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Identifying the Impact of Glial Engulfment and Aβ42-induced Nervous System Dysfunction

Zoë A. Paige

Honors Thesis

University of Vermont

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ABSTRACT

In mammals, microglia play the role of the resident macrophage in the central nervous system (CNS), whose duty is to engulf dead neurons, cellular debris, aging synapses and excess synaptic connections during development (Stevens et al., 2007). In *Drosophila* (fruit flies), cortex glia are a glial subset that play an analogous role to microglia. Draper, an engulfment receptor highly expressed within glia, has been identified as a principal player in engulfment (Freeman et al., 2003). However, the upstream activator of Draper in the *Drosophila* CNS remains ambiguous. This study explores the role of Macroglobulin complement protein (Mcr), a potential upstream activator of Draper, which is necessary for the engulfment of cellular debris and dead neurons (MacDonald et al., 2006, Ray et al., 2017, McLaughlin et al. 2019). Glial morphology and cross-talk with neurons, along with glial engulfment and regulation of Aβ plaques in the context of Alzheimer's Disease (AD), is woefully understudied. This project aimed to further elucidate the extent of this communication within normal and Aβ42-expressing *Drosophila.* Given the recent identification of the role of p38 kinases in multiple sclerosis, another glial-based neurodegenerative disease (Krementsov et al., 2014), the role of p38 kinases

in AD is explored through elimination of their glial expression in an Aβ42 background. Preliminary findings suggest that Mcr regulates engulfment through the Draper receptor in a cortex glia-specific fashion, and p38a contributes to the pathological phenotype observed in AD.

INTRODUCTION

Impacts of Drosophila Complement Proteins on Cell Death and Glial Engulfment: A Review of Previous Work

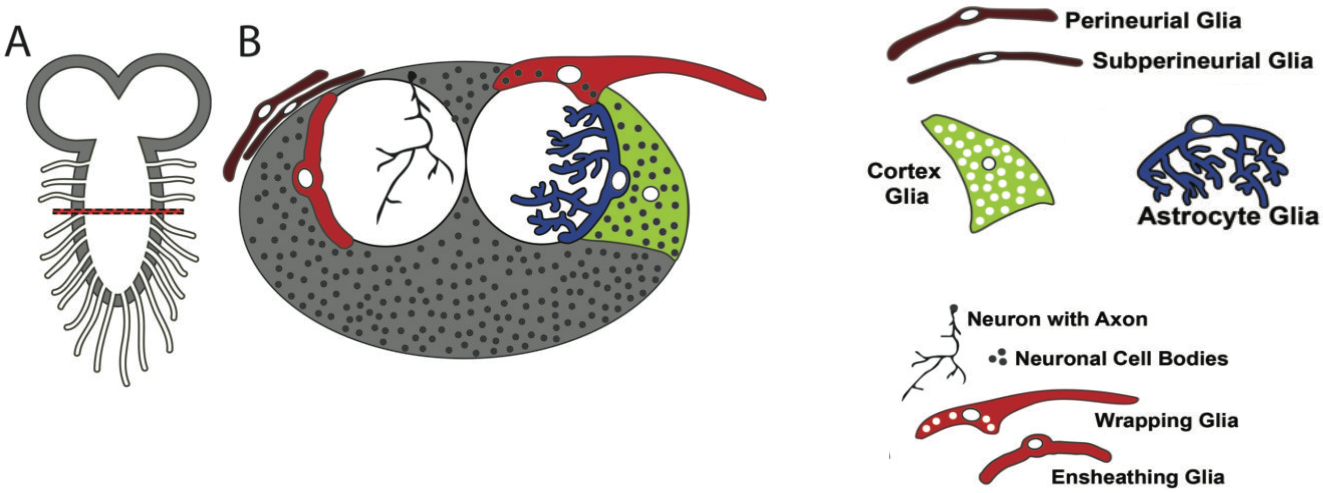
Part of the conversation between neurons and their glial counterparts has recently been elucidated in *Drosophila*. Dying neurons secrete Spätzle 5 (Spz5), which activates a receptor known as Toll-6 that is present on the surface of cortex glia, a *Drosophila* glial cell that surrounds neuronal cell bodies (**Figure 1)**. Toll-6 then activates a molecule known as dSARM, which leads to the activation of transcription factor, FoxO, that then goes on to upregulate an engulfment receptor referred to as Draper. Draper ultimately facilitates the process of engulfment of the original dying neuron by cortex glia (Mclaughlin et al., 2019, incorporated into model in Figure 8). Draper has been deemed necessary for the removal of dead cells and axonal debris during development, as well as for removal of injured axons which have begun to degenerate (MacDonald et al., 2006; Doherty et al., 2009). In Draper mutants, recruitment of glia to sites of neuronal injury and vital morphological changes, such as expansion of the glial membrane, cannot occur (MacDonald et al., 2006). When Draper is knocked down and phagocytic glia are inhibited, cellular debris may not be cleared (Doherty et al., 2009;Tasdemir-Yilmaz et al., 2014), which ultimately leads to neurodegeneration (McLaughlin et al., 2019).

Complement signaling has been implicated in the tagging of neurons or parts of neurons for glial removal in mammals (Iram et al., 2016; Schafer et al., 2012). In mammals, multiple molecules and receptors are involved in the complement cascade that leads up to the clearance of

cellular debris by microglia. Such mammalian signaling components include C1q, a protein that binds to a receptor called MegF10 that initiates the clearance of dying cells (Iram et al., 2016). In a healthy central nervous system, C1q activates C3, within a signaling pathway that tags synapses for elimination (Stevens et al., 2007). However, in the diseased brain, complement signaling can be skewed due to genetic and environmental factors. The complement protein, C1q has been shown to increase its expression along synapses prior to the accumulation of Aβ plaques in AD, and is essential to the neurotoxicity observed upon plaque accumulation. This suggests that genetic factors can cause inappropriate activation of the classical complement cascade (Hong et al., 2016).

Efficient and highly selective phagocytosis of dying cells is also necessary for processes of development and specialization of tissues, which require a signaling cascade capable of this level of specificity. In *Drosophila*, cortex glia (**Figure 1**) engulf dead and dying neuronal cell bodies, in a similar manner to the microglia of the mammalian model. The *Drosophila* counterpart to the MegF10 mammalian receptor has been established as Draper (Freeman et al., 2003). Recently, cortex glia-specific expression of Draper was shown to be indispensable to the process of clearing dead cells during larval development (McLaughlin et al., 2019), and in metamorphosis of the developing optic lobes (Nakano et al., 2019). While cortex glia are not the only phagocytic glial subtype in the CNS, sufficient Draper expression within cortex glia was found to be vital to the healthy development of the optic lobes (Nakano et al., 2019), as elimination of dead cells and pruning of synapses are essential developmental processes.

Figure 1: Schematic of the *Drosophila* Nervous System



Coutinho Budd et al., 2017

Figure 1. A. Brain and nerves from third instar larva (L3) with thoracic and abdominal segments. B. Cross section with select glial subtypes of the central nervous system. Compartmentalization of glial subtypes in *Drosophila* makes it possible to visualize how glial subtypes interact with subparts of a neuron, including the cell body, axons and synapses. (Coutinho-Budd et al., 2017)

Molecules that may Regulate Draper in the CNS

Thus far, the upstream regulatory molecule(s) that activates Draper in the CNS, initiating the engulfment process, have yet to be solidified. There are two main contending molecules whose relationship with Draper has been identified outside of the central nervous system. Additionally, some of these molecules have been found to exist within cortex glia.

