Final report

Our brains are in a constant state of physical change, with important components constantly being constructed and broken down. In abnormal circumstances, this balance can be shifted for longer or shorter periods of time, whether in brain injury, neurodegenerative disease, or in the case of stroke or infection. One role of glial cells is to keep the brain clean under both normal and pathological conditions. Much is known about how glial cells recognize the material or microbes to be degraded, but there is less underlying research about how these are degraded in glial lysosomes and how glia adapt to challenges such as injury by altered signalling and transcriptional responses. We completed two related projects and laid a solid foundation to a third one during the duration of our OTKA grant.

1. In the first three years of this grant period, we have gathered evidence by studying glial degradation pathways in a model organism, the fruit fly *Drosophila melanogaster*, that certain proteins of the autophagy pathway, the machinery responsible for the lysosomal degradation of intracellular material, regulate the degradation of axonal debris produced during injury, which is known as phagocytosis. This is a compelling scenario because phagocytosis takes up and breaks down extracellular material, unlike autophagy. This process, LC3-associated phagocytosis (LAP), was already known in macrophages, but only began to be characterized in the nervous system in parallel with their research. The experiments were performed by transecting a nerve running through the fruit fly wing, a simple, reproducible and easily visualizable model of traumatic injury. By manipulating glia and neurons separately and disrupting autophagy- and LAP-specific genes, we showed that the functions of autophagy-specific genes already known to contribute to LAP and LAP-specific genes are required for normal debris clearance. The corresponding proteins are also found on vesicles containing phagocytosed axon fragments. We were among the first two groups to describe LAP in glia.
   Inappropriate phagocytic activity in either macrophages or glial cells can be detrimental to vital functions. The absence of LAP after traumatic brain injury led to increased mortality in the long term, demonstrating the importance of debris degradation by LAP. One explanation for such lethal effects may be chronic inflammation, which may also lead to autoimmunity when macrophages are deficient in LAP. The enhancement of LAP through the overexpression of the Rubicon protein was able to yield faster clearance in the damaged nerve, which is a prerequisite

for recovery. Since LAP is already known to be involved in debris clearance in astrocytes in the mammalian brain, it could also serve as a potential therapeutic target in the future.

We published our findings in Nature Communications in May 2024 (Nat Commun. 2023 May 29;14(1):3077. doi: 10.1038/s41467-023-38755-4), Aron Szabo being first and co-corresponding author.

2. As an offshoot of this project, we started to analyse the effect of autophagy-related genes on the activation of glial signal transduction pathways following injury. This was already planned in the OTKA grant workplan. Reactive glia arise after nervous system perturbations and have distinct transcriptional signatures as exemplified by disease-associated microglia in neurodegeneration. They sense damage- and pathogen- associated molecular patterns (DAMPs and PAMPs), find-me and eat-me cues via dedicated receptors to mount an immune response. They also release various chemokines and cytokines to spread and control neuroinflammation. Upon CNS injury, these receptors trigger signalling pathways that include MAPK, JAK-STAT and NF-κB pathways. The transcriptomic changes in reactive glia show a great contribution from these signalling events.

   The JAK-STAT pathway is an important but less studied part of glial immunity, especially compared to the NF-κB pathway. Most interleukin and interferon receptors signal through the JAK-STAT pathway in mammals and induce the expression of either pro- or anti-inflammatory cytokines depending on the STAT paralog, both in peripheral immune cells and in glia. In neurodegenerative diseases, the JAK-STAT pathway contributes to neuroinflammation. Increased interferon-dependent microglial STAT1 activation is observed in models of Alzheimer's disease, causing complement-dependent synapse elimination. Stat92E transcription factor is the only known Drosophila orthologue of the Signal Transducer and Activator of Transcription (STAT) protein family.

   In the last two years of the grant period, we aimed to find pathways or interactors that modulate the activity of Stat92E in glia, inspired by the apparent caveat in the upstream regulation of Stat92E in injury responses in Drosophila. We used the same wing injury model as for the previous study on LAP. We investigated two glial signalling pathways that are known to be activated in glia after central nervous system injury, JNK signalling and Stat92E-regulated transcription (Fig. 1). The JNK activity reporter *TRE-EGFP* and *10XStat92E-dGFP* was measured after wing injury and *Atg5* silencing in glia. These reporters show strong upregulation upon injury in controls. Only Stat signalling was abrogated by *Atg5* knockdown. This effect was replicated by *Atg16* and *Atg13* RNAi, but was *Rubicon*-independent, thereby suggesting a

pathway that does not rely on LC3-associated phagocytosis. Accordingly, canonical autophagy is involved in glial Stat92E activation after injury.

