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## Ketone body supported respiration in murine isolated brain mitochondria is augmented by alpha-ketoglutarate and is optimized by neuronal SCOT expression

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### Abstract

Ketone bodies are increasingly examined as an alternative fuel source for the known decreases in glucose utilization that occur with neurodegeneration. Here, we established a protocol to maximize ketone body respiration in isolated brain mitochondria, while quantifying acetyl-CoA and energy charge via liquid chromatography-tandem mass spectrometry in control mice compared to mice with neuron-specific deletion of succinyl-CoA-3-oxoacid-CoA transferase (SCOT), required for CoA transfer from succinyl-CoA to AcAc to support its oxidation. Maximal ADP-dependent AcAc respiration occurred at 1 mM; however, the percent increase above basal was minimal (~15%). Alpha-ketoglutarate ( $\alpha$ KG) substantially increased AcAc-dependent respiration

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Author Contributions

Conceived and designed research (JPT,XCD,CSM,EMM,HDS,EF,PP,PAC), performed experiments (XCD,JA,EDQ,PP), analyzed data (XCD), interpreted results of experiments (JPT,XCD,CSM,EMM,HDS,EF,PP,PAC), prepared figures (XCD), drafted manuscript (XCD), edited and revised manuscript (XCD,JPT,CSM,EMM,HDS,EF,EDQ,KLF,PP,PAC), approved final version of manuscript (XCD,JPT,CSM,EMM,EF,PP,PAC).

Disclosures

The authors declare no conflicts of interest.

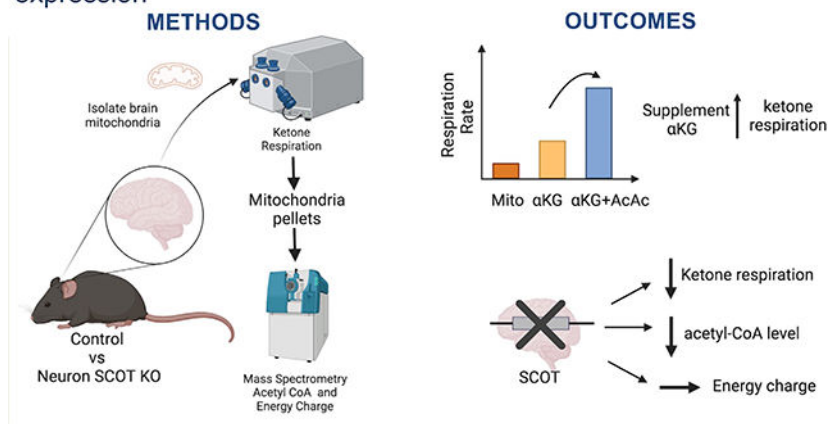
in isolated brain mitochondria, putatively through the generation of succinyl-CoA. Using mice with neuron-specific deletion of SCOT, we also examined brain mitochondrial respiration of AcAc and resulting acetyl CoA and energy charge (cellular energy availability via adenosine nucleotide ratios of ATP, ADP, and AMP). As expected, isolated brain mitochondria from SCOT-KO mice had lower AcAc State 3 respiration than control mice. Surprisingly, we did not find differences in mitochondrial energy charge between SCOT control and neuron SCOT-KO mice despite decreased acetyl-CoA level in SCOT-KO mice when AcAc was used as the substrate. In conclusion, we show that  $\alpha$ KG enhances ketone-supported respiration rate in isolated brain mitochondria, and ketone metabolism in neurons affects acetyl-CoA level in brain mitochondria but not energy charge. Future work will determine if diet, exercise, sex, or age impacts ketone-supported respiration rates in conjunction with differences in markers of brain health.

## New & Noteworthy

This paper established a protocol to maximize ketone body respiration in isolated brain mitochondria while quantifying acetyl-CoA and energy charge in control mice compared to mice with neuron-specific deletion of SCOT enzyme, required for ketone body oxidation. Findings are that alpha-ketoglutarate substantially increased AcAc-dependent respiration and neuron SCOT-KO had lower AcAc state 3 respiration with a decreased acetyl-CoA level.

## Graphical Abstract

Ketone body supported respiration in murine isolated brain mitochondria: role of alpha-ketoglutarate and neuronal SCOT expression



**CONCLUSION:**  $\alpha$ KG enhances ketone-supported respiration rate in isolated brain mitochondria, and neuron-specific SCOT knockout reduces ketone-supported respiration and acetyl-CoA levels but not energy charge

## Keywords

Brain; Ketone; Mitochondria respiration; SCOT

## Introduction

Studies investigating ketone metabolism as an alternative fuel source to enhance brain health and cognitive function in Alzheimer's disease, and to improve systemic metabolic health have increased over the last decade. During nutrient deprivation and limited carbohydrate availability, the liver catabolizes fatty acids and produces ketone bodies (KBs), which are then used by extrahepatic tissues as an alternative fuel, thus conserving limited glucose levels (1). Hepatic ketone synthesis, i.e., ketogenesis, includes producing acetoacetate (AcAc) and D- $\beta$ -hydroxybutyrate (D- $\beta$ OHB) from two acetyl-CoA molecules derived from  $\beta$ -oxidation of fatty acyl-CoAs. AcAc and D- $\beta$ OHB are transported across the hepatocyte membranes via monocarboxylate transporters 1 and 2 (MCT1 and MCT2) into the blood and into extrahepatic tissues to meet the energy demands of anabolism and catabolism throughout the body (2). Specific to our studies with main focuses on brain energetics, when the brain is deprived of its preferred fuel source, glucose, ketone utilization is increased to meet metabolic demands (3). Ketogenic diets, which increase daily KB levels (low in carbohydrates and high in fat and protein) were first used to treat epileptic children (4). Further, they have been shown to improve several other central nervous system (CNS) disorders, including Alzheimer's disease (AD), Parkinson's disease, hypoxia, and traumatic brain injury (4) (5).

Beyond serving as an alternative fuel for the brain, KBs play many "non-canonical" signaling roles in maintaining cellular homeostasis, including protein posttranslational modification (PTM), and modulating inflammation or oxidative stress responses (6). Also, KB metabolism increases intracellular acetyl-CoA levels, which controls key cellular processes such as energy metabolism, autophagy, and mitosis (7). Despite numerous studies investigating ketones and metabolism, limited studies have examined respiration with KB as a primary substrate or changes in oxidative metabolites that result from KB-supported metabolism in mitochondria. This critical knowledge gap impacts research questions, including if KB respiration in the brain can be altered by different interventions such as fasting and exercise and if enhanced KB utilization improves metabolite sensing and signaling. Previous studies have measured KB respiration in neuronal cells (8–10) but only a few have measured KB supported respiration in isolated mitochondria from brain tissue (11, 12). Notably, these few studies have only measured KB respiration and only one previous study measured ADP-dependent KB supported mitochondrial respiration (13). Moreover, we are not aware of studies that have used chemical inhibitors to investigate the entry of reducing equivalents (electrons) from KBs into the electron chain transport (ETC).

Therefore, our first objective was to use Oroboros respirometry in isolated brain mitochondria to develop a methodology to maximize KB supported respiration rates. We compared  $\alpha$ KG in the presence or absence of AcAc to explore if  $\alpha$ KG would increase succinyl-CoA to drive forward the near equilibrium reaction of succinyl-CoA:3-oxoacidCoA transferase (SCOT) necessary to produce AcAc-CoA for terminal oxidation. Altogether, we established a protocol to directly measure the KB oxidative respiration rate in isolated brain mitochondria. Second, we tested if KB metabolism impacts resulting brain mitochondrial levels of acetyl CoA and energy charge. We then determined if neuron-specific deletion of the fate-committing step in ketone oxidation, SCOT, would reliably alter KB-supported

respiratory responses and mitochondrial acetyl CoA and energy charge. Applying our protocol, we measured AcAc respiration in isolated brain mitochondria from SCOT control and SCOT-KO mice. After each experiment, we collected mitochondrial pellets from the respiratory chamber for metabolite and nucleotide concentration determination by mass spectrometry. Our results revealed a similar energy charge between SCOT control and SCOT-KO isolated mitochondria but a lower acetyl-CoA level in the mitochondria of the SCOT-KO than the control during respiration of AcAc.

