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REVIEW

Erebosis: An Emerging Concept of a New Type of Regulated Cell Death

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Abstract—This review briefly describes the discovery of a morphologically distinct form of regulated cell death in Drosophila, termed erebosis, which is characterized by organelle loss, nuclear flattening, and intracellular accumulation of Ance. The latter serves as a biomarker of this type of cell death but does not act as its trigger. Erebosis can be induced by amino acid deprivation accompanied by the reduced cytoplasmic viscosity, and its development is also associated with the increased heme oxygenase activity and decreased intracellular heme levels. We hypothesize that the heme-sensing protein E75 in *Drosophila melanogaster*, a homolog of mammalian REV-ERB proteins involved in circadian regulation, may provide a link between erebosis and renewal of enterocytes from the intestinal stem cells. Finally, we discuss the differences between erebosis and other forms of regulated cell death, as well as detection of ACE2 in the brains of mice in different models of Alzheimer's disease.

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INTRODUCTION

Cell death enables the body to eliminate damaged or diseased cells; such replacement with the healthy, young cells occurs continuously in the skin, intestines, and other tissues. More than 200 billion cells die in the human body every day, with half of these deaths occurring in the small intestine. Historically, cell death was classified primarily based on morphological features, distinguishing between apoptotic, autophagic, and necrotic morphotypes. In the modern NCCD (Nomenclature Committee on Cell Death) nomenclature, the term "necrosis" primarily characterizes the morphological outcome of cell death, whereas the regulated forms of cell death are classified according to their defining molecular mechanisms – such as necroptosis, pyroptosis, or ferroptosis. By 2022, scientists had identified and described 13 distinct cell death pathways [1], when, during the study of distribution of the angiotensin-converting enzyme Ance in the cells lining the *Drosophila melanogaster* intestine, a new type of cell death was accidentally discovered and named "erebosis" – from the Greek word "erebos", meaning "darkness" [2].

This type of cell death differed from those previously described in that the cells exhibited accumulation of Ance, progressive loss of the cellular organelles, cytoskeleton, and cell adhesion components, as well as nuclear flattening; consequently, the cells were difficult to visualize under the microscope, making

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them to appear dark. Inhibitors of apoptosis and autophagy did not affect this new type of cell death. In this brief review, we summarize the available information on this novel form of cell death, discuss its potential physiological significance for enterocyte regeneration, and examine molecular mechanisms that could integrate and explain the experimental data obtained over the past year.

DISCOVERY OF EREBOSIS

Ance – a marker of this type of death. Erebosis was discovered serendipitously during a study on localization of the angiotensin-converting enzyme Ance in the *Drosophila melanogaster* intestine. Ance belongs to the family of ACE-like metallopeptidases and is homologous to the mammalian ACE and ACE2 (angiotensin-converting enzyme 2) [2]. The ACE2 enzyme plays a crucial role in the renin-angiotensin-aldosterone system; studies on the ACE2-deficient mice have revealed that the enzyme exerts a protective effect in the models of ischemic kidney injury [3]. Subsequently, it was demonstrated that the enzyme also plays a protective role in the diabetes-related kidney diseases [4], including obesity-associated glomerulonephritis [5], and Alport syndrome (hereditary hemorrhagic nephritis) [6]. ACE2 injections are currently being investigated as potential therapeutic agents for various kidney diseases. It should be noted that ACE2 attracted significant attention in the early 2020s due to its role in the SARS-CoV-2 infection – specifically, it was shown that viral entry is mediated by the interaction of the spike protein with ACE2, which serves as a cell-surface receptor. Researchers conducted detailed studies not only of the interaction between the spike protein and ACE2 but also of the individual interactions of each with the heme molecule; binding of several heme molecules to ACE2 with micromolar-range constants was demonstrated with the help of surface plasmon resonance [7], suggesting that such interaction is likely non-specific. In the intestine, the ACE2 dimer forms a complex with the dimer of the neutral amino acid transporter (B0AT1 or SLC6A19) or the sodium-dependent amino acid transporter (SIT1 or SLC6A20), facilitating transport of these substrates into the enterocyte membrane [8].

Examination of the Ance expression patterns across various *Drosophila* organs revealed, via immunostaining, uneven labeling of the intestinal enterocytes; Ance is predominantly localized in the R1, R2, and R4 regions of the adult midgut [2]. Cell renewal occurs most actively in the R4 region; therefore, most studies on midgut renewal focus specifically on the R4 region or the posterior midgut. In addition to uneven intracellular Ance staining, a weak extracellular Ance signal was also observed [2]. Detection of Ance inside the cells was quite unexpected. Ance is a secreted protein, and the initial study demonstrated that its accumulation in erebotic cells is not associated with the sharp increase in the Ance transcription and depends on the presence of a signal peptide required for protein secretion [2]. Based on these data, the authors hypothesized that erebotic cells take up Ance from the extracellular space. More recent data (published in a 2026 preprint) regarding the transient permeabilization of the plasma membrane during erebosis are consistent with this model: pore formation is accompanied by the influx of extracellular Ance into the cell [9]. Thus, intracellular Ance likely originates from the pool of secreted soluble protein, although its molecular form within the erebotic cell itself has not been biochemically characterized.

Membrane permeability during erebosis. By labeling the cytosol and nucleus with green (GFP) and red (RFP) fluorescent proteins, it was possible to observe the step-by-step progression of erebosis – specifically, changes in permeability of both the nuclear and cytoplasmic membranes. An initial stage could be distinguished in which the cytosolically expressed GFP was absent from the cytosol, yet both green and red fluorescent proteins were present in the nucleus; subsequently, in the second stage, GFP (but not RFP) exited the nucleus; and finally, in the third stage, both GFP and RFP exited the nucleus (Fig. 1a). It was shown that the kinetics of Ance accumulation and release of cytosolic GFP coincide, suggesting formation of a pore in the plasma membrane. The cells were not necrotic, as addition of propidium iodide did not result in cell staining. In a preprint of the study published in 2026 [9] fluorescent dyes of varying molecular weights – and thus sizes – were used to determine dimensions of the formed pore; a Rhodamine B derivative was conjugated to dextrans with molecular weights of 150 kDa and 2000 kDa. It was found that the cells undergoing erebosis could be labeled with 150-kDa dextran but not with 2000-kDa dextran. To identify which pore-forming protein might be responsible for the observed effects, the authors knocked down Attacin-D, Torso-like, and Ninjurin A (NijA, an analog of mammalian NINJ1/2). The pore-forming proteins Gasdermin D and MLKL, which mediate membrane permeabilization during pyroptosis and necroptosis, respectively, are absent in *Drosophila*. Knockdown of the NijA gene inhibited erebosis. NINJ1 was discovered in 1996 [10] as a membrane protein that is activated in the neurons and Schwann cells surrounding the distal nerve segment following axonal injury to accelerate nerve regeneration. NINJ1 is also expressed in epithelial cells. Based on permeability of the pore to Ance (71 kDa) and GFP (27 kDa), it could be inferred that its diameter

