

A Pvr–AP-1–Mmp1 signaling pathway is activated in astrocytes upon traumatic brain injury

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2

April 8, 2024 (this version)

Reviewed preprint version 1

June 13, 2023

Posted to preprint server

March 28, 2023

Sent for peer review

March 28, 2023

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Abstract

Traumatic brain injury (TBI) caused by external mechanical forces is a major health burden worldwide, but the underlying mechanism in glia remains largely unclear. We report herein that *Drosophila* adults exhibit a defective blood-brain-barrier (BBB), elevated innate immune responses, and astrocyte swelling upon consecutive strikes with a high-impact trauma device. RNA sequencing (RNA-seq) analysis of these astrocytes revealed upregulated expression of genes encoding PDGF and VEGF receptor-related (Pvr, a receptor tyrosine kinase (RTK)), adaptor protein complex 1 (AP-1, a transcription factor complex of the c-Jun N-terminal Kinase (JNK) pathway) composed of Jun-related antigen (Jra) and kayak (kay), and matrix metalloproteinase 1 (Mmp1) following TBI. Interestingly, Pvr is both required and sufficient for AP-1 and Mmp1 upregulation, while knockdown of AP-1 expression in the background of Pvr overexpression in astrocytes rescued Mmp1 upregulation upon TBI, indicating that Pvr acts as the upstream receptor for the downstream AP-1–Mmp1 transduction. Moreover, dynamin-associated endocytosis was found to be an important regulatory step in downregulating Pvr signaling. Our results identify a new Pvr–AP-1–Mmp1 signaling pathway in astrocytes in response to TBI, providing potential targets for developing new therapeutic strategies of TBI.

Main Points

- The study provided RNA-seq data of astrocytes following traumatic brain injury (TBI)
- Genes involved in endocytic trafficking are upregulated in astrocytes after TBI
- A new Pvr–AP-1–Mmp1 pathway is activated in astrocytes following TBI.
- Inhibition of endocytosis in astrocytes upregulates the Pvr–AP-1–Mmp1 signaling.

eLife assessment

This study represents a **valuable** finding on the neuron-glia communication and glial responses to traumatic brain injury (TBI). The data supporting the authors' conclusions on TBI analysis, RNA-seq on FACS sorted astrocytes, genetic analyses on Pvr-JNK/MMP1 are **solid**. However, cellular aspects of the response to TBI, statistical analysis, and molecular links between Pvr-AP1 are **incomplete**, which could be further strengthened in the future by more rigorous analyses.

Introduction

Traumatic brain injury (TBI) is one of the leading causes for adult disability due to limited neurological recovery (Joy et al., 2019). About 43% of people discharged after hospitalization for acute TBI develop long-term locomotor disability (Ma et al., 2014). TBI triggers primary neural injuries via rapid physical forces that damage axons, glia and blood vessels (Chodobski et al., 2011; Daneshvar et al., 2015), followed by diverse secondary damage such as Wallerian axon degeneration (Büki and Povlishock, 2006) and blood-brain barrier (BBB) dysfunction (Chodobski et al., 2011) in the central nervous system (CNS). The outcome of TBI is affected by various factors including age and diet (Byrns et al., 2021; Katzenberger et al., 2016). In addition, glial cells are critical mediators in response to TBI in the mammalian brain (Mira et al., 2021). During TBI, astrocytes become active, a process characterized by swelling, scar tissue formation, and engulfment of neuronal debris (Liddelow et al., 2017; Sofroniew and Vinters, 2010), while microglia rapidly transform to their active state to phagocytose cell debris (Davalos et al., 2005).

Drosophila melanogaster is an ideal model organism for discovering new gene networks and investigating the molecular and cellular responses to CNS injury (Ayaz et al., 2008; Bier, 2005; Hoopfer et al., 2006; MacDonald et al., 2006). *Drosophila* astrocyte-like glia (henceforth astrocytes), the conserved counterparts for mammalian astrocytes, and ensheathing glia (ENG) constitute the major glial subclasses in the CNS (Doherty et al., 2009; Stork et al., 2014). A previous study revealed that *Drosophila* ENG perform the phagocytic function by the engulfment receptor Draper which interacts with downstream signaling components, including ced-6, Shark, Src42a, c-Jun N-terminal Kinase (JNK), and Mmp1 in the glial engulfment process in response to axotomy (Awasaki et al., 2006; Doherty et al., 2014; Lu et al., 2014; Lu et al., 2017; Purice et al., 2017). Although ENG function upon axotomy has been well characterized, the response of other types of glia such as astrocytes to neural injury remains unclear.

In mammals, signaling by platelet-derived growth factor (PDGF) directs cell migration, alters cell shape, and plays a role in wound healing (Hoch and Soriano, 2003; Sil et al., 2018). Meanwhile, vascular endothelial growth factor (VEGF) signaling is critical for cell proliferation and survival, and vascular endothelial cell mitogenesis and permeability (Apte et al., 2019; Sakurai et al., 2005). Pvr, short for PDGF- and VEGF receptor-related, is the sole *Drosophila* ortholog of the PDGF and VEGF receptor families (Cho et al., 2002). Pvr interacts with different signaling molecules in a context-dependent manner. For example, Pvr regulates thorax closure during metamorphosis via the c-Jun N-terminal Kinase (JNK) signaling pathway (Ishimaru et al., 2004), but the Pvr-mediated wound-induced epidermal cell migration does not depend on JNK (Wu et al., 2009). The JNK/Activator Protein-1 (AP-1) signaling pathway is involved in different cellular processes, including cell migration (Pastor-Pareja et al., 2004), organ size control (Willsey et al., 2016), and cell death (Moreno et al., 2002). The JNK effector AP-1 complex, composed of heterodimeric Jun/Jun-related antigen (Jra) and Fos/kayak (kay), promotes

transcription of genes including matrix metalloproteinase 1 (Mmp1) that functions in tissue remodeling, regulation of inflammatory processes, and metastasis of cancer cells (Egeblad and Werb, 2002 [↗](#); Page-McCaw et al., 2007 [↗](#); Parks et al., 2004 [↗](#)).

Previous studies have implicated a role for ensheathing glia, Draper pathway, and the innate immune pathway in response to TBI (Doherty et al., 2009 [↗](#); Lu et al., 2017 [↗](#); Purice et al., 2017 [↗](#); Katzenberger et al., 2013 [↗](#)). However, whether astrocytes also play a role in TBI response remains elusive. Herein, using a *Drosophila* TBI model induced with a high-impact trauma (HIT) device (Katzenberger et al., 2013 [↗](#)), we found that TBI caused multiple defects including upregulated expression of innate immune genes and astrocyte swelling. To uncover the molecular pathways activated by TBI, we performed RNA sequencing (RNA-seq) analysis of astrocytes sorted by fluorescence-activated cell sorting (FACS) to profile TBI-induced genes. Interestingly, we showed that the expression of Pvr, AP-1, and Mmp1 was upregulated following TBI. Knockdown of Pvr or AP-1 expression in astrocytes rescued TBI-induced Mmp1 upregulation, while Pvr overexpression promoted Mmp1 expression via AP-1 upon TBI. Our findings identify a new Pvr–AP-1–Mmp1 signaling pathway that acts predominantly in astrocytes in response to TBI, and shed light on the mechanisms of astrocyte responses following TBI.

Materials and Methods

Drosophila stocks and husbandry

Flies were maintained on standard cornmeal medium at 25°C otherwise indicated. The following *Drosophila* strains were used: *w¹¹¹⁸*, *UAS-mCD8::GFP*, *R86E01-Gal4* (#45914; Bloomington), *R54F03-Gal4* (#39157; Bloomington), *UAS-Redstinger* (#8547; Bloomington), *UAS-Jra* (#7216; Bloomington), *UAS-kay* (#7213; Bloomington), *UAS-Pvr* (#58429; Bloomington), *UAS-nLacZ* (#3955; Bloomington), *PCNA-GFP* (#25749; Bloomington) (Thacker et al., 2003 [↗](#)), and *TRE-dsRed* (Chatterjee and Bohmann, 2012 [↗](#)). *UAS-draper-RNAi*, *UAS-Pvr-RNAi*, *UAS-Jra-RNAi*, *UAS-kay-RNAi*, *UAS-Arpc2-RNAi*, *UAS-cpa-RNAi*, *UAS-cpb-RNAi*, *UAS-Stam-RNAi*, *UAS-Vps24-RNAi*, and *UAS-Chmp1-RNAi* flies were obtained from Tsinghua Fly Center (<https://thfc.zzb.org/en> [↗](#)). *yw UAS-CD8::GFP hs-FLP; tubP-Gal80 FRT-40A/CyO; alrm-Gal4 UAS-CD8::GFP UAS-Dcr2/TM6 Tb Hu* (alrm-Gal4 is a specific driver in astrocytes (Doherty et al., 2009 [↗](#)), a gift from Oren Schuldiner) and *FRT-40A* (a gift from Liqun Luo) were used for MARCM analysis of astrocytes. *UAS-shibire^{ts1}* (Pfeiffer et al., 2012 [↗](#)) was provided by Y. Zhong at Tsinghua University.

Traumatic brain injury (TBI) Assay

Construction and use of the HIT device were described previously (Katzenberger et al., 2013 [↗](#)). Deflection of the spring to 90° was the standard angle for strike. Mortality index at 24 h (MI₂₄) were determined by analyzing at least 120 flies (20 flies for each test).

Dye penetration assay

Dye penetration experiments were performed as previously described (Bainton et al., 2005 [↗](#)). 10 kD Texas red-conjugated dextran (Molecular Probes) at 2.5 mM was injected into the soft tissue between two abdominal segments of the exoskeleton of flies with and without TBI. Flies were allowed to recover in fresh food tubes and photographed 2 h later on a Zeiss microscope to detect the integrity of brain-eye barrier (BEB). To detect the integrity of BBB, fly brains were dissected at 2 h after injection and examined under an Olympus FluoView FV1000 microscope.

MARCM analysis

Mosaic analysis with a repressible cell marker (MARCM) analysis in astrocytes was carried out largely following previous publications (Jia et al., 2019 [↗](#); Lee and Luo, 1999 [↗](#)). Specifically, newly laid eggs were collected for a 4–5 h interval and cultured at 18°C. Clones were induced by heat shock at 24 h after larval hatching in a 37°C water bath for 15 min and flies were raised at 25°C after heat shock until immunohistochemistry analysis of adult brain.

