the case by 15 and even 13 days into adulthood (Figure 1F, G). By comparing the first two timepoints of each dissection set of age ranges, I found that Syd-1 levels began to decrease by 20.96% as early as 6 days into adulthood (Figure 1C). In each set of timepoints, Syd-1 decreased most sharply between the first two timepoints and then tapered more gradually (Figure 1C-G). To identify more precisely the relationship between age and Syd-1 levels, I determined the best-fit nonlinear regression model across all the dissection sets of age ranges. I found that a general negative semi-logarithmic relationship between age and Syd-1 levels best explained my data (Figure 1H). I therefore conclude that Syd-1 decreases throughout adulthood but does not do so at a consistent rate; instead Syd-1 experiences its largest, most rapid decline early on in adulthood and then decreases more gradually at later ages.

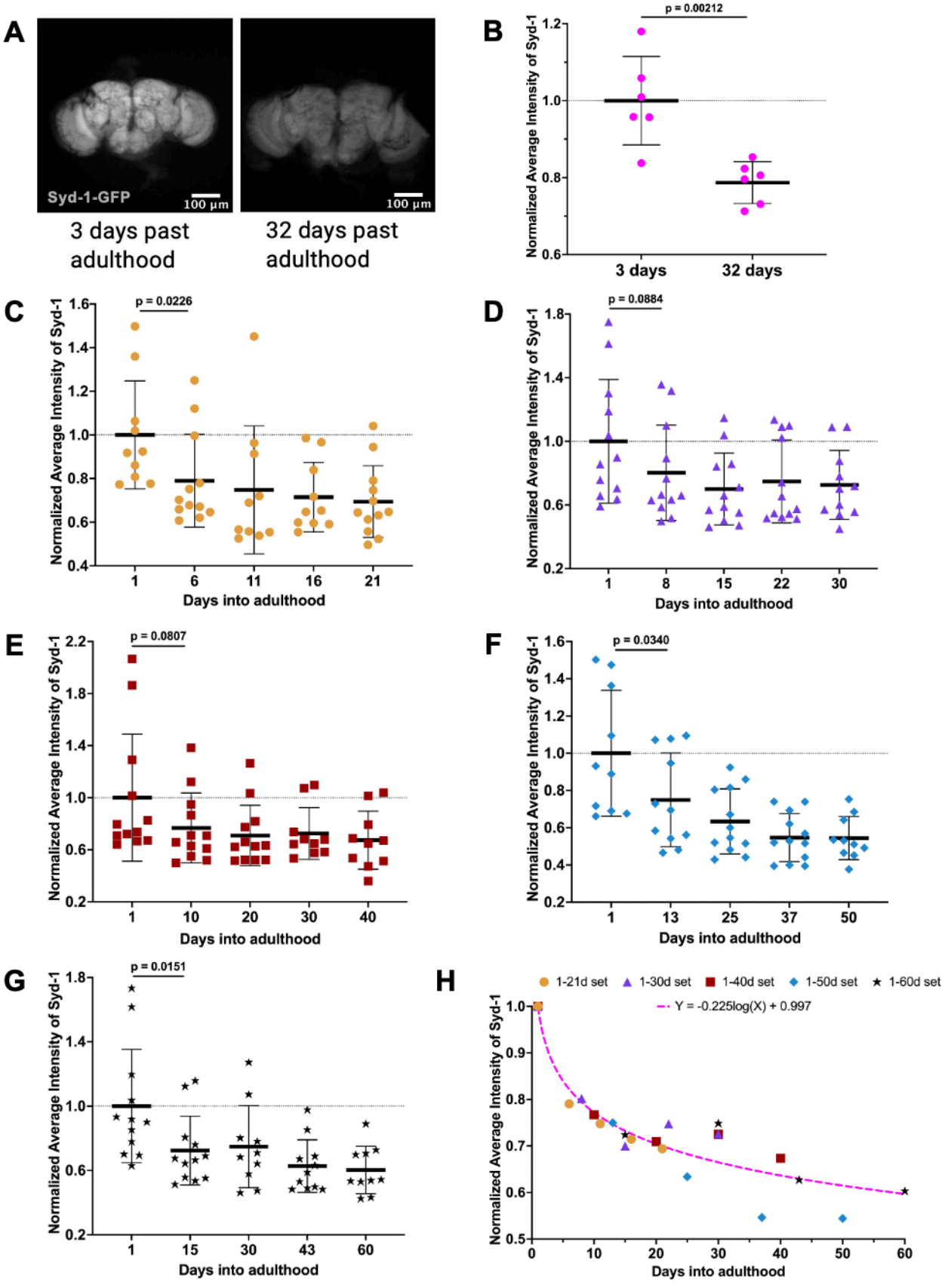

A
Syd-1-GFP
100 µm
100 µm
3 days past adulthood
32 days past adulthood
B
p = 0.00212
Normalized Average Intensity of Syd-1
3 days
32 days
C
p = 0.0226
Normalized Average Intensity of Syd-1
Days into adulthood
D
p = 0.0884
Normalized Average Intensity of Syd-1
Days into adulthood
E
p = 0.0807
Normalized Average Intensity of Syd-1
Days into adulthood
F
p = 0.0340
Normalized Average Intensity of Syd-1
Days into adulthood
G
p = 0.0151
Normalized Average Intensity of Syd-1
Days into adulthood
H
1-21d set
1-30d set
1-40d set
1-50d set
1-60d set
Y = -0.225log(X) + 0.997
Normalized Average Intensity of Syd-1
Days into adulthood

Figure 1. Syd-1 levels decrease most rapidly during youth and then more gradually as animals age.

Each data point represents 1 brain; the average intensity for each brain was normalized to the average Syd-1-GFP intensity of the control group (1-day-old). The normalized mean and SD for each age is shown. (A) Immunofluorescent images of brain-wide Syd-1-GFP intensity in 3-day-old and 32-day-old brains. Images acquired using Zeiss LSM 880 confocal microscope 10X lens. (B) Quantification of Syd-1-GFP intensity levels in 3-day-old and 32-day-old adults raised and maintained at 18°C. Each data point represents 1 brain, with n=6 per group. Significance determined with two-tailed t-test. (C-G) Quantification of Syd-1-GFP intensity levels at varying ages throughout adulthood with flies raised and maintained at 25°C. 5 independent data sets are shown. The normalized mean and SD for each age is shown; each data point represents 1 brain. Significance between the first 2 timepoints of each data set was determined using a one-tailed t-test. The average normalized Syd-1-GFP intensity is shown for adults aged to (C) 1 day (n=10), 6 days (n=12), 11 days (n=10), 16 days (n=10), and 21 days (n=12); (D) 1 day (n=12), 8 days (n=12), 15 days (n=12), 22 days (n=12), and 30 days (n=11); (E) 1 day (n=12), 10 days (n=12), 20 days (n=12), 30 days (n=10), and 40 days (n=10); (F) 1 day (n=10), 13 days (n=11), 25 days (n=12), 37 days (n=12), and 50 days (n=10); And (G) 1 day (n=12), 15 days (n=12), 30 days (n=10), 43 days (n=11), and 60 days (n=10). (H) The decrease in Syd-1 may trend toward a negative semi-log relationship between age (days into adulthood) and Syd-1 intensity, where log of age may explain the decrease in Syd-1 levels. Extra sum-of-squares F test was performed to evaluate the best-fit trendline for all combined aging data sets, shown in pink ($R2= 0.9179$, $F8,15=2.525$, $p= 0.0581$). Each individual data point shows the normalized average Syd-1-GFP intensity for that age.

Decreasing Syd-1 throughout development and adulthood prematurely increases Brp levels

My finding that Syd-1 decreases with age clearly contradicted my initial hypothesis but also suggested a new one: perhaps, instead, the age-dependent decrease in Syd-1 is what causes the age-dependent increase in Brp. My initial hypothesis was motivated by the observation that loss of *syd-1* during development significantly decreases the number of Brp puncta (Owald et al., 2010). However, loss of *syd-1* during development also increases the size of the Brp puncta that remain (Owald et al., 2010). I therefore revised my hypothesis: Syd-1 may normally restrict the

size of Brp puncta in adulthood, preventing too much Brp from accumulating. As flies age and Syd-1 decreases, Syd-1 becomes less able to inhibit Brp and so too much Brp accumulates. If my model holds true, then prematurely reducing Syd-1's dosage should prematurely allow excess Brp to accumulate. Because Syd-1 is required to recruit Brp during AZ assembly, a complete loss of Syd-1 is lethal. To ensure that flies would survive into adulthood, allowing me to measure Brp levels in their brains, I first tested the effect of simply reducing the dosage of *syd-1* by half. To do so, I raised wild-type flies and flies heterozygous for a deletion of *syd-1* at 25°C and compared their Brp levels at 10, 30, and 50 days into adulthood. I found no difference in Brp intensity at any timepoint (Figure 2A-C). These results suggest that halving the gene dosage of *syd-1* does not cause a premature increase of Brp, indicating that the *syd-1* gene is not haplo-insufficient.

