Succinate receptor 1 - an emerging regulator of myeloid cell function in inflammation

Grzegorz Krzak[1°], Cory M. Willis[1°], Jayden A. Smith[2],
Stefano Pluchino[1*] and Luca Peruzzotti-Jametti[1*]

[1]Department of Clinical Neurosciences and NIHR Biomedical Research Centre, University of Cambridge, Cambridge, UK;
[2] Cambridge Innovation Technologies Consulting (CITC) Ltd.

°Equal contribution.
*Correspondence: spp24@cam.ac.uk or lp429@cam.ac.uk.

Abstract

The rapidly evolving area of immunometabolism has shed new light on the fundamental properties of products and intermediates of cellular metabolism (metabolites), highlighting their key signaling roles in cell-to-cell communication. Recent evidence identifies the succinate—succinate receptor 1 (SUCNR1) axis as an essential regulator of tissue homeostasis. Succinate signaling via SUCNR1 guides divergent responses in immune cells, which are tissue- and context-dependent.

Herein, we explore the main cellular pathways regulated by the succinate—SUCNR1 axis and focus on the biology of SUCNR1 and its roles in the function of myeloid cells. Hence, we identify new therapeutic targets and putative therapeutic approaches aimed at resolving detrimental myeloid cell responses in tissues, including those occurring in the persistently inflamed central nervous system (CNS).

The succinate—SUCNR1 axis in inflammation

The last decade has seen an impressive increase in the understanding of the wider role that metabolites play in the regulation of cellular functions [1]. In innate immune cells, extensive intracellular metabolic reprogramming controls cell behavior, phenotype, and differential states of activation in response to external stimuli [2]. This is a fine-tuned process that requires the coordination of several metabolic networks that ultimately modulate intracellular and extracellular signaling pathways.

The metabolite succinate, an intermediate of the tricarboxylic acid (TCA) cycle, has emerged as a key modulator of innate immune responses in mammals (**Box 1**). Pro-inflammatory macrophages accumulate succinate intracellularly as a consequence of TCA cycle breaks [3].

Intracellular succinate acts as an **immunometabolite** to guide macrophage effector functions via the transcription of pro-inflammatory cytokines [3] and the production of reactive oxygen species (ROS) [4]. In chronic inflammation, such as in the case of rheumatoid arthritis, succinate is released into the extracellular compartment where it regulates cell-to-cell communication. In mouse disease models extracellular succinate acts both as a **metabokine** and **alarmin** to modulate immune cell function via binding to its cognate receptor SUCNR1 *in vitro* and *in vivo*, thus eliciting complex responses that are tissue- and context-dependent [5, 6].

Herein, we explore the main cellular pathways regulated by the succinate—SUCNR1 axis and focus on the biology of SUCNR1 and its roles in modulating adaptive and innate immune responses. We then focus our attention on myeloid cells, describing evidence of the dual role of SUCNR1 in these cells.

We anticipate that unravelling the complex mechanisms regulating this novel extracellular signaling pathway is crucial to understand the mechanisms sustaining innate immune responses in persistent inflammation. This will be instrumental in developing new therapeutic approaches aimed at resolving detrimental myeloid cell responses in tissues, such as those occurring in the persistently inflamed CNS.

Molecular Biology of SUCNR1

SUCNR1, initially named P2U2 due to its homology with the purinergic receptor P2Y2/P2U, was discovered in 1995 in a human megakaryocytic cell line [7]. In 2001, human *SUCNR1* and its mouse ortholog were identified using an expressed sequence tag data mining strategy for

novel **G-protein coupled receptors (GPCRs)** [8]. SUCNR1 was initially classified as GPR91, an orphan receptor, and in 2004, succinate was demonstrated as its cognate ligand [9]. Since then, SUCNR1 has been recognized as a key regulator of numerous and diverse physiological processes in cells [10].

The assessment of SUCNR1 molecular structure is key to understanding the variety of its intracellular signaling pathways [11, 12]. SUCNR1 has seven transmembrane domains connected by three hydrophilic extracellular loops, two N-glycosylation sites (Asn4 and Asn164), and one phosphorylation site (Ser326) [13]. These are sites of post-translational modifications, whose role in regulating receptor function and stability in different cell types remains largely unknown [8, 10].

As a GPCR, the activity of SUCNR1 is dependent on its coupling to different G-proteins. Specifically, SUCNR1 intracellular signaling relies on the associated α subunit type (i.e. $G\alpha_i$, $G\alpha_s$, and $G\alpha_q$). While the activation of the $G\alpha_q$ and $G\alpha_i$ subunits has been associated with pro-inflammatory polarization of myeloid cells (e.g. macrophages) [14, 15], $G\alpha_s$ subunit activation mostly results in an anti-inflammatory phenotype [16] (**Figure 1**). Therefore, both the post-translational modifications and α subunit expression dynamically regulate the activity of SUCNR1 in cells. This leads to a highly complex regulation that results in different, and somewhat opposing, signaling cascades in cells, as discussed below.

SUCNR1 expression in the CNS

Most **metabotropic receptors** are highly expressed in metabolically demanding organs, such as the kidneys, liver, and heart. Tissue-level expression data has identified SUCNR1 expression on human kidney *macula densa* cells [13], quiescent hepatic stellate cells of the rat liver [17], and cardiomyocytes of the rat heart [18]. In these cells, SUCNR1 regulates blood pressure and contributes to the pathophysiology of liver injury and cardiac hypertrophy [18]. SUCNR1 is also expressed in the musculoskeletal system [19] and in certain cancer and stem cells where it promotes proliferation, migration, and tissue remodeling [20, 21].

Despite the brain being the most metabolically active organ of the body, basal SUCNR1 expression is nearly undetectable in the healthy human and mouse CNS [22]. Publicly available transcriptomics databases of healthy human and mouse samples have confirmed that basal *SUCNR1/Sucnr1* expression is low throughout the CNS; with the highest expression observed in the olfactory bulbs, hippocampus, lateral ventricles, and cerebellum (**Table 1**) [23, 24].