## Materials and Methods

### Ethical approval and experimental protocol

All animal use was in accordance with the National Institutes of Health (NIH) *Guide for the care and use of laboratory animal* and conformed to the principles specified in protocols approved by the University of Kansas Medical Center and University of Minnesota Institutional Animal Care and Use Committees (IACUC).

### Isolated Brain Mitochondria

Wild-type C57BL/6J male mice (8 – 20 weeks of age) were fed *ad libitum* with a normal chow diet and housed on a 12:12 dark: light cycle. Neuron-specific SCOT knockout mice were developed by crossing the Synapsin 1-Cre driver to the floxed *Oxct1* allele on a C57BL/6NJ background (14). Wild-type C57BL/6J mice were used initially to establish the KB supported respiration protocol, with follow up studies focused on SCOT KO in C57BL/SNJ occurring separately. No between strain comparisons were performed. Mice were anesthetized and euthanized by phenobarbital injection and exsanguination followed by removal of the brain for isolation of mitochondria. The entire left hemisphere was transferred to pre-cooled glass Potter homogenizer with 8 mL of brain isolation buffer A (320 mM sucrose, 10 mM Tris-Cl, 1 mM EDTA, 2.5 g/L BSA). The tissue was homogenized with 4–6 strokes at 200 rpms. The resulting homogenate was centrifuged for 10 min at 1,000 x g at 4°C. Then, the supernatant was transferred to a new tube and centrifuged for 10 min at 10,000 x g at 4°C. After centrifugation, the supernatant was discarded, the mitochondrial pellet was resuspended in 6 mL isolation buffer B (320 mM sucrose, 10 mM Tris-Cl, 1 mM EDTA) and recentrifuged for 10 min at 9,000 x g at 4 °C. Afterwards, the pellet was collected and resuspended in 1600 µL of isolation buffer B. Isolated brain mitochondria were stored on ice until use.

### KB Respiration Protocol in Isolated Brain Mitochondria

Respiration of isolated brain mitochondria was determined using Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria). Experiments using isolated brain mitochondria from wild type C57BL/6J male mice were performed in modified MiR05 (110 mM sucrose; 60 mM K-MES; 0.5 mM EGTA; 3 mM MgCl<sub>2</sub>; 10 mM KH<sub>2</sub>PO<sub>4</sub>; 20 mM HEPES; 0.1% fatty acid-free BSA; and 20 mM glucose; adjusted to pH 7.1 with KOH). All experiments were performed at 37°C. 75–100 µg of mitochondria in buffer (25–30 µL) were added to each 2-ml respiration chamber. Substrate concentrations for respirometry experiment are shown as following: 2 mM Malate; 0.01 mM CoA; 2.5 mM Carnitine; 10 mM ADP; 1 mM αKG; 1 mM AcAc; 1 mM D-βOHB. Since KBs putatively provide

reducing agents to both complex I and II, we investigated the entry path of electrons from KBs to the ETC, leveraging malonic acid (5 mM Mna, complex II inhibitor) and rotenone (0.002 mM Rot, complex I inhibitor). Following all respirometry experiments, data was normalized to the mitochondrial protein via BCA assay. Acetoacetate was synthesized as previously described (15). Briefly, ethyl acetoacetate underwent base-catalyzed hydrolysis using an equimolar amount of sodium hydroxide at 60°C, followed by neutralization with HCl, sterile filtration and storage at -80°C. Chemical purity was assessed via <sup>1</sup>H-NMR and concentration was quantified via UHPLC-MS/MS.

### **Ketone Supported Respiration in SCOT Control and SCOT-KO**

30–32 weeks old C57BL/6NJ SCOT-Control and neuron-specific SCOT-KO male mice were placed on a reverse light cycle (dark 10:00 –22:00), with *ad libitum* access to water and normal chow diet. We used 30–32-week-old middle-aged mice as metabolism changes are often noticed of the middle-aged brain (16), to explore the ability of neuron cells to oxidize ketone bodies and determine the effects of ketone oxidation on acetyl CoA and energy charge in isolated brain mitochondria. Brain mitochondria were isolated as above, protein concentration determined by BCA, and 150 µg of mitochondrial protein was used. Respiration experiments were performed in the same modified MiR05 buffer as above at 37°C and substrate concentrations were the same as above. Briefly, following additions of different fuel substrates, brain mitochondria were added, and a baseline respiration value was reached. The respiratory flux was then assessed following sequential adding substrates. After each respiration experiment, contents of the reaction wells were transferred to microcentrifuge tubes and centrifuged at 8,000 x g at 4°C for 15 minutes. The supernatant was discarded, and the pellet was collected and frozen in liquid nitrogen and stored at -80°C until processing.

### **Acetyl CoA and energy charge analysis in isolated brain mitochondria via LC-MS/MS.**

Mitochondria were homogenized using pestle with cold 0.4 M perchloric acid, 0.5 mM EGTA extraction solution containing [ <sup>13</sup>C<sub>10</sub>, <sup>15</sup>N<sub>5</sub> ] ATP sodium salt (100 mM), [ <sup>13</sup>C<sub>10</sub>, <sup>15</sup>N<sub>5</sub> ] AMP sodium salt (100 mM), [1,2-<sup>13</sup>C<sub>2</sub>] acetyl-CoA lithium salt (5 mM), and [1,2,3-<sup>13</sup>C<sub>3</sub>] malonyl-CoA lithium salt (5 mM) purchased from Sigma. After incubation on ice for 10 min, samples were centrifuged at 15,000xg for 15 min at 4°C. The resulting supernatants were neutralized with freshly prepared 0.5 M K<sub>2</sub>CO<sub>3</sub>, vortexed, and centrifuged at 15,000xg for 30 min at 4°C. Final extracts were then analyzed by LC-MS/MS as previously described, with modifications (17). Briefly, analysis of energy acetyl CoA and energy charge was performed using a Vanquish LC system. Separation was achieved on a reverse-phase C18 column (Waters Xbridge, 150 × 2.1 mm, 3.5 µm) using the following mobile phases: A) 95% water/5% methanol with 4 mM dibutylammonium acetate (DBAA, Millipore Sigma) and B) 25% water/75% acetonitrile with 4 mM DBAA. The ion-pairing mobile phase additive DBAA provided adequate chromatographic separation. Samples were separated using the following binary gradients: 0–80% B for 8 min, 80–100% B for 5 min, 100% B for 3 min, and 100–0% B for 5 min. The flow rate was 100 µL/min, with a diverter valve used for the first 2 min to minimize matrix components entering the MS. The column was maintained at 30°C and the injection volume was 2 µL. The LC system was hyphenated to a Thermo Q Exactive Plus MS equipped with heated electrospray ionization.

The MS was operated in positive ionization mode with PRM mode used for quantitatively targeted analysis. The isolation window was set to  $m/z$  1.0; the resolution was 17,500, and the collision energy was 35 (arbitrary units). Retention times and  $m/z$  transitions for acetyl CoA are detailed in **Supplemental Table 1**. (DOI: [10.6084/m9.figshare.28653461](https://doi.org/10.6084/m9.figshare.28653461)). Energy charge was calculated from the concentration of quantified nucleotides using the following formula: Energy Charge =  $([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP])$ . (18)

### Statistical Analysis

Fig. 1, Fig. 2 and Fig. 3A&B were analyzed using one-way ANOVA, followed by a Fisher's LSD post hoc analysis. Fig. 3C was analyzed using unpaired  $t$  test to directly compare AcAc vs D- $\beta$ OHB supported induction of respiration. In Fig. 4E, Fig. 5, and Fig. 6, unpaired  $t$  test was used to examine SCOT control and SCOT KO under different substrate conditions. For Fig. 4B, 4D and 4F, the effect of genotype, substrates and their interaction were analyzed by a two-way ANOVA analysis, followed by a Fisher's LSD post hoc analysis. All data are presented as means  $\pm$  SEM and outliers are removed using Grubbs' test. Statistical significance was defined as  $P < 0.05$ , and all statistics were run using GraphPad Prism 10.0 (GraphPad Software Inc., La Jolla, CA).