Mcr is a complement-like opsonin that has been shown outside of the nervous system to induce autophagy through activation of the Draper receptor (**Figure 2**, Lin et al., 2017). Autophagy or "self-eating" is essential to the development of salivary glands and healing after tissue damage. Mcr knockouts display decreased recruitment of macrophages to laser-induced

epithelial wounds. The expression of constitutively active Src42A, a kinase known to phosphorylate Draper, is able to rescue the effects of Mcr knockdown in the salivary glands. This suggests that Mcr works through Draper to regulate macrophage recruitment and autophagy (Lin et al., 2017). The relationship between Mcr and Draper has yet to be elucidated within the *Drosophila* CNS, but it's impact on autophagy through Draper within salivary gland clearance and its resemblance to mammalian complement proteins, C1q/C3, make Mcr a promising target for further delineation of the phagocytic pathway in cortex glia.

Figure 2: Mcr is a fly complement protein that binds to Draper

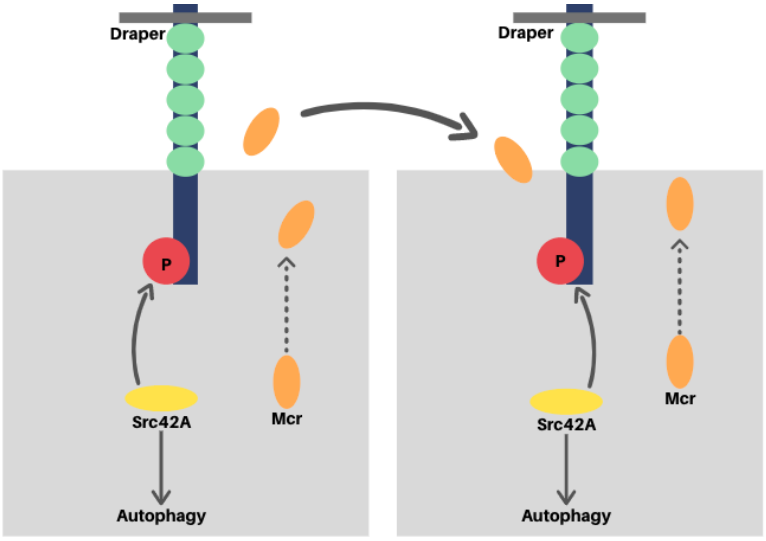


Figure 2. Mcr causes non-autonomous autophagy through the Draper receptor within dying salivary glands. Expression of Src42A (a known upstream activator of Draper) is able to rescue salivary gland clearance in Mcr mutants, suggesting that Mcr is upstream of Draper (modified from Lin et al., 2017)

An alternative molecule, the endoplasmic reticulum protein known as Pretaporter, has a relationship with Draper within the hemocytes of *Drosophila* (Kuraishi et al., 2009). Pretaporter is believed to migrate to the surface of cells to initiate the activation of Draper receptors during the process of cell death (Kuraishi et al., 2009). In animals expressing RNAi for Pretaporter or Draper--where the expression of these proteins had been deliberately knocked down--half the amount of phagocytosis occurred in the embryo compared to the control group (Kuraishi et al., 2009). In one study of the developing *Drosophila* optic lobes in the CNS, Pretaporter was determined to be a non-essential upstream activator of Draper, in that phagocytosis within the optic lobes could occur in its absence (Nakono et al., 2019). The finding by Nakono et al. (2019) suggests Pretaporter is a less plausible molecule than Mcr for upstream regulation of Draper-mediated engulfment in other parts of the CNS, or alternatively, that Pretaporter is not the main upstream activator of Draper in the CNS and another molecule compensates in its absence. Notably, Mcr has demonstrated adequate levels of mRNA expression in cortex glia (**Figure 3B**), further implicating its role as a potential upstream activator of Draper in the CNS.

Figure 3: Draper and Mcr are expressed in Cortex Glia

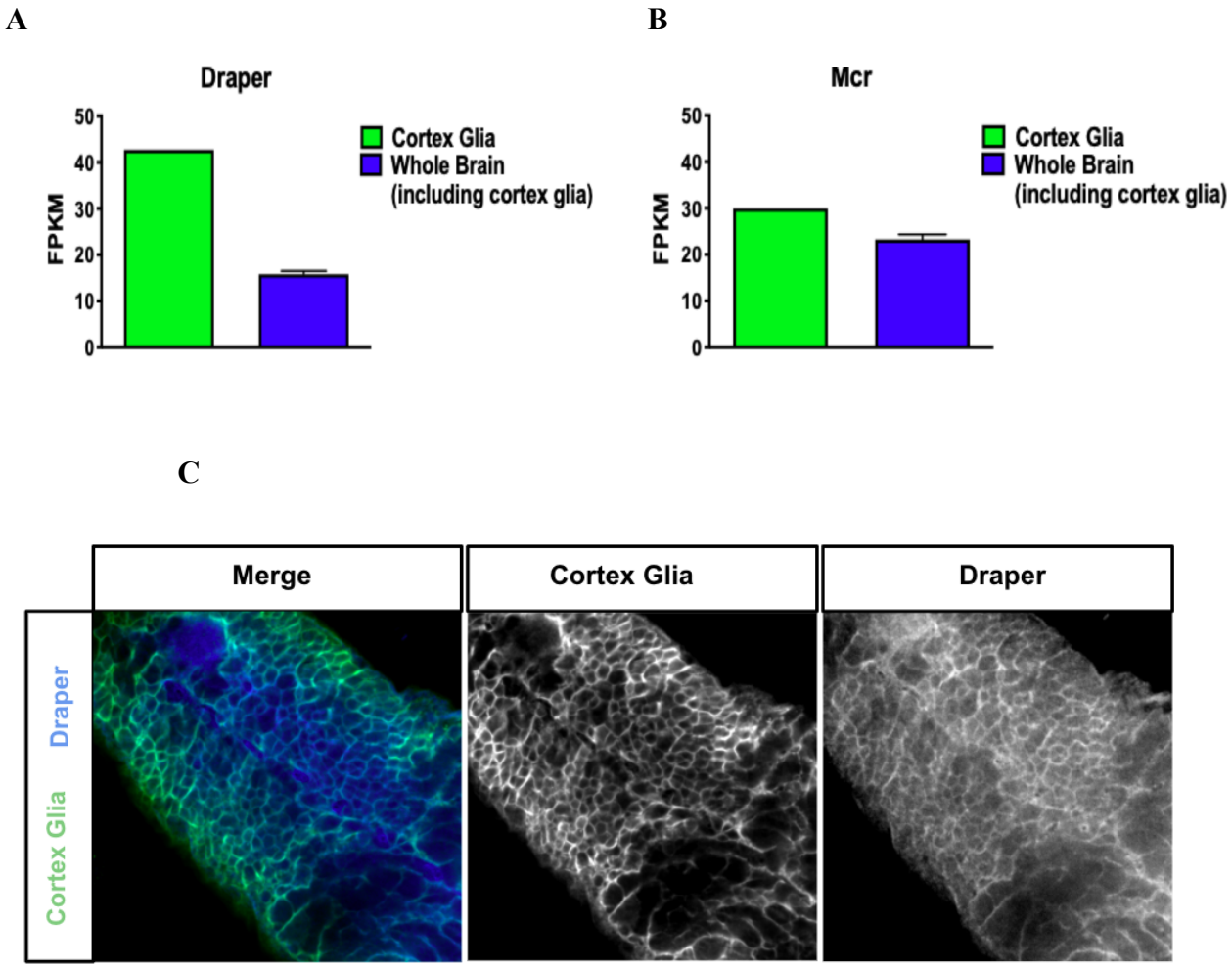


Figure 3. A-B: Unpublished RNA-sequencing data from the Coutinho-Budd lab, reported in fragments per kilobase million (FPKM). These data show that Draper (A) is more highly expressed in cortex glia than in the surrounding CNS, and that Mcr (B) is expressed in cortex glia as well. **C:** Staining for Draper and cortex glia show that Draper expression overlaps substantially with cortex glial staining, indicating that there are high levels of Draper expression within cortex glia.