Regardless of its low basal expression in the healthy CNS, it appears that SUCNR1 activation is key in the response of CNS cells to hypoxic-ischemic and inflammatory damage

(see **Figure 2**). For example, in rodent retinal ganglion cells (RGCs), the activation of the succinate—SUCNR1 axis leads to increased release of vascular endothelial growth factor (VEGF) and prostaglandin E_2 (PGE_2) *in vitro*, thus favoring vascularization via the activation of the mitogen-activated protein kinase – extracellular signal-regulated kinases1/2 (MAPK-ERK1/2) signaling pathway [25, 26]. In fact, the siRNA-mediated retinal down-regulation of *Sucnr1* in wild-type rats abolishes neovascularization in the presence of succinate, indicating that SUCNR1 expression in RGCs is a key regulator of the rodent retinal vascular network [27]. This could be of particular relevance in pathological settings, such as in **hypoxic-ischemic injury** [28]. While on the one hand, succinate—SUCNR1 signaling is beneficial, as it promotes neovascularization through PGE_2 dependent release of angiogenic factors [29], on the other hand succinate signaling through SUCNR1 can also become detrimental leading to vessel hyperproliferation, such as in the context of proliferative ischemic retinopathy [27].

The apical membrane of the retinal pigment epithelium (RPE) also expresses *Sucnr1* under homeostatic conditions [30, 31]. Here, SUCNR1 plays a putative role in iron homeostasis, as both the *in vivo* genetic disruption of the iron regulatory genes *Hfe* and *Hfe2* in mice*,* and the *in vitro* exposure of primary RPE cells to soluble iron, lead to increased SUCNR1 expression [30, 31]. This evidence is clinically relevant to **hemochromatosis,** an iron storage disorder associated with excessive iron accumulation. In fact, when human RPE cells are exposed *in vitro* to succinate and soluble iron, SUCNR1-dependent increased *VEGF* expression is observed [30]. Moreover, in a mouse model of juvenile hemochromatosis, enhanced bone-morphogenetic **(BMP)-6** signaling in combination with succinate has been shown to facilitate the interaction of pSmad4 with the *Sucnr1* promoter sequence, which promotes angioma formation through a *Sucnr1* and *Vegf* dependent mechanism [31].

Our group showed that active SUCNR1 expression and signaling is also present in both mouse and human induced neural stem cells (iNSCS) *in vitro* [32]. However, its precise roles in regulating endogenous NSC function, differentiation and metabolism [33] are still poorly understood. Similarly, rat astrocytes express *Sucnr1* [29] and, when exposed to succinate *in vitr*o, increased expression of pro-angiogenic factors (*Vegf, Ang1, Ang2*) and pro-inflammatory cytokines (*IL-1β* and *IL-6*) is described [29]. Succinate also increases the secretion of PGE_2, but not of VEGF, from RCA cells [29].

These data, together with the recent finding of genome-wide variation in *SUCNR1* function [34], suggest that SUCNR1 activity is a possible predisposing factor for the development of CNS-intrinsic dysfunctions, although this remains to be thoroughly investigated.

SUCNR1 signaling in adaptive and innate immune cells

Human hematopoietic progenitor cells (HPCs) are known to express SUCNR1 [35]. SUCNR1 has been linked with the maintenance of the self-renewing pool of HPCs, mostly via the activation of the MAPK signaling pathway [35, 36]. Megakaryocytes and erythroblasts also express SUCNR1 [37], as do many cells of the **adaptive** and **innate** immune systems across species (**Table 1**). The conserved expression of SUCNR1 in immune cells suggests a key role in immune system function, where acts as a regulator of both homeostatic and inflammatory cellular responses following exposure to pathogens or after tissue damage.

Within the adaptive immune system, SUCNR1 is detected on human T lymphocytes (both $CD4^+$ and $CD8^+$ subsets) and all major human B lymphocyte subsets [37, 38]. Although the exact function of SUCNR1 in T lymphocytes is yet to be fully characterized, a study on *ex vivo* expanded T cells from patients with systemic lupus erythematosus (SLE) suggests that T cells release large amounts of interleukin (IL)-10 and succinate following activation with oxidized mitochondrial DNA (mtDNA) *in vitro* [38]. In these oxidized mtDNA-stimulated SLE T cell - naïve B cell co-cultures, co-incubation with neutralizing anti-SUCNR1 antibodies partially inhibited the T cell-dependent activation of B cells and subsequent production of immunoglobulin G (IgG), relative to controls; which suggested that SUCNR1 expression on B cells is required for their activation [38]. Further exploring if succinate – in synergy with other cytokines and chemokines – is relevant to the enhancement and amplification of adaptive immune responses in other contexts, is open for investigation.

The functional effects of SUCNR1 activation in innate immune cells are not always straightforward, as they are both cell- and context-dependent [16, 39]. Within the innate immune system, SUCNR1 is expressed on dendritic cells (DCs), mast cells, and **mononuclear phagocytes (MPs)** [such as bone marrow-derived macrophages (BMDMs), tumor associated macrophages (TAMs), adipose tissue macrophages, and **microglia**] (details on species and cells are given in **Table 1**).

Amongst innate immune cells, human immature DCs show maximal expression and SUCNR1 activity controls their **chemotaxis** in a succinate-concentration dependent manner [40]. Here, SUCNR1 acts synergistically with **toll-like receptor** (TLR)-3 and 7 - but not TLR-2 or TLR-4 – to also increase the expression of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and IL-1β. Overall, this leads to an enhancement of antigen-specific presentation and subsequent $CD4^+$ T cell activation [40]. Of relevance, this response seems to be time limited**,** as SUCNR1 is swiftly downregulated once DCs become fully activated [40]. Also in mouse DCs, SUCNR1 enhances the lipopolysaccharide (LPS)-induced (i.e. TLR-4-dependent) production of pro-inflammatory cytokines [41]. Indeed, in mice with **experimental**

antigen-induced arthritis, SUCNR1-mediated chemotaxis guides mouse DCs into lymph nodes *in vivo*, leading to the expansion of pathogenic T helper **(Th)17 cells**, which in turn contributes to autoimmunity [41].