## Results

### KBs alone minimally drive respiration in brain mitochondria.

We determined maximal ADP-dependent respiration of isolated brain mitochondria from Wild-type C57BL/6J male mice in response to increasing concentrations of AcAc (0.1 –10 mM, Fig. 1A). Respiration of AcAc only increased above basal by 15% at a concentration ranging from 0.1 to 0.5 mM, decreasing after a concentration of 1 mM, and continued to decrease to 10 mM (~20– 35% less than basal at 8 mM: Fig. 1B). In contrast to other metabolic substrates, KB metabolism provides a surplus flux of acetyl-CoA (19). Other studies have shown AcAc alone inhibited  $\alpha$ -ketoglutarate dehydrogenase activity due to sequestration of free CoASH which inhibits KB utilization (20, 21). L-carnitine is another essential molecule involved in fatty acid metabolism that transports fatty acid chains into the mitochondrial matrix for utilization (22). Thus, we tested if low KB-supported respiration rate in brain mitochondria was due to the low availability of CoASH and carnitine. Since D- $\beta$ OHB is upstream of AcAc oxidation and generates reducing equivalents as NADH, we added D- $\beta$ OHB (1 mM) to examine if the extra step in breakdown is impacted by various treatments (Fig. 1C). Again, KB supported respiration rates in the isolated brain mitochondria were low: 5–10% increase for AcAc and 35%–40% increase for D- $\beta$ OHB (Fig. 1D). This increase with D- $\beta$ OHB may contribute to the production of NADH via  $\beta$ OHB dehydrogenase. Overall, our findings indicate that KB respiration in isolated mitochondria is relatively low when considering respiration rates of other mitochondrial substrates (23).

### $\alpha$ -ketoglutaric acid enhances respiration of AcAc.

Given the modest KB supported respiration rate after addition of CoASH and carnitine, we hypothesized that other substrates were needed for maximal mitochondrial KB respiration. Ketone body oxidation requires activation of AcAc by succinyl-CoA to generate AcAc-CoA

through the activity of SCOT. Thiolytic cleavage of AcAc-CoA generates acetyl-CoA that enters the TCA cycle for terminal oxidation. Succinyl-CoA is generated from  $\alpha$ KG in the presence of free CoASH by  $\alpha$ -ketoglutarate dehydrogenase. However, the common addition of malate to mitochondrial respiration experiments depletes endogenous  $\alpha$ KG by antiport with malate via the malate-aspartate shuttle, potentially limiting mitochondrial succinyl-CoA availability for AcAc activation via the SCOT reaction. Therefore, we posited that KB respiration in isolated mitochondria requires supplementation of succinyl-CoA. To test this, we added  $\alpha$ KG to our respiration protocol (Fig. 2A), expecting that it would increase succinyl-CoA and enhance KB respiration (Fig. 1E). Compared to adding AcAc alone (respiration 5–10% increase to basal: Fig. 1D), adding AcAc following the addition  $\alpha$ KG increases AcAc respiration 75% higher than basal (Fig. 2B). To determine the KB concentration for maximal mitochondrial respiration in the presence of  $\alpha$ KG, we again performed a titration of AcAc (Fig. 2C). Dose-dependent increase of AcAc respiration was observed up to 1 mM AcAc, with decreases observed at higher concentrations (Fig. 2D). Increasing  $\alpha$ KG concentrations failed to augment AcAc respiration further (Fig. 2E–F). To determine the extent to which succinyl-CoA synthetase-derived succinate drove the effect of  $\alpha$ KG on respiration, we used malonic acid, a complex II (succinate dehydrogenase) inhibitor, which diminished  $\alpha$ KG-dependent respiration only by 15% suggesting that  $\alpha$ KG sourcing of succinyl-CoA synthetase-derived succinate was a minor contributor (Fig. 2G–H). This suggests the  $\alpha$ KG effect is primarily complex I-dependent, via  $\alpha$ KG dehydrogenase-dependent NADH and succinyl-CoA. In summary, our experiments show that  $\alpha$ KG at 1 mM drives maximal respiration of AcAc. Finally, the addition of  $\alpha$ KG likely resulted in significant complex I-mediated respiration.

### Comparative respiration of AcAc and D- $\beta$ OHB in isolated brain mitochondria.

Since KBs putatively provide reducing agents to both complex I and II (24), we next investigated the entry path of electrons from KBs to the ETC using the protocol shown in Fig. 3A, leveraging malonic acid (Mna, complex II inhibitor) and rotenone (Rot, complex I inhibitor). Again, we observed increased respiration after the addition of AcAc and D- $\beta$ OHB compared to  $\alpha$ KG alone (Fig. 3B&3C). We then calculated this increased respiration using the respiration of  $\alpha$ KG alone and after the addition of KBs ( $\text{Respiration}_{(\alpha\text{KG} + \text{AcAc or D-}\beta\text{OHB})} - \text{Respiration}_{(\alpha\text{KG})}$ ) (Fig. 3D). We found no significant difference in respiration rates fueled by AcAc or D- $\beta$ OHB (Fig. 3D). Fig. 3D directly compares AcAc vs. D- $\beta$ OHB supported induction of respiration (Fig. 3D). A complex I inhibitor, rotenone (Rot), was added and elicited a bigger decrease than Mna for both substrates (Fig. 3B&3C), suggesting that  $\alpha$ KG + KB respiration are complex I driven via  $\alpha$ KG dehydrogenase and D- $\beta$ OHB dehydrogenase (Fig. 3D). To our surprise, Mna resulted in a bigger decrease in D- $\beta$ OHB supported respiration compared to respiration with AcAc in isolated brain mitochondria, suggesting that D- $\beta$ OHB was more complex II driven than AcAc via succinate (Fig. 3D). In summary, our findings suggest that  $\alpha$ KG is necessary for maximizing KB respiration in isolated brain mitochondria and KBs contribute to ETC mainly through complex I, however, D- $\beta$ OHB contributes more to complex II than AcAc.

### AcAc respiration requires SCOT.

As previously stated, ketone utilization is regulated by SCOT mediated covalent activation of AcAc by succinyl-CoA to form AcAc-CoA, which is converted to acetyl-CoA for entry into the TCA cycle (14). Since AcAc is downstream of D- $\beta$ OHB and focusing on the regulating step: the exchange between succinyl-CoA and AcAc via SCOT, we compared how control mice vs. SCOT-KO would respire with AcAc as a substrate and if loss of SCOT would differently impact metabolite pools within brain mitochondria. Since neuronal cells display high energy demand (25), we suspected that the lack of SCOT in neurons would impair AcAc respiration rate. Importantly however, mitochondria isolated from brain includes neuronal and non-neuronal sources including astrocytes and oligodendrocytes that still express the SCOT enzyme (26–29). First, we tested if  $\alpha$ KG was essential for AcAc respiration in middle-aged control and SCOT KO C57BL/6NJ mice (30–32 weeks) (Fig. 4) and again showed that without  $\alpha$ KG there was a modest minimal AcAc respiration (Fig. 5A&4B). No significant differences were noticed between the control and KO when comparing mitochondrial alone respiration and AcAc respiration. We then tested if AcAc would increase  $\alpha$ KG respiration (Fig. 5C). Both control and KO demonstrated significant increase in respiration ( $\alpha$ KG + AcAc) compared to the respiration with  $\alpha$ KG alone (2-fold increase (WT) and 68% increase (KO) to basal) (Fig. 5D). No significant difference was observed in respiration ( $\alpha$ KG) between the control and KO but there was a significant decrease in SCOT KO respiration ( $\alpha$ KG + AcAc) (Respiration 14.7% lower in SCOT KO to control). Since mitochondria were isolated from the whole left hemisphere of the brain and SCOT was eliminated only in neurons, we suspected residual AcAc respiration from other cell types but that a small decrease in AcAc respiration was attributable to lacking SCOT in neurons. As expected, controls showed a significantly higher AcAc respiration (Respiration ( $\alpha$ KG + AcAc) - Respiration ( $\alpha$ KG)) than SCOT-KO (Fig. 5E). We then used Rot and Mna in separate respiration experiments to distinguish how AcAc contributes to Complex I and Complex II, respectively. Like what we observed before, adding Rot had a larger inhibition of respiration than Mna, indicating respiration ( $\alpha$ KG + AcAc) was predominantly supported by complex I (Fig. 5F). However, no significant difference was notable between the control and KO in AcAc contribution to complex I or complex II (Fig. 5F). Again, maintenance of AcAc respiration in neuronal SCOT KO mitochondria is likely due to maintenance of SCOT expression in mitochondria isolated from other cell types in the brain.

