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Title: The cationic amino acid exporter Slc7a7 is induced and vital in tissue macrophages with sustained efferocytic activity

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Running title: Slc7a7 is vital upon efferocytosis

Key words: macrophages, microglia, efferocytosis, zebrafish

Summary statement:

The cationic amino acid exporter Slc7a7 is rapidly induced in tissue macrophages upon efferocytosis, allowing them to cope with the catabolism of numerous dead cells without compromising their own viability.

ABSTRACT

Most tissues harbor a substantial population of resident macrophages. Here we elucidate a functional link between the *Slc7a7* cationic amino acid transporter and tissue macrophages. We identified a mutant zebrafish devoid of microglia due to a mutation in the *slc7a7* gene. We found that in *Slc7a7* deficient larvae, macrophages do enter the retina and brain to become microglia, but then die during the developmental wave of neuronal apoptosis, which triggers intense efferocytic work from them. A similar macrophage demise occurs at other tissues and stages where macrophages have to engulf many cell corpses, whether due to developmental or experimentally triggered cell death. We found that *Slc7a7* is the main cationic amino acid transporter expressed in macrophages of zebrafish larvae, and that its expression is induced in tissue macrophages within 1-2 hrs upon efferocytosis. Our data indicate that *Slc7a7* is vital not only for microglia but also for any steadily efferocytic tissue macrophages, and that *slc7a7* gene induction is one of the adaptive responses that allow them to cope with the catabolism of numerous dead cells without compromising their own viability.

INTRODUCTION

Most tissues in vertebrates harbor resident macrophages. They are thought to be important for tissue homeostasis (Okabe and Medzhitov, 2016). They display quite diverse phenotypic traits among different tissues - which historically led to give them different names, e.g. "microglia" for the resident macrophages of the central nervous system (CNS). Many of these tissue resident macrophages do not derive from monocytes produced in the bone marrow. Instead, they originate from the yolk sac, and migrate to colonize embryonic tissues where they are ultimately maintained or self-renew throughout life (Perdiguerro and Geissmann, 2016).

In the past years, several studies aimed at obtaining a more comprehensive view of the molecular requirements for the early establishment of microglia and tissue-resident macrophage populations in general, using the zebrafish model. Thus, the CSF-1 receptor and its ligand IL34 were found essential for the attraction of yolk sac derived macrophages to the cephalic tissues, and the subsequent establishment of primitive microglia as well as epidermal macrophages (Herbomel et al., 2001; Wu et al., 2018). In addition, developmental neuronal cell death was found to be important as well for microglia establishment in the midbrain optic tectum (Xu et al., 2016). In parallel, we and others performed forward genetic screens for zebrafish larvae devoid of microglia (Demy et al., 2017; Meireles et al., 2014; Rossi et al., 2015; Shiao et al., 2013) - based on the convenient staining of microglia in live zebrafish larvae with neutral red that we had introduced (Herbomel et al., 2001). The mutations causing microglia absence in the resulting mutants published so far all affected microglia or its precursors cell-autonomously, and encoded proteins with very diverse functions: a Nod-like

cytoplasmic receptor (Nlr3l/Nlrp12); a transmembrane phosphate exporter (Xpr1b); a nuclear transcription regulator (Trim33); and a transmembrane cationic amino acid transporter (Slc7a7). For most of them, it is still unclear how the causative mutation led to microglia absence. We originally found the *slc7a7* mutant in a screen performed together with F. Peri's team, who have then published their study, which concluded that Slc7a7 identifies microglial precursors prior to entry into the brain (Rossi et al., 2015) and microglia thereafter. While our independent mapping of the mutation agreed on the nature of the causative mutation, our conclusions on the functional significance of Slc7a7 in microglia development are quite different. Therefore we present here our own study. In short, we show that *slc7a7* expression is induced in any efferocytic tissue macrophages in the zebrafish larva, and is vital for them whenever they have to provide a high rate of efferocytic activity. Our study also sheds light on how microglia acquires some of its long-term specific molecular signature.

RESULTS

In an F3 screen following ENU-based mutagenesis, we identified *cerise*^{NO067} – hereafter referred to as *cerise* - as a recessive mutant that displayed no microglia vitally stained by Neutral Red (NR) at 4 dpf (Fig. 1A,B). *Cerise* mutants are still devoid of microglia by 9 dpf (Fig. 1C) but otherwise properly developed, with a swim bladder and robust blood circulation (Fig. S1A,B), and can survive up to 17 days (data not shown).

We first mapped the *cerise* mutation by bulk segregant analysis of SSLPs (Geisler et al., 2007) to a 7.7 Mb interval on linkage group 7 (LG7). We then moved to SNP analysis based on whole-genome sequencing (Leshchiner et al., 2012) of pools of mutant and sibling embryos (3 pools: 20 siblings, 20 mutants, then 200 mutants). This analysis refined the location of the causative mutation within the previously defined region of LG7, and led to identify in the mutants a homozygous A>T transversion at the splice junction between intron 4 and exon 5 of the gene *solute carrier family 7, member 7 (slc7a7)* gene (Fig. 1D), confirming the results obtained by Rossi et al. (Rossi et al., 2015) in their independent characterization of the *cerise* mutant. This gene encodes the catalytic subunit of the heterodimeric transmembrane cationic amino acid (CAA) transporter y+LAT-1 (Torrents et al., 1999). **The *cerise* mutation changes the final AG of intron 4 into TG, which leads to exon 5 skipping and splicing of exon 4 to exon 6, as verified by mutant cDNA sequencing (data not shown). This causes a frameshift from the beginning of exon 6, leading to a premature stop codon nine codons downstream. Hence the predicted mutant protein comprises only the N-terminal half of the 501 amino acid WT Slc7a7 protein followed by 9 new amino acids, and thus lacks six of its twelve transmembrane domains.**

We evaluated the expression in macrophages/microglia of *slc7a7* and of the other genes encoding transmembrane CAA transport systems expressed at the plasma membrane

(Fotiadis et al., 2013), through an RNAseq analysis that we performed on fluorescent macrophages/microglia sorted from zebrafish larvae expressing mCherry under the control of a macrophage specific promoter (*mpeg1*) at 3 dpf (Fig. S2 and Table S1). We found *slc7a7* to be expressed at a high level in these cells, e.g. twice as much as the transmembrane receptor and macrophage lineage marker gene *csf1r*. In contrast, *slc7a6*, encoding the closely related y+LAT-2 transporter, was expressed very weakly. Of the three genes encoding monomeric CAA transporters - CAT-1 (*slc7a1*), CAT-2 (*slc7a2*) and CAT-3 (*slc7a3*) - only *slc7a2* and *slc7a3* were found expressed, but at a level 10-fold lower than *slc7a7* (Fig. S2 and Table S1). Thus, y+LAT1/Slc7a7 is the main CAA transporter in macrophages / microglia of zebrafish larvae.

