IL-1 β -mediated macrophage infiltration into the brain after peripheral tissue injury

Xiang Ke Chen

University of Hong Kong

Joseph Shiu-Kwong Kwan

Imperial College Healthcare NHS Trust

Gordon Tin-Chun Wong

University of Hong Kong

Kazi Md. Mahmudul Hasan

HK PolyU: The Hong Kong Polytechnic University

Zhen-Ni Yi

HK PolyU: The Hong Kong Polytechnic University

Alvin Chun-Hang Ma

HK PolyU: The Hong Kong Polytechnic University

Raymond Chuen-Chung Chang (✉ rccchang@hku.hk)

the University of Hong Kong <https://orcid.org/0000-0001-8538-7993>

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Abstract

Background: Infiltration of macrophages into the central nervous system (CNS) is involved in many neurological disorders, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and autism. Despite extensive studies into neuroinflammation associated macrophage infiltration into the CNS, its underlying mechanisms and pathological roles remain unclear, especially when triggered by peripheral inflammation.

Methods: To further elucidate the role and mechanism of peripheral inflammation in neurological disorders, we exploited *interleukin 1 beta (il1b)* mutant transgenic zebrafish (*Danio rerio*) with fluorescent protein expression restricted to macrophages to track the macrophage migration under peripheral inflammation following tail amputation.

Results: We found that macrophage infiltration into the brain of zebrafish embryo following peripheral tissue injury can be alleviated via genetically targeting *il1b*. In addition, through circulation-independent migration, macrophages infiltrate brains with evidence of increased apoptosis. We further identified the expression of *camk2g1* in the brains of zebrafish with hyperactive behavior following peripheral tissue injury. This *il1b*-regulated protein is associated with neuropsychiatry disorders.

Conclusion: These findings demonstrated that peripheral tissue injury induces *il1b*-mediated macrophage infiltration into the brain and a hyperactive behavior.

Background

Microglia, the tissue-resident macrophage, serves as a prime housekeeper of the central nervous system (CNS), where cellular debris, plaques and infectious agents are scavenged through phagocytosis. On the other hand disturbances in microglia are involved in a wide range of neurological disorders, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and autism [1]. Besides parenchymal microglia, perivascular and meningeal macrophages constitute the other predominant population of resident macrophages in the CNS that mediate immune responses at the interface between the brain and the periphery [2]. Strikingly, peripherally derived macrophages that are normally absent from the CNS due to the blood-brain barrier (BBB), are able to infiltrate the CNS under various pathological states, including CNS injury, AD, Parkinson's disease (PD) and anxiety [3–5]. Therefore, there is a growing interest in evaluating infiltrating as well as resident macrophages in the CNS as potential therapeutic target against various neurological disorders.

Neuroinflammation is characterized by high levels of pro-inflammatory cytokines and activation of microglia has long been recognized as the hallmark of multiple neurological disorders [6]. Peripheral macrophages are recruited by neuroinflammation [7] and infiltrate the CNS in the presence of a defective or even intact BBB [8,9]. These infiltrated macrophages can either promote or suppress microglial activation, microglial phagocytosis, and neuroinflammation according to the pathological conditions [5,10]. Upon infiltration into the CNS, macrophages can play opposing roles in the pathogenesis of

neurological disorders. For instance, these invading macrophages mediate cognitive dysfunction as well as seizures [11–13] but can also contribute to the removal of beta-amyloid plaques, neuroprotection and tissue repair in the CNS [14–16]. Despite these far-reaching roles of infiltrating macrophages in inflammatory neurological disorders, mechanisms regulating their recruitment and pathogenesis remain elusive.

Systemic inflammation is a pathological process characterized by activation of the innate immune system in response to peripheral mechanical trauma, infection/sepsis, or surgery [17]. Systemic inflammation elicits neuroinflammation and serves as a contributing factor to multiple neurological disorders, including post-traumatic stress disorder (PTSD), post-operative cognitive dysfunction (POCD) and other neurodegenerative diseases [18–20]. Different experimental models of systemic inflammation, such as surgery (laparotomy), lipopolysaccharides (LPS) or live bacteria injection have been used to elicit neuroinflammation and cognitive dysfunction [21–23]. Furthermore, infiltration of peripheral macrophages into the brain was reported previously in rodent models of inflammatory liver injury and AD with arthritis [24,25]. However, systemic and neuroimmune responses stimulated by sterile inflammation such as mechanical trauma is not well understood.

Here, we used live cells tracking and genetic manipulation in zebrafish (*Danio rerio*) and demonstrated that *il1b* mediated peripheral macrophage infiltration into the brain following peripheral tissue injury and consequently contributed to a hyperactive behavior.