### Metabolite levels and energy charge in isolated brain mitochondria fueled with AcAc.

Previously, KB metabolism has been shown to affect metabolite pools within various tissues that use KB as their fuel source (7). With neurons being the biggest energy consumer, we expected that lacking ketolysis in neurons would influence brain mitochondria metabolite profile. Providing brain mitochondria from the control and SCOT-KO with AcAc, we quantified the metabolite contents such as acetyl-CoA and calculated energy charge (EC) from ADP, AMP and ATP (18) with mass spectrometry. Energy charge is defined as  $(\text{ATP} + 0.5 \text{ ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$ , which is a measure of the relative concentrations of substrates and products of ATP-generating and ATP-utilizing reactions (18). Since  $\alpha$ KG is the co-substrate for maximizing AcAc respiration, we first evaluated the metabolite contents in the control and SCOT-KO with 1 mM of  $\alpha$ KG. A larger quantity of brain mitochondria (150  $\mu$ g) was used for a more accurate measurement of acetyl CoA and energy charge

(Fig. 4). We again used Mna and Rot to examine the impacts of  $\alpha$ KG on our experimental conditions (Fig. 6A). At the end of each respiration experiment, we measured respiration rate, and it showed no significant difference between the control and KO under following conditions:  $\alpha$ KG,  $\alpha$ KG+Mna,  $\alpha$ KG+Rot (Fig. 6B). Comparing respiration rate among three conditions, inhibiting complex I reduced the respiration rate ( $\alpha$ KG+Rot) greater than complex II ( $\alpha$ KG+Mna) (Fig. 6B). It again suggested that  $\alpha$ KG was primarily complex I driven via  $\alpha$ KG dehydrogenase which produces NADH. We then measured metabolite substrates: acetyl CoA and calculated energy charge using AMP, ADP, and ATP (18). Again, the control and KO showed no significant difference (Fig. 6D&6E).

To determine the role of neuronal SCOT in AcAc-dependent changes in acetyl-CoA and energy charge, we used AcAc and  $\alpha$ KG+AcAc to test any differences between the control and KO (Fig. 7A). Complex I and II inhibitors were added to examine the flow of electrons from  $\alpha$ KG+AcAc to the ETC within mitochondria. The mitochondrial respiration status showed no difference between the control and KO despite trends for lower respiration in KO without inhibitors and with Rot (Fig. 7B). As expected for  $\alpha$ KG+AcAc, acetyl-CoA levels were significantly decreased in the SCOT-KO across the three conditions, suggesting that diminished procession from AcAc to acetyl-CoA on brain mitochondria in which SCOT had been eliminated from neurons (Fig. 7C). This diminished acetyl-CoA pool phenotype are dependent on both  $\alpha$ KG and AcAc and not seen in either  $\alpha$ KG alone (Fig. 6C) or AcAc alone (Fig. 7C). Again, it suggests  $\alpha$ KG as a source of succinyl-CoA for the procession from AcAc to acetyl-CoA. Mitochondrial energy charge was not different between the control and KO (Fig. 7D).

## DISCUSSION

While brain ketone metabolism has been examined for decades, limited studies have focused on direct KB mitochondrial respiration, leaving a methodological gap that limits understanding. Previous studies have shown neuronal cell KB respiration (8–10) and other studies have also investigated how mitochondria from skeletal muscle (isolated mitochondria and permeabilized fibers) respire with ketone bodies as a primary substrate, revealing minimal increases in ADP-dependent respiratory rates (13). These findings do not track with the known capacity of ketone bodies to fuel metabolic tissues, suggesting a methodological gap for measuring KB supported respiration. To address this gap, we developed a new respiration protocol to maximize KB respiration in isolated brain mitochondria. Our findings suggest that mitochondrial respiration is minimally supported by AcAc alone but that the addition of  $\alpha$ KG before AcAc allows for the accumulation of succinyl-CoA, which drives the reaction forward, increasing respiration with both AcAc and D- $\beta$ OHB in mice isolated brain mitochondria. Furthermore, our findings using complex II inhibitor (malonic acid) suggests that D- $\beta$ OHB substrate contributes more electrons to complex II via succinate than AcAc but that both substrates primarily rely on complex I for respiration. We also showed mice with neuronal-specific deletion of succinyl-CoA transferase (SCOT) had lower KB-supported respiration than control mice but did not display differences in mitochondrial energy charge despite decreased acetylCoA levels when AcAc was used as the substrate.

Our results suggest that  $\alpha$ KG should be an addition to future studies examining KB respiration in metabolic tissues. The biochemistry of these effects is linked to succinyl-CoA availability driving forward a key near-equilibrium equation (SCOT) in ketone metabolism.  $\alpha$ KG dehydrogenase converts  $\alpha$ KG to succinyl-CoA via decarboxylation and succinyl-CoA is required for the conversion of AcAc to Acetoacetyl-CoA by the SCOT enzyme, an essential step in KBs utilization. AcAc is converted to AcAc-CoA via an equilibrium reaction catalyzed by succinyl-CoA through a CoA exchange with succinyl-CoA. Hydrolysis of AcAc-CoA releases more free energy than succinyl-CoA, favoring AcAc formation (30). Adding  $\alpha$ KG and increasing succinyl-CoA pulls AcAc toward the AcAc-CoA fate. SCOT-derived AcAc-CoA produces two acetyl-CoA which then enters the TCA cycle for final oxidation (30). As expected from our hypothesis, the addition of  $\alpha$ KG as a co-substrate maximized KB-supported respiration, but the maximal effect was reached at 1 mM of  $\alpha$ KG. However, adding  $\alpha$ KG may also drive up the production of succinyl-CoA which furnishes TCA cycle's succinyl-CoA synthetase with a substrate generating ATP (GTP) and succinate.  $\alpha$ KG-driven respiration is approximately 15% complex II-dependent (Fig. 2H) suggesting the engagement of succinyl-CoA synthetase to produce succinate, a complex II related substrate. The production of ATP could potentially affect ATP synthesis via decreasing  $\Delta G$ -ATP. However, this is likely not relevant in ADP abundant conditions (State 3). In addition, the accumulation of succinate could potentially inhibit SCOT enzymes capacity to produce AcAc-CoA + succinate, which could explain why increasing  $\alpha$ KG concentration above 1 mM does not provide no further increase AcAc supported respiration. Again, it suggests 1 mM  $\alpha$ KG maximizing AcAc-CoA production with the least inhibition from succinate. Other studies have shown that under the presence of complex I inhibition by rotenone, D- $\beta$ OHB improves oxygen consumption via complex II (31) but no studies have directly compared D- $\beta$ OHB with AcAc and its electron contribution to complex II. The addition of complex I inhibitor after inhibiting complex II completely eradicates the remaining mitochondrial respiration, suggesting there is no "extra mitochondrial" source of respiration in mitochondria isolated from brain tissue. Comparing the decrease induced by complex I inhibitor with complex II inhibitor, our results show that both AcAc and D- $\beta$ OHB primarily contribute electrons through complex I via production of NADH. Surprisingly, Mna resulted in a bigger drop in D- $\beta$ OHB compared to AcAc, suggesting that D- $\beta$ OHB was more complex II driven than AcAc via succinate. D- $\beta$ OHB should generate more NADH (Complex I substrate) via  $\beta$ OHB dehydrogenase, and thus, relative to AcAc, less of its  $\text{JO}_2$  should be attributable to Complex II. These results might be occurring due to adding  $\alpha$ KG as a co-substrate prior to the addition of AcAc or D- $\beta$ OHB. Further studies are needed to investigate the entry path of electrons into the ETC with only D- $\beta$ OHB and leveraging both malonic acid (Mna, complex II inhibitor) and rotenone (Rot, complex I inhibitor). As expected, increasing AcAc concentrations greater than 1 mM tended to decrease  $\text{JO}_2$ . This could be partially attributable to free CoASH sequestration-mediated inhibition of substrate activation. Moreover, acetyl-CoA accumulation at higher AcAc concentrations inhibit citrate synthase (32).