To confirm that the *cerise* phenotype was caused by Slc7a7 deficiency and have an alternative tool to knock this gene down, we designed two different splice blocking morpholinos (Mo) (Fig. 1D). Upon injection into wild-type (WT) embryos, the effect of the sp55 morpholino - closest to the mutation, and used by Rossi et al. (2015) - on microglia was only partial, whereas the sp22 morpholino fully phenocopied the *cerise* mutation (Fig. 1E-G). Accordingly, subsequent experiments were conducted using the sp22 Mo.

Transferring the *cerise* mutation into the transgenic *Tg(mfap4:mCherryF)* background, which specifically highlights macrophages/microglia confirmed the lack of primitive microglia from 4 dpf onwards (Fig. 1H,I and Fig. S1C,D). The same was found in *slc7a7*-sp22 morphants (Fig. 1J-M). At earlier stages (2 dpf), Slc7a7 deficient embryos display a normal production and overall deployment of primitive macrophages and are phenotypically unrecognizable from their controls (Fig. S1E,F).

Slc7a7-deficient macrophages die after colonizing the brain and retina

We noticed that the few microglial cells occasionally seen in the optic tectum (OT) of the mutants at 4 dpf, and even more so at 3 dpf (when the tectal microglia is just being established in the wt – see below), were more heterogeneous in size than wt microglia, and quite often occurred in small groups at the ventro-lateral corners of the OT (Fig. S3A-D, [arrows](#)) (this trait, not seen in other microglia mutants of our collection, is actually why we initially named this mutant *cerise*). Closer examination by VE-DIC microscopy revealed unusual unstained large vacuoles adjacent to or including the NR stained material (Fig. S3E-G), that have not been seen in wt larvae. Similar vacuoles appeared to be fluorescent in *cerise* *Tg(mfap4:mCherryF)* larvae, suggesting that they were remnants of dead/dying mCherry-positive macrophages/microglia (Fig. S3H-H’). As macrophage death in the CNS or elsewhere cannot be assessed by the usual markers of apoptosis (e.g. annexin V staining, caspase3 activation, TUNEL) because most of them contain engulfed dead cells that stain positive for all these markers, we turned to *in vivo* time-lapse confocal imaging. We followed the behaviour of

fluorescent tectal macrophage/microglial cells in transgenic morphant larvae from 3 dpf onwards and compared it to that in control larvae (Fig. 2A-C, Movies 1 and 2). While the latter displayed the typical ramified and dynamic morphology with little movement of the cell center acquired by that developmental stage (Li et al., 2012; Peri and Nüsslein-Volhard, 2008; Svahn et al., 2013), their counterpart in the morphant still displayed a more macrophage-like morphodynamics (Movies 1 and 2). Then, over the next 10 hrs, they disappeared from the tectum (Fig. 2A-C) - mostly after rounding up, becoming inert, and being engulfed by another macrophage/microglial cell, which a moment later also underwent the same fatal fate (Fig. 2C and Movie 2). These observations indicated that *Slc7a7*-deficient macrophages were actually able to colonize the OT but died once there, which could explain their inability to ever establish as a proper microglial population.

To further explore this prospect of a transient colonization of the CNS in *Slc7a7*-deficient embryos, we aimed at examining earlier developmental stages. The OT and the retina are the two CNS structures most densely populated with microglia in zebrafish larvae, but they acquire most of it at different times. While the main wave of OT colonization occurs between 2.5 and 3.5 dpf ((Herbomel et al., 2001; Xu et al., 2016); see also Fig. 3D), the retinal microglia number has already reached its plateau by 48 hpf (Demy et al., 2017; Herbomel et al., 2001). We therefore counted the number of macrophage/microglia cells in the retinae by 42 hpf in the progeny of *cerise* heterozygote carrier fish, and then genotyped the embryos. The results demonstrated that macrophages in homozygous mutant embryos were indeed able to colonize the retinae quite efficiently (Fig. 2E). An independent live follow-up of individual embryos documented the subsequent decrease of the retinal macrophage/microglia population in the mutants between 2 and 3 dpf (Fig. 2F). Following our previous observations in the OT, we hypothesized that these retinal macrophages also died. So we time-lapse imaged the retinal macrophages in morphant vs. control embryos from 54 hpf onwards. In the time-lapse sequences shown in Fig. 2G-H" and Movies 3 and 4, while the retinal macrophage number in the morphant was similar to the control at the onset of the imaging, it then steadily decreased, to virtually zero by 15 hrs later, with evidence of macrophage rounding and demise (Fig. S4A-A"). Interestingly, macrophages in interstitial tissues around the eye showed no sign of cell death (Fig. 2H', cyan arrowheads).

Thus, it appears that *Slc7a7*-deficient macrophages are able to normally colonize the OT and retina, but die there starting from 2.5 dpf. In the retina, where the microglial population has already reached its plateau by this stage, it results in a drop in cell number (Fig. 2E-F); in the OT, as the colonization wave and disappearance are concomitant, the microglial population never becomes substantial (Fig. 2D).