Draper regulates the clearance of Corozonin (Crz^+) neurons

Drosophila possess a subset of Corozonin (Crz) neurons that undergo programmed cell death within 6 hours post-onset of metamorphosis, or after pupal formation (APF) (Choi et al., 2006). This distinctive subset of neurons resides in the ventral nerve cord (VNC), in 8 pairs. They express the neuropeptide Crz, which may be stained for using an anti-Crz antibody, and should disappear completely by approximately 6 hrs APF. Previous studies have shown that the removal of dead cell bodies and neurites produced by programmed cell death of Crz neurons is suppressed by the loss of a single copy of Draper, in that the Crz^+ debris will remain in the VNC as it is unable to be phagocytosed. (Tasdemir-Yilmaz & Freeman, 2014). When Draper expression was knocked down specifically in astrocytes, clearance of Crz^+ dead cell bodies was unaffected, suggesting that Draper acts through another glial subtype in the clearance of Crz^+ cell bodies (Tasdemir-Yilmaz & Freeman, 2014), very likely cortex glia. As Draper is highly expressed within cortex glia in comparison to the whole brain (**Figure 3A, C**), and Mcr is thought to function upstream of Draper within salivary gland and epithelial cells (Lin et al., 2017), knocking down Mcr in cortex glia and examining the effects on the Crz^+ neurons in the VNC will determine whether cortex glial Mcr is necessary for cortex glial engulfment of cellular debris.

Alzheimer's Disease and Glial Engulfment

Glial engulfment has been shown to be a key regulator of neurodegenerative diseases, such as AD. Increased activity of phagocytic microglia and consequently synapse loss, appear to occur even prior to the accumulation of Aβ plaques (Hong et al., 2017; Boza-Serrano et al., 2018). Alzheimer's disease (AD) is a well-known neurodegenerative condition characterized by

the accumulation of Tau proteins (tangles) and beta amyloid (Aβ, plaques) in the brain, forming aggregations that impair neuronal communication and memory (Grundke-Iqbal et al., 1986; Hardy & Higgins, 1992). Neuroinflammation has been identified as a vital component to the onset of AD pathogenesis prior to plaque accumulation (Hong et al., 2017). Despite this relationship, the precise mechanisms that connect neuroinflammatory processes to memory loss remain elusive (Boza-Serrano et al., 2018). Most therapeutic interventions aim to address the neurotoxicity that is associated with Aβ-accumulation, plaques, and hyperphosphorylation of tau proteins; however, these treatments have not achieved significant clinical improvement, necessitating a greater focus on neuroinflammatory signaling and engulfment defects (Yiannopoulou et al., 2019).

Complement-mediated engulfment has been implicated in the early stages of AD in murine models (mouse models) (Hong et al. 2016). In the AD brain, abnormal pruning is activated, resulting in overactive synapse engulfment and decreased synapse density (Hong et al., 2016). Oligomeric Aβ deposits in the brain drive upregulation of C1q deposition on neurons along with increased phagocytic activity of microglia, in mammals (Hong et al., 2017).

In the same vein, overexpression of the *Drosophila* engulfment receptor, Draper has been shown to cause neuronal loss of dopaminergic and GABAergic neurons associated with memory formation and preservation, providing an important link between glial phagocytosis and neuronal loss (Hakim-Mishnaevski et al., 2019). Draper expression exists at baseline levels within the adult *Drosophila* brain, and this expression has been shown to increase in response to an acute olfactory nerve axotomy in order to promote clearance of neuronal debris (MacDonald et al., 2006). Draper expression has also been found to increase in response to neuronal expression of human Aβ42 (Ray et al., 2017).

In previous *Drosophila* models of AD, neuronal expression of human Aβ42 resulted in Aβ accumulation, locomotor dysfunction, and premature death (Ping et al., 2015), all pathological phenotypes that are exacerbated in Draper-null mutants. The removal of a single copy of Draper in an Aβ-background exacerbates pathological phenotypes, suggesting that glial expression of Draper is neuroprotective (Ray et al., 2017). Understanding the intricacies of glial phagocytic receptor regulation is thoroughly intertwined with elucidating AD pathophysiology.

P38 Kinase Involvement in AD Pathology

Another molecule that has been deemed vital to the process of phagocytosis of degenerating axons by microglia is p38 MAP kinase (MAPK). Axonal damage upregulates phosphorylation of p38 MAPK in microglia and has been deemed necessary for the onset of phagocytosis after axon injury (Tanaka & Yamashita, 2009). In addition, phagocytosis of Aβ plaques by microglia has been suggested to involve the activation of NFkB and p38 MAPK via the toll-like receptor, TLR4, (Kakimura et al., 2002), acting in a similar fashion as Toll-dependent activation of Draper in *Drosophila* (Mclaughlin et al., 2019). P38 kinases have been identified as pathological contributors to disease states in the context of another neuroinflammatory disease where glia play a key role, multiple sclerosis (MS) (Krementsov et al., 2014). P38α inhibition in all myeloid cells within a murine model of MS was shown to lessen the disease phenotype in females and not males (Krementsov et al., 2014).

Disease-associated microglia have been found to play a similar role in MS as they do in AD, in that they both rely on an apolipoprotein E (APOE) mediated switch from their homeostatic to neurodegenerative state (Krasemann et al., 2017), suggesting that p38 inhibition could exhibit similar positive effects in the context of AD. Furthermore, increased activity of p38

kinases has been noted in the brains of AD patients and is positively correlated to the amount of phosphorylated Tau observed (Hensley et al., 1999). Activation of p38 kinases has been shown to upregulate its downstream target, c-jun, which is known for its role as an apoptotic transcription factor that causes neuronal death in AD (Chang et al., 2010). Past studies have shown that down-regulation or knock-out of p38 MAPK in all cell types reduces the occurrence of Aβ plaques through promotion of BACE1 autophagy, an enzyme involved amyloid beta precursor protein processing (Schnöder et al., 2016). More specifically, p38 MAPK has been deemed necessary for the activation of microglia in the inflammatory response to Aβ-plaque accumulation and may lead to the impairment of phagocytosis of these plaques (Dong et al., 2016 ; Giovannini et al., 2008). In a murine model, inhibition of p38α appears to support activation of both microglia and astrocytes and facilitate the clearance of Aβ-plaques in early stages of AD (Colie et al., 2017).

What remains elusive is our understanding of cross-talk between activated glia and neurons in the context of p38, along with the role of the multiple isoforms of p38. *Drosophila* possess 3 p38 genes, which are homologous to the mammalian p38α: p38a with 80% conserved identity, p38b with 81%, and p38c with 65% (Han et al., 1998). The abundance of powerful genetic tools that may be utilized in *Drosophila* can be implemented to manipulate p38 kinase expression in an Aβ42-background and observe glial morphological changes to better understand cross-talk between neurons and glia in this context. Additionally, the low costs and short life cycle associated with the *Drosophila* model allow for efficient exploration of molecular modifiers of disease. A similar genetic manipulation of endogenous Mcr expression may be performed to elucidate how Mcr may regulate Draper activity in the context of an Aβ42-background.

METHODS

Homologous phagocytic signaling between the mammalian model and *Drosophila,* along with the relative ease of manipulating the genetic expression within fruit flies, make *Drosophila* ideal for elucidating the impact of potential upstream activators of Draper. The ability to manipulate Mcr expression and examine effects on engulfment and Aβ42 expression was vital for determining which molecule exhibits control over Draper in the CNS. In addition, various p38 kinase isoforms were knocked down in *Drosophila* glial cells in the presence of neuronally expressed Aβ42, and analysis of glial morphology was performed (in parallel with a lifespan assay performed by Dina Alter).