Materials And Methods

Zebrafish Maintenance and Transgenic Lines

Wild-type (WT) and transgenic zebrafish lines were maintained in 14:10 h light:dark cycle and fed living brine shrimp twice a day. Embryos from WT, *Tg(coro1a:DsRed)* [26], *Tg(mpx:mCherry)*, *Tg(meog1:GFP)*, and *Tg(kdrl:GFP;coro1a:DsRed)* obtained via crossing *Tg(kdrl:GFP)* and *Tg(coro1a:DsRed)* were raised at 28.5 °C and staged as previously described[27].

Generation of *il1b* mutant zebrafish by TALEN

il1b mutant zebrafish was generated via TALEN resulting in a loss of function as previously described [28]. Briefly, plasmid containing mRNA sequence of TALEN left or right arm were constructed separately using the FusX assembly system according to the design (**Additional File 1: Fig. S1A**). Equal volume of *in vitro* transcribed mRNAs of TALEN left and right arms were mixed and co-injected into the one-cell-stage zebrafish embryos. F1, F2, and F3 zebrafish embryos were obtained by outcrossing founder (F0) with WT zebrafish, incrossing F1 zebrafish and incrossing F2 zebrafish, respectively. Moreover, *il1b*^{Mut} reporter lines, such as *Tg(kdrl:GFP;coro1a:DsRed)*, was generated by crossing homozygous *il1b*^{Mut} with reporter lines. All *il1b* mutation was confirmed by restriction fragment length polymorphism (RFLP) assay using

il1b genotyping primer (listed in **Additional File 1: Table S1**) and NsiI-HF® restriction enzymes (NEB) as well as sanger sequencing (**Additional File 1: Fig. S1B and S1C**).

Modeling Peripheral Tissue Injury in Zebrafish Embryo

WT or transgenic zebrafish embryos at 3 dpf were anaesthetized using 0.16 mg/ml tricaine (Sigma-Aldrich) and then a tail amputation assay was applied in the position posterior to blood circulation of the caudal fin to model the peripheral tissue injury as previously described [29]. After amputation, zebrafish embryos were immediately transferred back to sterilized E3 medium (5 mM NaCl, 0.17 mM KCL, 0.33 mM CaCl, and 0.33 mM MgSO₄, pH 7.4) containing methylene blue.

RNA Extraction and Quantitative PCR

Total RNA was extracted from the tail region or the head region of zebrafish embryos using RNAiso Plus (Takara). cDNA was then synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) in accordance with manufacturer's protocol. Quantitative PCR was finally performed on ABI 7300 Real-Time PCR System with FastStart Universal SYBR Green Master (Roche) reagents (primers listed in **Additional File 1: Table S1**).

Whole-Mount RNA In Situ Hybridization

Zebrafish embryos at 3 or 4 dpf were applied for whole-mount in situ hybridization (WISH) using standard protocols described previously [30]. DIG-labeled antisense probes were made from the pGEM®-T Easy vector (Promega) containing following gene-coding sequences via *in vitro* transcription using DIG RNA Labeling Kit (Roche): previously described *pu.1* [31] while *il1b* (852 bp), *apoeb* (475 bp) and *mfap4* (607 bp) (primers listed in **Additional File 1: Table S1**).

Light-sheet Imaging and Flow Cytometry

Zebrafish embryos at 4–6 dpf were anesthetized using 0.16 mg/ml tricaine (Sigma-Aldrich) and then mounted in 1.5% low-melting agarose using glass capillary for fluorescence imaging by Zeiss Lightsheet Z.1 Selective Plane Illumination Microscope with or without a time-lapse mode. For flow cytometry, the head region of WT or *Tg(mepg1:GFP)* zebrafish embryos was isolated and then homogenized and filtered to get the cells suspension. Beckman Coulter FC 500 was utilized to quantify the number and percentage of GFP + cells under various conditions.

Acridine Orange, TUNEL and PH3 Staining

Acridine orange at 10 µg/ml (Sigma-Aldrich) and ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Millipore) were used to detect apoptotic cells in live and fixed zebrafish embryos, respectively, following the protocols described previously [32]. In addition, immunostaining together with phospho-Histone H3 (PH3) primary antibody (Cell Signaling Technology) was applied to detect the cells undergoing mitosis according to the previously described protocol [33].