Neutrophils infiltrate the brain and retina of *Slc7a7*-deficient larvae following microglia

demise

In the zebrafish embryo, beside macrophages, primitive myelopoiesis in the yolk sac also produces neutrophils. Unlike in mammals, these neutrophils then disperse, live and wander in the tissues like the macrophages, except for the CNS that they normally never enter (Le Guyader et al., 2008). However, in *cerise*/*slc7a7* mutants and morphants, we found a massive infiltration of neutrophils in the brain OT and the retinas by 4 dpf (Fig. 3A-C). Through time-lapse confocal imaging of GFP+ neutrophils in *Tg(mpx:GFP)* larvae, we found that this influx of neutrophils into the retinae and brain started by 2.5-3 dpf, when the macrophages/microglial cells were dying there (Fig. 3D-F and Movie 5). Time-lapse imaging of double transgenic lines highlighting macrophages in red and neutrophils in green confirmed the link between macrophage state and neutrophil recruitment, as the latter repeatedly interacted with the few macrophages remaining in the *cerise* brain (Fig. S5). **Past 4 dpf - by 6 and 9 dpf - neutrophils were less frequently observed in the CNS; whenever present, they always correlated with the presence of dying macrophages/microglia, with which they interacted (Fig. S5F-I)**".

Neither qPCR for the cytokines that might be released by the stressed/dying macrophages (IL1- β , TNF- α , CXCL8), nor immunohistochemistry for IL1- β (Vojtech et al., 2012), nor *slc7a7* MO injection into *Tg(IL1- β :GFP)* reporter embryos (Nguyen-Chi et al., 2014) allowed us to identify the molecular signals responsible for the attraction of neutrophils to the dying microglia in the CNS of mutant larvae, leaving them yet to be identified (not shown).

Slc7a7 is required for the survival of highly efferocytic tissue macrophages

Thus, microglial cells in *cerise* larvae begin to die by 2.5 dpf. This is also precisely the stage at which WT microglial cells become intensely vitally stained by neutral red (Herbomel et al., 2001). As a weak base, neutral red accumulates in the phagolysosomal compartment. In the primitive microglia it accumulates over time in the phagosomes containing apoptotic bodies that have become acidified (Fig. S6), The onset of intense NR staining of microglia by 56-60 hpf (Herbomel et al., 2001) thus actually reflects the beginning of the major wave of neuronal developmental cell death (DCD) and the engulfment of the numerous resulting corpses by the young microglia. As microglial cells in *cerise* embryos begin to die by that same stage, we hypothesized that *Slc7a7* could be vital for macrophages / microglia that have to eliminate numerous apoptotic cells. **Indeed, our imaging did indicate that before dying, *Slc7a7*-deficient microglia were capable of efferocytosis just like WT (Fig. S4B-C), and we occasionally witnessed them dying just after engulfing a neuronal or microglial cell corpse (Fig. 2C, Fig. S4D-F)**". We therefore examined other tissue macrophage populations that face such a situation at some point in zebrafish development. Beside microglia, we previously noted the similarly intense but more transient neutral red staining of the macrophages that eliminate the remnants of the hatching gland by 3 dpf (Fig. 4A) (Herbomel et al., 2001), and the

macrophages of the Caudal Hematopoietic Tissue (CHT, (Murayama et al., 2006)), which engulf many of the circulating primitive erythrocytes particularly from 6 to 12 dpf (Fig. 4B,C), as the latter become progressively replaced by erythrocytes produced from the definitive hematopoiesis. Therefore we examined these highly efferocytic macrophage populations in *cerise* mutants and their siblings. In the latter, at the hatching gland location at 3 dpf, WT mCherryF+ macrophages, despite a heavy efferocytic load, still showed numerous long pseudopodia /ramifications (Fig. 4D). In sharp contrast, in *cerise* mutants, no ramified macrophages could be seen, and the widely scattered mCherryF+ spots, mostly of subcellular size, were strongly suggestive of macrophage degeneration (Fig. 4E). Similarly, in the CHT, *cerise* mutants display an accumulation of subcellular sized mCherry+ spots and much fewer healthy macrophages than in WT controls (Fig. 4F-I)..

These data collected across tissues and stages altogether strongly suggested that *slc7a7* expression is vital for any tissue resident macrophages that even transiently face the challenge of intense efferocytic work.

We further explored this hypothesis by inducing large-scale efferocytic work for macrophages in *slc7a7* morphant vs. control embryos. To achieve this, we took advantage of the fact that blood circulation in zebrafish embryos is open until 2.5 dpf (due to its free flow in the yolk sac before the closure of the common cardinal vein) and thus readily accessible to all interstitial macrophages. We treated embryos at 30 hpf for 20 hrs with a low dose of phenylhydrazine (PHZ), which induces apoptosis of all circulating primitive erythrocytes (Ferri-Lagneau et al., 2012; Shafizadeh et al., 2004). Macrophages in *slc7a7*-sp22 morphants readily engulfed the apoptotic erythrocytes within a few hours as in WT control embryos, but on the next day, they had virtually disappeared from the *slc7a7* morphant embryos treated with PHZ, while their number had only slightly decreased in the control embryos (Fig. 4J-L).

Slc7a7 expression is induced in tissue macrophages upon efferocytosis

We next examined the expression of the *slc7a7* gene in wt larvae by whole-mount in situ hybridization (WISH) (Fig. 5). We noticed that the two tissue macrophage populations that showed a distinctly higher *slc7a7* expression by WISH were precisely the brain and retinal macrophages/microglia from 56 hpf onwards (i.e. the stage at which neuronal DCD begins) and the hatching gland associated macrophages by 3 dpf (Fig. 5A-C). From 3 dpf, again consistent with the death of *Slc7a7*-deficient macrophages, *slc7a7* mRNA signal was also lightly detected in the tail (Fig. 5D), in addition to the kidney tubules and gut epithelium as in mammals (Fig. 5E).

We then examined *slc7a7* expression by WISH following PHZ-mediated death and engulfment of circulating erythrocytes, and found that it was again induced in the engulfing macrophages [throughout the venous system](#) (Fig. 5F-K), enough to cause a 3-fold increase at the whole-

embryo level (Fig. 5L). These results altogether indicate that *slc7a7* expression is not only required in macrophages with sustained efferocytic activity, but also induced by the latter, suggesting that a high level of Slc7a7 is necessary in this situation.