We explored potential selective autophagy cargoes and found Su(var)2-10 (PIAS1), a negative regulator of Stat92E. PIAS1 functions as a SUMO ligase on STATs to inhibit their activity. Silencing *Su(var)2-10* in wing glia positively affected Stat92E activation exclusively following injury (Fig. 2).

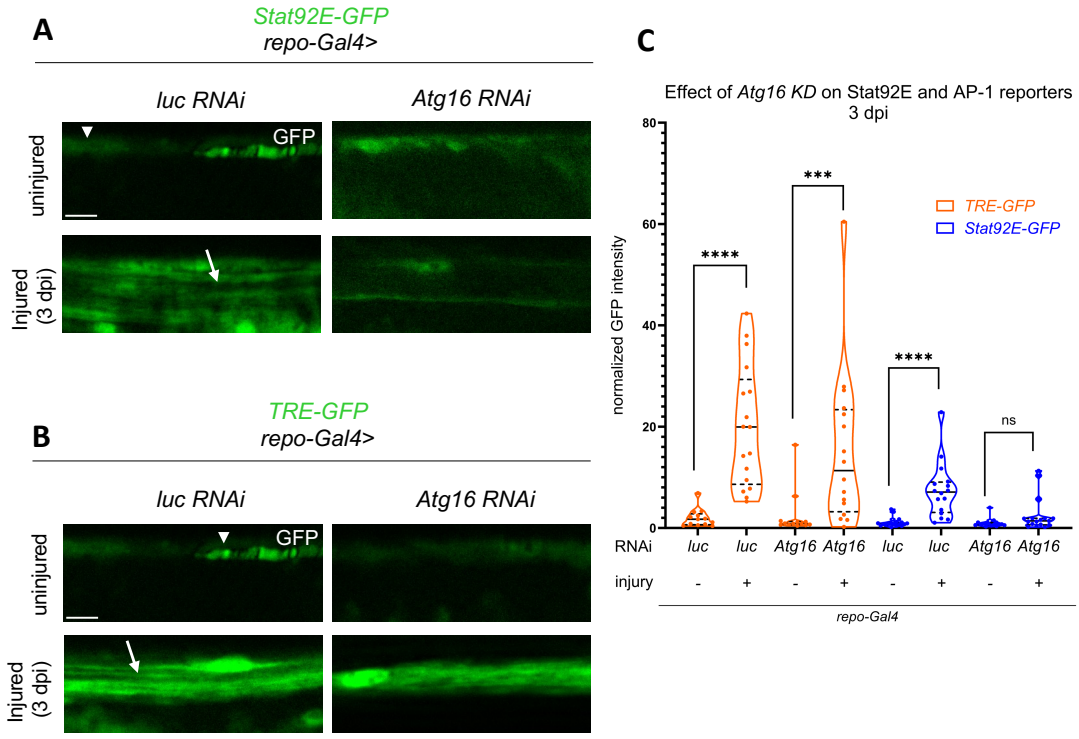

Figure 1. Stat92E-dependent transcription is abrogated by autophagy disruption in glia

Stat92E reporter is upregulated in glia after injury (arrows) that is abrogated by Atg16 RNAi (A). This effect is not observed in an AP-1 (TRE) reporter (B).

Interestingly, Su(var)2-10 levels were increased in *Atg16* silenced and *Atg101* mutant glial nuclei in the brain and upon lysosome inhibition in S2 cells (Fig. 2). Disruption of Su(var)2-10 SUMOylation by a specific mutation (CTD2) blocked its lysosomal degradation. An epistasis test revealed that autophagy may regulate Stat92E activity in glia by eliminating excess Su(var)2-10 to prevent Stat92E inhibition following injury. Stat92E upregulates several injury-responsive genes. We first concentrated on *drpr,* a prime target of Stat92E but failed to observe an injury- and autophagy-dependent transcription in wing glia. Instead, we found that *virus-induced RNA 1* (*vir-1*) is targeted and upregulated by Stat92E in glia upon injury that is promoted by autophagy (Fig. 3). This occurs in absence of viral infection, hinting to an unexpored mechanism of glial immunity. These findings indicate that glial reactivity after injury relies on Stat92E activation by autophagy via elimination an inhibitory element.