Besides being known as the energy source in the fasting state, KB are also heavily studied for signaling functions. Studies have shown that  $\beta$ OHB can function as a ligand for different G-protein coupled receptors to reduce lipolysis, reduce sympathetic tone,

and lower metabolic rate (33, 34). Less is known about how ketone utilization affects metabolite contents within mitochondria. Metabolites emanating from ketone metabolism likely regulate multiple mitochondrial signaling pathways and may provide an underlying context to the benefits of brain ketone metabolism (1). Here, we used the ketone respiration protocol we developed earlier and compared how control mice with Neuron-SCOT present vs. Neuron-SCOT KO differ in their acetyl CoA levels and energy status after providing AcAc as a fuel source. In the adult brain, neurons have the highest energy demand, and by knocking out the key enzyme SCOT for ketone utilization, we blunt neuronal ability to utilize AcAc, thus allowing us to test if significant changes in neuronal ketone utilization alter metabolite levels and energy charge. Since we used the entire half of the left hemisphere, we would expect residual ketone-supported respiration by other cell types that express SCOT but a decrease in ketone-supported respiration due to the absence of SCOT in neurons. Aligning with our hypothesis, we noticed a decline in AcAc-supported respiration in the SCOT-Neuron KO compared to the control. We observed an overall decrease in acetyl-CoA level in the SCOT-Neuron KO mitochondria using AcAc+ $\alpha$ KG as the substrate. Mounting evidence has shown that exogenous KB leads to increased levels of acetyl-CoA and the citric cycle intermediates in the brain (1, 35). The compromised neuronal ability to utilize AcAc in the SCOT-KO leads to lower production of acetyl-CoA, which might explain the overall decrease seen in SCOT-KO. However, this difference between the control and KO was not seen when AcAc alone was used (Fig. 7C). This again suggests that  $\alpha$ KG is an essential co-substrate for AcAc to be metabolized in brain mitochondria. One limitation for measuring acetyl-CoA level in isolated mitochondria is the possibility of acetyl-CoA being exported out of the mitochondria as acetyl-carnitine during the isolation process, thus the acetyl CoA levels reported here may not truly reflect levels in mitochondria in vivo. Interestingly, we also observed a disconnect of no significant differences in final respiration but lower acetyl-CoA level. This might be because acetyl-CoA was in excess and not a limitation in fueling the TCA cycle to fuel forward the respiration reaction.

We also measured AMP, ADP, and ATP concentration to calculate energy charge in isolated brain mitochondria following the respiration protocol in the O2k. Our results showed no significant difference in energy charge between WT and KO regardless of substrate conditions. Under physiological conditions, the energy charge value fluctuates around 0.75–0.90, stabilizing around 0.9 (36). In our case, mitochondrial energy charge was around 0.5 between WT and KO, indicating ATP depletion. This shift could be explained by mitochondrial shifting to a lower EC when its surrounding conditions are unfavorable, such as isolation from the in vivo environment.

Our study has other limitations. While we focused on ketone respiration in isolated brain mitochondria, ketone bodies enter cells via MCTs (MCT1 and MCT2) (2), a regulatory node ignored in isolated mitochondria. In addition, there is evidence that mitochondria exist in a reticulum in neurons and glia cells. Thus, the mitochondrial isolation process would disrupt the reticulum, and thus, we are quantifying respiration in mitochondria not truly representative of their morphology in-vivo (37).

In summary, the protocol and substrate conditions described above allows for a direct and enhanced measurement of maximal KB-supported respiration in isolated brain mitochondria

which will allow for future studies to investigate if and how brain mitochondria adjust to various interventions (ketogenic diets, intermittent fasting, exercise) or pathological states that may alter hepatic ketogenic production and utilization rates in the brain. Adding  $\alpha$ KG enhances ketone-supported respiration, enabling us to quantify better oxidative phosphorylation in brain mitochondria utilizing ketones. This method is meant to serve as a base platform upon which new modules can be tailored to expand its usage in other tissues, such as the heart, muscle, and kidney, expanding our capacity to understand ketone metabolism in mitochondria in relation to whole-body effects. We also demonstrate that neuronal loss of SCOT reduces acetyl CoA levels in isolated brain mitochondria compared to controls, but does not change energy charge, and only slightly lowers respiration in whole brain isolated mitochondria. Furthermore, the detailed analysis of acetyl CoA and energy status in isolated brain mitochondria following respiration is also a new method that provides a tool for relating respiratory results to mitochondrial fuel sources and energy status. Overall, our newly described substrate conditions for measuring KB-supported respiration set the stage for further work exploring the novel therapeutic potentials of ketone metabolism.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgement

The graphical abstract was created using bioRender (<https://BioRender.com>).

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## Data Availability

All data will be made available upon request.

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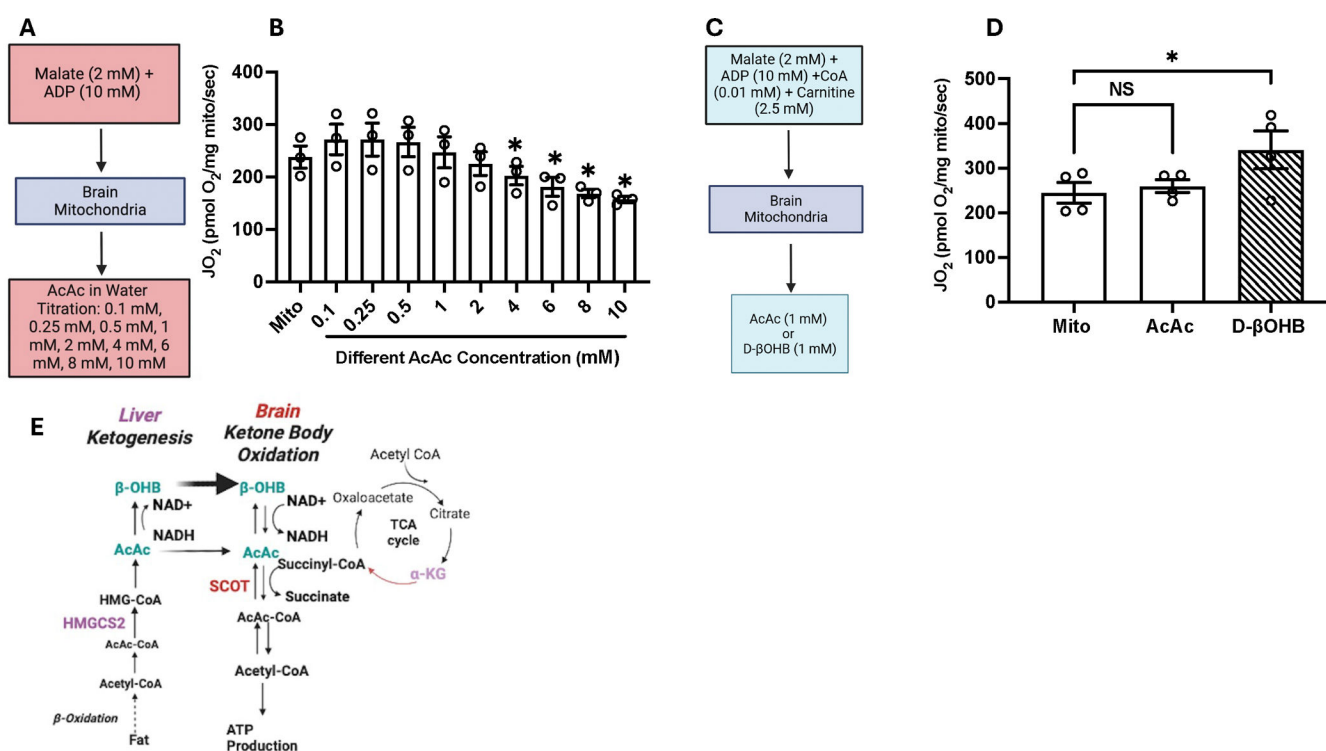
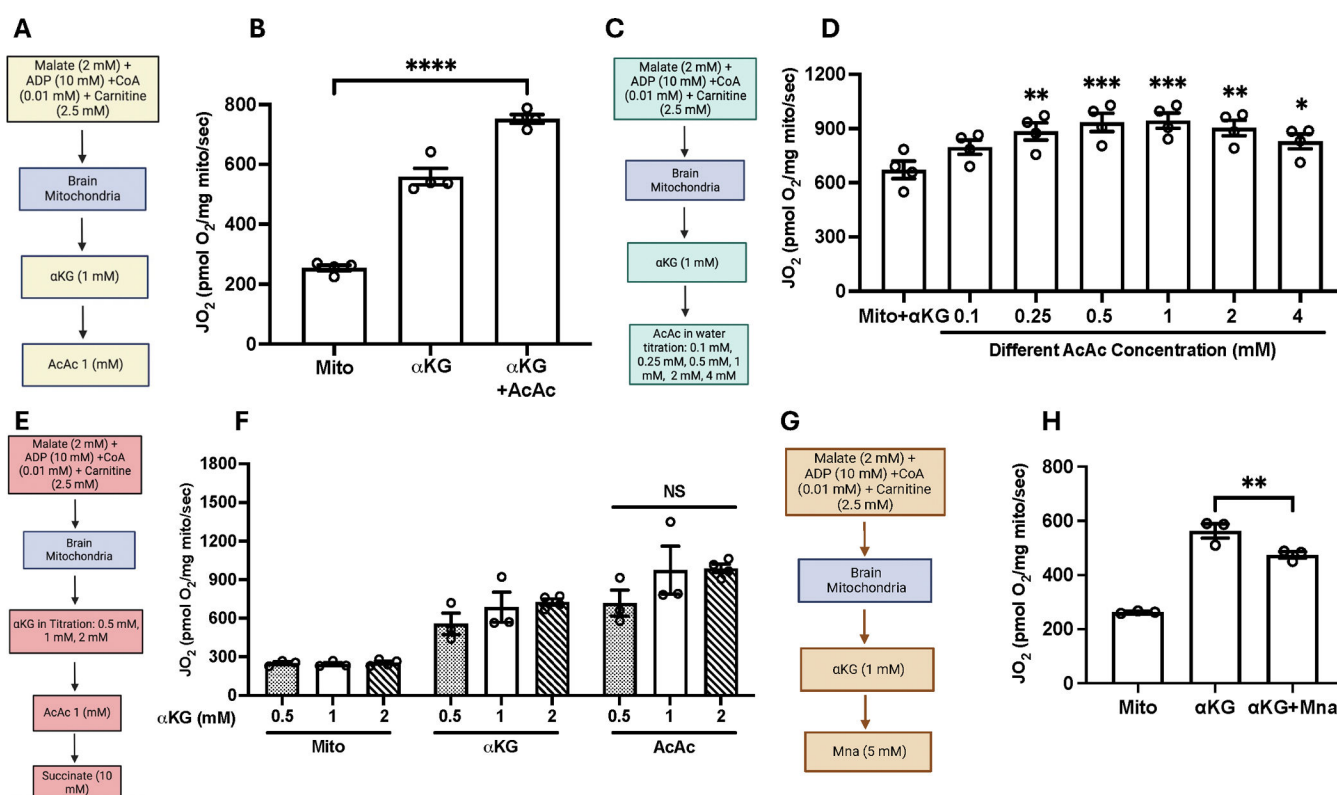