Drosophila Genetics

A) Genetic Manipulation: Controlling the Expression Mcr, Aβ42 and p38 Kinases

A gene encoding the transcriptional activator of yeast, Gal-4, can be inserted into the *Drosophila* genome under the control of a cell type specific promoter. The Gal-4 transcriptional activator binds to a UAS binding site, allowing us to express downstream genes of interest or express fluorescently-tagged proteins in a cell-type specific fashion (Brand & Perrimon, 1993).

Powerful binary drivers available in the *Drosophila* model allow us to perform simultaneous manipulations of expression in two different cell types without cross-reaction, producing offspring which express or lack the protein of interest (Brand & Perrimon, 1993). The Q-system functions in a similar manner to Gal4/UAS but is independent of the expression driven

by this Gal4/UAS binary expression system. QF2 is analogous to Gal4, and QUAS is analogous to UAS (Brand & Perrimon, 1993; Potter et al., 2010).

Our lab has developed a cortex glia-specific Gal4 driver. The Gal4/UAS system can be used to drive RNA-interference (RNAi) and knockdown of either p38 or Mcr expression in cortex glia. RNAi is a post-transcriptional gene silencing technology wherein antisense RNA can modify and knock down endogenous levels of protein expression (Fire et al., 1998). Our Mcr-RNAi lines were donated by Eric Baehrecke, the corresponding author on the paper describing the role of Mcr in the salivary glands (Lin et al., 2017). All p38 RNAi lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC). We obtained the transgenic fly line expressing human Aβ42 on the third chromosome from the BDSC, that was donated by Pascal Dijkers. Using the QF2/QUAS system in conjunction the neuron-specific promoter, Elav, Aβ42 can be expressed exclusively from neurons.

B.) Mcr and the Clearance of Dead Cells

To examine the impact of Mcr expression on cell death and engulfment, we overexpressed a flag-tagged Mcr specifically in cortex glia using a cortex glia-specific split gal4 driver expressing GFP, so that we could visualize cortex glia and we utilized death caspase-1 (DCP-1) expression as a marker of dead cells. To accomplish this, males from w;+/cyo;pUASt-Mcr-flag/TM6B were crossed with virgins of the genotype w;;wrapper-DBD-Nrv2-AD;UASCD8GFP/TM6B (the cortex glia-specific driver line). Virgin females are used due to the sperm-storing capability of the female fly, as it is essential to ensure the progeny are from the cross of interest. The genotype of the progeny tested was w;;PUASt-Mcr-flag/wrapper-DBD-Nrv2-AD UASCD8GFP, expressing flag-tagged Mcr in cortex glia. In order to examine the

impact of Mcr knockdown, we used the split gal-4 driver to drive Mcr-RNAi specifically in cortex glia. Males of the genotype w;UAS-Mcr-RNAi;Sb/TM6B were crossed with the driver line to produce progeny of the genotype w; UAS-Mcr-RNAi/+;wrapper-DBD,Nrv2-AD,UASCD8GFP/+, in which Mcr is specifically knocked down in cortex glia. A control was created by crossing w1118 (genetic strain control) with the driver line as a driver line-only negative control.

C.) P38 Knockdown in an AB-Background

In order to explore the glial morphology of p38 kinases in an Aβ-background, Aβ42 was expressed specifically from neurons while each of the p38 isoforms were knocked down in glial cells using the glial driver, Nrv2-Gal4. Female virgin animals from Elav-QF2; Nrv2-Gal4/CyO; QUAS-Aβ42/TM3[Sb] were crossed to males from one of several p38 RNAi lines. A control was created by crossing Elav-QF2; Nrv2-Gal4/CyO; QUAS-Aβ42/TM3[Sb] to w1118 males as a positive control.

D.) Mcr knockdown in the presence of Human Aβ42

A transgenic line was built to simultaneously express Mcr RNAi and Aβ42, by crossing virgin female animals with the genotype w; UAS-Mcr-RNAi;Sb/TM6B [Tb, Hu, e] with males of genotype w; Sp/CyO[dGV]: QUAS-Aβ42/TM3 [Sb, Tb-RFP]. Female and male animals with the genotype w; UAS-Mcr.RNAi/CyO[dGV]; QUAS-AB42/TM6B [Tb,Hu,e], were identified based on markers and crossed with one another in order to establish this line that could then be crossed to Elav-QF2;;Repo-Gal4 animals to knockdown Mcr in all glial subsets in the presence of Aβ42.

Alternatively, we could express Mcr in a subset of glial cells. For this, virgin females from Elav-QF2; Nrv2-Gal4/CyO[dGV]; Dr/TM3[Sb] were crossed with males from w; UAS-Mcr.RNAi/CyO[dGV]; QUAS-Aβ42/TM6B [Tb,Hu,e]. The animals examined possessed the genotype, Elav-QF2; Nrv2-Gal4/UAS-Mcr.RNAi; Dr/QUAS-Aβ42. These progeny animals would express Aβ42 in neurons and lack Mcr expression in ensheathing glia, astrocytes and cortex glia (driven by Nrv2-Gal4, **Figure 1**), primarily responsible for engulfment in the *Drosophila* CNS. Any existing expression of Mcr in perineurial glia and subperineurial glia remains intact, so as to preserve Mcr expression in glia that comprise the blood brain barrier of the *Drosophila* CNS.

In the creation of a positive control that expresses Aβ42 and has naturally occurring levels of Mcr, Elav-QF2; Nrv2-Gal4/CyO[dGV]; Dr/TM3[Sb] female virgins were crossed to males from w;;p{w+=QUAS-Aβ42.L}7 (on chromosome III). The progeny animals examined had the genotype, Elav-QF2; Nrv2-Gal4/+; QUAS-Aβ42/Dr. In the creation of a negative control that knocks down Mcr expression and lacks expression of Aβ42, female virgins from Elav-QF2; Nrv2-Gal4/CyO[dGV]; Dr/TM3[Sb] are crossed with males from w;;Mcr-IR;Sb/TM6B IV-3 CG7586, 100197. The progeny animals tested had the genotype Elav-QF2; Nrv2-Gal/UAS-McrRNAi; Dr;+.

Microdissection of the Fly CNS

In order to visualize the effects of Mcr expression on the prevalence of Crz engulfment, Aβ42 expression, and morphological changes in glia, microdissections of both larval, pupal, and adult brains were performed.

To visualize the impact of Mcr knockdown on the clearance of dead cells, dissections were performed on both wildtype animals and Mcr knockdown animals at L3 and pupal stages, 6 hours APF. Larvae were dissected in phosphate buffered saline (PBS), which ensures that a consistent pH is maintained. The brains were fixed in 4% formaldehyde diluted in PBS and washed in PBS with Triton-X (PTX). Larval dissections were used as a baseline comparison to ensure that the proper number of Crz neurons were present before metamorphosis, and compared to dissections performed 6 hours APF, to examine the clearance of Crz^{+} debris.

Mcr-RNAi and wildtype pupae of the correct genotype were identified at the onset of metamorphosis, when they first begin to cling to the vial wall, take on a pupal shape, and stop any movement. These animals were then moved to a separate vial, placed at 29 º C, and collected 6 hours later for dissection. The pupal case (now brown and opaque) was cut off and the animals were fixed with 4% formaldehyde in PBS and washed brain in PTX.

Adult brains were dissected in PTX and fixed in 4% formaldehyde in PTX. Brains were washed in PTX to ensure maximum permeability of the brain tissue for staining purposes.

Immunolabeling and Confocal Microscopy

Immunohistochemistry was used in order to identify proteins of interest (such as Mcr, Draper, and Aβ42) by tagging antigens with fluorescent antibodies. Primary and secondary

antibodies were applied to the fixed larval brain. Anti-cleaved DCP-1 can be used to stain the activated form of DCP-1 in order to visualize and quantify dead cells. Similarly, a protein known as Elav is involved in RNA processing specific to neurons, making it a neuronal marker. Elav staining was used concurrently with a DCP-1 stain and a glia marker to determine whether the cell death occurring was neuronal or glial cell death.