The activity of mast cells also relies on the presence of functional SUCNR1. In *Sucnr1*$^{-/-}$ mice, mast cells display a hyperreactive phenotype evidenced by increased TNF-α production in response to oxazolone-induced allergic contact dermatitis, relative to controls [42]. However, the hyperreactive response of mast cells within this context does not correlate with equally hyperreactive adaptive immune response, which are measured by either T cell infiltrates or expression of the **Th2-like** (anti-inflammatory) cytokines IL-4 and IL-13 [42]. These findings suggest a key physiological role for succinate—SUCNR1 signaling in mast cells that impact their differentiation and maturation capacity, although this warrants further studies.

Overall these data suggest that a coordinated expression and activation of SUCNR1 between multiple cell types of the adaptive and innate immune systems is necessary for modulating homeostatic and inflammatory responses via this novel metabolic signaling pathway.

Context-dependent role of SUCNR1 in MPs

While the succinate—SUCNR1 axis is rather defined in DCs and mast cells [40, 41], its role in the chemotaxis and activation of MPs remains quite controversial.

In *Sucnr1*$^{-/-}$ mice, macrophage infiltration into succinate-producing adipose tissue is significantly reduced *in vivo*, relative to controls [43]. However, the lack of a clear chemoattractant effect for succinate alone on macrophages *in vitro* questions the specificity of these findings. In fact, *Sucnr1*$^{-/-}$ macrophages show reduced migration only in response to tissue culture media derived from apoptotic/hypoxic adipocytes, which suggests that the presence of receptors other than SUCNR1 (e.g. reduced C-C chemokine receptor type I [CCR1] expression) play a major role in MPs chemotaxis [43]. Nevertheless, recent data demonstrated that succinate alone is able to promote the chemotaxis of SUCNR1-expressing TAMs in both syngeneic and xenogeneic lung cancer grafts models in mice, as this effect is abrogated by pre-treatment with anti-SUCNR1 antibodies *in vitro* suggesting a direct role of SUCRN1 in TAMs chemotaxis [21].

Engagement of SUCNR1 has also been linked to microglial chemotaxis, as suggested by the increased accumulation of microglia in the retina of *Sucnr1*$^{-/-}$ mice, relative to controls [44]. However, this phenotype only occurs when the hosts are globally deficient in *Sucnr1*,

suggesting that the SUCNR1-mediated control of retinal microglia chemotaxis may actually be dispensable [44].

Besides chemotaxis, the effects of the succinate—SUCNR1 axis on inflammatory responses of MPs have also led to conflicting results (described in **Figure 3,** Key Figure). For example, the pro-inflammatory effect of SUCNR1 in macrophages has been observed in alternatively activated (anti-inflammatory) macrophages derived from human peripheral blood mononuclear cells (PBMCs) [45]. Here, when macrophages are challenged *ex vivo* with IL-4 and IL-13 they significantly upregulate *SUCNR1*. However, subsequent stimulation with succinate, or the SUCNR1 agonist *compound 131*, decreases the expression of the anti-inflammatory cytokine IL-10 while upregulating the pro-inflammatory cytokine TNF-α, thus enhancing pro-inflammatory responses [45]. These findings suggest a prominent role for the succinate—SUCNR1 axis in maintaining inflammation by dampening anti-inflammatory responses. In addition, while previous evidence has shown that the absence of SUCNR1 on mouse peritoneal macrophages has no effect on intracellular IL-1β levels or the secretion of TNF-α and IL-6 (in response to 24 h stimulation with 10 ng/ml LPS [43]), recent data suggest instead that when BMDMs are stimulated for 24 h with higher doses (100 ng/ml) of LPS, or with the combination of LPS (1 ng/mL) and monosodium urate (180 ug/mL) *in vitro*, the absence of SUCNR1 leads to significantly lower IL-1β production [39]. It is also interesting to note that IL-1β treatment per se increased *Sucnr1* mRNA expression in BMDMs *in vitro* [39]. This finding anticipates a possible bidirectional crosstalk between pro-inflammatory mediators and the activity of SUCNR1, and the existence of a positive-feedback mechanism in inflammation. In this model, IL-1β would trigger the production and release of succinate from macrophages, while succinate in turn stimulates SUCNR1-expressing MPs to increase IL-1β, thus maintaining a chronic inflammatory state via an autocrine and paracrine loop [39]. Accordingly, *in vivo* data suggest that *Sucnr1*$^{-/-}$ bone marrow chimeric mice are protected from antigen-induced experimental arthritis, as early as 2 days after challenge*,* relative to controls [39].

However, recent work has challenged the prevalent pro-inflammatory role of SUCNR1 by showing that mouse BMDMs lacking SUCNR1 (*Sucnr1*$^{-/-}$) exhibit an increased pro-inflammatory phenotype relative to controls – characterized by increased release of IL-6, TNF-α and nitric oxide (NO) - in response to LPS (100 ng/mL) or LPS + interferon (IFN)-γ (10 ng/mL + 10 U/mL, respectively) for 24 h *in vitro* [46]. Since the *Sucnr1*$^{-/-}$ mice used to derived BMDMs from were identical in both the abovementioned studies [39, 46], discrepancies in the published results could be related to the use of different BMDM maturation protocols (i.e. recombinant M-CSF+IFN-γ pulse [39] *vs*. L-929 conditioned media [46]), as well as the age of the animals (age-matched [39] *vs.* an average of 28 weeks difference between WT and *Sucnr1*$^{-/-}$ mice [46]).

However, the exact mechanisms behind these apparent discrepancies are yet to be fully addressed.

Further data supporting the anti-inflammatory effect of SUCNR1 comes from the study of the inflamed CNS, where SUCNR1 stimulation can directly participate in resolution of inflammation *in vivo* [32]. Specifically, a delayed accumulation of succinate occurs in the in the cerebrospinal fluid (CSF), but not in the peripheral blood, of mice with **experimental autoimmune encephalomyelitis (EAE)**, a model of chronic MS [32]. We found that CSF succinate - most likely released by pro-inflammatory macrophages and microglia - signals to grafted NSCs via SUCNR1. NSCs, in response, initiate the secretion of prostaglandin E2 and the scavenging of extracellular succinate, which both contribute to the resolution of neuroinflammation [32].