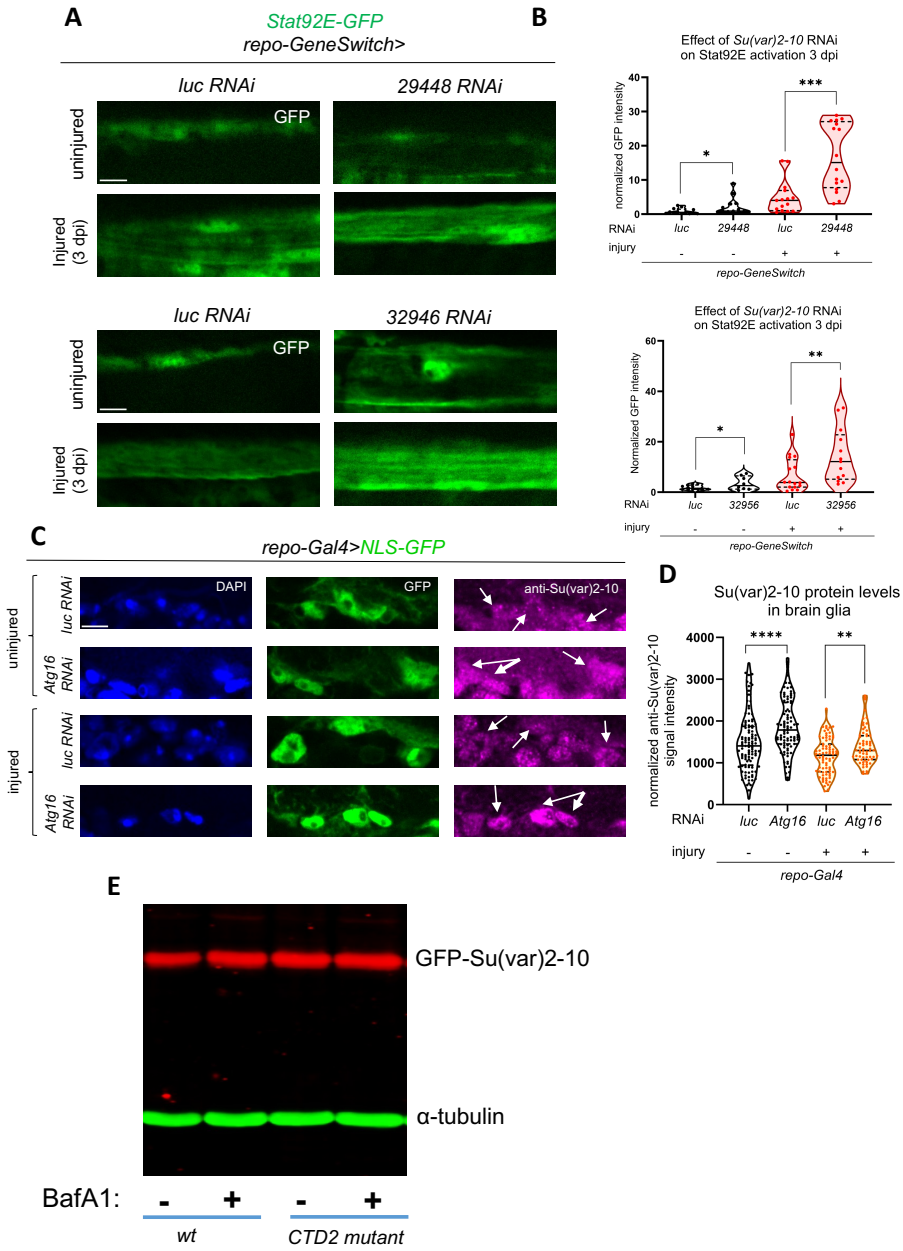

Figure 2. Autophagy clears Su(var)2-10 to promote Stat92E activation

Su(var)2-10 RNAi (29448 and 32946 constructs) relieves Stat92E-GFP repression in glia (A,B). In brain glia nuclei, Su(var)2-10 protein levels are elevated after *Atg16* RNAi (C,D). In S2 cell culture, Su(var)2-10 protein levels increase after lysosome inhibition (Bafilomycin A1) and in a CTD2 mutant disrupting Su(var)2-10 SUMOylation.

Next, we would like to understand the role *vir-1* plays in the glial injury response. In preliminary experiments, we subjected flies to traumatic brain injury and followed the survival of both wild-type and *vir-1* loss-of-function mutant animals over time. We see impaired survival of vir-1 mutants in injured conditions that suggests an important function of this gene in this context.

