Role of the central nervous system in zebrafish (*Danio rerio*) inflammation

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Running head: Nervous system and inflammation iin zebrafish

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Abstract

Inflammation is a crucial step in the development of chronic diseases in humans. Understanding the inflammation environment and its intrinsic mechanisms when it is produced by harmful stimuli may be a key element in the development of human-disease diagnosis. In recent decades, zebrafish (*Danio rerio)* have been widely used in research, due to their exceptional characteristics, as a model of various human diseases. Interestingly, the mediators released during the inflammatory response of both the immune system and nervous system, after its integration in the hypothalamus, could also facilitate the detection of injury through the register of behavioural changes in the fish. Although there are many studies that give well-defined information separately on such elements as the recruitment of cells, the release of pro- and anti-inflammatory mediators, or the type of neurotransmitters released against different triggers, to the best of our knowledge there are no reviews that put all this knowledge together. In the present review, the main available information on inflammation in zebrafish is presented in order to facilitate knowledge about this important process of innate immunity, as well as the stress responses and behavioural changes derived from it.

Keywords: Zebrafish (*Danio rerio)*; inflammation; nervous system regulation; mediators; pain.

1. Inflammation and zebrafish

Inflammation is a local temporary response triggered by the innate immune system in order to protect an organism against external damage. Among the most characteristic symptoms of inflammation can be found intensification of vascular permeability related to swelling, an increase in blood flow and heat sensation associated with redness, and a nerve fibre sensitisation link to functional disorders and pain (Bruce, 1913; Calixto, Otuki, & Santos, 2003; Carl Nathan, 2002). In general, the presence of different pathogens, irritants, injuries or trauma provide the basis for initiating this acute reaction, which can be developed in minutes to hours and last for days or weeks. Furthermore, depending on the timing, the trigger, the anatomical site (inflammation may affect any tissue) or the severity of the inflammation produced, the type of cells and the mediators involved will vary (Calder et al., 2013). Although there are some local or tissue-resident immune cells, such as microglia in the central nervous system (CNS) or Kupffer cells in the liver, with the onset of inflammation, circulating immune cells such as monocytes, granulocytes and T cells are recruited to the site of inflammation via secretion of inflammatory mediators (Ley, Laudanna, Cybulsky, & Nourshargh, 2007). In fact, the recruitment of immune cells is a necessary step to initiate the resolution process after an inflammatory response (Singer & Clark, 1999). In its attempts to restore physiological homeostasis, inflammation may persist for a long time, becoming a chronic response (Nathan & Ding, 2010). In humans, chronic inflammation is related to numerous diseases, such as cardio-vascular disease and diabetes (Dandona, Aljada, & Bandyopadhyay, 2004), Alzheimer's (Perry, Cunningham, & Holmes, 2007), cancer (Grivennikov, Greten, & Karin, 2010) and psoriasis (Candel et al., 2014). The effects of inflammation are closely linked to how the inflammation has been initiated, maintained and finished, and it should be deeply regulated, not only by the local environment in which it has been produced, but also by the rest of the organism (Abbas, Lichtman, & Pillai, 2019).

The zebrafish (*Danio rerio)* is a tropical teleost fish belonging to the minnow family (*Cyprinidae*) of the order Cypriniformes. It has been used as a model for different inflammation-related human diseases due to the high degree of genetic and functional homology that it shares with mammals (García-Moreno et al., 2019; Grunwald & Eisen,

2002). In addition, its rapid embryological development, transparent body and little size permit easy handling of specimens in the laboratory, requiring relatively low costs (Chakraborty, Hsu, Wen, Lin, & Agoramoorthy, 2009). Mechanisms of inflammation and homeostasis restoration have been studied by using nanoparticles, transgenic fish or drugs, such as 2,4,6-trinitrobenzenesulfonic acid (TNBS), used in mice to induce experimental models of Crohn's disease or inflammatory bowel disease, in order to develop new therapies for humans (Chakraborty et al., 2009; Duan et al., 2018; Geiger et al., 2013; Kizil, Kyritsis, & Brand, 2015; S. H. Oehlers et al., 2011; Y. Zhang et al., 2019). For instance, mechanisms of cell recruitment during tail fin regeneration after inflammatory events have been identified and coupled with research in brain regeneration (Petrie, Strand, Yang, Rabinowitz, & Moon, 2015). Another example is the use of silica nanoparticles in zebrafish embryos to reveal the involvement of the JAK1/TF signalling pathway during thrombus formation *in vivo* and the release of pro-inflammatory and procoagulant cytokines from endothelial cells (Duan et al., 2018). Moreover, anti-inflammatory drugs and natural products, such as indolealkylamines, have been associated with the suppression of TLR4/MyD88/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and TLR4/MyD88/MAPKs signalling pathways, combined with regulating lipid homeostasis, in an induced lipopolysaccharide (LPS)-inflammation model of zebrafish (Y. Zhang et al., 2019). The nociception mechanisms and pain that affect zebrafish behaviour have also been studied (Filby, Paull, Hickmore, & Tyler, 2010; Maximino et al., 2010; Norton & Bally-Cuif, 2010; Sneddon, 2009). However, to the best of our knowledge, such findings have never been presented together; doing so might lead to a better understanding of the mechanisms behind inflammation and the basic physiology associated with this important process in zebrafish.

Thus, in this review, because an injury or trauma produced in the skin of teleost fish may induce an inflammatory reaction that may be modulated by nerve fibres that are integrated in the wound microenvironment, we will focus specifically on the subsequent phases of skin inflammation and the crosstalk between inflammation and the nervous system in zebrafish. At the same time, the nerve fibres may inform the CNS about the stress being produced locally. Here, we will mainly focus on the relationship between the recruitment of immune cells at the inflammation site and the molecules that may mediate this reciprocity, as well as the effect of the inflammatory milieu on the CNS.

Thus, with this study, a different approach will be available that will allow us to understand how the immune and nervous systems are connected during inflammation.

2. Stages of inflammation – The background

The first defensive line in the body is composed of resident non-immune cells, such as skin keratinocytes, mucosal epithelial cells and vascular endothelial cells, which alert the immune system to the presence of inflammation-causing damage and modulate the inflammatory response (Al-Soudi, Kaaij, & Tas, 2017; Harris, 2014; Matsumoto et al., 2018) (Fig. 1). Upon exposure to an insult in a stage prior to the action of the immune system, local sensory nerve fibres report to the CNS and the motor nerve fibres the damage that has just been produced in order to initiate muscle reflex (Calixto et al., 2003; Hehn, Baron, & Woolf, 2012). Together with the immune cells, these tissue-resident cells initiate the inflammation process through both molecular and cellular mechanisms (Hawiger & Zienkiewicz, 2019). In general, there are two types of inflammation: acute and chronic. Acute inflammation is the initial response of the body to harmful stimuli, whereas prolonged inflammation is known as chronic inflammation. Acute inflammation is developed in five successive stages (Bordés Gonzalez, martínez Beltrán, García Olivares, & Guisado Barrilao, 1994).

2.1. First stage – Release of mediators

Inflammation starts with the release of numerous inflammatory preformed mediators by tissue-resident immune and non-immune cells, along with the retraction of the endothelial cells in order to increase blood flow, vasodilation and vascular permeability, which are associated with the redness and swelling symptoms (Bordés Gonzalez et al., 1994; Calder et al., 2013; Hawiger & Zienkiewicz, 2019) (Fig. 2). These events allow access to the site of inflammation by many large and soluble molecules and facilitate the recruitment of leucocytes from the systemic circulation (Kolaczkowska & Kubes, 2013; Larsen, G. L., & Henson, 1983; Medzhitov, 2008). These secreted factors include pro-inflammatory cytokines (mainly interleukin-1β [IL-1β], tumour necrosis factor α [TNF-α] and interleukin-6 [IL-6]), adhesion molecules, chemokines, proteolytic proteins, histamines, prostaglandins, leukotrienes, neuropeptides and neurotransmitters (Kolaczkowska & Kubes, 2013; Larsen, G. L., & Henson, 1983; Medzhitov, 2008). The activation of transcription factors is the action responsible for regulating the genes that encode all these mediators (Hawiger & Zienkiewicz, 2019).