Subsequent work confirmed the anti-inflammatory role of SUCNR1 *in vivo*, showing that SUCNR1 in mouse macrophages can induce a prevalent anti-inflammatory phenotype [16]. Specifically, in a LysM^{Cre}*Sucnr1*$^{fl/fl}$ myeloid cell-specific *Sucnr1* conditional knockout mouse line, a significant increase of CD11b^{+}/CD11c^{+}/CD206^{-}pro-inflammatory macrophages and pro-inflammatory genes (*Il12b*, *Tnf*, and *Nos2*) was reported in white adipose tissue (WAT) *in vivo*, relative to controls [16]. *In vitro*, treatment with IL-4 (30 ng/mL) induced *Sucnr1* in mouse peritoneal macrophages [while treatment with LPS (250 ng/mL) had the opposite effect (decreased *SUCNR1* expression) in macrophages derived from human PBMCs] [16]. Most importantly, the SUCNR1-dependent induction of an anti-inflammatory phenotype in macrophages occurred in response to IL-4 via an alternative cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) phosphorylation signaling cascade subsequent to $G\alpha_s$ stimulation [16].

Finally, recent evidence in syngeneic and xenogeneic lung cancer grafts models in mice provides further confirmation on the role of succinate—SUCNR1 signaling in skewing macrophages into anti-inflammatory TAMs [21]. In this setting, peritoneal macrophages treated with succinate (1 mM) for 24 h showed a significant increase in the anti-inflammatory mRNAs *Arg1*, *Fizz1*, *Mgl1*, and *Mgl2 in vitro,* relative to controls, which was completely abolished by siRNA mediated *SUCNR1* downregulation. Furthermore, succinate released by the tumor induced a significant increase in the number of VCAM1^{+}/CD11c^{+}/CD11b^{low-} TAMs *in vivo*, suggesting that the succinate–SUCNR1 axis is indispensable to induce anti-inflammatory TAMs [21].

Altogether, these findings suggest that succinate, produced (and released) by pro-inflammatory MPs, act on SUCNR1 via competing feedback loops that regulate both pro-

inflammatory and anti-inflammatory MPs. However, the exact circumstances and mechanisms guiding the prevalence of either response still remain to be fully elucidated.

SUCNR1 as potential pharmacological target for chronic neuroinflammation

While the role of acute inflammation is well-known, the transition towards a chronic state of inflammation has now become a therapeutic focus, particularly in the context of inflammatory and degenerative neurological diseases [47, 48]. Under persistent neuroinflammation, the activation of innate immune cells leads to a state of 'sterile' inflammation, wherein the pro-inflammatory phenotype of MPs continues in the absence of triggering stimuli, thus leading to the constant damage of neurons and oligodendrocytes [5].

As strong evidence supports a key role for the succinate—SUCNR1 axis in regulating the phenotype and function of innate immune cells and the response of CNS cells to damage, the pharmacological targeting of this pathway is therapeutically relevant for certain conditions associated to persistent (detrimental) innate immune responses in the CNS, such as progressive multiple sclerosis. Unfortunately, the expression of SUCNR1 on a wide range of CNS and immune cells, as well as the seemingly disparate functional effects that can result from activating this pathway, complicate the study and putative development of therapeutics targeting SUCNR1. Major considerations regarding the cell- and context-specific effects of SUCNR1 signaling must be taken when designing and interpreting experimental results, as should the CNS-permeability of any prospective succinate analogs [46, 49]. Ideally, attention to these considerations will not only lead to a better understanding of the succinate—SUCNR1 axis, but also inform the development of therapies to target chronic-persistent neuroinflammation.

Most of the work in this area has been related to the identification of SUCNR1 agonists. By employing a structural model based on the crystal structure of the closely-related P2Y1 receptor (27% homology to SUCNR1), SUCNR1's binding site was characterized and virtual screening was used to identify its putative ligands [45]. Several non-metabolite agonists were identified, with the most potent candidates having structures based on the succinate backbone with an amide-linked hydrophobic moiety capable of occupying a side-pocket at the SUCNR1 binding site [45]. Certain agonists were determined to have 10-100 times higher potency than succinate, exhibiting specificity for either human or mouse SUCNR1, and displaying similar signaling effects to succinate in human *ex vivo* macrophages [45]. Further structure-function studies of this agonist series identified nanomolar potency ligand candidates with excellent *in*

vitro stability, but high hydrophilicity [50]. The most potent succinate analog is *cis*-epoxysuccinic acid with an **EC_{50}** of 2.7 μM; this compound is not succinate dehydrogenase-active but has the same effect as the canonical SUCNR1 ligand, as it increases blood pressure in rats upon intravenous administration [51]. For reference, succinate's EC_{50} for the activation of SUCNR1 has been reported in the range of 17-56 μM [9, 45], while typical plasma concentrations of succinate in humans are on the order of 2-30 μM (see **Clinician's Box**) [45]. Of note, the xanthone natural products vinaxanthone and xanthofulvin have also been found to be positive allosteric modulators of SUCNR1, enhancing the receptor's affinity for succinate [52].

Based on the predominant role of SUCNR1 as a positive regulator of innate immune cell chemotaxis and chronic inflammation, it is not a stretch to consider that SUCNR1 antagonists are protective in environments where inflammation is exacerbated, such as in certain autoimmune conditions, including progressive multiple sclerosis. Several small molecule inhibitors of human SUCNR1 were developed from a single hit identified in a high-throughput *in vitro* screen of a commercial compound library [53]. Through structure-activity relationship-guided systematic modification of the **naphthyridine-based** hit, potent and selective antagonists were identified, most notably those labeled "2c" and "4c". These compounds exhibited promising **IC_{50}** values of 30 nM and 7 nM, respectively, and were >1,000 times more selective for human SUCNR1 over hGPR99 (which shares 33% protein sequence homology) [53]. While both 2c and 4c exhibit poor oral bioavailability, intraperitoneal administration into succinate-treated Wistar rats of either compound readily ameliorated hypertension relative to controls [53]. Other compounds synthesized in the study traded improved oral bioavailability for decreased affinity/selectivity [53]. Assays employing another compound in the series — *2d* — demonstrated the ability of the SUCNR1 inhibitor to suppress type I collagen induction in rat hepatic stellate cells subjected to activation by high glucose (700 mg/dL) or high succinate (3 mM) conditions modeling non-alcoholic steatohepatitis (**NASH**) *in vitro* [54]. An alternative series of SUCNR1 inhibitors was identified through high-throughput screening with one compound — *NF-56-EJ40* — demonstrating an impressive selectivity for human SUCNR1 (IC_{50} = 25 nM), over rat SUCNR1 (IC_{50} >100 μM) [46]. The structural basis of this species-selectivity was elaborated through crystallography and molecular modeling, with comparisons to the structurally similar P2Y1 receptor providing leads for prospective allosteric inhibitors. While this series of antagonists demonstrated poor permeability due to their highly polar zwitterionic nature, systematic optimization of the structural scaffold including incorporation of an internal salt bridge yielded potent inhibitors with good bioavailability and oral exposure [55].

