1

2

3

4 **Selective linkage of mitochondrial enzymes to intracellular**
5 **calcium stores differs between human induced pluripotent**
6 **stem cells, neural stem cells and neurons**

7

8 Huanlian Chen¹, Ankita Thakkar¹, Abigail C. Cross¹, Hui Xu¹, Aiqun Li^{2,4}, Dan Pauli²,
9 Scott A. Noggle², Laken Kruger³, Travis T. Denton³, Gary E. Gibson^{1*}

10

11

12 ¹ Burke Neurological Institute, Brain and Mind Research Institute, Weill Cornell
13 Medicine, White Plains, NY

14 ² The New York Stem Cell Foundation Research Institute, New York, NY

15 ³ Department of Pharmaceutical Sciences, Washington State University, College of
16 Pharmacy and Pharmaceutical Sciences, Spokane, WA

17 ⁴ Current address: Department of Genetics and Genomic Sciences, Icahn School of
18 Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY

19

20 *To whom correspondence should be directed

21 Gary E. Gibson, Brain Mind Research Institute, Weill Cornell Medical College, Burke
22 Neurological Institute, 785 Mamaroneck Avenue, White Plains, NY, USA. 10605

23

24 ggibson@med.cornell.edu; TEL 914-597-2291

25

26

27

28

29 **Abbreviations used:**

30 AP: alkaline-phosphatase

31 APOE4: epsilon 4 allele of Apolipoprotein E gene

32 AD: Alzheimer's disease

33 *t*-BHP: *tert*-butyl-hydroperoxide

34 BRCS: bombesin or bradykinin releasable calcium stores

35 BSS: balanced salt solution

36 CESP: carboxyethyl succinyl phosphonate

37 DMAP: dimethyl acetyl phosphonate

38 ER: endoplasmic reticulum

39 HBSS: Hank's Balanced Salt Solution

40 IP₃: inositol 1,4,5-trisphosphate

41 IP₃R: inositol 1,4,5-trisphosphate receptors

42 iPSC: induced pluripotent stem cells

43 KGDHC: alpha-ketoglutarate dehydrogenase complex

44 NEM: neural expansion media

45 NIM: neural induction media

46 NSC: neural stem cells

47 PDHC: pyruvate dehydrogenase complex

48 PS-1: presenilin-1

49 PTP: permeability transition pore

50 RRID: research resource identifier (see scicrunch.org)

51 RyR: ryanodine receptors

52 SNAP: S-nitroso-N-acetylpenicillamine

53 SP: trisodium succinyl phosphonate

54 STIM: stromal interaction molecule

55 TCA: tricarboxylic acid cycle

56

57 **Abstract**

58 The coupling of the endoplasmic reticulum (ER) with mitochondria modulates
59 neuronal calcium signaling. Whether this link changes with neuronal development is
60 unknown. The current study first determined whether ER calcium stores are similar
61 during development of human neurons, and then tested if the ER/mitochondrial coupling
62 varied with development. The release of ER calcium to the cytosol by the IP₃ agonist
63 bradykinin was determined in human induced-pluripotent stem cells (iPSC), neural stem
64 cells (NSC) and neurons. The concentration dependence for the release of ER calcium
65 was similar at different stages of development. Metabolism changes dramatically with
66 development. Glycolysis is the main energy source in iPSC and NSC whereas
67 mitochondrial metabolism is more prominent in neurons. To test whether the coupling of
68 mitochondria and ER changed with development, bombesin or bradykinin releasable
69 calcium stores (BRCS) were monitored after inhibiting either of two key mitochondrial
70 enzyme complexes: the alpha-ketoglutarate dehydrogenase complex (KGDHC) or the
71 pyruvate dehydrogenase complex (PDHC). Inhibition of KGDHC did not alter BRCS in
72 either iPSC or NSC. Inhibition of PDHC in neurons diminished BRCS whereas decreased
73 KGDHC activity exaggerated BRCS. The latter finding may help understand the
74 pathology of Alzheimer's disease (AD). BRCS is exaggerated in cells from AD patients
75 and KGDHC is reduced in brains of patients with AD. In summary, a prominent
76 ER/mitochondrial link in neurons is associated with selective mitochondrial enzymes.
77 The ER/mitochondrial link changes with human neuronal development and plausibly
78 links ER calcium changes to AD.

Key words: Alzheimer's disease, mitochondria, endoplasmic reticulum, development, stem cells, alpha-ketoglutarate dehydrogenase complex, pyruvate dehydrogenase complex, calcium stores, tricarboxylic acid cycle

Introduction

The physiological and morphological connections between mitochondria and the endoplasmic reticulum (ER) are critical in the regulation of neuronal calcium signaling (Csordás *et al.* 1999; Hajnóczky *et al.* 1994; Rizzuto *et al.* 1993; Rizzuto *et al.* 1998; Karagas & Venkatachalam 2019) and in Alzheimer's disease (AD). The IP₃ receptors (IP₃R) on the ER appear to be in close proximity to the mitochondria (Seitaj *et al.* 2018). The relative reliance on mitochondria and glycolysis varies in human induced pluripotent stem cells (iPSC), neural stem cells (NSC) and neurons. iPSC and NSC are very glycolytic, whereas metabolism in neurons is more mitochondrial (Intlekofer & Finley 2019; Khacho *et al.* 2019). However, little is known about calcium signaling during various stages of development of human neurons. Thus, these experiments test whether cytosolic calcium in human iPSC, NSC or neurons increases similarly in response to potassium depolarization or to IP₃ agonists such as bradykinin (BRCS).

Considerable data suggest that releasable ER calcium stores are abnormal in AD. The calcium stores that are released by agonists that increase IP₃ formation, such as bradykinin or bombesin are exaggerated in cells from AD patients, as well as in cell and animal models of AD. Bombesin or bradykinin releasable calcium stores (BRCS) are exaggerated in fibroblasts from patients with both genetic and non-genetic forms of AD (Ito *et al.* 1994), cultured fibroblasts from presenilin-1 (PS-1) knock-in mice (Leissring *et al.* 2000) and cultured neurons from 3×Tg AD mice (Stutzmann *et al.* 2004; Stutzmann *et*

al. 2006). The abnormal proteins resulting from presenilin mutations interact with the IP₃ receptor Ca²⁺ release channel and alter its stimulatory effect in response to IP₃ (Cheung *et al.* 2008; Cheung *et al.* 2010). These calcium changes are likely pathologically important, therefore, modulating Ca²⁺ release is a reasonable therapeutic target (Schrack *et al.* 2019).