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159 *2.2. Second stage – Effect of mediators*

160 The inflammatory exudate and the blood flow are able to produce a local tumour in the
161 affected area, where all mediators previously released trigger the production of
162 immunoglobulins, kinins, acute-phase reactants and coagulation factors while
163 generating complement activation (Bordés Gonzalez et al., 1994) (Fig. 3). Neutrophils
164 recruited at the inflammation place shortly secrete chemokines that activate tissue-
165 resident cells and recruit more leucocytes to the damaged area, amplifying inflammation
166 (Kolaczkowska & Kubes, 2013; Sommer, Torraca, & Meijer, 2020). Histamine released
167 by mastocytes and serotonin secreted from serotonergic neurons are responsible for
168 vascular permeability increasing (Cole et al., 1995; Larsen, G. L., & Henson, 1983;
169 MacGlashan, 2003). In addition, the cytokines TNF-α and IL-1β released by the
170 resident cells are able to induce postcapillary venule endothelial cells to express E-
171 selectin and increase the expression of ligands for leucocyte integrins, and to stimulate
172 cells to secrete chemokines, such as C-X-C motif chemokine ligand 8 (CXCL8) (also
173 known as IL-8) and chemokine (C-C motif) ligand 2 (CCL2), while binding to
174 neutrophils and monocyte receptors, respectively (Burhans, Hagman, Kuzma, Schmidt,
175 & Kratz, 2019; Imitola et al., 2004). TNF-α and IL-1β are also able to act on the
176 hypothalamus in order to induce an increase in body temperature (fever) (Blomqvist &
177 Engblom, 2018). Otherwise, IL-1β and IL-6 may induce hepatocytes to produce acute-
178 phase reactants, including c-reactive protein, serum amyloid P component (SAP) and
179 fibrinogen, which are secreted into the blood. In addition, TNF-β can stimulate the
180 expression of tissue factor by endothelial cells in order to activate coagulation and, at
181 the same time, can inhibit thrombomodulin expression, which is an inhibitor of
182 coagulation (Burhans et al., 2019; Duvigneau et al., 2019). The activation of both
183 intrinsic and extrinsic clotting pathways has been studied in lines of zebrafish through
184 screening to detect mutants with defective blood coagulation and with the use of several
185 anticoagulants, such as warfarin and hirudin (Jagadeeswaran, Gregory, Johnson, &
186 Thankavel, 2000).

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188 *2.3. Third stage – Cellular recruitment*

189 The third stage of acute inflammation is mainly characterised by cell migration from
190 surrounding tissues to the inflammation site (Bordés Gonzalez et al., 1994;
191 Kolaczkowska & Kubes, 2013; Ley et al., 2007) (Fig. 4). This migration consists of the

rolling of circulating leucocytes along the endothelium through the union of selectins and their ligands, the subsequent adhesion of leucocytes to the endothelium and the final extravasation or diapedesis (Bordés Gonzalez et al., 1994; Kolaczkowska & Kubes, 2013; Ley et al., 2007). All these leucocyte behaviours have been studied by direct microscopy with fluorescently marked leucocytes in zebrafish, because there are at least nine transgenic lines (Herbomel, Thisse, & Thisse, 1999, 2001). The ensemble of adhesion molecules and chemokines released in the vascular endothelium are responsible for this phase (Calder et al., 2013). One practical example of this process was shown in a study on the effect of carrageenin in the skin of zebrafish. In this study, oedema formation was observed to result from the release of the pro-inflammatory proteins and the recruitment of leucocytes at the inflammation site caused by carrageenin (Huang et al., 2014). Similarly, the migration/infiltration and proliferation of the immune cells in the skin of zebrafish was reported in another study in which fluorescent light was able to induce the early expression of skin genes associated with inflammation (Gonzalez et al., 2018). In addition, the genetic inhibition of TNF-α and tumour necrosis factor receptor 2 (TNFR2) in an *in vivo* study of psoriasis with a zebrafish model demonstrated the rapid infiltration of neutrophils into the skin, but not of macrophages, and hyperproliferation of keratinocytes through the activation of a hydrogen peroxide (H_2O_2)/NF-κB/Dual Oxidase 1 (Duox1) positive feedback inflammatory loop (Candel et al., 2014). As previously mentioned, infiltrating neutrophils are the first cells recruited at the site of inflammation, followed by monocytes. The monocytes then differentiate into macrophages and adopt a pro-inflammatory M1-like phenotype, becoming 'classically activated M1' macrophages. The M1 macrophages show very high antigen presentation capacities and expression of reactive oxygen species (ROS), TNF-α and IL-1β (Block, Zecca, & Hong, 2007; Kolaczkowska & Kubes, 2013; Medzhitov, 2008; Nathan, 2006; Nguyen-Chi et al., 2015).

Activated neutrophils exert their function through the release of their granule contents, which is a combination of ROS, myeloperoxidase, and proteases (such as elastase, proteinase 3 and cathepsin G) (Kolaczkowska & Kubes, 2013; Korkmaz, Horwitz, Jenne, & Gauthier, 2010; Larsen, G. L., & Henson, 1983; Medzhitov, 2008; Nathan, 2006). Neutrophils can also contribute to the recruitment and action of antigen presenting cells, including macrophages, dendritic cells and endothelial cells (Nathan,

2006). In cases where the initial cause of the inflammation was a pathogen, neutrophils and macrophages may introduce the pathogens into their cytoplasm inside vesicles (named phagosomes) originated during the phagocytosis process. One step of this process is to destroy the pathogen through the use of ROS or the proteolytic enzymes present in cell granules and in lysosomes, which fuse their membrane with that of the phagosomes, giving rise to phagolysosomes (Kolaczkowska & Kubes, 2013). Apart from phagocytosis, neutrophils may also kill microbes by extruding their DNA and granule contents by forming extracellular threads called neutrophil extracellular traps, in which bacteria and fungi are trapped and killed (Kolaczkowska & Kubes, 2013).

2.4 Fourth step – Regulation of inflammation

In order to avoid the negative effects of excessive inflammation, it is very important that the activation of inhibitory mechanisms, including those carried out by the nervous system, integrate into the inflammatory process until a balance is reached, (Bordés Gonzalez et al., 1994; Nathan & Ding, 2010; Pinho-Ribeiro, Verri Jr, & Chiu, 2017) (Fig. 5). Through this process, the phenotype of macrophages is progressively converted to the M2-like phenotype, so that they become 'alternatively activated M2' macrophages – the process includes intermediate phenotypes in which both M1 and M2 markers are expressed (Nguyen-Chi et al., 2015; Wiegertjes, Wentzel, Spaink, Elks, & Fink, 2016) – switching from the production and secretion of pro-inflammatory molecules to anti-inflammatory, such as cytokines and prostaglandins. Lipoxins (endogenous anti-inflammatory molecules) cease the recruitment of neutrophils to the inflammation place in favour of monocyte recruitment (Chandrasekharan & Sharma-Wali, 2015). In addition, resident and recruited macrophages participate in the scavenging of foreign particles and cellular debris, thereby collaborating to initiate the tissue repair process (Kolaczkowska & Kubes, 2013; Larsen, G. L., & Henson, 1983; Medzhitov, 2008; Nathan, 2006). The rest of the neutrophils are phagocyted by macrophages since they are known to be short-lived and soon to undergo apoptosis. Nonetheless, a recently characterised subpopulation of neutrophils in zebrafish seems to have a longer lifespan than 'normal' neutrophils and are able to return to the circulation after activation, thereby having an anti-inflammatory effect (Ellett, Elks, Robertson, Ogryzko, & Renshaw, 2015; Harvie & Huttenlocher, 2015). The combination of differently coloured fluorescent leucocytes in zebrafish offers further insights into the *in vivo* microanatomy and the visualisation of the cellular physiology of the host (Hall,

Flores, Storm, Crosier, & Crosier, 2007; Meijer et al., 2008). For instance, using a zebrafish tail-amputation model with fluorescently labelled macrophages (mCherry) and TNF-α, it was shown that pro-inflammatory (green fluorescent protein, GFP^+) and anti-inflammatory macrophages (GFP^-) accumulated in the damaged tissue and that anti-inflammatory macrophages that remained were associated with the injury until regeneration was completed, unlike pro-inflammatory macrophages, which withdrew from the damaged area. This model also made it possible to study neutrophil reverse migration at different stages of the inflammatory response (S. K. Yoo & Huttenlocher, 2011; Sa Kan Yoo et al., 2010).