Follow-up studies of the therapeutic utility of SUCNR1 antagonists have yet to be reported in the scientific literature, but several of the co-authors of the naphthyridine-based antagonist study are listed as inventors on various patents describing a complementary series of SUCNR1

inhibitors with prospective applications in treating NASH and related conditions. Indeed, both fibroblast growth factor 21 and its recombinant peptide analog lower α-smooth muscle actin production through inhibition of the succinate—SUCNR1 signaling axis, thus reducing liver fibrosis in mouse models [56]. The mitochondrial fission-activating type 2 diabetic drug metformin has likewise been ascribed an inhibitory role in succinate—SUCNR1 signaling, ameliorating inflammation and fibrosis in a mouse model of NASH [57].

RNA interference-mediated downregulation of SUCNR1 has also been used to explore the physiological ramifications of succinate—SUCNR1 signaling (and thus its potential therapeutic modulation). SUCNR1 is alleged to be a key target of miR-758, with oxidized low-density lipoprotein-induced overexpression of this miRNA associated with suppression of SUCNR1 and its downstream signaling pathways, resulting in damage to human vascular endothelial cells *in vitro* [58]. Alternatively, short hairpin RNA-mediated knockdown of *Sucnr1* expression in a rat model of retinopathy attenuated the avascular area, abnormal neovascularization, and loss of pericytes through regulation of VEGF relative to controls [59]. One novel approach envisions an enhanced-affinity recombinant SUCNR1 mutant able to sequester elevated concentrations of succinate in the gut, such as that associated with inflammatory bowel disease [II].

There may be a significant opportunity to therapeutically intervene in conditions of chronic inflammation through amelioration of SUCNR1 overactivity in the innate immune system. The benefits of such an approach are perhaps most immediately achievable in metabolic conditions such as NASH, obesity, and type-2 diabetes, which are marked by a strong inflammatory basis to their pathology and in which GPCR-targeting drugs have shown significant potential [60, 61]. Nonetheless, the need to elucidate the pathological relevance of the succinate—SUCNR1 axis in human disease remains (given that preclinical animal models of GPCR signaling modulation have not always successfully translated) [60, 62].

Beyond systemic metabolic conditions, applications for SUCNR1 inhibitors in the treatment of neuroinflammatory diseases are feasible and worth pursuing. In conditions of elevated CNS succinate, potent and selective antagonists offer the potential to restore homeostasis to the succinate—SUCNR1 axis in resident or infiltrating MPs, alleviating the chronic inflammation driving neurodegeneration and disability. Towards this goal, there is an unmet need for orally bioavailable SUCNR1-inhibiting compounds capable of effectively permeating the blood-brain barrier (e.g. those possessing a low molecular weight and moderate lipophilicity while minimizing interactions with brain efflux transporters). The structural and pharmacokinetic optimization of SUCNR1 antagonists (described above) points to substantial opportunities for novel compounds to be explored in clinical studies, pending *in vivo* validation in appropriate animal disease models. Notably, while the apparent limited activity of SUCNR1 in a non-

pathological context suggests that such a compound might have a high therapeutic index, a greater understanding of the systemic effects of pharmaceutical modulation of the succinate—SUCNR1 axis are essential in ruling out any unanticipated off-target effects.

Concluding Remarks

Overall, SUCNR1 has thus far been largely overlooked as a putative therapeutic target, perhaps due to its complex and tissue/context-dependent functions. While large pharmaceutical companies have previously filed patents on methods for screening SUCNR1 modulators or the use of SUCNR1 as a marker/target for immune cells, these have been seemingly neglected. While several research groups and small companies further the development of more bioavailable agonists and antagonists, future therapeutic exploitation of the succinate—SUCNR1 axis will necessitate a greater elucidation of the complexities of this pathway.

The succinate—SUCNR1 axis is seemingly relevant in conditions of stress and damage, while its role in homeostatic conditions is negligible (see **Box 2**). This might be advantageous in developing candidate therapeutics to treat certain pathologies as the off-target effects of SUCNR1 modulation are likely to have minimal effects on non-activated SUCNR1 cells in the host, although this remains to be rigorously tested (see **outstanding questions**). In our view, understanding the complex role of SUCNR1 in regulating neuro-immune interactions in health and disease will uncover the right window of opportunity to modulate this axis, in order to promote anti-inflammatory and pro-regenerative responses that can resolve chronic neuroinflammation.

Acknowledgements

The authors wish to acknowledge the contribution of past and present members of the Pluchino laboratory, who have contributed to (or inspired) this review. We are grateful to Giovanni Pluchino for critical insights into this manuscript. This work has received support from the National MS Society (USA; grant RG-1802-30200 to SP), the Italian Multiple Sclerosis Association (AISM, grant 2018/R/14 to SP), the United States Department of Defense (DoD) Congressionally Directed Medical Research Programs (CDMRP) (grant MS-140019 to SP), and the Bascule Charitable Trust (RG 75149 and RG 98181 to SP). L.P.J. was supported by a senior research fellowship Fondazione Italiana Sclerosi Multipla (FISM cod. 2017/B/5 and financed or co-financed by the ‘5 per mille’ public funding), a clinical research career

development fellowship from the Wellcome Trust (220711/Z/20/Z) and the Addenbrooke's Charitable Trust (RG 97519).

Disclaimer Statement

S.P. is cofounder, CSO, and shareholder of CITC Ltd and iSTEM Therapeutics, and cofounder and Non-Executive Director at asitia Therapeutics. L.P.-J. is shareholder and Director of Strategy and Innovation at CITC Ltd. J.A.S. is a Project Manager and Senior Research Associate at CITC Ltd, Head of Research at iSTEM Therapeutics and CSO of asitia Therapeutics.