In order to refine the temporal link between efferocytosis and upregulation of Slc7a7 expression in macrophages, we aimed at inducing efferocytosis at precise anatomical locations and time. To do so, we exposed the fish embryos to a low concentration of CuSO₄ (50 μM), which has been shown to rapidly and specifically induce apoptosis of the sensory cells of the neuromasts of the lateral line within the epidermis (Fig. 6A), causing the immediate recruitment of 5-6 macrophages per neuromast to engulf the dead cells (Carrillo et al., 2016; Olivari et al., 2008). We treated the fish embryos at 54 hpf for 30 min. and monitored *slc7a7* expression at each neuromast of the posterior lateral line by WISH every 30 min over the next 6 hours (Fig. 6B). The CuSO₄ treatment efficiently destroyed the sensory cells (Fig. 6C-E), hence recruiting macrophages (Fig. 6C'-E'), which started to express *slc7a7* strongly enough to be detected by WISH (Fig. 6C''-E'',F,G), with a peak by 1.5 hr post treatment (hpt), and virtually no more expression by 4 hpt (Fig. 6B), even though recruited macrophages were still present in the area (see also Carrillo et al., 2016).

Taken together, our results thus show that the cationic amino acid exporter Slc7a7 is vital for and induced in tissue macrophages with sustained efferocytic activity.

DISCUSSION

In this study, we have found that Slc7a7-deficient zebrafish lack microglia because Slc7a7 is vital for tissue macrophages with sustained efferocytic activity. Thus, macrophages that have entered the brain and retina to become microglia start to die when the main wave of neuronal DCD occurs, triggering intense efferocytic work from these macrophages to eliminate the numerous neuron corpses. In the optic tectum - the most routinely documented brain compartment in zebrafish microglia studies - the initial entry of Slc7A7-deficient macrophages can be easily overlooked because the main developmental influx of macrophages into the OT is triggered by signals emanating from the dying neurons (Xu et al., 2016). Therefore when the neuronal DCD wave begins by 2.5 dpf, the attracted macrophages immediately have to engulf many neuron corpses, and die right away if they are Slc7a7-deficient. In support of this scenario, we had initially noticed that the few neutral red labelled microglial spots found in the mutant OT, usually together with signs of microglial demise, often occurred in the ventrolateral corners of the OT (hence the name *cerise* that we gave to this mutant). A recent study precisely showed that these are indeed the main sites of macrophage entry into the OT (Xu et al., 2016).

In contrast, in the retina, the macrophage/microglia number has already reached its plateau by 2 dpf; therefore in *Slc7a7*-deficient embryos, the demise of this already established population when the neuronal DCD begins is much more evident than in the OT.

In both the OT and the retina, we found a substantial infiltration of neutrophils following the demise of macrophages / microglia there. This neutrophil infiltration cannot be merely due to the accumulation of neuronal corpses in absence of microglia to engulf them, since it does not occur in other microglia-deficient mutants such as the *CSF1R*-deficient *panther* (Herbomel et al., 2001; Wu et al., 2018). It is most likely due to signals emanating from the dying microglia itself, because these infiltrating neutrophils do interact with the dying microglia, and up to 9 dpf we found them only when- and wherever dying/dead microglial cells were present. A similar neutrophil infiltration was reported in one particular mutant, deficient in *Nlr3l* (Shiau et al., 2013), in which macrophages showed an early inflammatory phenotype, with a clear induction of *il1-β* and *cxc18*. In contrast, we found no induction of these cytokine genes in *cerise* embryos in toto, nor any sign of *il1-β* induction in *Slc7a7*-deficient macrophages in situ using a *Tg(il1-β:GFP)* reporter line (Nguyen-Chi et al., 2014). So the molecular signals attracting neutrophils to the dying microglia in the CNS remain to be identified.

Since our initial characterization of zebrafish primitive microglia (Herbomel et al., 2001), its intense vital staining by Neutral Red has been widely used as an easy readout for microglia number, notably in forward and reverse genetic screens (Kuil et al., 2019, and references therein). Here we clarify that this strong NR staining actually reflects the specific accumulation of neutral red over time in the already acidified phagosomes containing apoptotic bodies. Therefore beyond microglia, it is a very convenient live staining method to spot any episode of high efferocytic activity among the various tissue macrophage populations of the developing fish. We have thus identified two such episodes, and the correlative demise of the corresponding macrophage populations in the *cerise* mutant – i) the engulfment by 3 dpf of the hatching gland cells that died following the release by apocrine secretion of their protease-loaded granules into the medium to trigger hatching; and ii) the engulfment of many primitive erythrocytes once the definitive ones are produced, peaking from 5-6 dpf onwards. These examples further indicate that *Slc7a7* is vital not specifically for microglia but for all tissue macrophages that undergo a phase of high efferocytic activity. This can also readily explain our observation that by 48 hpf, i.e. before the above three prominent developmental apoptosis events, mutant embryos already have 10 % less macrophages overall, and 25 % less macrophages in the retina than their non-mutant siblings. Indeed, before 48 hpf, macrophages do have to engulf corpses resulting from more limited developmental apoptosis events: e.g. among the pro-erythroblasts by the onset of blood circulation (Herbomel et al., 1999), among the newly born neurons of cranial ganglia by 30-35 hpf (Herbomel et al., 2001), and within the still neuroepithelial retina by 36 hpf (Cole & Ross, 2001).

We also found that *slc7a7* expression is induced in efferocytic macrophages in vivo, and our assay of macrophage recruitment to chemically damaged neuromasts showed that *slc7a7* induction occurs within 1-2 hrs following macrophage contact with apoptotic cells, and is over 2 hrs later. For macrophage populations whose efferocytic activity is sustained over many hours, or days as the primitive microglia, *slc7a7* expression becomes continually stimulated accordingly. It is conceivable that such continual stimulation may eventually turn into a stable expression feature of such a tissue macrophage population, even once their sustained efferocytic activity has subsided. We note indeed that in comparative transcriptome studies, *slc7a7* was found expressed at a high level in adult zebrafish microglia relative to brain tissue (Oosterhof et al., 2017), and in adult mouse microglia relative to peritoneal macrophages (Hickman et al., 2013). Interestingly, we found a developmental expression pattern very similar to *slc7a7* for at least two other genes, coding for lysosomal proteins involved in protein and lipid catabolism, cathepsin B and prosaposin (P.H., unpublished data), that will then also remain highly expressed in adult microglia (Oosterhof et al., 2017). This suggests that a whole subset of microglial markers or traits may well be acquired through - and thus dependent on - an episode of high and sustained efferocytic activity – namely the main neuronal DCD wave.