2.5. Fifth step – Reparation

Finally, mechanisms of homeostasis are activated to terminate inflammation and initiate the total or partial repair of the tissues damaged by the offending agent or by the self-inflammatory response. Endocrine functions are mainly responsible for the development of this last stage (Bordés Gonzalez et al., 1994) (Fig. 6). In terms of the immune system, the alternatively activated M2 macrophages still in the inflammation focus secrete various growth factors in order to attract keratinocytes, fibroblasts and blood vessels into the wound and increase the phagocytic activity of leucocytes (Colton, 2009; Ponomarev, Maresz, Tan, & Dittel, 2007; Shaw & Martin, 2009; Singer & Clark, 1999). In fact, hydrocortisone treatment in zebrafish points to the existence of fibroblast-stimulating signals from inflammatory cells, and after their activation, granulation tissue formation and vascularisation are promoted (Leibovich & Ross, 1975). Interestingly, the same study in which this was found hinted that re-epithelialisation did not require an inflammatory response, being independent of wound debridement by neutrophils and chemotactic signals from macrophages (Dovi, He, & Dipietro, 2002; Martin et al., 2003). Nonetheless, the inflammation process seems to indicate an evolutionary conservation of crucial molecular mechanisms in different vertebrate classes, which should be carefully analysed for a better understanding.

3. Neuromodulation and integration of the CNS in the control of inflammation

The crosstalk between the immune system and the CNS to maintain or recover homeostasis is largely developed by the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS), alerting to inflammation after its onset (Hanoun, Maryanovich, Maria, Arnal-Estapé, & Frenette, 2015). Although the anatomical

organisation of fish CNS does not closely resemble that of mammalians, the main functions are highly conserved across vertebrates, and brains of teleost contain equivalents to most mammalian cell types (Kunst et al., 2019; Machluf, Gutnick, & Levkowitz, 2011). In addition, in a study using a retinal-injury model of zebrafish, an abundance of neurogenic zones was found in the adult zebrafish brain, along with a high regenerative capacity, which are associated with the correlation between DNA demethylation and regeneration-associated gene expression, which has not been found in mammals (Grandel & Brand, 2013; Powell, Grant, Cornblath, & Goldman, 2013). Furthermore, adult zebrafish are able to regenerate all parts of their body successfully, including their fins, heart and liver (Keightley, Wang, Pazhakh, & Lieschke, 2014; Shi, Fang, Li, & Luo, 2015), a property that has been extensively studied because of its possible value for other vertebrates, including humans.

The zebrafish hypothalamus is a complex structure responsible for integrating environmental signals, processing within the endocrine systems and triggering the systemic responses needed to regain and/or maintain homeostasis; moreover, its neuronal populations have been shown to be functionally analogous to their mammalian counterparts (Machluf et al., 2011; Markakis, 2002). Against external neuroendocrine disruptors, the hypothalamus is able to operate through direct innervations with synaptic neurotransmission and through the secretion of neuromodulators into the surrounding tissue or vasculature (Andersson & Tracey, 2012) (Fig. 1). Indirectly, external disturbances may be processed via the ANS (Kiba, 2002). The ANS comprises two branches that start from the spinal cord: the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). The SNS prepares the body for alert situations by activating cardio-vascular organs, increasing blood flow to skeletal muscles and lungs, and suppressing gastro-intestinal activity. By contrast, the PNS is unrelated to stress situations, decreasing muscle contractility and heart rate and increasing gastro-intestinal function (Kiba, 2002). Fibres that transduce action potentials are non-myelinated slow-conducting postsynaptic neurons, called C-fibres, which are involved in heat, cold and mechanical pain, and Aβ/Aδ-myelinated presynaptic neurons, which mediate mechanosensation and mechanical pain with faster conduction (Basbaum, Bautista, Scherrer, & Julius, 2009). Interestingly, the buoyancy, dilution of chemicals and relatively stable thermal environment provided by the aquatic environment may be related to a higher percentage of Aδ-fibres than C-fibres in teleost fish due to their dual

role in mediating reflex escape behaviour and prolonged noxious stimulation (Sneddon, 2002). By contrast, in mammals there is a large amount of C-fibres involved in noxious stimulation and inflammatory pain due to their advance during evolution and the increased chance of injury (Sneddon, 2002; Sneddon, Braithwaite, & Gentle, 2003).

Thus, upon activation by external damage, and before starting the immunological stages of the inflammation, pain stimuli are led to neuron bodies within the dorsal root ganglia and relayed to the spinal cord and hypothalamus in order to be processed (Pinho-Ribeiro et al., 2017). In higher vertebrates, pain is processed through the ascending afferent pathways, the spinothalamic pathway and the ventrobasal, medial and lateral areas, which project to the somatosensory cortex, allowing the location and intensity of pain from the inflamed area to be perceived (Colloca et al., 2017). In later stages, in which the inflammation becomes chronic, several mediators called pyrogens (such as IL-1β, TNF-α and IL-6), released from the local area of inflammation, reach the thermoregulatory centre in the anterior hypothalamus, through portions lacking a blood-brain barrier (specific areas of the surfaces of the cerebral ventricles, where the metabolism of araquidonic acid is activated, producing prostaglandins, mainly prostaglandin E_2) (Biddle, 2006; King & Wood, 1957; Milton & Wendlandt, 1970) (Fig. 3-5). In addition, magnocellular neurons located in the paraventricular and supraoptic nuclei of the anterior hypothalamus project their axons directly to the neurohypophysis (posterior lobe of the pituitary) and release oxytocin and arginine–vasopressin (AVP) into the general blood circulation, inducing peripheral vasoconstriction and muscular contractions (Markakis, 2002) (Landgraf & Neumann, 2004). All these physiological changes are related to fever and an increase in body temperature (Dinarello, 2015).

