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Research article

Live imaging reveals a new role for the sigma-1 (σ_1) receptor in allowing microglia to leave brain injuries

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HIGHLIGHTS

- Role of σ_1 *in vivo* is to allow microglia to leave neuronal injuries.
- σ_1 Agonists block microglial responses to neuronal injuries.
- σ_1 Agonists do not affect microglial motility and their ability to collect dying neurons.

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ABSTRACT

Microglial cells are responsible for clearing and maintaining the central nervous system (CNS) microenvironment. Upon brain damage, they move toward injuries to clear the area by engulfing dying neurons. However, in the context of many neurological disorders chronic microglial responses are responsible for neurodegeneration. Therefore, it is important to understand how these cells can be "switched-off" and regain their ramified state. Current research suggests that microglial inflammatory responses can be inhibited by sigma (σ) receptor activation. Here, we take advantage of the optical transparency of the zebrafish embryo to study the role of σ_1 receptor in microglia in an intact living brain. By combining chemical approaches with real time imaging we found that treatment with PB190, a σ_1 agonist, blocks microglial migration toward injuries leaving cellular baseline motility and the engulfment of apoptotic neurons unaffected. Most importantly, by taking a reverse genetic approach, we discovered that the role of σ_1 *in vivo* is to "switch-off" microglia after they responded to an injury allowing for these cells to leave the site of damage. This indicates that pharmacological manipulation of σ_1 receptor modulates microglial responses providing new approaches to reduce the devastating impact that microglia have in neurodegenerative diseases.

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1. Introduction

In the CNS, immune responses depend on the recruitment and activation of microglia, the resident CNS phagocytes. Under physiological conditions, microglia display low cell body displacement and scan the surrounding environment with their fine branches [15]. In the presence of brain injuries, both in mouse and fish, microglia move toward damage by following ATP signaling via P2Y12 receptors [5,20]. Here, microglia release different factors such as cytokines (IL-1β, TNF-α, IL-6), chemokines (MCP-1), excitatory neurotransmitters (glutamate), complement factors, prostaglandins and reactive oxygen species [10]. To date, while it is clear that microglial responses are important for clearing the damaged area; it is also evident that these cells have to leave injuries to allow subsequent tissue repair [11,14]. Unfortunately, our knowledge on how they move away from damage is limited due to an inability to study these events *in vivo*. In addition, in the context of many neurological disorders, chronic microglial activation is known to be detrimental as it leads to widespread cell death and neurodegeneration [6,23]. For example, involvement of microglia in the Alzheimer's disease has also been shown upon accumulation of amyloid-β (Aβ) and this determines a cascade of events in glia cells leading to neuronal death [8,16]. Therefore,

Abbreviations: Aβ, amyloid-β; IL, interleukin; TNF, tumor necrosis factor.

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molecular factors involved in the modulation of microglial responses to CNS stimuli (e.g., ischemia, stroke, Aβ, etc.) are of great interest, and sigma (σ) receptors are among them. Two subtypes of σ receptors, namely σ_1 and σ_2, have been identified in the early 1990s [18], and although their mechanisms of action are not fully understood, these receptors are important targets for pharmaceutical research. While the σ_2 subtype is investigated in cancer related research [13,1], neuroprotective and neuroregulative functions have been proposed for σ_1 receptors, with σ_1 ligands reported to protect against brain ischemia and to potentiate neurite outgrowth [24]. Lately, σ_1 receptors have been found in microglia, and recent studies with primary cultures of microglia show that σ receptor agonists are able to decrease microglia activation [7]. The σ receptors non-selective agonists di-*orto*-tolyl-guanidine (DTG), and 5-ethoxy-2-[2-(morpholino)ethylthio]benzimidazole (afobazole) were shown to decrease microglia migration in response to ATP by reducing ATP-evoked increases in Ca^{2+} [4]. While afobazole, was shown to reduce neuronal and glial cell injury *in vitro* following ischemia, very recently, another σ_1 receptor agonist 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) has been shown to reduce neonatal excitotoxic brain lesions by inhibiting microglia activation [26]. Therefore, σ_1 receptors appear to be important targets for modulating microglia responses to brain insults. To further investigate the role of σ_1 receptors in modulating microglial activity, we selected the well characterized σ_1 receptor ligand PB190 (4-methyl-1-[4-(6-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)butyl]piperidine) [3] and studied its effect by live imaging of optically transparent zebrafish embryos. Zebrafish embryonic brain has been previously shown to be a powerful system to study microglia *in vivo* as zebrafish microglia cells and their responses to damage are very well conserved and comparable to their murine counterpart [12,17,20].

