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Targeting mitochondrial metabolism in neuroinflammation: towards a therapy for progressive multiple sclerosis?

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Abstract:	The lack of effective treatment options for chronic neurological conditions, such as multiple sclerosis, highlights the need to re-evaluate disease pathophysiology in the process of identifying novel therapeutic targets. The persistent activation of mononuclear phagocytes is one of the major drivers of neurodegeneration and it sustains central nervous system damage. Mitochondrial metabolism influences the activity of mononuclear phagocytes and the metabolites that they produce play key signalling roles in inflammation. However, how changes in immune cell metabolism sustain a chronic state of neuroinflammation is not fully understood. We hypothesise that novel molecular and cellular therapies for chronic neuroinflammation should target mitochondrial metabolism in innate immune cells to prevent secondary neurological damage and the accumulation of irreversible disability in patients.

Highlights

- Mononuclear phagocytes (MPs) - including resident microglia and blood-borne macrophages - control local immune surveillance in the central nervous system.
- In chronic neurological conditions, such as multiple sclerosis (MS), MPs are persistently activated and responsible for several maladaptive responses that include neurotoxicity and inhibition of remyelination.
- Cell metabolism guides the activation of MPs towards a pro-inflammatory or anti-inflammatory phenotype and function, depending on the local microenvironment.
- Specific metabolites act as intracellular and extracellular signalling molecules regulating neuroimmune interactions and inflammation.
- Neural Stem Cells (NSCs) interfere with MPs metabolism via selective uptake of specific metabolites and secretion of metabolic enzymes packed into extracellular vesicles.

Targeting mitochondrial metabolism in neuroinflammation: towards a therapy for progressive multiple sclerosis?

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Abstract

The lack of effective treatment options for chronic neurological conditions, such as multiple sclerosis, highlights the need to re-evaluate disease pathophysiology in the process of identifying novel therapeutic targets. The persistent activation of mononuclear phagocytes is one of the major drivers of neurodegeneration and it sustains central nervous system damage. Mitochondrial metabolism influences the activity of mononuclear phagocytes and the metabolites that they produce play key signalling roles in inflammation. However, how changes in immune cell metabolism sustain a chronic state of neuroinflammation is not fully understood. We hypothesise that novel molecular and cellular therapies for chronic neuroinflammation should target mitochondrial metabolism in innate immune cells to prevent secondary neurological damage and the accumulation of irreversible disability in patients.

The cellular biology of the chronically inflamed brain

The central nervous system (CNS) has long been considered an immune-privileged or immunologically unique organ because of the absence of a route to the lymph nodes for the antigen-presenting cells that reside in the parenchyma and perivascular spaces of the healthy CNS. Furthermore, the CNS generally lacks a cell-mediated response to instilled antigens, and a robust response to intraparenchymal CNS antigens can be elicited by peripheral immunization [1]. However, the presence of a functional **meningeal lymphatic system** in the CNS suggests that current dogmas regarding brain tolerance and the immune privilege of the brain should be revisited [2], and highlights a very close relationship between the CNS and immune system in both health and disease [3, 4].

In physiological conditions, the CNS is continuously guarded by resident microglia and blood-borne immune cells such as macrophages, dendritic cells and T cells (including regulatory T cells) that detect damaging agents that would disrupt homeostasis and optimal functioning [5]. Among the sentinels that patrol the CNS, those called **mononuclear phagocytes (MPs)** [6] are in charge of mediating local immune surveillance by residing within specific anatomical niches [1]. These resident MPs include both microglia, which constitute 5–10% of total brain cells and are the only true CNS parenchymal MPs [7], and macrophages, which reside in the perivascular spaces, the leptomeninges and the choroid plexus [8].

The activation of CNS resident MPs, and especially that of microglial cells, is a salient feature of the neuroinflammation that is prominent in almost all neurodegenerative diseases, including Alzheimer's disease (AD) (**Box 1**), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) [9, 10]. **Multiple Sclerosis (MS)** is classically defined as a secondary neurodegenerative disease, as neuronal death occurs secondarily to a type of myelin loss evolving on a background of inflammation, consisting of T lymphocytes, B lymphocytes, and plasma cells [11, 12]. Further, in MS, the local activation of resident MPs is coupled with the recruitment of blood-circulating MPs to areas of CNS and blood brain barrier damage (**Box 2**).

Resident and CNS-infiltrating MPs play different roles and contribute to MS differently during the course of disease [13]. In **Relapsing Remitting (RR) MS**, active lesions predominate and the most common lesion types (patterns I and II) [14] show a diffuse perivascular and parenchymal T cell infiltration and a background of MPs. MPs are also present in the **normal appearing white matter (NAWM)** and in

both cases they express pro-inflammatory markers, including CD40, CD86, CD64 and CD32 [15], while downregulating the expression of homeostatic markers, such as P2RY12 [16]. Late in the disease, correlative evidence exists that the immune response progressively shifts from adaptive to innate, with the activation of MPs increased in patients with progressive MS and higher clinical disability [17]. In **progressive MS**, MPs are present in both subpial cortical lesions and in **smouldering plaques** [18]. Subpial cortical lesions are characterised by demyelination of the superficial cortex with sparse MPs in association with inflammation in the overlying leptomeninges. Smouldering plaques are frequently and almost exclusively found in progressive MS [18], and represent the slow expansion of pre-existing lesions now characterised by accumulation of inflammatory MPs leading to active demyelination [16, 19]. In progressive MS, the extent of MP activation is further increased by retrograde neurodegeneration after axonal damage in white matter lesions [20, 21].

Mechanistically, the release of mediators of inflammation by either resident or infiltrating pro-inflammatory MPs triggers the activation of additional microglial cells and causes damage to essential components of mitochondria in neurons, such as mtDNA and enzymes of the mitochondrial respiratory chain. This worsens the unsteady balance between the energy demands and supply, thus inducing a chronic state of *virtual hypoxia* in chronically demyelinated axons [22]. Pro-inflammatory MPs also impair glutamate transport in astrocytes, which in turn further increases the extent of direct neuronal and oligodendrocyte excitotoxicity [23]. Consequently, active demyelination and neurodegeneration are clearly associated with a dominant pro-inflammatory phenotype of MPs.

Immune processes and cell metabolism are conserved biological functions linked to diseases that span multiple areas of medicine. Recent evidence suggests that metabolic changes in innate immune responses influence both the effector phase as well as the resolution of inflammation by modulating immune cell fate and function [24]. As such, alterations of certain metabolic configurations of immune cells might contribute to dysfunctional immune responses, a typical feature of autoimmunity [24]. Unfortunately, how the innate immune system is organised in progressive MS and which role cell metabolism plays in MP function during chronic neuroinflammation are outstanding questions that still need to be answered.

Here we anticipate that the changes in the metabolism of macrophages and microglia play a major role in the maintenance of the persistent state of neuroinflammation that contributes to disease progression in MS.

We believe that identifying the metabolic determinants of such a steady activation of CNS-compartmentalised innate immune responses will help pinpoint a range of druggable metabolic pathways through which to interfere with neuroinflammation, reducing concomitant neurodegeneration and accumulation of irreversible disabilities in MS patients [25].