4. Communication mediators between inflammation and the nervous system

Sympathetic nerve fibres play an essential role in maintaining the homeostasis niche against stress and dangerous stimuli, forming a complex framework in the local microenvironment, established in order to start a defensive response with the resident cells (Katayama et al., 2006; Yamazaki & Allen, 1990). Upon activation by an inflammatory signal, sensory nerves are able to release cytokines, neuropeptides and neurotransmitters from their peripheral terminals, which modulate the effects on the vasculature and on the function of innate and adaptive immune cells (Deutschman & Tracey, 2014) (Fig. 2-6). Furthermore, the dendritic cells, neutrophils, macrophages,

mast cells and T cells (all of which express receptors for neuronal mediators) all produce cytokines (small, secreted proteins that have a specific effect on the interactions of and communications between cells) which affect the SNS (Nathan & Ding, 2010). This bidirectional communication allows the control of the inflammation in subsequent stages by the activation of specific neuronal networks in the SNS (Nathan & Ding, 2010; Pinho-Ribeiro et al., 2017). Many of the mammalian cell pathways and mediators of both the immune system and nervous system are highly conserved in zebrafish (Bollaerts, Van Houcke, Andries, De Groef, & Moons, 2017). Likewise, inflammatory molecules, such as interleukins, chemokines and growth factors, as well as neurohormones, neuropeptides and the inhibitory or excitatory neurotransmitters released by GABAergic, glutamatergic, cholinergic, dopaminergic or serotonergic neurons, seem to be highly evolutionarily conserved among various mammalian species (H. Meijer & P. Spaink, 2011; Hall et al., 2009; Panula et al., 2010; S. Zhang & Cui, 2014). Some of these pathways are conserved even across invertebrates, such as fruit flies, in which toll-like receptors were discovered. However, few studies point to the effect of all these known mediators in an inflammatory response in zebrafish (Oosterhof, Boddeke, & van Ham, 2015). Although most of these mediators have functionally equivalent orthologs in fish, there is a relatively low sequence-based homology between fish and mammals (Savan & Sakai, 2006). Interestingly, an ancient genome duplication has occurred in teleosts and that is the reason why it is estimated that 30% of genes have a duplicate variant that may exhibit redundancy and share the same function (Postlethwait et al., 2000). The roles of these mediators can vary depending on the amount, location and timing of production (Savan & Sakai, 2006). For instance, chemokine signalling molecules can differ substantially between mammals and teleosts, and even among mammals, where some chemokines are not conserved at the sequence level (Bajoghli, 2013; Nomiyama et al., 2008). In zebrafish, although many functional homologs remain to be identified, 63 chemokines and 24 receptors due to the genome duplication have been described, representing a high number of chemokines and receptors in comparison to the similar molecules described in other species (DeVries et al., 2006). Among some pro-inflammatory stimuli, cytokines such as TNF and IL-1 or bacterial toxins may induce the expression of the C-X-C chemokine receptor type 4 (CXCR4) and its ligand C-X-C motif chemokine ligand 12 (CXCL12) (also known as stromal cell-derived factor 1) (Imitola et al., 2004). Both CXCR4 and CXCL12 are expressed in the ventricular zone of the adult zebrafish brain and seem to

play a role during homeostatic neuronal migration (Diotel et al., 2010). The chemokine CXCL8 has two homologs, CXCL8a and CXCL8b, which are also involved in neutrophil migration towards a peripheral wound during inflammation and after injury (de Oliveira et al., 2013; S. H. B. Oehlers et al., 2010). Other mediators, like those involved in arachidonic acid and therefore in the synthesis of eicosanoids, such as cyclooxygenase-2 (COX-2) and leukotriene C4 (LTC4), participate in the stimulation of inflammation (Funk, 2001; Ishikawaa, Griffinb, Banerjeeb, & Herschman, 2007).

When some of these mediators inform the CNS of the onset of inflammation, high-conserved neuropeptides, such as corticotropin-releasing hormone (CRH), substance P, calcitonin gene-related peptide, and orexins, bring support to the release of other pro-inflammatory mediators, facilitating vasodilation and thus promoting immune cell recruitment in the initial phase of inflammation (Chiu et al., 2013; Hehn et al., 2012; Pavlov & Tracey, 2012; Peter & Fryer, 1983). Subsequently, the activation of the HPA axis via afferent nerves may also terminate inflammation through the release of glucocorticoids from the adrenal cortex (Sternberg, 2006). In zebrafish, neurotransmitters, known to play a significant role in various physiological and behavioural processes, may also regulate leucocyte trafficking to sites of inflammation, due to the conservation of a large variety of circuits and receptors (Panula et al., 2010). In response to an inflammatory response, the nervous system acts by releasing acetylcholine (ACh) through the efferent vagus nerve, inhibiting the production of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-18) via activation of the homopentameric receptor α7-nicotinic (α7nAChR) in macrophages and reducing inflammation (Zila, Mokra, Kopincova, Kolomaznik, & Javorka, 2017). These macrophages seem to be located in the spleen, although there is controversy about how ACh reaches the organ, since there is hardly any direct connection between the vagus nerve and the spleen (Zila et al., 2017). Populations of $CD4^+$ T cells that express the β2 adrenergic receptor could act as mediators in this connexion, but there is still no satisfactory explanation for this (Inoue et al., 2019). In mammals, ACh is degraded by two cholinesterases – acetylcholinesterase (AChE) and butyrylcholinesterse (BuChE) – but the genome of the zebrafish encodes only the gene for AChE, which was detected in the brain (Bertrand et al., 2001; Radić, Pickering, Vellom, Camp, & Taylor, 1993; Soreq & Seidman, 2001). Thus, AChE is involved in the termination of nerve impulses through catalysis of the neurotransmitter ACh (Dix et al., 2007). In zebrafish, dopamine

is released principally from the midbrain and hypothalamus, and homologs of both D1- and D2-receptor subtypes have been described (Narayanan, Guarnieri, & DiLeone, 2010; Panula et al., 2010; Schweitzer & Driever, 2009). Dopamine or agonist receptors of dopamine have been reported to modulate activation, proliferation and cytokine production by immune cells (Basu & Dasgupta, 2000; Sarkar, Basub, Chakrobortya, Dasgupta, & Basu, 2010; Torres-Rosas et al., 2014). Consistent with this, the deficiency of dopamine is tightly associated with immune system abnormalities and CNS inflammation, so the zebrafish has been used as a model of biomedical research in order to study the molecular mechanisms of Parkinson's disease and attention deficit hyperactivity disorder in humans (Fontana et al., 2019; Perry, 2012; Wüllner & Klockgether, 2003). Glutamate is an inhibitor neurotransmitter involved in the ATP-dependent recruitment of microglia against an injury, through the binding of the glutamate receptor NMDA, the subsequent influx of Ca^{2+} ions and the release of ATP (Sieger, Moritz, Ziegenhals, & Prykhozhij, 2012). Otherwise, after stressful behaviours, noradrenaline (NA) is synthesised in neurons of the locus coeruleus and in the medulla oblongata of zebrafish (Rink & Wullimann, 2002). In the SNS, NA regulates heart function and blood pressure (Brodde, Bruck, Leineweber, & Seyfarth, 2001; Feng et al., 2019; Zimmerman, 1981). Depending on the time point of activation relative to inflammation, low concentrations of NA are related to pro-inflammatory effects, while high concentrations of NA have predominantly anti-inflammatory effects (Pongratz & Straub, 2013). These pro- and anti-inflammatory effects are mediated through α-adrenergic receptors and β-adrenergic receptors, respectively (Pongratz & Straub, 2013). In addition, NA seems to be involved in the adhesion molecule expression of venular endothelial cells, facilitating leucocyte trafficking (C. Scheiermann, Y. Kunisaki, D. Lucas, A. Chow, J. Jang, D. Zhang, D. Hashimoto, M. Merad, 2013; Viswanathan & Dhabhar, 2005).

5. Inflammation, pain and behaviour

As we have seen, most of the basic anatomical regions, cells and neurotransmitters are conserved between zebrafish and mammals, despite a few important differences. For instance, fish have nociceptive pathways and respond to inflammation, and therefore, to noxious stimuli in the stage prior to the immune response, but the debate over whether fish feel pain, considered a higher cognitive process, has continued since the discovery

of nociceptors in trout in the early 2000s (Malafoglia, Bryant, Raffaeli, Giordano, & Bellipanni, 2013; Rose, 2002). The matter depends less on the detection of noxious stimuli by fish, but rather on how they respond at an emotional level (Woodruff, 2018).