Herein, by combining chemical treatments with live imaging in fish embryos, we show that the administration of σ_1 agonists PB190 and DTG significantly reduces microglial migration toward injuries, while base line motility and engulfment of neurons remain unaffected. Taking advantage of reverse genetics in zebrafish, we find that the role of σ_1 receptor *in vivo* is to resolve microglial activation allowing for these cells to leave the site of damage after that they have responded to an injury. These results show that chemical tools acting at the level of σ_1 receptor modulate microglial activities and can be used in the context of brain injuries to control specific microglial functions.

2. Materials and methods

2.1. Transgenic fish lines and fish maintenance

Fish were kept at 26.5 °C in a 14 h light/10 h dark cycle. Embryos were raised at 28.5 °C in E3-solution. To avoid pigmentation, 0.003% PTU was added at 1 dpf. Microglia were visualized using pu1::Gal4-UAS::TagRFP [20] and Ca^{2+} using beta-actin::GCaMP3.1 [20]. For live imaging, only 3.5 dpf old zebrafish embryos were used. They were anesthetized in 0.01% tricane and embedded in 1.5% low melting agarose.

2.2. Confocal imaging and laser injuries

Imaging was done using an Olympus FV 1000 and an Andor Spinning Disk Confocal with 20x/NA0.7 and 40x/NA1.15 objectives. Embryos were anesthetized in 0.01% tricane and embedded in 1.5% low melting agarose. In general, 30–40 z-stacks spanning 45–60 μm of the fish brain were captured and flattened by maximum projection in Imaris (Bitplane) and Fiji. Lesions were generated using a 532 nm laser coupled to the FV 1000 and a pulsed 355 nm laser coupled to the Andor Spinning Disk using the protocol described in [20]. In particular, ablations were done at anatomical points that can be identified easily. Ablations were confirmed under the microscope and they were previously shown to lead to the death of approximately 200 neurons without either damaging the blood–brain barrier or perturbing normal larval development [20]. Images were analysed using both Imaris and Fiji. Statistical analysis was performed using Prism6 (GraphPad). For all values a nonparametric distribution was assumed.

2.3. Genetic and pharmacological perturbations

Morpholino oligonucleotides (Sigma1: 5′-GAACATTATTGT-TTTACTCACCCGC-3′) were obtained from Gene Tools Inc., and injected at a concentration of 0.9 mM with 0.2% Phenolred (Sigma) and 0.1 M KCl (Sigma) into one-cell stage embryos. Knock-down was checked using a σ_1 polyclonal antibody (Santa Cruz sc-22948). Acridine orange staining was performed by incubating zebrafish larvae in 10 μg/mL acridine orange (Sigma) in E3 for 60 min followed by several washes in fresh E3 medium.

2.4. Reagents

All chemicals used in this study were of analytical grade. PB190 was synthesized according to the previously reported procedure [3] and used at a concentration of 100 μm. DTG was purchased from Aldrich and used at a concentration of 100 μm. The chemicals have been added to the E3 fish medium plus 1% DMSO.

2.5. Immunoblotting

Western blot analysis was performed in accordance to the method described by Westerfield [25]. Proteins extracts produced from 3.5 dpf old embryos were separated in 10% polyacrylamide gels and transferred to Hybond ECL membranes (Amersham Pharmacia Biotech) by wet-blotting. For detection, we have followed the standard method of ECL Western blotting detection (Amersham Pharmacia Biotech). The polyclonal against σ_1 receptor (Santa Cruz, SC-22948) was added at a concentration of 1/100.