The activities of key mitochondrial enzymes decline in AD and these abnormalities may modulate the mitochondrial/ER interactions that accompany AD. In non-human tissues, inhibition of one of these enzymes, the alpha-ketoglutarate dehydrogenase complex (KGDHC) exaggerates BRCS in an AD-like manner. The precise mechanism is unknown, but reductions in KGDHC diminish GTP (Kiss *et al.* 2013), and GTP is known to modulate ER/mitochondrial interactions (Hajnóczky *et al.* 1994). Furthermore, diminished KGDHC causes release of mitochondrial proteins (Banerjee *et al.* 2016) and post-translational modification of non-mitochondrial proteins (Yang *et al.* 2019). The activity of the pyruvate dehydrogenase enzyme complex (PDHC), which links glycolysis and mitochondrial metabolism, also declines with AD, but the relation to the ER/mitochondrial interactions has never been examined.

Thus, the current experiments test the interactions of KGDHC with BRCS in human neurons at different stages of development and, additionally, compare the consequences of diminished KGDHC and PDHC activities in neurons. The results reveal that the consequences differ between the two enzymes and between human iPSC, NSC and neurons. The results support the hypothesis that treating the mitochondrial deficit related to AD may ameliorate calcium abnormalities in AD.

Results

Generation and characterization of iPSC, NSC and iPSC-derived neurons

All of the cell types were confirmed with their cell-specific markers (iPSCs: OCT4 and SSEA4; NSC: Nestin, SOX2, SOX1 and PAX6, iPSC derived neurons: MAP2, neurofilament and synaptophysin) (Figure-1). The identity of the iPSC-derived neurons was confirmed by depolarization of voltage gated calcium channels by 50 mM KCl. K^+ depolarization increased intracellular calcium response in iPSC-derived neurons, but not in either iPSC or NSC (Figure 2). Thus, both the antibody marker studies and the depolarization indicated that our robust neuronal differentiation protocol yielded highly homogenous neurons.

BRCS in iPSC, NSC and iPSC-derived neurons

The dose response to bradykinin determined whether BRCS varied with differentiation. Cells at each stage of differentiation had a dose dependent response to bradykinin. The patterns of change in iPSC (Figure 3) and neurons (Figure 5) were very similar. The $[Ca^{2+}]_i$ peaked and then went back to the basal $[Ca^{2+}]_i$ within two minutes after bradykinin addition. However, the NSC responded differently than the other cells. The $[Ca^{2+}]_i$ did not return to basal $[Ca^{2+}]_i$ even after 5 min of bradykinin addition (Figure 4).

The consequences of reducing KGDHC on BRCS in iPSC, NSC and iPSC-derived neurons

Cells were treated with the specific KGDHC inhibitor, trisodium succinyl phosphonate (SP, 500 μ M), before adding bradykinin. First, the inhibitory effect of SP on

KGDHC was confirmed with a histochemistry assay. In iPSC, 500 μ M SP inhibited KGDHC activity by 67% after a one-hour treatment and by 75% after 24 hours of treatment (Figure 6). In NSC, SP inhibited KGDHC activity by 89% after one hour of treatment and by 94% after 24 hours of treatment (Figure 7). In iPSC-derived neurons, 500 μ M SP inhibited KGDHC activity by 87% after one hour of treatment and by 83% after 24 hours of treatment (Figure 8). Reducing KGDHC with SP for one hour and 24 hours did not alter BRCS in iPSC (Figure 6) nor NSC (Figure 7). However, reducing KGDHC in iPSC-derived neurons exaggerated BRCS by 69% and 144% after a one-hour or 24-hour treatment, respectively (Figure 8).

Consequences of reducing PDHC on BRCS in iPSC-derived neurons

iPSC-Derived neurons were treated with the specific PDHC inhibitor, dimethyl acetyl phosphonate (DMAP, 5 or 100 μ M) for one hour (Park *et al.* 2000). First, we confirmed the inhibitory effect of DMAP with histochemistry. In these neurons, 5 μ M or 100 μ M DMAP inhibited PDHC activity by 75% and 76%, respectively (Figure 9A). 5 μ M or 100 μ M DMAP inhibited BRCS by 44.8% and 46.4%, respectively (Figure 9).

Consequences of reducing PDHC or KGDHC on resting cytosolic free calcium

The BRCS change in iPSC-derived neurons did not appear to be related to alter resting cytosolic free calcium, since changes in cytosolic free calcium were minimal when reducing KGDHC or PDHC in the absence IP₃ agonists (Figure 10).

Discussion

These experiments are the first to report that the release of ER calcium stores and their relation to mitochondria differ in iPSC, NSC and neurons. Although the cells at all stages of development had IP₃ releasable stores, the patterns were markedly different in NSC compared to iPSC and iPSC-derived neurons. Previous studies only showed the cytosolic calcium changes in iPSC-derived neurons for the study of AD (Prè *et al.* 2014; Duan *et al.* 2014), motor or dopaminergic neurons (Hartfield *et al.* 2014; Dafinca *et al.* 2016) or other neurodegenerative diseases (Hartfield *et al.* 2014; Schöndorf *et al.* 2014; Rabenstein *et al.* 2017; Prè *et al.* 2014). In addition to establishing differing patterns of BRCS at different stages of development, the experiments in this manuscript demonstrate that the mitochondrial link to BRCS varies. Reducing KGDHC in human iPSC-derived neurons exaggerated BRCS, but had no impact in NSC or iPSC. This difference may relate to the fact that mitochondria have a very different role in iPSC, neural stem cells and neurons. iPSC and neural stem cells primarily use glycolysis for energy, whereas neurons depend on mitochondria. BRCS are sensitive to select free radicals, and free radical production is much higher when cells use mitochondria for energy (Khacho *et al.* 2019; Intlekofer & Finley 2019). Thus, differences in ROS may underlie the differences in the sensitivity of the various cell types to inhibition of PDHC or KGDHC.