The role of cell metabolism in innate immune activation

In MPs, cellular polarisation has been traditionally categorised as having either *toxic* (M1-like, classically-activated, pro-inflammatory) or *protective* (M2-like, alternatively-activated, anti-inflammatory) phenotype and function [26]. This first classification was in part based on one important metabolic feature: M1 macrophages produce nitric oxide (NO), whereas M2 macrophages produce polyamines [27]. On the one hand, the nitric oxide synthase (NOS) family of enzymes catalyse the conversion of L-arginine to L-citrulline plus toxic NO in M1-like macrophages [28]. On the other hand, the arginases convert L-arginine into L-ornithine and urea in M2-like macrophages [29]. Despite the attractive simplicity of this dichotomy, accumulating evidence suggests that MPs polarisation is multidimensional, with an overlap in gene expression and metabolism after pro- or anti-inflammatory stimuli, as opposed to occurring on a simplified linear spectrum [30]. Nonetheless, these initial studies have highlighted a key concept in MPs biology: the link between MP activation and metabolism [31].

Many of the current lines of evidence investigating how metabolism affects the pro-inflammatory status of MPs come from *in vitro* experiments on mouse bone marrow derived macrophages (BMDM) challenged with lipopolysaccharides (LPS). These seminal observations have uncovered that pro-inflammatory MPs, differently from anti-inflammatory MPs (**Box 3**), are characterised by a switch to **aerobic glycolysis,** similar to the Warburg effect in cancer cells [32], upregulation of the **pentose phosphate pathway (PPP)** and increased **fatty acid synthesis (FAS)** due to the existence of enzymatic break points in the **tricarboxylic acid (TCA) cycle** (**Figure 1**).

Switching cell metabolism towards aerobic glycolysis in MPs begins with an increased glucose uptake, mediated by the upregulation of glucose transporter type 1 (GLUT1), which enables the efficient uptake of glucose in an environment where nutrients are restricted by inflammation [33]. Glucose is used in the glycolytic pathway to produce adenosine triphosphate (ATP) and pyruvate. Key regulators of these changes are the protein kinase mammalian target of rapamycin (mTOR) and the transcription factors Myc and hypoxia-inducible factor (HIF)-1α, which induces a glycolytic gene expression profile [34]. Pyruvate is then used to produce lactate via lactate dehydrogenase (LDH), which is then released in high concentrations in the extracellular environment.

Along with glycolysis, the PPP is induced in pro-inflammatory MPs. Glucose-6-phosphate (G6P) is used to provide 3-phosphoglycerate for the serine biosynthetic pathway (required for the synthesis of amino acids for cytokines), and to generate ribose 5-phosphate for the synthesis of nucleotides and nicotinamide adenine dinucleotide phosphate (NADPH) [35]. These changes are partially dependent on the inhibition of the carbohydrate kinase-like protein (CARKL), a key enzyme that bridges glycolysis and the PPP [36]. NADPH is an essential cofactor for the production of reactive oxygen species (ROS) by NADPH oxidases, for inducible nitric oxide synthase (iNOS), and for the production of the antioxidant glutathione, which protects the cell against ROS-mediated damage [35]. The inhibition or deletion of NADPH oxidase is sufficient to switch microglial activation from a pro-inflammatory to an anti-inflammatory state [37].

In pro-inflammatory mouse BMDMs, TCA cycle breaks appear at two major points: one at the level of succinate dehydrogenase (SDH), which catalyses the oxidation of succinate to fumarate, and another at the level of isocitrate dehydrogenase (IDH), the enzyme that converts isocitrate to α-ketoglutarate (αKG). These breaks lead to the accumulation of specific metabolites (e.g. succinate, malate, citrate and itaconate), and to the reduction several others (e.g. fumarate and αKG) [31, 38].

The main source of succinate accumulation in pro-inflammatory MPs appears to be glutamine. LPS-activated MPs increase the uptake of glutamine and initiate glutaminolysis to replenish αKG in the TCA cycle and maintain global metabolic flux [32]; αKG can be then converted into succinate within the TCA cycle [39]. Succinate

is also produced from succinic semialdehyde through the enzyme succinate semialdehyde dehydrogenase, an intermediate reaction of the gamma-aminobutyric acid (GABA)-shunt [40].

The malfunction of the SDH increases succinate concentrations by blocking its conversion to fumarate. Nonetheless, a large increase in malate is also seen, which is dependent on an enhanced arginosuccinate shunt that feeds into fumarate and malate (linking the urea and the TCA cycles) [38]. Pro-inflammatory macrophages use the arginosuccinate shunt because of the break after SDH, and inhibition of aspartate aminotransferase (AAT), a key enzyme in the shunt, suppresses NO and interleukin (IL)-6 production [38].

The progressive accumulation of succinate starts then driving the activity of the SDH, thus promoting mitochondrial ROS production from the NADH-ubiquinone oxidoreductase (complex I) via a process termed **reverse electron transport (RET)** [41]. Essentially, while in resting MPs SDH typically receives electrons from succinate and passes them on to complex III for forward **electron transport chain (ETC)** flux, in pro-inflammatory MPs electrons transfer from SDH to complex I. This depends on the presence of a high proton-motive force and increased succinate concentrations, and results in mitochondrial ROS generation. Mitochondrial ROS then stabilize HIF-1α, thereby promoting pro-inflammatory cytokine IL-1β expression.

The second break in the TCA cycle in LPS activated MPs depends on a 7-fold relative reduction of the enzyme IDH activity compared to resting MPs, which leads to the accumulation of isocitrate and the upstream metabolites cis-aconitate and citrate [38]. Cis-aconitate is converted by the enzyme cis-aconitate decarboxylase 1 (CAD), coded by the *immunoresponsive gene 1* (*Irg1*), to itaconate [42, 43], which has major antimicrobial activity (e.g. by inhibiting the citrate-lyase expressed by different bacterial strains) and a key anti-inflammatory role (see **Box 4**).

Another important metabolite that increases in LPS-stimulated macrophages is malonyl-coenzyme A (CoA) [44]. One of its roles is to inhibit fatty acids from associating with carnitine by regulating the enzyme carnitine acyltransferase, thereby preventing them from entering the mitochondria, where **fatty acid oxidation (FAO)** occurs [45]. Preventing FAO reduces the fuel to the TCA cycle, hence contributing to the decrease of oxidative phosphorylation. On the contrary, FAS is enhanced in pro-inflammatory MPs via different mechanisms. First, mitochondrial citrate is exported to the cytosol by the citrate carrier (CIC), where it is converted to acetyl-CoA, an

important intermediate for the synthesis of fatty acids [46]. Second, mTOR activation upregulates lipid synthesis [47] and the NADPH derived from the PPP is used as a co-factor for *de novo* FAS, ultimately favouring the expansion of the endoplasmic reticulum (ER) and Golgi to support cytokine secretion [24] (**Figure 1**).

Understanding how cell metabolism is regulated in inflammatory MPs, and which metabolic pathways are responsible for the abovementioned changes, holds the promise of unveiling novel targets to interfere with the persistent activation of microglia/macrophages in the CNS of patients with progressive MS.

Metabolites as signalling molecules in inflammation

Growing evidence suggests that metabolites are not mere intermediaries for energy production but can also shape inflammatory responses. These 'immunometabolites' function both as intracellular signalling molecules [48], as well as extracellular signals of inflammation, acting as *de facto* 'metabokines' (**Figure 2**).

Intracellular metabolite signalling depends on the ability to trigger post-translational modifications of proteins, including acetylation, malonylation, succinylation, succination (by fumarate) and glutarylation [49]. Moreover, intracellular metabolites regulate the activity of metabolic enzymes capable of binding specific mRNAs within RNA-enzyme-metabolite (REM) networks [50]. For example, the glyceraldehyde-3-phosphate (G3P) dehydrogenase (GAPDH), which normally blocks the translation of several mRNAs including the tumour necrosis factor (TNF)-α, during inflammatory activation is preferentially used as a glycolytic enzyme, thereby allowing TNF-α translation and secretion [51, 52].