Mass Spectrometry-based Proteomics

Total protein was extracted using cell lysis buffer (Sigma-Aldrich) from the head region of zebrafish embryos. After purification and trypsin (Promega) treatment, the peptides were desalted using Pierce C18 Spin Columns (Thermo fisher). Proteomics was then performed on Thermo Fisher Orbitrap Fusion Lumos Mass Spectrometer coupled with Dionex UltiMate 3000 RSLCnano. Label-free relative quantification was processed with Progenesis Q1 software (<http://www.nonlinear.com/progenesis/qi/>) and the abundance of various proteins was quantified based on three independent experiments and normalized according to the housekeeping protein (gapdh).

Behavioral Assay of Locomotor Activity

il1b^{Mut} Zebrafish larvae at 7 dpf/4 dpa and their siblings with or without amputation were transferred into the 35 mm Petri dish containing 2 mL E3 medium. The protocol of locomotor activity was adapted from the Locomotion Assay with slight modification [34]. Zebrafish larvae from four groups was recorded for a 30-min period using a camera at the same time. A 10-min period after 10-min acclimation was analyzed using Fiji - Image J (<https://imagej.net/Fiji>) with tracking plugin and the total travel distance, active swim time and mean velocity were calculated.

Statistical analysis

Data are presented as mean (M) $\pm$ standard deviation (S.D.). Unpaired t-test, one-way analysis of variance (ANOVA), and two-way ANOVA with Tukey's HSD with were performed where appropriate using Statistical Package for the Social Sciences (SPSS) Version 14.0 and a p-value less than 0.05 was considered statistically significant.

Results

Peripheral tissue injury induced neuroinflammation, macrophage infiltration and apoptosis in the brain

Peripheral tissue injury from tail amputation in zebrafish embryos is widely utilized to investigate acute and sterile innate immune responses to the mechanical trauma-induced inflammation [26]. Tail amputation was performed at 72 hours post-fertilization (hpf), and at 24 hours post-amputation (hpa), elevated inflammatory cytokines/chemokines, including *il1b*, *il34*, *il6*, *tnfa* and *ccl2* but not *il8* and *il10* mRNA, were detected in the tail region, indicating a peripheral/systemic inflammation (Fig. 1A). Increased mRNA expression of *il1b*, *il34*, *il6*, *il8*, *il10* but not *tnfa*, and decreased mRNA expression of *ccl2* were detected in the head region at 24 hpa, suggesting neuroinflammation (Fig. 1B and 1C). The number of leukocytes labeled with *coro1a* (*coro1a*⁺), largely macrophages/microglia and neutrophils [35], were markedly increased in the brain at 4 days post-fertilization (dpf)/24 hpa, 5 dpf/48 hpa and 6 dpf/72 hpa (Fig. 1D-1E). Specifically, the number of *mpeg1*-expressing (*mpeg1*⁺) macrophages increased in the brain, while no difference in the number of *mpx*-expressing (*mpx*⁺) neutrophils was found in the brain after amputation (Fig. 1F-1H). Similarly elevated number of macrophages in the brain was also identified in acute response to amputation at 3 hpa and 6 hpa due to a redistribution, where *mpeg1*⁺ macrophages

were recruited from caudal hematopoietic tissue (CHT) and aorta-gonad-mesonephros (AGM) to the brain and injury site while the total number of macrophages remained unchanged (**Additional File 1: Fig. S2A-S2C**). To investigate the potential mechanism underlying recruitment of macrophages to the brain, apoptosis was also examined in the brain as circulatory macrophages are more susceptible than microglia to cell death and apoptosis during CNS injury [36]. Acridine orange-labeled (AO+) apoptotic cell phagocytosed by *coro1a* + macrophages/microglia increased in the brain at 24 hpa (Fig. 1I and 1J). Collectively, peripheral tissue injury elicited neuroinflammation, infiltration of macrophages and apoptosis in the brain under systemic inflammation.