The cellular basis for the selective effects of inhibiting PDHC and KGDHC on BRCS in neurons is unknown. The selective effect makes it unlikely that this is a consequence of altered mitochondrial buffering of cytosolic calcium. Inhibiting PDHC will block acetyl-coA entry into the tricarboxylic acid cycle, which will deplete the substrates of the tricarboxylic acid cycle. On the other hand, blocking KGDHC may lead

to accumulation of substrates such as alpha-ketoglutarate that leave the mitochondria and serve as signaling molecules throughout the cell. The current results in human neurons confirm our previous studies in rodent neurons that showed that inhibition of KGDHC exaggerates BRCS in an AD-like manner. Our previous studies in mouse cells showed that diminishing KGDHC increased BRCS under conditions that increased the mitochondrial NAD⁺/NADH ratio and elevated cytochrome c release (Gibson *et al.* 2012; Chen *et al.* 2017). The precise mechanism is unknown, but reductions in KGDHC diminish GTP (Kiss *et al.* 2013), and GTP is known to modulate ER-mitochondrial interactions (Hajnóczky *et al.* 1994). Furthermore, diminished KGDHC activity causes release of mitochondrial proteins (Banerjee *et al.* 2016) and post-translational modification of non-mitochondrial proteins (Yang *et al.* 2019). No other studies have examined the consequences of inhibiting PDHC on BRCS.

The relation of the increased BRCS with KGDHC inhibition and the elevation in BRCS in cells from AD patients and models of AD is not clear. The exaggerated BRCS with AD is well documented, and the current experiments further document a connection to KGDHC activity. Since KGDHC activity declines in AD brain, the decline may underlie the BRCS change in AD. The reduced KGDHC activity may also further exaggerate the increase in calcium in the AD cells. The exaggerated release of calcium from ER by activation of either ryanodine receptors (Stutzmann *et al.* 2004; Stutzmann *et al.* 2006) (Flucher *et al.* 1993) or IP₃ receptors (Ito *et al.* 1994) (Chakroborty & Stutzmann 2014; Cheung *et al.* 2008; Del Prete *et al.* 2014) (Popugaeva & Bezprozvanny 2013) (Shilling *et al.* 2014) is among the most reproducible abnormalities that support a critical role of calcium in AD (Peterson C 1985). FAD mutant PS1 (M146L) and PS2

(N141I) interact with the IP₃R Ca²⁺ release channel and exert profound stimulatory effects on its gating activity in response to saturating and sub-optimal levels of IP₃ (Cheung *et al.* 2008). In addition, the membrane hyperpolarization associated with ER-calcium release is enhanced in mutant PS1 mice and reduces spiking activity and responsivity to synaptic inputs (Stutzmann *et al.* 2006; Oddo *et al.* 2003; Stutzmann 2007). The increased calcium leads to premature and severe defects in synaptic plasticity, behavior and cognitive function (Lacampagne *et al.* 2017). The calcium dysregulations seen in AD are not merely accelerated or amplified signaling changes, inevitable in old age, but rather, are novel and pathogenic changes to fundamental calcium signaling patterns (Stutzmann 2007). Reductions in KGDHC activity similar to those in AD would exaggerate these calcium effects in AD.

The lack of understanding of the molecular basis for the exaggerated BRCS in AD tissues makes it difficult to describe a molecular mechanism linking KGDHC and PDHC to the exaggerated BRCS. Our previous studies show that one possibility is the excess oxidants in AD cells. Select oxidants such as *t*-BHP (*tert*-butyl-hydroperoxide) or SNAP (S-nitroso-*N*-acetylpenicillamine) can cause AD-like changes in cytosolic calcium concentrations. AD fibroblasts have higher ROS production and BRCS (with *t*-BHP) than aged controls (Huang *et al.* 2010). Oxidant induced modification of protein thiol groups may underlie the AD-related exaggeration of the BRCS. These oxidants can alter BRCS by activating RyR (ryanodine receptor) transnitrosation of thiol containing proteins (Huang *et al.* 2005; Arnelle & Stamler 1995). Consistent with this idea are recent studies which show post-translational modifications of the ryanodine receptor by phosphorylation, oxidation and nitrosylation in brains of AD patients, and in two murine

models of AD (Lacampagne *et al.* 2017). Whether this is also true of the IP₃ receptor is not known. An increased number of ryanodine receptors appears important in mice with mutated presenilin; increased ROS expression in neurons contributes to increased calcium release, synaptic decline and increased RyR2 expression seen in the hippocampus and cortex. This increased IP₃ receptor-evoked calcium release may involve the increase of stromal interaction molecule 1 (STIM1) expression and the decrease of STIM2 expression in the soma. These STIMs translocate to plasma membrane–ER contact sites, where they bind to Orai1 channels and open a Ca²⁺ influx pathway to slowly replenish ER Ca²⁺ stores using extracellular Ca²⁺ (Mustaly-Kalimi *et al.* 2018; Schrank *et al.* 2019).

The effects of increased cytosolic calcium following release from ER calcium stores on the mitochondria is not clear. Mitochondria serve as an acute calcium buffer, so the exaggerated BRCS likely increases mitochondrial calcium concentration. In heart, increased intracellular Ca²⁺ concentration results in excess uptake of Ca²⁺ by the mitochondria and increased respiratory rate by the TCA cycle. However, this also leads to mitochondrial calcium overload as well as metabolic dysfunction (Schrank *et al.* 2019). Prolonged increases of Ca²⁺ release open the mitochondrial permeability transition pore (PTP) to release apoptotic signaling molecules (e.g., cytochrome C) (Schrank *et al.* 2019; Mustaly-Kalimi *et al.* 2018).

These results demonstrate that the relation of ER calcium to mitochondria changes with development. The results provide evidence in iPSC-derived human neurons that diminishing the activity of KGDHC causes AD-like changes in release of calcium from the endoplasmic reticulum.

