Localization of SUCLA2 and SUCLG2 subunits of succinyl CoA ligase within the cerebral cortex suggests the absence of matrix substrate-level phosphorylation in glial cells of the human brain

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Abstract We have recently shown that the ATP-forming SUCLA2 subunit of succinyl-CoA ligase, an enzyme of the citric acid cycle, is exclusively expressed in neurons of the human cerebral cortex; GFAP- and S100-positive astroglial cells did not exhibit immunohistoreactivity or in situ hybridization reactivity for either SUCLA2 or the GTP-forming SUCLG2. However, Western blotting of post mortem samples revealed a minor SUCLG2 immunoreactivity. In the present work we sought to identify the cell type(s) harboring SUCLG2 in paraformaldehyde-fixed, free-floating surgical human cortical tissue samples. Specificity of SUCLG2 antiserum was supported by co-localization with mitotracker orange staining of paraformaldehyde-fixed human fibroblast cultures, delineating the mitochondrial network. In human cortical tissue samples, microglia and oligodendroglia were identified by antibodies directed against Iba1 and myelin basic protein, respectively. Double immunofluorescence for SUCLG2 and Iba1 or myelin basic protein exhibited no co-staining; instead, SUCLG2 appeared to outline the cerebral microvasculature. In accordance to our previous work there was no co-localization of SUCLA2 immunoreactivity with either Iba1 or myelin basic protein. We conclude that SUCLG2 exist only in cells forming the vasculature or its contents in the human brain. The absence of SUCLA2 and SUCLG2 in human glia is in compliance with the presence of alternative pathways occurring in these cells, namely the GABA shunt and ketone body metabolism which do not require succinyl CoA ligase activity, and glutamate dehydrogenase 1, an enzyme exhibiting exquisite sensitivity to inhibition by GTP.

Keywords Krebs cycle · Citric acid cycle · Substrate-level phosphorylation · Succinyl CoA ligase · Microglia · Oligodendrocytes

Introduction

Succinyl CoA ligase (SUCL) is a heterodimeric enzyme, composed of an invariant α subunit encoded by SUCLG1 and a substrate-specific β subunit, encoded by either SUCLA2 or SUCLG2. This dimer combination results in either an ATP-forming (EC 6.2.1.5) or a GTP-forming
SUCL (EC 6.2.1.4). The enzyme catalyzes the conversion of succinyl-CoA and ADP (or GDP) to CoASH, succinate and ATP (or GTP) in the mitochondrial matrix, as part of the citric acid cycle (Johnson et al. 1998a). ΔG for this reaction is 0.07 kJ/mol and therefore it is reversible (Li et al. 2013).

In frequently used laboratory animals such as rodents, SUCLA2 and SUCLG2 exhibit quantitative differences in a tissue-specific expression, as opposed to humans where differential expression in some tissues appears to be qualitative (Johnson et al. 1998b; Lambeth et al. 2004). Characteristically, in the human brain SUCLA2 is found only in the neurons, while SUCLG2 is not found either in neurons or in glial fibrillary acidic protein (GFAP)- and S100-positive astroglial cells (Dobolyi et al. 2013).

Mindful of the weak immunoreactivity for SUCLG2 in Western blotting experiments from post mortem human brain samples (Dobolyi et al. 2013), we sought to identify the cell type(s) harboring mitochondria with this protein. In our previous study, we identified neurons and astrocytes using the markers Nissl and GFAP/S100, respectively (Dobolyi et al. 2013). In the present work we extended our investigation by including the microglial marker ionized calcium-binding adapter molecule 1 (Iba1) and oligodendroglial marker myelin basic protein.

Materials and methods

Cell cultures Fibroblast culture from skin biopsies from a control subject was prepared. Cells were grown on poly-L-ornithine coated 25 mm round glass coverslips for 5–7 days, at a density of approximately $8 \times 10^5$ cells/coverslip in RPMI1640 medium (GIBCO, Life technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 2 mM glutamine and kept at 37 °C in 5% CO₂. The medium was also supplemented with penicillin, streptomycin and amphotericin (item A5955, Sigma-Aldrich St. Louis, MO, USA).