ranges from 16 to 50 nm. The pore formed by NINJ1 has a diameter of approximately 35 nm, which falls within the experimentally observed range. However, we must emphasize that the pore opened by NINJ1 is irreversible, which contradicts the results observed in erebosis, as erebosis is not accompanied by the cell lysis, and the cell retains its barrier function. NINJ1 oligomerization and pore opening are induced by the cell swelling and hypotonic shock, when osmolarity of the medium drops from 360 mOsm to 76 mOsm [11]. Hypotonicity is sufficient to open the NINJ1 pore, which leads to the plasma membrane rupture during various types of cell death, including ferroptosis [12].

INFLUENCE OF PHYSICAL PROPERTIES OF THE CYTOSOL ON INTRACELLULAR PROCESSES

Dependence of erebosis and enterocyte renewal on cytosol viscosity. In 2026, a laboratory led by Sa Kan Yoo determined that erebosis is initiated by the change in the *Drosophila* diet: specifically, reducing dietary sugar had no effect, whereas a 90% reduction in amino acid content triggered erebosis [13]. Researchers conducted extensive work, screening all possible amino acids, and determined that the effect was not due to a specific amino acid but rather total quantity of amino acids, which established a specific concentration, density, and, most importantly, viscosity of the medium. A concentration of approximately 200 mM of any single amino acid was sufficient to inhibit erebosis. The researchers selected amino acids that are not metabolized but are capable of inhibiting erebosis – such as α-aminoisobutyrate – as well as arabinose and deoxyribose (which are not metabolized by *Drosophila*); these substances generated a similar “effective” cytosolic viscosity that suppressed erebosis. Viscosity was assessed using the FRAP (fluorescence recovery after photobleaching) method by measuring diffusion rate of GFP in the cytosol in the presence of various non-metabolizable amino acids and saccharides. Thus, it was concluded that cytosolic viscosity is the key factor determining the extent to which a cell is susceptible to this new type of cell death. Furthermore, it has been shown that erebosis is reversible in its early stages (it could be slowed down) and that, overall, erebosis is a much slower process than apoptosis or other types of cell death. Time-lapse imaging revealed that the early phase of erebosis lasts for 5 minutes, whereas the intermediate and late phases together take approximately 12 h [2].

Erebosis has been proposed as a novel mechanism of cell death during the homeostatic turnover of intestinal enterocytes (Fig. 1b), because switch to a low-amino-acid diet led to accelerated enterocyte turnover [13]. It should be emphasized that the original article describing the erebosis phenomenon noted that erebotic cells are located adjacent to the clusters of intestinal stem cells that generate new cells [2]. This fact made it possible to hypothesize that erebosis is not an accidental form of cell death caused by injury, but rather a normal element of intestine function. It should be noted that the adult *Drosophila* intestine, specifically the posterior region of the midgut, has become an important model for stem cell research [14]. An intestinal stem cell divides asymmetrically, giving rise to a new stem cell and an enteroblast, which subsequently differentiates into an enterocyte (Fig. 1b). Prior to the discovery of erebosis, it was assumed that the aging enterocytes died via apoptosis, yet no direct evidence for this had ever been obtained. Thus, erebosis could indeed be regarded as a form of cell death executed by enterocytes, characterized by preservation of the dying cell acting as a cellular barrier until the new enterocyte matures (Fig. 1b).

Effect of cytosol viscosity on tubulin polymerization-depolymerization. It should be noted that in recent years, there has been growing interest in the influence of biophysical properties of the cytosol on biochemical processes and structural rearrangements within cells. Various models of cytoplasm have been proposed to explain the effect of its consistency on cellular metabolism. The cytoplasm contains macromolecules (proteins, nucleic acids, lipids, etc.) at concentrations of 100-300 mg/mL, occupying between 10% and 40% of the total cell volume. Size of these components ranges from small globular proteins to extensive networks formed by organelles and cytoskeleton polymers. Ribosomes alone account for about 20% of this volume. Biophysical studies have shown that these components form a porous viscoelastic material that allows small molecules to pass through via diffusion but hinders movement of the larger particles [15]. Cytoskeleton stability is determined, in particular, by the stability of tubulin organization. Microtubules are essential for organization of the Golgi apparatus, endoplasmic reticulum, and mitochondria [16]. The rates of both microtubule polymerization and depolymerization vary linearly and inversely with cytoplasmic concentration; however, extensive data indicate that these effects stem from the changes in cytoplasmic viscosity rather than from the cellular stress responses or macromolecular crowding effect *per se*. The authors succeeded in reproducing these effects by varying viscosity of the medium [17]. The results indicate that, even under normal conditions, cytoplasmic viscosity physically slows down microtubule assembly and disassembly processes, demonstrating importance of the cytoplasmic viscosity for the rate of biochemical reactions