Box 1

Intracellular succinate signaling in mammals

In response to *in vitro* stimuli, such as lipopolysaccharide (LPS) and hypoxia, myeloid cells switch their metabolism from oxidative phosphorylation (OXPHOS) to aerobic glycolysis for energy production (i.e. Warburg effect) in a similar manner to tumor cells**.** Inflammatory MPs display a dysfunctional mitochondrial respiratory chain, and a breakdown of the Krebs cycle (also known as TCA cycle) at the succinate dehydrogenase (SDH), which results in intracellular succinate accumulation [3]. Accumulated succinate is then oxidized by SDH to initiate reactive oxygen species (ROS) generation via complex I by reverse electron transport (RET) [63]. In mouse BMDMS, succinate acts as a stabilizing signal for the transcription factor hypoxia inducible factor 1 alpha (HIF-1α) via prolyl hydroxylases enzymes (PHDs) inhibition, to induce transcription of interleukin (IL)-1β and drive a pro-inflammatory phenotype [3].

Intracellular succinate can also affect chromatin structure and function. Succinate promotes protein succinylation of the lysine residues altering protein conformation and changing chromatin-histone interactions [64]. Histone succinylation can then be regulated by the mitochondrial sirtuin 5 (SIRT5)-dependent desuccinylation, which also suppresses SDH activity [65]. Finally, succinate accumulation also results in the epigenetic reprograming by inhibiting tet methylcytosine dioxygenase 2 (TET2) and jumonji c domain-containing (JMJD) protein families, leading to the histone and DNA methylation [66].

Box 2

Constitutive SUCNR1 knockout mice

Constitutive knockout technology provides a powerful *in vivo* tool to understand the functional and behavioral phenotype of gene deletion. To this extent, *Sucnr1*$^{-/-}$ mice have been useful as they display normal development and maturation, despite a significantly lower body weight, compared to their WT littermates [67]. *Sucnr1*$^{-/-}$ mice are fertile, show no overt blood pressure anomalies, no abnormal brain vascularization [29, 68], no alteration of food intake, nor deposition of fat or lean mass compared to WT littermates [19]. However, *Sucnr1*$^{-/-}$ mice do show deficits in conditions of stress and disease. Their endurance on a forced treadmill running test is significantly impaired, as indicated by an overall lower average running speed and decreased muscle strength [19]. When fed a high fat diet (HFD), *Sucnr1*$^{-/-}$ mice show increased fat deposition, hyperglycemia, reduced insulin secretion, and augmented hepatocyte damage compared to WT littermates [67]. Moreover, *Sucnr1*$^{-/-}$ mice intravenously supplemented with succinate show abnormal cardiac parameters related to the function of the left ventricle, such as decreased systolic volume and ejection fraction [69]. In the future, to circumvent the well-known limitations of global gene knockout (such as compensatory mechanisms and off-target

cellular effects), cell-specific gene knockout targeting strategies should be used to provide more accurate insights into the function of SUCNR1 [16].

Figure and Table legends

Figure 1. Diverse signaling functions of the G-alpha subunit of succinate receptor 1 (SUCNR1) in mammals. SUCNR1 activation of the $G\alpha_i$ subunit leads to inhibition of adenylyl cyclase (AC), which results in decreased cyclic adenosine monophosphate (cAMP) concentrations, decreased activity of PKA, and ultimately the release of pro-inflammatory cytokines [14, 15]. By contrast, SUCNR1 activation of the $G\alpha_s$ subunit has the opposite effects, leading to increased protein kinase A (PKA) activity and regulation of transcription of anti-inflammatory mediators via cyclic AMP response element binding protein (CREB) activity [16]. Finally, SUCNR1 activity via the $G\alpha_q$ subunit enhances instead the activity of PLC, which produces the secondary messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). While IP_3 increases Ca^{2+} in the cytosol leading to activation of Ca^{2+}-dependent mitogen-activated protein kinase – extracellular signal-regulated kinases1/2 (MAPK-ERK1/2) pathways, DAG activates protein kinase C (PKC). Both of these pathways lead to pro-inflammatory cascades (such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), MAPK-ERK1/2, and p38), nitric oxide (NO) production, and prostaglandin E_2 (PGE_2) secretion [40, 70]. Of note, recent evidence suggests that phospholipase C (PLC) activation in human cells is also mediated by the βγ subunits of SUCNR1 [71]. Red and green arrows indicate downregulation and upregulation, respectively. Adenosine triphosphate (ATP), Krüppel-like factor 4 (KLF4).

Figure 2. SUCNR1 Expression in non-immune cells in the mammalian CNS. During cell therapy to suppress chronic inflammation, excessive succinate (SUC) accumulation in the cerebrospinal fluid (CSF) activates succinate receptor 1 (SUCNR1) on grafted neural stem cells (NSC) leading to the release of prostaglandin E_2 (PGE_2), SUC uptake, and down-regulation of pro-inflammatory mononuclear phagocytes (MP) (*bottom left insert*) [32]. In Hypoxia-ischemia, SUCNR1 is expressed in cortical neurons and astrocytes, where it induces pro-angiogenic and inflammatory factors secretion (*bottom center insert*) [29]. Within the retina, microglia, retinal ganglion cells (RGC), and the retinal pigmented epithelium (RPE) express SUCNR1 [25, 26, 30]. Global loss of *Sucnr1*$^{-/-}$ leads to microgliosis in healthy mice (*i*), SUCNR1 on RGC leads to increased expression of pro-angiogenic and pro-inflammatory factors during hypoxia-ischemia (*ii*), and SUCNR1 on RPE induces vascular endothelial growth factor (VEGF) release through bone morphogenetic protein 6 (BMP6)-Smad signaling in hemochromatosis (*iii*) [31].
Bruch's membrane (BM), iron (Fe^{3+}), interleukin (IL)-1β, interleukin 6 (IL-6), solute carrier 13A3/5 (SLC13A3/5), angiopoietin 1 (Ang1), angiopoietin 2 (Ang2), prostaglandin-endoperoxidase synthase 2 (PTGS2), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK).