**Figure 1.**

KBs minimally drive respiration following Malate, ADP, CoA and Carnitine (A) Brain mitochondrial respiration protocol: Malate+ ADP+ AcAc (B) AcAc titrations were performed to determine maximal ketone-supported respiration (C) Respiration protocol: Malate +ADP + CoA + Carnitine + AcAc or D- $\beta$ OHB (D) CoA and Carnitine were added to determine impact AcAc and BOHB supported respiration. (E) Succinyl-CoA maybe one key regulator in ketone supported respiration. All data are represented as  $\pm$  SEM and evaluated via one-way ANOVA, followed by a Fisher's LSD post hoc analysis. \* $P < 0.05$

**Figure 2.**

Optimize αKG and AcAc concentration for AcAc supported respiration (A) Respiration protocol: Malate + ADP+ CoA + Carnitine + α ketoglutaric acid (αKG) + AcAc. (B) ADP-dependent (State III) respiration rate fueled by AcAc in isolated brain mitochondria in the presence of αKG. (C) Respiration protocol: Malate + ADP+ CoA + Carnitine + αKG + AcAc (D) AcAc titrations were performed to determine AcAc saturation in the presence of αKG. (E) Respiration protocol: Malate + ADP+ CoA + Carnitine + different concentration αKG + 1 mM AcAc (F) Different concentration αKG were tried to maximize ketone respiration while minimizing αKG effects on brain mitochondria (G) Respiration protocol: Malate + ADP+ CoA + Carnitine + αKG + inhibitor (H) Respiration rate was measured in the absence of KBs and Malonic acid was added to measure complex II supported respiration fueled by αKG. n=3–4 for all groups. All data are represented as ± SEM and one-way ANOVA, followed by a Fisher's LSD post hoc analysis. \*P<0.05; \*\*P<0.01; \*\*\*P=0.0001; \*\*\*\*P<0.0001.

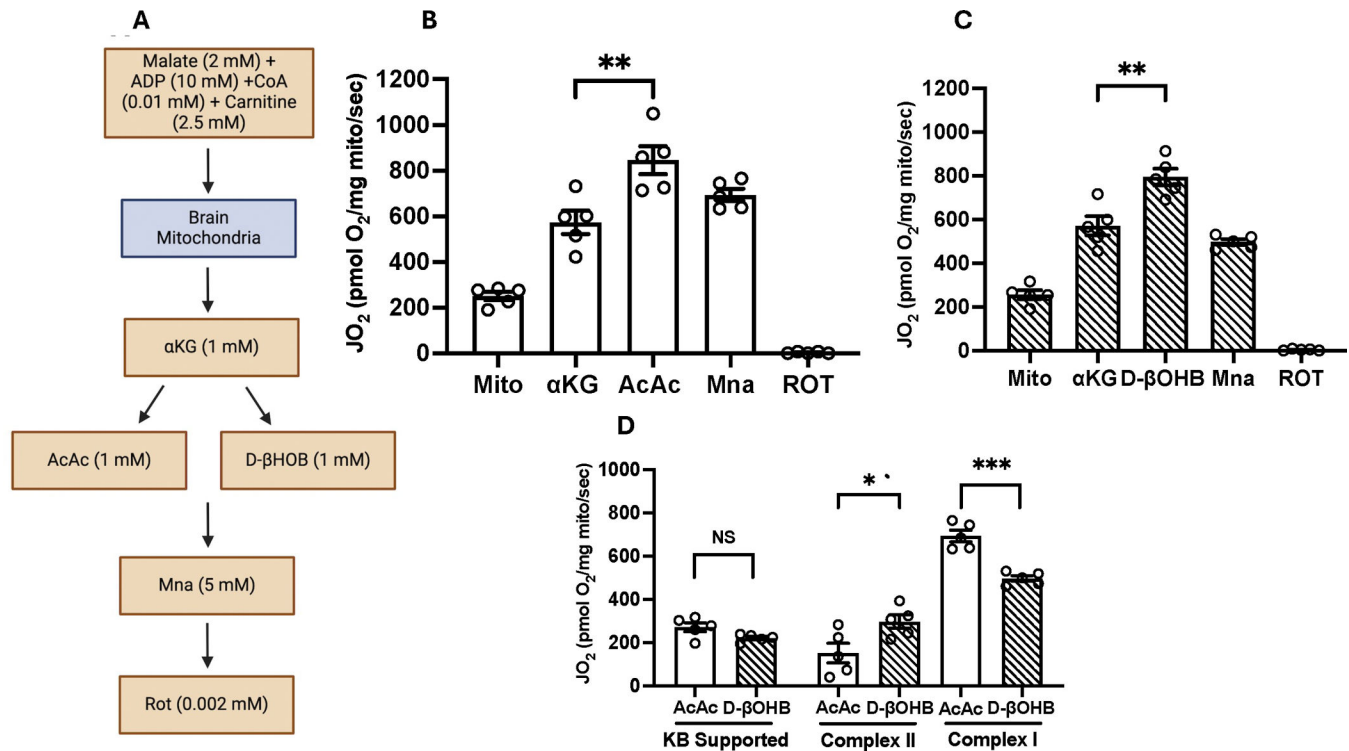


**Figure 3.**

KBs supported respiration protocol (A) Respiration protocol: Malate + ADP+ CoA + Carnitine + αKG + AcAc or D-βOHB + Mna + Rot (B) ADP-dependent (state III) respiration rate supported by AcAc in isolated brain mitochondria were measured. (C) ADP-dependent (state III) respiration rate supported by D-βOHB in isolated brain mitochondria were measured. (D) AcAc or D-βOHB supported respiration calculated using Respiration (AcAc or D-βOHB)-Respiration(αKG); Complex I calculated using: Respiration (Mna)-Respiration (Rot); Complex II calculate using Respiration (AcAc or D-βOHB)-Respiration. N=5 for all groups. Data are represented as ± SEM. Mna, Malonic acid, Rot, Rotenone. Fig. B and Fig. C are analyzed via one-way ANOVA, followed by a Fisher's LSD post hoc analysis. Fig. D is analyzed by by unpaired T test for each comparison between AcAc vs. D-βOHB. \*P<0.05; \*\*\*P =0.0001; \*\*\*\*P<0.0001.