3. Results

3.1. Microglial response to neuronal injuries can be specifically blocked by the usage of σ_1 receptor agonists

In order to study microglial responses to neuronal cell death in the living zebrafish brain, we developed a UV pulsed cutting laser attached to a Spinning Disk Confocal microscope to induce neuronal cell death without damaging the blood–brain barrier nor perturbing normal larval development [20]. Systemic ablation of neuronal cells leads to microglial branching and migration toward the site of injury (Fig. 1C_1–C_3; [20]. To further investigate the role of σ_1 receptors in modulating microglial responses to injuries, we selected PB190 (4-methyl-1-[4-(6-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl) butyl]piperidine; [3], a well characterized σ_1 receptor ligand to study its effect on microglia by using live imaging. PB190 was shown to behave as a σ_1 receptor agonist both *in vitro* and *in vivo* [22]. Moreover, it displays better σ_1 pharmacological profile compared to DTG (K_i = 26.2 nM for σ_1 receptor and K_i = 40 nM for σ_2 receptor, respectively) [2] and afobazole (K_i = 5.9 mM for σ_1 receptor) [19], with subnanomolar σ_1 receptor affinity (K_i = 0.42 nM) and 86-fold higher selectivity toward the σ_2 subtype. In addition, selectivity against other CNS receptors (e.g., serotonergic and dopaminergic) was shown for this compound [21]. Treatment of zebrafish larvae with the σ_1 specific agonist PB190, as well as the reference compound DTG, significantly reduced microglial reaction