259

260

Methods

Culture of iPSC, NSC and iPSC-derived neurons

We selected iPSC that were generated from fibroblasts (AG6842) obtained from the Coriell Institute (Camden, NJ). The fibroblasts were collected from a 75 year-old male donor, who was from a family bearing A246E PS1 mutation, but he did not have the mutation and was APO $\epsilon 3/\epsilon 3$. Fibroblasts were reprogrammed using four high-titer retroviral constructs prepared by the Harvard Gene Therapy Core Facility that encoded human Oct4, KLF4, SOX2 and c-Myc, respectively (Sproul *et al.* 2014). The iPSC colonies were initially selected by morphology, passaged several times to remove transformed cells and expanded before characterization. After iPSCs were expanded to multi-well format, they were characterized using a variety of quality control assays including karyotype and fingerprinting for cell-line genetics, alkaline-phosphatase (AP) enzymatic activity for reprogramming process, immunostaining for pluripotency markers, qPCR for endogenous pluripotent markers and viral transgene silencing, as well as APOE and PSEN1 genotyping (Sproul *et al.* 2014) .

iPSC were cultured on dishes and plates with pre-coating (one hour at 37 °C) of Geltrex matrix solution (1:100 dilution) and the cells were cultured with StemFlex Medium for one or two days (ThermoFisher Scientific, Grand Island NY). iPSC were harvested with StemPro Accutase (ThermoFisher Scientific) for one minute at 37 °C and then cultured with StemFlex medium containing ROCK Inhibitor, Stemolecule Thiazovivin (1 μ M; Reprocell, Beltsville, MD) for the first 24 hours of culture. After 24 hours, the medium was replaced with StemFlex Medium without ROCK Inhibitor, and medium was then exchanged daily. iPSC were cryopreserved in Synth-a-Freeze

cryopreservation medium (ThermoFisher Scientific) and experiments were only performed using iPSC from passage 30 to 35.

NSC were induced from iPSC with a seven-day incubation with PSC Neural Induction Medium (NIM, Neurobasal Medium and Neural Induction Supplement; ThermoFisher Scientific) on Geltrex pre-coated plates. Media was changed daily. After induction, the NSC were harvested with StemPro Accutase for 5 min at 37 °C and cultured with Neural Expansion Medium (NEM, Neurobasal Medium, Advanced DMDM/F-12 and Neural Induction Supplement; ThermoFisher Scientific) containing Rock inhibitor Y27632 (5 µM, Sigma) for the first 48 hours of culture. NSC were cryopreserved in Synth-a-Freeze cryopreservation medium and neural differentiation was only preformed using passage five NSC.

For neuronal differentiation, the dishes or plates were pre-coated with Poly-L-ornithine (100 µg/mL) at 37 °C for two hours and Laminin (3 µg/mL) at 37 °C overnight. iPSC-derived neurons were differentiated from NSC (passage 5) for 28 days with Neuronal Differentiation Medium (Neurobasal Medium, 1 × B-27 supplement with antioxidant, 1 × GlutaMAX supplement, 2 × CultureOne supplement, 200 µM ascorbic acid, 1 × Anti-Anti, 20 ng/mL BDNF, 20 ng/mL GDNF; ThermoFisher Scientific) containing Rock Inhibitor Y27632 (5 µM) for the first 48 hours of differentiation at the seeding density of 2.5×10^4 cell/cm². The media was exchanged partially by removing half of the medium and replacing it with Neuronal Differentiation Medium twice weekly. Cells were typically used for neuronal markers characterization and BRCS after four weeks in culture. Differentiation was done at least 15 times.

Immunostaining of cell specific cell markers

iPSC, NSC and iPSC-derived neurons were cultured on Delta T dishes (Bioprotechs, Butler, PA) at different seeding densities and culture times (iPSC and NSC, 2×10^5 cell/dish for two days; iPSC-derived neurons, 1×10^5 cell/dish for four weeks). After culture, the cells were fixed with Image-iT Fixative Solution (4% formaldehyde; ThermoFisher Scientific) at room temperature for 15 min. After fixation, the cells were rinsed with DPBS (ThermoFisher Scientific) and blocked with blocking buffer (0.1% Triton X-100, 1% BSA in DPBS) at room temperature for one hour. After blocking, the cells were incubated with primary antibodies in blocking buffer at 4 °C overnight. The next day, the cells were rinsed with DPBS and incubated with secondary antibodies in blocking buffer at room temperature for one hour, following by staining with DAPI solution (ThermoFisher Scientific) at room temperature for 15 min. After DAPI staining, the dishes were mounted with ProLong Gold Antifade Mountant (ThermoFisher Scientific). The images were taken using a 20× objective under a Nikon 80i epifluorescence microscope. The table shows the details of primary and secondary antibodies.

Primary Antibody	Vendor	RRID	Cat #	Dilution/dose
OCT4	ThermoFisher Scientific	AB_2534182	A13998	1:400
SSEA4	ThermoFisher Scientific	AB_2533506	414000	10 µg/mL
Nestin	BD Biosciences	AB_396354	611658	1:1000
SOX2	ThermoFisher Scientific	AB_2533841	8-1400	3 µg/mL
SOX1	R&D systems	AB_2239897	AF3369	1:100
PAX6	ThermoFisher Scientific	AB_2533534	42-6600	1:200
MAP2	ThermoFisher	AB_2533001	13-1500	2 µg/mL

	Scientific			
Synaptophysin	EMD Millipore	AB_94786	MAB329	1:500
Neurofilament	ThermoFisher Scientific	AB_10984147	MA5-14981	1:100
Secondary Antibody	Vendor	RRID	Cat #	Dilution/dose
Alexa Fluor 488 donkey anti-mouse	ThermoFisher Scientific	AB_141607	A21202	1:2000
Alexa Fluor 488 donkey anti-goat	ThermoFisher Scientific	AB_2534102	A11055	1:2000
Alexa Fluor 594 donkey anti-rabbit	ThermoFisher Scientific	AB_141637	A21207	1:2000
Alexa Fluor 594 donkey anti-goat	ThermoFisher Scientific	AB_2534105	A11058	1:2000

