Abstract

Glial cells, the resident immune cells of the brain, are important for brain health as they represent a critical function both in brain injury as well as in brain development and maintenance. Following brain injury, glia quickly respond by cleaning up neuronal and synaptic debris. In addition to their role as the cleaning cell of the brain, recent research has shown that during development, glia also shape and fine-tune neural connections by eliminating weak synapses fated for removal. However, whether glial cells also continue to remodel synaptic architecture in the adult brain is unclear. Moreover, synaptic loss is an early hallmark of Alzheimer's disease (AD), but whether glial cells play a role in this pathophysiology remains mysterious. In this study, we investigate the role of glial cells in AD-related synaptic loss using a *Drosophila melanogaster* model of AD. First, we will establish when synaptic loss occurs in AD model flies. Subsequently, we will explore the role of the glial engulfment receptor Draper, which is essential for glial clearance of neuronal debris after injury, and evaluate its effect on synaptic loss in the AD fly model. For these studies, we will use immunohistochemistry and western blotting techniques, as well as the power of *Drosophila* genetics. In this study, we aim to identify glial signals that may contribute to early synapse loss in this model and identify molecular targets, which can potentially be manipulated to prevent or delay the progression of AD.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, which is implicated by hallmark aggregations of extracellular amyloid beta protein. Mutations in the amyloid precursor protein (APP), causing a truncated version of the protein have been identified as a risk factor for Alzheimer's disease. However, attention is also shifting to the immune cells of the brain called microglia. As the resident immune cells of the brain, they are actively surveying the brain parenchyma for injury (1). Upon injury, they quickly respond, by changing their morphology and protein expression to engulf cellular and synaptic debris through active phagocytosis (2). In addition, microglia are critical for proper brain development as they shape synaptic connections during development. It has been shown that developing mice deficient for one essential microglial engulfment receptor displayed immature and underdeveloped synaptic connectivity due to deficits in synaptic pruning (3,9). In *Drosophila*, the glial engulfment receptor Draper has been shown to be essential in glial engulfment of neuronal debris following injury (4). Upon axon injury, glial cells recruit their membranes at the site of injury and upregulate Draper. When *draper* is knocked down or glial phagocytic activity is inhibited, axon fragments persist and are not cleared after injury (11). While Draper is specific to *Drosophila*, it is highly conserved and its mammalian homolog MEGF10 shows similar function in glial engulfment in mammals (8). This positions Draper as an essential and specific glial receptor for the interaction between glial cells and damaged/dying neurons and allows us to use the powerful *Drosophila* model to investigate conserved pathways. In improving our understanding of Alzheimer's disease, recent data suggests that amyloid beta aggregation is not the very first or critical molecular symptom of Alzheimer's disease (7). In mice, synaptic loss and dysfunction has been observed before any sign of hallmark aggregation (5), however whether glial cells are involved in this process is not clear. In addition, defects in glial cells have been shown to accumulate in the aging brain potentially leading to disease (6, 10). We will use a *Drosophila* model of Alzheimer's Disease, in which human Abeta is expressed in a pan neuronal fashion…… Using this model, we aim to examine 1) whether synapse loss occurs in AD model flies, 2) whether this synapse loss coincides with changes in the expression of the glial engulfment receptor Draper, and 3) whether synapse loss is rescued in a *draper* null background. By investigating the very initial pathophysiology observed in Alzheimer's Disease and its associated signals, we hope to shed light on a potentially powerful mechanisms to slow down or even halt Alzheimer's Disease before it leads to progressive neurodegeneration and cognitive decline.

Methods

Fly Lines
w+/-; elav-Gal4/ UAS-ABarc; +
w+/-; elav-Gal4/ w118; +
w+/-; elav-Gal4/BRP-SNAP; UAS-ABarc
w+/-; elav-Gal4/ BRP-SNAP; +
w+/-; elav-Gal4/ BRP-SNAP; Drpr Δ5 rec 9/UAS-ABarc Drpr Δ5 rec 9
Crosses were set up and maintained at 25o C. Flies were collected every 2 days (M, W, F) and incubated at 30o C.

Adult Fly Brain Fixation
Fix Solution 1- 1x PBS, 0.01% Triton-X, 4% Paraformaldehyde
Wash Solution 1- 1x PBS, 0.01% Triton X
Fix Solution 2- 1x PBS, 0.1% Triton-X, 4% Paraformaldehyde
Wash Solution 2- 1x PBS, 0.1% Triton X
Adult flies from chosen groups were anesthetized and heads removed. Heads were fixed in Fix Solution 1 for 20 minutes at room temperature with rocking. Heads were washed in wash solution 1 for 3 x 2 minutes.
Brains were dissected in wash solution 1 in a standard dissection plate. Newly dissected brains were transferred to new tubes with wash solution 1. Wash solution 1 was pipetted out and replaced with Fix Solution 2. Brains were fixed for 20 minutes at room temperature with rocking. Brains were washed 3 x 2 minutes in wash solution 2.