***il1b* mediated infiltration of macrophages into the brain in response to peripheral tissue injury**

To define the role of systemic inflammation in peripheral tissue injury, a transcription activator-like effector nucleases (TALEN)-mediated mutant line of *il1b* [28] was established (**Additional File 1: Fig. S1A-S1C**). While somatic and stable (*il1b*^{+/-} or *il1b*^{-/-}) *il1b* mutants displayed normal gross development, an *il1b* knockout mutation (*il1b*^{Mut}) significantly decreased mRNA expression of cytokines regulated by *il1b*, including *il6*, *il8*, and *tnfa* but not *il34*, *il10* and *ccl2* in *il1b*^{-/-} zebrafish embryos (**Additional File 1: Fig. S1D**). Basal levels of macrophages/microglia were measured in *il1b*^{+/-} zebrafish before tail amputation at 72 hpf and the number of *pu.1*-labeled myeloid progenitors in CHT remained unchanged (**Additional File 1: Fig. S1E and S1F**). In addition, the number of neutral red-labeled (neutral red+; lysosome marker) microglia mildly decreased in both *il1b*^{+/-} and *il1b*^{-/-} zebrafish embryos compared with the *il1b*^{+/+} siblings (**Additional File 1: Fig. S1G-S1H**). However, the number of *mpeg1* + macrophages/microglia in the brain and CHT as well as the mRNA expression of *mpeg1* and *mpx* in the head remained unchanged in *il1b*^{+/-} zebrafish embryo compared with wild-type siblings (**Additional File 1: Fig. S1I-S1K**). While *il1b*^{Mut} showed no impact on early development of macrophages/microglia in the brain and CHT at 72 hpf, the number of macrophages in CHT at 4 dpf and the number of macrophages/microglia in brain at 5 dpf of heterozygous *il1b*^{Mut} declined (Fig. 2A-2E). Upon tail amputation, peripheral tissue injury-induced infiltration of macrophages alleviated in *il1b*^{Mut} at 5 dpf/2 dpa (Fig. 2A-2C). Moreover, the number of macrophages in CHT was also reduced after amputation in both heterozygous *il1b*^{Mut} and wild-type siblings at 1 dpa, possibly due to the redistribution of macrophages (Fig. 2D and 2E). To separate infiltrating macrophages from microglia in the brain, macrophage-specific (*mfap4*) and microglia-specific (*apoeb*) markers were used [37]. The number of both *mfap4*+ and *apoeb*+ cells were elevated in the wild-type siblings but not in homozygous *il1b*^{Mut} zebrafish after amputation (Fig. 2F-2I). With all these results, it is most likely that *il1b* mediates infiltration of both macrophages and microglia into the brain of zebrafish embryos after peripheral tissue injury.

Macrophages infiltrated the brain through circulation independent migration after peripheral tissue injury

A time-lapse imaging was applied to investigate the migration routes of *il1b*-mediated macrophages infiltration into the brain at 4 dpf when the microglia ceased to invade the brain during development [32].

Using *Tg(kdrl:GFP;coro1a:DsRed)* double-transgenic zebrafish line to visualize cerebral blood vessels (endothelial cells) and pan-leukocytes (Fig. 3A), circulation-independent migration of macrophage infiltration into the brain were observed at 4 dpf/1 dpa while no macrophage was found to move out from the blood stream. A schematic diagram was shown to present the distribution of active macrophages around the brain (Fig. 3B). Specifically, peripheral macrophages located in the neighboring tissues infiltrate the brain through the lateral periphery of the hindbrain after tail amputation. In contrast, such infiltration could not be seen after amputation in CTRL, *il1b^{Mut}* (both *il1b^{+/-}* and *il1b^{-/-}* showing similar results) and *il1b^{Mut}+* (**Additional File 1: Video S1-S4**). Macrophages distributed around mid-hindbrain boundary (MHB) failed to infiltrate the brains of zebrafish with or without tail amputation (Fig. 3C-3F, **Additional File 1: Video S5**). In addition, compared to CTRL (Fig. 3C), more active macrophages/microglia were found within the brain of amputated zebrafish (Fig. 3D; **Additional File 1: Video S6**), which might be due to the infiltration of macrophages through the lateral periphery of the hindbrain (Fig. 3D; **Additional File 1: Video S7**). Therefore, this indicated that peripheral tissue injury induced *il1b*-regulated infiltration of macrophages from neighboring tissues through the lateral periphery of the hindbrain. Besides macrophages infiltration into the brain through circulation-independent migration, *il1b*-mediated cerebral blood vessel narrowing was observed. The diameter of cerebral branch vessels reduced while the main vessels increased after tail amputation, which were ameliorated in heterozygous *il1b^{Mut}* (**Additional File 1: Fig. S3A and S3B**). As expected, a declined blood flow was detected in the cerebral branch vessels by using *Tg(kdrl:GFP;gata1:DsRed)* with *gata1*-labeled (*gata1*+) erythrocytes (**Additional File 1: Fig. S3C**). Surprisingly, circulatory macrophages were found to be trapped within the narrow cerebral branch vessels, which might also contribute to an increased number of macrophages in the brain following amputation (**Additional File 1: Fig. S3D**). While infiltration of macrophages into the brain was accomplished via circulation-independent migration, the response of cerebral blood vessels and blood flow to peripheral tissue injury might participate in other pathological processes, such as apoptosis, that affected infiltration of macrophages to the brain.