Intracellular metabolites can also affect chromatin structure and function. In pro-inflammatory MPs, glycolysis is induced and its end-product lactate inhibits class II histone deacetylase (HDAC), thus increasing acetylation and regulating gene expression [53]. Conversely, higher NAD^+/NADH ratios influence the activities of the class III HDACs sirtuin 1 and 6, which use oxidized NAD^+ as a substrate for the deacetylation process, but also for the inhibition of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and protein lysine de-succinylation [54, 55]. Mitochondria and mitochondrial metabolites also play a key role in the acetylation process. Cytosolic acetyl-CoA, which is partially derived from glycolysis and glutamine, is mostly regulated by the mitochondria via the CIC.

Cytosolic acetyl-CoA is used for histone acetylation, thereby increasing the transcription of glycolytic genes such as hexokinase 2, phosphofructokinase, and LDH [56].

The balance between αKG, succinate, fumarate, and 2-hydroxyglutarate (2HG) is instead critical for the activity of αKG-dependent dioxygenases involved in histone and DNA demethylation. αKG is used as a substrate for the enzymes of the ten-eleven translocation (TET) family, which catalyse DNA demethylation and the Jumonji C (JmjC)-domain-containing histone demethylase (KDMs) [57]. These enzymes use αKG and molecular oxygen to oxidize their substrate (methyl cytosine) and reduce methylation, producing succinate and carbon dioxide as by-products. Accumulation of succinate and fumarate leads to the inhibition of DNA demethylases and KDMs via product inhibition [58, 59]. As consequence, a distinct DNA and histone hypermethylation profile is observed in SDH and fumarase hydratase-deficient tumours, where succinate and fumarate accumulate, respectively, and in IDH mutant tumours, where 2HG accumulates [58]. The effect of all these epigenetic changes is that in pro-inflammatory BMDMs both histone methylation and histone acetylation are present at the promoter and enhancer levels, potentiating pro-inflammatory gene transcription [57]. These epigenetic modifications mediated by metabolites are extremely important for MPs polarisation [60], as well as for the acquisition of the specialised phenotype linked with trained immunity (**Box 5**).

Extracellular metabolites can also act as key signalling molecules, both at organismal level and within specific inflammatory microenvironments [61].

At organismal level, metabolites of dietary tryptophan produced by the microbiome (e.g. kynurenine) suppress the microglial expression of NF-κB dependent transcripts in an aryl hydrocarbon receptor (AHR)-dependent manner [62]. In turn, microglia-derived transforming growth factor (TGF)-α signalling limits the activity of neurotoxic A1 astrocytes in mice with **experimental autoimmune encephalomyelitis (EAE),** as a model of MS [62]. Ketone bodies, such as the beta-hydroxybutyrate (BHOB), which are produced from the liver after food deprivation [63], have alos major anti-inflammatory effects via hydrocarboxylic acid receptor 2-HCAR2 (GPR109A) signalling on mouse BMDMs and human monocytes *in vitro*, and in mouse models of NLR family pyrin domain containing 3 (NLRP3)-driven inflammation *in vivo* [64]. While also short-chain fatty acids can be sensed by both

GPR43 and GPR109A to promote inflammatory resolution and mediate anti-inflammatory effects *in vivo* in models of colitis, arthritis and asthma [65]; medium chain fatty acids act through the GPR84 to induce a pro-inflammatory response in human and rat polymorphonuclear leukocytes (PMNs) and macrophages both *in vitro* and *in vivo* [66].

Within specific microenvironments, metabolites secreted by inflammatory cells act as feed-back mechanisms of local immune regulation by affecting the duration and magnitude of the inflammatory response. For example, extracellular succinate is abundantly detected in synovial fluid from patients with rheumatoid arthritis (RA) and coronary sinus blood from patients with myocardial infarction, where its concentration correlates with the extent of damage [67]. Extracellular succinate acts on the succinate receptor SUCNR1/GPR91, a G protein–coupled cell surface sensor with pleiotropic roles in different tissues/organs [68], which plays a key functional role in mast cells [69], dendritic cells (DCs) [70], monocytes [71] and macrophages/microglia within the immune system [70, 72].

Activation of DCs via GPR91 favours their maturation and migration to lymph nodes, augments proinflammatory cytokine production in response to TLR signalling, and enhances antigen-specific activation of T helper cells [70]. GPR91 on inflammatory macrophages is a main checkpoint of a feed-forward loop of cell activation, where it senses extracellular succinate to up-regulate a HIF-1α–dependent innate pathway potentiating IL-1β production to exacerbate experimental RA in mice [71].

By contrast, several metabolites released within the inflammatory microenvironment have anti-inflammatory properties. Among these, lactate acts through the lactate receptor GPR81 to inhibit adenylyl cyclase and suppress innate immunity [73], while fumarate may influence the GPR109A/HCAR2 signalling pathway to reduce microglial activation [74] (**Figure 2**).

MS therapeutics and their effect on mitochondrial metabolism

Cell metabolism is emerging as a highly relevant therapeutic target to interfere with the activation of both macrophages [75] and microglia [76] and possibly with the accumulation of irreversible disabilities in patients with chronic neuroinflammatory diseases.

Several ways to modify MPs metabolism can be envisaged, including the development of specific therapeutics that target intracellular metabolic pathways. Intracellular inhibition of SDH in mouse BMDMs with dimethyl malonate (DMM), a cell permeable pro-drug that generates malonate, has been proven effective in decreasing IL-1β expression, while boosting the induction of the anti-inflammatory cytokine IL-10 in response to LPS *in vitro* [77]. Similarly, a new cell-permeable itaconate derivative, 4-octyl itaconate, decreases the production of inflammatory cytokines in LPS stimulated mouse BMDMs and human macrophages via activation of nuclear factor erythroid 2–related factor 2 (NRF2) [78] (see also **Box 4**). Similarly, the metabolic modulator metformin, a frontline treatment for type II diabetes mellitus acting via both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms, decreases the production of the pro-form of the inflammatory cytokine IL-1β in response to LPS in mouse BMDMs via complex I inhibition [79].

Several disease modifying treatments (DMTs) tested in MS are able to interfere with key metabolic mitochondrial pathways.

The methyl ester of fumaric acid dimethyl fumarate (DMF; *Tecfidera, Biogen Idec.*) was recommended for approval in the European Union as an orally administered disease modifying therapy in adult patients with RR MS or psoriasis by the European Medicines Agency (EMA; www.ema.europa.eu). DMF binds to Kelch-like ECH-associated protein 1 (Keap1) to enable the nuclear translocation of NRF2 [80] and mediates the transcription of anti-oxidative genes such as the hemoxygenase-1 (HO1) and the quinoline oxidoreductase-1 (NQO1) [81]. DMF is also an agonist of the GPR109A/HCAR2, a G protein-coupled membrane receptor that modulates microglial activation [74]. Recent data also suggest that the main mechanism of action of DMF in RR MS depends on the succination and inactivation of the catalytic cysteine of the glycolytic enzyme GAPDH, thereby downregulating aerobic glycolysis in activated myeloid and lymphoid cells [82].