Figure 3, Key Figure. Pro- and anti-inflammatory effects of succinate receptor 1 (SUNCR1) activation in mammalian macrophages. Current evidence suggests that the succinate—SUCNR1 axis on macrophages induces tissue- and disease-specific polarization. A-B) Pro-inflammatory effects are seen in rheumatoid arthritis, where a 'positive-feedback' loop between interleukin (IL)-1β and succinate maintains chronic inflammation [39], and in human peripheral blood mononuclear cells (PBMCs) derived alternatively activated macrophages, where stimulation of SUCNR1 induces tumor necrosis factor (TNF)-α while suppressing IL-10 [45]. C-D) Anti-inflammatory effects have been described in the adipose tissue, where activation of SUCNR1 promotes the response of these cells to type 2 cytokines [16], and in tumor-associated macrophages (TAMs), where the succinate—SUCNR1 axis promotes an anti-inflammatory gene signature and chemotaxis within the lung cancer microenvironment [21]. Arginase 1 (Arg 1), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), found in inflammatory zone protein 1 (Fizz1), lipopolysaccharides (LPS), toll-like receptor 4 (TLR4).

Table 1. SUCNR1 expression and signaling pathways in mammalian neural and immune cells.

Glossary

Alarmin: endogenous molecule rapidly released as a result of disease, trauma, or infection that is able to both recruit and activate various types of immune cells.

Adaptive immune system: antigen-dependent immune response involving clonal expansion and activation of T and B lymphocytes. Such immune response takes days to weeks to be established and involves an immune memory that recognizes previously encountered pathogens to provide long-lasting immune response.

Bone-morphogenetic protein (BMP)-6: a secreted signaling protein able to induce the growth of cartilage and bone.

Chemotaxis: the movement of the phagocytotic cells towards increasing ligand gradient to elicit inflammatory response, antigen presentation, and phagocytosis.

Experimental antigen-induced arthritis: an experimental animal model that reproduces aspects of clinical rheumatoid arthritis by immunization of a joint with methylated bovine serum albumin.

Experimental autoimmune encephalomyelitis (EAE): the most common murine model of multiple sclerosis induced either by injected myelin protein or by myelin-activated T cells.

G-protein coupled receptors (GPCRs): the largest family of membrane receptors with diverse intracellular signaling properties resulting from G protein subunit activity.

Half maximal effective concentration (EC_{50}): concentration of a drug needed to induce half of a maximum biological response (e.g. upon a ligand binding to a receptor).

Half maximal inhibitor concentration (IC_{50}): concentration of a drug needed to reduce a biological response by 50%.

Hemochromatosis: a disease of iron homeostasis causing accumulation of iron in the body.

Hypoxia-ischemia injury: brain injury (e.g. stroke) resulting from cardiac arrest and consequently leading to the oxygen deprivation in the brain (brain hypoxia).

Immunometabolite: intermediate product generated during cellular metabolism that can act on intracellular and extracellular signaling pathways to shape and modulate immune responses.

Innate immune response: an immediate, nonspecific immune response mediated by myeloid cells. Such response is based on the myeloid cell ability to recognize evolutionary-conserved molecular features of pathogens (such as bacterial LPS) to elicit immune response.

Metabokine: signaling product derived from cellular metabolism that binds to and regulates dedicated cell surface G-protein coupled and ionotropic receptors.

Metabotropic receptors: membrane-bound receptors that act via signaling on secondary messengers such as G-proteins. Metabotropic receptors are also knowns as G protein-coupled receptors.

Microglia: Resident MPs that are responsible for CNS surveillance due to its 'immune-privileged' status behind two anatomical 'barriers' (i.e. the blood-brain-barrier and the blood-cerebrospinal-fluid-barrier), that restrict the free movement of circulating peripherally derived immune cells and soluble factors, such as metabolites, into the CNS parenchyma.

Mononuclear phagocytes (MPs): innate immune myeloid cells that reside either within the tissue- (e.g. microglia) or blood-born macrophages.

Naphthyridine-based: molecular entities based on a fused double-ringed aromatic diazanaphthalene core.

Non-alcoholic steatohepatitis (NASH): an inflammatory liver disease marked by the accumulation of fat in the organ, often accompanied by fibrosis.

T helper (Th)17 cells: a subset of pro-inflammatory $CD4^+$ T cells so-called due to their characteristic production of the pro-inflammatory cytokine interleukin (IL)-17.

Th2-like: a generally anti-inflammatory condition resembling that generated by T helper (Th)2 cells, a subset of $CD4^+$ T cells characterized by their production of the cytokines interleukin (IL)-4, IL-5, IL-10, and IL-13.

Toll-like receptor: a class of single-pass membrane-spanning receptor proteins that commonly recognize structurally conserved molecular signals of infection and thus play a significant role in innate immunity.

Clinician‘s box

The physiological concentration of plasma and serum succinate in healthy human individuals can vary from 2 to 20 µM [11] and 2 to 30 µM [9, 45], respectively. Such concentrations under physiological conditions are insufficient to activate SUCNR1 signaling (EC_{50} for succinate-induced activation of SUCNR1 is 17-56 µM [45]).

A several-fold increase in succinate concentration in local tissues and biological fluids can be detected in patients diagnosed with rheumatoid arthritis (RA) (74 fold increase in the synovial fluid in RA patients) [72], myocardial infarction (succinate concentration in blood: 2.73 ± 0.11 µM vs 1.38 ± 0.14 µM control) [73], obesity (succinate in serum of obese 43.93 ± 6.16 µM vs. 23.2 ± 1.57 µM in lean individuals) [74], lung cancer (serum concentration of lung cancer patients: 0.53 ± 0.03 mM vs 0.30 ± 0.02 mM in healthy individuals) [21], and Crohn’s disease (2 fold increase of succinate in serum) [75]. Additionally, increased expression of SUCNR1 mRNA and protein is characteristic in the intestines during Crohn’s disease [75] and lung cancer tissue [21].

Therapeutically, direct brain succinate infusion (12 mmol/L) by microdialysis catheters in patients with traumatic brain injury enhances brain metabolism by recycling NADH to NAD^+ leading to improved post-injury recovery [76].