Apoptosis but not proliferation contributed to the *il1b*-mediated macrophage number increase in the brain

Recruitment of macrophages/microglial is an essential step in the clearance of apoptotic neurons through phagocytosis, where the dying cells release various “find me” signals [38]. Thus, elevated apoptotic signaling molecules in the brain may contribute to the infiltration of macrophages/microglia. We further examined whether apoptosis is the mechanism underlying *il1b*-mediated phagocytes recruitment as *il1b* is a direct triggering factor for neuronal apoptosis [39]. Unlike the increase in apoptotic cells in the brain after tail amputation in wild-type embryos, tail amputation in homozygous *il1b^{Mut}* zebrafish did not trigger any significant increase in the number of AO + and TUNEL-labeled (TUNEL+) apoptotic cells. The results were consistent with the unchanged number of macrophages/microglia in the brain, in which apoptotic cells in the *il1b^{Mut}* remained the same as in CTRL group (Fig. 4A-4D). Moreover, a reduced number of TUNEL + apoptotic cells were also found in the CHT of zebrafish with tail

amputation, probably due to the redistribution of leukocytes, while *il1b*^{Mut} also resulted in a reduced number of apoptotic cells in CHT (Fig. 4E and 4F). In addition to *il1b*-induced apoptosis, we observed that neutral red + and LysoTracker + lysosome, an organelle for phagocytosis, decreased in the macrophages/microglia in the brain following tail amputation. Mutation of *il1b* (*il1b*^{-/-}) also led to a reduction of *mpeg1*+ & LysoTracker + colocalized cells (**Additional File 1: Fig. S4A-S4D**). Accumulation of lysosomes and macrophages at the injury site was also reduced in heterozygous and homozygous *il1b*^{Mut} (**Additional File 1: Fig. S4E-S4G**). Importantly, the LysoSensor + acidified lysosomes were reduced only after tail amputation indicating an impaired phagocytosis, and this could contribute to the increased apoptotic cells in the brain (**Additional File 1: Fig. S4H-S4I**). To eliminate the potential influence of glial cell proliferation on the increased number of macrophages/microglia in the brain, phospho-Histone H3 (PH3) was utilized to label proliferating cells. PH3 + cells in the midbrain remained unchanged after tail amputation, which indicates that increased number of macrophages/microglia was not due to the proliferation (Fig. 4G and 4H). On the contrary, the decreased number of macrophages/microglia in homozygous *il1b*^{Mut} might attribute to the reduced proliferation as shown by the decreased number of PH3 + cells (Fig. 4G and 4H). In CHT, the number of PH3 + cells decreased after tail amputation in both wild-type siblings and *il1b*^{Mut} (Fig. 4I and 4J). Collectively, increased apoptosis but not cell proliferation contributed to *il1b*-mediated infiltration of macrophages/microglia into the brain.

***il1b* -mediated proteomic changes and disordered behavior in response to peripheral tissue injury**

To further investigate the potential effect of peripheral tissue injury on the brain and mechanisms underlying *il1b*-mediated infiltration of macrophages, proteomic analysis was performed with the head of zebrafish embryo (Fig. 5A). Despite the limited number of proteins identified, 29 out of 803 (3.6%) proteins were found in the head showing significant changes after tail amputation (AMPU) vs control (CTRL), which included 27 increased and 2 decreased proteins (Fig. 5B). We tracked these 29 proteins in various proteomic analysis including *il1b*^{Mut} vs CTRL, *il1b*^{Mut} + AMPU vs *il1b*^{Mut}, *il1b*^{Mut} + AMPU vs CTRL (data not shown) and *il1b*^{Mut} + AMPU vs AMPU (data not shown), and excluded proteins that did not respond to *il1b*^{Mut} at different conditions (Fig. 5C). Finally, 21 out of the 29 (72%) proteins were found to be regulated by *il1b* in AMPU, with the expression increased after amputation and alleviated in *il1b*^{Mut} (Fig. 5D). The function of these proteins was classified to explore the effect of peripheral tissue injury-induced *il1b* on the brain. In accordance with previous findings, apoptosis inducing factor mitochondria associated 4 (*aifm4*) and apolipoprotein Eb (*apoeb*), the specific marker of pro-apoptosis and microglia were found. Proteins involved in neuroprotection and other biological processes, such as development and metabolism, in response to *il1b*-mediated damage to the brain were also identified. Importantly, *camk2g1*, orthologous to human *CAMK2G* that is involved in multiple psychiatry disorders [40] is also on the list (Fig. 5D). Since the *il1b*-mediated increased expression of *camk2g1* may result in psychiatry disorders, locomotor behavior of zebrafish was recorded at 7 dpf/4 dpa. A hyperactive behavior characterized by increased travel distances and active swim time without affecting average velocity (data not shown) was observed in amputated wild-type siblings but not in homozygous *il1b*^{Mut} zebrafish embryos. This indicates that peripheral tissue injury induced an *il1b*-mediated hyperactive behavior

(Fig. 5E and 5F). In summary, peripheral tissue injury induced *il1b*-mediated induction of *camk2g1* in the brain, which together with the other 20 proteins may contribute to the observed hyperactive behavior.