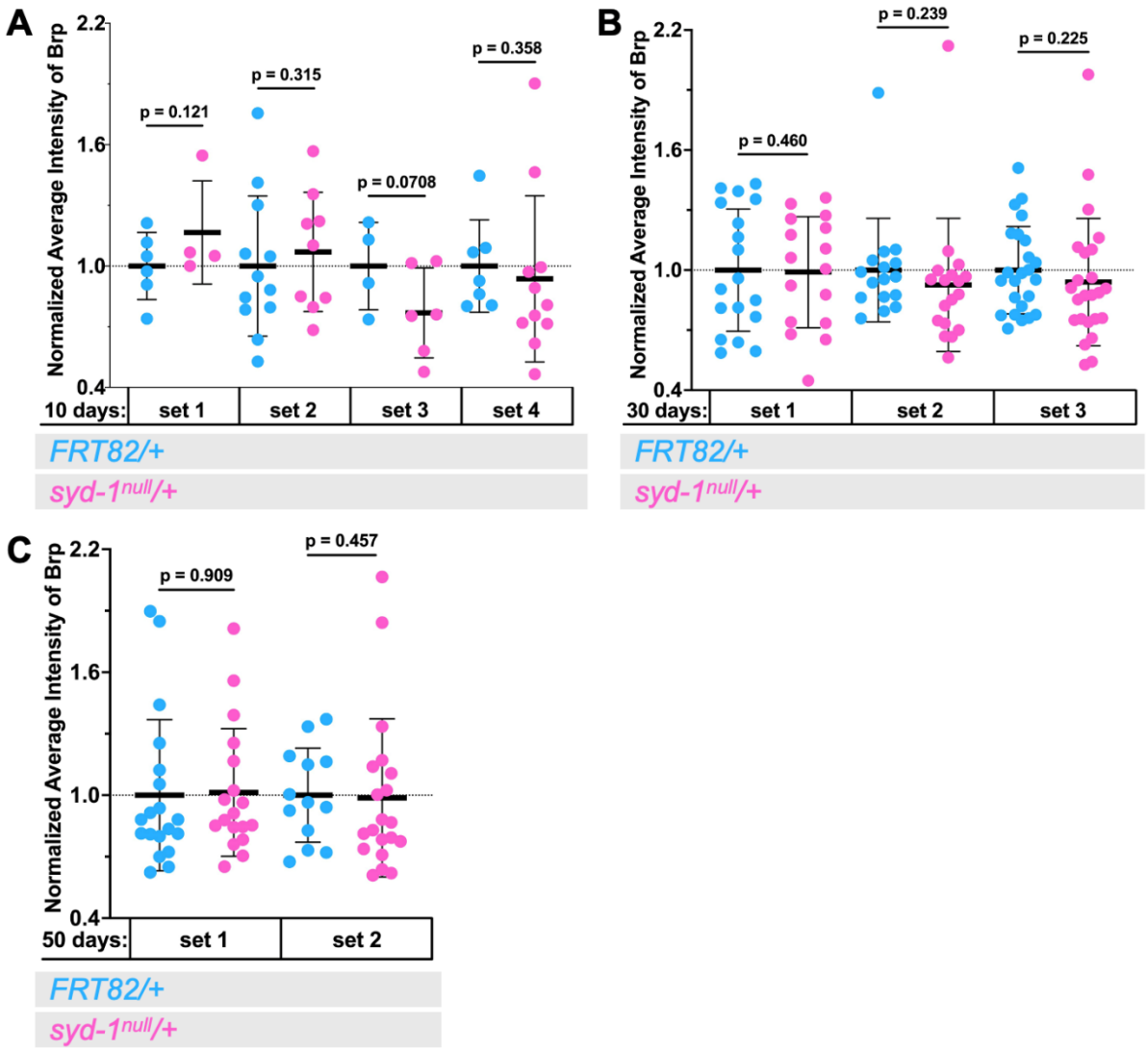


Figure 2. Reducing the dosage of *syd-1* by half is not sufficient to increase Brp levels in adults.

Quantification of the normalized average intensity of Brp for each age along with its mean and SD is shown. Each data point represents an individual brain; the average intensity for each brain was normalized to the average intensity of the control group (*FRT82/+*). Different colors represent different genotypes, where *FRT82/+* (light blue) is the appropriate control expressing normal levels of Syd-1. P-values are shown above each comparable set of dissections, where a one-tailed t-test was used to determine significance if $p<0.05$. Flies expressing normal levels of Syd-1 (*FRT82/+*) and half the normal levels of Syd-1 (*syd-1^{null}/+*, pink) were maintained at 25°C and dissected at (A) 10 days (set 1: *FRT82/+* n=6, *syd-1^{null}/+* n=4), (set 2: *FRT82/+* n=12, *syd-1^{null}/+* n=9), (set 3: *FRT82/+* n=4, *syd-1^{null}/+* n=6), (set 4: *FRT82/+* n=7, *syd-1^{null}/+* n=11), (B) 30 days (set 1: *FRT82/+* n=18, *syd-1^{null}/+* n=16) days (set 2: *FRT82/+* n=20, *syd-1^{null}/+* n=20) (set 3: *FRT82/+* n=24, *syd-1^{null}/+* n=24), and (C) 50 days (set 1: *FRT82/+* n=20, *syd-1^{null}/+* n=18) (set 2: *FRT82/+* n=13, *syd-1^{null}/+* n=20) into adulthood.

To decrease Syd-1 levels by more than half but not enough to cause lethality, I used RNA interference (RNAi). I used a pan-neuronal Gal4 driver to express *syd-1RNAi* in all neurons during development and adulthood. RNAi generally works better at higher temperatures, so these flies were kept at 29°C. I found that using *syd-1RNAi* on chromosome 2 (*syd-1RNAi on 2*, which comes from the Vienna *Drosophila* Resource Center and requires co-expression with Dicer-2) was lethal to flies, producing no results. Because this *syd-1RNAi* transgene apparently reduces Syd-1 to too low a dosage for survival, I then analyzed whether flies expressing a different RNAi transgene, *syd-1RNAi* on chromosome 3 (*syd-1RNAi on 3*, which comes from the Bloomington *Drosophila* Stock Center and does not require Dicer-2), might survive long enough for me to analyze their Brp levels. Indeed, *syd-1RNAi on 3* flies survived to adulthood. I found that 10-day-old adults expressing *syd-1RNAi on 3* showed an 18.85% increase in Brp intensity compared to wild-type adults (Figure 3A). This result is consistent with my model that the decrease in Syd-1 during adulthood might cause age-dependent Brp accumulation. However, because I disrupted Syd-1 throughout development as well as adulthood, the observed premature accumulation of Brp might instead have been caused by the changes in Syd-1 levels during development.