Biotin, or vitamin B7, is a co-enzyme for three carboxylases (including the pyruvate carboxylase) of the TCA cycle in the mammalian brain involved in energy production [83] and it increases the levels of cellular ATP in conditions of virtual hypoxia [84, 85]. The results of a first double-blind, controlled, randomized phase III trial of daily oral MD1003 (*Qizenday*, Medday Pharmaceuticals; a highly concentrated formulation of biotin) described a significant reduction of progression of

neurological symptoms in >150 people with progressive MS compared with placebo treated controls [86]. A second study involved 93 patients with MS who had visual loss due to inflammation of the optic nerve. On December 2017, the EMA's Committee for Medicinal Products for Human Use (CHMP) concluded that the clinical data from these two trials enrolling> 250 patients were not sufficient to assess the effectiveness or the safety of biotin. This press release was released jointly with a letter from Medday Pharmaceuticals, the biotech company developing the pharmaceutical grade high dose biotin, notifying the EMA of the withdrawal of the marketing authorization application of *Qizenday* for the treatment of people with progressive MS. A further multi-centre, double-blind, controlled, randomized phase III trial of daily *Qizenday* is currently underway and will continue to recruit participants (>600 patients) with primary or secondary progressive MS from North America and Europe, including study centres in Edinburgh, London and Manchester. The estimated completion date for this trial is September 2019 (ClinicalTrials.gov Identifier: NCT02936037).

Lipoic acid is an antioxidant and cofactor for at least five enzyme systems. Two of these (the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase) are key enzymes of the TCA cycle. The results of a double-blind, controlled, randomized phase II/III trial of daily oral lipoic acid described a significant reduction in the annualized percent change brain volume, and suggested a clinical benefit in people with secondary progressive MS, while maintaining favourable safety, tolerability, and compliance [87].

Teriflunomide (*Aubagio, Genzyme Therapeutics*) is the active metabolite of the pyrimidine synthesis inhibitor leflunomide. It selectively and reversibly inhibits dihydro-orotate dehydrogenase, a key mitochondrial enzyme in the *de novo* pyrimidine synthesis pathway, leading to a reduction in proliferation of activated T and B lymphocytes [88]. The results of the Teriflunomide Multiple Sclerosis Oral (**TEMSO**) double-blind, controlled, randomized phase III trial described a significant reduction of progression of disability (at the higher dose) [89] in people with RR MS.

Finally, periodic fasting mimicking diet (FMD) has been proposed as an alternative approach to increase protection of multiple systems in mice and possibly humans [90]. FMD ameliorates CNS damage and behavioural outcomes in EAE mice, by increasing the corticosterone levels and regulatory T (T_{reg}) cell numbers and reducing the levels of pro-inflammatory cytokines, T_H1 and T_H17 cells, and antigen-presenting

cells (APCs) [91]. These effects are possibly related to an arrest B and T cell development and concomitant selective localisation of mature B and T cell numbers in the bone marrow seen in fasting mice [92, 93]. Preliminary results of the randomized, parallel-group, three-arm Pilot Trial to Test the Feasibility of Prolonged Fasting and Ketogenic Diet in Relapsing-remitting Multiple Sclerosis (IGEL) (ClinicalTrials.gov Identifier: NCT01538355) showed an increase in the β-hydroxybutyrate concentrations in plasma, a reduction in the number of autoimmune lymphocytes and a reduction of relapses and expanded disability status scale in the FMD and ketogenic diet groups [91].

While the evidence that all the above therapeutics for MS interact with key metabolic and mitochondrial pathways is clear, whether these therapies affect also the metabolism of inflammatory MPs in progressive MS is yet to be elucidated.

Competition for common metabolic substrates with immune cells via exogenous stem cell therapies

While the results for targeting mitochondrial metabolism in MS are exciting and metabolic modulators appear to be well tolerated and have minimal side effects, the delivery of these compounds to CNS-confined innate immune cells may be a challenge for the success of these therapies in progressive MS. In fact, the selective targeting of chronically activated MPs might be a crucial step to treat neuroinflammatory conditions in which inflammation is compartmentalised within the CNS and is coupled by neurodegeneration.

Ourselves and others have attempted to solve this key issue by investigating how new experimental cellular medicines able to target the chronically inflamed brain may be beneficial for treating neuroinflammatory conditions in animal disease models [94].

Syngeneic neural stem cells (NSCs) injected through the blood stream (intravenously, IV), or locally into the CNS via the cerebrospinal fluid (CSF) circulation (intracerebroventricularly, ICV) in rodents and non-human primates with EAE express functional cell adhesion molecules (e.g. CD44), integrins (e.g. α4β1) and chemokine receptors (e.g. CCR2, CX3CR1, CXCR4) to enter the inflamed CNS and selectively accumulate around perivascular spaces where inflammatory cells co-reside [95]. By establishing a wide range of cell-to-cell interactions, transplanted NSCs are able to (i) induce the apoptosis of Th1 and Th17 T cells through Fas ligand

(FasL), TNF-related apoptosis-inducing ligand (TRAIL) and Apo-3 ligand (APO3L); (ii) reduce the proliferation of T cells through NO and prostaglandin E2 (PGE2); and (iii) reduce the T cell receptor (TCR) dependent activation of T cells through the inhibition of IL-2 and IL-6 signalling [95-104]. Thus, NSC grafts exert major immunomodulatory properties in neuroinflammation [105]. However, while much is known on the anti-inflammatory properties of grafted NSCs on the adaptive immune system, the molecular effect of NSCs grafts on innate immune responses in the context of chronic neuroinflammation are not yet fully understood [106] (**Figure 3**).

To this aim, we have recently developed a multidisciplinary approach that allowed the characterization of the main mechanisms of chronic neuroinflammation, and analysed the metabolic determinants of the protracted and persistent activation of mouse MPs, both *in vitro* and *in vivo* in EAE [107]. Within the same study, we also sought to understand to which extent experimental NSC therapeutics could interfere with the cellular metabolic processes responsible for chronic neuroinflammation. Our work shows that the inflammatory metabolite succinate significantly increases in the CSF - but not in the peripheral blood - of mice with late stage chronic EAE, indicating its compartmentalisation in the CNS. The intracerebroventricular transplantation of somatic or directly-induced NSCs (iNSCs) had overlapping therapeutic and anti-inflammatory effects, which correlated with a striking reduction in the number of MPs in the CNS, and reduced the amount of succinate in the CSF only [107].

We also demonstrated that transplanted NSCs were able to *'sense and respond to'* the extracellular succinate released by MPs via GPR91. In fact, the activation of GPR91 on NSCs induced the release of the anti-inflammatory PGE2 and promoted the scavenging of succinate with consequential anti-inflammatory effects. Finally, loss of *Gpr91* function in NSCs led to significantly reduced anti-inflammatory activities on pro-inflammatory BMDMs *in vitro* and *in vivo* after transplantation in EAE mice [107].

NSCs are also able to secrete extracellular vesicles (EVs) that behave as fully independent metabolic units harbouring intact and functional metabolic enzymes (e.g. asparaginase-like 1 protein) capable of depleting the microenvironment (or the intracellular compartment of a target cell) of key metabolites used for the survival or proliferation of immune cells (e.g. asparagine for T cells) [108].

Along with the above intrinsic behaviours, NSCs can also change their intrinsic metabolism (i.e. arginine) in response to priming with inflammatory Th1 cytokines

[interferon (IFN)-γ, TNF-α and IL-1β], and increase the secretion of extracellular arginase-1, which is responsible of anti-proliferative effects on lymph node cells [109]. Finally, NSC grafts also provide local paracrine support, via the *in situ* release of growth factors and cytokines [106] including TGF-β2, inhibiting the differentiation and activation of inflammatory monocyte-derived cells (MCs) in the CNS of EAE mice, thus favouring their switch toward an anti-inflammatory phenotype [110].

Part drugs and part devices, NSCs work as naturally occurring disease-modifying agents endowed with a highly refined machinery thereby (i) sensing diverse signals (including the extracellular metabolic and inflammatory milieu); (ii) migrating to specific sites in the body; (iii) integrating inputs to make decisions; and (iv) executing complex response behaviours (**Figure 3**) [111].