Diagnostically, ^{13}C-labeled succinate and ^{99m}Tc- and ^{18}F-labelled SUCNR1 compounds [77], which allow for human *in vivo* nuclear magnetic resonance (NMR) or positron emission tomography (PET) tracing of succinate and SUCNR1 expression (e.g. in the CNS), might open the possibility of using both succinate and SUCNR1 as putative biomarkers of chronic inflammatory disease, such as progressive multiple sclerosis [76].

Table 1. SUCNR1 expression and signaling pathways in neural and immune cells in rodents and humans.

Cell type (SUCNR1 expression)	Function of SUCNR1	Activated G protein	Activated Pathway	Studied model	References
Retinal ganglion cells	Increased expression of pro-angiogenic factors (*Vegf, Ang1, Ang2*) and pro-inflammatory cytokines (*IL-1β, IL-6*); increased secretion of PGE2 and VEGF	unknown	MAPK-ERK1/2; PGE_2-EP_4-dependent mechanism	*Ex vivo Sucnr1*$^{-/-}$ mice *In vitro* rat RGC-5 cells	[29]
	Vascularization in diabetic retinopathy mediated by VEGF induction and secretion	unknown	MAPK-ERK1/2 - C/EBP β (c-Fos, HIF-1α); MAPK-ERK1/2 – COX2 – PGE_2	*In vitro* rat RGC-5 cells *In vitro* rat primary retinal ganglion cells	[25, 26, 59]
	Retinal angiogenesis in development, ischemia, and proliferative ischemia; production of pro-angiogenic factors AGP1, AGP2, and VEGF	unknown	unknown	*In vitro* rat RGC-5 cells	[27]
	Axonal growth and visual system development	unknown	MAPK-ERK1/2	*Ex vivo Sucnr1*$^{-/-}$ mice	[78]
Cortical neurons	*unknown*	unknown	unknown	*Ex vivo Sucnr1*$^{-/-}$ mice	[29]
Retinal pigment epithelium (RPE)	Iron homeostasis; VEGF secretion; increased expression in juvenile hemochromatosis	Possibly Gαi	BMP6-Smad1/5/8-pSmad5	*In vitro* primary mouse RPE cells *In vitro* human RPE cell lines ARPE-19 and HRPE	[30, 31]
Astrocytes	Increased expression of pro-angiogenic factors (*Vegf, Ang1, Ang2*) and pro-inflammatory cytokines (*IL-1β, IL-6*); increased secretion of PGE2	unknown	PGE_2-EP_4-dependent mechanism	*In vitro* rodent brain cortex astrocytes	[29]
Neural stem cells	Increased succinate uptake; increased *Slc13A3/5* and *Ptgs2*;	unknown	P-p38 MAPK	*In vivo* mice EAE model *In vitro* mouse NSCs/iNSCs	[32]

	release of PGE2				
Erythroblasts (EBs) (mRNA, protein)	unknown	unknown	unknown	*In vitro* human EBs from hematopoietic $CD34^+$ cells	[37]
Platelets (mRNA, protein)	Stimulates aggregation, enhances platelets-derived eicosanoid release	$G\alpha_I$ and $G\beta\gamma$	cAMP–PKA pathway Src kinase activation and $PI3K\beta$/Akt1	*In vitro* human platelets	[14, 71]
T cells ($CD4^+$) (protein)	Possibly regulates T cell-dependent B cell activation	unknown	unknown	*Ex vivo* human umbilical cord blood	[37] [38]
B cells ($CD8^+$)(protein) B cells ($CD19^+$, IgD^+CD27^-, IgD^+CD27^+, and IgD^-CD27^+)(protein)	IgG and IgM secretion in synergy with IL-10 from naïve B cells	unknown	unknown	*Ex* vivo human blood from patients with systemic lupus erythematosus	
Immature monocyte-derived dendritic cells (iMoDCs) (mRNA)	Promotes chemotaxis, pro -inflammatory augmentation, T cell activation enhancement	$G\alpha_q$	MAPK-ERK1/2	*Ex vivo* human iMoDCs *Ex vivo Sucnr1*$^{-/-}$ mice DCs	[40]
Bone marrow-derived DCs (BMDCs)(mRNA)	Promotes pro-inflammatory phenotype, and chemotaxis	unknown	unknown	*In vitro* mice BMDCs *In vivo Sucnr1*$^{-/-}$ mice antigen-induced arthritis (AIA) model	[41]
Bone marrow-derived macrophages (BMDMs)	Modulates inflammatory response	unknown	unknown	*In vivo* Sucnr1$^{-/-}$ mice model *In vitro* mice BMDMs	[39, 43, 46]
Monocyte-derived macrophages (M2 [IL-4], M1 [INFy + LPS]) (mRNA)	Enhances chemotaxis in the WAT	unknown	unknown	*In vitro* human blood monocytes	[43]
Peripheral blood mononuclear cells (M2 [IL-4, IL-13]) (mRNA)	Decreases the expression of IL-10, TLR4 and TLR5 while upregulating TNF-1α	unknown	unknown	*Ex vivo* human blood mononuclear cells	[45]
Adipose tissue macrophages (ATMs)(mRNA, protein)	Promotes anti-inflammatory phenotype via IL-4	$G\alpha_s$	PKA–CREB–KLF4 pathway	*In vivo* LysM-Cre *Sucnr1*$^{fl/fl}$ mice *In vitro* human monocyte THP1 cell line	[16]

Tumor-associated macrophages (TAMs)(mRNA, protein)	Promotes tumor metastasis via IL-6	unknown	PI3K-HIF-1a pathway	*In vivo* mice cancer models *In vitro* mouse peritoneal macrophages *In vitro* mice *Sucnr1*$^{-/-}$ peritoneal macrophages	[21]
Mast cells (MCs)(mRNA)	Hyperreactive phenotype of Sucnr1$^{-/-}$ MCs	unknown	unknown	*In vitro* human MCs *In vitro* mouse bone marrow derived MCs *In vitro* mice *Sucnr1*$^{-/-}$ dermatitis model	[42]

Resources

[I] See patent WO2018167800 - FUSED BICYCLIC COMPOUNDS, COMPOSITIONS AND APPLICATIONS THEREOF; https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2018167800.

[II] See patent WO2019053731 - SUCCINATE-REGULATING POLYPEPTIDES AND USE THEREOF; https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2019053731.

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