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Hypoxia-ischemia and sexual dimorphism: modeling mitochondrial dysfunction using brain organoids

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Abstract

Hypoxic–ischemic encephalopathy (HIE) is a leading cause of neurodevelopmental morbidities in full-term infants. There is strong evidence of sexual differences in hypoxic-ischemic (HI) injury where male neonates are at higher risk as they are subject to more pronounced neurological deficits and death than females. The cellular and molecular mechanisms underlying these sexual discrepancies in HI injury are poorly understood. Mitochondrial dysregulation has been increasingly explored in brain diseases and represents a major target during HI events. In this review, we discuss (1) different mitochondrial functions in the central nervous system (2), mitochondrial dysregulation in the context of HI injury (3), sex-dependent mitochondrial pathways in HIE and (4) modeling of mitochondrial dysfunction using human brain organoids. Gaining insight into these novel aspects of mitochondrial function will offer valuable understanding of brain development and neurological disorders such as HI injury, paving the way for the discovery and creation of new treatment approaches.

Keywords Mitochondria, Hypoxic-ischemic encephalopathy, Sexual differences, Brain organoid

Hypoxic-ischemic encephalopathy – A significant disease burden with sex associated factors

Hypoxic-ischemic encephalopathy (HIE) is one of the most serious birth complications affecting newborns. HIE results from deprivation of oxygen or blood flow to the fetal brain during the prenatal, intrapartum or postnatal period [1, 2]. Approximately 40–60% of affected infants either die within two years of birth or suffer from severe disabilities, including cerebral palsy, epilepsy, severe learning and intellectual disabilities, developmental cognitive and motor impairments, and later psychiatric disorders [3]. HIE accounts for 23% of infant mortality worldwide and affects 0.7–1.2 million infants annually [4]. Literature reports indicate favorable survival and neurodevelopment outcome in female compared to male infants affected by HIE [5–8]. Male infants are 2 times more likely to experience prenatal anoxia, infection and hemorrhage and 1.8 times more likely to suffer cerebral birth trauma [9, 10]. Males show an increased risk for neurodevelopmental disorders such as language and speech disorders, autism, dyslexia, attention deficit-hyperactivity disorder, learning disabilities and cerebral palsy compared to females [8, 9, 11, 12]. While male infants are more vulnerable to ischemic insult and display greater long-term deficits than females [13], the pathways underlying these sex differences remain poorly understood. This review offers a fresh perspective on the role of mitochondria in sexual differences and male vulnerability

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in HIE and explores new insights and uncovers aspects that have not been previously addressed in the literature concerning human cells. Recent work using human pluripotent stem cells in innovative and relevant human cell-based models affords an opportunity to expand and improve understanding of the role of mitochondria in HIE and sexual dimorphism.

General considerations

Mitochondria are membrane-bound organelles integral to normal cellular function as they are the powerhouse of cells. They are also responsible for the synthesis of heme and phospholipids, apoptotic activation, cell death, and calcium homeostasis [14]. Mitochondria comprise a double-membrane system, an inner and outer mitochondrial membrane separated by an intermembrane space. The inner membrane contains numerous folds called cristae which extend into the interior (matrix) of the organelle. The outer membrane is permeable to small molecules thanks to porins which form channels that allow the free diffusion of molecules smaller than about 6000 daltons into the intermembrane space. Consequently, the intermembrane space contains ions and small molecules in proportions quite similar to those in the cytosol. The inner membrane is a functional barrier that limits the passage of small molecules between the intermembrane space and the mitochondrial matrix while maintaining the proton gradient that drives oxidative phosphorylation [15]. Mitochondria have their own genetic system within the matrix constituted by mitochondrial DNA (mtDNA). Mitochondrial genomes are circular DNA molecules, present in several copies per organelle. Thirteen proteins are encoded by human mtDNA and are involved in electron transport, oxidative phosphorylation, 16 S, 12 S RNA and 22 tRNAs required for translation of proteins encoded by the organelle genome.

Role of mitochondria during brain development

Brain development occurs in interconnected stages: the formation of brain cells (neurogenesis and neurulation), their movement to appropriate locations (migration), the growth of axons and dendrites to establish connections with other neurons (neuronal differentiation and pathfinding), the creation of synapses for communication between cells (synaptogenesis), the refinement of these synapses (maturation and pruning) and the development of supportive tissue that ensures effective communication among neurons (gliogenesis or myelination) [16].

Neurulation marks the early stages of brain development where the neural tube forms from the ectoderm during early embryonic development and leads to development of the central nervous system (CNS) [17]. After neurulation, neurogenesis occurs, where neural progenitor cells proliferate and differentiate into different types of neurons and glial cells [18]. As development progresses, neuronal maturation takes places, including axon and dendrite growth, synaptic formation and refinement. During brain formation, a precise communication between the vascular and neural compartments is crucial for proper development and function. The vascularization process involves the formation of the neurovascular unit (NVU), which is composed of astrocytes, endothelial cells, pericytes and neurons and plays a crucial role in the blood-brain barrier (BBB) and homeostasis [19]. In human embryos, the cranial vasculature begins forming around 21 days post-fertilization. Vascularization of the neural tube progresses in alignment with the closure of the neural tube [20].