In response to a painful stimulus, such as an inflammatory insult, there is a withdrawal reflex, an emergency movement away from it, in which the nociception pathway transmits the message from the injured limb to the spinal cord before the order is sent directly back to the hurt limb (Broom, 2001). As the brain receives this information afterwards, it is possible that there is no awareness of pain (Broom, 2001). However, tissue injury and inflammation are intimately related to an increase in pain sensation, which is a long-term consolidated experience (Pinho-Ribeiro et al., 2017). In mammals, nociceptor peripheral nerve terminals have receptors and ion channels that detect molecular mediators released during inflammation, leading to pain sensitivity or 'hyperalgesia', a topic up for debate in relation to fish (Shi et al., 2015). For instance, when a noxious substance (e.g. acetic acid or bee venom) was injected in trout, the painful stimuli reduced the motivation of the fish to feed for hours (Sneddon et al., 2003). Moreover, the injection of pain killers at the same site as the noxious substance restored their natural behaviour and physiology, possibly exemplifying the highly conservative nature of vertebrate physiology (Mettam, Oulton, McCrohan, & Sneddon, 2011). In addition, after exposure to electric stimuli or a positive reward, trout learned to avoid the shock location or look for the reward after just a few exposures. In fact, in case of conflict between reward and shock, they were willing to pay a pain cost in order to access the resources (Dunlop, Millsopp, & Laming, 2006).

Due to the zebrafish's characteristics and the conservation of nociceptive mechanisms across vertebrates, the fish has been used as a model for inflammation studies, but also for pain and nociception studies (Sneddon, 2009). The behavioural responses of zebrafish larvae have been studied in this context, revealing a dose-dependent increase in locomotor activity against potentially painful stimuli, such as temperature or chemical substances, which can be treated with common analgesics (Lopez-Luna, Al-Jubouri, Al-Nuaimy, & Sneddon, 2017a; Prober et al., 2008; Wolman & Granato, 2012). In fact, zebrafish specimens exposed to continuous stressors, such as netting and handling, showed a transient rise in body temperature related to a real fever response, which is often referred to as 'emotional fever' (Rey et al., 2015). In addition, when skin

and muscle of zebrafish larvae were burned, axons innervating the damaged area were dramatically affected, even presenting an up-regulation of homeostatic-related gene expression, such as c-Jun, vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide 1b, in enteric neurons as a possible additional effect of the nociceptive mechanism (Malafoglia et al., 2014). In contrast, adult zebrafish reduced their locomotor activity after injections of formalin or acetic acid, which may be reversed by opioids (e.g. morphine or buprenorphine) but not when co-administered with an opioid antagonist (Curtright et al., 2015; Lopez-Luna, Al-Jubouri, Al-Nuaimy, & Sneddon, 2017b; Magalhães et al., 2017; Steenbergen & Bardine, 2014; Taylor et al., 2017). High concentrations of morphine (1 mg/L) are able to produce immunosuppression and down-regulation of inflammation-related genes in zebrafish inflammation models produced by LPS, while lower concentrations (100 ng/L–100 µg/L) produce exacerbation of inflammation by down-regulating multixenobiotic resistance transporter genes that control LPS efflux (Mottaz et al., 2017).

6. Conclusion and future perspectives

In this review, we have shown the chemical and cellular development of inflammation, the nexus of inflammation and the nervous system in zebrafish, as well as their implications for the welfare of specimens. Due to the high degree of conservation between teleost and mammals at both the molecular and genetic level, research in zebrafish may help to overcome the limitations of mammalian models and contribute to the development of regenerative therapies. For that reason, both inflammation studies and research that implicates its nexus with the nervous system, such as pain-and-behaviour tests and screening tools for anti-inflammatory drugs, in zebrafish should be taken into account. Thus, it may be necessary to adapt existing zebrafish behaviour tests in larvae and adults to test the effectiveness of inflamed-noxious stimuli and to develop new standardised protocols to provide support for further research.

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Conflicts of interest

The authors declare no conflict of interests.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Figure legends

Figure 1. Schematic representation of skin and nervous system background of zebrafish in a previous stage of an inflammatory reaction. Resident non-immune cells, immune cells and neurons involved are shown. Key cells and molecules involved are explained on the right box. Skin keratinocytes, Langerhans cells and vascular endothelial cells wander around the different skin layers and alert to the presence of an inflammation trigger in order to initiate the inflammatory reaction. C and Aδ fibres warn the central nervous system of the damage produced.

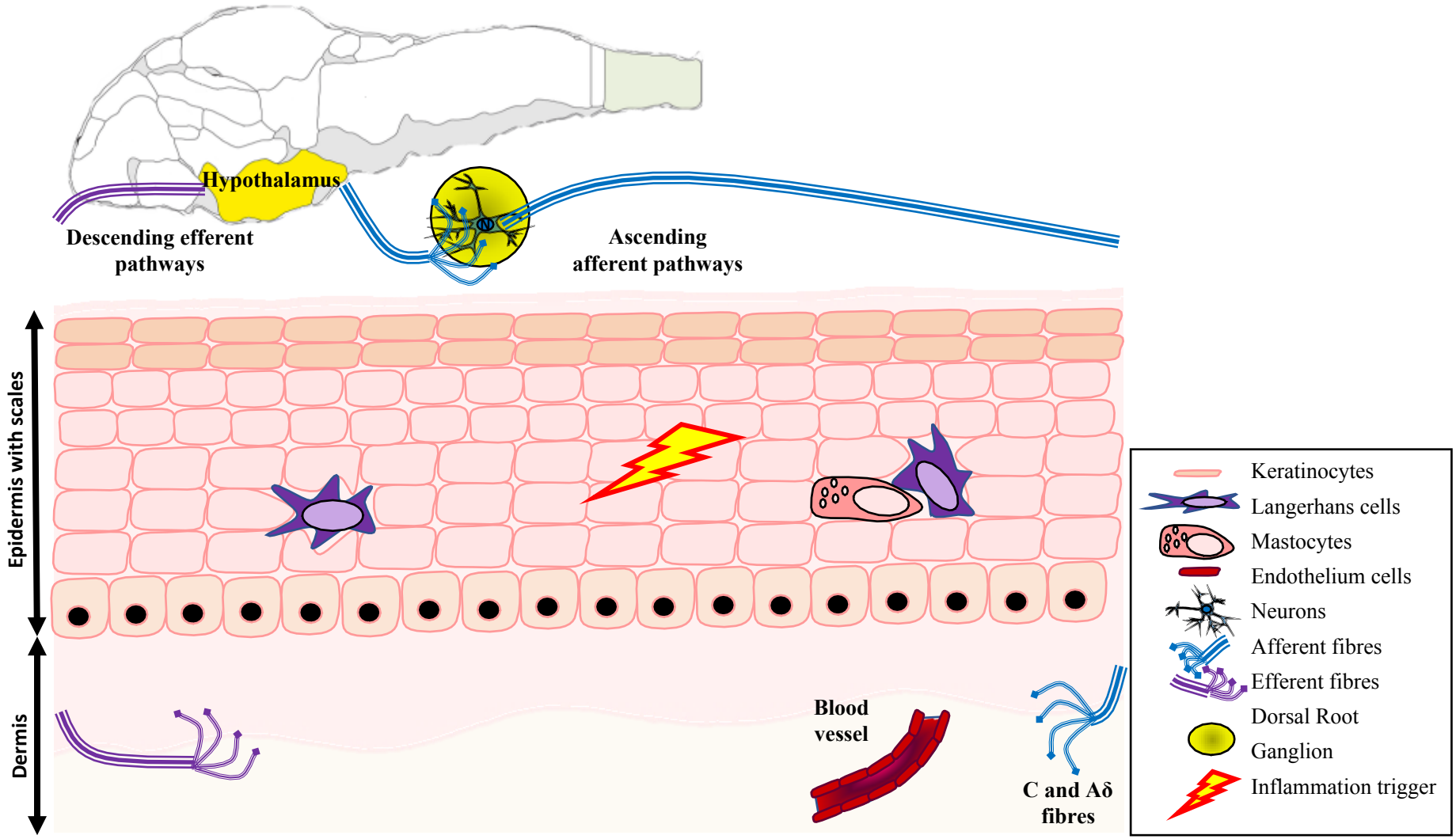
Figure 2. Schematic representation of the first stage of the inflammation produced in the skin of zebrafish and its nexus with the central nervous system. Key cells and molecules involved are explained on the right box. Preformed mediators released by resident cells and nerve fibres allow the access of many large and soluble molecules to the inflammation site. Redness and swelling begin to be visible in the inflammation site.