Discussion

Infiltration of peripheral macrophages into the CNS has been well documented in various central neurological disorders, such as neurodegenerative diseases, psychiatry disorders and traumatic brain injuries [3–5]. However, little is known about the mechanisms responsible for the infiltration and its pathological roles in systemic inflammation-mediated neurological disorders. Here we demonstrated a dynamic interplay of peripheral tissue injury, systemic inflammation, neuroinflammation, apoptosis and macrophage infiltration using a zebrafish model. Most importantly, we demonstrated that *il1b* is a key regulator in mediating the infiltration of peripheral macrophages into the brain from neighboring tissues through circulation-independent migration, a process which is probably triggered by the accumulation of apoptotic cells in the brain [38]. The proteomic analysis also identified an elevation in an *il1b*-regulated protein, *camk2g1* following peripheral tissue injury. This protein is elevated in the brain affected by neuropsychiatry disorders, and its increase accompanied with neuroinflammation, infiltrating macrophages and neuronal apoptosis might contribute to the hyperactive behavior of zebrafish observed in this study [3,40].

Many studies have reported that circulatory macrophages infiltrate the brain following peripheral inflammation and further contribute to the CNS diseases. However, they were limited to the several relatively rare disease states including arthritis and liver injury [24,25,41]. Our work provided experimental evidence of systemic inflammation induced infiltration of macrophages into the brain in response to peripheral tissue injury, and suggests a potential role of infiltrated macrophages in the development of peripheral trauma-induced neurological disorders such as PTSD and POCD [3,42]. Furthermore, we confirmed that *il1b* but not *tnfa*, the latter being generally recognized as a cytokine in systemic inflammation-induced infiltration of macrophages and neurological disorders, served as the key proinflammatory regulator under peripheral tissue injury [24,25]. While microglia-secreted CCL2, a chemokine attracting macrophages into the CNS [24,43], remained unchanged in this model, our results demonstrated that the accumulation of apoptotic cells in the brain triggered by *il1b* impaired phagocytosis, as characterized by reduced lysosomes in macrophage/microglia, and may contribute to the recruitment of macrophages/microglia following peripheral tissue injury [36,39,44]. These observations suggest that the mechanisms of the systemic inflammation mediated neurological disorders may vary between pathological conditions, which warrant further studies. It is widely believed that breakdown of BBB promotes circulatory macrophage infiltration, though infiltrating macrophages can be found in the CNS with intact BBB and migrating through the cerebrospinal fluid (CSF) [9,45]. In this current study, circulation-independent infiltration of macrophages was observed, where macrophages invaded the brain from the neighboring tissues through the lateral periphery of the hindbrain, specifically between metencephalon (or cerebellum) and myelencephalon, differs from the microglial invasion to the zebrafish midbrain during development [46]. This suggests that in addition to circulation, other less

studied migration routes might be involved in the infiltration of macrophages into the brain under specific disease states.

Involved in the pathogenesis of multiple neurological disorders, infiltration of macrophages into the brain might result in seizure or sickness behaviour or anxiety in response to peripheral inflammation and neuroinflammation, respectively [3,24,47]. We demonstrated that peripheral tissue injury results in hyperactive behavior marked by elevated expression of *camk2g1* in the context of both systemic inflammation and neuroinflammation. More importantly, *il1b* mutation mitigated the increased levels of *camk2g1* and the hyperactive behavior. *CaMK2G*, the human ortholog of *camk2g1*, is associated with major neuropsychiatry disorders. Correlation between polymorphism in *CaMK2G* and human memory performance was reported previously [48]. In addition, abnormal levels of alternative *CaMK2G* splicing was found in the brain of patients with autism spectrum disorder (ASD) [49]. More importantly, the elevated *CaMK2G*-encoding protein (γ CaMKII) level in the brain has been detected in rodent models of anxiety, schizophrenia and major depressive disorder (MDD) [50–52]. Thus, hyperactive behavior observed in our peripheral tissue injury model might represent the *camk2g1*-related neuropsychiatry disorder. While the roles of *CaMK2G* in macrophages infiltration and subsequent impact on neurological functions require further investigations [53], the *il1b*-mediated elevated expression of *camk2g1* together with infiltration of macrophages, neuronal apoptosis and neuroinflammation might serve as the potential mechanism underlying peripheral tissue injury-induced neuropsychiatry disorders.