Immunohistochemistry analysis and confocal microscopy

Brain tissues were dissected out from adult after specific treatments. Fixation and staining of adult brains were performed as previously described (Jia et al., 2019 [↗](#)). Brains were mounted on slides in Vectashield mounting media (Vector Labs). Primary antibodies were used at the following dilutions: chicken anti-GFP (ab13970, Abcam) at 1:1000, mouse anti-Repo (8D12, DSHB) at 1:100, mouse anti-Elav (9F8A9, DSHB) at 1:100, mouse anti-Mmp1 (3A6B4, 3B8D12, and 5H7B11, DSHB) each at 1:20 for all three antibodies, rabbit anti-Pvr (McDonald et al., 2003 [↗](#)) at 1:1000, rabbit anti-Dcp-1 (Asp216, Cell Signaling Technology (CST)) at 1:100, rabbit anti-pH3 (06-570, Millipore) at 1:1000, and mouse anti-β-gal (40-1a, DSHB) at 1:100. All secondary antibodies (Alexa Fluor 488, 568 or 633 labeled; Molecular Probes) were used at a dilution of 1:1000. The nuclei dye To-Pro-3 Iodide (T3605, ThermoFisher) was also used at a dilution of 1:1000. The cell-permeant Mitotracker (M22426, ThermoFisher) was used at a concentration of 100 nM. For soma diameter analysis, we measured the average of long and short axes of astrocytes. All images were collected using an Olympus FV1000 laser scanning confocal microscope and processed with ImageJ. Serial images of 25–30 μm (one image per 1 μm slice) were projected for presentation and statistical analysis.

Astrocyte preparation for RNA-seq analysis

Flies were collected at 3 days after eclosion and treated with TBI. Brains from adult flies (*R86E01-Gal4/UAS-Redstinger*) with no injury, 4 h after injury and 24 h after injury were dissected out in freshly prepared adult hemolymph saline (AHS) buffer, as previously described (Yang et al., 2016 [↗](#)). Briefly, a total of ~50 brains for each group was immediately transferred to an Eppendorf tube (1.5 mL) containing 200 μL AHS with Pronase (1 mg/mL; P5147, SigmaAldrich) and Dispase (1 mg/mL; LS02104, Worthington Biochemical Corporation). After tissue digestion for 30 min at 25°C, the enzyme medium was removed, followed by two brief, gentle washes with fresh AHS containing 2% PBS. Brain samples were then triturated by gently pipetting in 1 mL AHS through four fire-polished Pasteur pipettes with descending pore sizes (400 μm, 300 μm, 200 μm, and 100 μm) for a total of ~20 min. Next, the dissociated cells were centrifuged for 10 min at 100 × g and the AHS was replaced with the same buffer to minimize any RNA content released from damaged cells during the trituration process. Finally, dissociated tissues were filtered through 45 μm filter and then put on ice. FACS sorting was performed according to the following gating procedure with 70 μm nozzle by BD FACS Aria: (1) an initial SSC-A/FSC-A gate to minimize debris; (2) a SSC-A/pe-Texa Ted-A gate to choose only red fluorescence positive cells. At this step, 1600 cells were sorted directly into ice-cold cell lysis buffer (oligo-dT₃₀VN primer, ribonuclease inhibitor, dNTP mix) and stored at -80°C until all samples were ready for RNA extraction and cDNA amplification.

cDNA library preparation

A total of 5 biological replicates were collected for each condition (no injury, 4 h after injury, and 24 h after injury). Cell samples were sent to the Anoroad Company for RNA extraction. An Oligo-dT primer was introduced to the reverse transcription reaction for first-strand cDNA synthesis, followed by PCR amplification to enrich the cDNA and magbeads purification step was used to purify the products. Briefly, Smart-Seq2 method was used to amplify cDNA. Concentration and integrity of cDNA was assessed using Qubit 3.0 Fluorometer (Life Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies) to ensure the cDNA length was around 1–2 kbp. After quality

control, 40 ng cDNA was used to be fragmented at 350 bp by Bioruptor® Sonication System (Diagenode Inc.). To construct Illumina library, end repair, 3' ends A-tailing, adaptor ligation, PCR amplification and library validation were performed. PerkinElmer LabChip® GX Touch and Step OnePlus™ Real-Time PCR System were used for library quality inspection. Qualified libraries were loaded on Illumina HiSeq platform for PE150 sequencing.

RNA-seq and data analysis

A total of 539 million reads (average 35.9 million reads per sample) was obtained. The fastq data were first processed with Trimmomatic (Bolger et al., 2014) to remove Illumina adaptor. The remaining data was aligned against the UCSC *D. melanogaster* genome (dm6) using Hisat2 (Kim et al., 2015; Kim et al., 2019; Pertea et al., 2016) with the annotation file of the same version. On average, 92% of the initial reads were mapped to dm6. HTSeq was used to calculate the raw read counts of genes for each sample. DESeq2 (Love et al., 2014) was used to normalize the data. Transcripts were considered as significantly differentially expressed if their adjusted *p* values were less than 0.01 ($p < 0.01$) by the Benjamini-Hochberg method. Following normalization of the data, DESeq2 was used to perform pairwise comparisons between the control and experimental groups using parametric tests. Genes with absolute value of logarithmic fold change ($\log_2FC$) > 1.0 were qualified for the final set of DEGs. Volcano plots and heatmap were constructed for visualization of the resulting expression intensity data following the identification of DEGs and RNA-Seq data normalization by the adjusted *p* value calculations.

Quantitative reverse transcription-PCR analysis

RT-qPCR was performed largely based on previous protocols (Zhao et al., 2020). Flies were treated with the same conditions as the RNA-seq experiment. RNA for antimicrobial peptides (AMPs) analysis was extracted from whole fly brains by TriZol. RNA for confirming RNA-seq findings was isolated from more than 10000 astrocytes sorted by FACS using the PicoPure RNA isolation kit (KIT0204, Life Technologies). cDNA was generated with SuperScript III First-Strand Synthesis SuperMix (11752250, Invitrogen). For each gene, at least three pairs of qPCR primers were screened by standard curve analysis, and the best primer pairs were chosen based on their efficiency and specificity. Real-time qPCR was carried out by KAPA Library Quantification Kit (KR0389, KAPA Biosystems). Primer sequences are provided in Table S1.

Thermogenetic inhibition of endocytosis

For inhibition of endocytosis in astrocytes using *UAS-Shi^{ts}*, crosses were reared and treated by HIT device at 23°C to avoid unintended inactivation. For thermogenetic inactivation, flies after TBI were undergone intermittent temperature treatment at 32°C (32°C for 30 min, and then back to 23°C for 30 min, for four rounds, four hours in total) until dissection.

Statistical analysis

All statistical comparisons were performed using GraphPad Prism software, with significance indicated by *** ($p \leq 0.001$), ** ($p \leq 0.01$), * ($p \leq 0.05$), and ns ($p > 0.05$). One-way ANOVA was used to compare multiple group means. Student's t-tests were used for statistical comparisons between two groups.

Results

TBI is induced in *Drosophila* adults by a high-impact trauma (HIT) device

To analyze how brains respond to TBI, we applied consecutive strikes to adult flies using a HIT device to induce brain injury as described previously (Katzenberger et al., 2015). To quantify the degree of injury, we analyzed the mortality index at 24 h after TBI (MI₂₄), defined as the percentage of dead over the total flies tested. Consistent with a previous report (Katzenberger et al., 2013), MI₂₄ of 3-day-old adult flies was $20 \pm 3.18\%$ after 4 HIT strikes (Figure 1A). MI₂₄ after 6 strikes was $71.88 \pm 7.44\%$, suggesting that increasing the number of strikes results in a higher MI₂₄ rate (Figure 1A). Given that TBI has been shown to disrupt the formation of the blood-brain barrier (BBB) in mammals and flies and blood-eye barrier (BEB) in flies (Rodríguez-Baeza et al., 2003; Sangiorgi et al., 2013; Katzenberger et al., 2015; Saikumar et al., 2020), we examined BBB/BEB integrity 2 h after injecting tetramethylrhodamine-conjugated dextran (molecular weight = 10,000 Da) into adult fly abdominal segments (Bainton et al., 2005). The dye crossed the barrier and penetrated the retina and brain, indicating that both BBB and BEB were disrupted after HIT strikes (Figures 1B–G). The percentage of flies exhibiting dextran-permeable BBB increased as the number of strikes increased from 0 to 6, with six strikes resulting in severe BBB disruption (Figure 1G) but maintaining a moderate viability providing enough samples for phenotypic analysis. Based on these results, six HIT strikes administered at 5 min intervals were applied to induce TBI throughout the present study.

In addition to disruption of BBB/BEB integrity, we also analyzed TBI-induced secondary damage such as local and systemic immune responses. Given that Toll and immune deficiency (Imd) are the two crucial innate immune response pathways in *Drosophila* (Valanne et al., 2011), and their common downstream genes encode antimicrobial peptides (AMPs) that protect flies from TBI injury (Alphen et al., 2022), we analyzed the expression of AMPs in adult fly brains at different time points after TBI. As shown by the real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) results, expression of the AMP genes *AttC*, *DiptB* and *Mtk* was significantly upregulated at 1 h and reached a peak at 4 h after injury (Figure 1H), consistent with previous reports (Barekat et al., 2016; Katzenberger et al., 2013). Taken together, these results suggest that HIT strikes induce TBI reflected by BBB/BEB disruption and elevated expression of innate immune response genes.

TBI induces neuronal but not glial apoptosis in adult fly brains

Given that neurons and glia have been shown to undergo cell death during both primary and secondary injury responses upon TBI in humans (Ng and Lee, 2019), we performed immunostaining with an antibody against Death caspase-1 (Dcp-1), the homolog of human caspase-3, to detect apoptotic cells in our TBI model. Interestingly, the number of Dcp-1⁺ puncta was significantly increased in adult fly brains at 24 h after TBI (Figures 1I and J), with a 2.47-fold increase compared to the number in uninjured controls (Figure 1K). Furthermore, we found that almost all Dcp-1⁺ puncta were co-localized with Elav rather than Repo, suggesting that neurons but not glia are apoptotic (Figures 1L–M). These results suggest that, as in mammals, TBI also induces neuronal apoptosis in fruit flies.