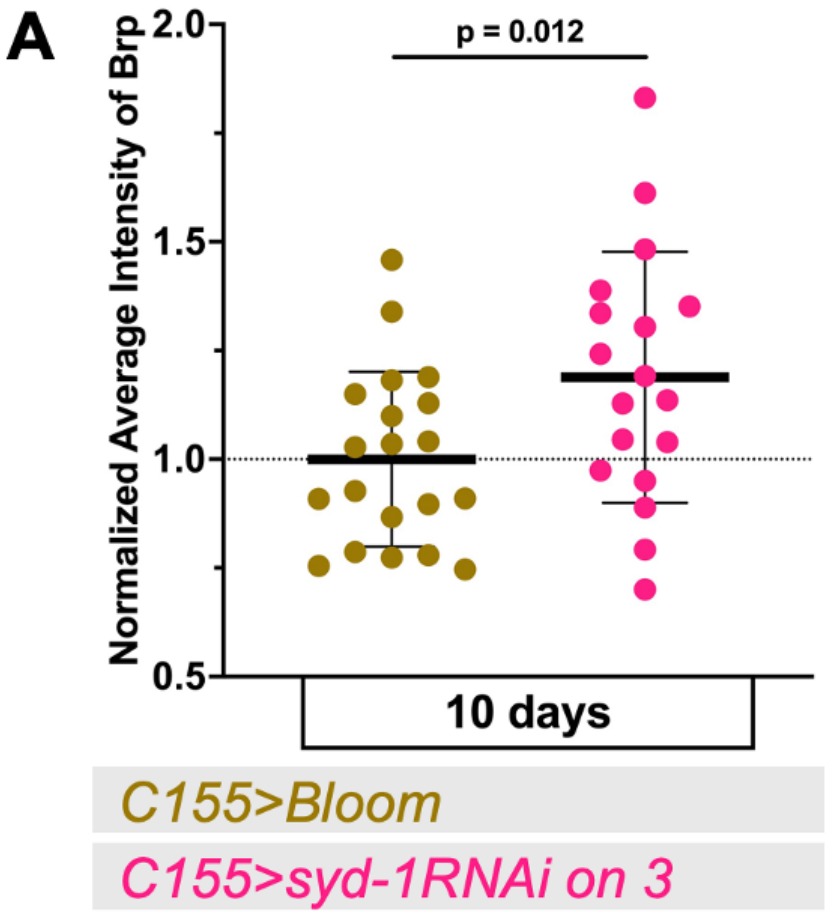


Figure 3. Expressing *syd-1RNAi* to reduce *syd-1* during development and adulthood increases Brp levels in young adults.

Quantification of the normalized average intensity of Brp for each age along with its mean and SD is shown. Each data point represents an individual brain; the average intensity for each brain was normalized to the average intensity of the control group (*C155>Bloom*). Different colors represent different genotypes, where *C155>Bloom* (gold) represents the appropriate control expressing normal levels of Syd-1. The p-value is shown above, where a one-tailed t-test was used to determine significance if $p<0.05$. (A) Using the C155-Gal4 driver, flies expressing normal levels (*C155>Bloom*, n=20) or less than half the normal levels of *syd-1RNAi* on chromosome 3 (pink) (*C155>syd-1RNAi on 3*, n=20) were maintained at 29°C and dissected at 10 days into adulthood.

The C155-Gal4/Gal80^{ts} system does not temporally control gene expression throughout adulthood

To distinguish whether the increase in Brp is caused by decreasing Syd-1 exclusively during adulthood or during development, I employed the Gal4/UAS/Gal80^{ts} system to temporally control expression of *syd-1RNAi*. Gal80^{ts} prevents Gal4 from activating transcription at lower temperatures (18°C) but becomes inactivated at higher temperatures (25-29°C), allowing Gal4 to activate transcription of the gene of interest (Elliot & Brand, 2008). To validate this, I first created progeny that expressed the components of Gal4, Gal80^{ts}, and UAS-syt-GFP. After raising them at 18°C, 25°C, or 29°C, I transferred adult progeny from 18 to 25°C or 18 to 29°C to confirm that I could cause GFP to be exclusively expressed in adulthood. I hypothesized that by using the Gal4/UAS/Gal80^{ts} system, Gal80^{ts}'s activity at 18°C would cause brain-wide GFP intensity to be lower in flies expressing Gal80^{ts} than in flies expressing the Gal4 activator alone, but at the higher temperatures of 25°C or 29°C, the inactivation of Gal80^{ts} would cause Gal80^{ts} flies to have the same levels of brain-wide GFP intensity as flies expressing the Gal4 activator alone.

I first tested whether the pan-neuronal C155-Gal4 driver would allow me to temporally restrict GFP expression to adulthood. When raised and maintained at 18°C, 5-day-old C155-Gal4 Gal80^{ts} adults (*C155;G80^{ts}>sytGFP*) showed an 81.41% decrease in GFP intensity compared with adults with C155-Gal4 only (*C155>sytGFP*), indicating that Gal80^{ts} was working as predicted (Figure 4A). However, Gal80^{ts} also exhibited significantly decreased GFP expression in flies raised and maintained at 25°C, suggesting that either 25°C was too low a temperature to reliably inactivate Gal80^{ts} or that C155-Gal4 was too weak to overcome even a reduced amount

of Gal80^{ts} (Figure 4A). My ultimate goal was to be able to keep expression off at 18°C during development and then turn expression on in adulthood by shifting to a higher temperature (25°C or 29°C). To test whether C155-Gal4 might be suitable for this, I shifted C155-Gal4 Gal80^{ts} flies from 18 to 25°C or 18 to 29°C for varying amounts of time during adulthood. Unfortunately, I found that neither temperature shift restored GFP expression to the expected level, as GFP intensity was still significantly lower in C155-Gal4 Gal80^{ts} adults (Figure 4B). These results suggest that either Gal80^{ts} is not completely inactivated at high temperatures or the C155-Gal4 driver may not stay on consistently throughout adulthood. In case of the latter, I would need to use a different, stronger driver that maintained its function throughout adulthood.

The Nsyb-Gal4/Gal80^{ts} system temporally controls gene expression exclusively during adulthood

I next tested the Nsyb-Gal4 driver under the same set of circumstances as C155-Gal4. I found that 1-day-old Nsyb-Gal4 G80^{ts} adults (*NsybG4;G80ts>sytGFP*) showed an 89.99% and 56.01% decrease in GFP intensity at 18°C and 25°C, respectively, from controls that expressed the Nsyb-Gal4 driver alone (*NsybG4>sytGFP*), but showed no significant differences in GFP intensity at 29°C (Figure 4C). At 12 and 25 days into adulthood, 25°C Nsyb-Gal4 G80^{ts} adults were still significantly lower in GFP intensity while 29°C Nsyb-Gal4 G80^{ts} adults showed no changes from controls (Figure 4C). When shifted from 18-25°C or 18-29°C, Nsyb-Gal4 G80^{ts} adults shifted to 29°C generally exhibited higher GFP intensities than those shifted to 25°C (Figure 4D). From these results, I concluded that the Nsyb-Gal4 driver, paired with the G80^{ts} repressor, was sufficient to temporally control gene expression starting from adulthood.