Mitochondria play a critical role in the developing brain, including different stages such as neurulation, specification, proliferation and survival of CNS progenitors, neurogenesis, neural migration and finally neurite outgrowth and synaptogenesis [21]. Neurulation, the process which converts the neural plate into the neural tube, involves organelle biogenesis, including the formation of ribosomes and mitochondria. This process requires the synthesis of nucleic acids, epigenetic modifications and changes in precursor cell transcriptomes. Nucleic acid synthesis and epigenetic modifications are partially dependent on mitochondrial folate-mediated one-carbon metabolism (FMOCM), but also on the enzyme dihydroorotate dehydrogenase located in the inner mitochondrial membrane [22]. Thus, mitochondrial FMOCM is crucial in meeting the high demand for nucleic acids during the early stages of neural development. The involvement of mitochondria in nuclear DNA methylation is connected not only to the activities of the folate/methionine cycle, but also to mtDNA haplotypes. According to recent studies, epigenetic histone methylation is regulated by mtDNA heteroplasmy [23]. Following neurulation, neural stem cells (NSCs) continue to proliferate and begin to differentiate into more refined areal identities. Early to-mid corticogenesis depends critically on mitochondria and metabolic adaptations with proliferation and survival of developmental stem cell/progenitors [24–26].

Neurogenesis is regulated by mitochondrial biogenesis and bioenergetics. Indeed, as metabolism shifts from glycolysis to mitochondrial OxPhos, NSCs transform into neural progenitor cells and then differentiate into various types of neuronal cells [27–29]. Additionally, mitochondrial structure and dynamics are vital for neurogenesis. Some studies have demonstrated that altering the fission and fusion dynamics of the mitochondrial network by targeting the fission protein Drp1 and the fusion proteins MFN1/2 disrupts neurogenesis and differentiation [30, 31]. Recent studies have shown that mitochondrial metabolism dictates the pace of neuronal development

[32]. Mitochondrial dynamics and metabolism follow specific timelines during the development of cortical neurons. In the neurons of newborns, both the number and metabolic activity of mitochondria are low, and gradually increase as the neurons mature. Improved mitochondrial metabolism in human neurons accelerates maturation, resulting in greater neurite complexity, increased excitability, and enhanced synaptic function [32].

Mitochondria contribute to synaptic functions in several ways. First, by producing energy and ATP to sustain the continuous release of neurotransmitters and to maintain ion gradients, both of which necessitate energy. Second, mitochondria play a crucial role in regulating Ca^{2+} levels within the presynaptic terminal which is essential for neurotransmitter release [14]. Additionally, the tricarboxylic acid cycle participates in the metabolism of the primary excitatory neurotransmitter, glutamate, and the primary inhibitory neurotransmitter, GABA. Synaptic activity produces reactive oxygen species (ROS) which pose a potential threat to cellular health. Mitochondria help buffer and neutralize ROS and thereby safeguard the fragile components within synapses against oxidative harm. However, impaired mitochondria themselves can generate ROS. In general, deficiencies in mitochondrial function and structure can impact synaptic transmission. Mitochondria are known to generate ATP and to take up Ca^{2+} which can both impact presynaptic release properties. Maintaining presynaptic calcium levels and managing the size of the synaptic vesicle pool are essential for effective synaptic transmission [33].

The NVU and the immature brain begin their development early in fetal life, with the formation of BBB components and neural networks that lay the foundation for brain function and maturation. Physiologically, the NVU is essential for controlling brain blood flow, the BBB, and neuro-immune responses, which are impaired in HIE [34]. The role of mitochondria in BBB function has been relatively underexplored. However, brain endothelial cells exhibit a comparatively higher mitochondrial context, indicating that these cells likely possess a distinct metabolic requirement that depends more on oxidative respiration than other cell types [35]. Mitochondria are the primary source of ATP, which is essential for the functioning of the BBB, synaptic signaling and neuronal activity. The brain's high metabolic demand requires mitochondrial oxidative phosphorylation to generate energy, especially in neurons and endothelial cells. The NVU supports energy susbstrates crucial for meeting metabolic demands, not only through intracellular communication but also via the mitochondria [36, 37]. Mitochondria primarily function as crucial signaling organelles in the vascular endothelium rather than merely producing ATP [38, 39], thus facilitating the release of vasoactive factor from the endothelium to regulate and preserve the integrity of the BBB and brain homeostasis [40, 41].

The development of neurons in the immature brain is heavily influenced by mitochondrial function, which can be driven by sexual hormones. Androgens and estrogens shape the developing brain by influencing various factors, including cell survival and death, neural and glial development, neuronal migration, myelination, synaptogenesis, synaptic pruning and neurochemical profiles [42]. Sexual hormones such as estrogen significantly influence neuronal development, contributing to sex-specific brain differentiation and function. For instance, estrogen has a protective role in the regulation of oxidative stress [43], NADH-linked respiration rate [44] and metabolic pathways [45]. Estrogens influence the expression of genes including miRNAs and IncRNAs, which control mitochondrial functions such as metabolism, OxPhos, apoptosis, UPR^{mt}, and mitochondrial fission and fusion [46]. Under physiological conditions, sex-dependent mitochondrial function results in less oxidative damage to mitochondrial DNA in females than in males [42] and a higher respiration rate in females [44]. Sex-related differences in vasculature could explain sex-specific vulnerability in brain diseases. Estrogen suppression of NADPH oxidase activity and reduction in mitochondrial ROS production were found in endothelial cells, as were increased levels of enzymes involved in the respiratory chain and the tricarboxylic acid cycle [47–49], leading to reduced apoptosis of endothelial cells and maintained energy metabolism. Higher NADH-linked respiration rates in female mice are associated with an increase of PDHc activity [50]. A positron emission tomography brain imaging study in humans revealed that women exhibit higher overall cerebral glucose metabolism than men, indicating enhanced glucose-dependent mitochondrial energy production in females [51]. Cikic et al. found that mitochondrial proteins and mitochondrial DNA in female rat arteries are more highly expressed than in male rat arteries. These elevated mitochondrial proteins in female rats result in greater mitochondrial respiration and dilation of arteries than in male rats [52]. Transporters such as ATP synthase, TOM20, TOM40 are expressed more in female than in male rat cerebral microvessels [52]. This study also found a greater abundance of proteins involved in degradation of mitochondria in male cerebral microvessels. Estrogen can also reduce BBB permeability [53] and decrease peripheral infiltration [54] in female rodents. Ischemic injury to blood vessels triggers thrombo-inflammation, which exhibits sexual dimorphism [55].