TBI leads to astrocyte activation

In addition to the TBI-induced neuronal apoptosis mentioned above, glial cells also become reactive after nervous system injury (Burda and Sofroniew, 2014). It has been shown that ENG phagocytose neuronal debris upon axotomy (Doherty et al., 2009), but whether astrocytes respond to TBI remains unknown. To explore the potential role of astrocytes in the TBI response,

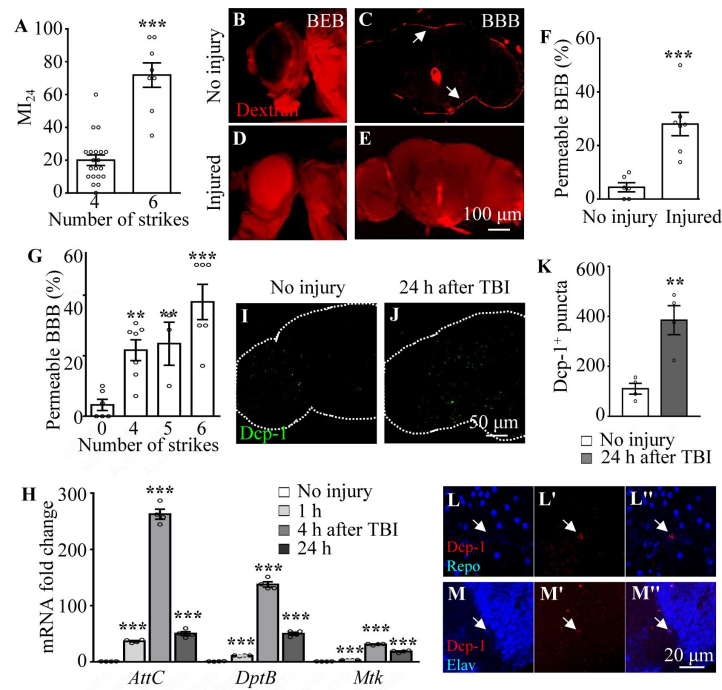


Figure 1.

TBI is induced in adult flies by a high impact trauma (HIT) device.

(A) MI₂₄ is the mortality index calculated from the percentage of dead over total flies. Increasing the number of strikes from 4 to 6 increases the MI₂₄ value. Error bars denote standard error of the mean (SEM; ***p < 0.001, Student's t-test). (B-E) The intactness of blood-eye-barrier (BEB) and blood-brain-barrier (BBB) before or after 6 strikes is indicated by the accumulated fluorescence intensity at the border of the eye (B, D) and brain (C, E), respectively. The examination was carried out at 2 h after fluorescent dye injection. Arrows indicate the hemolymph exclusion line. Scale bar = 100 μm. (F) The percentage of permeable BEB with or without TBI (***p < 0.001). (G) The percentage of permeable BBB following different numbers of strikes. Error bars denote SEM (**p < 0.01 and ***p < 0.001, Student's t-test). (H) Histogram showing mRNA expression levels of innate immune response genes of the AMP family (*Attc*, *DptB* and *Mtk*) in the adult brain. Expression was increased in all cases following treatment. The *RPL28* gene served as an internal reference. Results are means ± SEM (***p < 0.001, Student's t-test). (I, J) The number of Dcp-1⁺ puncta (green) increased in injured adult brains 24 h after TBI. Scale bar = 50 μm. (K) Quantification of Dcp-1⁺ puncta. Error bars denote SEM (**p < 0.01, Student's t-tests). Results are means ± SEM. (L-M'') Apoptotic debris labelled with anti-Dcp-1 antibodies (Red). Glial and neuronal nuclei are labelled with anti-Repo (L) and anti-Elav (M) antibodies (Blue), respectively. Scale bar = 20 μm.

R86E01-GAL4 (Kremer et al., 2017 [↗](#)), an astrocyte-specific driver, was used to express *UAS-mCD8::GFP* to label astrocyte plasma membrane. In uninjured fly brains, GFP intensities in the cell bodies and processes of astrocytes were widely distributed throughout the brain (**Figures 2A** [↗](#) and **2D** [↗](#)). While no significant change in GFP intensity was detected in brains at 4 h after TBI (**Figures 2B** [↗](#) and **2D** [↗](#)), the GFP intensity was significantly increased and the cell bodies of astrocytes were significantly enlarged at 24 h after TBI in the central brain region, especially in the antennal lobes (ALs) (**Figures 2C** [↗](#) and **2D** [↗](#)). Of note, these results were not affected by the presence of one or two UAS transgenes as the efficiency of *R86E01-GAL4* in driving the expression of either was comparable as shown by the similar GFP levels (**Figures 2A** [↗](#)–C, I, and J).

In mammals, the hallmarks of astrocyte activation are hypertrophy of astrocytes and upregulated level of the glial fibrillary acidic protein (GFAP) (Pekny et al., 2004). Given that there's no GFAP homolog in *Drosophila* (Doherty et al., 2009 [↗](#)), we examined morphological changes in astrocytes at single-cell resolution following TBI by mosaic analysis with a repressible cell marker (MARCM) on adult fly brains at 3–4 days after eclosion. The cell bodies of these astrocytes were located at the interface between neuropil and cortex regions, or in neuropils, and their cellular processes infiltrated into neuropils (**Figure 2E** [↗](#)). Interestingly, astrocytes in injured brains exhibited bright and distinct GFP⁺ accumulations associated with their cellular processes at 24 h after TBI (**Figures 2F** [↗](#) and **2G** [↗](#)). Quantitatively, the number of these accumulations per square nanometer in the cellular processes was significantly increased at 24 h after TBI; the increase in the number of accumulations might be responsible for the increase in GFP intensity triggered by TBI (**Figure 2G** [↗](#)). Importantly, the diameter of astrocytes cell bodies was also increased at 24 h after injury (**Figure 2H** [↗](#)). Taken together, these results show that astrocytes become reactive in adult brains at 24 h after TBI.

Axotomy induces expression of injury-related proteins such as extracellular proteases the matrix metalloprotease (MMP) (Purice et al., 2017 [↗](#)). In addition to the morphological changes in astrocytes, we observed upregulated expression of *Mmp1* in TBI-treated brains (**Figures 2I** [↗](#)–J", 2M–N", and 2Q). These *Mmp1*⁺ signals were colocalized with membrane-bound *mCD8::GFP* driven by the astrocytes-specific driver *R86E01-GAL4* or ENG-specific driver *R56F03-GAL4* (**Figures 2K** [↗](#), 2L, 2O, and 2P), with a more dominant expression in ENG. These results suggest that *Mmp1* might be produced from both types of glia after TBI.

Transcriptional profiling of astrocytes in adult flies reveals elevated expression of injury-related genes upon TBI

To further investigate how astrocytes respond to TBI, we analyzed transcriptional changes in astrocytes by RNA-seq (**Figure 3A** [↗](#)). Due to the tiny soma and morphological complexity, the analysis of a purified population of glial cells has been difficult and technically challenging. We have optimized a protocol for purifying astrocytes from adult *Drosophila* of *R86E01-Gal4>UAS-Redstinger* by fluorescence-activated cell sorting (FACS) (see details in Materials and Methods). As FACS removes most cell masses and RFP-negative cells, the remaining RFP⁺ cells are mostly viable, as shown by MitoTracker staining, and purity was 92.96% (RFP⁺ and MitoTracker⁺ cells/total cells) in uninjured brains (**Figures S1A–C** [↗](#)). Further analysis of the RNA-seq data showed that the astrocyte markers *Eaat1* (110359 reads) and *alrm* (186413 reads) were highly expressed, while *nSyb*, a neuronal marker, was hardly detected (125 reads) at 4 h after TBI, verifying the purity of FACS-sorted astrocytes. A principal component analysis (PCA) revealed that the variance between different groups (no injury vs. 4 h and 24 h after injury) was greater than the variance within groups (**Figure S1D** [↗](#)). From analysis on five biological replicates for each time point (no injury, 4 h, and 24 h after injury), we obtained 539 million reads (an average of 36 million reads per sample) and aligned the RNA-seq reads to the *Drosophila* genome. We found that 93.67 ± 0.89% of reads mapped to the *Drosophila* genome (dm6 version). Using a cutoff of ≥ 2-fold differential

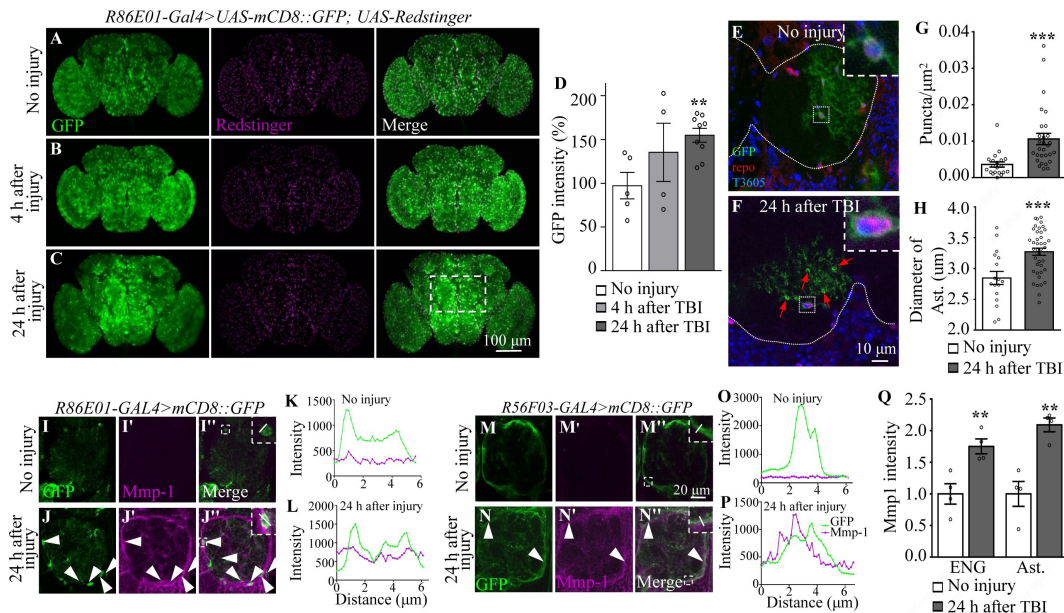


Figure 2.

TBI alters glial morphology and upregulates Mmp1 expression.