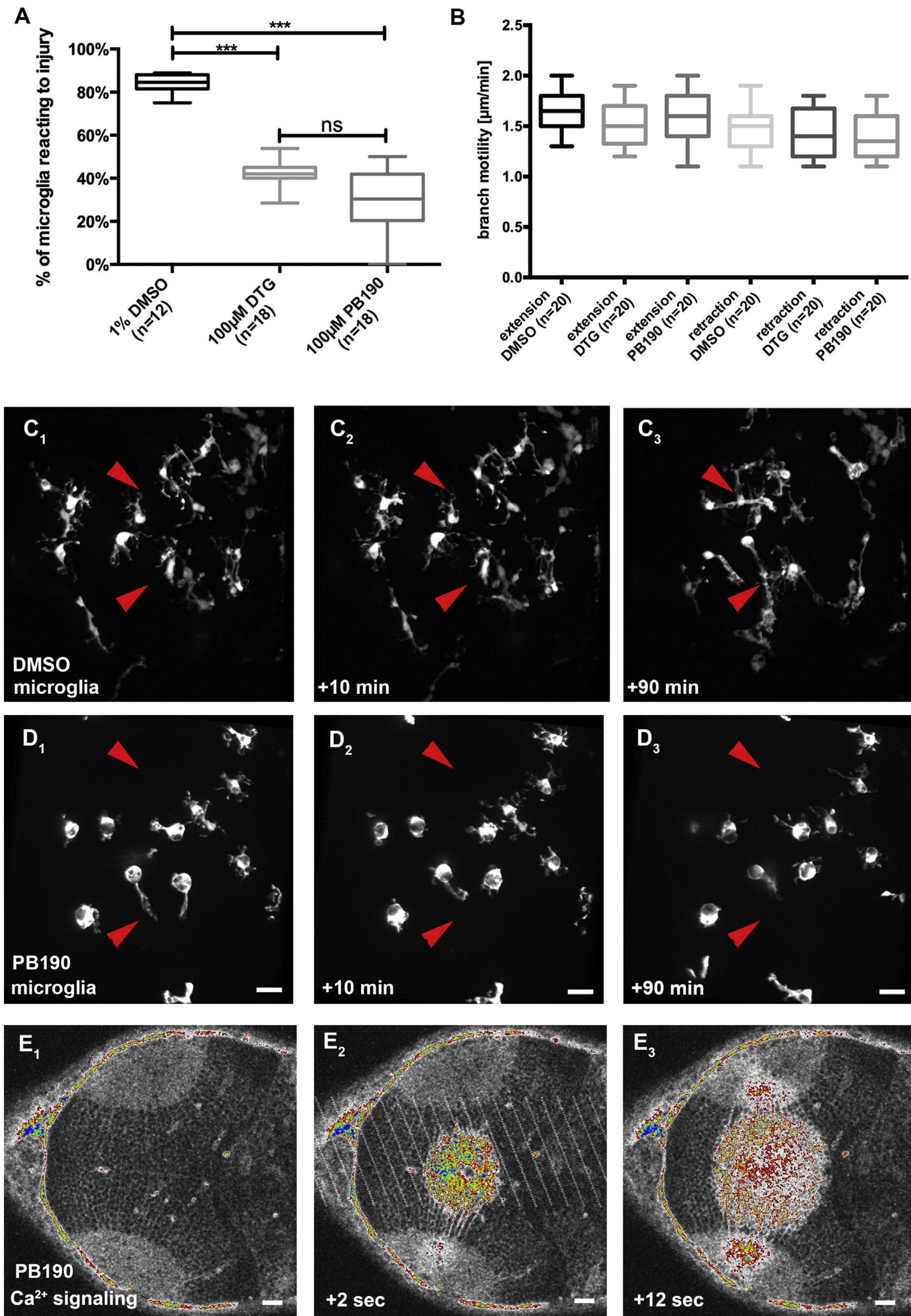

Fig. 1. σ_1 Receptor is important for microglial reaction toward injury.
(A) Percentage of microglia reacting to injury in DMSO compared to DTG and PB190 treated zebrafish larvae. (B) Speed of microglia branch extension and retraction in DMSO, DTG and PB190 treated embryos. (C) Time course of microglia (pU1::Gal4-UAS::TagRFP) reaction toward injuries in DMSO treated embryos. (C1) before, (C2) 10 min after and (C3) 90 min after cut. (D) Time course of microglia (pU1::Gal4-UAS::TagRFP) reaction toward injuries in PB190 treated embryos. (D1) before, (D2) 10 min after and (D3) 90 min after cut. (E) Dorsal views of a 3,5 dpf larval brain showing the time course of a Ca^{2+} wave (beta-actin::GCaMP3.1) forming upon central brain injury in PB190 treated zebrafish larvae. (E1) before, (E2) 2 s after and (E3) 12 s after cut. Scalebar for all images 20 µm. Images (C) and (D) were obtained using an Andor Spinning Disk Confocal with a 40x/NA1.15 objective. Images (E) were produced using an Olympus FV 1000 with a 40x/NA1.15 objective. Statistical analysis was performed using a Kruskal-Wallis test with a Dunn's multiple comparison test.