Mitochondrial dysregulation in the context of HI injury

Dysregulation in mitochondria following HI injury is summarized in Fig. 1. HI conditions leading to inadequate oxygen supply to cells make them especially vulnerable to mitochondrial dysfunction. Mitochondria play a crucial role in brain development and in injury response in the developing brain [56]. HI events play a significant role in the production of ROS. Under normal conditions, cellular antioxidant mechanisms effectively eliminate these ROS. One way this happens is through the action of superoxide dismutase, which converts superoxide radicals generated during cellular respiration into hydrogen peroxide. This hydrogen peroxide is then further broken down by glutathione peroxidase and catalase [57]. However, during HI injury, the mitochondria produce ROS at a rate that exceeds the capacity of antioxidant defenses, resulting in damage to cellular DNA, lipid peroxidation in mitochondria, disruption of calcium homeostasis [58] and depolarization of the mitochondrial membrane [59]. There are some differences in immature and mature brain in the regulation of the mitochondrial membrane permeability transition pore (mPTP), with a developmental shift of cyclophilin D (CypD), a regulator of mPTP. Wang et al. demonstrated that CypD/mPTP is crucial for the development of brain injury in adult mice, while Bax-dependent mechanisms dominate in the immature brain [60]. A mitochondrial permeability transition occurs after HI injury, but it is unclear whether it is involved in the development of injury in the immature brain [61].

HI injury is also linked to a rise in apoptosis, or programmed cell death. This brain injury results in elevated levels of ROS and calcium influx, which cause a membrane permeability transition and the release of cytochrome C from the mitochondrial intermembrane space. This triggers the activation of the apoptosome and initiates caspase-mediated apoptosis [56, 62, 63]. Autophagy plays a crucial role in maintaining mitochondrial function during HI injury, helping to avoid cellular damage by removing damaged mitochondria and promoting cell survival. Activation of autophagy has been noted following cerebral ischemia [64].

Disruption of mitochondrial quality control after vascular and oxygen impairment may result in NVU dysfunction such as neuronal death, endothelial injury, neuroglial response, BBB breakdown and neuroinflammation [65, 66]. Furthermore, oxidative stress induces pro-inflammatory polarization of neuroglia, neuronal apoptosis and endothelial damage [67]. Excessive mitochondrial fission triggers amplification of the inflammatory cascade, leading to the activation of hyperactive microglia and astrocytes.

Mitochondrial dysregulation is key factor in HI events, and there is considerable evidence supporting the existence of basic differences in brain metabolism between the sexes and thus in mitochondrial function [42].

Sex-dependent mitochondrial pathways in HIE

Mitochondrial energy metabolism

During HI brain injury, oxygen restriction leads to depression of mitochondrial respiration and consequently ATP depletion [68–70]. The early recovery period in the immature rat is characterized by an incomplete restitution of ATP and phosphocreatine, and increased levels of 2-deoxyglucose and lactate [71]. In addition, a compensatory increase of anaerobic glucose cycling to

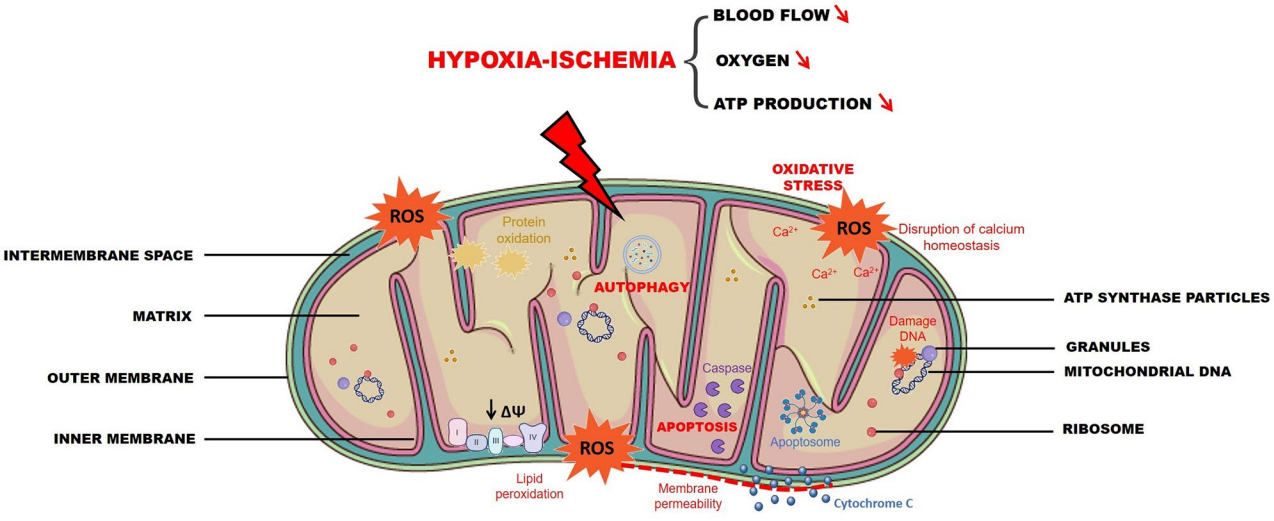

Fig. 1 Mitochondrial dysregulation during HI events. Mitochondria play a crucial role in HI, injury with production of ROS species, apoptosis, increased membrane permeability, lipid peroxidation, autophagy, protein oxidation and disruption of calcium homeostasis. Created using BioRender.com and SMART Servier Medical Art

lactate is observed in this early recovery period [71, 72]. This shift of the brain's energy production toward anaerobic glycolysis reflects mitochondrial dysfunction, altered metabolic pathways and a high metabolic demand.