Thus our conclusion is quite different from that of Rossi et al. (2015) who claimed that *slc7a7* expression identifies microglial precursors prior to their entry into the brain. This conclusion was drawn from an embryo-wide photoconversion at 24 hpf of Kaede protein expressed from a transgenic *slc7a7* locus; at 4 dpf they found photoconverted Kaede in microglial cells. However, the photoconverted Kaede in the microglial cells at 4 dpf appeared to delineate not the entire cells but an intracellular compartment - most likely phagolysosomal (Rossi et al., 2015, Fig. 4P-R), and the ~ 4 *slc7a7*+ macrophages that can be detected by 24 hpf (Rossi et al., 2015, Fig. 3K) could hardly account for the amount of photoconverted Kaede seen in most or all microglial cells at 4 dpf (Rossi et al., 2015, Fig. 4N). Our own interpretation of these data is that, owing to the high diffuse *slc7a7* expression seen throughout the head at 24 hpf (Rossi et al., 2015, Fig. 3I), the global photoconversion performed at 24 hpf notably photoconverted Kaede throughout the brain (Rossi et al., 2015, Fig. 4K,N), and this photoconverted Kaede accumulated in the phagolysosomal compartment of the microglial cells when the latter massively engulfed dead neurons during the neuronal DCD wave.

Why is *Slc7a7* vital for highly efferocytic macrophages ? Sustained efferocytosis requires macrophages to catabolize the content of numerous cell corpses at a high rate, and then recycle or expell the resulting metabolites. These challenges involve adaptations of the macrophage metabolism that are only beginning to be uncovered (Han and Ravichandran, 2011). Since *Slc7a7* is the main cationic amino acid transporter expressed in the macrophages, and is known to act mainly as an exporter (Fotiadis et al., 2013), *Slc7a7* deficiency would predictably lead to a large excess of cationic amino acids in efferocytic

macrophages. An excess of arginine could conceivably be detrimental to the cell if it is used to produce excess NO via NO synthases (NOS); however we found that zebrafish primitive macrophages do not express any *nos* gene (Table S1). So the basis of a potential toxicity of excess intracellular cationic amino acids for the macrophage would remain to be identified.

In humans, inborn SLC7A7 deficiency causes a disease named Lysinuric Protein Intolerance (LPI) (Torrents et al., 1999), the primary symptoms of which are due to the absence of SLC7A7 in the basolateral membrane of gut enterocytes (where it normally allows the transfer of dietary cationic amino acids from the gut to the blood) and kidney tubules (where it allows reabsorption of cationic amino acids). However, beside these common symptoms, LPI patients suffer from variable, quite diverse types of all too often fatal symptoms, at least two of which suggest an involvement of macrophages - pulmonary alveolar proteinosis and hemophagocytic lymphohistiocytosis (Mauhin et al., 2017; Ogier de Baulny et al., 2012). It has been very difficult to study the etiology of these symptoms due to the lack of a viable mouse model until very recently (Bodoy et al., 2019). In addition to the symptoms listed above, a recent update of LPI patients follow-up pointed that over half of the patients presented with cognitive disorders (Mauhin et al., 2017). This has been explained so far by chronic or episodic hyperammonemia (due to a urea cycle disorder consequent to low serum arginine). Our study suggests an alternative or additional possible cause. As nothing is known about microglia in LPI patients, it is conceivable that as in the zebrafish, the first wave of microglia died in utero during the developmental neuronal death episodes, and that its demise contributed to later cognitive disorders.

MATERIALS AND METHODS

Zebrafish lines and embryos

Wild-type (WT), transgenic and mutant zebrafish embryos were raised at 28°C in Embryo Water (Volvic® water containing 0.28 mg/mL Methylene Blue [M-4159; Sigma] and 0.03 mg/mL 1-phenyl-2-thiourea [P-7629; Sigma]), and staged according to Westerfield (Westerfield, 1993).

AB & Tübingen WT fish (ZIRC), and the transgenic lines *Tg(mfap4:mCherry-F)^{ump6}* (Phan et al., 2018), *Tg(mpeg1:Gal4FF)^{gl25}* (Palha et al., 2013), *Tg(UAS:Kaede)rk8* (Hatta et al., 2006), *Tg(mpeg1:mCherry-F)^{ump2}* (Nguyen-Chi et al., 2014), *Tg(UAS-E1b:Eco.NfsB-mCherry)^{c264}* (Davison et al., 2007), *Tg(elavl3:EGFP)^{knu3}* (Park et al., 2000), *Tg(mpx:GFP)ⁱ¹¹⁴* (Renshaw et al., 2006), *Tg(gata1:dsRed)^{sd2}* (Traver et al., 2003), *Tg(mpeg:GFP-CAAX)^{sh425}* (Keatinge et al., 2015), *Tg(lyz:DsRed2)^{nz50}* (Hall et al., 2007) have been used in this study. The *cerise^{NO067}* mutant was obtained by ENU chemical mutagenesis on a Tübingen WT background, and maintained by outcross of heterozygous carriers with WT/Tg fish. [The fish were housed in the](#)

Institut Pasteur fish facilities accredited by the French Ministry of Agriculture. Work on fish was performed in compliance with French and European regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, February 6th, 2013). All experiments performed on >5dpf larvae were approved by the Ethics Committee #89 (CETEA Institut Pasteur) and registered under the reference DAP190108.