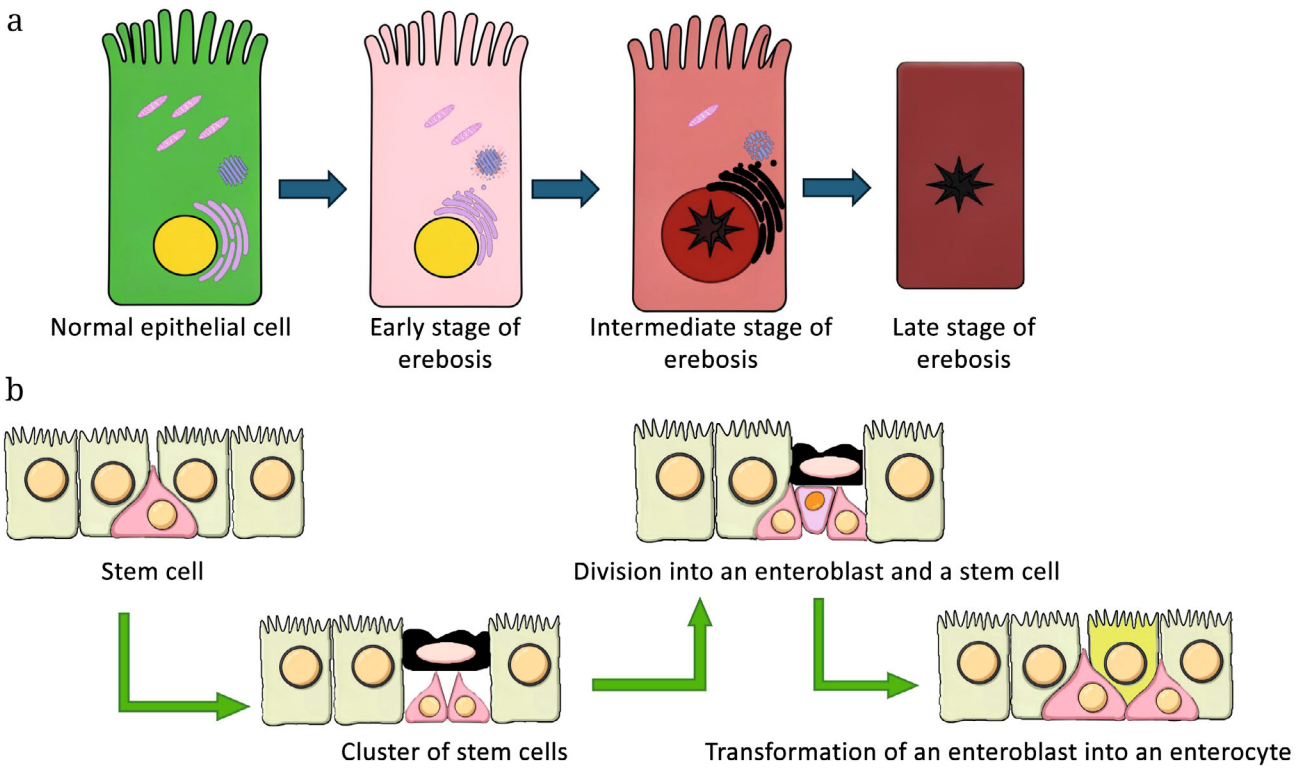

Fig. 1. Erebosis. a) Stages of erebosis. A normal epithelial cell is shown, expressing cytosolic GFP (indicated in green) as well as nuclear GFP and RFP (indicated in yellow). The cell also contains a nucleus, organelles (mitochondria, ER, and Golgi apparatus), intercellular junctions, and microvilli. During the early stage of erebosis, the cytosolic GFP fluorescence signal is lost, whereas the nuclear GFP and RFP signals are retained. During the intermediate stage, the nuclear GFP fluorescence signal is lost, while the nuclear RFP remains detectable. During the late stage, the nuclear RFP fluorescence signal is also lost. Ance immunostaining is shown in red, with the signal becoming progressively more intense as erebosis proceeds. b) Stages of enterocyte renewal. The stem cell is located at the base; at the onset of erebosis, stem cells accumulate near the erebotic cell. Subsequently, the stem cell divides into an enteroblast and a stem cell; the enteroblast next differentiates into an enterocyte, while the erebotic cell exits the enterocyte layer.

in a living cell [17]. It should be noted that cytoskeleton is compromised during erebosis, and its destabilization could also be attributed to the decrease in cytosolic viscosity.

THE ROLE OF HEME IN EREBOSIS

Heme as a metabolic brake on erebosis. Recent studies have significantly expanded our understanding of erebosis, enabling a shift from describing the predominantly morphological features of the process to elucidation of its metabolic regulation. Morikawa et al. used the ability to distinguish the initial stage of erebosis based on the loss of green fluorescent protein and manually selected 8 GFP+ normal enterocytes and 8 GFP− erebotic enterocytes from the R4 region of the *Drosophila* intestine for sequencing [18]. Silencing of the genes that differed between the cluster of normal cells and the cluster of erebotic cells had no effect on erebosis; therefore, a detailed analysis of the cluster of normal cells was undertaken to identify differences among the cells within it. Activation of the proteasome genes was observed in three cells; consequently, these cells within the normal cluster were classified as pre-erebotic. Analysis of the differentially expressed genes in the pre-erebotic versus normal cells identified the heme oxygenase *Ho* (a homolog of mammalian HMOX1) and the *Drosophila* heme exporter gene *Mrp5* as regulators of the initiation of erebosis [18]. Intracellular heme has been shown to act as a negative regulator (a “metabolic brake”) of erebosis. This pattern was experimentally confirmed: overexpression of *Mrp5*, which leads to the reduction in the intracellular heme levels, enhanced erebosis, whereas knockdown of the *Ho* or *Mrp5* genes, as well as addition of 5-aminolevulinic acid (a heme synthesis precursor) suppressed it. Thus, reduction in the cytoplasmic heme pool appears to be one of the earliest known metabolic events capable of initiating the erebosis program.

The effect of heme is not associated with oxidative processes. Involvement of heme naturally raises the question of the potential role of oxidative

stress in the regulation of erebosis. Free heme exhibits pro-oxidant properties; however, its degradation by heme oxygenase yields both compounds with antioxidant activity **(**such as bilirubin and carbon monoxide) and Fe^{2+} ions, which could stimulate formation of reactive oxygen species. Nevertheless, experimental evidence argues against oxidative stress acting as a direct trigger for erebosis. Increase in the intracellular heme content resulting from the *Ho* or *Mrp5* knockdown was not accompanied by the elevated levels of reactive oxygen species or accumulation of lipid peroxides [18]. Furthermore, the antioxidant *N*-acetylcysteine did not inhibit the development of erebosis. Consequently, despite the close link between the heme metabolism and cellular redox homeostasis, regulation of erebosis by heme appears to occur independently of oxidative stress [18]. This feature fundamentally distinguishes erebosis from ferroptosis – a regulated form of cell death characterized by iron-dependent peroxidation of membrane phospholipids as a key event. Nevertheless, shared regulatory nodes linked to the heme and iron metabolism may exist between these processes.