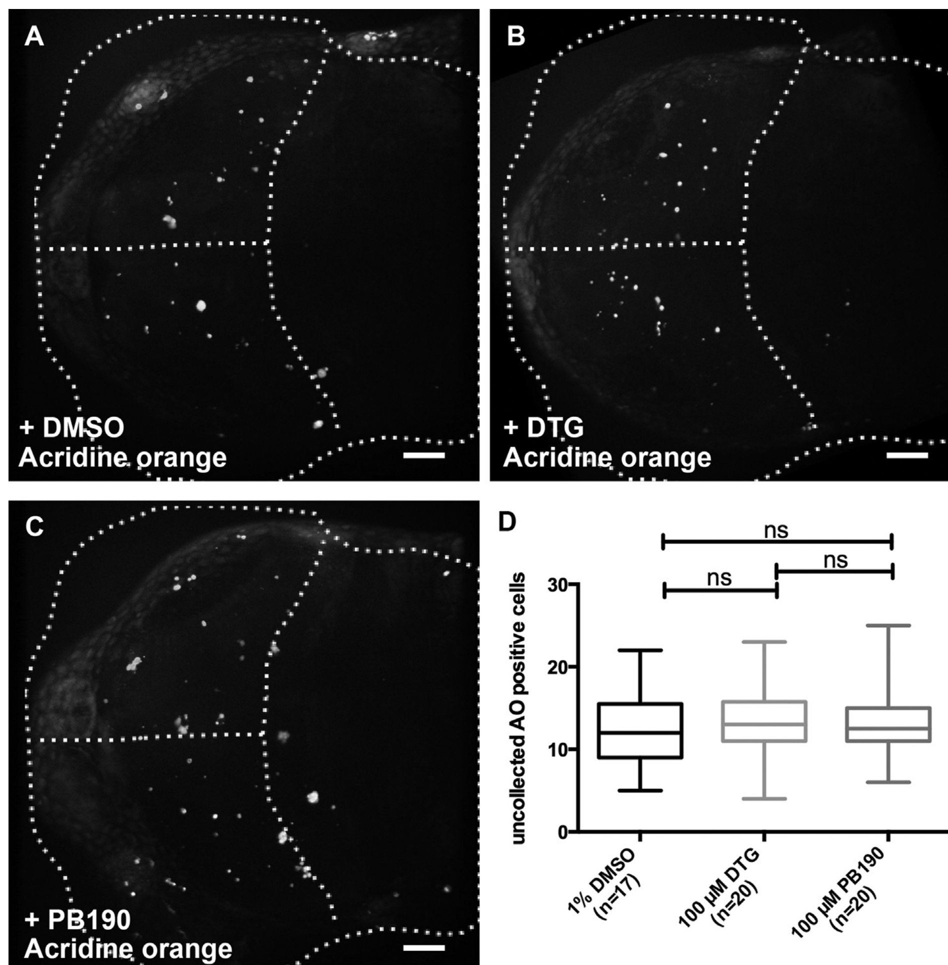

Fig. 2. Treatment with σ_1 agonist does not lead to a clearance phenotype.
(A) Acridine orange staining of 3,5 dpf zebrafish larvae treated with DMSO. (B) Acridine orange staining of 3,5 dpf zebrafish larvae treated with DTG. (C) Acridine orange staining of 3,5 dpf zebrafish larvae treated with PB190. (D) Quantification of uncollected acridine orange positive cells in a 3,5 dpf larvae treated with DMSO, DTG or PB190. Scalebar for all images 20 μm. All images were obtained using an Andor Spinning Disk Confocal with a 20x/NA0.7 objective. Statistical analysis was performed using a Kruskal–Wallis test with a Dunn's multiple comparison test.

toward damage (Fig. 1A, Fig. 1D_1–D_3). While in DMSO controls, microglia responded to a nearby injury by redirecting their branching toward damage, in treated embryos they failed to do so even 90 min after damage (Fig. 1A compare Fig. 1C_1–C_3 with D_1–D_3 and compare movie 1 with movie 2). This drug treatment did not influence Ca^{2+} signaling which was shown to be necessary and sufficient for microglial reactions (Fig. 1E; [20]. Interestingly, even though treatment with σ_1 agonists blocks the reaction of microglia toward neuronal injuries, the cellular baseline activity of these cells, defined in terms of branch extension and retraction speed, did not change significantly in treated larvae compared to controls (Fig. 1B and movie 1 and movie 2), indicating that these chemicals do not affect general microglial motility. During brain development microglia collect apoptotic neurons, a function which is of great importance for brain development. Labeling of apoptotic cells *in vivo* by using acridine orange allowed assessing microglial behavior in response to neuronal cell death. In embryos treated with both DTG and PB190 microglia appeared more roundish (Fig. 1D). However, they collected apoptotic neurons at a normal rate as shown by equal numbers of uncollected apoptotic cells in drug treated and DMSO control embryos (Fig. 2A–D). Thus, we conclude that DTG and PB190 are specifically blocking microglial responses to neuronal injuries without affecting other important aspects of microglial biology, such as branching dynamics and engulfment of apoptotic neurons.

3.2. σ_1 Receptor is involved in "switching-off" microglia allowing for these cells to leave the site of damage

Next, we wanted to establish if the phenotype that we observe in the living zebrafish brain using DTG and PB190 is indeed due to σ_1 receptors activation *in vivo*. For this, we combined drug treatment with reverse genetics and knocked-down the σ_1 using a morpholino that blocks translation as confirmed by using an antibody against the σ_1 receptor (Fig. 3A). The knock-down of the σ_1 receptor completely abolished the effect of PB190, and indeed in treated morphant brains microglia can respond to an injury by migrating to the site of damage (Fig. 3C). To investigate the functional requirement of σ_1 in microglia, we examined the knock-down phenotype upon brain injuries. We found that in σ_1 morphant embryos subjected to an injury microglia respond at a normal rate (Fig. 3C). However, while in control embryos the first microglia leave the site of damage after around 249 min, in morphant embryos microglia remain significantly longer with the first cells leaving after around 420 min (Fig. 3B). Interestingly, in PB190 treated embryos, a fraction of the microglia (24%) still responds to injury. These microglia that have reached the site of damage in PB190 treated embryos stay only shortly and leave after around 74 min (Fig. 3B). All together these findings point to a clear role for the σ_1 receptor in modulating microglial responses to damage. In particular, we find that the role of this receptor is to allow microglia to move away from damage.