Figure 3. Schematic representation of the second stage of the inflammation produced in the skin of zebrafish and its nexus with the central nervous system. Key cells and molecules involved are explained on the right box. Chemokines activate tissue-resident cells and facilitate the recruitment of leucocytes from the systemic circulation to the damaged area. Proinflammatory cytokines increase the local temperature and activate coagulation. Histamine and serotonin released by nerve fibres and mastocytes increase vascular permeability and produce local tumour in the affected area. Redness and swelling are visible in the site where the inflammation was initiated.

Figure 4. Schematic representation of the third stage of the inflammation produced in the skin of zebrafish and its nexus with the central nervous system. Key cells and molecules involved are explained on the right box. Leucocyte are recruited to the inflammated area while macrophages polarizate to M1-like phenotype.

Figure 5. Schematic representation of the fourth stage of the inflammation produced in the skin of zebrafish and its nexus with the central nervous system. Key cells and molecules involved are explained on the right box. The polarization of macrophages from M1-like to M2-like phenotype and the release of anti-inflammatory molecules by M2-macrophages and nerve fibres are evident. These activate inhibitory mechanisms that compensate the effects of the proinflammatory molecules released previously. Redness and swelling begin to be less visible in the site where the inflammation was initiated.

Figure 6. Schematic representation of the fifth stage of the inflammation produced in the skin of zebrafish and its nexus with the central nervous system. Key cells and molecules involved are explained on the right box. Hormones released in the central nervous system that activate the endocrine system and molecules released by the immune cells activate the damage repairment in the affected tissue, through the activation of the homeostasis mechanism. Redness and swelling begin to disappear in the site where the inflammation was initiated.


Hypothalamus
Descending efferent pathways
Ascending afferent pathways
Epidermis with scales
Dermis
Blood vessel
C and Aδ fibres
Keratinocytes
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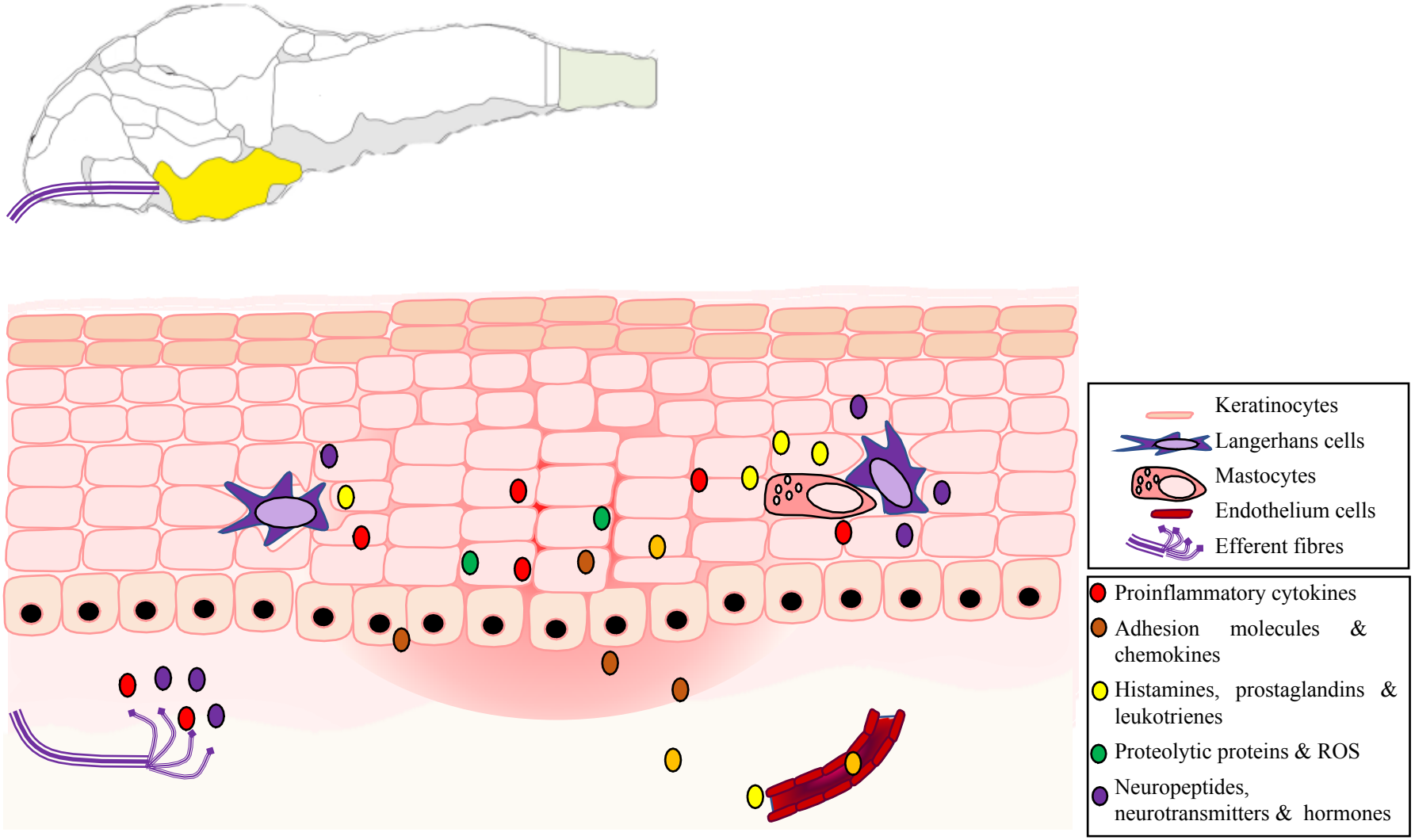
Mastocytes
Endothelium cells
Neurons
Afferent fibres
Efferent fibres
Dorsal Root
Ganglion
Inflammation trigger