Mapping and identification of the mutation

1500 *cerise* homozygous mutant larvae were collected along with a few of their siblings after neutral red based screening at 4 dpf. We first mapped the mutation to a 7.7 Mb interval on linkage group 7 (LG7) by bulk segregant analysis of sequence-length polymorphism (SSLP) markers, as described previously (Geisler et al., 2007). Then whole genome Illumina HiSeq2000 sequencing (Leshchiner et al., 2012) of 20 siblings and 220 mutants followed by SNP analysis led to the identification of a mutation within this region, a AG>TG change at the splice acceptor of *slc7a7* intron 4 (GenBank: [BC110115.1](#)). To confirm the mis-splicing induced by the *cerise* mutation, total RNA was isolated with Trizol reagent [Invitrogen, 15596-026] according to manufacturer's instructions, and retrotranscribed into cDNA by M-MLV reverse transcriptase [Invitrogen, 28025-013]. A PCR was performed to amplify the full mutant *slc7a7* cDNA using :

Forward primer 5'- AAATAACATGACCTGCCCGC -3'

Reverse primer 3'- AATCCCGTCAGCAGCTTTTG -5'

The 2395bp amplicon was then fully sequenced (Eurofins Genomics) using :

Sequencing primer 1 5'- TGTGACCACGACAATGAACG -3'

Sequencing primer 2 5'- TCGCTGGTAGTGTGGACAAT -3'

Sequencing primer 3 5'- ATCATCACTGGCCTGGTCAA -3'

Sequencing primer 4 5'- TGCTTCAAGGCTGTTCTTCG -3'

The result was that exon 5 was skipped and exon 4 spliced to exon 6; the rest of the mRNA was as predicted for WT.

Morpholino injections

Two splice blocking antisense morpholinos (Mos) against *slc7a7* RNA were synthesized by Gene Tools :

Sp22 (targeting the exon2/intron2 donor site) : 5'-ATACATCCAACCTCACAGATGCAGGC-3'

Sp55 (targeting the exon5/intron5 donor site) : 5'-AAAGTGTTTATTACTCACCACAGCC-3'

1-5 nL of 0.6 mM Mo solution was microinjected in one to two-cell-stage embryos.

Neutral Red vital staining of microglia and Sudan Black staining of neutrophils

Highly efferocytic cells, including microglia, were revealed in live embryos and larvae by adding Neutral Red [N-4638; Sigma] to Volvic Water at a final concentration of 5 µg/mL for 2 hrs, or 2.5 µg/ml for ~5 hrs when optimal signal-to-noise ratio was desired. After incubation in the dark at 28°C, larvae were rinsed in Volvic Water, anesthetized, and observed under a stereomicroscope or a compound wide-field microscope (see below). Sudan Black staining of neutrophils in formaldehyde fixed embryos was done as previously described (Le Guyader et al., 2008).

Neuromast damage with CuSO₄

Neuromast hair cell damage was achieved by incubating 54 hpf larvae in 50 µM CuSO₄ [1.02791; Sigma] for 30 min, as previously described (Olivari et al., 2008). Treated larvae were then rinsed 3 times with Embryo Water. After WISH, *slc7a7* RNA signal was manually assessed for each neuromast (SO1-L7) using a Macrofluor microscope (Leica), and/or imaged on a Reichert Polyvar2 wide-field microscope (see below).

Ablation of erythrocytes with Phenylhydrazine

The day of the experiment, Phenylhydrazine (PHZ) [P26252; Sigma] was dissolved in water at a concentration of 5 mg/mL for stock solution. Dechorionated live embryos at 30-35 hpf were then soaked in 5 µg/mL PHZ in Embryo Water overnight in the dark so as to induce massive death of primitive erythrocytes (Pelster and Burggren, 1996). The embryos were washed the next day with Volvic® water and processed for further experiments.

Whole-mount mRNA *in situ* hybridization and immunohistochemistry

Embryos and larvae were anesthetized with 0.16 mg/ml Tricaine [A-5040; Sigma] at the stage of interest, then fixed overnight at 4°C in 4% methanol-free Formaldehyde [Polysciences, Cat#: 040181]. Whole-mount *in situ* hybridization (WISH) was performed according to (Thisse and Thisse, 2004). Whole-mount immunohistochemistry (WIHC) was performed as described previously (Murayama et al., 2006), omitting the acetone treatment. The primary antibodies used were rabbit anti-zebrafish L-plastin and PU.1 polyclonal antibodies (at 1:5000 and 1:800 dilution, respectively) (Le Guyader et al., 2008), and the 4C4 mouse monoclonal antibody (Becker & Becker, 2001), used as pure hybridoma supernatant. The secondary antibodies were Cy3-coupled anti-rabbit antibody [111-166-003; Jackson ImmunoResearch] at 1:800 dilution, Cy3-coupled anti-mouse antibody [115-166-003; Jackson ImmunoResearch] at 1:500 dilution, and Alexa488-coupled anti-rabbit antibody [A11070; Molecular Probes] at 1:500 dilution.

SNP genotyping of fixed embryos

After WISH or WIHC and counting of macrophages / microglial cells in a clutch of *cerise* and sibling embryos, the tail tip of each embryo was cut using a sharp scalpel and isolated in a PCR tube for genotyping. Genomic DNA was extracted by boiling at 90°C for 30 min. in 40 µL of Extraction Buffer (25 mM NaOH, 0.2 mM EDTA), then the pH was neutralized by adding Tris-HCl. Genotyping was performed by qPCR using forward primers ending at and including the *cerise* mutation :

WT-complementary forward primer 5'-CCCTCTGGTGGTTATTTTTTCA-3'

mutant-complementary forward primer 5'- CCCTCTGGTGGTTATTTTTTCT-3'

The reverse primer 5'-CCACAGCATCACTGTCCAG-3' was designed so as to generate a 142 nt amplicon, optimal for qPCR reaction. qRT-PCR was performed using Takyon Rox SYBR Master mix blue dTTP kit [Eurogentec, Cat#: UF-RSMT-B0701], and carried out for three biological replicates with measurements taken from three technical replicates on an Applied Biosystems 7300 Real Time PCR system (Thermofisher). Mutants and siblings were then sorted by comparing their amplification curves upon qPCR with the WT and mutant set of primers ($Ct_{mutant} - Ct_{WT}$).

Microscopy and image analysis

Low-magnification bright-field images were acquired using video cameras mounted on a Leica Macrofluor driven by the Metavue (Metamorph) software or a Zeiss Macrofluor driven by the Zen software (Zeiss).