Antibody Staining
Primary antibody master mixes were prepared at the following concentrations, diluting the antibody in wash solution 2:
Mouse Anti-Draper 1:400; Guinea Pig Anti- Draper 1:400; Mouse Anti-AB 1:2000; Mouse Anti-Bruchpilot 1:50.
Secondary antibody master mixes were prepared at the following concentration, diluting the antibody in wash solution 2:
Mouse 488 1:400; Guinea Pig 546 1:400 (Jackson Immuno).
Brains were incubated in primary antibody with shaking at 4oC overnight. Brains were washed 4 x 30 minutes in wash solution 2. Brains were then incubated in secondary antibody for 2 hours at room temperature with shaking and then washed 4 x 30 minutes in wash solution 2.

BRP-SNAP Staining
SNAP Tag mix was prepared at a concentration of 1 μM in 1x PBS with 0.3% Triton X. Brains were permeabilized for 1 x 10 min in 1x PBS with 0.3% Triton X for 10 min prior to staining. Brains were stained for 15 minutes at room temperature in a glass dissection dish with rocking. Brains were washed 1 x 5 min in 1x PBS & 0.3% Triton X and 1 x 5 min in 1x PBS.

Mounting
Brains were transferred to the glass well of a dissection dish and the wash solution was carefully removed with a pipette. A drop of Vectashield (Vector Labs) was then immediately dispensed onto the brains. Brains were incubated for 30 minutes at 4oC or overnight if desired. Slide preparation required placing a strip of clear tape onto a clean microscope slide. A template was used to trace and cut 4 wells into the tape. Brains were carefully transferred from the dissection dish to their appropriate well on the microscope slide. Excess Vectashield was carefully removed and brains were aligned and oriented with antennal lobes facing up. Small drops of Vectashield were added once all brains were oriented and a coverslip was gently dropped onto the wells. The coverslip was

sealed around the border with a layer of CoverGrip (Biotium).

Confocal Microscopy and Quantification
Images were taken on a Zeiss LSM 700 confocal microscope using a 40X oil immersion lens. For Draper staining, 11 slices through the Z plane were taken per brain at an interval of 1 μm. For Bruchpilot staining, 35 slices were taken through the Z plane at an interval of 0.5 μm. quantification was completed using the Volocity software (Perkin & Elmer). For Draper staining, the 5 slices, which depicted the same anatomical region in each sample, were quantified. Two standard circles were used to measure regions within the cortex above the antennal lobes. A threshold for background fluorescence was set in addition to a restriction of voxels 0.2 um in size or greater. For Bruchpilot staining, the 20 slices, which depicted the same anatomical region, were quantified. The antennal lobes were traced out, cropped and quantified. A threshold for background fluorescence was set in addition to a restriction of voxels 0.2 um in size or greater. Data was analyzed and graphed in GraphPad Prism 6.

Western Blot Protocol
Whole heads were pulled and collected in 1X loading buffer (10 heads per 30 uL of 1x loading buffer). Samples were homogenized using a pestle and centrifuged for 10 minutes at 4oC. Supernatant was collected and boiled for 10 minutes at 95oC and frozen at -80oC.
10x running buffer was diluted to 1x and chilled at 4oc prior to use. 12 uL of sample was loaded into each well of a 10% Tris-HCl gel. 1.5 μL of protein ladder was loaded into edge lanes. Gel was run at a constant 125V for 2 hours.
The wet transfer sandwich was prepared after the fiber pads, filter paper, and nitrocellulose membrane were soaked in transfer buffer. Gel sandwich was placed back in gel and ran at 100V for 90 minutes.
Membrane was soaked in methanol and then blocked with SEA Block (Calbiochem) for 30 min. Membrane was incubated overnight at 4oC with primary antibodies of the following concentrations: Rabbit Anti-Draper 1:2000 and Sheep Anti-Tubulin 1:4000. Membrane was washed 3 x 10 min in 1xPBS-Tween. Secondary antibodies (Rabbit 790 1:2000 & Sheep 680 1:2000) were prepared in 1X PBS and applied to membrane for 2 hours at room temperature. Membrane was washed 2x5 minutes in 1xPBS-Tween and 2x 5 minutes in 1xPBS. Membrane was visualized on Li-Cor Odyssey Imager.