Keratinocytes
Langerhans cells
Mastocytes
Endothelium cells
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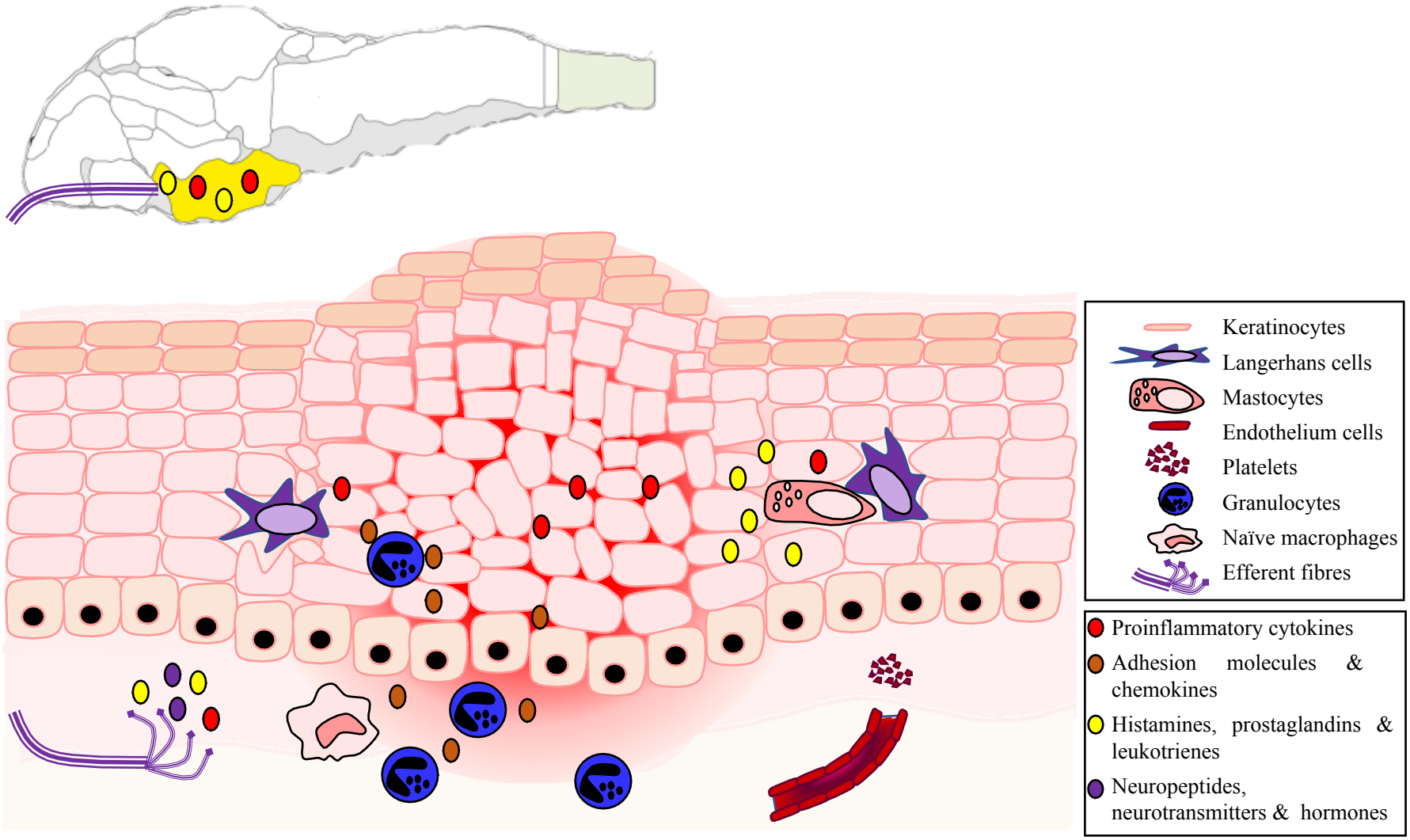
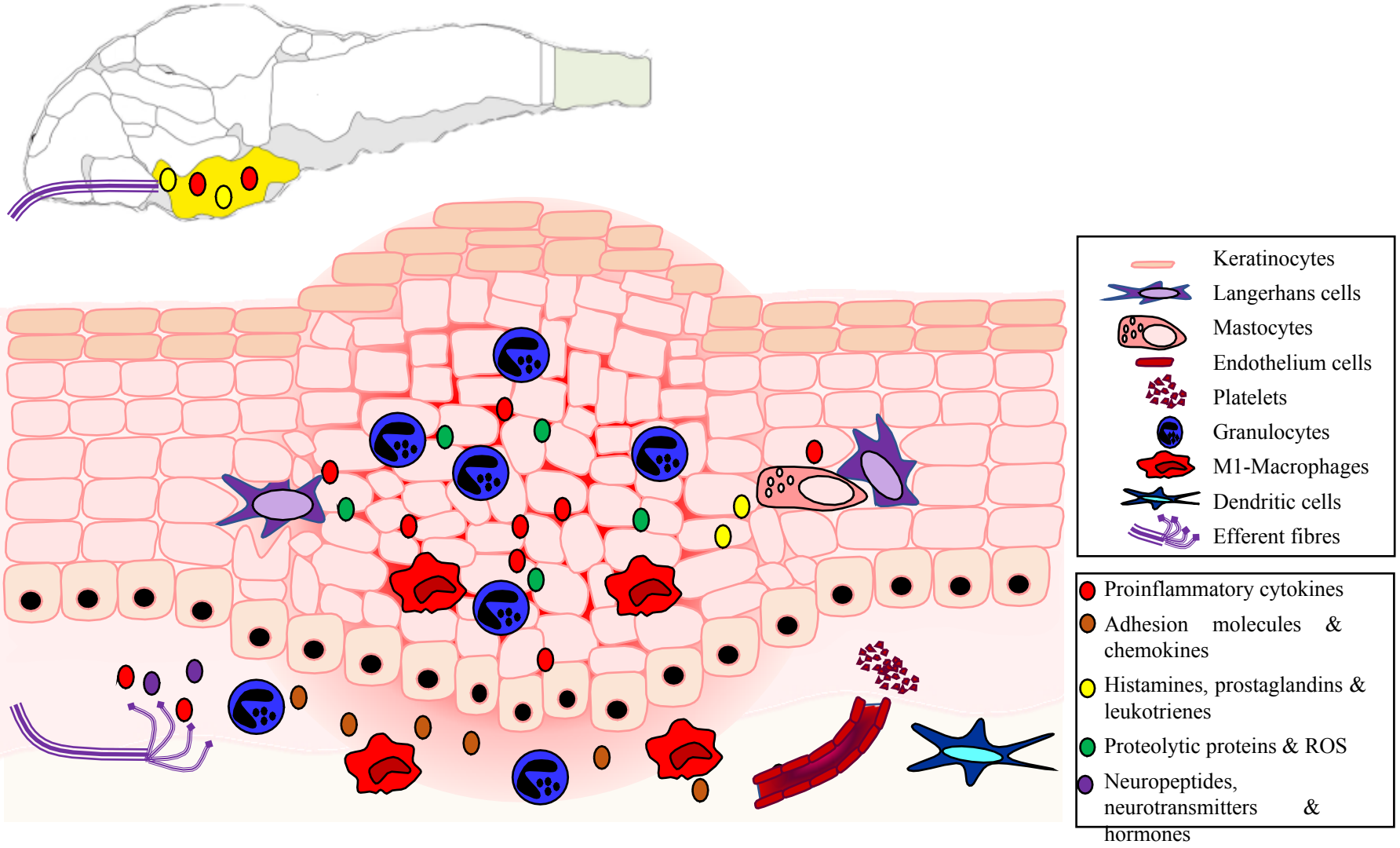
Proinflammatory cytokines
Adhesion molecules & chemokines
Histamines, prostaglandins & leukotrienes
Proteolytic proteins & ROS
Neuropeptides, neurotransmitters & hormones


Keratinocytes
Langerhans cells
Mastocytes
Endothelium cells
Platelets
Granulocytes
Naïve macrophages
Efferent fibres
Proinflammatory cytokines
Adhesion molecules & chemokines
Histamines, prostaglandins & leukotrienes
Neuropeptides, neurotransmitters & hormones