Histochemical assay of KGDHC and PDHC

To estimate relative KGDHC and PDHC activity in intact cells, histochemical assays of these two enzymes were performed based on our previous study (Park *et al.* 2000). iPSC, NSC and iPSC-derived neurons were seeded on 24-well plates at a seeding density of 5×10^4 cells/well for different culture times (iPSC and NSC for 2 days, and iPSC-derived neurons for 28 days) in 0.5 mL of growth medium (details in culture section). On the day of each experiment, the medium was aspirated, and the cells were washed once with balanced salt solution (BSS) [140 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose and 10 mM HEPES (pH 7.4)]. The cells were then treated with SP (trisodium succinyl phosphonate, 100, 200 and 500 μ M) or DMAP (5 or 100 μ M) in 0.5 mL of BSS for 60 min at 37 °C. At the end of the treatment, the buffer was aspirated and the well was washed with 200 μ L of Hank's Balanced Salt Solution (HBSS) containing 0.05% (v/v) Triton X-100. Wells were incubated with 200 μ L of either complete assay mixture or assay mixture without substrates as a negative control. Samples were

incubated with the assay mixture for 40 min. After incubation, the treatment medium was aspirated and the cells were washed with Ca^{2+} - and Mg^{2+} -free HBSS. The dark blue formazan product was solubilized with 10% (w/v) SDS in 0.01 N HCl overnight in a CO_2 incubator at 37 °C. The absorbance was read at 570 nm with a Spectra Max 250 model plate reader (Molecular Devices, Sunnyvale, CA).

Measurement of K^+ depolarization

Cells were loaded with 2 μM fura-2 AM (ThermoFisher Scientific) in BSS for one hour at 37 °C and rinsed twice with BSS (Gibson *et al.* 2002). Then, 2 mL of BSS was added to each dish and $[\text{Ca}^{2+}]$ was monitored on the stage of an inverted Olympus IX70 microscope at 37 °C with a Delta Scan System (Photon Technology International, Edison, NJ). Excitation wavelengths were alternated between 350 and 378 nm (band pass 4 nm) and emission was monitored at 510 nm with a Hamamatsu C2400 SIT camera (Hamamatsu, Hamamatsu City, Japan) at 5-second intervals. Basal $[\text{Ca}^{2+}]$ was measured for one minute. 50 mM KCl (Sigma) was added to depolarize voltage gated calcium channels to allow entry of extracellular calcium, which increases cytosolic calcium. The signal was measured for another 5 minutes. Each value was the average of 32 images taken within 5 seconds. Standard images of fura-2 solutions with minimum and maximum $[\text{Ca}^{2+}]$ were taken at the end of each day's experiment to calculate the intracellular calcium concentrations (Huang *et al.* 1991).

Measurement of BRCS

Cells were loaded with 2 μ M fura-2 AM in BSS for one hour at 37 °C and rinsed twice with Ca^{2+} -free BSS (140 mM NaCl, 5 mM KCl, 1.5 mM MgCl_2 , 5 mM glucose, 10 mM HEPES, 0.1 mM CaCl_2 , 1mM EGTA, pH 7.4) (Gibson et al., 2002). Then, 2 mL of Ca^{2+} -free BSS was added to each dish and $[\text{Ca}^{2+}]$ was monitored with the Delta Scan System. Basal $[\text{Ca}^{2+}]$ was measured for one minute. Bradykinin (100, 200 and 500 nM; Sigma) was added to release calcium from internal calcium stores and the signal was measured for another 5 minutes. Each value was the average of 32 images taken within 5 seconds. Standard images of fura-2 solutions with minimum and maximum $[\text{Ca}^{2+}]$ were taken at the end of each day's experiment to calculate the intracellular calcium concentrations (Huang *et al.* 2010).

Statistics

Microsoft EXCEL (RRID: SCR_016137) was used to prepare graphics and IBM SPSS Statistics 25 (RRID:SCR_002865) was used to test the statistical significance for one-way ANOVA with Student Newman Keul's test.

Acknowledgements and conflicts. This research was funded by NIH 2P01AG014930-15A1 and the Burke Foundation. None of the authors have a conflict of interest.

Figure legends

Figure 1. Immunocytochemical characterization of iPSC, NSC and iPSC derived neurons.

NSC were differentiated from iPSC (passage 30-35) after 7 days in culture. NSC were cultured for five passages before initiating neuronal differentiation. Neurons. Cells were differentiated for four weeks. Cell specific markers were confirmed in cells at the three stages of differentiation. Cells were cultured on Delta T dishes at different seeding densities and culture times (iPSC, 2×10^5 cell/dish for two days; NSC, 2×10^5 cell/dish for two days; neurons, 1×10^5 cell/dish for four weeks). Markers were tested by immunocytochemistry (iPSCs: OCT4 and SSEA4; NSC: Nestin, SOX2, SOX1 and Pax6, iPSC derived neurons: MAP2, neurofilament and synaptophysin).

Figure 2. Characterization of voltage gated calcium channels in iPSC-derived cells

Functional characterization of the neurons was done by assessing the responses of $[Ca^{2+}]_i$ to depolarizing concentrations of K^+ (KCl, 50 mM) in iPSC, NSC and iPSC derived neurons. Cells were loaded with Fura-2 AM (2 μ M) in BSS for 60 min. After loading, the cells were rinsed with BSS and $[Ca^{2+}]_i$ was measured for one min of basal response and for another 5 min after adding KCl (50 mM): (A) The temporal response of $[Ca^{2+}]_i$ after adding KCl at 1 min. (B) the percentage of cell response to KCl in the three types of cells. Values are means $\pm$ SEM (n=106, 92 and 72 cells in iPSC, NSC, and iPSC-derived neurons, respectively). The error bars for the iPSC and NSC were too small to show. *** indicates values vary significantly ($p < 0.05$) from the other groups by ANOVA followed by Student Newman Keul's test.