The effect of heme is linked to Dpp signaling. The Decapentaplegic (Dpp) signaling pathway is a highly conserved developmental regulatory pathway in *Drosophila melanogaster* mediated by Dpp, a secreted morphogen homologous to the vertebrate bone morphogenetic proteins (BMP2 and BMP4). Increase in the intracellular heme levels was accompanied by the enhanced Dpp signaling, whereas decrease in the level of phosphorylated Mad (a key effector of this pathway) was observed in the erebotic cells. Genetic suppression of the Dpp signaling components, including Tkv, Put, and Med, led to the enhanced erebosis, whereas activation of Dpp or of the constitutively active form of the Tkv receptor suppressed it. Moreover, activation of the Dpp signaling inhibited the development of erebosis – a process triggered by overexpression of the *Mrp5* gene and the consequent depletion of the intracellular heme pool. These results point to the existence of a heme–Dpp axis, in which sufficient intracellular heme levels maintain a signaling state that prevents the onset of erebosis, whereas drop in the heme levels removes this inhibitory signal. The specific molecular mechanism by which intracellular heme enhances the Dpp signaling has not yet been established. The authors hypothesize that it may involve heme binding to regulatory proteins [18].

Heme sensor proteins in Drosophila. In the case of vertebrates, one could speculate about the involvement of the transcription factor Bach1 (Bach1 protein [BTB and CNC homology 1]), a heme-dependent transcriptional repressor [19, 20], based on the fact that the *Drosophila* Dpp is a homolog of the vertebrate BMP2 and BMP4, and BMP2 is a target gene of the transcription factor Bach1 according to the ENCODE ChIP-seq data. Therefore, this hypothesis should certainly be considered in future studies of erebosis in mammals. However, in Drosophila, the Bach1 ortholog (CnC) is also an ortholog of Nrf1 and Nrf2 and lacks a heme-binding domain [21]. A potential Bach1/2 ortholog, the protein encoded by the Dmel\CG15725 gene, was recently described in the FlyBase, but there is no information regarding the presence of a heme-binding sequence in it.

One well-characterized heme-sensing protein in Drosophila is the nuclear receptor E75, whose closest mammalian homologs are REV-ERBα and REV-ERBβ. E75 contains a tightly bound heme, and redox state of the heme iron regulates interaction of E75 with the transcription factor DHR3 [22]. In mammals, heme also serves as a physiological ligand for REV-ERBα and REV-ERBβ; its binding promotes recruitment of the NCoR corepressor and enhances transcriptional repression [23]. These proteins are directly involved in circadian regulation. It is well established that enterocyte renewal is regulated by circadian rhythms [24], including in *Drosophila* [25]. Therefore, it is possible that the heme effect observed during the study of erebosis and, consequently, during generation of enterocytes from the stem cells is linked to this heme-sensing protein. Another interesting property of this protein in *Drosophila* is that heme coordination via a thiolate linkage results in a chemically reactive heme capable of interacting with both CO and NO [22].

Binding of REV-ERBα to the proximal promoter regions of the *Bmp2* and *Bmp4* genes in rats was demonstrated using chromatin immunoprecipitation combined with PCR [26]. Collectively, the results of this study demonstrated that the decrease in the circadian system activity is accompanied by the increased expression of several genes of the BMP family, and that REV-ERBα acts as a transcriptional repressor of this signaling pathway.

The mammalian BMP superfamily comprises at least 20 proteins, of which only a few, including BMP2, BMP4, BMP6, and BMP7, are linked to circadian regulation. In *Drosophila*, Dpp, which is involved in circadian rhythm regulation, transmits signals via Mad, whereas in mammals, BMP signaling is mediated by various proteins of the SMAD family. The study [27] demonstrated that treating breast cancer cells with BMP-6 (100 ng/mL) induced the HMOX1 transcription via recruitment of Smad1/5 to the Smad-responsive element within the HMOX1 promoter. Regarding the *Drosophila* heme oxygenase gene (*Ho*), there is no information in the literature regarding the presence of an element responsive to phosphorylated Mad or other downstream components of the Dpp signaling pathway.

Thus, there is evidence regarding regulation of the BMP2 and BMP4 expression by the heme-sensing transcription factor REV-ERBα in mammals [26], as well as regarding regulation of the HMOX1 via BMP6–Smad1/5 signaling [27]. However, these observations were obtained in different experimental systems and do not conclusively demonstrate existence of a unified REV-ERB–BMP–HMOX1 axis. At the same time, E75 is of interest as a potential heme sensor, linking changes in the intracellular heme levels to the regulation of erebosis. However, an important limitation of this hypothesis must be considered: in mammals, REV-ERBα acts as a transcriptional repressor of BMP family genes [26], whereas in erebosis, increase in the intracellular heme levels is accompanied by the enhanced Dpp signaling [18]. Thus, a simple linear model in which heme binding to E75 directly enhances Dpp signaling is inconsistent with the mode of action of REV-ERBα in mammals. If E75 is indeed involved in the heme-dependent regulation of Dpp signaling, its effect could be indirect, for example through repression of a negative regulator of Dpp or through other intermediate regulatory components. Moreover, the possibility that the link between intracellular heme and Dpp signaling is mediated independently of E75 cannot be excluded. Therefore, involvement of E75 should be regarded as a testable hypothesis rather than an established component of the heme–Dpp axis.