Weis et al. investigated the impact of HI injury on respiratory complexes in the hippocampus and cerebral cortex of male and female rats [73]. The activity of the complexes was evaluated 2 and 18 h after HI injury and there was pronounced inhibition of complex I-III, II and IV activities in all groups 18 h after HI events. However, complex II activity was greater in females subjected to HI injury than in males. This higher complex II activity could be due to the protective effect of estrogen [74], which maintains oxidative phosphorylation in the face of compromising stress. Estrogens are involved in mitochondrial biogenesis, with an increase in mtDNA copy number after 24, 48 and 72 h [75], and an increase in the number and size of mitochondria in pregnant bonnet monkeys, an effect believed to be caused by elevated E2 [76]. Sexual differences significantly influence mitochondrial function, ATP production and responses to metabolic substrates such as ketone bodies. Ketone bodies are primarily produced in the mitochondrial of liver cells and can be used to produce ATP [77]. Differences between genders in the metabolic response to fasting have also been noted in humans. During short-term fasting, the rise in plasma fatty acids and ketone body levels [78–80], along with the reduction in plasma glucose concentration [81, 82], tends to be more pronounced in women compared to men. These data suggest an advantageous metabolic profile for women during diminished glucose blood levels. Women have a greater ability to utilize fatty acids and ketone bodies more efficiently and are better able to maintain ATP production during periods of glucose depletion than men.

Demarest et al. found that inhibition of respiratory chain activity is associated with a decrease in mitochondrial mass in female but not male rats and with depolarized mitochondrial membranes in both sexes [83]. They confirmed that brain mitochondrial electron transport chain impairment is greater in males than females after HI events, but found an increase in complex I-dependent proton leak (state 4 respiration) in female animals. This increase in state 4 respiration in females could be a compensatory response to decreased ROS production in females or increased uncoupling proteins [83].

Overall, these data suggest greater brain mitochondrial electron transport chain impairment in males than females after HI events resulting in energy failure, increased ROS production, neuroinflammation, mitochondrial-mediated cell death, calcium dysregulation and long-term cognitive and neurological deficits.

Oxidative stress

The production and detoxification of ROS/RNS (reactive nitrogen species) are crucial in both normal cellular function and CNS injury recovery, with emerging evidence indicating sex differences. Studies show higher ROS damage in males measured by F2-isoprostane levels, both in human preterm twins and adults with traumatic brain injury [84, 85]. Animal research indicates sex-dependent oxidative damage with higher levels and activity of mitochondrial antioxidant enzymes in females than in males [86–88]. These differences may be due to the neuroprotective effect of female hormones. This suggests that females are more resilient to ROS/RNS injury due to stronger antioxidant defenses, while males might benefit more from antioxidant treatments after CNS injury. This neuroprotection by female hormones is also found in the immature brain. For instance, 17-β estradiol protects the immature rat brain against oxidative stress and neurodegeneration [89] and after chronic hypoxia exposure [90].

Mitochondria are a primary source of cellular ROS/RNS production. Under pathological conditions, elevated levels of ROS/RNS can damage proteins, lipids, and nucleic acids, which need to be repaired to meet cellular energy requirements and ensure cell survival. It is hypothesized that nitric oxide synthase (NOS) induction through calcium influx serves as a key regulator of cellular energy demand. Nitric oxide (NO), produced by NOS, is freely diffusible and competes with oxygen at complex IV, temporarily inhibiting the rate of oxidative phosphorylation [91, 92]. Interestingly, the induction of neuronal NOS (nNOS) is higher in male animals after cerebral ischemic injury [93, 94]. Pharmacologically inhibiting or genetically knocking out nNOS provides neuroprotection in male mice, but increases infarct sizes in female mice [93]. This implies that NO production after injury plays a beneficial role in females. However, it is uncertain whether this benefit arises from vasodilation and the restoration of cerebral blood flow or from another mechanism.

The antioxidant enzyme glutathione peroxidase GPx is considered the most crucial enzyme in neurons and glia for detoxifying hydrogen peroxide (H_20_2). Borras et al. showed that mitochondria from females produce approximately half the amount of H_2O_2 in both synaptic and non-synaptic brain mitochondria compared to males. Female brain mitochondria also exhibit four times less oxidative damage to mitochondrial DNA compared to males [88]. Demarest et al. [83] demonstrated a deficit in GPx antioxidant activity and protein oxidation at 20–24 h at the beginning of secondary HI injury in males. Complex I is known to produce ROS following HI injury [95] in males and these discrepancies in mitochondrial respiration may indicate sex differences in the production of ROS following HI events.

Morken et al. reported lower astrocytic mitochondrial metabolism in male rat pups, whereas that of females was reduced longer and encompassed both neurons and astrocytes following HI injury [96]. This diminution of astrocytic mitochondrial metabolism could lead to deep perturbation of maintenance of neuronal function and support of neuronal viability from astrocytes in males, and indicates sexual differences in mitochondrial metabolism [97]. Overall, Morken et al. found differential alterations in oxidative stress in female and male rats using magnetic resonance spectroscopy following HI injury.