Figure 3. BRCS in iPSC

iPSC were cultured on Delta T dishes for one or two days. The cells were loaded with Fura-2 AM (2 μ M) in BSS for 60 min for dye loading. The media were changed to calcium free BSS and the calcium measurements were initiated. Bradykinin (100, 200 or 500 nM) was added after 1 min of basal $[Ca^{2+}]_i$ measurement. The left panel shows the tracings and the right panel shows the integration of the $[Ca^{2+}]_i$ peak over the 5 min interval after bradykinin addition. Values are means $\pm$ SEM (n=355 and 250 cells in one and two days of culture, respectively). Different letters indicate values vary significantly (p<0.05) from the other groups by ANOVA followed by Student Newman Keul's test.

Figure 4. BRCS in NSC

NSC (passage 5) were cultured on Delta T dishes for two days. After two days of culture, the cells were loaded with Fura-2 AM (2 μ M) in BSS for 60 min (Gibson *et al.* 2002). After loading, the media were changed to calcium free BSS. The calcium measurements were initiated and bradykinin (100, 200 or 500 nM) was added after 1 min of basal $[Ca^{2+}]_i$ measurement. The top panel shows the tracings and the bottom panel shows the integration of the $[Ca^{2+}]_i$ peak over the 5 min interval after bradykinin addition. Values are means $\pm$ SEM (n=490 cells). Different letters indicate values vary significantly (p<0.05) from the other groups by ANOVA followed by Student Newman Keul's test.

Figure 5. BRCS in iPSC-derived neurons

NSC (passage 5) were cultured on Delta T dishes. After four weeks of culture, the cells were loaded with Fura-2 AM (2 μ M) in BSS for 60 min. After loading, the media were changed to calcium free BSS, and the calcium measurements were initiated. Bradykinin (100, 200 or 500 nM) was added after 1 min of basal $[Ca^{2+}]_i$ measurement. The top panel shows the tracings taken and the bottom panel shows the integration of the $[Ca^{2+}]_i$ peak over the 5 min interval after bradykinin addition. Values are means $\pm$ SEM (n=435 cells). Different letters indicate values vary significantly ($p < 0.05$) from the other groups by ANOVA followed by Student Newman Keul's test.

Figure 6. Inhibiting KGDHC did not alter BRCS in iPSC.

iPSC were cultured in 24-wells plate at a seeding density of 1×10^5 cells/well for two days. After two days of culture, cellular KGDHC activity was measured by histochemistry (Panel A). iPSC were cultured on Delta T dishes for one or two days. After culture, the cells were pre-treated with trisodium succinyl phosphonate (SP, 500 μ M) for one hour (in BSS) or 24 hours (in completed media). After 1 hour of Fura-2 loading, the media were changed to calcium free BSS, and the calcium measurements were initiated and bradykinin (500 nM) was added after 1 min of basal $[Ca^{2+}]_i$ measurement. (Panel B). Values are means $\pm$ SEM (n=261 and 272 cells for one hour and 24 hours of treatment, respectively). Different letters indicate values vary significantly ($p < 0.05$) from the other groups by ANOVA followed by Student Newman Keul's test (Panel C).

Figure 7. Reducing KGDHC did not alter BRCS in NSC

NSC were cultured on 24-well plate at the seeding density of 1×10^5 cell/well for two days. After two days of culture, KGDHC activity was measured by histochemistry (Panel A). NSC were cultured on Delta T dishes for two days. After culture, the cells were pre-treated with trisodium succinyl phosphonate (SP, 500 μ M) one hour (in BSS) or for 24 hours (in completed media). After 1 hour of Fura-2 loading, the media were changed to calcium free BSS, and the calcium measurements were initiated and bradykinin (500 nM) was added after 1 min of basal $[Ca^{2+}]_i$ measurement (Panel B). Values are means $\pm$ SEM (n=208 and 205 cells for one hour and 24 hours of treatment, respectively). Different letters indicate values vary significantly ($p < 0.05$) from the other groups by ANOVA followed by Student Newman Keul's test (Panel C).

Figure 8. Reducing KGDHC exaggerated BRCS in neurons

(A) iPSC-derived neurons were cultured on 24-well plates at the seeding density of 5×10^4 cell/well for four weeks. After four weeks of culture, KGDHC activity was measured by histochemistry (Panel A). Neurons were cultured on Delta T dishes for four weeks. After culture, the cells were pre-treated with SP (500 μ M) for one hour (in BSS) or 24 hours (in completed media). After 1 hour of Fura-2 loading, the media were changed to calcium free BSS, and the calcium measurements were initiated and bradykinin (500 nM) after 1 min of basal $[Ca^{2+}]_i$ measurement (Panel B). Values are means $\pm$ SEM (n=627 and 603 cells for one hour and 24 hours of treatment, respectively). Different letters indicate values vary significantly ($p < 0.05$) from the other groups by ANOVA followed by Student Newman Keul's test. (Panel C).

Figure 9. Reducing PDH diminished BRCS in iPSC derived neurons

iPSC-derived neurons were cultured on 24-well plates at the seeding density of 5×10^4 cells/well for four weeks. After four weeks of culture, the activity of PDHC was measured by histochemistry (Panel A). Neurons were cultured on Delta T dishes for four weeks. After culture, the cells were pre-treated with DMAP (5, 100 μ M) for one hour (in BSS). After 1 hour of Fura-2 loading, the media were changed to calcium free BSS, and the calcium measurements were initiated and bradykinin (500 nM) after 1 min of basal $[Ca^{2+}]_i$ measurement.(Panel B). Values are means $\pm$ SEM (n= 840 cells). Different letters indicate values vary significantly ($p < 0.05$) from the other groups by ANOVA followed by Student Newman Keul's test (Panel C).

Figure 10. Reducing KGDHC or PDHC on basal calcium in iPSC, NSC, and iPSC derived neurons

Basal $[Ca^{2+}]_i$ in iPSC (A), NSC (B), and iPSC derived neurons (C)(D) were calculated from the same experiments in Figure 6-9. Values are means $\pm$ SEM. Different letters indicate values vary significantly ($p < 0.05$) from the other groups by ANOVA followed by Student Newman Keul's test.