Altogether these exciting findings are in line with the emerging concept of short-range intercellular communication within inflammatory microenvironments guiding cellular responses and neuroimmune interactions [112].

Most of the concepts behind this novel provocative perspective of *homeostatic cell competition* at the level of the stem cell graft-host environment niche are not fully unexpected. Cell competition is a well-established model in cancer, where tumour cells consume high levels of glucose and release lactate and the tryptophan-metabolizing enzyme indoleamine 2,3-dioxygenase (IDO), to mediate local immunosuppression in the tumour microenvironment via the consumption of tryptophan and release of kynurenine [113, 114]. Nonetheless, IFN-γ-mediated induction of IDO is recognised as the sole direct mechanism of human mesenchymal stem cell (MSC)-mediated suppression of adaptive immune responses both directly on T cells, as well as through the differentiation of monocytes into M2-like immunosuppressive macrophages *in vitro* [115, 116].

Evidence linking metabolism, mitochondrial dynamics, and proteostasis as key regulators of stem cell function is emerging [117]. It is therefore not completely unrealistic to speculate that a metabolic control of stemness confers grafted cells an intrinsic capacity of interfacing with the environment [118]. This also implies that biologically flexible stem cell grafts release diverse '*batches*' of signalling molecules (e.g., short peptides, enzymes, nucleic acids, microRNAs, etc.) either secreted or packed into EVs with the potential of targeting different types of tissues; and largely accounts for the established therapeutic plasticity of a broad range of stem cell

therapeutics across several tissues [106, 119]. As such, the progressive exhaustion of NSCs within brain germinal niches, as a major component of brain aging and related diseases, is being proposed as a possible explanation of the emerging connection between metabolic dysfunction, anatomical and structural damage, depression and other cognitive and mental health problems in the chronically inflamed brain [120, 121].

The next challenge is to identify those metabolic processes and intracellular pathways playing a role in neuroinflammation and develop druggable therapeutic approaches that will recondition the chronically inflamed brain towards neuroprotection and tissue regeneration.

Concluding remarks

Here we hypothesise that characterising the metabolic determinants of CNS-compartmentalised innate immune responses has concrete chances to identify a range of therapeutic targets to interfere with the accumulation of irreversible disabilities in people with progressive MS.

We believe that this novel wave of "metabolic therapeutics" targeting the maladaptive metabolism of immune cells might help treating a range of neurological conditions in which the innate immune system becomes dysfunctional. However, important issues still need to be addressed (see **Outstanding Questions Box)** to ensure the successful application of these therapies in the clinic (see **Clinician's Corner Box**).

However, cell metabolism is highly fluid and adaptable, thus leading to several limitations intrinsic to the proposed hypothesis.

First, it is extremely hard to snapshot the metabolic processes of neuroinflammation *in vivo* without introducing experimental biases.

Second, small molecules targeting cell metabolism might be have ubiquitous effects - most of which being likely *off target* - on both neuronal and non-neuronal cells. However, one could argue that in pathological conditions in which specific cells adopt prevalent metabolic pathways (e.g. aerobic glycolysis in inflammation) the selectivity of compounds targeting these metabolic routes would be greatly increased (i.e. cellular selectivity based on metabolic demand).

Lastly, it is also plausible that significant beneficial effects might be achieved only with combination therapies targeting (different and/or complementary) metabolic

pathways, as well as other biological processes (e.g. neurodegeneration and failure of remyelination in the case of progressive MS). For example, potentiating oxidative phosphorylation and the TCA cycle might have dual beneficial effects in progressive MS by reverting energy deficiency in neurons [122], while counteracting the Warburg effect in MPs.

In this perspective, exogenous stem cell therapeutics may overcome most of these limitations for small molecules and biologics, being *de facto* capable to adapt their own phenotype and function, while secreting and up-taking multiple therapeutic small molecules in response to the local tissue microenvironment [111, 112]. A careful choice of the cellular sources (e.g. allogeneic *vs.* autologous, CNS-specific *vs.* non-specific) and delivery features (e.g. timing and route) must therefore be made to balance the potential of cell-replacement of damaged cells with the homeostatic modulation of MPs metabolism by the graft [105].

The final aim of these molecular and cellular therapies targeting mitochondrial immune cell metabolism will be to interrupt the persistent activation of innate immune responses in the CNS in a way that favours neuroprotection and ultimately promotes tissue regeneration.

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Conflict of Interest

SP owns >5% of CITC Ltd.

Box 1. Disease-associated microglia (DAM) in neurodegeneration

Ex vivo single-cell RNA sequencing (scRNA-seq) analysis of Alzheimer Disease-transgenic (Tg-AD) mouse brains discovered disease-associated microglia (DAM), a subset of microglia showing a unique transcriptional and functional signature [123]. DAM seem to be a common response signature to CNS disease that leads to the upregulation of specific genes, most of which are involved in lipid metabolism pathways, via a two-step activation process of microglia that is ultimately dependent on the triggering receptor expressed on myeloid cells 2 (TREM2) [123]. TREM2 is a sensor for a broad range of lipids and lipoproteins that activates microglial phagocytosis, and negatively regulates autophagy via mTOR, a master regulator of glycolysis [124]. In TREM2-deficient mouse models or human samples of AD, microglial clustering around β-amyloid plaques is impaired and the rare human polymorphism R47H of TREM2 shows one of the highest odds ratios (that is, 2.9–4.5) for susceptibility to late-onset AD [125]. These data suggest that the long-term protective function of microglial cells in neurodegenerative conditions is linked with TREM2 functions at different levels, including the maintenance of proper lipidic and glycolytic metabolic states.

Box 2. Infiltrating MPs in neuroinflammation

The contribution of blood-circulating MPs to CNS damage during EAE in mice commonly entails the local recruitment of $GR1^{+}/LY6C^{+}/CX3CR1^{low}/CCR2^{+}$ monocytes (equivalent to human $CD14^{++}/CD16^{-}$ monocytes) to sites of injury, with a CCR2 dependent mechanism [126]. Shortly after infiltration into the intact CNS parenchyma, monocytes downregulate their CCR2 and upregulate the CX3C chemokine receptor 1 (CX3CR1), becoming almost indistinguishable from activated CNS resident MPs [127]. CNS resident macrophages and microglia express several common markers (e.g. CX3CR1 and CD11b), and only recently specific markers indicative of a microglial homeostatic signature, such as the transmembrane protein 119 (TMEM119), the P2Y purinoceptor 12 (P2RY12) and the Sal-like protein 1 (SALL1), have been described [128, 129].

In mice with EAE, resident MPs seem to play a key pathogenic role, as early changes in vascular permeability are associated with **perivascular inflammatory cuffing** and parenchymal microglial activation that precede the arrival of blood-derived monocytes accompanying demyelination [130]. However, circulating MPs also play

an important role in the pathophysiology of disease, as it has been shown that in a transgenic mouse line expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) in peripheral helper T (Th) cells, GM-CSF leads to invasion of MPs into the CNS and the spontaneous development of neurological deficits [131]. A current hypothesis suggests that in EAE, while tissue-resident MPs clear tissue debris, contribute to recovery, and protect the CNS, MPs entering through the blood vasculature are instead detrimental and instruct demyelination [132, 133].

Box 3. Metabolism of anti-inflammatory MPs

Functional polarisation of MPs towards an anti-inflammatory phenotype can be achieved *in vitro* by IL-4 (or IL-13) stimulation.