Staining for the neuropeptide corazonin gene with rabbit anti-Crz antibody was performed. Anti-Crz staining allows for the visualization of the 8 pairs of Crz^{+} neurons and Crz^{+} debris, pre- and post-apoptosis, in the CNS. The CNS of Mcr-RNAi and control animals were stained with primary antibodies used at the following concentrations: chicken anti-GFP, 1:1000, mouse anti-Draper, 1:300, and Rat anti-Elav, 1:100, and Ms anti-Crz, 1:500. Preabsorbed secondary antibodies were used to avoid cross-reactivity between mouse and rat antibodies. Secondary antibodies used were donkey anti-chicken alexafluor488, 1:100, donkey anti-rabbit alexafluor405, 1:100, donkey anti-rat Cy3, 1:100, and donkey anti-mouse Cy5, 1:100.

P38 larval brains were stained using primary antibodies chicken anti-GFP, 1:1000, rabbit anti-RFP, 1:1000, and Rat anti-Elav, 1:100. Secondary antibodies used were donkey anti-rabbit Cy3, 1:100, donkey anti-chicken alexafluor488, 1:100, and donkey anti-rat Cy5, 1:100.

Adult brains expressing Mcr-RNAi in an Aβ42-background were stained using primary antibodies at the following concentrations; rabbit anti-dsRed, 1:1000, mouse anti-Aβ42, 1:500 and rat anti-Elav, 1:100. Secondary antibodies used were donkey anti-rabbit Cy3, 1:100, donkey anti-mouse Cy5, 1:100, and donkey anti-rat 405, 1:100. All brains were mounted on a glass slide with Vectashield and stored in a refrigerator at 4 degrees Celsius. Imaging was performed on a spinning disk confocal microscope.

RESULTS

The initial approach taken to explore the impacts of Mcr knockdown on the prevalence and clearance of cell death was through overexpression and knockdown of Mcr in cortex glia and quantification of DCP-1 puncta. This method proved difficult, as the DCP-1 antibody produced a great deal of background, making it very challenging to decipher which staining was background and what were actual cell death puncta (**Figure 4**).

Figure 4: Manipulation of Mcr expression and Assessment of Cell

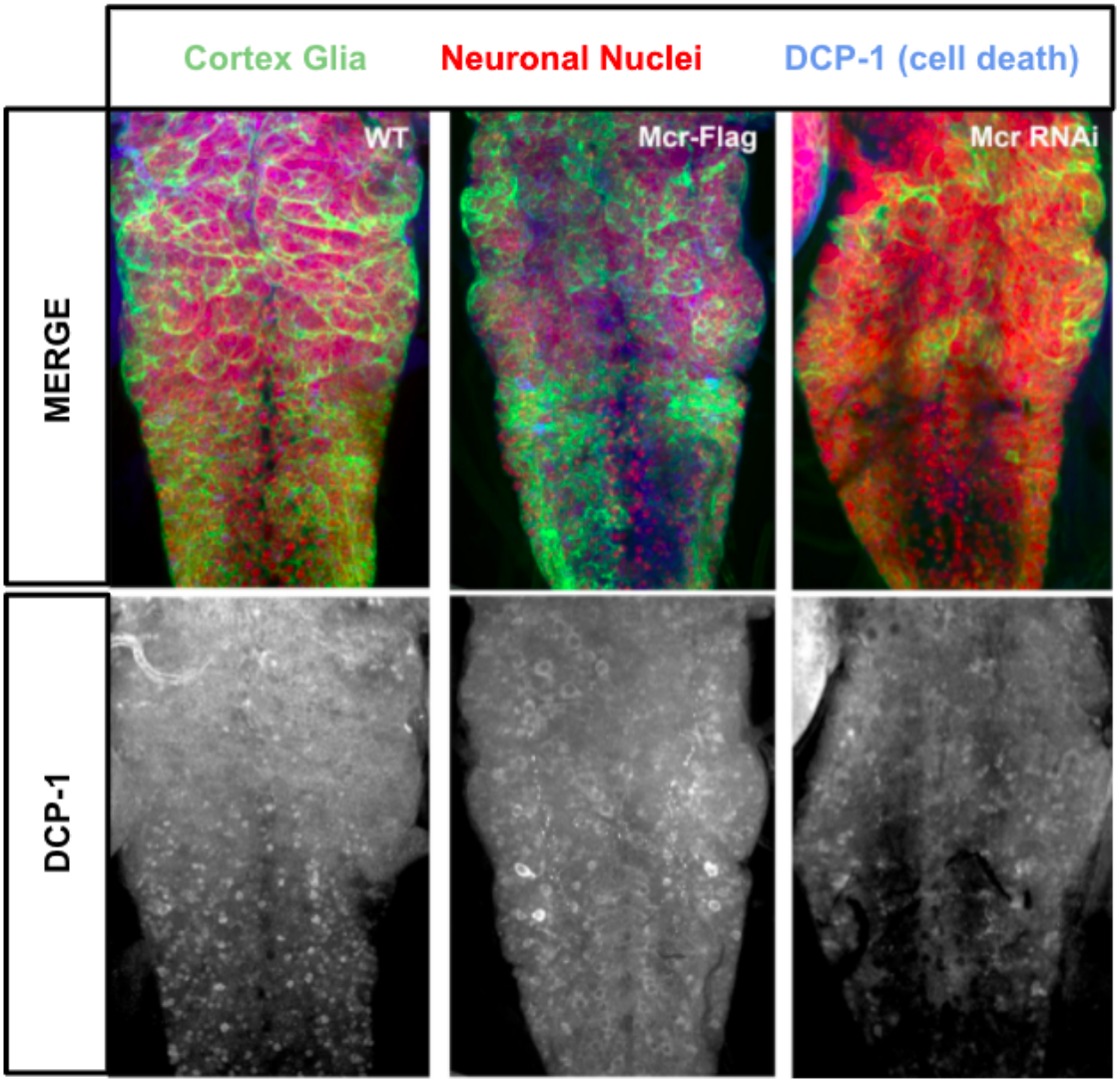


Figure 4. This figure compares ventral nerve cords from wildtype control animals (left), expression of flag-tagged Mcr (middle), and Mcr-RNAi (right). It is difficult to parse out which puncta represent cell death using this DCP-1 stain, so we were unable to make any inferences about the prevalence of dead cells.

In an effort to devise a more clear-cut method of analyzing the role of Mcr in engulfment, we used a method that capitalizes upon the presence of 8 pairs of distinctive Crz^{+} neurons that are known to be cleared at 6 hours APF. If Mcr is necessary for glia engulfment of neuronal debris, then we would expect to see a greater number of cell bodies left over in the pupal CNS. Preliminary findings suggest that Mcr plays a role in cortex glial engulfment of dying neurons. In wildtype animals at L3, anti-Crz antibodies will label 8 pairs of Crz^{+} neurons (**Figure 5A, quantified in E**). By 6 hours APF, nearly all of the Crz^{+} debris are cleared from the neuropil (**Figure 5B, E**). These neurons still develop normally in the absence of Mcr (**Figure 5C, E**); however, when Mcr was knocked down in a cortex glial-specific fashion, cellular debris of Crz+ neurons that is typically cleared in wildtype animals remained in the VNC (**Figure 5D, E**). The difference in the number of Crz^{+} cell bodies that remained in the VNC was substantial (9 cell bodies remained in the Mcr knockdown animal, and an average of 2 in the wild type animals).