References:

- Arnelle, D. R. and Stamler, J. S. (1995) NO⁺, NO[•], and NO⁻ Donation by S-Nitrosothiols: Implications for Regulation of Physiological Functions by S-Nitrosylation and Acceleration of Disulfide Formation. *Archives of Biochemistry and Biophysics* **318**, 279-285.
- Banerjee, K., Munshi, S., Xu, H. et al. (2016) Mild mitochondrial metabolic deficits by α -ketoglutarate dehydrogenase inhibition cause prominent changes in intracellular autophagic signaling: Potential role in the pathobiology of Alzheimer's disease. *Neurochemistry international* **96**, 32-45.
- Chakroborty, S. and Stutzmann, G. E. (2014) Calcium channelopathies and Alzheimer's disease: Insight into therapeutic success and failures. *European Journal of Pharmacology* **739**, 83-95.
- Chen, H., Xu, H., Potash, S., Starkov, A., Belousov, V. V., Bilan, D. S., Denton, T. T. and Gibson, G. E. (2017) Mild metabolic perturbations alter succinylation of mitochondrial proteins. *Journal of Neuroscience Research* **95**, 2244-2252.
- Cheung, K.-H., Mei, L., Mak, D.-O. D., Hayashi, I., Iwatsubo, T., Kang, D. E. and Foskett, J. K. (2010) Gain-of-function enhancement of IP3 receptor modal gating by familial Alzheimer's disease-linked presenilin mutants in human cells and mouse neurons. *Sci Signal* **3**, ra22-ra22.
- Cheung, K.-H., Shineman, D., Müller, M. et al. (2008) Mechanism of Ca²⁺ disruption in Alzheimer's disease by presenilin regulation of InsP3 receptor channel gating. *Neuron* **58**, 871-883.
- Csordás, G., Thomas, A. P. and Hajnóczky, G. (1999) Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. *The EMBO Journal* **18**, 96-108.
- Dafinca, R., Scaber, J., Ababneh, N. a. et al. (2016) C9orf72 Hexanucleotide Expansions Are Associated with Altered Endoplasmic Reticulum Calcium Homeostasis and Stress Granule Formation in Induced Pluripotent Stem Cell-Derived Neurons from Patients with Amyotrophic Lateral Sclerosis and Frontotemporal Dementia. *STEM CELLS* **34**, 2063-2078.
- Del Prete, D., Checler, F. and Chami, M. (2014) Ryanodine receptors: physiological function and deregulation in Alzheimer disease. *Molecular Neurodegeneration* **9**, 21.
- Duan, L., Bhattacharyya, B. J., Belmadani, A., Pan, L., Miller, R. J. and Kessler, J. A. (2014) Stem cell derived basal forebrain cholinergic neurons from Alzheimer's disease patients are more susceptible to cell death. *Molecular neurodegeneration* **9**, 3-3.
- Flucher, B. E., Andrews, S. B., Fleischer, S., Marks, A. R., Caswell, A. and Powell, J. A. (1993) Triad formation: organization and function of the sarcoplasmic reticulum calcium release channel and triadin in normal and dysgenic muscle in vitro. *Journal of Cell Biology* **123**, 1161-1174.
- Gibson, G. E., Chen, H.-L., Xu, H., Qiu, L., Xu, Z., Denton, T. T. and Shi, Q. (2012) Deficits in the mitochondrial enzyme α -ketoglutarate dehydrogenase lead to Alzheimer's disease-like calcium dysregulation. *Neurobiology of Aging* **33**, 1121.e1113-1121.e1124.

- Gibson, G. E., Zhang, H., Xu, H., Park, L. C. H. and Jeitner, T. M. (2002) Oxidative stress increases internal calcium stores and reduces a key mitochondrial enzyme. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1586**, 177-189.
- Hajnóczky, G., Lin, C. and Thomas, A. P. (1994) Luminal communication between intracellular calcium stores modulated by GTP and the cytoskeleton. *Journal of Biological Chemistry* **269**, 10280-10287.
- Hartfield, E. M., Yamasaki-Mann, M., Ribeiro Fernandes, H. J., Vowles, J., James, W. S., Cowley, S. A. and Wade-Martins, R. (2014) Physiological characterisation of human iPS-derived dopaminergic neurons. *PloS one* **9**, e87388-e87388.
- Huang, H.-M., Chen, H.-L. and Gibson, G. E. (2010) Thiamine and Oxidants Interact to Modify Cellular Calcium Stores. *Neurochemical Research* **35**, 2107-2116.
- Huang, H.-M., Chen, H.-L., Xu, H. and Gibson, G. E. (2005) Modification of endoplasmic reticulum Ca²⁺ stores by select oxidants produces changes reminiscent of those in cells from patients with Alzheimer disease. *Free Radical Biology and Medicine* **39**, 979-989.
- Huang, H.-M., Toral-Barza, L. and Gibson, G. (1991) Cytosolic free calcium and ATP in synaptosomes after ischemia. *Life Sciences* **48**, 1439-1445.
- Intlekofer, A. M. and Finley, L. W. S. (2019) Metabolic signatures of cancer cells and stem cells. *Nature metabolism* **1**, 177-188.
- Ito, E., Oka, K., Etcheberrigaray, R., Nelson, T. J., McPhie, D. L., Tofel-Grehl, B., Gibson, G. E. and Alkon, D. L. (1994) Internal Ca²⁺ mobilization is altered in fibroblasts from patients with Alzheimer disease. *Proc Natl Acad Sci U S A* **91**, 534-538.
- Karagas, N. E. and Venkatachalam, K. (2019) Roles for the Endoplasmic Reticulum in Regulation of Neuronal Calcium Homeostasis. *Cells* **8**, 1232-1263.
- Khacho, M., Harris, R. and Slack, R. S. (2019) Mitochondria as central regulators of neural stem cell fate and cognitive function. *Nature Reviews Neuroscience* **20**, 34-48.
- Kiss, G., Konrad, C., Doczi, J. et al. (2013) The negative impact of α -ketoglutarate dehydrogenase complex deficiency on matrix substrate-level phosphorylation. *Faseb j* **27**, 2392-2406.
- Lacampagne, A., Liu, X., Reiken, S. et al. (2017) Post-translational remodeling of ryanodine receptor induces calcium leak leading to Alzheimer's disease-like pathologies and cognitive deficits. *Acta Neuropathologica* **134**, 749-767.
- Leissring, M. A., Akbari, Y., Fanger, C. M., Cahalan, M. D., Mattson, M. P. and LaFerla, F. M. (2000) Capacitative calcium entry deficits and elevated luminal calcium content in mutant presenilin-1 knockin mice. *J Cell Biol* **149**, 793-798.
- Mustaly-Kalimi, S., Littlefield, A. M. and Stutzmann, G. E. (2018) Calcium Signaling Deficits in Glia and Autophagic Pathways Contributing to Neurodegenerative Disease. *Antioxidants & Redox Signaling* **29**, 1158-1175.
- Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B. P. and LaFerla, F. M. (2003) Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiology of Aging* **24**, 1063-1070.
- Park, L. C. H., Calingasan, N. Y., Sheu, K.-F. R. and Gibson, G. E. (2000) Quantitative α -Ketoglutarate Dehydrogenase Activity Staining in Brain Sections and in Cultured Cells. *Analytical Biochemistry* **277**, 86-93.