Anti-inflammatory mouse macrophages are characterised by high levels of arginase-1 activity, which is needed to metabolize arginine to proline, a component of collagen which is required for tissue repair and resolution of inflammation [134]. Although arginine metabolism is one of the discriminative criteria between pro- and anti-inflammatory mouse macrophages, human data question the translational value of this dogma, as neither arginase-1 nor Ym1 are upregulated *in vitro* in alternatively activated human monocytes and monocyte-derived macrophages [135, 136].

During the past years, accumulating evidence suggests that both human and murine anti-inflammatory MPs rely on glucose and mainly glutamine uptake *in vitro* [60, 137]. In mouse BMDMs, IL-4 signalling co-opts the Akt-mTORC1 pathway to trigger the uptake of glucose and to regulate anti-inflammatory genes that include (e.g. arginase 1 and macrophage galactose N-acetyl-galactosamine specific lectin 2) via phosphorylation of ATP-citrate lyase (or ACL) [60]. Glucose is used to support hexosamine biosynthesis and to generate uridine diphosphate (UDP)-N-acetylglucosamine (UDP-GlcNAc), which is necessary for the post-translational modification of lectin mannose receptors [138].

Glutamine is very important for M2 polarization which activates glutamine catabolism and UDP-GlcNAc-associated modules in BMDMs. Correspondingly, glutamine deprivation (or inhibition of N-glycosylation) decreases M2 polarization *in vitro* [38].

Anti-inflammatory macrophages downregulate flux through the PPP by inducing CARKL, which inhibits this pathway resulting in reduced NADPH-mediated glutathione levels [36].

IL-4 stimulation of signal transducer and activator of transcription 6 (STAT6) results in PPARγ-coactivator-1β (PGC-1β), leading to mitochondrial biogenesis, preservation of TCA cycle functionality and increased FAO [139]. Short-chain fatty acids diffuse passively into the mitochondria, whereas medium- and long-chain fatty acids are imported through conjugation to carnitine palmitoyltransferase (CPT). Irreversible inhibition of the CPT1 with *etomoxir* is sufficient to inhibit expression of classic M2 genes [140], while CPT2 is dispensable for macrophage polarization [141]. Anti-inflammatory MPs can then effectively use the acetyl-CoA derived from FAO to drive forward flux in the ETC.

The translation factor eIF5A, which is regulated by polyamines and undergoes a specific posttranslational modification called hypusination, is an interesting cellular therapeutic target to affect M2 polarisation in mouse macrophages using small molecules [142]. However, further experiments are needed to understand how to modify eIF5A activity in order to promote an efficient anti-inflammatory pro-reparative activity of MPs. Understanding how the metabolism of anti-inflammatory MPs is regulated will help identify novel pathways that control the activation of MPs to favour neuroprotection and promote tissue regeneration.

Box 4. Itaconate has a key role in the inflammatory response

Itaconate plays a key role in the inflammatory response of macrophages being necessary for their early antimicrobial response. However, the observation that BMDMs from *immunoresponsive gene 1* (*Irg1*)$^{-/-}$ mice are deficient in itaconate production, while secreting more IL-12, NO, IL-6, IL-1β and IL-18, suggested that the main role of itaconate is to limit the detrimental cellular effects of prolonged inflammatory activation [143].

The major anti-inflammatory effects of itaconate during MP activation have been attributed to the inhibition of succinate oxidation by SDH [143]. Yet, itaconate is only a weak SDH inhibitor and this inhibition alone is not sufficient to account for its pronounced immunoregulatory effects. It is now well established that itaconate has other effects including the induction of **electrophilic stress** [144] and the alkylation of Keap1 through dicarboxypropylation [78]. While in normal conditions Keap1 binds to the transcription factor NRF2 facilitating its ubiquitination and proteolysis, itaconate blocks this process and favours the translocation of NRF2 to the nucleus and the transcription of anti-oxidant genes. The induction of itaconate is also

found after the stimulation of MPs with IFN-β and one of its major effects is to limit the expression of with type I interferons from activated cells via a negative feedback loop [78]. These data point at itaconate or specific derivatives [78, 145] as useful metabolic therapeutics to lessen chronic MP activation in neuroinflammation.

Box 5. Metabolism and trained immunity in MPs

The innate immune system quickly adapts after a previous challenge through functional and epigenetic reprogramming, a process that has been termed *trained immunity* or *innate immune memory* [146]. Priming human peripheral blood mononuclear cells (PBMCs) with *Candida albicans* (or beta-glucan) results in increased cytokine production upon re-stimulation with LPS (in contrast to the pre-stimulation with LPS which induces tolerance) [147]. Induction of trained immunity by beta-glucan causes activation of the mTOR pathway and HIF-1α activity associated with specific transcriptional and epigenetic (e.g. H3K4me3 and H3K27ac) signature [148]. In a trained cell, histone methylation tags the promoters of inflammatory genes allowing for an accelerated and stronger response upon re-stimulation. Several other pathways are differentially regulated in trained MPs, including increased 2HG concentrations, consumption of aspartate, accumulation of mevalonate and increased cholesterol synthesis pathway [149]. The inhibition of histone methyltransferases or histone acetyltransferases, or the blockage of mevalonate generation all prevent the induction of trained immunity [149]. The interplay between cell metabolism and epigenetic changes is an extremely hot topic in science that will likely unveil why maladaptive responses are maintained in the persistently inflamed tissues.

Figure Legends

Figure 1. Metabolic pathways in MPs during inflammation. The metabolism of pro-inflammatory MPs significantly differs from that of anti-inflammatory MPs, and it characterised by increased aerobic glycolysis, upregulation of the PPP and increased FAS due to the existence of enzymatic break points in the TCA cycle. Anti-inflammatory MPs instead rely on glycolysis and glutamine uptake, but also downregulate the PPP and increase FAO to feed the TCA cycle.

Abbreviations: ACL, ATP-citrate lyase; ATP, adenosine triphosphate; C, mitochondrial complex; CAD, cis-aconitate decarboxylase 1; CIC, citrate carrier; FA, fatty acid; FAS, fatty acid synthesis; G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT1, glucose transporter type 1; IDH, isocitrate dehydrogenase; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NADP, Nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; PGI, glucose-6-phosphate isomerase; PGs, prostaglandins; PPP, pentose phosphate pathway; RET, reverse electron transport; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TCA, tricarboxylic acid; TLR4, toll like receptor 4.

Figure 2. MPs metabolites act as intracellular and extracellular signalling molecules in inflammation. Intracellular metabolite signalling leads to post-translational modifications of proteins, regulates the activity of metabolic enzymes capable of binding specific mRNAs (e.g. GAPDH), guides mRNA expression (e.g. IL-1β) and ultimately affects chromatin structure and function. When released in the extracellular space, metabolites can act as 'metabokines', which bind to specific receptors to convey pro-inflammatory (*red*) or anti-inflammatory (*green*) signals to immune cells.

Abbreviations: AHR, aryl hydrocarbon receptor; BHOB, beta-hydroxybutyrate; C, mitochondrial complex; CIC, citrate carrier; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPR, G protein–coupled receptor; HAT, histone acetyltransferases; HDACs, histone deacetylase; HIF1-α, Hypoxia-inducible factor-1α; IFNs, interferons; IL-1β, interleukin-1 beta; KMD, histone demethylase; NAD, nicotinamide adenine dinucleotide; NRF2, nuclear factor erythroid 2–related factor 2;

RET, reverse electron transport; ROS, reactive oxygen species; SDH, succinate dehydrogenase.

Figure 3. How stem cells mediate homeostatic cell competition with MPs.