POSSIBLE SIGNS OF EREBOSIS IN ALZHEIMER'S DISEASE

Erebosis is a form of cell death previously reported only in *Drosophila* intestinal enterocytes, cells characterized by rapid turnover. Accumulation of the Ance enzyme serves as a biomarker for the cells in the state of erebosis. Given that Ance and ACE2 belong to a related family of metallopeptidases, it was of interest to examine data related to the changes in the ACE2 levels in neurodegenerative diseases. In the study [28], the elevated ACE2 staining has been demonstrated in the hippocampus of the aged wild-type male mice (C57BL/6J strain), female mice with Alzheimer's disease-like neuropathology (3xTg-AD strain), and humans suffering from the disease. The ACE2-positive cells are hippocampal neurons that frequently contain phospho-tau protein in the mice with Alzheimer's-like neuropathology. Administration of phospho-tau protein into the hippocampus of young adult wild-type mice increases hippocampal ACE2 expression.

The 3xTg-AD mice serve as a model of Alzheimer's disease, carrying three human mutant genes associated with the condition. In these mice, formation of amyloid plaques and accumulation of hyperphosphorylated tau protein begin at 6 months of age, and pronounced pathological changes in the hippocampus are observed in the females after 12 months. Since neuropathological changes are more consistently pronounced in the female 3xTg-AD mice than in the males, the female 3xTg-AD and the control mice were used in this study. Similar to the results obtained for the C57BL/6J mice, weak ACE2 staining was observed in the hippocampus of the 2-month-old 3xTg-AD mice and their age-matched controls (B6129SF2/J strain). Some ACE2-positive cells exhibited fragmented or shrunken nuclei, lacked staining for NeuN, a protein marker used in neuropathology to reliably identify mature post-mitotic neurons in the central and peripheral nervous systems, and tested positive in the TUNEL assay, indicating cell damage or death. Intensity of the ACE2 staining was higher in the 14-month-old 3xTg-AD mice and in the control animals. It is important to note that the levels of ACE2 staining were higher in the 14-month-old 3xTg-AD mice than in the age-matched control mice.

To determine whether the ACE2 accumulation occurs during other forms of cell death, brain sections were used from the 2-month-old male CD-1 mice that had undergone 90-minute occlusion of the right middle cerebral artery to induce an ischemic lesion. The brain samples for sectioning were harvested 24 hours after arterial occlusion. Various forms of cell death, including necrosis and apoptosis, were observed in the ischemic penumbra – the region surrounding the infarct core. As expected, the TUNEL-positive cells were detected in the ischemic penumbra; however, no ACE2 staining was observed in this region [28]. Thus, based on the ACE2 staining, the authors concluded that erebosis may occur in the Alzheimer's disease models, but not in the ischemic stroke model.

It should be noted that ACE2 exists in two forms – membrane-bound and soluble – and their expression levels vary depending on the disease states and under the influence of drugs. The membrane-bound form is a full-length type I transmembrane protein with its active site facing the extracellular space, where it converts the pro-inflammatory and vasoconstrictive angiotensin II into the protective peptide angiotensin-(1-7). The soluble form is generated by proteolytic shedding of the ectodomain of the membrane-bound ACE2 by sheddases (primarily ADAM10 and ADAM17), a process known as ectodomain shedding [29]. A soluble form of ACE2 is present in blood in negligible amounts, and a significant increase in its levels correlates with poor prognosis for the heart disease [30] and coronaviral infection [31].

In the *Drosophila* undergoing erebosis, accumulation of the intracellular Ance is likely linked to the influx of secreted extracellular Ance across

a transiently permeable plasma membrane [2, 9]. However, this situation cannot be directly compared to the soluble form of mammalian ACE2: Ance is a secreted protein, whereas soluble ACE2 is primarily generated through proteolytic cleavage of the ectodomain of the membrane-bound ACE2. Nevertheless, the data regarding the changes in the ratio of soluble to membrane-bound ACE2 in Alzheimer's disease are of interest in the context of identifying potential molecular markers of an erebosis-like state. Only one study compared the absolute amount and the ratio of soluble and membrane-bound ACE2 fractions in the *postmortem* parietal cortex samples from the patients with the clinically and neuropathologically confirmed diagnosis of Alzheimer's disease with those from the control group [32]. The ratio of the soluble fraction to the membrane-bound fraction was found to be significantly higher in the patients; moreover, absolute amount of the soluble fraction was inversely correlated with the *antemortem* global cognitive function.

Two studies on the role of ACE2 in the Alzheimer's disease published to date do not so much suggest possible existence of erebosis in this condition as highlight the need for the detailed investigation of the role of ACE2 in the cerebral cortical organoids while monitoring changes in the cellular morphology within the Alzheimer's disease models.

DISTINCTIONS BETWEEN EREBOSIS, FERROPTOSIS, AND OTHER TYPES OF CELL DEATH

Table 1 compares erebosis with apoptosis, autophagy-dependent cell death (ADCD), necroptosis, pyroptosis, and ferroptosis. For the established forms of regulated cell death, terminology and key molecular criteria are presented in accordance with the NCCD recommendations [33, 34]. Modern nomenclature is based primarily on the molecular mechanisms required to execute a specific type of cell death, whereas morphological features, kinetics, and sensitivity to pharmacological inhibitors are considered supplementary comparative characteristics [33]. Erebosis is not yet included in the NCCD nomenclature and is presented in the table as an emerging concept of cell death for comparative analysis. Absence of a detectable immune response links erebosis to apoptosis; however, caspase inhibitors do not suppress erebosis, as demonstrated upon its discovery in 2022 [2]. In the initial study, the erebotic cells did not accumulate propidium iodide, a finding that argued against the persistent loss of plasma membrane integrity characteristic of necrotic death [2]. However, subsequent studies revealed that erebosis involves a transient, non-lytic permeabilization of the plasma membrane, accompanied by the efflux of cytoplasmic proteins and influx of the extracellular macromolecules into the cell [9]. No autophagosome formation was observed in the case of erebosis [2]. Morphological features, kinetics, initiating conditions, and known molecular characteristics of erebosis distinguish it from the forms of cell death listed in Table 1. However, the execution mechanism of erebosis has not yet been established; therefore, its status as a distinct form of regulated cell death requires further confirmation.