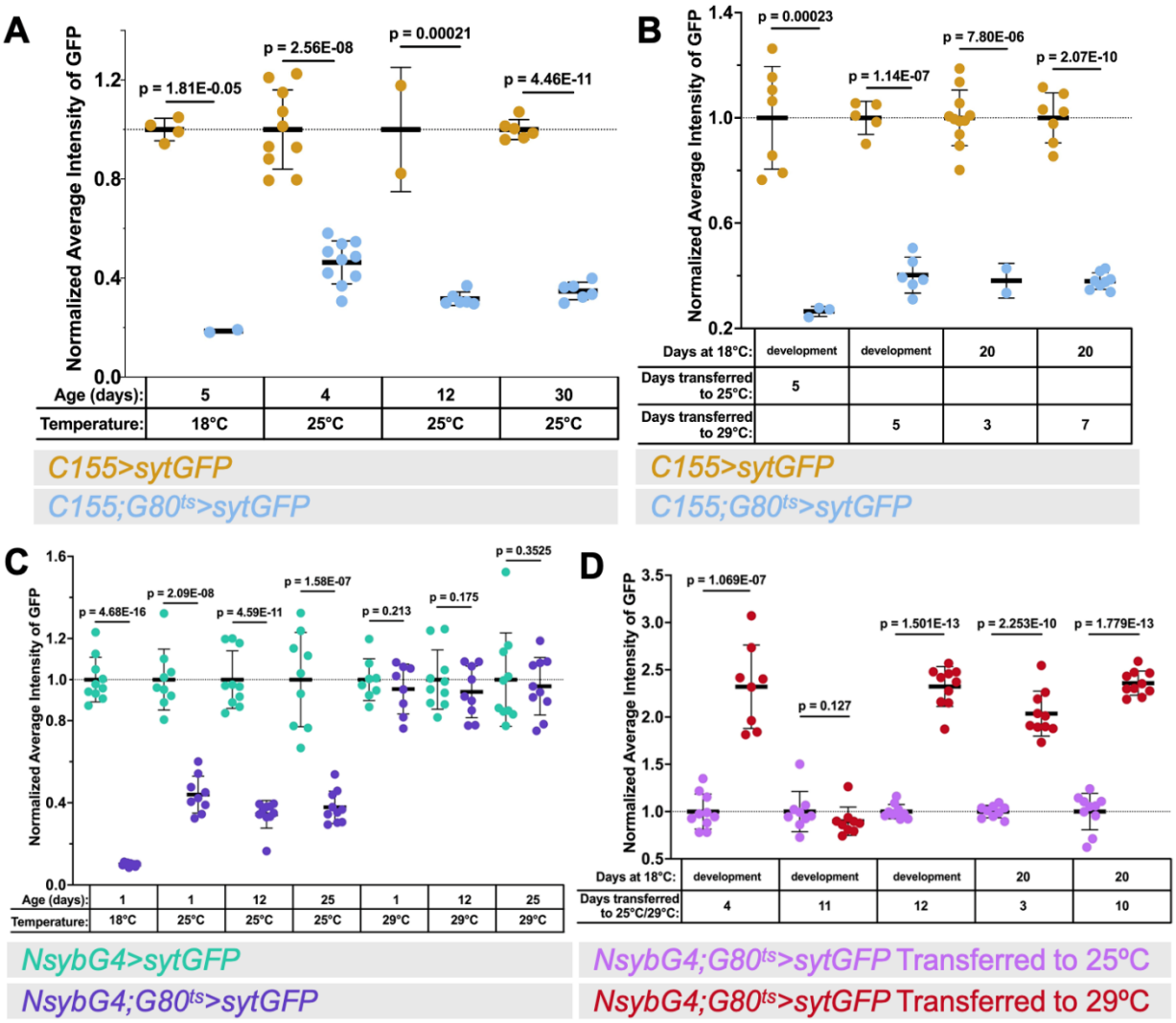


Figure 4. The Gal4/Gal80^{ts} system using the Nsyb-Gal4 driver is more effective at temporally controlling gene expression than C155-Gal4.

Quantification of GFP intensity is shown for (A-D). Mean and SD for each age is shown, with each data point representing an individual brain; the average intensity for each brain was normalized to the average intensity of the control group (*C155>sytGFP*, *NsybG4>sytGFP*, or *NsybG4;G80^{ts}>sytGFP* at 25°C). P-values are shown above each comparable set of dissections, where a one-tailed t-test was used to determine significance if $p<0.05$. (A) Adults expressing the C155-Gal4 driver alone (*C155>sytGFP*, gold) or with the Gal80^{ts} repressor (*C155;G80^{ts}>sytGFP*, blue) were raised at the same temperature the entire time: 5 days at 18°C (*C155>sytGFP* n=4; *C155>G80^{ts}* n=2), 4 days at 25°C (*C155>sytGFP* n=10; *C155;G80^{ts}>sytGFP* n=10), 12 days at 25°C (*C155>sytGFP* n=2; *C155;G80^{ts}>sytGFP* n=6), and 30 days at 25°C (*C155>sytGFP* n=6; *C155;G80^{ts}>sytGFP* n=6). (B) *C155>sytGFP* and *C155;G80^{ts}>sytGFP* adults were initially raised at 18°C, but then transferred to either 25°C or 29°C at varying ages throughout adulthood. Adults were transferred to 25°C or 29°C starting from day 1 and dissected after 5 days (25°C: *C155>sytGFP* n=7; *C155;G80^{ts}>sytGFP* n=3) (29°C: *C155>sytGFP* n=5; *C155;G80^{ts}>sytGFP* n=6), or transferred to 29°C starting from day 20 and dissected after either 3 (*C155>sytGFP* n=11; *C155;G80^{ts}>sytGFP* n=2) or 7 days (*C155>sytGFP* n=7; *C155;G80^{ts}>sytGFP* n=8). (C) Adults expressing the Nsyb-Gal4 driver alone (*NsybG4>sytGFP*, purple) or with the Gal80^{ts} repressor (*NsybG4;G80^{ts}>sytGFP*, green) were raised at the same temperature the entire time: 1 day at 18°C (*NsybG4>sytGFP* n=10; *NsybG4;G80^{ts}>sytGFP* n=10), 25°C (*NsybG4>sytGFP* n=9; *NsybG4;G80^{ts}>sytGFP* n=9), or 29°C (*NsybG4>sytGFP* n=8; *NsybG4;G80^{ts}>sytGFP* n=8), 12 days at 25°C (*NsybG4>sytGFP* n=10; *NsybG4;G80^{ts}>sytGFP* n=10) or 29°C (*NsybG4>sytGFP* n=10; *NsybG4;G80^{ts}>sytGFP* n=9), and 25 days at 25°C (*NsybG4>sytGFP* n=9; *NsybG4;G80^{ts}>sytGFP* n=10) or 29°C (*NsybG4>sytGFP* n=10; *NsybG4;G80^{ts}>sytGFP* n=10). (D) *NsybG4;G80^{ts}>sytGFP* flies were initially raised at 18°C and then transferred to 25°C (yellow) or 29°C (magenta) starting from day 1 of adulthood for 4 days (25°C n=10; 29°C n=8), 11 days (25°C n=9; 29°C n=9), or 12 days (25°C n=10; 29°C n= 10), or kept at 18°C for 20 days into adulthood and then transferred to 25°C or 29°C for 3 days (25°C n=9; 29°C n=10) or 10 days (25°C n=10; 29°C n=10).

Decreasing Syd-1 levels primarily during adulthood prematurely increases Brp levels

I employed the Gal4/UAS/Gal80^{ts} system by raising progeny expressing the Nsyb-Gal4 driver, Gal80^{ts} repressor, and *syd-1RNAi on 3* (*NsybG4;G80^{ts}>syd-1RNAi on 3*) at 18°C during development and then transferring them to 29°C starting from day 1 of adulthood. Compared to wild-type adults (*NsybG4;G80^{ts}>Bloom*), 10-day-old *NsybG4;G80^{ts}>syd-1RNAi on 3* adults

demonstrated a 20.70% increase in Brp intensity (Figure 5A). While consistent with my model, I wanted to confirm that these results were exclusively due to the decrease in Syd-1, and not caused by potential off-target effects of the *syd-1RNAi on 3* transgene.

To test this, I performed the same experiment using the *syd-1RNAi on 2* transgene, anticipating that expressing it in neurons would no longer cause lethality if restricted to adulthood. Indeed, I found that using *syd-1RNAi on 2* starting from day 1 of adulthood does not cause lethality and increases Brp intensity by 30.71% in 10-day-old adults (*NsybG4;G80^{ts}>syd-1RNAi on 2*) compared to wild-type (*NsybG4;G80^{ts}>7702RNAi*) (Figure 5B). I conclude that decreasing Syd-1 during adulthood is sufficient to increase Brp levels, consistent with my model in which Syd-1 is required to limit Brp in adults.

I next wondered whether continuing to decrease Syd-1 levels throughout adulthood would cause a more extreme accumulation of Brp in even older flies. Alternatively, since older wild-type flies normally have lower Syd-1 levels, further decreasing Syd-1 may not have an added effect. I chose two later timepoints, 14 days and 30 days, to distinguish whether decreasing Syd-1 continued to increase Brp levels at older ages. I found that 14-day-old *NsybG4;G80^{ts}>syd-1RNAi on 2* adults did not show significant differences in Brp intensity from wild-type, and 30-day-old *NsybG4;G80^{ts}>syd-1RNAi on 2* adults showed a 14.14% increase in intensity from 30d wild-type adults (Figure 5C). I conclude that decreasing Syd-1 levels specifically during adulthood appears to lead to the greatest increase in Brp accumulation at a young age (10 days), but this effect diminishes as the flies age, becoming less pronounced at 14 and 30 days. This is consistent with my previous findings that the age-dependent decrease in Syd-1 is most pronounced in early adulthood before stabilizing with age and supports my model

that reducing Syd-1 levels prematurely during adulthood hinders its ability to anchor Brp at AZs, consequently allowing for the premature increased accumulation of Brp.