Figure 5: Knockdown of Mcr in cortex glia impairs the developmental clearance of corazonin (Crz) Neurons

A-D **E**

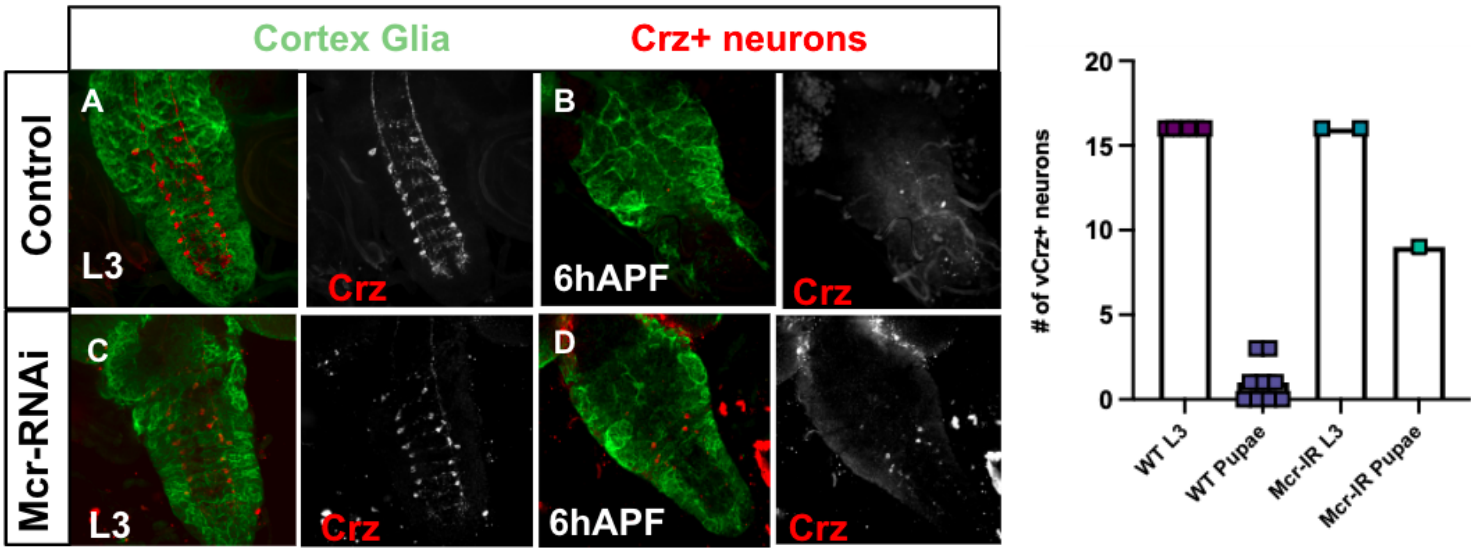


Figure 5. A:WT L3 larva contain 16 Crz^{+} neurons (red, 8 pairs) that undergo programmed cell death and are typically cleared by 6 hrs after pupal formation (APF). **B**: At 6 hrs APF, minimal Crz^{+} cellular debris remains (red). **C**: Cortex glial knockdown of Mcr still show 16 Crz^{+} neurons at L3, but impaired clearance of Crz^{+} debris at 6hrs APF **(D). E)** Quantification. WT L3: n=6, WT Pupae: n=9, Mcr-RNAi L3: n=2, Mcr RNAi Pupae: n=1.

Drosophila expressing Aβ42 often develop a rough eye phenotype due to neurodegeneration within the eye. The severity of the rough eye phenotype characteristic of the expression of Aβ42 in neurons is more severe in animals which possess two copies of Aβ42 (homozygous), whereas animals with one copy of Aβ42 display a normal eye phenotype (**Figure 6A**).

In the interest of examining the role of p38 kinases in an Aβ42-background, Dina Alter performed a lifespan assay to determine the viability of animals expressing various *Drosophila*

p38 homologs. Knockdown of p38a RNAi was found to significantly increase viability. However, knockdown of either p38b or p38c did not exhibit any significant effects on viability (**Figure 6B**). In our baseline analysis of p38 knockdown on glial morphology we observed a slight change in the morphology of cortex glial cell bodies in the p38a knockdown, where they often appear rounder and more prominent than in either p38b or C knockdown (**Figure 6A**).

Figure 6: Knockdown of p38a kinase significantly prolongs lifespan in an Aβ42-Background

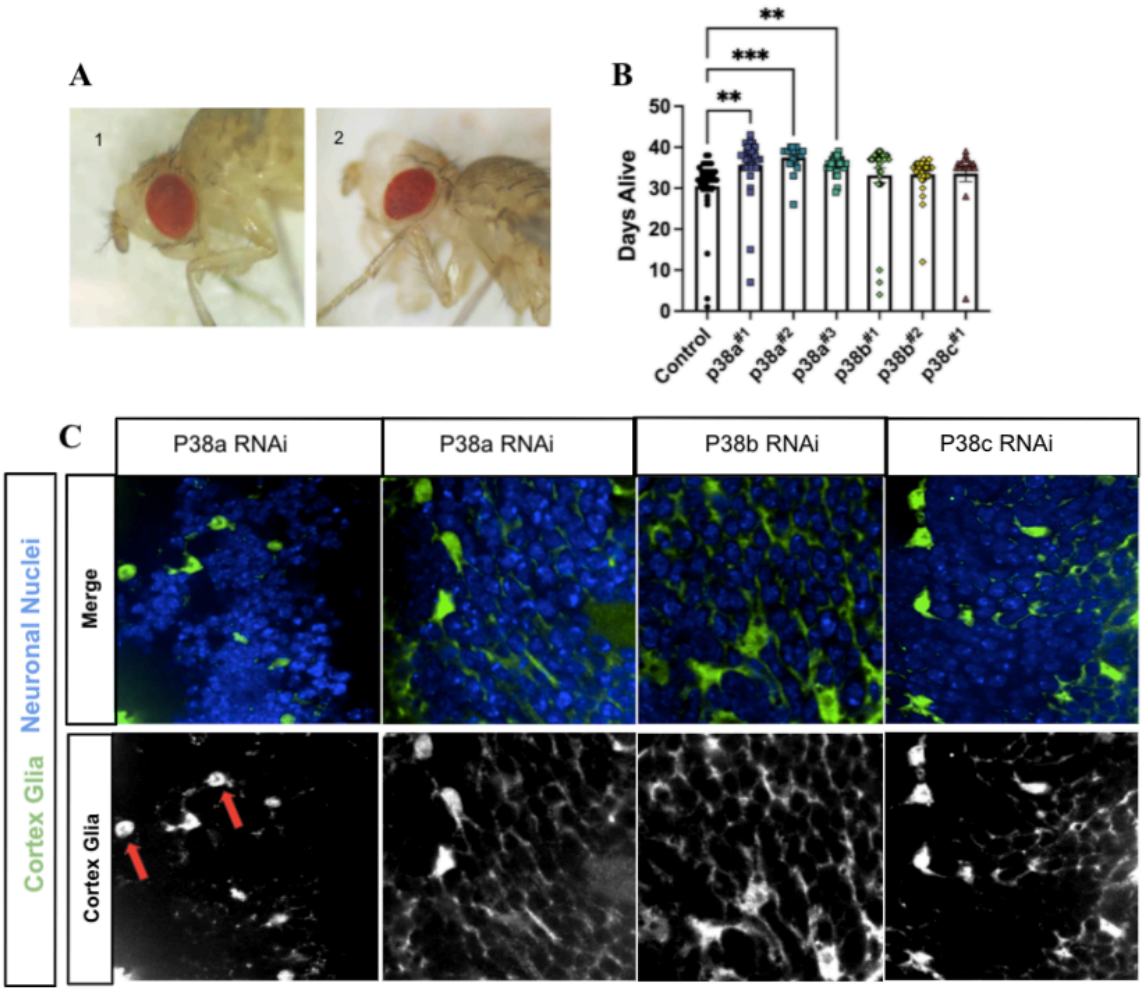


Figure 6. A. (1) One copy of Aβ42 results in a normal eye phenotype, (2) whereas two copies of Aβ42 induces a rough eye phenotype, indicating more neurodegeneration. **B**. Quantification of viability (days lived) for flies expressing Aβ42 in neurons with a glial driver alone (control), or knockdown of either p38a, p38b, or p38c (performed by Dina Alter). All three p38 RNAi lines tested significantly increased viability, while p38b and p38c had no significant effect. Control: n=37, p38a RNAi #1: n=34, p38a RNAi #2: n=24, p38a RNAi #3: n=30, p38b RNAi #1: n=28, p38b RNAi #2: n=33, p38c RNAi #1: n=17. **C.** Glial morphology after knockdown of the 3 *Drosophila* p38 genes. Some of the cortex glial cell bodies of the p38a RNAi brain appear to be more rounded and much brighter in comparison to the surrounding staining (as indicated by the red arrows), indicative of altered glial morphology.