Another study reported that genes related to oxidative stress pathways were significantly upregulated in male but not in female rats [98]. An X chromosome-linked inhibitor of apoptosis (XIAP) has been shown to reduce oxidative stress following HI events in mice, especially females [99], suggesting a genetic advantage in managing oxidative stress in females leading to sex-based differences in HI injury outcomes.

To conclude, males are more vulnerable to oxidative stress with higher production of ROS, greater mitochondria permeability increase leading to more release of mitochondrial proteins such as cytochrome C, apoptosis-inducing factor (AIF), EndoG and Smac/Diablo which activate a cascade of signaling events [100]. These factors contribute to worsened outcomes in males compared with females, with greater neuronal damage and long-term deficits in brain function.

Mitochondrial-mediated apoptosis

Apoptosis is a form of programmed cell death crucial to the development and homeostasis of cells. One of the most extensively researched areas in sexually dimorphic neurotrauma and neuroprotection is the difference in cell death pathways between sexes. There are sexual differences in the apoptotic cascade; two pathways may be preferentially activated [62]. The first pathway, the caspase-dependent pathway, comes into play following increase of nNOS activation and involves APAF-1 and the formation of an apoptosome which binds with caspases (3, 6, 7 and 9) leading to chromatin condensation and DNA fragmentation. This caspase-dependent pathway tends to predominate in females [101–103]. The other pathway, the caspase-independent pathway, involves reduction in NAD and activation of ADP-ribose and PARP-1 with release of AIF and endonuclease G and eventually cell death [104, 105]. This second pathway seems to predominate in males [101]. Several studies indicate that when caspase activation is similar in both sexes, females might be more resistant to caspase-dependent cell death compared to males [106, 107].

During HI brain injury, the AIF-mediated caspase3-independent cell death pathway is more prominent in males, whereas in females caspase3-dependent cell death is more pronounced. Li et al. have shown that AIF upregulation significantly worsens HI brain injury in neonatal male mice compared to female mice [108]. This sex difference is associated with activation of apoptotic cell death and increased neuronal cell death.

Male rats tend to have more caspases activated and this can be associated with a greater apoptotic event and thus a vulnerability profile in males with early brain damage [109].

Cytochrome C release from mitochondria is an initiating step in the apoptotic process. The pattern of cytochrome C release is dimorphic. In response to HI injury, female neurons display a moderate and sustained cytochrome C release associated with greater cleavage and activation of caspase 3 leading to apoptosis [102, 110]. After activation, caspase-3 is translocated into the nucleus to cleave PARP-1 leading to caspase-dependent cell death. Interaction between PARP-1 and estrogen receptor-α (ER-α) may decrease such activation [111]. Zhang et al. investigated sexual differences in neuronal survival and transduction signaling in male and female rat primary cortical neuronal cultures. They found higher levels of phosphor-ERK1 and AKT in female cortical cultures, demonstrating enhanced neuronal survival or decreased apoptosis in the female rat brain [112].

Another explanation could be X-linked endogenous apoptotic inhibitors with advantageous genetic inheritance for females after HI injury. As a typical example, neuronal XIAP overexpression led to significant neuroprotection from pathological caspase activation and tissue loss in the mouse neonatal brain after HI injury [99, 113]. Also, the inhibitor of apoptosis protein (IAP) in females may be profitable during HI events, leading to less severe cerebral damage than in males [114]. The IAP family are potent inhibitors, which can bind to caspase-9, thereby hampering cell death.

Autophagy

Autophagy is a cellular degradation and recycling process and can be activated in response to distinct challenges such as survival-promoting removal of pathogens, degradation of damaged proteins and organelles, or programmed cell death.

Sex differences in autophagy have been observed in both in vitro and in vivo models. For instance in a study published in 2013, after cardiac ischemia, male, but not female, rats showed significantly reduced levels of anti-apoptotic protein Bcl 2. In addition, pro-apoptotic protein Bax was markedly reduced in female rats but not in males [115]. Weis et al. demonstrated that autophagy pathways show a region- and sex-specific pattern in the rat brain following neonatal HI injury [116]. *A* significant study on sex differences in autophagy revealed fundamental differences in neuronal cultures following

nutrient deprivation [117]. The study found decreased viability in XY cells, along with increased LC3-II protein levels, compared to XX cells. Additionally, XX neurons exhibited phospholipase A2-mediated increases in lipid droplet formation after nutrient deprivation, which was not observed in XY neurons. This suggests that autophagy may be more harmful to XY cells under stress, while the relative resilience of XX cells to nutrient deprivation may be due to their enhanced ability to synthesize and use free fatty acids as alternative energy sources.

Grischuk et al. provide evidence of sex differences in autophagy after ischemic stroke in a mouse model [118]. Five key autophagy proteins, P62, LC3, Atg7, beclin1 and Pulk1, show differential expression in response to cerebral ischemia in male and female mice. 24 h after a stroke event, males showed a significant increase in Beclin1 and LC3-II and a decrease in p62, suggesting stimulation of autophagy, whereas levels in females were decreased compared to sex male shams. Beclin1-independent autophagy is mediated by Atg7 and leads to caspase-dependent apoptosis [118] which could be a pathway utilized by female mice.

Consistently, Acaz-Fonseca et al. [119, 120] found that Ucp-2 and HIF1α were upregulated in male rats after stroke compared to females. These upregulations of genes were associated with enhanced levels of LC3-II after stroke only in male rats.