- Peterson C, G. G., Blass JP (1985) Altered Calcium Uptake in Cultured Skin Fibroblasts from Patients with Alzheimer's Disease. *New England Journal of Medicine* **312**, 1063-1065.
- Popugaeva, E. and Bezprozvanny, I. (2013) Role of endoplasmic reticulum Ca²⁺ signaling in the pathogenesis of Alzheimer disease. *Frontiers in Molecular Neuroscience* **6**.
- Prè, D., Nestor, M. W., Sproul, A. A. et al. (2014) A time course analysis of the electrophysiological properties of neurons differentiated from human induced pluripotent stem cells (iPSCs). *PloS one* **9**, e103418-e103418.
- Rabenstein, M., Peter, F., Joost, S., Trilck, M., Rolfs, A. and Frech, M. J. (2017) Decreased calcium flux in Niemann-Pick type C1 patient-specific iPSC-derived neurons due to higher amount of calcium-impermeable AMPA receptors. *Molecular and Cellular Neuroscience* **83**, 27-36.
- Rizzuto, R., Brini, M., Murgia, M. and Pozzan, T. (1993) Microdomains with high Ca²⁺ close to IP₃-sensitive channels that are sensed by neighboring mitochondria. *Science* **262**, 744-747.
- Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A. and Pozzan, T. (1998) Close Contacts with the Endoplasmic Reticulum as Determinants of Mitochondrial Ca²⁺ Responses. *Science* **280**, 1763.
- Schöndorf, D. C., Aureli, M., McAllister, F. E. et al. (2014) iPSC-derived neurons from GBA1-associated Parkinson's disease patients show autophagic defects and impaired calcium homeostasis. *Nature Communications* **5**, 4028.
- Schrank, S., Barrington, N. and Stutzmann, G. E. (2019) Calcium-Handling Defects and Neurodegenerative Disease. *Cold Spring Harbor Perspectives in Biology*.
- Seitaj, B., Bultynck, G. and Ivanova, H. (2018) Modulation of IP₃ Receptor Stability and Its Implication in Cell Death and Disease. *Messenger* **6**, 9-21.
- Shilling, D., Müller, M., Takano, H., Daniel Mak, D.-O., Abel, T., Coulter, D. A. and Foskett, J. K. (2014) Suppression of InsP₃ Receptor-Mediated Ca²⁺ Signaling Alleviates Mutant Presenilin-Linked Familial Alzheimer's Disease Pathogenesis. *The Journal of Neuroscience* **34**, 6910.
- Sproul, A., Jacob, S., Prè, D. et al. (2014) Characterization and Molecular Profiling of PSEN1 Familial Alzheimer's Disease iPSC-Derived Neural Progenitors. *PloS one* **9**, e84547.
- Stutzmann, G. E. (2007) The Pathogenesis of Alzheimers Disease—Is It a Lifelong “Calciumopathy”? *The Neuroscientist* **13**, 546-559.
- Stutzmann, G. E., Caccamo, A., LaFerla, F. M. and Parker, I. (2004) Dysregulated IP₃ signaling in cortical neurons of knock-in mice expressing an Alzheimer's-linked mutation in presenilin1 results in exaggerated Ca²⁺ signals and altered membrane excitability. *J Neurosci* **24**, 508-513.
- Stutzmann, G. E., Smith, I., Caccamo, A., Oddo, S., Laferla, F. M. and Parker, I. (2006) Enhanced ryanodine receptor recruitment contributes to Ca²⁺ disruptions in young, adult, and aged Alzheimer's disease mice. *J Neurosci* **26**, 5180-5189.
- Yang, Y., Tapias, V., Acosta, D. et al. (2019) Succinylation Links Metabolic Reductions to Amyloid and Tau Pathology. *bioRxiv*, 764837.

Figure-1

Specific markers identify iPSC, NSC, and neurons

Figure-2

Figure-3

iPSC after 1 day of culture

bioRxiv preprint doi: <https://doi.org/10.1101/2020.06.20.162040>; this version posted June 20, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

iPSC after 2 days of culture

Figure-4

NSC after 2 days of culture

bioRxiv preprint doi: <https://doi.org/10.1101/2020.06.20.162040>; this version posted June 20, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure-5

Neurons after 28 days of culture

bioRxiv preprint doi: <https://doi.org/10.1101/2020.06.20.162040>; this version posted June 20, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure 6

A.

In situ KGDHC activity

bioRxiv preprint doi: <https://doi.org/10.1101/2020.06.20.162140>; this version posted June 20, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

B.

C.

Figure 7

A.

In situ KGDHC activity

bioRxiv preprint doi: <https://doi.org/10.1101/2020.06.20.162040>; this version posted June 20, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

B.

C.

Figure 8

A.

In situ KGDHC activity

bioRxiv preprint doi: <https://doi.org/10.1101/2020.06.20.162040>; this version posted June 20, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

B.

C.

Figure 9

Figure-10

A. SP in iPSC

B. SP in NSC

C. SP in neurons

D. DMAP in neurons