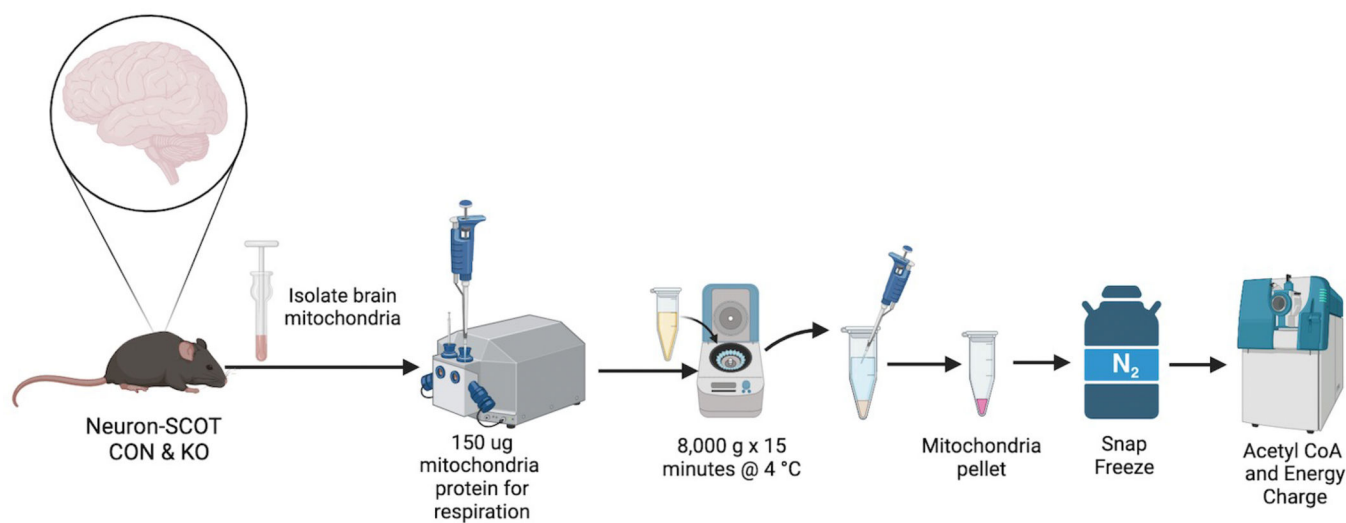
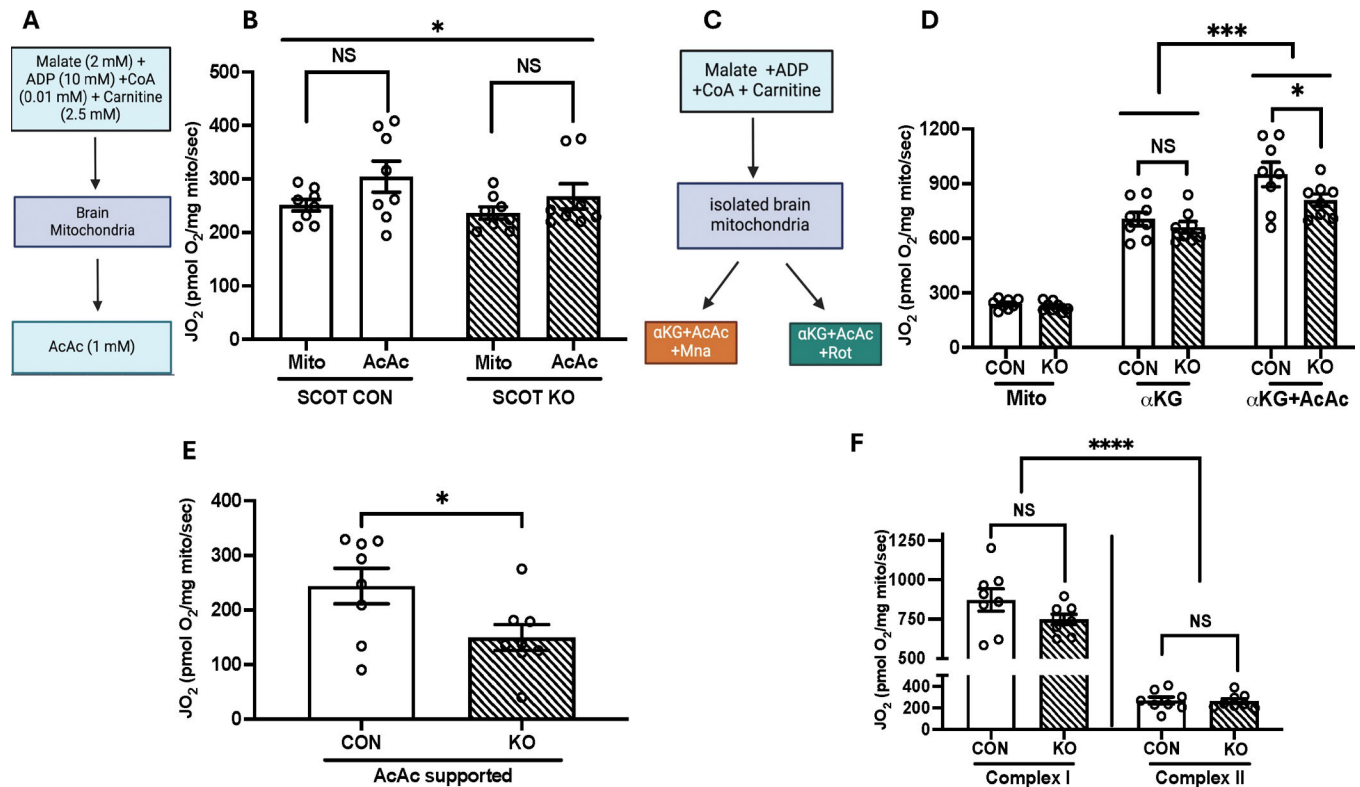


**Figure 4.** Overview of experimental design: process for measuring respiration, metabolite level, and energy charge in isolated brain mitochondria.

**Figure 5.**

WT vs SCOT KO AcAc Supported Respiration. (A) Brain mitochondrial respiration protocol: Malate + ADP + CoA + Carnitine + AcAc (B). WT and KO show minimal AcAc supported respiration. (C). Brain mitochondrial respiration protocol: Malate + ADP + CoA + Carnitine +  $\alpha KG$  + AcAc + Mna or Rot. (D). ADP-dependent (state III) respiration rate supported by AcAc in isolated brain mitochondria were measured (E). AcAc supported respiration (Respiration $_{\alpha KG + AcAc}$  - Respiration $_{\alpha KG}$ ) in WT vs KO (F). Complex I (Respiration $_{\alpha KG + AcAc}$  - Respiration $_{Rot}$ ) and Complex II (Respiration $_{\alpha KG + AcAc}$  - Respiration $_{Mna}$ ) supported respiration in WT vs KO. N=8 for all groups. Data are represented as  $\pm$  SEM. Mna, Malonic acid, Rot, Rotenone. For Fig. B, Fig. D, and Fig. F, data are analyzed by two-way ANOVA analysis, followed by a Fisher's LSD post hoc test. For Fig. E, data is analyzed via unpaired T test. \* $P < 0.05$ ; \*\*\* $P = 0.0001$ ; \*\*\*\* $P < 0.0001$ .