Deprivation of nutrients and oxygen is a common mechanism in HI injury and in starvation. Sex differences in adaptation to famine have been investigated and can give insight into male vulnerability in the context of HI events. During starvation, neurons from males more readily undergo autophagy and die, while neurons from females mobilize fatty acids, accumulate triglycerides, form lipid droplets and survive longer [117]. These findings support the notion of male vulnerability in HI injury, with increased autophagy processing and reduced neuronal survival, compared to females.

Further research into the roles and mechanisms of autophagy therapy in HI events could lead to the development of effective preventive and therapeutic strategies targeting autophagy [121]. All these studies, mostly done on rodents, demonstrate sex-associated factors in HIE at the mitochondrial level, including energy metabolism, oxidative stress, apoptosis and autophagy (Fig. 2; Table 1).

Modeling mitochondrial dysfunction in brain organoids

Brain organoids have shown great potential in recapitulating many aspects of in vivo models [123], including modeling sex-related differences using right extrinsic signals during differentiation [124–126]. However, the ability of organoids to recapitulate sex-dependent mitochondrial responses observed in in vivo models is still an emerging area of research. Mitochondrial function in brain organoids can mimic certain aspects of mitochondrial biology seen in vivo, however, the study of sex-specific differences may depend on factors such as the sex of the cells used, culture conditions including hormonal influence (estrogen, testosterone), and the use of appropriate cell types that reflect sex-dependent interactions (neurons, astrocytes).

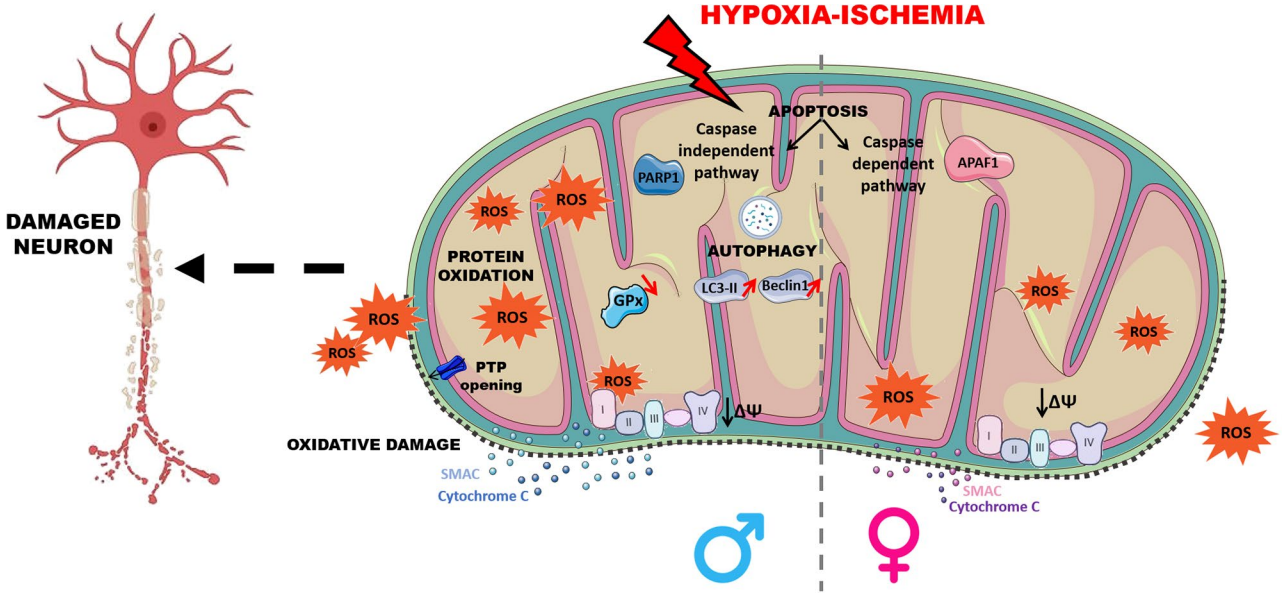

Fig. 2 Sex differences in mitochondria signaling pathways in the context of HI injury. Evidence of sex differences in mitochondrial dysfunction regarding oxidative damage, autophagy, protein oxidation and apoptosis pathways. Inspired by [122] and created using BioRender.com and SMART Servier Medical Art

Table 1 Comparative table on sex-dependent mitochondrial pathways

PATHWAYS	MALE	FEMALE	REFERENCE
MITOCHONDRIAL ENERGY METABOLIMS	Pronounced inhibition of complex acivities especially for complex II from mitochondrial reSpiratory complex and no decrease of mitochondrial mass.	Greater activity for complex II from mitochondrial respiratory complex and a decrease of mitochondrial mass.	[49]
OXIDATIVE STRESS	Higher ROS damage (F2-isoprostane). Higher induction of neuronal NOS after cerebral ischemic injury. GPx deficit antioxidant activity and protein oxidation after HI injury. Low astrocytic mitochondrial metabolism. Upregulation of genes involved in oxidative stress pathways.	Higher levels and activity of mitochondrial antixodiant enzymes. Less oxidative damage to mitochondrial DNA.	[50-54] [59-61] [65]
APOPTOSIS	Caspase-indenpendent pathway predominent. AIF upregulation significantly aggravates HI brain injury. More caspase activated associated with greater apoptotic event.	Caspase dependent pathways predominent. Moderate and sustained cytochrome C release. Higher levels of phospho-ERK1 and AKT leading to enhanced neuronal survival or decreased apoptosis. Interaction between PARP-1 and ER-α decrease activation of caspase-3. X-linked endogenous IAP.	[40] [74-81]
AUTOPHAGY	Reduced levels of anti-apoptotic protein Bcl2 after cardiac ischemia. Decreased cellular viability with increased LC3-II protein levels after nutrient deprivation. After stroke event, significant increase in Beclin1, LC3-II and decrease in p62. Upregulation of Ucp-2 and HIF1α associated with enhanced level of LC3-II after stroke.	Better basal autophagy activity. Pro-apoptotic protein Bax reduced after cardiac ischemia. After nutrient deprivation, neurons exhibited phospholipases A2-mediated which increases lipid droplet formation leading to enhance ability to synthetize and use free fatty acids as alternative energy sources	[82-87]