Immunocytochemistry of cell cultures Fibroblasts cultures were first treated with 1 μM Mitotracker Orange (MTO) for 5 min in their culture media, at 37 °C in 5% CO₂. Subsequent immunocytochemistry of the cultures was performed by fixing the cells with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, followed by permeabilization by 0.1% TX-100 (in PBS) for 10 min and several washing steps in between with PBS at room temperature. Cultures were treated with 10% donkey serum overnight at 4 °C followed by bathing in 1% donkey serum and 0.175 μg/ml anti-SUCLG2 (Atlas Antibodies AB, AlbaNova University Center, Stockholm, Sweden, Cat. No. HPA046705) for 1 h, at room temperature. This antibody was raised by immunizing rabbits with a recombinant fragment corresponding to the amino acid region 363–432 of human SUCLG2 (NP_003839). Therefore, it should not be able to distinguish among transcript variants (Dobolyi et al. 2013).

Human brains Human brain samples were collected in accordance with the Ethical Rules for Using Human Tissues for Medical Research in Hungary (HM 34/1999) and the Code of Ethics of the World Medical Association (Declaration of Helsinki). Surgical brain samples were obtained from tissue removed during brain surgeries at the National Institute of Clinical Neurosciences, Budapest, Hungary in the framework of the Human Brain Tissue Bank and Laboratory, Semmelweis University, Budapest. Prior written informed consent was obtained from the patients that included the request to conduct neurochemical analyses. The protocols including analyses of tissue samples were approved by institutional ethics committee of the Semmelweis University. The surgical patients underwent the removal of brain tumors. The medical history of the subjects was obtained from medical or hospital records, interviews with family members and relatives, as well as from pathological and neuropathological reports. All personal identifiers had been removed and samples were coded before the analyses of tissue. Immunolabeling was performed using frontal cortical sample of a 58 years old man and temporal cortical samples from 64 to 66 years old women.

Tissue collection for immunolabeling For immunocytochemistry, brains were cut into 5–10 mm thick coronal slices and immersion fixed in 4% paraformaldehyde in 0.1 M PBS for 3–5 days. Subsequently, the blocks were transferred to PBS containing 0.1% sodium azide for 2 days to remove excess paraformaldehyde. Then the blocks were placed in PBS containing 20% sucrose for 2 days for cryoprotection, after which the blocks were frozen and cut into 50 μm thick serial coronal sections on a sliding microtome. Sections were collected in PBS containing 0.1% sodium azide and stored at 4 °C until further processing.

Double labeling of SUCLA2 and SUCLG2 with glial markers in brains sections Every fifth free-floating brain section of human temporal and frontal cortical blocks was immunostained for SUCLA2, SUCLG2, and for glial markers. First, the glial markers were applied. Oligodendrocytes were labeled with a mouse anti-myelin basic protein antibody (1:100, Abcam, Cambridge, UK; cat. number: ab24567) while microglia were stained with goat anti-Iba1 (1:500, Abcam; cat. number: ab107159). Thus, the sections were incubated in the primary antibodies for 24 h at room temperature, then in Alexa
594 donkey anti-mouse or anti-goat secondary antibodies (1:500; Molecular Probes, Eugene, OR) for 2 h and washed. Subsequently, rabbit anti-SUCLA2 (Proteintech Europe Ltd, Manchester, UK, Cat. No. # 12627-1-AP) and anti-SUCLG2 antisera were applied using 1:1,000 dilution followed by incubation of the sections in biotinylated anti-rabbit secondary antibody (1:1,000 dilution; Vector Laboratories, Burlingame, CA) and then in avidin-biotin-peroxidase complex (1:500; Vector Laboratories) for 2 h. Subsequently, the labeling was visualized by incubation in fluorescein isothiocyanate (FITC)-tyramide (1:8,000) and H2O2 in 100 mM Trizma buffer (pH 8.0 adjusted with HCl) for 6 min. Finally, all sections were mounted on positively charged slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) and coverslipped in antifade medium (Prolong Antifade Kit, Molecular Probes).