Conclusion

Although peripheral injury/inflammation has long been linked to neurological disorders, the underlying mechanisms remain unclear. The present study demonstrated that peripheral tissue injury induces *il1b*-mediated macrophage infiltration into the brain and a hyperactive behavior, which may contribute to development of therapeutic strategies against macrophage infiltration in neurological disorders following peripheral injury.

Abbreviations

AD (Alzheimer's disease); AGM (aorta-gonad-mesonephros); aifm4 (factor mitochondria associated 4); ALS (amyotrophic lateral sclerosis); AMPU (amputation); AO (acridine orange); apoeb (apolipoprotein Eb); ASD (autism spectrum disorder); BBB (blood-brain barrier); CHT (caudal hematopoietic tissue); CSF (cerebrospinal fluid); CTRL (control); dpf (days post-fertilization); F0 (Founder); hpa (hours post-amputation); hpf (hours post-fertilization); *il1b* (interleukin 1 beta); LPS (lipopolysaccharides); MDD (major depressive disorder); PD (Parkinson's disease); PH3 (phospho-Histone H3); POCD (post-operative cognitive dysfunction); PTSD (post-traumatic stress disorder); RFLP (restriction fragment length polymorphism); TALEN (transcription activator-like effector nucleases); WISH (whole-mount in situ hybridization); WT (wild-type)

Declarations

A. Ethics approval and consent to participate

Experimental protocols involving zebrafish were approved by the Committee of the Use of Laboratory and Research Animals (CULATR) of the University of Hong Kong and Animal Subjects Ethics Sub-Committee (ASESC) of The Hong Kong Polytechnic University.

B. Availability of data and materials

All data supporting this study are available from the corresponding author upon reasonable request.

C. Competing interests

The authors declare no competing interests.

D. Funding

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E. Acknowledgement

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F. Authors' contributions

XC, ACM, and RCC designed experiments and wrote manuscript; XC, JSK, GTW, KMH, ZY performed experiments and analyzed data.

All authors read and approved the final manuscript.

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Figures

Figure 1

Infiltration of macrophages into the brain in response to peripheral injury (A-B) qPCR results presented mRNA levels of various inflammatory cytokines in the region of tail/injury site (A) and the head (B) compared to the control (CTRL) (three independent experiments, n = 30 each). *, p < 0.05; **, p < 0.01 compared with CTRL based on unpaired t-test. (C) Representative images of *il1b* whole-mount in situ hybridization (WISH) in CTRL and amputation (AMPU). Scale bar, 100 μ m. (D) Representative images of

Tg(*coro1a*:DsRed) in CTRL and AMPU. Scale bar, 50 μ m. Asterisk, pigment. (E) Number of *coro1a*⁺ cells in the brain at 4-6 days post fertilization (dpf) under CTRL (n = 9 each) and AMPU (n = 9 each). **, p < 0.01 compared with CTRL based on unpaired t-test. (F) Representative images of Tg(*mpeg1*:GFP) in CTRL and AMPU. Scale bar, 50 μ m. (G) Representative images of Tg(*kdr1*:GFP; *mpx*:mCherry) in CTRL and AMPU. Scale bar, 50 μ m. Asterisk, pigment. (H) qPCR results showed mRNA levels of *mpeg1* and *mpx* in the brain compared to the CTRL (three independent experiments, n = 30 each). **, p < 0.01 compared with CTRL based on unpaired t-test. (I-J) Representative images of acridine orange (AO) stained Tg(*coro1a*:DsRed) in CTRL (n = 18) and AMPU (n = 18) and the number of AO⁺ and *coro1a*⁺ cells. Scale bar, 80 μ m (Merged), 40 μ m (Enlarged). **, p < 0.01 compared with CTRL based on unpaired t-test. Red arrow, AO⁺ and *coro1a*⁺ cells.