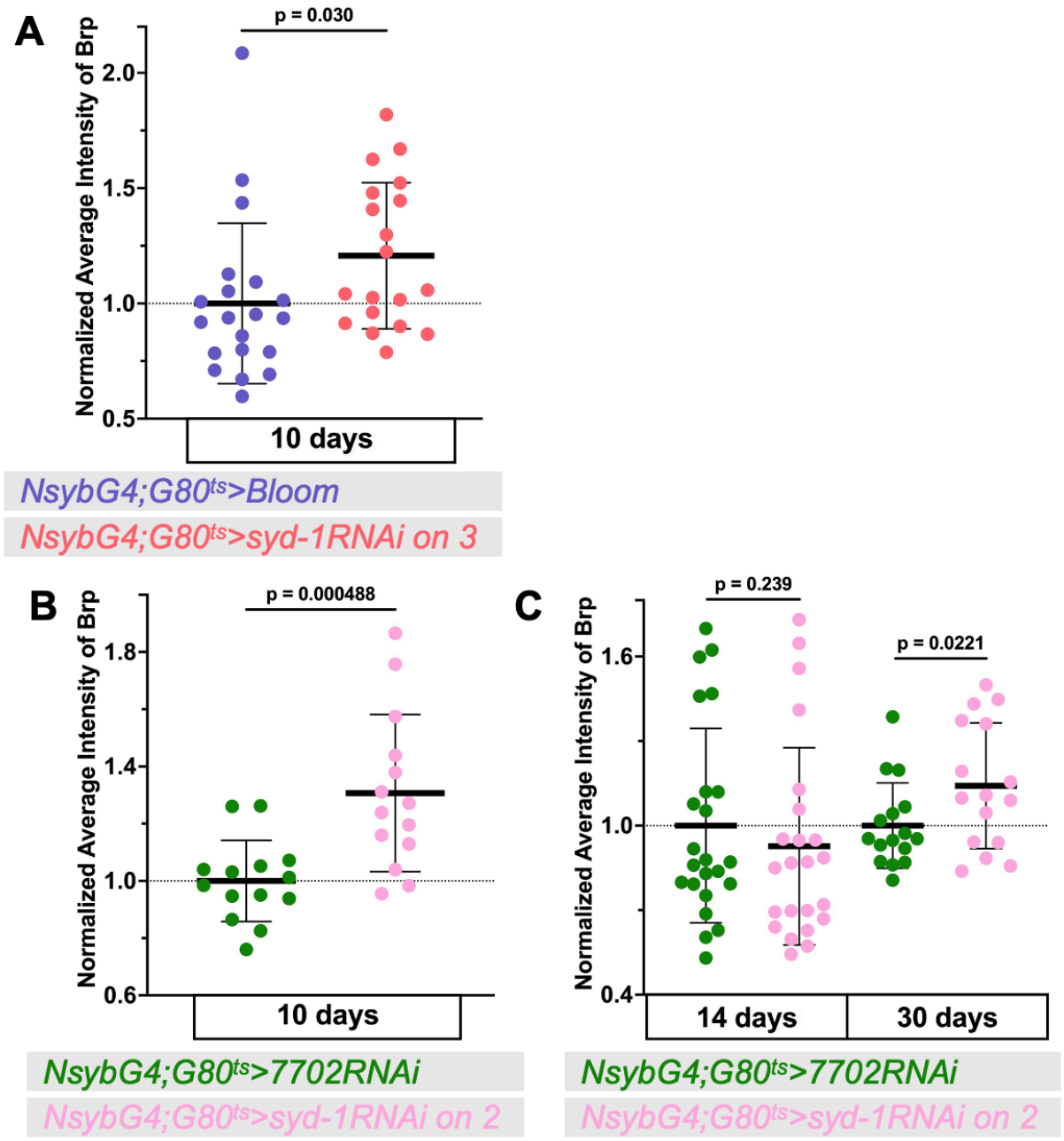


Figure 5. Restricting *syd-1RNAi* expression to adulthood prematurely increases Brp levels in younger adults.

Quantification of the normalized average intensity of Brp for each age along with its mean and SD is shown. Each data point represents an individual brain; the average intensity for each brain was normalized to the average intensity of the control group (*NsybG4;G80^{ts}>Bloom* or *NsybG4;G80^{ts}>7702RNAi*). Different colors represent different genotypes, where every control group expressed normal levels of Syd-1. P-values are shown above each comparable set of dissections, where a one-tailed t-test was used to determine significance if $p<0.05$. Using the NsybGal4 driver and Gal80^{ts} repressor, Syd-1 levels were decreased specifically during adulthood by raising flies at 18°C and then transferring them to 29°C starting day 1 of adulthood. (A) Comparison between 10-day-old dissected *NsybG4;G80^{ts}>Bloom* (purple, n=20) and *NsybG4;G80^{ts}>syd-1RNAi on 3* (coral, n=20). (B-C) Comparison between adults expressing normal levels of Syd-1 (*NsybG4;G80^{ts}>7702RNAi*, green) and adults expressing less than half the normal levels of Syd-1 using *syd-1RNAi* on chromosome 2 (*NsybG4;G80^{ts}>syd-1RNAi on 2*, light pink) at 10 days (*NsybG4;G80^{ts}>7702RNAi* n=14, *NsybG4;G80^{ts}>syd-1RNAi on 2* n=14), 14 days (*NsybG4;G80^{ts}>7702RNAi* n=23, *NsybG4;G80^{ts}>syd-1RNAi on 2* n=23), and 30 days (*NsybG4;G80^{ts}>7702RNAi* n=16, *NsybG4;G80^{ts}>syd-1RNAi on 2* n=16).

Doubling the dosage of *syd-1* is not sufficient to reduce Brp accumulation in aging adults

If the decrease in Syd-1 is solely responsible for age-dependent Brp accumulation, then overexpressing Syd-1 should be sufficient to prevent the increase in Brp. I first tested whether doubling the number of copies of endogenous *syd-1* by using a duplication of the *syd-1* region (*sydDup*), would be sufficient to prevent the age-dependent increase in Brp in adult flies. Since doubling the number of copies of the endogenous *brp* gene impairs memory in young 3-day-old flies (Gupta et al., 2016), doubling the dosage of *syd-1* may cause Brp levels to decrease, resulting in strengthened memory. I found that neither 10-day-old nor 30-day-old *sydDup* adults have less Brp than wild-type flies (Figure 6A), indicating either that Syd-1 alone may not be sufficient to lower Brp levels—as in other genes are also required to prevent age-dependent Brp accumulation—or that doubling the *syd-1* copy number is not enough to prevent a drop in Syd-1 protein levels in adulthood.

Overexpressing Syd-1 either during development and adulthood or specifically during adulthood is not sufficient to reduce Brp accumulation

I next used pan-neuronal Gal4 to significantly overexpress Syd-1 using *UAS-syd-1^{WT}*. I raised flies at 18°C and transferred them to 25°C starting from day 1 of adulthood, but since I was not using Gal80^{ts}, Syd-1 was overexpressed in all neurons throughout both development and adulthood. I found that this overexpression was not sufficient to reduce Brp levels in 10-day-old, 28-day-old, or 30-day-old adults (Figure 6B-C), again suggesting that other genes may be contributing to the accumulation of Brp. However, I wanted to rule out one final possibility: that by overexpressing Syd-1 during development, I was increasing the recruitment of Brp during synapse assembly and masking a potential later ability of Syd-1 overexpression to prevent Brp accumulation. To avoid any possible developmental effects on adult Brp levels, I again employed the Gal4/UAS/Gal80^{ts} system—this time to significantly overexpress Syd-1 specifically during adulthood. I created *NsybG4;G80^{ts}>UAS-syd-1^{WT}* progeny, raised them at 18°C, and then transferred flies to 29°C at various timepoints during adulthood to initiate overexpression of Syd-1. I found that overexpressing Syd-1 primarily during adulthood was not sufficient to reduce Brp in 10-day-old or 30-day-old adults (Figure 6D). Similarly, *NsybG4;G80^{ts}> UAS-syd-1^{WT}* adults shifted from 18 to 29°C for 10-11 days starting from day 10, 15 or 20 of adulthood did not reduce Brp (Figure 6E). Because significantly increasing Syd-1 levels is not sufficient to reduce Brp levels, these findings suggest that *syd-1* is not the only gene responsible for the age-dependent increase in Brp and that other components are involved in this process. As these results, along with all other previous results, were found using brain-wide fluorescence intensity methods, it may also be possible that overexpressing Syd-1 has effects on individual Brp puncta that this staining method cannot detect.