ROS (reactive oxygen species); NOS (nitric oxide synthase); GPx (glutathione peroxidase); HI (hypoxic-ischemic); AIF (apoptosis-inducing factor); ER-α (estrogen receptor-α); IAP (inhibitor of apoptosis protein)

CORRECTIONS DANS LA TABLEAU
METABOLIMS => METABOLISM
acivities => activities
reSpiratory => respiratory
indenpendent => independent
antixodiant => antioxidant
predominent => predominant
phospholipase A2-mediated which increases liquid droplet => phospholipase A2-mediated increases in liquid droplet
leading to enhance ability => leading to enhanced ability
synthetize => synthesize

The use of brain organoids as a promising tool allows investigation of mitochondrial health and dysfunction in specific brain diseases [127]. In 2021, Duong et al. reported deep analysis of mitochondrial health, including genetics, function and morphology, using a functional human-derived cerebral organoid model [128]. Pamies et al. investigated the neurotoxicity of some chemicals on mitochondrial health linked to the increased incidence of autism, attention deficit and hyperactivity disorders using a multicellular human brain spheroid model [129]. Inak et al. used cerebral organoids from iPSC affected by mutations in the mitochondrial complex IV *SURF1* and recapitulated neurogenesis defects and a failure in the development of mature neurons [130]. They reported a link between mitochondrial function and a common neurodegenerative disease by using organoids generated from *PITRM1*-knockout hiPSCs [131]. Srikanth et al. evaluated the effect of an isogenic *DISC1* mutation and its role in neuropsychiatric disease, in a cerebral organoid model, by examining morphology and gene expression [132]. In their 2020 study, Winanto et al. uses spinal cord organoids to investigate the contribution of motor neuron pathology in the context of mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) [133]. Pacitti and Bax established a novel in vitro model of mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) using cerebral organoids [134].

Overall, brain organoids offer a valuable model for studying sex differences (sex chromosomes or hormonal influence) with a focus on mitochondrial dysfunction in the context of HI injury.

To model HI injury in human brain organoids, cells can be exposed to low oxygen and glucose conditions for different times of incubation. Some studies have induced hypoxic injury in human brain organoids by using 1% oxygen for 48 h, 3% and 5% oxygen for 24 h or 1% and 8% oxygen for several days [135]. Oxygen glucose deprivation (OGD) of human neural organoids resulted in severe, permanent damage, even with a reoxygenation step [136]. By modulating the percentage of oxygen to which brain organoids are exposed, but also using glucose deprivation in cell culture media, the percentage of oxygen can be associated with the severity of HI injury resulting in different outcomes correlated with the parameters of oxygen and glucose deprivation protocol. The extent of mitochondrial dysfunction such as energy failure could be correlated with HI severity and is a key

determinant of the intensity of cellular injury and tissue recovery [137]. Currently, there is no study on deep cellular mechanisms of mitochondria dysregulation using brain organoid technology in the context of HI injury. Additional research is required to explore the detailed mechanisms of mitochondrial dysregulation during HI events, particularly in brain organoid models. A brain organoid model could be useful for evaluation of mitochondrial biogenesis, mitophagy or oxidative stress and for analysis of the link between OGD and mitochondrial dysfunction.

Deciphering sexual dimorphism by modeling mitochondrial dysfunction using brain organoids

There are two main sources of sexual differentiation: cell-intrinsic chromosomal complement and circulating sex hormones [120]. Brain organoids have the potential to improve our understanding of both aspects by studying sex-related cellular/molecular mechanisms in the context of HI injury.

It is possible to generate female and male human brain organoids using genetics or sex hormones and to study sexual differences associated with HI injury. The impact of sex hormones on brain development has mostly been studied in mice [138]. However, brain sexualization differs between rodents and humans and, furthermore, studies in nonhuman primates have shown divergent mechanisms [139]. Exposure to androgens has been studied in 3D brain organoids in an attempt to understand morphological differences between human males and females. The upshot is that increased neuron numbers in both female and male brain organoids impact brain size [140]. Males have on average a larger brain than females [141–143]. However, the mechanisms underlying this discrepancy remain to be elucidated. Kelava et al. used brain organoids to demonstrate that the sex chromosomal complement has no effect on neurogenesis, but that androgens, which are sex steroids, increase proliferation of cortical progenitors and the neurogenic pool [140]. These findings showed that brain organoids are a relevant model for studying the origin of sex-related brain differences in humans. This study demonstrates how early hormonal influence impacts brain development and might have long-term effects on sex-specific conditions.

Brain organoids offer an innovative tool for preclinical development of pharmacological drugs [135]. Some preclinical studies targeting mitochondria by using pharmacological agents or genetic modification have demonstrated neuroprotective effects in HI injury [144–147]. However, these in vivo and in vitro preclinical studies did not evaluate potentially different responses in females and males. Targeting mitochondria and tailoring pharmacological drugs according to the sex of neonates could be a promising approach in the context of HI injury. At the core of personalized medicine, it is essential to understand sex differences in drug disposition and response to improve pharmacological drugs.