Results
Figure 5: Bruchpilot fluorescence levels are decreased in young AB flies.
Figure 8: Draper is not essential for AD synaptic loss. w+/-; elav-Gal4/BRP-SNAP; UAS-ABarc in left column; w+/-; elav-Gal4/ BRP-SNAP; Draper null/UAS-ABarc Draper null in right column. Synapses were stained using SNAP-tagged version of Bruchpilot

Discussion

Glial cells are a multi-faceted cell type playing essential roles in brain development, injury, and maintenance. Their significant contribution to the brain microenvironment makes them a prime candidate to investigate what effect they may have on early Alzheimer's disease (AD), which is best characterized by synaptic loss that occurs before the onset of symptoms like memory loss. As glial cells are capable of pruning and engulfing synapses during development, this study aimed to investigate how glial cells may be involved in synaptic loss in the context of Alzheimer's disease. More specifically, the highly conserved glial engulfment receptor Draper has previously been shown to be crucial for glial engulfment activity following neuronal injury (4,11) and its mammalian homolog, MEGF10, is known to be involved in synaptic pruning (8). Therefore, we hypothesized that Draper may also be upregulated and

involved in AD related synaptic loss. For this study, we used a *Drosophila* model of Alzheimer's disease, in which human Aß is expressed pan-neuronally in the fly brain. In humans and mouse models of Alzheimer's disease, this protein forms aggregates and leads to destruction of neurons. More specifically, the expressed Aß protein in our fly model contained the arctic mutation, which is known to cause severe protein aggregation in human disease (14). In our model, we observed the formation of Aß-aggregates in the fly brain shortly after eclosion, and they increased in size and number with age (Figure 1). In addition, we observed motor deficits in the Aß-expressing flies and a reduced lifespan compared to age-matched controls (data not shown). The advantages of this model system in *Drosophila* are the ability to selectively express human Amyloid Beta in all neurons by utilizing a binary expression system (UAS/Gal4 system) with the pan-neuronal Elav-Gal4 driver. The limitations of this *Drosophila* AD model are that the current model does not allow for adult specific Aβ expression. Instead, Aβ is expressed throughout development, thereby quickly forming aggregates after eclosion. This is in stark contrast to human Alzheimer's Disease pathology which requires decades for Aβ plaques to form.

By utilizing immunohistochemistry techniques, Draper and the presynaptic protein Bruchpilot were visualized and quantified in both experimental Aß-expressing and age matched non-Aß expressing control groups. Bruchpilot staining revealed that synapse levels were decreased in young (1-4 day old) Aβ flies (Figure 4 & 5), while Draper staining in young (1-3 day old) Aβ flies was increased in the olfactory antennal lobe region of the adult fly brain (Figure 2 & 3). These studies suggest that Draper may play a role in glial cell mediated synaptic loss in AD. To further investigate whether Draper is involved in early synapse loss in AD, an Aß- expressing fly line in a Draper null mutation background was created. This allowed us to explore whether the removal of Draper would result in an increase or rescue of synapses. Staining with a fluorescent molecular probe against an endogenous SNAP-Tag attached to Bruchpilot allowed for more specific and thorough staining in a control group (w+/-; elav-Gal4/BRP-SNAP; UAS-Aßarc) and experimental Draper null group (w+/-; elav-Gal4/ BRP-SNAP; Draper null/UAS-Aßarc Draper null). This study revealed that Draper removal conferred no increase or rescue of synapses (Figure 8). Taken together, this preliminary data suggests that Draper is not essential in inducing synaptic loss in AD. However, this does not eliminate the possibility that glial cells contribute to synaptic loss. Alternative glial engulfment pathways such as the Crk/Mbc/dCed-12 may be implicated in synaptic loss. The Crk/Mbc/dCed-12 pathway acts in a semi non-redundant fashion in parallel to the Draper signaling pathway in the activation of glial cells and phagocytosis of cellular debris (12). While Draper is required for initial glial activation in response to injury, this study suggests that it is not the key player for AD-related synaptic loss. Other mediators in the glial engulfment pathways, such as the Crk/Mbc/dCed-12 pathway, should be investigated in future research. In addition, although Draper was seen to be upregulated in young Aβ flies but does not rescue synaptic loss when knocked down, it may be upregulated for a myriad of other biological reasons. Glial cells are known to engulf Aβ plaques and prune synapses during development (3, 8, 13) and this could be the reasons why we observed a Draper upregulation in our *Drosophila* AD model. Future work should include refining the AD model in Drosophila in order to better mimick the pathophysiology of AD. For example, in our experimental group, Aß was expressed throughout development and plaques form a few days after eclosion. In order to create a more realistic AD model, Aβ could be expressed in adult flies only without perturbing development. This may mimick the human age-related disease more closely. This may be accomplished through the use of a weaker neuronal driver than Elav-Gal4, or through genetically inhibiting Aß expression during the fly's development.

In conclusion, this study provides a preliminary depiction of the role of Draper in glial mediated synaptic loss in the context of AD. Further research would include repeating these experiments and investigating other engulfment pathways, which may be implicated in the earliest symptoms of AD.