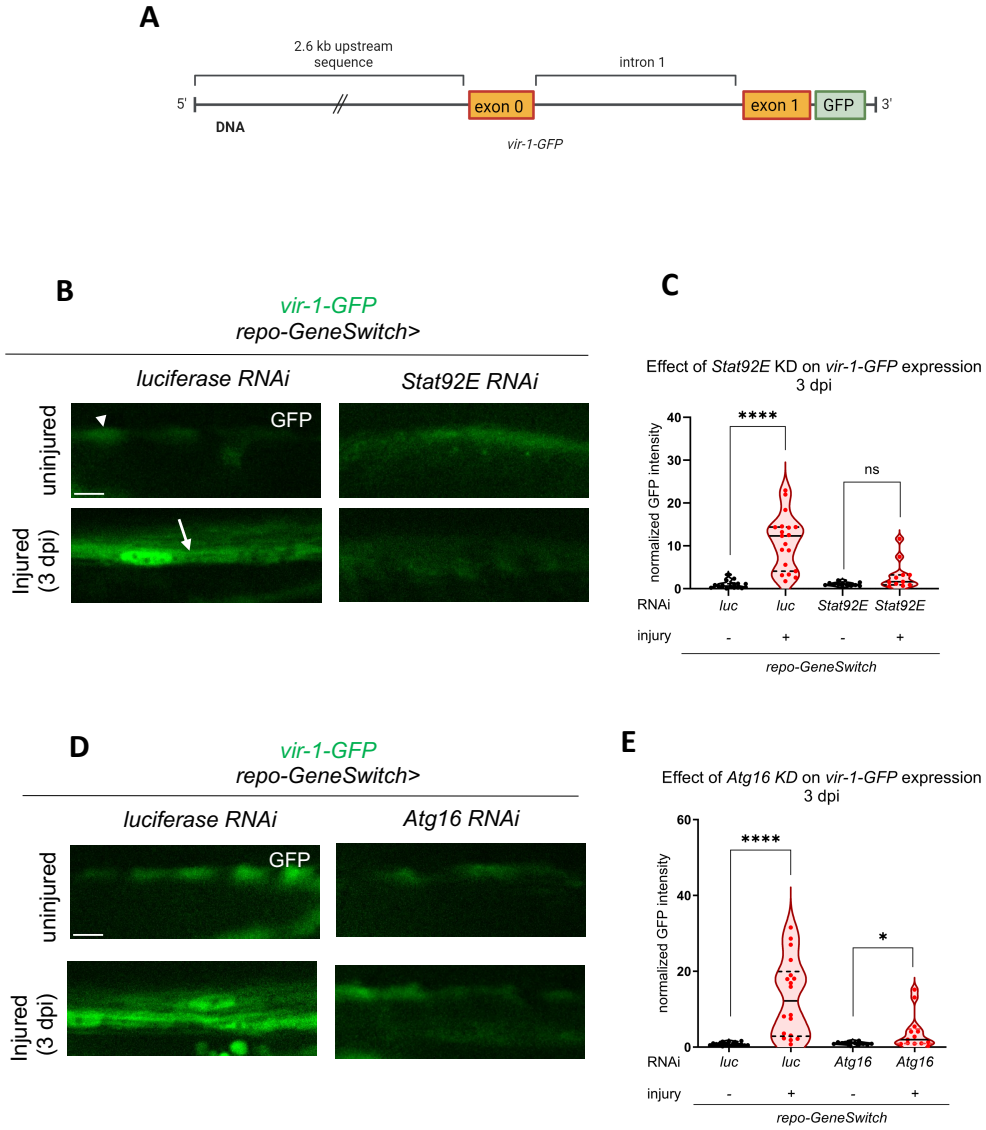

Figure 3. *vir-1* is a glial target of autophagy-regulated Stat92E following injury

A-C. *vir-1* is a STAT92E target that is upregulated after injury. D-E. *Atg16* RNAi in glia disrupts *vir-1* upregulation after injury.

Furthermore, we have preliminary RNA sequencing results from wings proficient or deficient in *Atg5* only in glia. This shows hundreds of genes with altered regulation after injury that depends on glial *Atg5,* including Stat92E targets. We found antimicrobial peptides that bear Stat binding sites and are not induced after injury upon Atg5 silencing in glia. This signifies the importance of autophagy in neuroinflammation.

We will upload a preprint from this study in August to bioRxiv and submit it to Cell Reports in September.

3. As a last aim in the OTKA workplan, we envisioned to generate a novel Drosophila Alzheimer's disease model by expressing the toxic Aβ42 Arctic peptide independently of the Gal4-UAS system and only in adults in a conditional manner. This would form the basis of evaluating the role of canonical autophagy or LAP in Aβ42 aggregate clearance. We established this fly model that achieves both inducible expression of Aβ42 in neurons via the Q system and allows for manipulation of genes by Gal4 in the glial domain. We created a *QUAS-Aβ42^{Arctic}* transgenic line by inserting the construct into attP landing sites. We drove this construct into all neurons with *elavC155-QF2* and observed considerable expression by immunostaining that showed formation of small Aβ42 aggregates. We built genotypes that by a single cross will knock down genes in glia that may mediate Aβ42 clearance (e.g. LC3-associated endocytosis/phagocytosis or autophagy) and we will track Aβ42 abundance that is emmitted from neurons. This will be followed by physiological assays to assess the *in vivo* importance of Aβ42 clearance by *Atg* genes.
   This project part suffered a setback as compared to the work plan (years 3 and 4) due to intense efforts to elucidate the role of autophagy in Stat activation (point 2) that will result in an earlier publication in the coming months.