(A–C) Representative images of astrocyte membranes labelled with *mCD8::GFP* driven by the astrocyte driver *R86E01-Gal4*. Co-expression of *UAS-Redstinger* labels astrocyte nuclei. GFP intensities are increased at 4 h (B) and 24 h (C) after injury. The rectangle defined by white dash lines denotes antennal lobes. Scale bars = 100 μm. (D) Quantification of GFP intensity at different timepoints after TBI. Results are means ± SEM (***p* < 0.01, Student's *t*-test). (E, F) MARCM (*y w UAS-CD8::GFP hs-FLP; tubP-Gal80 FRT-40A/CyO; alrm-Gal4 UAS-CD8::GFP UAS-Dcr2/TM6 Tb Hu* cross with *FRT-40A* flies) analysis reveals single astrocytes with evenly distributed GFP⁺ cellular processes without injury (E) and an increased number of GFP⁺ puncta at 24 h after injury (F). Arrows denote GFP⁺ accumulations in astrocyte processes. White dots demarcate neuropils. Anti-Repo antibodies labelled glial nuclei. TO-PRO labelled all nuclei. Insets show the morphology of a glial soma before and after injury. Scale bar = 10 μm. (G) Quantification of GFP⁺ puncta in astrocyte processes before and after injury. (H) Quantification of the astrocyte soma diameter surrounding ALs after injury. Results are means ± SEM (***p* < 0.001, Student's *t*-test). (I–J'') Representative images of ALs co-labelled with GFP and Mmp1 (magenta) in uninjured and injured adult brains. (J–J'') GFP-labelled astrocyte membranes (green) overlapped with Mmp1 (magenta) after injury. (N–N'') After injury, Mmp1 co-localizes with GFP-labelled ENG (*R56F03-Gal4*) membranes (green). Arrows show Mmp1-GFP colocalization in L'' and P''. Scale bar = 20 μm. (K, L) Graph showing fluorescence intensity per pixel in each channel for the inset in I'' and J'' of the astrocyte membrane and Mmp1 along a line drawn through a cell body. (O, P) Graph showing fluorescence intensity per pixel in each channel for the inset in M'' and N'' of the ENG membrane and Mmp1 along a line drawn through the process of ENG. (Q) Quantification of Mmp1 intensity in I–J'' and M–N''. Ast is the short of astrocytes. Results are means ± SEM (***p* < 0.01, Student's *t*-test).

expression (adj. *p* value <0.01, Benjamini-Hochberg procedure), we identified 522 and 357 genes with altered expression at 4 h (416 upregulated and 106 downregulated) and 24 h (229 upregulated and 128 downregulated) after injury, respectively (**Figures 3B–D**; Table S2).

Gene Ontology (GO) analysis of these upregulated genes revealed that a significant proportion were associated with endosomal transport, actin cytoskeleton, and wound healing (**Figure S2**). Furthermore, we detected upregulated expression of components functioning in the innate immune response after TBI. For instance, expression of the components Relish and Dif of the Toll and Imd pathways was upregulated in astrocytes after TBI (**Figure 3D**), in agreement with a previous finding that Relish is necessary for TBI-induced secondary injuries (Swanson et al., 2020). In addition, expression of injury-related genes including *chic*, *Jra*, *kay*, *rl*, *Cad96Ca*, *Idgf4*, *Stam*, *draper*, *Pvr*, *spict*, *Mmp1* and *NijA*, and receptor genes including *Pvr*, *draper*, *AdoR*, *PGRP-SA*, and *NimA* were upregulated in astrocytes at 4 h or 24 h after injury (Table S3). A set of endocytic trafficking genes was also identified, including *Arpc1*, *Arpc2*, *cpa*, *cpb*, *Stam*, and *Chmp1*. Subsequent RT-qPCR experiments confirmed the upregulation of all six genes tested at 4 h and/or 24 h after TBI (**Figure 3E**), supporting the robustness of the RNA-seq analysis. As a control, the astrocytes-specific *alrm* gene showed no upregulation upon TBI by RT-qPCR and RNA-seq. Taken together, these findings validate the approach of RNA-seq transcription profiling in detecting genes functionally related to injury responses in astrocytes following TBI.

Pvr and AP-1 are required for the upregulation of Mmp1 after TBI

Among the candidates exhibiting upregulated expression in astrocytes upon TBI, three receptors (*Pvr*, *AdoR* and *Draper*), all acting upstream of and activating the JNK pathway (Ishimaru et al., 2004; Macdonald et al., 2013; Poernbacher and Vincent, 2018), emerged as new factors with uncharacterized functions in astrocytes (**Figure 3D**). The *Drosophila* RNA interference (RNAi) Screening Center Integrative Ortholog Prediction Tool (DIOPT) identified orthologs of these three receptors, subunits of transcriptional factor AP-1 (composed of *Jra* and *kay*) of the JNK pathway, *Mmp1*, and endocytic trafficking genes in mouse and human (Table 1) (Hu et al., 2011). These proteins mediate essential injury responses in vertebrates (Borea et al., 2016; Motz and Coukos, 2013; Shi et al., 2021). To examine a potential role of the receptors and their downstream signaling components in astrocytes after TBI, we quantified the immunostaining intensity of these proteins from projected images of the whole brain (including 20–30 slices with 1 μ m per slice). As our RNA-seq analysis revealed elevated expression of JNK-related injury genes such as *AP-1* and *Mmp1* (**Figure 3D** and Table 1), we analyzed the expression of *Mmp1*, a transcriptional target of AP-1 (Purice et al., 2017), as a readout for the activity of JNK signaling in response to TBI. We found that upregulated *Mmp1* expression induced by TBI was potently suppressed by knocking down the expression of *Pvr*, *Jra* or *kay* (**Figures 4A–D**, 4F), indicating that *Mmp1* upregulation is mediated by *Pvr* and AP-1, and predominantly occurs in astrocytes. We note that the location and intensity of *Mmp1* signals are variable among different individuals (**Figure 4**), probably because the brain damage caused by HIT is not stereotypical.

Even though flies with *Draper* knockdown in astrocytes exhibited high mortality, analysis of escapers indicated that *Mmp1* upregulation upon TBI was not completely inhibited by *Draper* knockdown (**Figure 4E** and 4F). Knockdown of the other receptor *AdoR* in astrocytes caused a weak inhibition of AP-1 and *Mmp1* upregulation (**Figure S3A–A''**, S4G–H'', S4I and S4J); *TRE-dsRed* that expresses the red fluorescent protein *dsRed* under the control of 4 $\times$ AP-1 motifs (Chatterjee and Bohmann, 2012) was used as a reporter for AP-1 activity. As *Pvr* showed the strongest regulation on *Mmp1* expression among the three receptors (**Figure 4** and S3), we focused on the potential role of *Pvr* in astrocytes following TBI in the present study. It is noteworthy to mention that although *Draper* expression was upregulated upon axotomy (MacDonald et al., 2006) and TBI (**Figure 3D**), inhibiting *Draper* expression in astrocytes did not suppress the *Mmp1* upregulation after TBI (**Figure 4E** and 4F). Taken together, these results suggest that *Pvr*, but not *Draper*, and AP-1 are required for upregulated *Mmp1* in astrocytes after TBI.

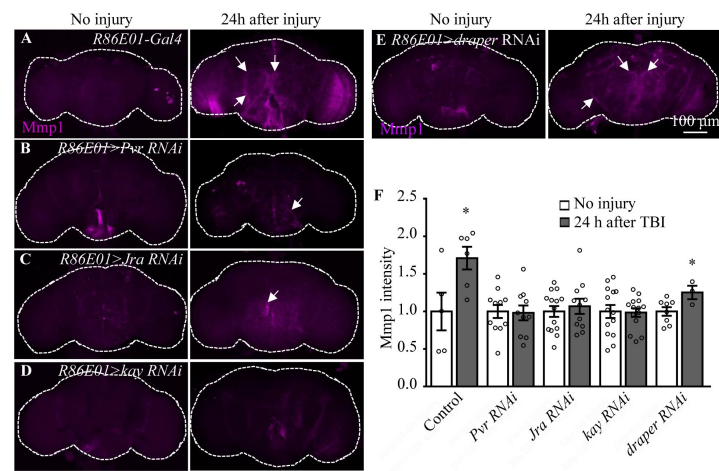


Figure 4.

Pvr, Jra and kay are required for Mmp1 upregulation after TBI.

(A–F) Knockdown of Pvr, Jra or kay by *R86E01-Gal4* driven RNAi in astrocytes suppresses Mmp1 upregulation after TBI. Scale bar = 100 μ m. All images are projections of 25–30 μ m immunostaining slices (one image per 1 μ m slice). Arrows show Mmp1 signals. (F) Quantification of Mmp1 intensity in different genotypes. Results are means $\pm$ SEM (**p* < 0.05 by Student's *t*-test).

Genes in <i>Drosophila</i>	Conserved genes in mouse	Conserved genes in human	4 h after injury (FC)	24 h after injury (FC)
Pvr	pdgfr/vegfr	VEGFR	13.83	2.79
AdoR	Adora2a	ADORA2B	37.53	7.73
Mmp1	Mmp14	MMP14	26.35	4.79
Jra	Jun	JUN	3.58	1.18
kay	FOSL2	FOS	6.23	1.77
cpa	Capza2	CAPZA1	3.43	1.72
cpb	Capzb	CAPZB	2.01	1.25
Stam	Stam2	STAM	2.14	1.46
Chmp1	Chmp1b	CHMP1B	2.16	0.92
Vps24	Chmp3	CHMP3	2.11	0.87

Table 1.

Upregulated genes related to receptors and endocytosis.

Pvr positively regulates AP-1 signaling and Mmp1 expression in astrocytes after TBI

We next investigated the genetic pathway by which Pvr functions in astrocytes in response to TBI. We examined the activity of AP-1 using the *in vivo* reporter TRE-dsRed (Chatterjee and Bohmann, 2012). In uninjured brains, we detected low TRE-dsRed levels, suggesting a basal AP-1 activity (Figure 5A–A). Similar to Mmp1, TRE-dsRed levels were robustly upregulated at 24 h after TBI, suggesting activation of JNK/AP-1 signaling (Figure 5B–B). Importantly, TBI-induced expression of TRE-dsRed and Mmp1 was suppressed or further upregulated when inhibiting or overexpressing Pvr expression in astrocytes, respectively (Figure 5C – F). Overexpression of Jra or kay alone did not affect expression of TRE-dsRed or Mmp1 (Figure S3A–F, 5I and 5J), suggesting that overexpressing a single subunit of the AP-1 complex is not sufficient to upregulate AP-1 activity. While Pvr overexpression caused an increase in TRE-dsRed and Mmp1 expression levels, Jra or kay RNAi knockdown in the background of Pvr overexpression suppressed the increase (Figure 5G–L), indicating that Jra and kay act downstream of Pvr to mediate the injury response upon TBI. These results together show that Pvr is a receptor acting upstream of AP-1 signaling that promotes Mmp1 expression during the injury response in astrocytes (Figure 5M).