Image processing Fluorescent sections were evaluated using a Bio-Rad 2100 Rainbow Confocal System (Bio-Rad Laboratories, Inc, CA, USA). For the human brain specimens, pictures were taken at the frequencies of the two dyes and also in the far red range. This latter image was subtracted from both of the other two images in order to cancel-out significant autofluorescence of the sections which was detected in all channels. Subsequently, the contrast and sharpness of the images were adjusted using the “levels” and “sharpness” commands in Adobe Photoshop CS 8.0. Full resolution was maintained until the photomicrographs were finally cropped at which point the images were adjusted to a resolution of 300 dpi.

Results

The specificity of SUCLG2 immunoreactivity in human fibroblast cultures

We have used a new antibody directed against the human SUCLG2 protein that does not distinguish among various isoforms (see “Materials and Methods”) as in (Dobolyi et al. 2013). The specificity of SUCLG2 immunoreactivity was validated using human fibroblast cells, see Fig. 1. The mitochondrial network was selectively stained by loading cells with Mitotracker Orange (MTO, 1 μM) prior to fixation (panel a). Fibroblasts were subsequently labeled with the procedure of SUCLG2 immunostaining (panel b), and there was a co-localization with the decoration obtained by MTO (panel c). Note that only a minority of the mitochondrial network was stained by the SUCLG2 antiserum, as opposed to SUCLA2 immunoreactivity in the same cells, in which the majority of the mitochondrial network was stained (Dobolyi et al. 2013).
SUCLA2 and SUCLG2 labeling in the cerebral cortex

SUCLA2 immunoreactivity appeared in cells located throughout the cerebral cortex (Fig. 2a). The cellular distribution of SUCLA2 labeling suggested that SUCLA2 immunoreactivity is restricted to mitochondria (see Fig. 3a), exactly as described by us previously (Dobolyi et al. 2013). Furthermore, the distribution of SUCLA2-immunoreactive cells corresponded to that of neurons in accordance with the previously described neuronal expression of this protein. In these sections, the walls of large blood vessels were also labeled with SUCLA2, see upper left corner of figure panel 2a. The distribution of SUCLA2 immunoreactivity differed markedly from that of myelin basic protein as the latter one was striking in the white matter and labeled radially arranged structures in accordance with the established localization of myelinated fibers within the cortex (Fig. 2b). Indeed, double labeling confirmed the absence of co-localization between SUCLA2 and myelin basic protein (Figs. 2c, 3b). The distribution of Iba1-labeled microglial cells was relatively even within the examined brain section corresponding to the role of this cell type in immune defense (Fig. 2e). Microglial cells had small somata and several processes (Fig. 3a). Thus, the morphology of SUCLA2 and Iba1-labeled cells was different. Consistent with this, no co-localization of these markers were found in any cortical regions examined (Fig. 3a).