We also examined concurrent expression of Mcr-RNAi and Aβ42 in adult *Drosophila* brains to determine if knockdown of Mcr expression impacted prevalence of Aβ42 or glial morphology. Our preliminary data suggest that Mcr knockdown leaves a greater amount of Aβ42 expression in the adult central brain, and glia appear to be slightly hypertrophied (**Figure 7A, B**); however, these data need to be quantified. In contrast, Aβ42 expression in the optic lobes appears to be highly expressed in both control and Mcr-RNAi brains (**Figure 7C, D**), likely due to higher expression of the Elav-QF2 driver in optic lobe neurons, which is consistent with the observable rough eye phenotype indicating neurodegeneration (**Figure 6A**).

Figure 7: Mcr knockdown may impact Aβ42 expression and glial morphology

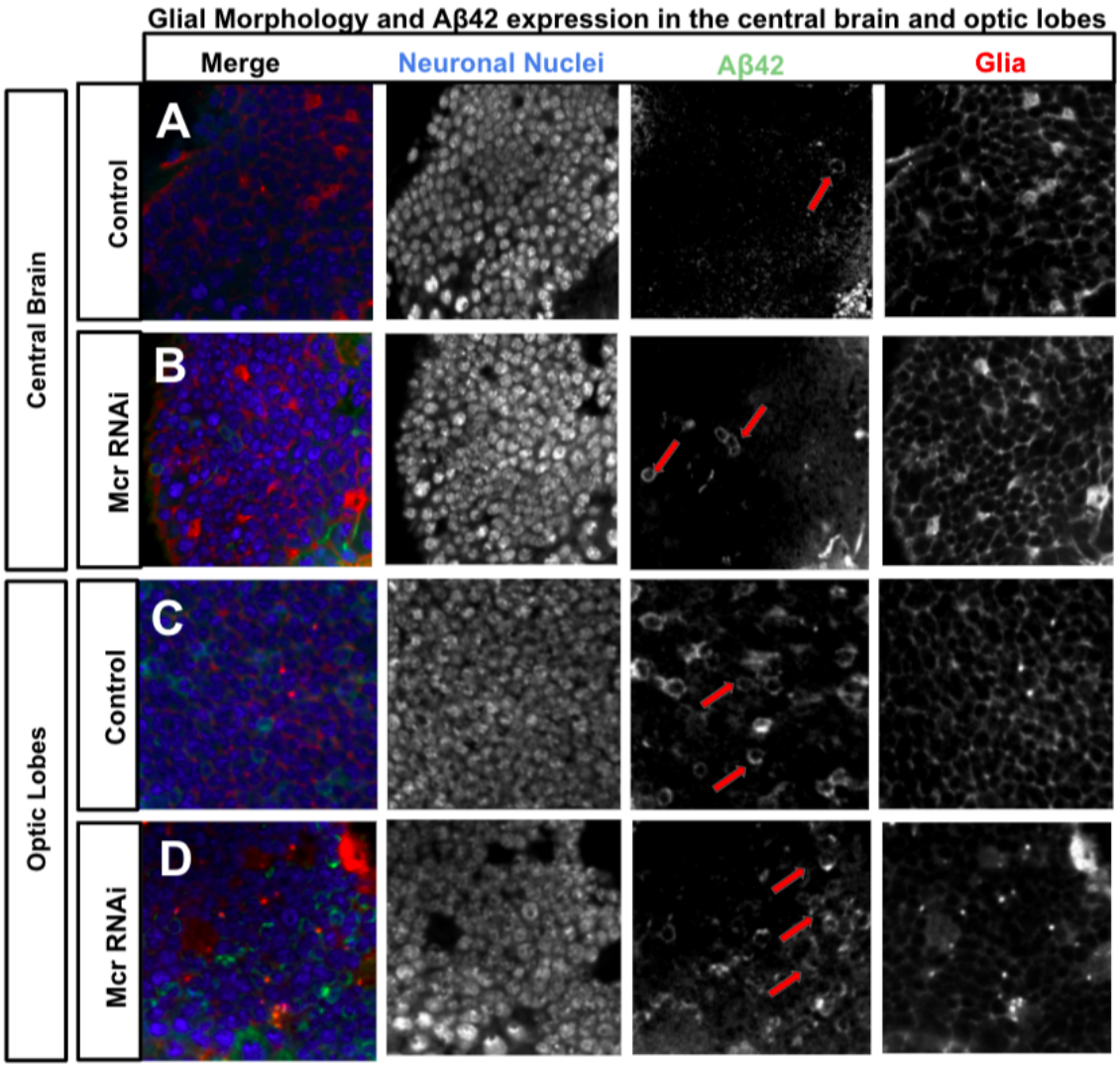


Figure 7. A-B: The adult central brain. Control brains (A) appear to show less Aβ42 accumulation than Mcr knockdown (B) in the central brain. **C-D:** The adult optic lobes show high Aβ42 expression in both control (C) and Mcr RNAi (D) animals.

DISCUSSION

The purpose of the present study was to explore potential molecular modifiers of glial engulfment and Aβ42-induced phenotypes using the *Drosophila* model. Given that high levels of background were observed in our brains stained for DCP-1, we determined that DCP-1 puncta were very difficult to quantify as the determination of actual dead cells versus background staining was very subjective. We turned to the clearance of Crz^{+} neurons as a means of examining engulfment by cortex glia. These preliminary findings suggest that Mcr knockdown impairs cortex glial engulfment of neuronal debris (**Figure 5)**. Bearing in mind that the Draper receptor is necessary for engulfment of debris in the CNS during this developmental stage (Tasdemir-Yilmaz, & Freeman, 2014), this finding suggests that Mcr plays a role in Draper signaling and function. This research was primarily motivated by the paper by Lin et al. (2017), who found that Mcr was the upstream activator of Draper in the *Drosophila* salivary gland, and by our finding that Mcr was adequately expressed within cortex glia and Draper was highly expressed within cortex glia (Coutinho-Budd Lab, unpublished data).

It is possible that Mcr is acting directly on the Draper engulfment receptor, to initiate the process of engulfment within cortex glia. Another potential role of Mcr in its contribution to the engulfment of dead cells is through binding to the neuron and contributing the opsonization for removal or the "eat me signal" (**Figure 8**). In mammals, the complement proteins c1q/c3 opsonize neurons for removal by microglia (Stevens et al., 2007), and it is possible that deposition of Mcr could be contributing to the tagging of neurons for removal by microglia in response to either neuronal axotomy or Aβ42-induced neurotoxicity (**Figure 8**).