Keratinocytes
Langerhans cells
Mastocytes
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Granulocytes
M1-Macrophages
Dendritic cells
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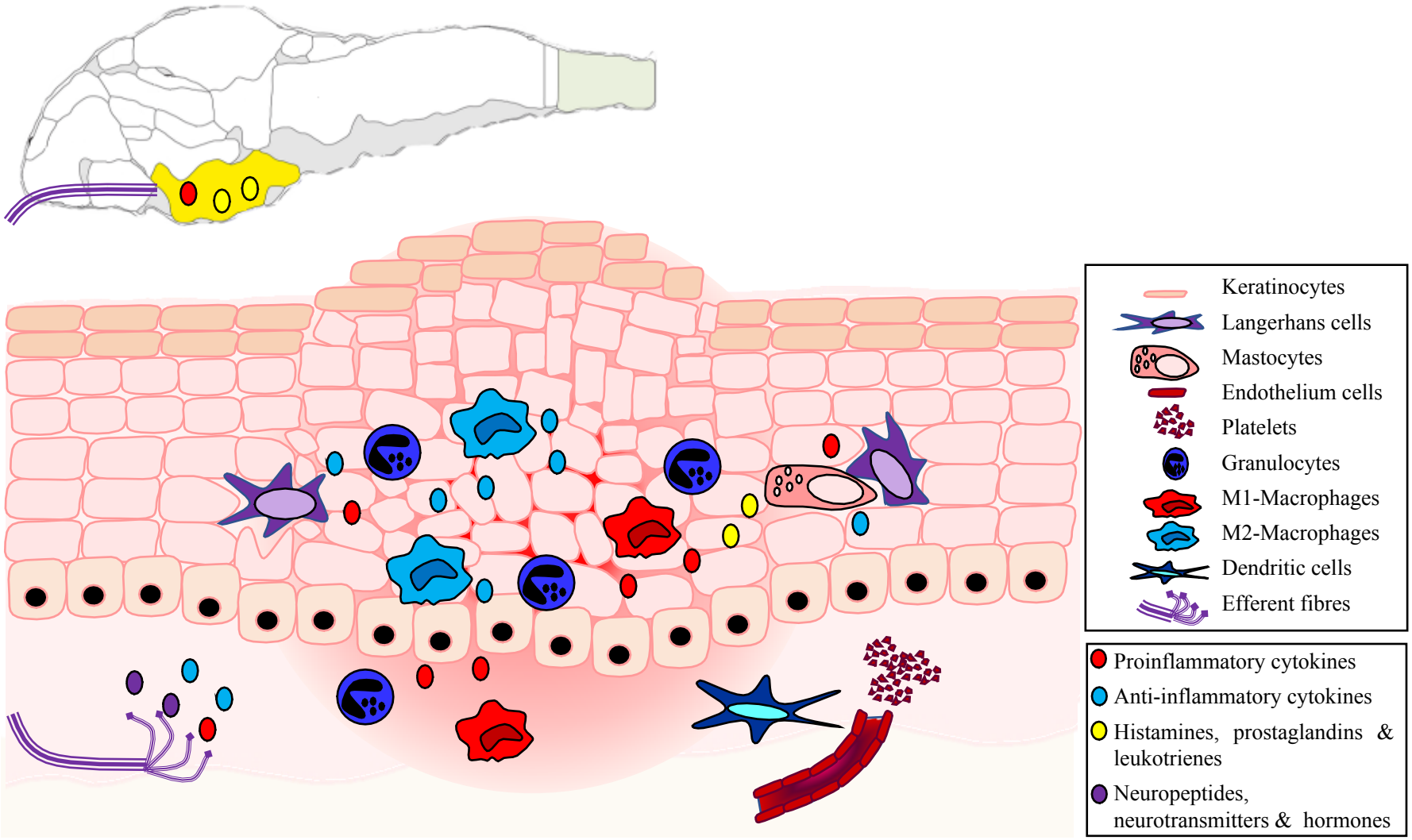
Adhesion molecules & chemokines
Histamines, prostaglandins & leukotrienes
Proteolytic proteins & ROS
Neuropeptides, neurotransmitters & hormones


Keratinocytes
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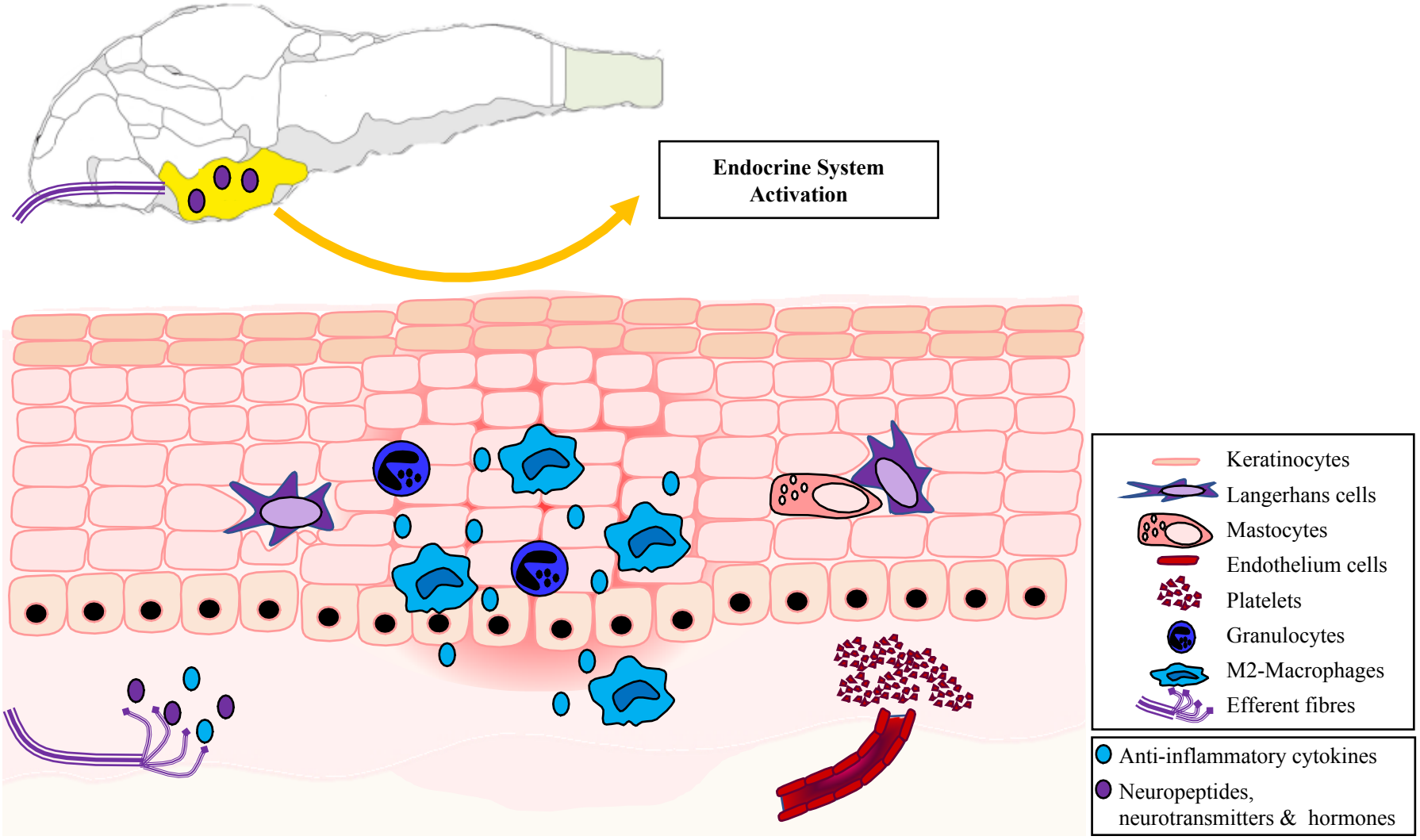
Platelets
Granulocytes
M1-Macrophages
M2-Macrophages
Dendritic cells
Efferent fibres
Proinflammatory cytokines
Anti-inflammatory cytokines
Histamines, prostaglandins & leukotrienes
Neuropeptides, neurotransmitters & hormones


Endocrine System Activation
Keratinocytes
Langerhans cells
Mastocytes
Endothelium cells
Platelets
Granulocytes
M2-Macrophages
Efferent fibres
Anti-inflammatory cytokines
Neuropeptides, neurotransmitters & hormones