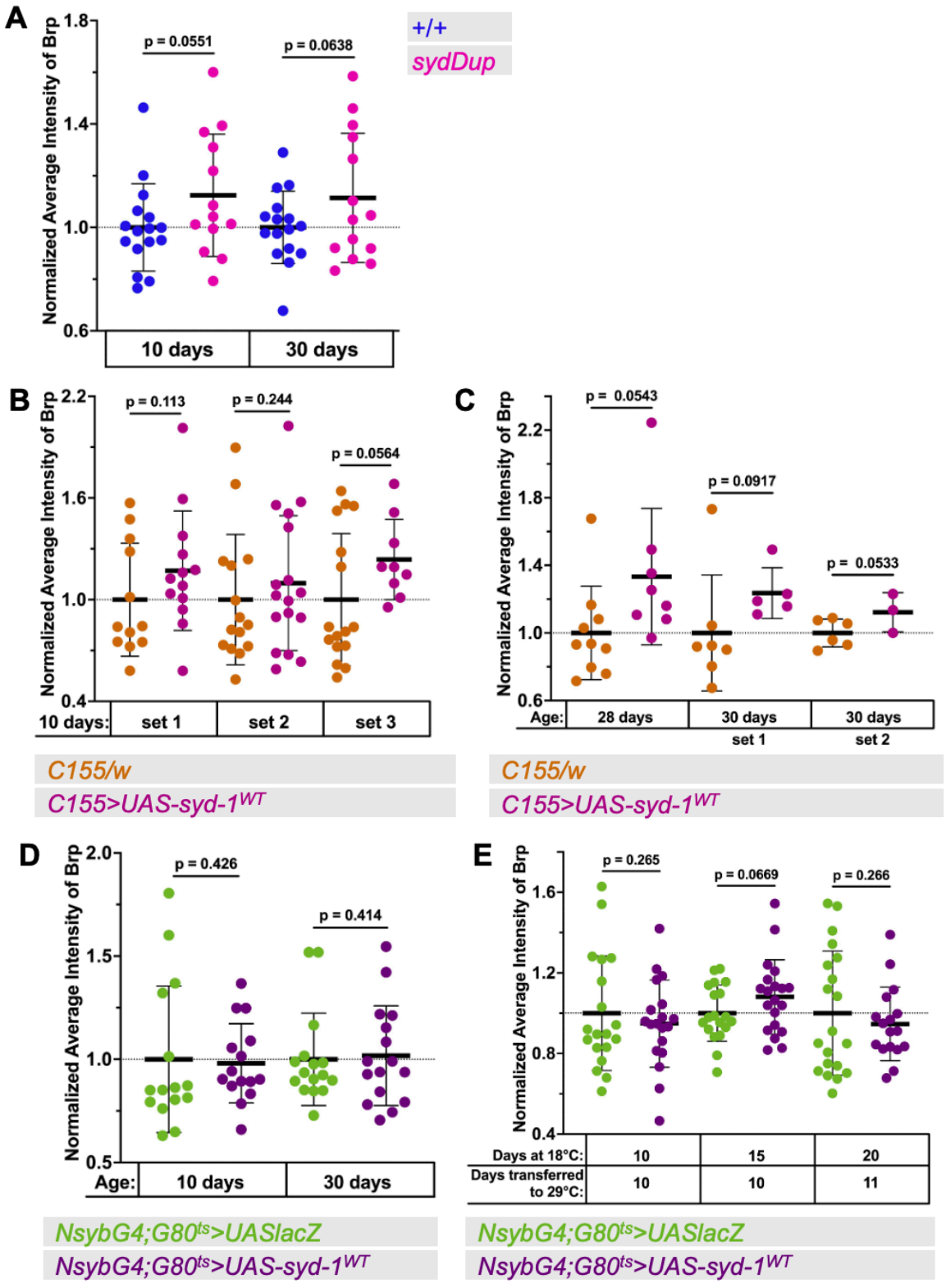


Figure 6. Increasing Syd-1 levels during development and/or adulthood had no effect on the age-dependent increase in Brp.

Quantification of the normalized average intensity of Brp for each age along with its mean and SD is shown. Each data point represents an individual brain; the average intensity for each brain was normalized to the average intensity of the control group (+/+, *C155/w*, and *NsybG4;G80ts>UASlacZ*). Different colors represent different genotypes, where every control group expressed normal levels of Syd-1. P-values are shown above each comparable set of dissections, where a one-tailed t-test was used to determine significance if $p<0.05$. (A) Adults expressing normal levels of Syd-1 (+/+, dark blue) or double the levels of Syd-1 (*sydDup*, hot pink) were maintained at 25°C and dissected at 10 days (+/+ n=16, *sydDup* n=14) or 30 days (+/+ n=16, *sydDup* n=14). (B-C) Flies expressing normal levels of Syd-1 (*C155/w*, orange) or overexpressing Syd-1 (*C155>UAS-syd-1^{WT}*, magenta) during development and adulthood were raised at 18°C and then transferred to 25°C starting day 1 of adulthood. Adults were then dissected at 10 days (set 1: *C155/w* n=12, *C155>UAS-syd-1^{WT}* n=13), (set 2: *C155/w* n=15, *C155>UAS-syd-1^{WT}* n=17), (set 3: *C155/w* n=16, *C155>UAS-syd-1^{WT}* n=9), 28 days (*C155/w* n=10, *C155>UAS-syd-1^{WT}* n=8), and 30 days (set 1: *C155/w* n=7, *C155>UAS-syd-1^{WT}* n=5) (set 2: *C155/w* n=6, *C155>UAS-syd-1^{WT}* n=3). (D) Flies expressing normal levels of Syd-1(*NsybG4;G80ts>UASlacZ*, lime green) or overexpressing Syd-1 (*NsybG4;G80ts> UAS-syd-1^{WT}*, dark purple) primarily during adulthood were raised at 18°C and then transferred to 29°C starting day 1 of adulthood. Adults were then dissected after 10 days (*NsybG4;G80ts>UASlacZ* n=15, *NsybG4;G80ts> UAS-syd-1^{WT}* n=15) or 30 days (*NsybG4;G80ts>UASlacZ* n=16, *NsybG4;G80ts> UAS-syd-1^{WT}* n=16). (D-E) *NsybG4;G80ts>UASlacZ* and *NsybG4;G80ts> UAS-syd-1^{WT}* were raised and maintained at 18°C and then transferred to 29°C at either 10, 15, or 20 days into adulthood. 10-day-old (*NsybG4;G80ts>UASlacZ* n=20, *NsybG4;G80ts> UAS-syd-1^{WT}* n=20) or 15-day (*NsybG4;G80ts>UASlacZ* n=20, *NsybG4;G80ts> UAS-syd-1^{WT}* n=20) adults at 18°C were then dissected after spending 10 days at 29°C. 20-day-old adults (*NsybG4;G80ts>UASlacZ* n=20, *NsybG4;G80ts> UAS-syd-1^{WT}* n=17) at 18°C were then dissected after spending 11 days at 29°C.

Discussion

While the underlying mechanisms behind age-dependent Brp accumulation are not yet fully understood, the components involved in synaptic assembly are well studied and provide a starting point for investigating their potential roles in Brp accumulation during adulthood. I hypothesized that changes during adulthood to Syd-1, a presynaptic component required to recruit Brp to AZs during development, cause the age-dependent accumulation in Brp indicative of age-dependent memory decline. I found that Syd-1 levels decline with age, suggesting a model in which the decrease in Syd-1 is responsible for the age-dependent increase in Brp. My finding that disrupting *syd-1* expression in young adults causes a premature increase in Brp levels supports this model. Although this provides evidence that Syd-1 is necessary to prevent Brp accumulation during adulthood, I found that overexpressing Syd-1 is not sufficient to reduce Brp in either young or old adults. These results suggest a revised model in which additional components work together with Syd-1 to regulate Brp levels during adulthood; age-dependent changes to these components, in addition to the decrease in Syd-1, then cause the age-dependent accumulation of Brp.

The age-dependent decrease in Syd-1 was observed at a brain-wide level

I quantified the overall levels of Syd-GFP and Brp throughout the central regions of the brain by averaging the overall intensity of each per total brain area. However, previous work (in young adults) has shown that different brain regions exhibit varying ratios of Brp to Syd-1 intensity (Fulterer et al., 2018). Investigating whether the age-dependent decrease I observed in Syd-1-GFP levels is uniform across the brain or varies by brain region, as well as testing whether manipulating Syd-1 levels by expressing *syd-1RNAi* or overexpressing Syd-1^{WT} has greater

effects on Brp expression in brain regions with higher Syd-1 to Brp ratios than in regions with lower ratios, would enhance our understanding of Syd-1's role in Brp accumulation.