However, erebosis is much less well-characterized in many respects than the other types of cell death listed in Table 1. The most significant difference lies in the role of ninjurin. Proteins of the ninjurin family (Ninjurin A/NijA in *Drosophila melanogaster* and Ninjurin-1/NINJ1/2 in mammals) are involved in several processes. NINJ1 is considered an effector of plasma membrane rupture in several forms of lytic cell death.

It has been shown that during ferroptosis, NINJ1 oligomerizes and promotes the late stage of plasma membrane rupture and release of the damage-associated intracellular molecules [35]. Notably, absence of NINJ1 reduces the plasma membrane rupture in macrophages and fibroblasts undergoing ferroptosis. Importantly, NINJ1 acts primarily downstream of the key lipid peroxidation processes; that is, NINJ1 appears not to initiate the ferroptotic program but rather participates in its terminal membrane phase. In pyroptosis, gasdermin D initially forms pores, and NINJ1 executes only the terminal stage [36]. During necroptosis, cleavage of the sialic-acid-binding Ig-like lectin 12 (SIGLEC12) generates a 20 kDa fragment homologous to NINJ1; this cleavage is necessary and sufficient to induce terminal plasma membrane rupture (PMR) [37]. It is worth emphasizing that the key mammalian homologs of *Drosophila* NijA are the NINJ1 and NINJ2 proteins, and the latter does not possess the ability to rupture the plasma membrane [38].

During erebosis in *Drosophila melanogaster,* ninjurin accumulates as membrane clusters and facilitates transient efflux of the cytoplasmic proteins and influx of the extracellular macromolecules into the cell, without causing complete membrane destruction, as discussed in the preprint [9]. This behavior is of particular interest when compared to the function of NINJ1 in mammals. It could be hypothesized that the proteins of Ninjurin family serve as universal effectors of plasma membrane remodeling that can be recruited by various cell death programs. However, ultimate outcome of their activation is highly context-dependent. In ferroptosis and pyroptosis, NINJ1 promotes irreversible disruption of plasma membrane integrity and cell lysis. It is currently unknown whether NijA mediates a controlled,

Table 1. Comparison of erebosis with selected forms of regulated cell death

Cell death type	Apoptosis (extrinsic and intrinsic)	Autophagy-dependent cell death ADCD	Necroptosis	Pyroptosis	Ferroptosis	Erebosis
Morphological features: cell shape and size, nucleus, mitochondria	cell shrinkage, formation of plasma membrane blebs, chromatin and nuclear condensation and fragmentation, formation of apoptotic bodies; the plasma membrane retains its integrity during the actual process of apoptosis [33, 34]	pronounced autophagic vacuolization and accumulation of autophagosomes/ autolysosomes; the specific morphology depends on the model. Presence of autophagosomes *per se* is not a sufficient criterion for ADCD [33, 39, 40]	necrotic morphotype: swelling of the cell and organelles, followed by loss of plasma membrane integrity [33, 41]	cell swelling and membrane permeabilization; the characteristic chromatin condensation differs from that seen in apoptosis, and typical apoptotic nuclear fragmentation is absent [33, 36, 42]	reduced-size mitochondria with increased membrane electron density and reduction or disappearance of cristae; the nucleus usually remains relatively preserved until late stages [33, 43, 44]	flattened cells, a marked reduction in cytoplasm and actin filaments, loss of organelles, and flattening of the nucleus, which initially increases in size before the loss of the nucleolus [2]
Characteristic kinetics*	the latent period depends heavily on the stimulus and cell type and could range from tens of minutes to many hours. Following MOMP/ effector caspase activation, the terminal phase develops rapidly, within minutes to tens of minutes** [45, 46]	it depends heavily on the model. In the larval midgut of *Drosophila*, intense formation of Atg8a-positive autophagosomes is observed after puparium formation; pronounced histolytic remodeling occurs over the next few hours [39]	it typically develops over the course of several hours; terminal damage to the plasma membrane occurs following accumulation of the phosphorylated MLKL (mixed lineage kinase domain-like protein) [pMLKL] on the membrane above the lytic threshold [41, 47]	following gasdermin activation, the terminal phase develops rapidly: cellular changes preceding PMR occur within 10-20 min [42]; the time to onset depends on the stimulus and the type of inflammasome	it typically develops over the course of several hours; the kinetics depend on the mode of induction and the cellular model. In the HT-1080 cells treated with erastin, accumulation of lipid ROS begins after approximately 2 hours, while overt cell death commences at around 6 h [43]; direct inhibition of GPX4 by the agents such as RSL3 typically induces a more rapid onset of ferroptosis	loss of cytosolic GFP could occur within 5 min; the subsequent progression of the process is significantly slower, with nuclear GFP/ RFP signals persisting for more than 12 h [2]

Table 1 (*cont.*)

Cell death type	Apoptosis (extrinsic and intrinsic)	Autophagy-dependent cell death ADCD	Necroptosis	Pyroptosis	Ferroptosis	Erebosis
Initiating signals/ conditions	intrinsic: DNA damage, ER stress, oxidative stress, growth factor deprivation, and other cellular stresses; extrinsic: activation of death receptors FAS, TRAIL-R1/ R2, TNFR1, and other receptor systems [33, 34]	context-dependent: hormonal signals (e.g., ecdysone during *Drosophila* larval midgut degradation), severe nutrient deprivation, hypoxia/ischemia, and experimental hyperactivation of autophagy (e.g., Tat-Beclin 1); in certain models, ADCD occurs under conditions of cytotoxic stress when the apoptotic machinery is impaired [33, 39, 40, 48]	signaling via TNFR1, FAS, TLR3/4, ZBP1, and other systems; activation of the RIPK3–MLKL-dependent pathway is particularly likely when CASP8 activity is insufficient or inhibited [33, 41]	PAMPs (pathogen-associated molecular patterns)/ DAMPs and other innate immune signals activating inflammasomes; cytosolic LPS and other signals triggering non-canonical pyroptotic pathways [33, 36]	impairment of cystine transport, GSH depletion, inhibition or inactivation of GPX4, and other disturbances leading to the uncontrolled iron-dependent peroxidation of membrane phospholipids [33, 43, 44]	amino acid deficiency and reduced effective viscosity of the cytosol [13]; reduction in intracellular heme content [18]
Determining mechanism/key effectors	intrinsic: BAX/ BAK – MOMP – cytochrome c/ APAF1 – CASP9 – CASP3/7; Extrinsic: death receptor/ FADD – CASP8/10 – CASP3/7 [33, 34].	cell death is functionally dependent on the autophagic machinery or its components [33, 39]	RIPK3–MLKL; RIPK1 is an essential upstream component only in certain contexts [33, 41]	gasdermins; in the canonical and non-canonical pathways, primarily GSDMD, which is activated by the corresponding inflammatory caspases [33, 36]	iron-dependent peroxidation of phospholipids containing long-chain polyunsaturated fatty acids; GPX4/GSH is one of the key protective systems; sensitivity to ferroptosis is also regulated by other lipid and iron metabolism systems [43, 44]	the effector mechanism has not been identified. Dpp signaling activity is functionally linked to the process [18]; involvement of NijA in the transient membrane permeabilization is based on the preliminary data [9]