Disruption of endocytosis and endocytic trafficking enhances Pvr, AP-1, and Mmp1 upregulation upon TBI

How was the Pvr signalling induced by TBI regulated? It has been shown that VEGFR2 endocytosis proceeds in a clathrin-dependent manner (Simons et al., 2016). During clathrin-dependent endocytosis, scission needs Cdc42-interacting protein 4 (Cip4) to recruit dynamin and Wiskott-Aldrich syndrome protein (WASP), the activator of the Arp2/3 complex that mediates actin polymerization and promotes the movement of newly-formed clathrin-coated vesicles (Brüser and Bogdan, 2017). VEGFRs are transmembrane RTKs that are activated when they locate at the cell membrane (Simons et al., 2016). We first investigated whether endocytosis is involved in Pvr signaling upon TBI. *Shibire* (*shi*) encodes a GTPase called dynamin which is responsible for fission of the vesicle from the membrane (van der Bliek and Meyerowitz, 1991). We analysed the effect of endocytosis on Pvr signaling by blocking the activity of dynamin with a temperature sensitive mutant, *shi^{ts}*, in astrocytes. Interestingly, both Pvr (Figures 6A, 6B, and 6E) and TRE-dsRed (Figures 6C, 6D, and 6F) intensities were increased at 4 h after TBI when dynamin activity was blocked at the non-permissive 32°C. As a control, expression of nLacZ under the same conditions as mutated dynamin did not exhibit a significant difference in the intensities of Pvr and TRE-dsRed at the permissive 23°C and the non-permissive 32°C after TBI (Figures 6A, 6B, 6E, and 6F). These results suggest that Pvr signaling is facilitated when endocytosis is blocked.

In addition to Pvr, results from our transcription profiling analysis also revealed upregulation of the expression of actin dynamics and endocytic trafficking genes including *Arpc2*, *cpa*, and *cpb* following TBI (Tables 1 and S2). To explore whether endocytic trafficking genes are involved in the injury response in astrocytes, we analyzed Mmp1 and TRE-dsRed expression when inhibiting the expression of *Arpc2*, *cpa*, or *cpb*. Interestingly, TBI-induced Mmp1 levels were further increased after knockdown of each of the three (Figures 7A–D, 7O); consistently, TRE-dsRed expression was also increased after knockdown of *cpa* or *cpb* (Figures 7H–K, 7P). These results indicate that genes involved in endocytosis and endocytic trafficking normally inhibit induction of AP-1 and Mmp1 by TBI in astrocytes.

In addition, we also observed upregulated expression of a set of endosomal sorting genes, including *Stam*, *Chmp1*, and *Vps24* that encode components of the endosomal sorting complex required for transport (ESCRT) in astrocytes in response to TBI (Tables 1 and S2). These genes are implicated in the formation of intraluminal vesicles (ILVs) from multivesicular bodies (MVBs).

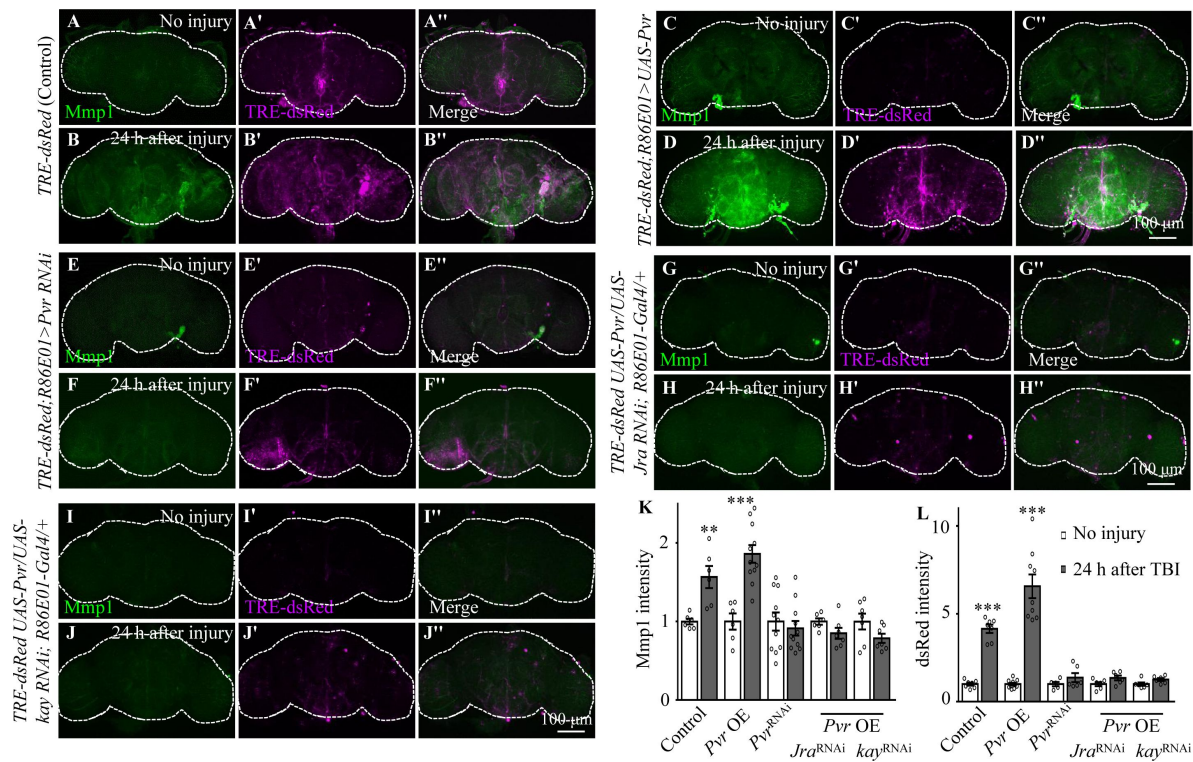


Figure 5.

Pvr is required for AP-1 upregulation after TBI.

(A–B'') Adult brains showing increased *TRE-dsRed* (an AP-1 reporter) expression at 24 h after TBI. (C – D'') Overexpression of Pvr in astrocytes leads to upregulated dsRED expression compared with control flies. Scale bar = 100 μm. (E – F'') Knockdown of Pvr in astrocytes causes downregulation of dsRed expression compared with control flies after TBI. (G–H'') Overexpression of Pvr and knockdown of *Jra* at the same time in astrocytes causes downregulation of dsRed and Mmp1 after TBI compared with controls. (I–J'') Overexpression of Pvr and knockdown of *kay* in astrocytes at the same time causes downregulation of dsRed and Mmp1 after TBI compared with controls. (K, L) Quantification of Mmp1 (K) and dsRed (L) intensity. Results are means ± SEM ***p* < 0.01, ****p* < 0.001, Student's *t*-tests.

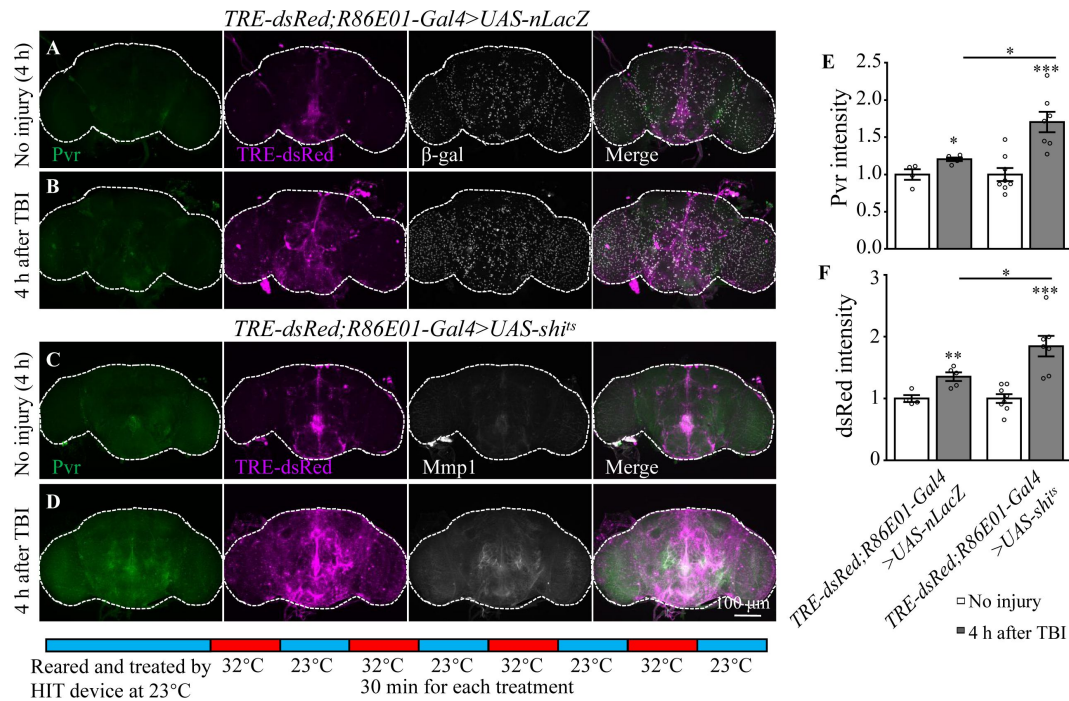


Figure 6.

Inhibition of dynamin activity increases the expression levels of Pvr and dsRed after TBI.

(A, B) Representative Pvr immunostaining (green), dsRed (magenta) and β -gal intensity (gray) in control animals (*TRE-dsRed;R86E01-Gal4>UAS-nLacZ*) at 4 h after TBI. All images are projections of 25–30 μ m immunostaining slices (one image per 1 μ m slice). (C, D) Representative Pvr immunostaining (green), dsRed (magenta) and Mmp1 intensity (gray) in *shi^{ts}*-expressing animals (*TRE-dsRed;R86E01-Gal4>UAS-shi^{ts}*) at 4 h after TBI. For thermogenetic inactivation of dynamin, flies after TBI were incubated at intermittent temperature at 32°C (32°C for 30 min, and then back to 23°C for 30 min, for four rounds, four hours in total) until dissection. (E, F) Quantification of Pvr and dsRed intensity in different genotypes. Results are means $\pm$ SEM (* p < 0.05).