My finding that Brp accumulates in part because of an age-dependent decrease in Syd-1 raises another question: what causes the age-dependent decrease in Syd-1? Bhukel et al. (2019) provide evidence that Brp accumulation is regulated by a signal from neurons in the MB region of the brain. In particular, they found that prematurely decreasing autophagy, the intracellular process of degrading old or damaged substances, within the MB led to a premature increase in Brp in all neurons (Bhukel et al., 2019). Because age-dependent memory decline is associated with a reduction of autophagy, these findings suggest a model in which an age-associated decline in MB neuron autophagy causes MBs to activate a brain-wide signal that increases Brp accumulation in all neurons, causing weakened memory (Bhukel et al., 2019). Based on my results, one possibility that this MB signal increases Brp by decreasing Syd-1 levels. To test this, future studies could disrupt autophagy within MB neurons and assess whether that decreases levels of Syd-1 on both a regional level and brain-wide level. This work would lead to a more conclusive mechanism for age-dependent Brp accumulation by investigating the upstream processes that may control Syd-1 levels and whether Syd-1 is regulated cell autonomously or non-cell autonomously.

Syd-1 is necessary but not sufficient to maintain Brp levels throughout adulthood

Although I found that prematurely reducing the *syd-1* gene dosage using RNAi prematurely increased Brp levels in young flies, simply halving *syd-1* was not sufficient to produce the same phenotype. Since I observed that Syd-1 levels normally decline by only 20-30%, I would have expected that prematurely decreasing Syd-1 by half should have caused an increase in Brp. However, I did not directly test how effective reducing the *syd-1* gene dosage

was at reducing Syd-1 protein levels (we do not have an anti-Syd-1 antibody, and the endogenous Syd-1:GFP protein trap is homozygous lethal, preventing me from using it to test the effects of gene dosage). One interpretation is that reducing the *syd-1* dosage by half may have little or no effect on Syd-1 protein levels, which would explain why halving *syd-1* did not affect Brp levels in younger flies.

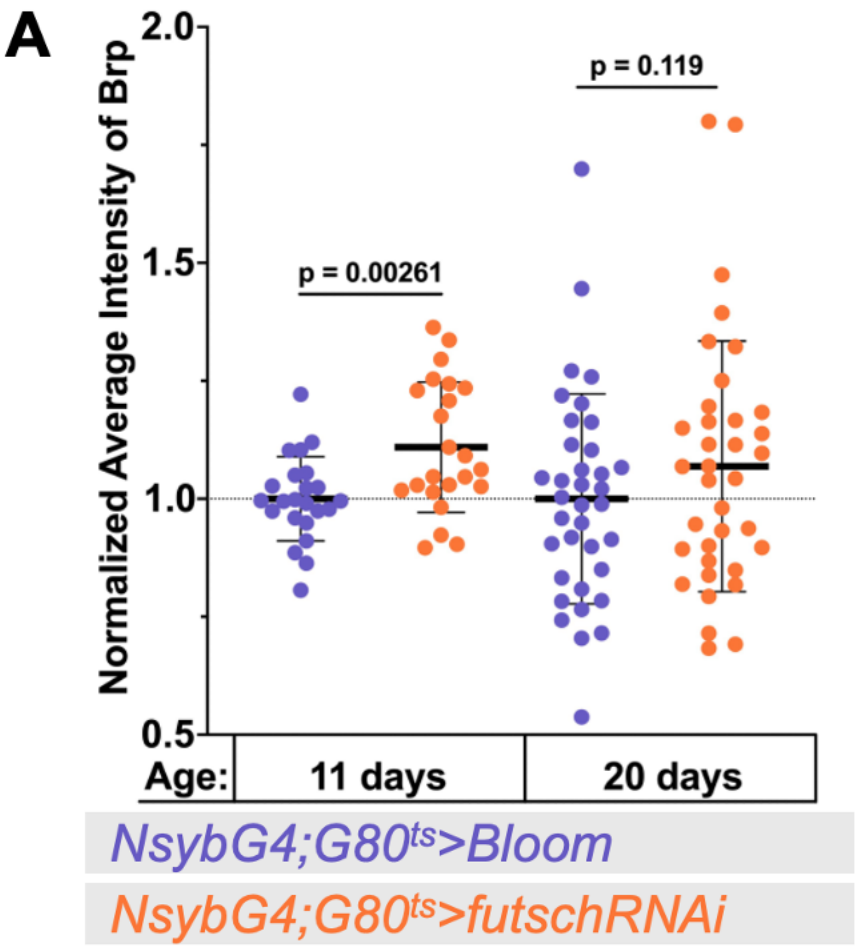
I was similarly unable to quantify the effects of *syd-1RNAi* transgene expression on Syd-1 levels. However, my finding that two quite different *syd-1RNAi* transgenes caused similar increases in Brp in 10-day-old flies, suggests that this effect was caused by the standard, expected mechanism of reducing Syd-1 expression. I conclude that Syd-1 is necessary to maintain Brp levels as flies age. In comparison, 14-day-old flies expressing *NsybG4;G80ts>sydRNAi on 2* exhibited no change in Brp while 30-day-old flies exhibited an increase in Brp at approximately half the magnitude of 10-day-old flies. One possibility is that the sample size for assessing Brp intensity at 14 days was not sufficient to detect an increase in Brp. But, in any case, it seems that prematurely decreasing *syd-1* leads to the greatest rise in Brp in younger adults, and has less of an effect as flies age and more Brp naturally accumulates.

While Syd-1 is necessary to prevent a Brp increase, I found that overexpressing Syd-1 is not sufficient to reduce Brp levels in either younger or older adults. Because I did not monitor Syd-1 levels, it is theoretically possible that my overexpression system did not actually increase Syd-1 expression. In the future, it would be useful to distinguish how Syd-1 levels change in response to Syd-1^{WT} overexpression (I used a transgene that tags Syd-1^{WT} with 3xFLAG tag that could be monitored with an anti-FLAG antibody), in order to allow for the more precise genetic manipulation of *syd-1* and interpretation of results. However, because this 3xFLAG-tagged Syd-1 has previously been shown to be expressed and localized correctly, I interpret my results to

mean that Syd-1 overexpression is not sufficient to reduce Brp. This suggests that other genes are likely involved in the process of age-dependent Brp accumulation. I have already identified a second gene, *futsch/MAP1B* (see below), that prematurely increases Brp levels when disrupted in young adults. I therefore speculate that manipulating the expression of other genes, in addition to overexpressing *syd-1*, will be necessary to prevent Brp levels from increasing with age.

The components that interact with Syd-1 during development provide a starting point to identify other gene candidates. Spinophilin (Spn) is a known antagonist of Syd-1 and is thought to promote Brp recruitment by competing with Syd-1 to bind Nrx-1 in order to tune the appropriate amount of Brp for AZ maturation (Ramesh et al., 2023). Reducing *syd-1* during development increases and prolongs the normal increase in Brp that occurs during memory formation, whereas reducing *Spn* has the opposite effect (Ramesh et al., 2023). Their antagonism continues into adulthood: the impaired middle-term memory experienced by *Spn* mutants is rescued by eliminating one copy of *syd-1* (Ramesh et al., 2023). This suggests the need to test a revised model, where an age-dependent increase in Spn, in addition to the age-dependent decrease in Syd-1, allows Spn to bind more frequently to Nrx-1 than Syd-1, resulting in increased Brp recruitment to AZs as flies age.

As mentioned above, I have also found that expressing *futschRNAi* to reduce *futsch* expression specifically during adulthood increases Brp levels by 10.94% in 11-day-old flies (Supplemental Figure 1); similar to my finding for *syd-1RNAi*, *futschRNAi* has no effect on Brp levels in older flies. Futsch is a presynaptic protein known to link microtubules to AZs, and loss of *futsch* throughout life leads to problems with memory and premature neurodegeneration (Dent, 2020; Lepicard et al., 2014). This strengthens the possibility that other presynaptic proteins, necessary for Brp recruitment during development, might also collaborate with Syd-1 to sustain Brp levels into adulthood. Future work should decipher the interactions between these proteins during adulthood and whether manipulating the levels of multiple interacting presynaptic proteins is sufficient to prevent the age-dependent accumulation of Brp.



Supplemental Figure 1. Using *futschRNAi* to reduce *futsch* expression starting from adulthood increases Brp in younger adults only.