Wide-field video-enhanced (VE) Nomarski / differential interference contrast (DIC) and fluorescence microscopy were performed as described previously (Herbomel & Levraud, 2004; Murayama et al., 2006), through the 40x/1.00 water-immersion objective of a Nikon 90i microscope and the 40x/1.00 oil-immersion objective of a Reichert Polyvar 2 microscope. Images were obtained from a HV-D20 3-CCD camera (Hitachi), digitized through a GVD-1000 DV tape recorder (Sony), then still images were collected using the BTVpro software (Bensoftware, London).

For fluorescence confocal imaging, embryos and larvae were mounted as previously described (Demy et al., 2017). Images were then captured at selected times on an inverted Leica SP8 set-up allowing multiple point acquisition, so as to image mutants and their siblings in parallel. Image stacks were processed with the LAS software to generate maximum intensity projections, or were exported into the Photoshop (Adobe) or Imaris software (Bitplane) for further analyses.

RNAseq analysis of macrophages in zebrafish larvae

Zebrafish larvae from three different transgenic backgrounds highlighting macrophages - *Tg(mpeg1:mCherry)*, *Tg(mpeg1:Gal4; UAS:NfsB-mCherry)*, and *Tg(mpeg1:mCherry; mpx:gfp)*

– were anesthetized at 3 dpf with Tricaine, then dissociated into single-cell suspensions as previously described (Covassin et al., 2006). The red fluorescent macrophages were sorted by FACS, collected directly in lysis buffer, and RNA was extracted using a miRNeasy Micro kit [217084; Qiagen]. Sequencing was performed on Illumina HiSeq2500 by ZF-Screens (Leiden, Netherlands) on three biological replicates: Tg(mpeg1:mCherry) (RNA extracted from 9480 cells of 92 embryos), Tg(mpeg1:Gal4/UAS:NfsB-mCherry) (RNA extracted from 10875 cells of 146 embryos), Tg(mpeg1:mCherry/mpx:gfp) (RNA extracted from 10004 cells of 196 embryos). 10 Mreads of 50 nt (0.5 Gb) were obtained for each replicate. The resulting data were analysed with the DESeq software.

Statistical analysis

Statistical analyses and graphic representations were done using Prism software. Distributions were normalized by log, and an analysis of variance (Two-way ANOVA) was performed. qPCR samples were analyzed using the $\Delta\Delta\text{Ct}$ method; the mean Ct value of housekeeping gene (*ef1a*) was used for normalization; the 2 $\Delta\Delta\text{Ct}$ values were graphed with the geometric mean \pm 95% confidence intervals to estimate the fold change.

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Competing interests

The authors declare no competing or financial interest

Author contributions

P. Herbomel conceived and supervised the project, and obtained the funding. M. Tauzin isolated the *cerise* mutant, gave it its name, and conducted the initial phenotypic study. D L Demy, M. Carrère and R. Noche conducted most of the subsequent study, with the help of M. Le Bris and C. Baek during their respective Master internship. D L Demy performed the initial mapping of the mutation; I. Leshchiner & W. Goessling identified the *cerise* mutation in *slc7a7* via deep sequencing of mutant vs. sibling genome pools. P. Herbomel and D L Demy wrote the paper.

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FIGURE LEGENDS

Fig. 1. The *cerise* phenotype of microglia absence is caused by a mutation in the *slc7a7* gene and is fully mimicked by the *sp22* morpholino.

(A, B) Dorsal view of the microglia in the optic tectum of live 4 dpf larvae stained with Neutral Red (NR) in siblings (A) and *cerise* mutants (B).

(C) Quantification of NR+ tectal microglia in *cerise* sibling (white dots, n=112) and mutant (black dots, n=66) larvae from 4 to 9 dpf.

(D) Exon structure (green boxes) of the *slc7a7* gene, with position of the *cerise* mutation (red star) and of the splice junctions targeted by the *sp22* and *sp55* morpholinos (red dashes).

(E, F) Dorsal view of the tectal microglia in live NR-stained *sp55* (E) and *sp22* (F) morphant larvae at 4 dpf.

(G) NR+ tectal microglia cell counts (at 4 dpf) show that unlike *sp55*, the *sp22* morpholino fully mimicks the absence of microglia of *cerise* mutant larvae. [This experiment was done 7 years after the one shown in \(C\); the overall stronger mutant phenotype here is likely due to the selection of carriers leading to clear mutant phenotypes over fish generations.](#)

(H-M) Dorsal view (rostral to the top) of mCherry+ tectal microglia in *Tg(mfap4:mCherryF)* zebrafish larvae at 4 dpf in control siblings (H), *cerise* mutants (I), uninjected controls (J), *sp22* morphants (K), and at 7 dpf in uninjected controls (L) and *sp22* morphants (M).

Error bars in (C,G) show mean \pm s.e.m. Scale bar, 100 μ m. Sib, sibling; mut, mutant; fb, forebrain; ot, optic tectum; cb, cerebellum; hb, hindbrain; NI, non-injected; Mo, morpholino; NR+, Neutral Red positive; Nb, number

Fig. 2. Macrophages/microglia in *Slc7a7* deficient larvae die within the brain and retina from 2.5 dpf onwards

(A-B') Dorsal view (rostral to the top) of the tectal microglia in live *Tg(mpeg1:Gal4; UAS:Kaede)* zebrafish larvae at the beginning (A, B) and end (A', B') of a 10 hrs time-lapse confocal imaging session started at 72 hpf of a non-injected control (A, A') and a *sp22* morphant (B, B'). (C) Selected time points (maximum projections) of the *in vivo* time-lapse confocal imaging of *Tg(mpeg1:Gal4; UAS:NfsB-mCherry)* tectal microglia started at 72 hpf of a *sp22* morphant, dorsal view, rostral to the top. Grey or blue arrowheads, and green arrowheads, point at two series of macrophages that die, are engulfed by another, which dies in turn, and so on...; see also Movie 2. (D) *In vivo* quantification of mCherry+ macrophages/microglia in the OT of *Tg(mfap4:mCherryF)* *cerise* siblings (white dots) and mutants (red dots) examined sequentially at 48 and 72 hpf.