The immune-modulatory properties of grafted NSCs on innate immune responses in the context of chronic neuroinflammation depend on the expression of chemokine receptors, cytokine receptors, and receptors for metabolites, which guide their pathotropism and response to inflammation. In the local inflammatory microenvironment, NSCs engage in a homeostatic cell competition with MPs by (i) changing their own metabolism and increasing the secretion of extracellular arginase-1; (ii) releasing EVs that harbour functional metabolic enzymes; (iii) depleting the extracellular milieu of inflammatory immunometabolites including succinate; and (iv) secreting a range of anti-inflammatory molecules.

Abbreviations: Asrgl1, asparaginase-like 1; BMP, bone morphogenetic protein; C, mitochondrial complex; CCR2, C-C chemokine receptor type 2; CX3CR1, C-X3-C motif chemokine receptor 1; CXCR4, C-X-C chemokine receptor type 4; EP4, Prostaglandin E2 receptor 4; EVs, extracellular vesicles; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPR, G protein–coupled receptor; HIF1-α, hypoxia-inducible factor-1α; HO-1, heme oxygenase-1; IFN, interferon; IFN-γR1, interferon γ receptor 1; IL-1β, interleukin-1β; LIF, leukemia inhibitory factor; mTOR, mammalian target of rapamycin; NF-κβ, nuclear factor-κβ; NO, nitric oxide; PGE2, prostaglandin E2; RET, reverse electron transport; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SLC, solute carrier; TGF, transforming growth factor; TN-C, tenascin-c; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor.

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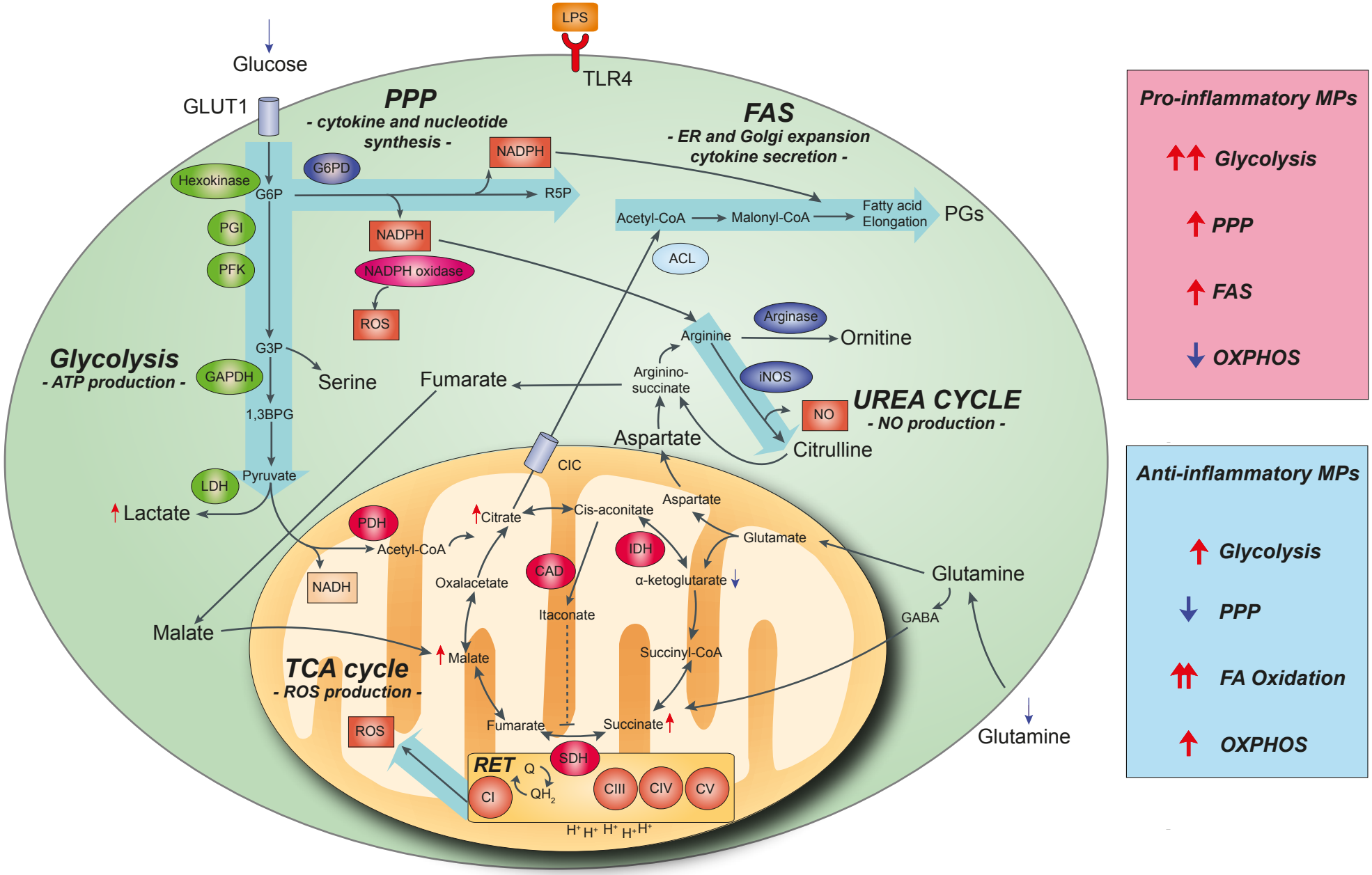
Outstanding Questions Box

- Is the persistent MP activation seen in chronic neuroinflammatory conditions a cause (*outside-in model*) or a result of the ongoing neurodegeneration (*inside-out model*)?
- What is the role of MPs metabolism in maintaining chronic neuroinflammation in progressive MS?
- Given the sexual dimorphism in microglial function and dysfunction, and the sex bias in CNS disorders with microglial pathology, how will the development of MPs-directed therapeutics differ to address pathways and targets that may differ in men and women?
- What is the role of the process of aging in the course of secondary progressive MS, in the metabolism of MPs?
- Is the region-dependent identity of MPs affecting their bioenergetic and immunoregulatory pathways in the brain?