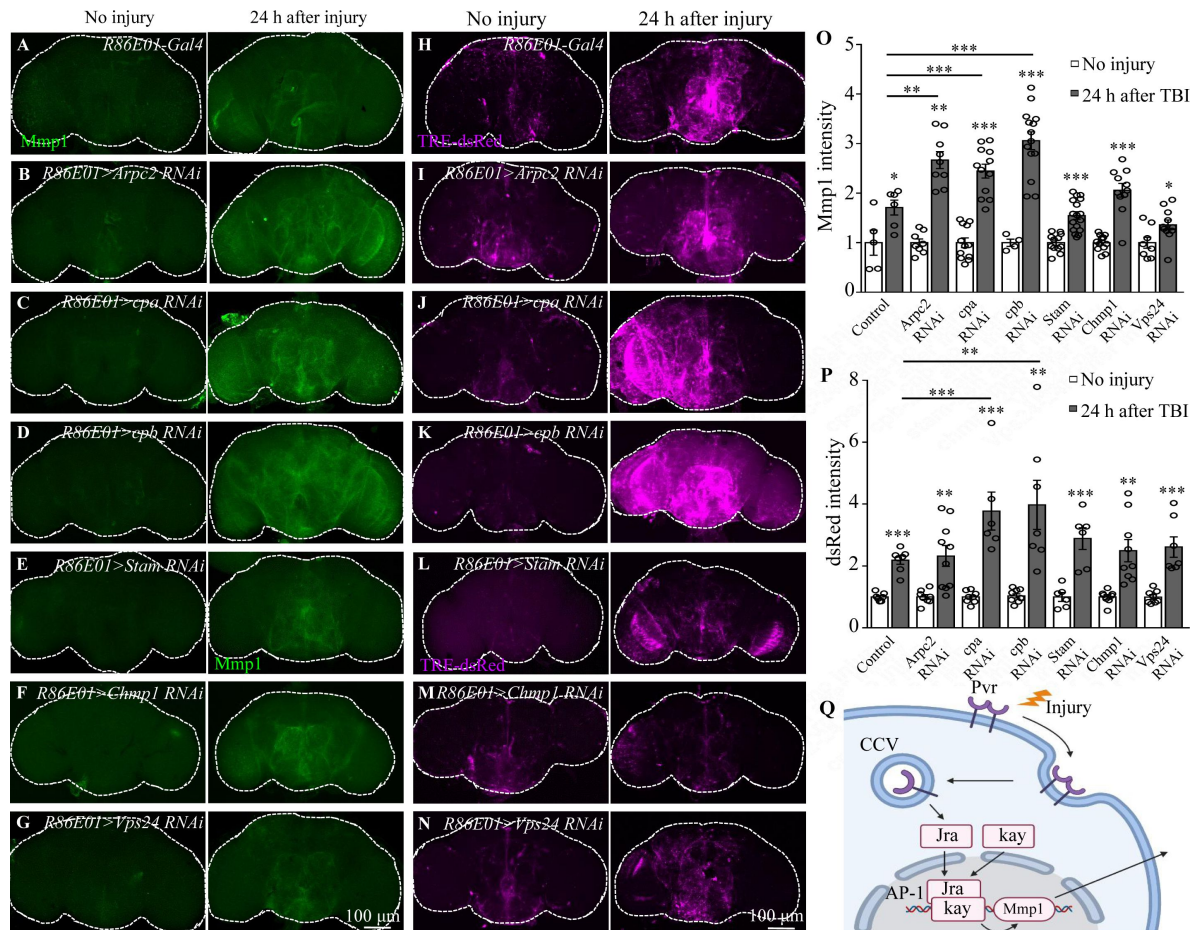


Figure 7.

Knockdown of endocytic trafficking-related genes up-regulates expression of AP-1 and Mmp1 upon TBI

(A, H) Representative Mmp1 immunostaining (magenta) and dsRed intensity (magenta) in control animals (*R86E01-Gal4*) at 24 h after TBI. (B–D, I–K) Knockdown of *Arpc2*, *cpa*, and *cpb* in astrocytes results in further upregulation of Mmp1 and dsRed upon TBI. All images are projections of 25–30 μm immunostaining slices (one image per 1 μm slice). (E–G, L–N) Knockdown of *Stam*, *Chmp1* or *Vps24*, components of ESCRT, in astrocytes had no influence on Mmp1 and dsRed upregulation compared with control after TBI (A, H). All images are projections of 25–30 μm immunostaining slices (one image per 1 μm slice). (O, P) Quantification of Mmp1 and dsRed intensity in different genotypes. Results are means $\pm$ SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, Student's *t*-tests). (Q) Schematic diagram of Pvr-AP-1-MMP1 signaling activated by TBI in astrocytes. AP-1 transcription factor complex is composed of Jra and kay.

Inhibiting expression of the ESCRT-0 subunit Stam and the ESCRT-III subunits Chmp1 or Vps24 in astrocytes however did not result in a significant difference in Mmp1 or TRE-dsRed expression levels compared with controls following TBI (**Figure 7E–G**, 7L–N, 7O and 7P), suggesting that endosome sorting in MVB may not affect Pvr signaling activity.

Discussion

Using a previously established a *Drosophila* TBI model, we found that TBI causes neuronal apoptosis, BBB/BEB disruption, and astrocyte swelling. Astrocytes respond to TBI by elevated Pvr expression, which in turn enhances the expression of the downstream effectors AP-1 and Mmp1. Our findings identify a new signaling pathway upregulated in astrocytes following TBI.

Unlike vertebrates, in which astrocytes become reactive (reactive astrogliosis) and actively clear neuronal and synaptic debris via phagocytosis upon axon injury (Liddelow and Barres, 2017), axon transection in *Drosophila* shows no immediate astrocytic response to injury (Doherty et al., 2009; Purice et al., 2017). Instead, *Drosophila* ENG express key components of the phagocytic machinery and engulf neuronal debris in response to axotomy (Doherty et al., 2009). Here, we examined for the molecular and cellular changes of astrocytes after TBI. Upon TBI, astrocytes exhibited apparent damage response including enlarged soma and altered cellular processes containing more membrane-bound GFP⁺ accumulations (**Figure 2A** – J). These findings suggest that astrocytes are activated upon TBI, demonstrating a critical role for astrocytes in the injury response.

The innate immune response in *Drosophila* relies on the activation of multiple signaling pathways including NF- κ B/Relish-mediated Imd and Doral/Dif-mediated Toll signaling pathways. Interestingly, our RNA-seq data revealed elevated expression of genes *Relish* and *Dif* in both pathways upon TBI (**Figure 3D**). However, the expressions of AMPs were minimum in astrocytes before TBI and no AMPs genes were identified in differentially expressed genes. Thus, AMPs genes were not activated in astrocytes, though upregulated AMP expressions were detected by bulk RNA-seq after TBI. In addition, expression of AP-1 (composed of Jra and kay) and Mmp1 was also upregulated, indicating that the AP-1 signaling pathway is activated in astrocytes following TBI. Even though upregulated Mmp expression in reactive glial responses is an evolutionarily conserved feature of *Drosophila* and mammalian glia (Cunningham et al., 2005; Purice et al., 2017), transmembrane receptors on astrocytes that regulate Mmp1 expression have not been identified. In mammals, knockdown of VEGF signaling in keratinocytes reduced angiogenesis and delayed wound healing (Rossiter et al., 2004). In *Drosophila*, studies of wound healing showed that Pvr likely acts upstream of actin regulatory proteins that promote actin polymerization to lead cells migration (Brock et al., 2012; Wu et al., 2009).

Through a combination of RNA-seq and genetic analyses, we identified Pvr as a new receptor in astrocytes; Pvr promotes Mmp1 expression via the transcription factor complex AP-1. At present, we do not know how Pvr promotes expression of AP-1 and Mmp1. But previous findings show that Pvr phosphorylates JNK to trigger the downstream signaling containing AP-1 during thorax closure in metamorphosis (Ishimaru et al., 2004). During development, the midline glia survival and migration are regulated by the ventral unpaired median (VUM) and the midline precursor 1 (MP1) midline neurons, the sources of Pvr ligands, i.e., PDGF- and VEGF-related factor 1-3 (Pvf1-3) (Learte et al., 2008). Additionally, epidermal cell-derived Pvf1 may be sequestered in the blood and exposed upon injury to bind Pvr receptors on wound-edge epidermal cells and initiate the extension of cell processes into the wound gap (Wu et al., 2009). Specifically, Pvr ligands bind to Pvr receptor to induce autophosphorylation of tyrosine residues, leading to JNK phosphorylation. Phosphorylated JNK (Bsk) then phosphorylates Jun/Jra transcription factors, triggering Jun/Jra association with Fos/kay, to form the AP-1 complex (Bogoyevitch and Kobe, 2006). In addition, glial AP-1 is essential for recovery, ensuring brain integrity and animal survival in the early post-

TBI period (Byrns et al., 2021 [↗](#)). Given that Pvr and AP-1 genetically interact in astrocytes upon TBI, it is likely that Pvr acts upstream of AP-1 and regulates its activity via JNK phosphorylation following brain injury. We speculate that Pvr ligands that may secret from injured cells to activate Pvr-positive astrocytes after TBI; activated Pvr then increases Mmp1 expression via AP-1 and secretion to the intercellular space to participate in injury response.

In addition to Pvr, our RNA-seq data also identified other receptors potentially functioning in astrocytes in response to TBI. Specifically, AdoR expression was upregulated after TBI (Table S2), and knockdown of AdoR expression in astrocytes led to inhibition of AP-1 after TBI (**Figure S3G–J** [↗](#)), though at a weaker extent compared with Pvr knockdown. In mammals, A_{2B}R, a subtype of AdoR, was shown to stimulate VEGF production in various cell types (Borea et al., 2016 [↗](#)). Pvr promotes the expression of adenosine deaminase-related growth factor A (Adgf-A) which inactivates extracellular adenosine, the ligand of AdoR (Zurovec et al., 2002 [↗](#)). Furthermore, Pvr and AdoR collaborate in differentiating cells to maintain the expression of Adgf-A (Mondal et al., 2011 [↗](#)). Thus, it is possible that Pvr and AdoR act independently or in a coordinated manner to regulate the injury response to TBI in astrocytes in *Drosophila*.