Figure 2

il1b modulates infiltration of macrophages and microglia into the brain (A-B) Representative images of 4 dpf Tg(*mpeg1*:GFP) in CTRL (n = 9), AMPU (n = 9), *il1b* mutation (*il1b*^{Mut}) (n = 9) and *il1b*^{Mut}+AMPU (n = 9); and the number of *mpeg1*⁺ cells in the brain at 4-5 dpf. Scale bar, 40 μ m. *, p < 0.05; **, p < 0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD. (C) Representative flow cytometry result indicated the percentage of *mpeg1*⁺ cells in the head. (D-E) Representative images and the

number of mpeg1+ cells in caudal hematopoietic tissue (CHT) of 4 dpf Tg(mpeg1:GFP) in CTRL (n = 9), AMPU (n = 9), il1bMut (n = 9) and il1bMut+AMPU (n = 9). Scale bar, 50 μ m. **, p < 0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD. (F-G) Representative images and the number of apoeb+ cells in the brain of CTRL (n = 20), AMPU (n = 20), il1bMut (n = 20) and il1bMut+AMPU (n = 20). Scale bar, 40 μ m. **, p < 0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD. (H-I) Representative images and the number of apoeb+ (microglia) and mfap4+ (macrophage) cells in the brain of CTRL (n = 20), AMPU (n = 26), il1bMut (n = 20) and il1bMut+AMPU (n = 20). Scale bar, 40 μ m. **, p < 0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD.

Figure 3

Macrophages infiltrated into the brain through circulation independent migration. (A) Representative image of the brain of Tg(kdrl:GFP;coro1a:DsRed). Kdrl (White) and coro1a (Magenta) as the specific markers represent the endothelia cells/blood vessels and macrophages/microglia. Scale bar, 80 μ m. (B) Schematic diagram of distribution of active coro1a+ cells in the brain under CTRL, AMPU, *il1b^{Mut}* and *il1b^{Mut}+AMPU*. M, midbrain. H, hindbrain. (C-F) Time-lapse imaging of active coro1a+ cells in the brain

under CTRL (C), AMPU (D), *il1b*Mut (E) and *il1b*Mut+AMPU (F) presenting three different distribution patterns. T, time. Scale bar, 40 μ m. Red arrows, active *coro1a*+ cells.

Figure 4

Apoptosis contributed to infiltration of macrophages into the brain (A-B) Representative images and the number of AO+ and *coro1a*+ cells in the brain of CTRL (n = 6), AMPU (n = 6), *il1b*Mu (n = 6)t and *il1b*Mut+AMPU (n = 6). Scale bar, 40 μ m. **, p < 0.01 compared with CTRL based on two-way ANOVA with

Tukey's HSD. (C-D) Representative images and the number of TUNEL+ cells in the brain of CTRL (n = 14), AMPU (n = 15), *il1b*Mut (n = 12) and *il1b*Mut+AMPU (n = 14). Scale bar, 40 μ m. **, p < 0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD. (E-F) Representative images and the number of TUNEL+ cells in the CHT of CTRL (n = 7), AMPU (n = 7), *il1b*Mut (n = 6) and *il1b*Mut+AMPU (n = 6). Scale bar, 40 μ m. **, p<0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD. (G-H) Representative images and the number of phospho-histone H3 (PH3)+ cells in the brain of CTRL (n = 12), AMPU (n = 12), *il1b*Mu (n = 12)t and *il1b*Mut+AMPU (n = 12). Scale bar, 40 μ m. **, p < 0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD. (I-J) Representative images and the number of PH3+ cells in the CHT of CTRL (n = 12), AMPU (n = 12), *il1b*Mut (n = 12) and *il1b*Mut+AMPU (n =12). Scale bar, 40 μ m. **, p < 0.01 compared with CTRL based on two-way ANOVA with Tukey's HSD.

Figure 5

Proteomic analysis and behavioral responses to peripheral tissue injury (A) Schematic diagram of proteomic analysis of the brain region of zebrafish embryos. (B) Targeted proteins were identified in AMPU:CTRL (three independent experiments, n = 50 each) based on $p < 0.05$ (unpaired t-test), fold change >1.5 or <0.6 and labeled in magenta. (C) Tracking targeted proteins in il1bMut:CTRL and il1bMut+AMPU:il1bMu (three independent experiments, n = 50 each). (D) Heatmap presented the responses of targeted protein under CTRL, AMPU, il1bMut and il1bMut+AMPU. Asterisk, key proteins identified. (E-F) Responses image of locomotor activity, the total travel distance and active swim time in CTRL (n = 5), AMPU (n = 5), il1bMut (n = 5) and il1bMut+AMPU (n = 5) were calculated based on five independent experiments. **, $p < 0.01$ compared with CTRL based on two-way ANOVA with Tukey's HSD.

Figure 6

Diagram

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Video1CTRL.mp4](#)
- [Video2AMPU.mp4](#)
- [Video3il1bMut.mp4](#)
- [Video4il1bMutAMPU.mp4](#)
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- [Video6Pattern2.mp4](#)
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