Table 1 (*cont.*)

Cell death type	Apoptosis (extrinsic and intrinsic)	Autophagy-dependent cell death ADCD	Necroptosis	Pyroptosis	Ferroptosis	Erebosis
Molecular signs/ markers	activated/ cleaved CASP3/7; for the intrinsic pathway – MOMP and cytochrome c release; for the extrinsic pathway – CASP8 activation [33, 34]	there is no specific single marker. Atg8/LC3-positive autophagosomes and autolysosomes confirm autophagy activation but do not prove ADCD; functional dependence of the cell death on the autophagic machinery is required [33, 39, 40]	pMLKL, its oligomerization and translocation to the plasma membrane; pRIPK3 – an additional marker [33, 47]	cleavage of gasdermin and formation of its pore-forming N-terminal fragment – most commonly GSDMD-N – and activation of the corresponding inflammatory caspases serve as additional markers of a specific pathway [33, 36]	lipid peroxidation and signs of dysfunction in the systems that prevent ferroptosis, including GPX4. There is no single universal, specific biomarker [33, 44]	intracellular accumulation of Ance in *Drosophila*, likely associated with the uptake of secreted extracellular Ance [2, 9]; the molecular form of Ance following its entry into the cell has not been directly characterized
Pharmacological inhibitors/ modulators***	Z-VAD-FMK (pan-caspase inhibitor) [34]	there is no universal specific inhibitor. In *Drosophila*, chloroquine partially suppressed premature cell elimination caused by disrupted Dpp signaling [40, 49]	necrostatin-1 and the more specific necrostatin-1s are RIPK1 inhibitors in the RIPK1-dependent models of necroptosis [50]	VX-765 is a CASP1 inhibitor in canonical CASP1-dependent models; experiments have also shown that disulfiram blocks GSDMD pore formation [36, 51]	ferrostatin-1, liproxstatin-1, iron chelators, such as deferoxamine [43, 44]	5-aminolevulinic acid is an experimental suppressor of erebosis via increase in the intracellular heme pool [18]; increasing effective viscosity of the cytosol with amino acids or non-metabolizable solutes also inhibits erebosis [13]
Plasma membrane rupture (PMR)	terminal plasma membrane rupture is absent during the process of apoptosis itself; however, secondary necrosis may occur if the dead cell is not promptly eliminated [33, 34]	it is not a universal defining feature; state of the plasma membrane depends on the specific ADCD model [33]	MLKL-dependent membrane permeabilization followed by terminal PMR; in human cells, SIGLEC12 acts downstream of MLKL. Upon cleavage of SIGLEC12 by the protease TMPRSS4, a 20-kDa fragment	Gasdermin pores mediate early membrane permeabilization; terminal PMR in many models occurs with participation of NINJ1 [36]	following critical peroxidation of membrane lipids, terminal loss of the plasma membrane integrity ensues; NINJ1 is required for efficient late-stage membrane permeabilization	terminal PMR and lysis are not observed; in the initial study, erebotic cells did not accumulate propidium iodide [2]. More recent data indicate transient, non-lytic membrane permeabilization, involving influx of

Table 1 (*cont.*)

Cell death type	Apoptosis (extrinsic and intrinsic)	Autophagy-dependent cell death ADCD	Necroptosis	Pyroptosis	Ferroptosis	Erebosis
			is generated that is homologous to NINJ1; this cleavage is necessary and sufficient for PMR induction [37]		and PMR, but acts at a later stage relative to the key events of ferroptosis [35]	extracellular macromolecules and efflux of cytoplasmic macromolecules; involvement of NijA in this transient permeabilization is based on preliminary data [9]
Immunological consequences	it is usually not accompanied by marked inflammation [33, 34]	context-dependent; a universal ADCD immunological profile has not been established [33]	often pro-inflammatory due to membrane permeabilization, PMR, and release of DAMPs [33, 41]	typically, strongly pro-inflammatory; associated with the release of IL-1β, IL-18, and DAMPs [33, 36]	context-dependent; release of DAMPs and immunostimulatory effects are possible [44]	not established
Physiological role	embryonic and post-embryonic development, tissue homeostasis, regulation of cell populations, elimination of damaged, superfluous, or potentially dangerous cells [33, 34]	context-dependent tissue remodeling during development; one of the best-characterized examples is removal of the *Drosophila* larval midgut during metamorphosis [33, 39, 40]	protection against certain pathogens, particularly those capable of inhibiting apoptosis; involvement in inflammatory and pathological tissue processes [33, 41]	innate antimicrobial defense and elimination of infected cells with the release of inflammatory mediators [33, 36]	no single universal physiological function has been established; ferroptosis is involved in tumor suppression and immune regulation, as well as in pathological tissue damage [44]	the proposed role is homeostatic renewal of the intestinal enterocytes in the adult *Drosophila melanogaster* [2, 13, 18]

Note. Classification of the established forms of regulated cell death and their key molecular characteristics are presented in accordance with the NCCD 2018 recommendations [33]; information on apoptosis has been further refined in accordance with the NCCD 2023 recommendations [34]. Morphological features, kinetics, and pharmacological sensitivity serve as additional comparative characteristics and do not, in themselves, allow for unequivocal determination of the mechanism of cell death. Erebosis is not currently included in the official NCCD nomenclature and is presented in the table as an emerging concept of cell death.