**Fig. 2** Immunocytochemical identification of succinyl CoA ligase subunits, oligodendroglia, and microglia in the human cerebral cortex. The distribution of SUCLA2 (a), myelin basic protein (b), SUCLG2 (d) and Iba1 (e) immunoreactivities are shown in low magnification. c Double labeling (panels a and b combined) show somewhat overlapping but still fundamentally different distributions of SUCLA2 and myelin basic protein. f Double labeling (panels d and e combined) demonstrate the distinct distributions of SUCLG2 and Iba1 in the cerebral cortex. Scale bar= 500 μm
SUCLG2 immunoreactivity was found in all layers of the human cerebral cortex. Its distribution was, however, characteristic of blood vessels suggesting endothelial and/or pericytic localization. However, we must note that perivascular staining due to the presence of SUCLG2 in astrocyte end-feet is a viable possibility. The distribution of SUCLG2 immunoreactivity was clearly different from that of myelin basic protein (Fig. 2b) and Iba1-positive microglia (Fig. 2e), which was also confirmed by double labeling (Fig. 2f). Analysis of high magnification images revealed that SUCLG2 immunoreactivity present in structures resembling mitochondria appeared in capillary-forming cells. This is best depicted in the lower left corner (two white arrowheads) and upper right corner (single white arrowhead) of figure panel 3d; note the SUCLG2 decoration pattern forming a tubular structure around the red blood cells (stained green because of the immunohistochemical procedure, detailed in “Materials and Methods”) in a longitudinal (lower left corner) and cross-section (upper right corner) mode delineating a capillary. No co-localization was found between SUCLG2 and the microglia marker Iba1 (Fig. 3c) and oligodendroglial marker myelin basic protein (Fig. 3d). On the other hand, SUCLG2 expression has been demonstrated in microglial cells of post-mortem brains of Alzheimer’s disease patients and age-matched controls (Ramirez et al. 2014).

In humans, SUCLG2 exhibits transcript variants. However, the possibility that the human glial transcript variant of SUCLG2 is not recognized by existing antibodies has been ruled out in (Dobolyi et al. 2013): all transcripts are identical in the region 1–396, except in position 220 (Dobolyi et al. 2013). The antibody used in (Dobolyi et al. 2013) to identify SUCLG2 was raised by immunizing rabbits with a recombinant fragment corresponding to a region within the N terminal amino acids 1–204 of human SUCLG2 which is common among all transcript variants. Furthermore, in (Dobolyi et al. 2013), PCR and in situ hybridization for all known variants have been performed for human brain material; SUCLG2 mRNA was deduced to be very scarce in the human brain, while the same probes tested robustly positive in human fibroblasts.

**Discussion**

The present article is a follow-up on the lead provided by our previous work showing that SUCLG2 is very weakly expressed in the human brain, and it is not found in either neurons or astrocytes (Dobolyi et al. 2013). Here, the most important finding is that SUCLG2 expression was further confirmed to be absent from microglia and oligodendroglia, but it was detected in cells forming the microvasculature, most likely endothelial cells and/or pericytes.
An additional finding is that SUCLG2 staining in human fibroblasts covered only a small fraction of the mitochondrial network. On the other hand, SUCLA2 staining covered the majority of mitochondria in the same cells (Dobolyi et al. 2013). It would be interesting to see a triple-colocalization study of SUCLG2, SUCLA2 and mitotracker orange in the same cell; a non-overlapping SUCLA2-SUCLG2 staining would signify the presence of two different mitochondrial populations regarding succinyl CoA ligase activity, one representing the ATP-forming population, and the other the GTP-forming tier. This would not be too surprising; ATP is associated with catabolic pathways, while GTP with anabolism (Pall 1985). GTP is very valuable in the mitochondrial matrix, as it cannot be transported through the inner mitochondrial membrane of higher organisms (Pfaff et al. 1986) because it cannot be processed further by the irreversible α-ketoglutarate dehydrogenase complex (Chinopoulos 2013). When succinyl CoA ligase operates in the opposite direction towards ATP or GTP formation (a process termed ‘substrate-level phosphorylation’), it yields succinate that can be either further processed by succinate dehydrogenase, and advance in the citric acid cycle, or act as a metabolic signal in inflammation (Mills and O’Neill 2014).

In the human brain, there are at least two biochemical pathways that can mediate a by-pass of succinyl CoA ligase: i) the ‘GABA shunt’, and ii) ketone metabolism.