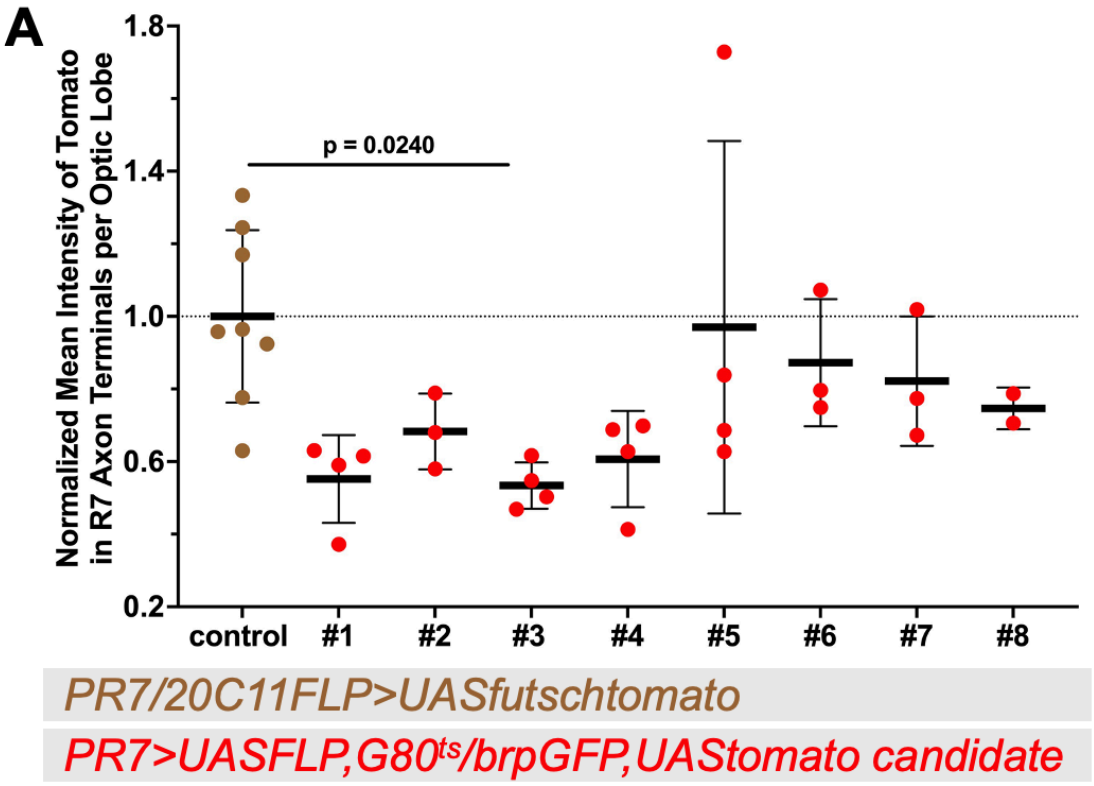
Quantification of the normalized average intensity of Brp for each age along with its mean and SD is shown. Each data point represents an individual brain; the average intensity for each brain was normalized to the average intensity of the control group (*NsybG4;G80^{ts}>Bloom*). Different colors represent different genotypes, where *NsybG4;G80^{ts}>Bloom* is the appropriate control expressing normal levels of Syd-1. P-values are shown above each comparable set of dissections, where a two-tailed t-test was used to determine significance at 11 days and a one-tailed t-test was used to determine significance at 20 days if $p<0.05$. Using the NsybGal4 driver and Gal80^{ts} repressor, Futsch levels were decreased specifically during adulthood by raising flies at 18°C and then transferring them to 29°C starting day 1 of adulthood. (A) Comparison between adults expressing normal levels of Futsch (*NsybG4;G80^{ts}>Bloom*, purple) and adults expressing less than half the normal levels of Futsch using futschRNAi (*NsybG4;G80^{ts}>futschRNAi*, orange) at 11 days (*NsybG4;G80^{ts}>Bloom* n=23 , *NsybG4;G80^{ts}>futschRNAi* n=23) and 20 days (*NsybG4;G80^{ts}>Bloom* n=36, *NsybG4;G80^{ts}>futschRNAi* n=36).

A brain-wide decrease in *syd-1* expression leads to the brain-wide increase in Brp levels

Although prematurely reducing the dosage of *syd-1* prematurely increases Brp levels when visualizing the entire central brain, I did not test whether the increase in overall Brp intensity was due to increased Brp puncta size, increased number of Brp puncta, and/or the localization of Brp at AZs as opposed to other regions along the axon. As a loss of *syd-1* during development leads to a decreased number but increased size of individual Brp puncta (Owald et al., 2010), I speculate that something similar may occur in aging adults as *syd-1* expression is decreased. To investigate how manipulating *syd-1* expression during adulthood may induce changes to Brp at an individual AZ level, I am in the process of refining the "STaR" method in which endogenous Brp can be tagged with GFP in defined subsets of neurons using site-specific recombination catalyzed by FLP recombinase (Chen et al., 2014). Because Chen et al. (2014) developed these methods by focusing on labeling R7 photoreceptor neurons located in the optic lobe, I have been further refining these tools by (1) adding the Gal4/Gal80^{ts} temporally controlled gene expression system to restrict manipulating *syd-1* expression to adulthood; and (2) replacing the R7-specific 20C11-FLP transgene with a UAS-FLP transgene that theoretically tags Brp with GFP in any neurons that express Gal4 . I have developed a recombinant strain of flies that contain both *UAS-FLP* (required to label Brp) and *Gal80^{ts}* (required to repress target gene expression at lower temperatures) so that I will be able to manipulate Syd-1 and label Brp in defined sets of central brain neurons specifically during adulthood (Supplemental Figure 2). Additionally, I have developed a recombinant strain of flies that contain both the *20C11-FLP* and *Gal80^{ts}* transgenes so that I can also manipulate Syd-1 and label Brp in R7 photoreceptor neurons specifically during adulthood.

Because I—like previous investigators—observed the premature increase in Brp in the central brain and did not assess Brp in optic lobes (Gupta et al., 2016; Ramesh et al., 2023; Turrel et al., 2022), it seems best to use STaR to assess individual Brp puncta in central brain neurons rather than in R7 photoreceptors. I therefore screened candidate Gal4 drivers (from the Bloomington Drosophila Stock Center but suggested to me by Chris Doe's lab) and found two that are expressed in central brain regions of young adults; the next step is to test whether these drivers stay consistently on throughout adulthood. In the future, it is possible that pairing a central brain Gal4 driver to the recombinant strain including both the Gal4/Gal80^{ts} system and components required to label individual AZs and neurons may reveal the specific molecular changes that account for the increase in Brp intensity as a result of reducing *syd-1* expression during adulthood.

We are working toward a molecular understanding of what changes in adulthood lead to changes in synaptic structure and function in old age. By demonstrating that Syd-1 plays a role in facilitating the age-dependent accumulation of Brp, this has directed us toward specific research avenues in the future that may help identify the molecular mechanisms responsible for age-dependent memory decline. Given that aging inherently brings a higher risk of chronic diseases and health concerns, acquiring the means to delay memory decline could alleviate one of the many challenges associated with growing older (National Institute on Aging).



Supplemental Figure 2. Successful STaR recombinant lines exhibit decreased tomato intensity in R7 axon terminals at 18°C because they express the Gal80^{ts} repressor.

Quantification of the normalized average tomato intensity of R7 axon terminals per optic lobe in each brain along with its mean and SD is shown. Each data point represents an individual brain; the average tomato intensity for each brain was normalized to the average tomato intensity of the control group (*PR7/20C11FLP>UASfutschtomato,* gold). Different colors represent different genotypes, where *PR7/20C11FLP>UASfutschtomato* is the appropriate control that portrays normal tomato levels in R7 axon terminals when maintained for 35 days at 25°C. The p-value is shown above between the control group and recombinant candidate #3, where a one-tailed t-test was used to determine significance if $p<0.05$. (A) Comparisons between 35-day-old control group raised at 25°C expressing normal levels of tomato intensity in R7 axon terminals (*PR7/20C11FLP>UASfutschtomato* n=8) and each 1 to 3-day-old recombinant candidate (*PR7>UASFLP,G80^{ts}/brpGFP,UAStomato, red*) raised at 18°C (#1 n=4, #2 n=3, #3 n=4, #4 n=4, #5 n=4, #6 n=3, #7 n=3, #8 n=2). Recombinant candidate #3 was chosen to maintain a stock that included both *UASFLP* and the Gal80^{ts} repressor as it demonstrated that at 18°C, the Gal80^{ts} repressor was working to inhibit activation of *UAStomato*, which would normally label axon terminals.

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