* The indicated kinetic features depend on the cell type, nature and intensity of the stimulus, and experimental model, and do not constitute a classification criterion.

** The term “caspase-independent apoptosis” was used in earlier literature to describe the caspase-independent cell death following MOMP. In the current NCCD nomenclature, this outcome is not classified as a distinct type of apoptosis. Following MOMP, caspase inhibition could significantly delay cell death but does not necessarily ensure long-term cell survival [34, 52].

*** Pharmacological sensitivity serves as an auxiliary experimental criterion. Inhibitors may have non-specific effects, and blocking one cell death pathway could, under certain conditions, lead to a switch to another pathway.

temporary alteration of the membrane permeability preserving the overall membrane framework during erebosis, or if another, as-yet-unidentified driver is responsible for forming a transient pore.

Available data do not support classifying erebosis as a form of ferroptosis, as erebosis is not accompanied by the detectable increases in lipid peroxidation and allows the dying cell to maintain its structural integrity for an extended period; in contrast, the defining event of ferroptosis is iron-dependent oxidation of the membrane phospholipids, leading to the loss of plasma membrane integrity. Diverse role of heme is particularly noteworthy: reduction in the intracellular heme levels triggers erebosis in *Drosophila melanogaster*, whereas ferroptosis is driven by the iron-dependent lipid peroxidation occurring when the membrane repair systems are impaired [44].

CONCLUSION

A detailed mechanistic comparison of erebosis with other types of regulated cell death is currently quite difficult, as erebosis has not yet been conclusively identified in mammals. It has been well characterized morphologically in the *Drosophila* enterocytes and differs significantly from other forms of cell death (Table 1). Studies published in 2026 demonstrated that erebosis depends on cytoplasmic viscosity, heme oxygenase activity, and intracellular heme levels. Additionally, the data presented in a preprint indicate formation of the transient membrane pores involving ninjurin. However, all the publications regarding *Drosophila* originate from a single laboratory in Japan, making it difficult to assess the extent to which these results could be replicated in other laboratories. Undoubtedly, identifying new targets linked to the role of heme in the mechanism of erebosis would greatly aid in elucidating mechanistic details of this process. One potential heme sensor involved in the regulation of erebosis is E75, a homolog of the mammalian REV-ERB proteins. Investigating the role of E75 in the heme-dependent regulation of erebosis using genetic approaches and Dpp signaling analysis could help to determine its place in this process. If E75 is not involved in this regulation, it would be necessary to search for other heme-sensing proteins that link changes in the intracellular heme levels to the initiation of erebosis. The use of *Drosophila* models is justified by the fact that *Drosophila* possesses far fewer heme-sensing proteins than humans or mammals. In mammals, potential candidates for the role of heme-sensing proteins in this process include not only the previously mentioned Bach1 but also the heme-regulated inhibitor kinase (HRI) and various other transporters and proteins.

Morphological description of this new type of cell death and the use of Ance immunostaining as a biomarker constitute reliable findings when visualization is performed at the cellular level. However, detection of the ACE2-positive neurons in the models of Alzheimer's disease [28] does not, in itself, provide sufficient evidence for the existence of erebosis in mammals, as accumulation of ACE2 was not accompanied by full characterization of the complex of morphological and molecular features of erebosis described in *Drosophila*. In the *postmortem* study reported in [32], the membrane-bound and soluble ACE2 fractions from the parietal cortex were analyzed, but neither form was visualized at the cellular level.

It is quite surprising that, despite the vast number of publications regarding the role of ACE2 at the molecular level in various pathologies – and particularly in the context of COVID-19 – no one has ever reported a new type of cell death. Interest in the role of ACE2 in COVID-19 and its association with other membrane proteins has even led to determination of the detailed structures of its complexes with the amino acid transporters B0AT1 (SLC6A19) and SIT1 (SLC6A20) [8]. ACE2 functions as a chaperone for the B0AT1 transporter, forming a functional heterotetrameric complex with it. The *in vivo* role of B0AT1 has been well characterized; studies have shown that uptake of the neutral amino acids (particularly isoleucine) mediated by this transporter is adapted not only to circadian rhythms but also to the levels of dietary protein and amino acid intake, specifically regarding expression and functional activity in the proximal small intestine [53]. These observations are notably similar to the conclusions reported by the Japanese authors. *Drosophila* possesses a homolog of B0AT1, the DmNAT1 transporter, which was overlooked by the Japanese researchers studying erebosis, even though both visualization methods and specific inhibitors for it are available.

Overall, the morphology of erebosis is unusual and intriguing, but the process itself requires thorough investigation: specifically, the mechanism by which Ance enters an enterocyte, and the extent to which the intracellular form of ACE2 might serve as a biomarker for this type of cell death in mammals should be elucidated.

Abbreviations

ACE2	angiotensin-converting enzyme 2
ADCD	autophagy-dependent cell death
Ance	angiotensin-converting enzyme *Drosophila melanogaster* (homolog to mammalian ACE/ACE2 family)
Bach1	Bach1 protein (BTB and CNC homology 1)

BMP	bone morphogenetic protein (Dpp in *Drosophila melanogaster*)
DAMPs	damage-associated molecular patterns
GFP	green fluorescent protein
HMOX1	heme oxygenase 1 (Ho in *Drosophila melanogaster*)
MOMP	mitochondrial outer membrane permeabilization
NINJ1	ninjurin 1
Nrf1/2	nuclear factor erythroid 2-related factor 1/2
PMR	plasma membrane rupture
RFP	red fluorescent protein
RIPK	receptor-interacting protein kinase
SIGLEC12	sialic-acid-binding Ig-like lectin 12

Contributions

D. M. Hushpulian and S. V. Nikulin – manuscript writing; A. A. Zakharyants – collection and analysis of the literature data, discussion of the results; B. Thomas and S. V. Kazakov – critical review and scientific consultation; I. G. Gazaryan – manuscript writing, conceptualization, and scientific supervision. All authors – editing of the manuscript and approval of the final version.

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Ethics approval and consent to participate

This work does not contain any studies involving human and animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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