The ‘GABA shunt’ (Fig. 4) is a pathway commencing from glutamate (which could arise from α-ketoglutarate through glutamate dehydrogenase) being converted to GABA by the cytosolic glutamate decarboxylase (4.1.1.15), encoded by either GAD65 or GAD67 (Soghomonian and Martin 1998). GABA may also arise by metabolism of putrescine (Sequerra et al. 2007; Jakoby and Fredericks 1959; De Mello et al. 1976; Seiler et al. 1973), or enter the cytoplasm from the extracellular space through transporters in the plasma membrane. GABA transaminates with glutamate to yield succinate, and thus enter the citric acid cycle. Succinate semialdehyde will get dehydrogenated by the NAD+-dependent succinate semialdehyde dehydrogenase (EC 1.2.1.24), encoded by aldehyde dehydrogenase 5 family, member A1 (ALDH5A1) to yield succinate, and thus enter the citric acid cycle. Succinate semialdehyde can also be converted to gamma-hydroxybutyrate by succinic semialdehyde reductase, encoded by AKR7A2 (Picklo et al. 2001), an enzyme that is primarily localized to astrocytes and microglia of the human cerebral cortex, hippocampus and midbrain (Picklo et al. 2001; Ris and von Warburg 1973; Hoffman et al. 1980).

The enzymes required for a complete ‘GABA shunt’ are present in glial cells in the adult human brain; astrocytes express glutamate dehydrogenase (both isoforms 1 and 2, see below) (Spanaki et al. 2014), GAD67 (but not GAD65), GABA-T as well as the GABA_A and GABA_B receptors (Lee et al. 2011a; Schwab et al. 2013) reviewed in (Velez-Fort et al. 2012). The intensity of immunostaining for GAD67 and GABA-T in these cells is comparable or greater to that observed for known inhibitory neurons. The GABA transporter GAT-3 is exclusively expressed in astrocytes (Minelli et al. 1996); GAT-1 and -2 are found in astrocytic processes and other neuronal- and non-neuronal elements (Conti et al. 1998, 1999). As a result of the concerted action of these enzymes and transporters, cultured human astrocytes maintain an intracellular GABA level of 2.32 mM (Lee et al. 2011b). On the same line, cultured human microglia express the mRNA and protein for GABA-T, in

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Fig. 4 Illustration of the pathways bypassing succinyl CoA ligase in human glia. KGDH ketoglutarate dehydrogenase complex, GLUD glutamate dehydrogenase, GAD glutamate decarboxylase, GABA γ-aminobutyric acid, EC space extracellular space, GABA-T GABA transaminase, SSA succinate semialdehyde, SSADH succinate semialdehyde dehydrogenase, SSAR succinate semialdehyde reductase, GHB γ-hydroxybutyric acid, SDH succinate dehydrogenase, OXCT1 succinyl-CoA:3-ketoacid-coenzyme A transferase 1
addition to GABA_A and GABA_B receptors (Lee et al. 2011a). The NAD⁺-dependent succinate semialdehyde dehydrogenase encoded by ALDH5A1 is expressed in both neurons and glial cells in the adult human brain (http://www.proteinatlas.org/ENSG00000112294/tissue).

Regarding ketone bodies metabolism, it is well established that 3-hydroxybutyrate and acetoacetate can replace glucose as the major source of brain energy in situations such as starvation (Robinson and Williamson 1980). Ketone bodies are not only a product of the liver, but also of astrocytes: astrocytes produce ketone bodies from fatty acids (Auestad et al. 1991), and the branched-chain amino acids valine, isoleucine and leucine (Bixel and Hamprecht 1995). A critical enzyme in ketone body metabolism is the mitochondrial succinyl-CoA:3-ketoacid-coenzyme A transferase 1 (EC 2.8.3.5) (Hersh and Jencks 1967), encoded by OXCT1. This enzyme catalyzes the reversible transfer of CoA from succinyl CoA to acetoacetate yielding acetoacetyl CoA and succinate (Fig. 4). OXCT1 is expressed in both neurons and glial cells in the human brain (http://www.proteinatlas.org/ENSG00000083720/tissue). From the reaction catalyzed by succinyl-CoA:3-ketoacid-coenzyme A transferase 1, it is apparent that there can be interconversion of succinate to succinyl CoA and vice versa, obviating the need for succinyl CoA ligase.