Figure 1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PGI
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NADPH
NADPH oxidase
ROS
Acetyl-CoA
Malonyl-CoA
Fatty acid Elongation
PGs
ACL
Arginase
Arginine
Ornitine
Glycolysis
- ATP production -
GAPDH
G3P
Serine
Fumarate
Arginino-succinate
iNOS
NO
UREA CYCLE
- NO production -
1,3BPG
Aspartate
Citrulline
CIC
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Pyruvate
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Citrate
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OXPHOS
Anti-inflammatory MPs
Glycolysis
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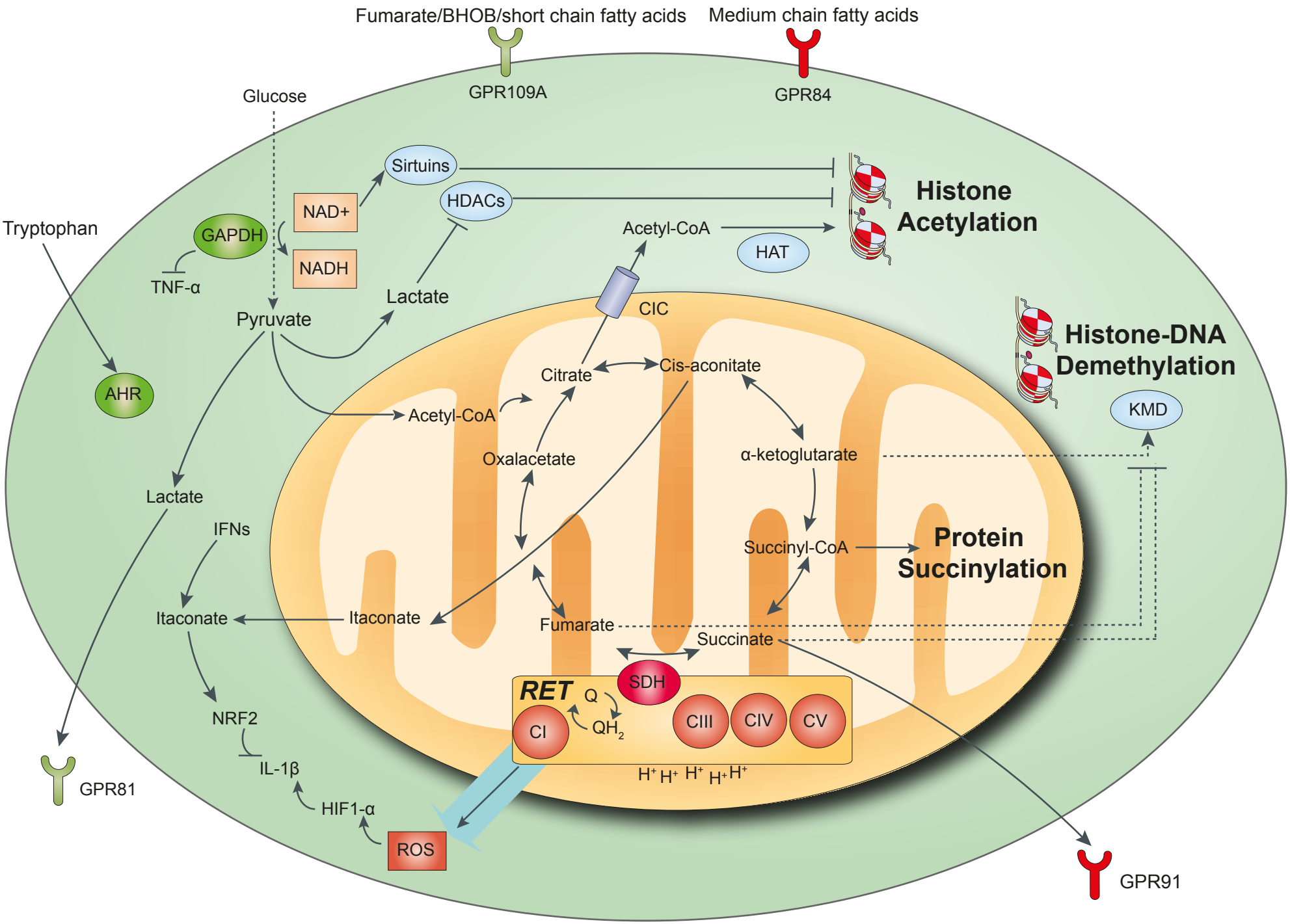
FA Oxidation
OXPHOS

Figure 2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AHR
Pyruvate
Lactate
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HAT
Histone Acetylation
CIC
Citrate
Cis-aconitate
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Oxalacetate
α-ketoglutarate
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Protein Succinylation
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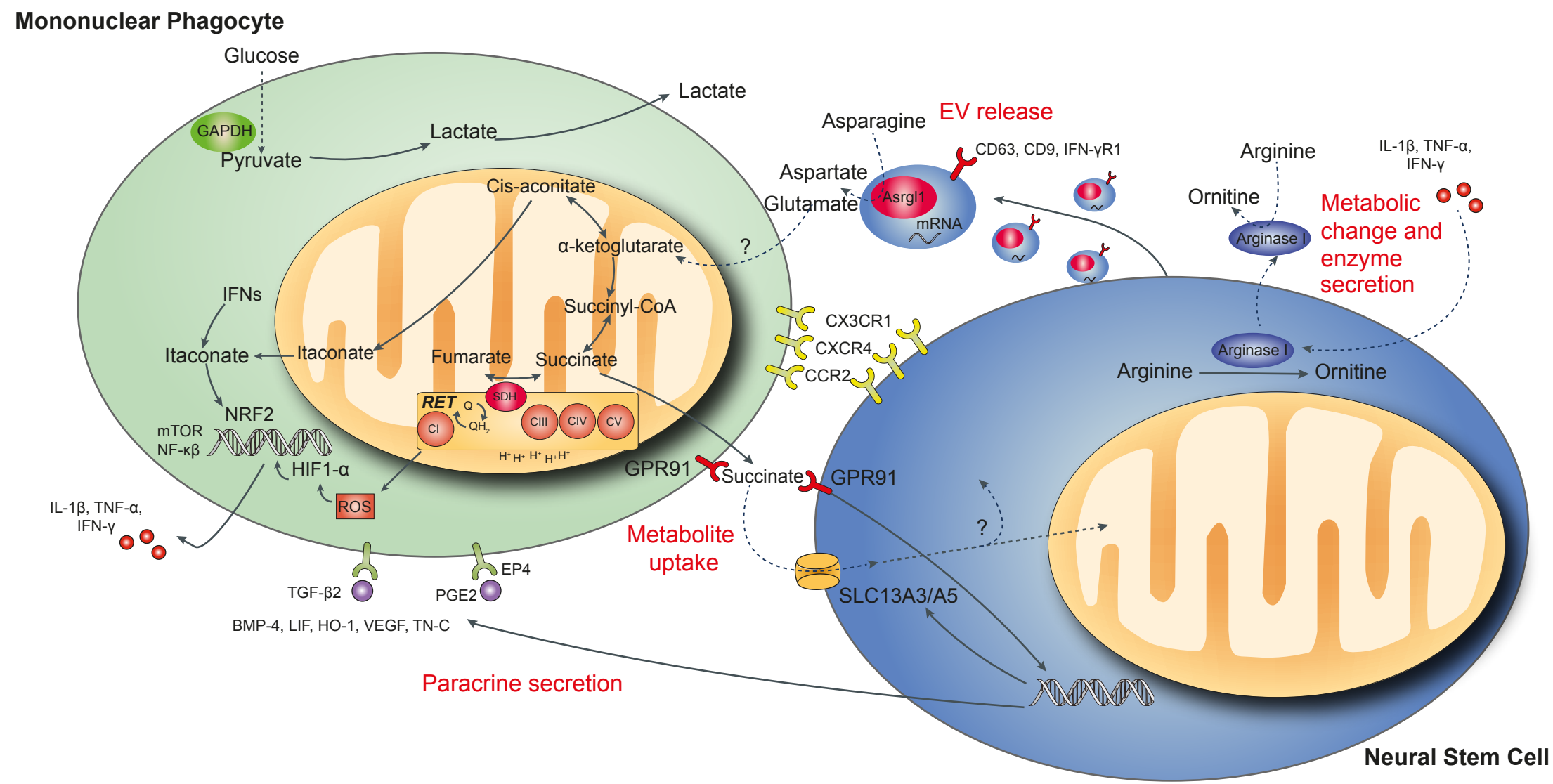
Figure 3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HIF1-α
ROS
RET
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SDH
CI
CIII
CIV
CV
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IL-1β, TNF-α,
IFN-γ
TGF-β2
PGE2
EP4
BMP-4, LIF, HO-1, VEGF, TN-C
Paracrine secretion
Asparagine
EV release
CD63, CD9, IFN-γR1
Aspartate
Glutamate
Asrgl1
mRNA
CX3CR1
CXCR4
CCR2
GPR91
Succinate
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Metabolite
uptake
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SLC13A3/A5
Arginine
Ornitine
Arginase I
IL-1β, TNF-α,
IFN-γ
Metabolic
change and
enzyme
secretion
Arginase I
Arginine
Ornitine
Neural Stem Cell