Concluding remarks and future perspectives

HIE is a brain disease characterized by deprivation in oxygen and blood flow during the newborn period. This leading pediatric neurological condition features sex-related differences, with a vulnerability pattern for male compared to female neonates. These discrepancies can be explained by several factors, such as sex hormones, the X chromosome, the apoptotic cascade and immunity. There is evidence of sex-associated differences in mitochondrial dysfunction affecting metabolism, oxidative stress, apoptosis and autophagy in the context of HI injury. Mitochondria and neuroinflammation are key contributors to HI injury. For instance, the opening of the mPTP leads to the release of accumulated ROS and Ca^{2+} which damage biological macromolecules and activate inflammatory responses which promote neuronal damage [148]. There is a deep cross-talk between mitochondrial stress and neuroinflammation, and activation of microglia, which collectively exacerbates neuronal injury. Key proteins have been found to be central in mitochondrial stress and show potential as promising targets for regulating neuroinflammation during stroke [149]. Furthermore, the pathological signaling triggered by various mitochondrial damage-associated molecular pattern molecules (DAMPs) released from the mitochondria of damaged or stressed cells during HI events intensifies inflammation across different tissue types [150].

Mitochondrial DNA is particularly susceptible to free radicals due primarily to its proximity to ROS [151] and may be damaged and involved in the pathophysiology of HI conditions [152]. Damaged mitochondria release mitochondrial DNA into the extracellular space, where it can function as DAMPs and trigger inflammation [56]. Sharma et al. study found sex differences in mtDNA content in response to oxygen glucose deprivation/Reox, with a significant increase in mtDNA for XY primary cerebellar neurons (CGN) at 2 h of OGD compared to XX CGN [153].

Targeting the mitochondria would be an innovative approach to neuroprotection in HI injury. Mitochondria-targeted antioxidants could be a valuable way of reducing oxidative stress, a major pathway in neuronal injury during HI events. Nie et al. evaluated the effect of N-acetylcysteine (NAC) in combination with hypothermia in male and female neonatal rats after severe HI injury and demonstrated differential benefits for males versus females. Only females treated with hypothermia + NAC 50 mg/kg showed improvement in the short-term infarct volume [154]. Ganguly et al. investigated the effect of the antioxidant MitoQ on placental and fetal oxidative

stress in a rat model of prenatal hypoxia. MitoQ treatment was more effective in placentae from females than males [155]. Metabolic modulators are a promising therapy for the treatment of HI injury. For instance, nicotinamide (NAD) supplementation prevents ATP depletion and cerebral ischemia in male rats [156], but also reduces infarct volume in female rats [157]. There are many articles demonstrating the neuroprotective role of ketone bodies and a ketogenic diet in HI in vivo models, however they do not include sex-specific responses to this treatment [158–160]. Other relevant approaches may be used to attenuate HI brain lesions, such as mitochondrial transplantation [161–163], stem cell transplant [164, 165] or gene therapy [166], and these should be investigated in both sexes.

Using new relevant tools such as human brain organoids, it should be possible to evaluate this new therapeutic approach to this brain disease, including sex-related differences.

Refining organoid models to more accurately replicate the specific conditions of HI injury involves the fine-tuning of oxygen and nutrient supply to the organoids or the creation of a more controlled hypoxic environment to mimic the ischemic conditions observed during HI events. Integrating specific mitochondrial markers, such as reporter genes, into 3D models could help track mitochondrial dysfunction in HI injury in real time [167]. Since mitochondrial dysfunction can affect various cerebral cell types, a more complex model and longer culture period would offer insights into how glial cells interact with neurons during mitochondrial impairment in HI injury. Improved vascularization in brain organoids will be important since ischemia involves disruption of blood flow [135]. This could involve the use of endothelial cells to promote the formation of blood vessels and vessel-like structures within organoids [168]. Genetic engineering using CRISPR-based gene editing could be a valuable way to study the impact of mitochondrial mutations in the context of HI injury in human brain organoids and sexual dimorphism [169, 170]. CRISPR-based gene editing could also be used for gene therapy of mitochondrial dysfunction in HI injury [171, 172].

Taking into account this gender dimension in the research content will improve the scientific quality and relevance of the results [173]. Considering sex as a biological variable in scientific studies is required to promote rigorous, reproducible and responsible biomedical research and to improve the generalizability and translational potential of preclinical findings for clinical discovery [174]. There is a need for sex-stratified clinical trials in HIE to account for sex-related differences, as males and females differ in their response to drug treatment [175], brain injury and long-term outcomes. Different therapeutic strategies might work differently, such as antioxidants [154, 155], erythropoietin [176], caffeine [177] and therapeutic hypothermia [178].

Mitochondrial biomarkers such as the oxidative stress biomarkers inducible nitric oxide synthase (iNOS), IL-6 and NADPH oxidase 2 (NOX2) could be used for early diagnosis or therapeutic monitoring of HI injury [179]. As women and men differ in specific drug pharmacokinetics, pharmacodynamics, and molecular mechanisms, it is essential to take sex into consideration in studies to evaluate appropriate doses, administration, and adverse events and to establish clear therapeutic goals.

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AM was responsible for project administration, conceptualization, funding acquisition and editing of the manuscript. RGB was responsible for writing the manuscript. CLD and HH were responsible for editing of the manuscript. All authors approved the submitted version.
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Data availability
All data are available on the PubMed website.

Declarations

Ethics approval and consent to participate
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Consent for publication
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