Figure 8

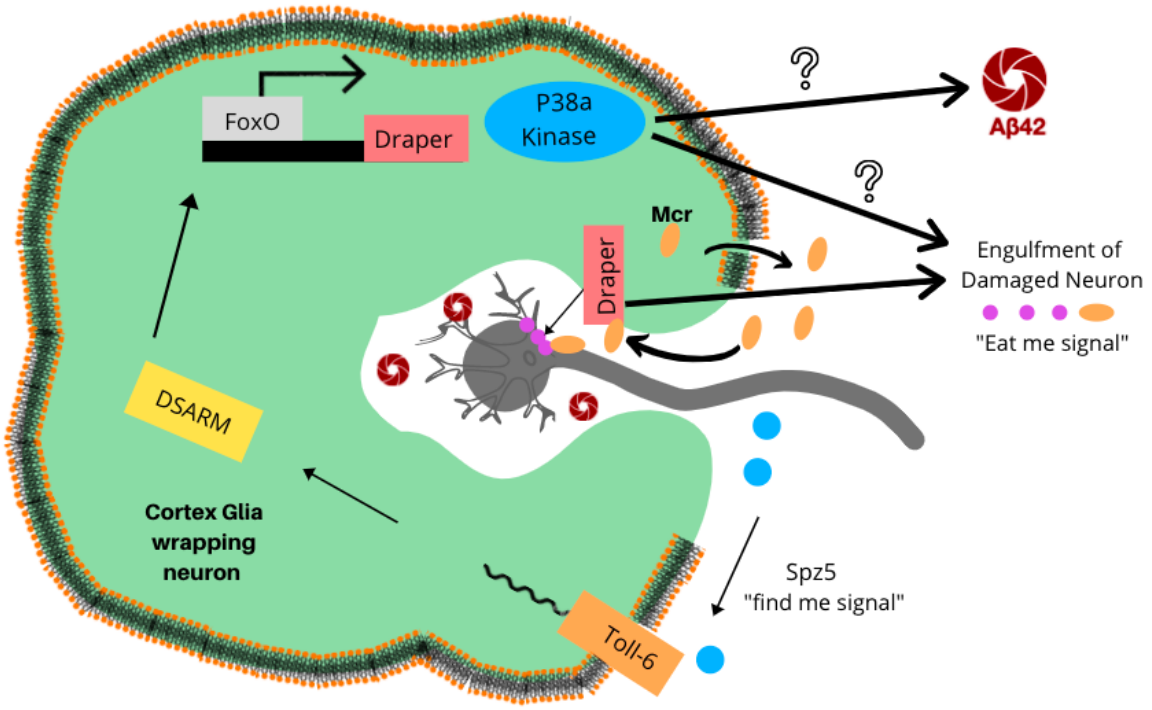


Figure 8. Model for the proposed mechanism of cortex glial engulfment of dead and dying neurons, or after toxicity induced by Aβ42 plaque accumulation. Damaged neurons release Spz5 as a "find me signal" that activates a transmembrane toll receptor, Toll-6. Toll-6 activates the molecule dSARM, which activates transcription factor, FoxO, resulting in the expression of Draper. The engulfment receptor, Draper, appears to be activated by Mcr, which is secreted from cortex glia. Mcr may also contribute to the "eat me signal" present on the damaged neuron. P38a kinase is potentially necessary for glial engulfment according to previous literature and was found to exhibit disease mitigating effects found in this study.

In the future, the Mcr knockdown engulfment assay utilized in this study must be expanded to a far greater number of animals. One issue that was encountered when trying to expand this assay to a greater number of animals was in the use of an alternative Elav driver line (Elav-QF2; Sp/CyO; Repo-Gal4,UASCD8GFP) that had been crossed with our Mcr-RNAi line. After performing many dissections of this cross, we discovered that the driver line was not always expressing GFP, which could potentially also mean that Mcr-RNAi wasn't being driven

in these crosses. This issue may have been due to contamination of the line (introduction of animals from other lines), cross-mating between progeny of the cross of interest, or errors in the creation of the driver line itself.

In order to further delineate the relationship of Mcr with the engulfment receptor, Draper, it will be important to measure Draper concentration within Mcr-RNAi and Mcr-overexpression crosses with a Western Blot. This will help to determine whether Mcr is acting directly on the Draper receptor and inducing upregulation of Draper, or whether it is contributing solely to the tagging of the neuron for removal.

Based on the lifespan assay performed in this study and previous research using the mammalian model, p38a contributes to the pathological phenotype observed in AD. P38α has previously been deemed necessary for the phagocytosis of degenerating neurons after axotomy *in vitro* (Tanaka & Yamashita, 2009), and may be necessary for heat shock protein-induced activation and engulfment of microglia in response to Aβ plaques (Kakimura et al., 2002). We would have liked to have performed a lifespan assay on animals expressing Mcr-RNAi subtypes in the presence of neuronally expressed Aβ42, but we did not have enough time at the end of the semester to do so. Time-limitations brought about by Covid-19 limited the number of dissections and breadth of experiments we were able to perform.

In the future, employment the same engulfment assay used to examine the effects of Mcr knockdown in our study (**Figure 5**) would be helpful to examine the impact of p38a RNAi on engulfment of Crz^{+} neurons. An assay such as this would allow us to solidify the role of p38a in glial engulfment of neuronal debris. As the *Drosophila* genes p38a and p38b are homologous to mammalian p38α with 80% identity, and 81% conserved identity respectively, it would be

interesting to look at a concurrent knockdown of p38a and p38b. There is a possibility that the p38a gene may be compensating for the loss of p38b when we knock down expression, leading to no difference in lifespan. Furthermore, it is possible that knockdown of the two most heavily conserved *Drosophila* genes would offer greater neuroprotection from Aβ42. Our data indicates that there may be a subtle change in p38a morphology in baseline conditions wherein p38 expression is knocked down in the absence of Aβ42 expression (**Figure 6**). The cortex glial cell bodies appear to take on a more rounded morphology, and the staining is brighter when contrasted with the rest of the brain (**Figure 6**). It will also be essential to examine adult p38a knockdown brains (identified by our lifespan assay) with an Aβ42-background for changes in glial morphology and relative Aβ42 load and see whether *Drosophila* p38a knockdown mitigates disease through the regulation of Aβ42 expression or role in glial engulfment. Based on the findings of the p38a knockdown in our lifespan assay we would expect to see a lesser degree of Aβ42 expression in the p38a knockdown brains.

Ray et al., (2017) performed a lifespan assay with Draper mutants in an Aβ42-background and identified Draper as a mitigator of disease progression. Loss of Draper in an Aβ42-background produced a significantly more severe phenotype than the Aβ42-background or Draper null mutant alone. In a similar manner to our p38 lifespan assay, it will be important to perform a lifespan assay on Mcr RNAi mutants with an Aβ42-background to see if Mcr expression is impacting the pathological phenotype observed in AD. Additionally, we could measure Aβ42 load in Mcr mutants versus controls and examine glial morphology in a greater number of animals. Measuring Aβ42-load would help us to determine whether impaired engulfment induced by Mcr may impact the clearance of Aβ42 plaques.

Significance

Determining which molecules are involved in the upstream activation of the Draper receptor in the CNS--thus, establishing which molecules are indirectly responsible for the clearance of dead cells, aging synapses and Aβ42 plaques by glia--is essential to glean a holistic understanding of neurodegenerative disease. These pathways are relevant to diseases of aging, such as Alzheimer's, Huntington's, multiple sclerosis, and other neurodegenerative diseases. Communication and signaling resulting in the engulfment of cellular debris is indispensable to the maintenance of a healthy CNS making these proteins potential targets for the regulation of disease states. This project identifies Mcr as a potential molecular modifier of engulfment by cortex glia in the *Drosophila* CNS and implicates its role in mediating Aβ42 expression. We also identify p38a kinase as playing a role in the production of pathological phenotype observed in AD. These findings lay the groundwork for further exploration of these molecular modifiers in an Aβ42-background, using the *Drosophila* model as well as higher level model organisms.

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