Our RNA-seq profiling also revealed upregulation of Draper. Previous studies revealed a role for Draper-mediated phagocytosis of axonal debris in ENG after axotomy (Lu et al., 2017 [↗](#); MacDonald et al., 2006 [↗](#)); axotomy induces Wallerian-like degeneration (Büki and Povlishock, 2006 [↗](#); Tang-Schomer et al., 2010 [↗](#)). Specifically, damage-associated molecular pattern (DAMP) proteins released from injured neurons bind to Draper and activate the JNK and JAK/STAT pathways in ENG (Doherty et al., 2014 [↗](#); Freeman, 2015 [↗](#); MacDonald et al. 2013 [↗](#)). Here, our findings showed that Draper does not affect Mmp1 expression in astrocytes upon TBI. Although an increase in Draper expression was detected in astrocytes by RNA-seq, knockdown of its expression did not significantly suppress Mmp1 upregulation after TBI. Thus, Draper activates JNK/AP-1 and JAK/STAT signaling in ENG in response to axotomy (Doherty et al., 2014 [↗](#); MacDonald et al. 2013 [↗](#)), whereas astrocytes respond to TBI via Pvr-mediated AP-1 signaling. Despite different pathways are activated in different types of glia upon brain injury, they may cooperate in response to TBI.

Endocytosis and trafficking of VEGFR, the mammalian Pvr homolog, regulate the specificity as well as the duration and strength of the signaling output (Simons et al., 2016 [↗](#)). Once activated on the plasma membrane and translocated into the cytoplasm, VEGFRs are either shuttled to lysosomes for degradation or recycled back to the plasma membrane to replenish the receptor pool (Simons et al., 2016 [↗](#)). Our findings showed that disruption of endocytosis by disrupting dynamin activity and inhibiting the expression of Arpc2, cpa, or cpb that regulate endocytic transport into early endosomes enhanced the AP-1 and Mmp1 upregulation in astrocyte following TBI (**Figures 7A–D** [↗](#), 7H–K, 7O, 7P). As receptor endocytosis attenuates the strength or duration of many signaling processes by physically reducing the concentration of cell surface receptors accessible to the ligand (Jékely et al., 2005 [↗](#)), blocking dynamin-mediated endocytosis and endocytic trafficking potentially results in accumulated Pvr receptors on the plasma membrane to activate the downstream effector AP-1, thereby enhancing the astrocytes response to TBI. On the other hand, ubiquitinated RTKs are recognized by ESCRT-0, followed by sequential recruitment of ESCRT-I, ESCRT-II, and ESCRT-III onto the membranes of MVBs to form intraluminal vesicles (ILVs) (Goh and Sorkin, 2013 [↗](#)). Knockdown of the ESCRT-0 subunit Stam and the ESCRT-III subunits Chmp1 and Vps24 did not affect the expression of Pvr downstream signaling components (**Figure 7E–G** [↗](#), 7L–N, 7O and 7P), suggesting that Pvr signal transduction was not regulated by endosomal sorting.

Considering the importance of VEGF signaling in a variety of cellular processes, including wound repair (Folkman and Klagsbrun, 1987 [↗](#)), elucidating the downstream signaling mechanisms, as well as defining the full array of Pvr targets in healthy and diseased brains, will be critical for dissecting the protective or detrimental roles of VEGF in the context of brain injury. Given that the

Pvr receptor, AP-1, and the MMP family of secreted proteases are all highly conserved across species, our work provides new insights into how VEGF signaling in glia mechanistically contributes to the TBI response in mammals.

Acknowledgements

This work was supported in part by the Ministry of Science and Technology of China (2019YFA0707100 and 2021ZD0203900 to Y.Q. Zhang), the National Natural Science Foundation of China (31921002, 31830036, and 31861143031 to Y.Q. Zhang, 32170962 to M. Ho and 31500839 to C.X. Mao), the Chinese Academy of Sciences Strategic Priority Research Program B grants (XDBS1020100 to Y.Q. Zhang). We thank Bloomington and Tsinghua stock centers for providing fly stocks. We are also grateful to Zhiguo Ma, Oren Schuldiner, Sean Speese, Yong Zhang, Zhiyong Liu, and the members of the Yong Q. Zhang lab for discussion and suggestions.

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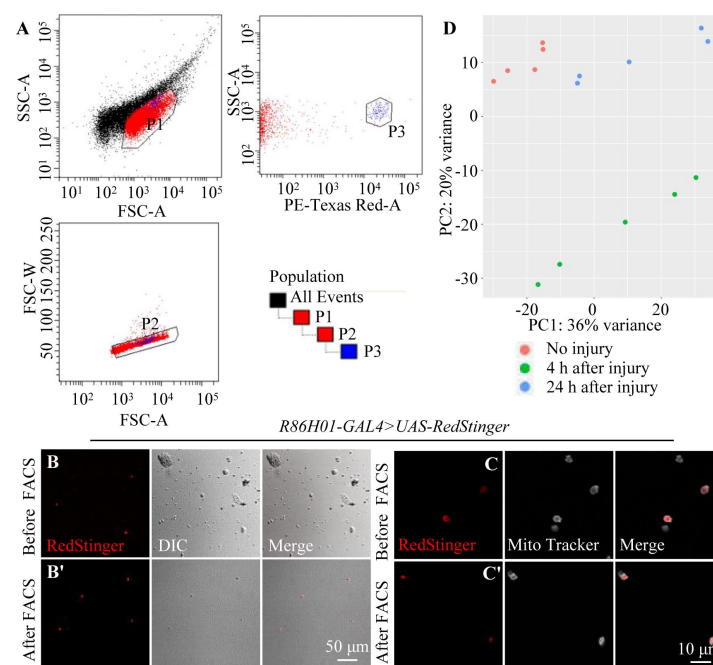


Figure S1.

Sorting of adult astrocytes by FACS.

(A) To discriminate between healthy and damaged or dying cells, side scatter (SSC-A, log) and forward scatter (FSC-A, log) were plotted. Higher SSC-A signals indicate increased granularity and therefore likely dying cells. Thus, one should define the sorting gate as the population with the lower SSC-A signal (P1). (P2) Exclude cell clusters by sorting only events with a low FSC width (FSC-W) versus similar area (FSC-A) signal to get exact single cell. (P3) To gate for RFP⁺ cells, plotted the signal from the excitation of cells with the 568-nm laser (PE-Texas Red-A, log) against the signal from the SSC-A. The population with the higher PE-Texas Red-A signal was the candidate cells cluster. The strategy for FACS is shown at the lower right corner. (B–C') A population of cells with strong RFP signal was purified from adult flies of *R86E01-Gal4>UAS-Redstinger*. (B and B') Confocal images of representative astrocytes before and after sorting by FACS. Scale bar = 50 μ m. (C and C') As cell-permeant MitoTracker labels mitochondria of live cells, cells after FACS both labelled by RFP and MitoTracker. Scale bar = 10 μ m. (D) A Principal Component Analysis of all biological replicates (uninjured versus injured) revealing genes expressed at absolute value of logarithmic fold change ($\log_2$ FC) > 1.0.

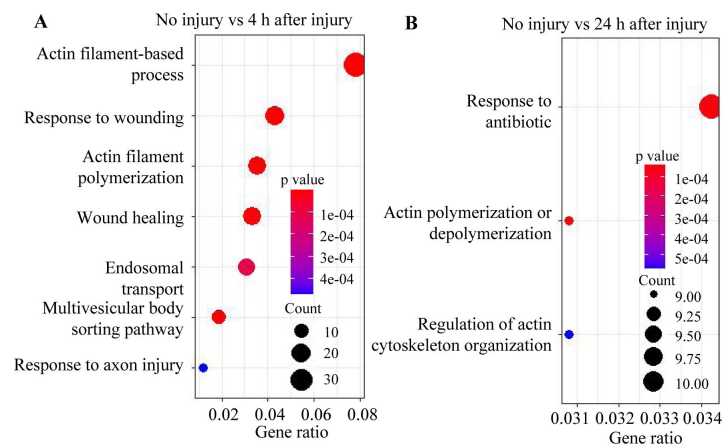


Figure S2.

GO analysis of differentially expressed genes at 4 h and 24 h after injury.

Scatterplot showing GO functional enrichment results for differentially expressed genes of astrocytes in the brain at 4 h (A) and 24 h (B) after injury, $p < 0.01$.

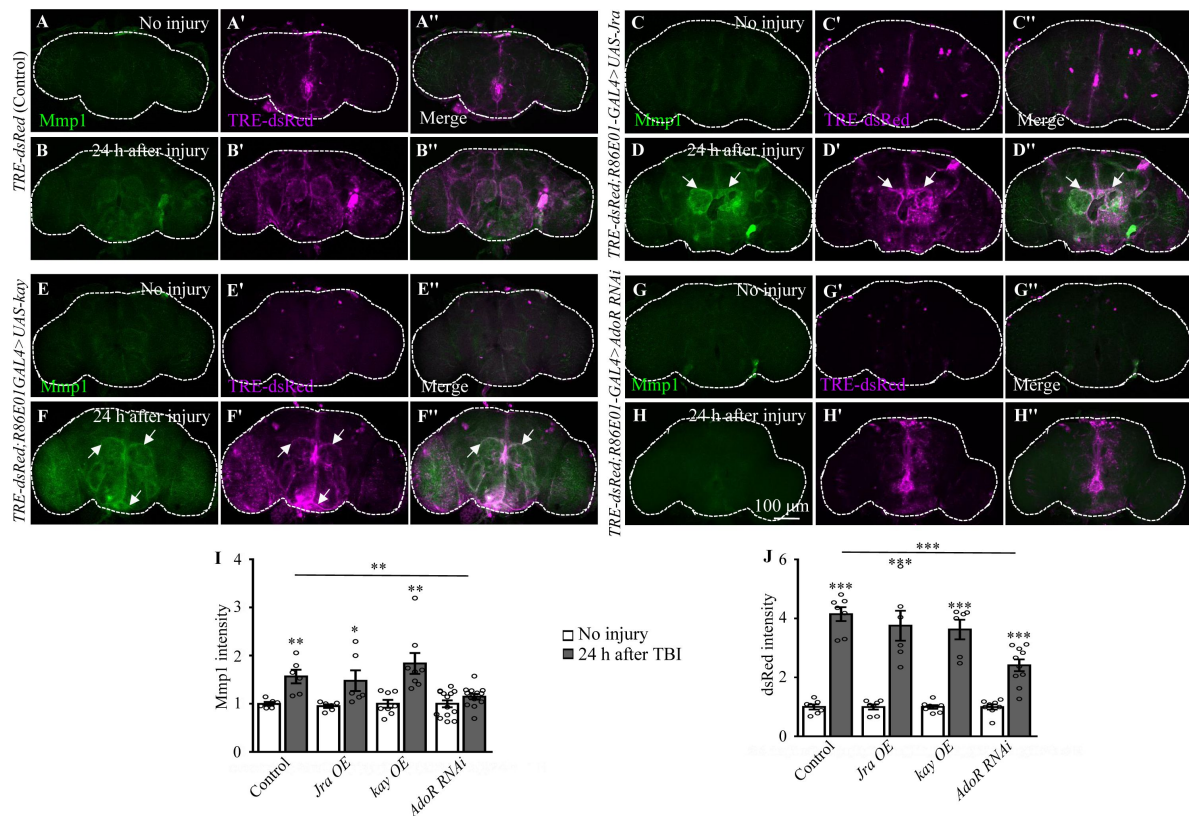


Figure S3.