From the above, it is evident that human glia can accommodate for the lack of succinyl CoA ligase with the ‘GABA shunt’ and/or ketone metabolism, and thus maintain production of reducing equivalents by the citric acid cycle. However, it would be erroneous to consider that the ‘GABA shunt’ and ketone bodies metabolic pathways evolved in these cells in response to the lack of succinyl CoA ligase. Glia benefit from shuttling succinyl CoA towards GABA formation and ketone body metabolism: i) GABA is an effective immunomodulatory molecule (Jin et al. 2013); in human astrocytes and microglia, GABA suppresses the reactive response of the inflammatory stimulants lipopolysaccharide and interferon-γ by inhibiting the induction of inflammatory pathways mediated by NFkB and P38 MAP kinase (Lee et al. 2011a); ii) Ketone bodies produced by the astrocytes may promote neuroprotection by supplying them to the neurons (Guzman and Blazquez 2001).

Alternatively, in the absence of succinyl-CoA ligase, succinyl-CoA emerging from the α-ketoglutarate dehydrogenase complex in glial mitochondria could serve as cofactor for lysine succinylation, a wide-spread post-translational modification (Zhang et al. 2011), or advance towards porphyrin synthesis. Furthermore, the absence of a GTP-forming succinyl CoA ligase and, likewise, of an ATP-forming succinyl CoA ligase, since ATP can transphosphorylate to GTP by the concerted action of SUCL with a mitochondrial isoform of a nucleotide diphosphate kinase known as nm23-H4 (Kadmas et al. 1991; Kowluru et al. 2002)) is compatible with the high level of expression of glutamate dehydrogenase (GLUD) in human glia (Plaitakis and Zaganas 2001): In the human brain, GLUD is encoded by either GLUD1 or GLUD2 (Plaitakis and Zaganas 2001). GLUD1 is potently inhibited by GTP (IC₅₀=0.20 μM), (Plaitakis and Zaganas 2001). GLUD2 is refractory to GTP, but it is strongly activated by ADP and leucine (Spanaki et al. 2012). However, GLUD2 expression is much lower than GLUD1 (Zaganas et al. 2012). Both GLUD isoforms are mostly expressed in the astrocytes; oligodendroglia or myelinated fibers do not express GLUD2 (Spanaki et al. 2010). To the best of our knowledge, there are no published data on GLUD2 expression in microglia.

Although the above considerations in conjunction with our results point to the direction of a truncated citric acid cycle in human glia, experimental evidence that support or refute this concept in glia within the adult human brain are as of yet, lacking; substrate-specific metabolism of in situ astrocytic mitochondria deduced by isotopomeric analysis of 13C distributed among various metabolites has been extensively addressed but only in cell-specific cultures from rodent brain originating from fetal materials that mature on entirely artificial conditions (Westergaard et al. 1994; Schousboe et al. 1997; Hassel 2000; McKenna and Sonnewald 2005). Therefore, extrapolations from such experiments on what could be actually happening in glia within the adult human brain—as suggested in Fig. 4—must be exercised with caution.

A potential ‘downside’ for glial cells lacking the capacity for nucleotide-forming SUCL (substrate-level phosphorylation) is that their mitochondria are likely to engage in cytosolic ATP consumption (Chinopoulos et al. 2010; Kiss et al. 2013, 2014; Chinopoulos 2011a), during natural fluctuations of their membrane potentials (Gerencser et al. 2012) should they become depolarized. By the same token, glial mitochondria should also be vulnerable in terms of relying on in-house- ATP reserves during energy crisis, such as during brain ischemia (Chinopoulos 2011b; Chinopoulos and Adam-Vizi 2010).

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Conflict of interest The authors declare no conflict of interest.

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