(E) Quantification of L-plastin+ macrophages in the retinas of *cerise* progeny at 42 hpf, immunostained for L-plastin and subsequently genotyped as homozygous WT (+/+), heterozygous (+/-) or homozygous *cerise* mutants (-/-). Error bars show mean±s.e.m.

(F) *In vivo* quantification of mCherry+ macrophages/microglia in the retina of *Tg(mfap4:mCherryF)* *cerise* siblings (white dots) and mutants (red dots) examined sequentially at 48 and 72 hpf.

(G-H') Lateral view of the retinal microglia of live *Tg(mpeg1:Gal4; UAS:NfsB-mCherry)* larvae at the beginning (G,H) and end (G', H') of a 15 hrs time-lapse imaging session started at 54 hpf of a non-injected control (G,G') and a sp22 morphant (H,H'). See also Movies 3 and 4.

Scale bars, 75 µm. r, retina; l, lens; NI, non-injected; h, hours; Mo, morpholino; Nb, number

Fig. 3. Neutrophils are recruited to the CNS of *Slc7a7* deficient larvae following macrophage/microglia death

(A-C) Dorsal view of control (A) and *cerise* (B) larvae at 4 dpf stained for neutrophils with Sudan Black (SB), and corresponding SB+ cell counts in the OT and retinas (C). Error bars show mean±s.e.m.

(D-F) Dorso-lateral view of live *Tg(mpx:GFP)* control (D) and *slc7a7* sp22 morphant (E) larvae at 3 dpf, and close-ups on GFP+ cells within the morphant retina at 3 dpf (F). See also Movies 5 and 6.

(G, H) Dorsal view, rostral to the top, of the OT of sibling (G) and mutant (H) *Tg(mpeg1:mCherry; mpx:GFP)* larvae at 4 dpf, with macrophages/microglia in red, and neutrophils in green (maximum projection of a confocal z-stack spanning the entire OT); see also Movies 7 and 8.

Fig. 4. Tissue macrophages other than microglia also die upon sustained efferocytosis in *cerise* mutants

(A-C) Lateral view of the hatching gland (hg) at 3 dpf (A) and CHT at 7 dpf (B, C) in live WT larvae vitally stained with Neutral Red (A, B), or in a *Tg(gata1:DsRed; mpeg1: GFP)* larva (C).

(D-I) Comparative lateral views of the red fluorescence of sibling (D, F, G) and mutant (E, H, I) *Tg(mfap4:mCherry)* or *Tg(mpeg1:mCherry)* larvae, showing the hatching gland (hg) at 3 dpf (D, E), the CHT at 3 dpf (F, H), and the CHT at 6 dpf (G, I).

(J-L) Lateral view of the global macrophage population at 54 hpf in live *Tg(mpeg1:mCherryF)* control (J) and *slc7a7* morphant (K) embryos treated with PHZ at 30 hpf for 20 hrs, and corresponding quantification (L). Error bars show mean±s.e.m. 2-way ANOVA. ****P<0.0001; ns, non significant (P>0.05). Scale bars, 50 µm; y, yolk sac; ca, caudal artery; cv, caudal vein.

Fig. 5. Efferocytic macrophage populations strongly express *Slc7a7*.

Whole-mount *in situ* hybridization for *slc7a7* mRNA in WT embryos. (A) dorsal and (B-K) lateral views. (A) Optic tectum (OT), rostral to the top, 3 dpf. hb, hindbrain. (B) retina, 60 hpf,

with its concentric neuronal cell layers - ganglion cell layer (gcl), inner nuclear layer (inl) - and photoreceptor layer (pr); l, lens; inset, close-up on a *slc7a7*⁺ microglial cell, showing the signal-free efferophagosomes within the cell. (C) hatching gland (hg), 3 dpf; (D) CHT, 3 dpf; this lower *slc7a7* mRNA signal was seen when the anterior part was overexposed (inset). (E) pronephric tubules (pink arrowhead, also visible in C) and intestinal bulb and tract (cyan arrowhead), 3 dpf. (F-L) Induction of *slc7a7* expression in macrophages following PHZ treatment. Lateral views at 50 hpf in control (F-H) or PHZ treated (I-K) embryos. (F, I, I') Head, with *slc7a7*⁺ macrophages in hyaloid vasculature behind the lens (l) in I, I'; (G, J-J'') Yolk sac, with *slc7a7*⁺ macrophages in the blood circulation valley (duct of Cuvier, dc) in (J-J''). The higher magnification views in (I', J', J'') show the *slc7a7*-negative efferophagosomes within the *slc7a7*⁺ macrophages; in I', images of three macrophages taken at slightly different foci were superimposed on the main image so as to show them in focus. (H, K), Trunk-tail, with *slc7a7*⁺ macrophages in the posterior cardinal vein (pcv) and caudal vein plexus (cvp) in K. (L) Global quantification of *slc7a7* RNA expression by qPCR on control and PHZ-treated embryos. Error bars show s.e.m. * P < 0.05 (see Materials & Methods).

Fig. 6. *Slc7a7* expression is maximally induced 1-2 hrs following efferocytosis.

(A) Location of the neuromasts of the posterior lateral line - named L1 to L7 in rostro-caudal direction - in the zebrafish embryo at 48 hpf.

(B) Fraction of neuromasts showing a clear *slc7a7* mRNA signal by whole-mount *in situ* hybridization at successive time points after a 30 min exposure to CuSO₄. The number of embryos screened for each time point (n) is indicated above the curve.

(C-G) VE-DIC/Nomarski (C,D,E,F), confocal (C',D',E') and bright-field observation (C'',D'',E'',G) of neuromast morphology (neuromast location circled in red), *Tg(mpeg1:GFP)* macrophage recruitment, and *slc7a7* ISH signal at 0 (C-C''), 2 (D-D'') and 4 (E-E'') hours after exposure to CuSO₄. Close-up observation at neuromast L4 at 2 hpt shows that *slc7a7* is expressed by macrophages recruited at the damaged neuromast (F,G). Scale bars, 50 μm