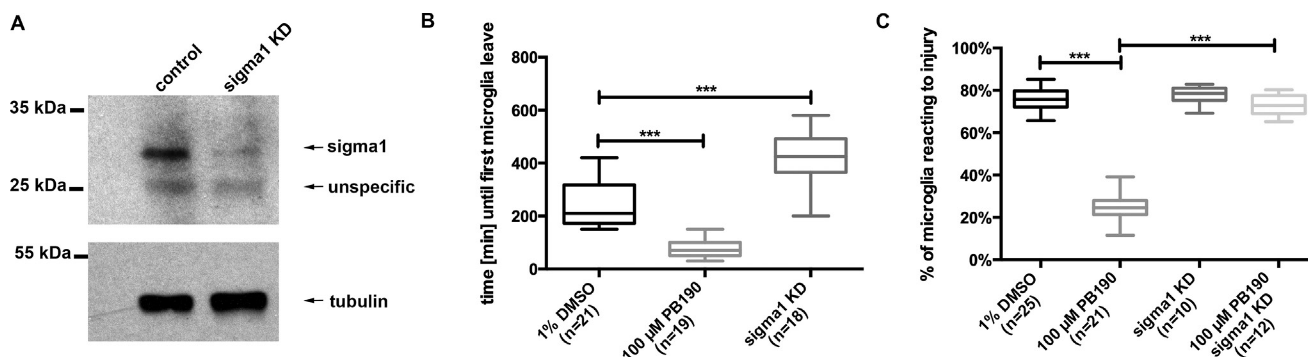

Fig. 3. The σ_1 receptor allows for microglia to leave the site of damage.
(A) σ_1-Mo mediated knock-down on protein level. (B) Quantification of time until first microglia cell leaves the site of injury in DMSO compared to PB190 treated embryos with larvae lacking their σ_1 receptor. (C) Percentage of microglia reacting to an injury in DMSO and PB190 treated compared to larvae lacking their σ_1 receptor and PB190 treated σ_1 knock-down embryos. Statistical analysis was performed using a Kruskal–Wallis test with a Dunn's multiple comparison test.

Thus, modulation of σ_1 receptor activity can be used to reduce the devastating impact of neuroinflammation and microglial responses in the context of many neurodegenerative diseases.

4. Discussion

In this study, we show that the role of σ_1 *in vivo* is to "switch-off" microglia reaction toward injuries allowing for these cells to leave the site of damage. In σ_1 receptor knock-down microglia remain much longer at the site of damage, while, in brain treated with σ_1 agonist, these cells move away quickly. Moreover, we show that the action of σ_1 on microglia is really specific to damage responses. Indeed, in embryos treated with σ_1 agonists microglia do not respond to injury but they maintain other activities such as branching and engulfment of apoptotic neurons that are generated during development. To the best of our knowledge, this is the first *in vivo* real-time imaging study of σ_1-modulated microglial responses.

Given the importance of microglia both in development and in the context of many neurological disorders, many studies have focused on these cells and their response to brain damage. However, to better understand microglia and their molecular modulators, such as the σ_1 receptor, it is crucial to preserve the normal morphology and dynamics of this cellular network and to work in the context of the intact brain. The work described here takes full advantage of the optical transparency of the small zebrafish brain. The embryo is alive under the microscope and can be manipulated both genetically and pharmaceutically by simply adding chemical compounds to the medium. In this way, the role of molecules involved in modulating microglial functions and behaviors can be tested directly *in vivo*. In this way, we found that treatment with the high-affinity and highly selective σ_1 receptor agonist PB190 or reference compound DTG, drastically reduces migration of microglial cells toward damage (Fig. 1A and Fig. 1D_1–D_3). This effect is very specific and restricted to injury responses, as these two compounds do not affect other aspects of the microglial behavior, such as scanning the brain and collecting apoptotic neurons. Most importantly, we also show that application of these chemicals does not affect signaling transmission within the brain. Indeed, we have found that Ca^{2+} signaling, necessary for ATP release and microglial reactions to brain injuries [20], is not affected in σ_1 receptor agonist treated brains.

Finally, in this study, we identify the role of σ_1 receptor *in vivo*, which is to switch off microglia allowing for these cells to leave the site of damage. Indeed, morphant microglia move to sites of injury as expected but remain there significantly longer. Interestingly, it has been shown that ATP attracts microglia toward damage via activation of P2Y12 on microglia [9]. It has also been shown that the σ-receptor agonist afobazole inhibits microglial response mediated by P2Y and P2X purinergic receptors [4]. Therefore, activation of σ-receptor at the site of damage might reduce microglial response to ATP allowing for these cells to move away. Thus, one key finding of this study is the fact that after traumatic injury, activation of σ-receptor using specific agonists, might resolve inflammation and prevent chronic microglial responses. The demonstration that microglial responses can be modulated pharmacologically by σ_1 receptor activation could unlock new approaches for future therapeutic applications.

All in all, the present study provides strong support to the crucial role that σ_1 receptors play in the modulation of microglia, and strongly encourages further investigation on σ_1 receptors for the prevention of chronic neuroinflammation, shedding light on novel opportunities to reduce the devastating impact that microglia have in many neurodegenerative diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2015.02.004.

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