Overexpression of Jra or kay does not enhance the expression of TRE-RFP and Mmp1 in adult brain after TBI.

(A, B'') Fly brains showing TRE-RFP expression before and 24 h after TBI. (C–F'') overexpression of Jra or kay in astrocytes shows no apparent changes of RFP and Mmp1 staining signals compared with control flies with or without brain injury (*TRE-dsRed/UAS-Jra* or *kay*; *R86E01-Gal4/+*). Scale bar = 100 μ m. (G, H) Knockdown of AdoR in astrocytes inhibits the expression of Mmp1 after TBI. However, Knockdown of AdoR in astrocytes does not inhibit the expression of dsRed. (I, J) Quantification of Mmp1 and dsRed intensity before and after injury in different genotypes. Data are present as means $\pm$ SEM; * p < 0.05, ** p < 0.01, *** p < 0.001 by Student's t tests.

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Reviewer #1 (Public Review):

Li et al report that upon traumatic brain injury (TBI), Pvr signalling in astrocytes activates the JNK pathway and up-regulates the expression of the well-known JNK target MMP1. The FACS sort astrocytes, and carry out RNAseq analysis, which identifies pvr as well as genes of the JNK pathway as particularly up-regulated after TBI. They use conventional genetics loss of

function, gain of function and epistasis analysis with and without TBI to verify the involvement of the JNK-MMP1 signalling pathway downstream of PVR. They also show that blocking endocytosis prolongs the involvement of this pathway in the TBI response.

The strengths are that multiple experiments are used to demonstrate that TBI in their hands damaged the BBB, induced apoptosis and increased MMP1 levels. The RNAseq analysis on FACS sorted astrocytes is nice and will be valuable to scientists beyond the confines of this paper. The functional genetic analysis is conventional, yet sound, and supports claims of JNK and MMP1 functioning downstream of Pvr in the TBI context.

For this revised version the authors have removed all the unsupported claims. This renders their remaining claims more solid. However, it has resulted in the loss of important cellular aspects of the response to TBI, limiting the scope and value of the work.

The main weakness is that novelty and insight are both rather limited. Others had previously published that both JNK signalling and MMP1 were activated upon injury, in multiple contexts (as well as the articles cited by the authors, they should also see Losada-Perez et al 2021). That Pvr can regulate JNK signalling was also known (Ishimaru et al 2004). The authors claim that the novelty was investigating injury responses in astrocytes in *Drosophila*. However, others had investigated injury responses by astrocytes in *Drosophila* before. It had been previously shown that astrocytes - defined as the Prospero+ neuropile glia, and also sharing evolutionary features with mammalian NG2 glia - respond to injury both in larval ventral nerve cords and in adult brains, where they proliferate regenerating glia and induce a neurogenic response (Kato et al 2011; Losada-Perez et al 2016; Harrison et al 2021; Simoes et al 2022). The authors argue that the novelty of the work is the investigation of the response of astrocytes to TBI. However, this is of somewhat limited scope. The authors mention that MMP1 regulates tissue remodelling, the inflammatory process and cancer. Exploring these functions further would have been an interesting addition, but the authors did not investigate what consequences the up-regulation of MMP1 after injury has in repair or regeneration processes.

The statistical analysis is incorrect in places, and this could affect the validity of some claims.

Altogether, this is an interesting and valuable addition to the repertoire of articles investigating neuron-glia communication and glial responses to injury in the *Drosophila* central nervous system (CNS). It is good and important to see this research area in *Drosophila* grow. This community together is building a compelling case for using *Drosophila* and its unparalleled powerful genetics to investigate nervous system injury, regeneration and repair, with important implications. Thus, this paper will be of interest to scientists investigating injury responses in the CNS using *Drosophila*, other model organisms (eg mice, fish) and humans.

<https://doi.org/10.7554/eLife.87258.2.sa1>

Reviewer #3 (Public Review):

In this study, authors used the *Drosophila* model to characterize molecular details underlying traumatic brain injury (TBI). Authors used the transcriptomic analysis of astrocytes collected by FACS sorting of cells derived from *Drosophila* heads following brain injury and identified upregulation of multiple genes, such as Pvr receptor, Jun, Fos, and MMP1. Additional studies identified that Pvr positively activates AP-1 transcription factor (TF) complex consisting of Jun and Fos, of which activation leads to the induction of MMP1. Finally, authors found that disruption of endocytosis and endocytotic trafficking facilitates Pvr signaling and subsequently leads to induction of AP-1 and MMP1.

Overall, this study provides important clues to understanding molecular mechanisms underlying TBI. The identified molecules linked to TBI in astrocytes could be potential targets for developing effective therapeutics. The obtained data from transcriptional profiling of astrocytes will be useful for future follow-up studies. The manuscript is well-organized and easy to read.

However, the connection suggested by the authors between Pvr and AP-1, potentially mediated through the JNK pathway, lacks strong experimental support in my view. It's important to recognize that AP-1 activity is influenced by multiple upstream signaling pathways, not just the JNK pathway, which is the most well-characterized among them. Therefore, assuming that AP-1 transcriptional activity solely reflects the activity of the JNK pathway without additional direct evidence is unwarranted. To strengthen their argument, the study could benefit from direct evidence implicating the JNK pathway in linking Pvr to AP-1. This could be achieved through genetic studies involving mutants or transgenes targeting key components of the JNK pathway, such as Bsk and Hep, the Drosophila homologues of JNK and JNKK, respectively. Alternatively, employing p-JNK antibody-based techniques like Western blotting, while considering the potential challenges associated with p-JNK immunohistochemistry, could provide further validation. This important criticism regarding the molecular link between Pvr and AP-1 has been overlooked.

<https://doi.org/10.7554/eLife.87258.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Recommendations for the authors:

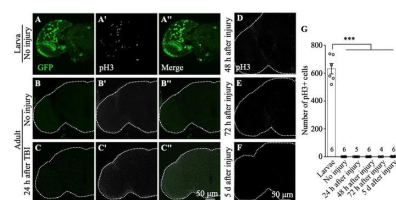
Reviewer #1 (Recommendations For The Authors):

To resolve and further test the claim that TBI did not induce cell proliferation:

How many brains did they analyse? Sample sizes must be provided in Figure S1.

As per reviewer's suggestion, we removed one of the unsupported claims shown in Figure S1. The original Figure S1 is shown below with the sample number added.

Author response image 1.



The authors could either improve the TBI method or the detection of cells in S-phase, mitosis or cycling. They could use PCNA-GFP or BrdU, EdU or FUCCI instead and at least provide evidence that they can detect cells in S-phase in intact brains. Timing is critical (ie cell cycle is longer than in larvae) so multiple time points should be tested. Or they could use pH3 but test more time points and rather large sample sizes. If they are not able to provide any evidence, then their lack of evidence is no evidence. The authors should consider removing pH3 and PCNA-GFP related claims instead.

We have removed pH3 and PCNA-GFP related results and claims.

Other unsupported claims:

Figure 2A-C is not very clear what they are showing, but it is not evidence of astrocyte hypertrophy. It does not have cellular resolution and does not show the cell size, membranes, nor number

(1) We have avoided the term “hypertrophy” and changed the description throughout the text to “astrocyte swelling”.

(2) Images in the resolution of Figure 2E and 2F were able to show the enlarged soma of astrocytes, suggesting swelling.

What is the point of using RedStinger in Figure 2?

We used RedStinger to label the astrocyte nuclei.

Figure S5 is not convincing, as anti-Pvr does not look localised to specific cells. Instead, it looks like uniform background. If they really think the antibody is localised, they should do double stainings with cell type specific markers. If the antibody does not work, then remove the data and the claim. They could test with RNAi knock-down in specific cell types and qRT-PCR which cells express pvr instead.

We have removed the claim that “Pvr is predominantly expressed in astrocytes” and changed the description to “Immunostainings using the anti-Pvr antibodies revealed that endogenous Pvr expression is low in the control brains, yet significantly enhanced upon TBI. Reducing Pvr expression, but not Pvr overexpression, in astrocytes blocked the TBI-induced increase of Pvr expression (Figure S5)”.

Figure S6: it is unclear what they are trying to show, but these data do not demonstrate that astrocytes do not engulf debris after TBI, as there isn't sufficient cellular resolution to make such claim. Firstly, they analyse one single cell per treatment. Secondly, the cell projections are not visible in these images, and therefore engulfment cannot be seen. The authors could remove the claim or visualise whether astrocytes phagocytose debris or not either using clones or with TEM.

We agree with the reviewer that our images do not have the resolution to make this claim. We have removed Figure S6 and corresponding text description.

On statistics:

The statistical analysis needs revising as it is wrong in multiple places, eg Fig. 1F,G,H; Figure 2D. They only use Student t-tests. These can only be used when data are continuous, distributed uniformly and only two samples are compared; if more than 2 samples, distributed uniformly, then use One-Way ANOVA and multiple comparisons tests. If data are categorical, use Chi-Square.

We have double checked and compared the experimental group to the control separately using the Student t-tests throughout the study.

Other points for improvement:

Figure 2E,F: what are GFP puncta and how are they counted?

I. Each GFP puncta looks like a little circle, likely representing a functional or dysfunctional structure. The biology of the GFP puncta is currently unknown.

II. We used the ImageJ to quantify the GFP puncta:

- (1) Image- type-8 bits
- (2) Process-subtract background (Rolling ball radio:10)
- (3) Image-Adjust-Threshold-Apply
- (4) Analyze-Measure-set measurements-choose “area” “limit to threshold”-OK
- (5) Count the puncta number in the choosing area.
- (6) Get the number of puncta per square micron.

| *All genotypes must be provided (including for MARCM clones), currently they are not.*

We have shown the full genotype in the corresponding legend.

| *Figure 7O,P indicate on figure that these are RNAi*

We have revised the labels to RNAi in Figure 7O,P.

| **Reviewer #2 (Recommendations For The Authors):**

| *Several typos are present in the text.*

We have read the manuscript carefully and corrected typos throughout.