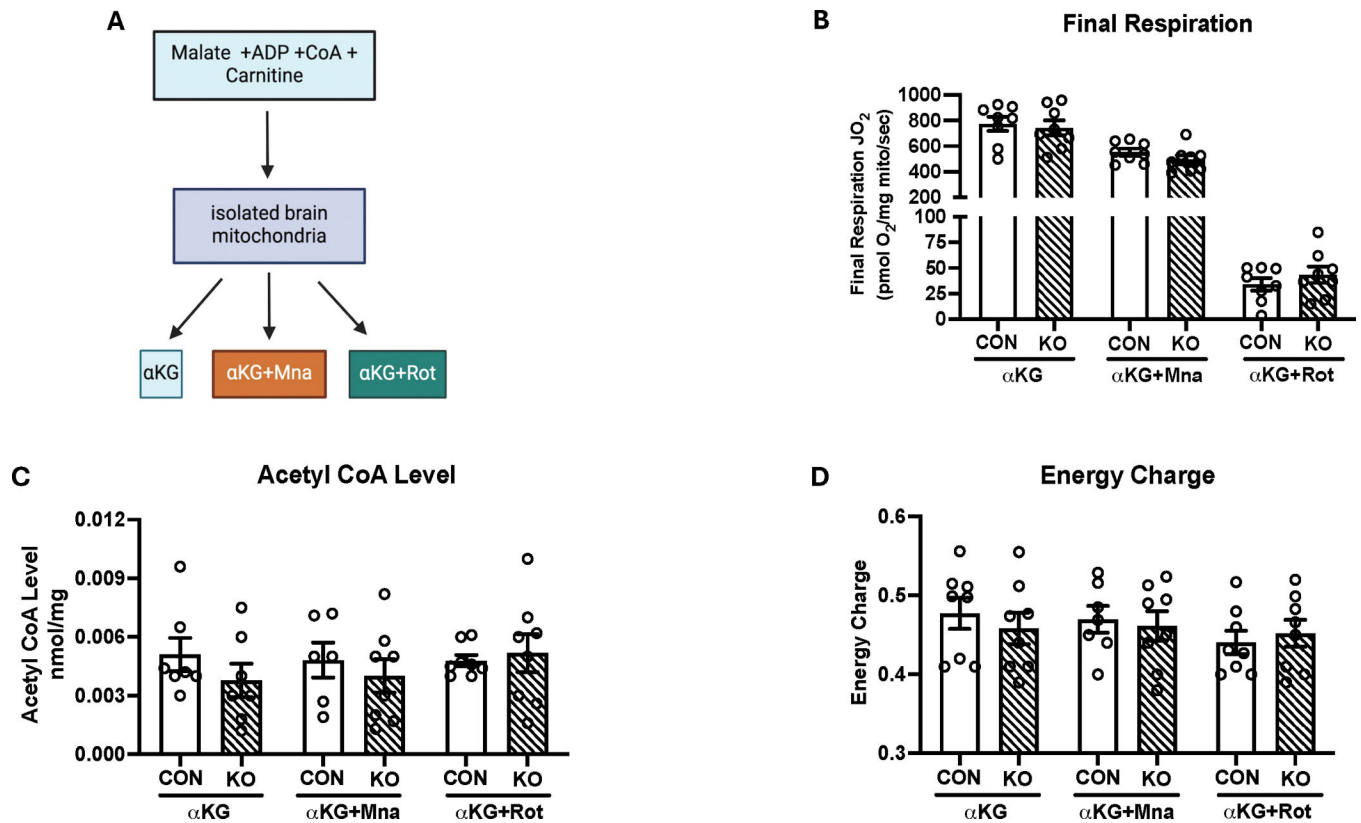


**Figure 6.**

No significant genotype difference in  $\alpha$ KG Supported Respiration and metabolite profile.

(A). Brain mitochondrial respiration protocol: Malate +ADP + CoA + Carnitine +  $\alpha$ KG vs  $\alpha$ KG +Mna vs  $\alpha$ KG + Rot (B). Final respiration status prior to metabolite analysis (C). Acetyl-CoA level for all three conditions (D). Energy charge for all three conditions calculated using AMP, ADP, and ATP. N=8 for all groups. All data are represented as  $\pm$  SEM. Mna, Malonic acid, Rot, Rotenone. All data are analyzed via unpaired T test to compare the control and SCOT KO in each condition.

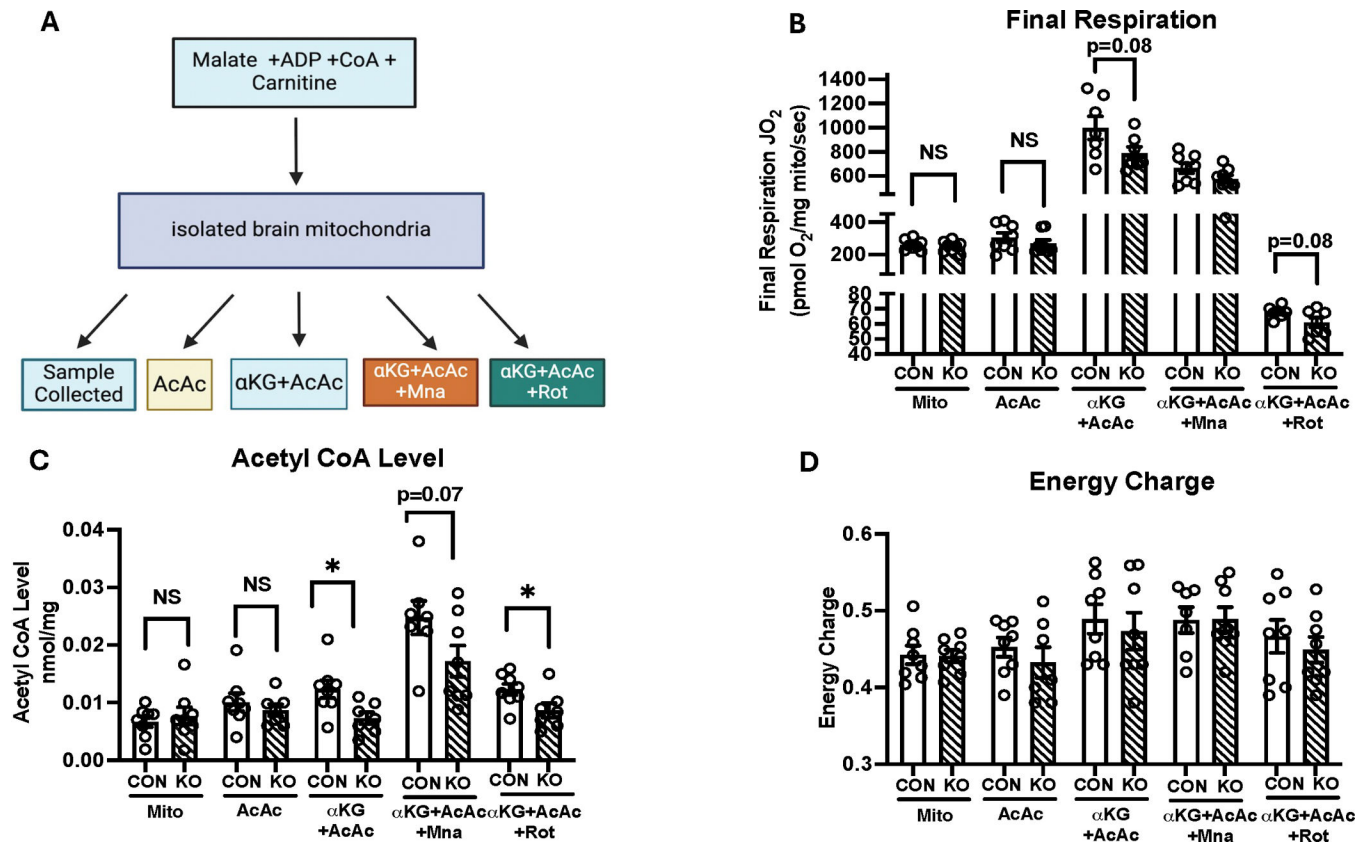


**Figure 7.**

SCOT KO has a lower acetyl-CoA level using  $\alpha$ KG +AcAc as the fuel source. (A) Brain mitochondrial respiration protocol: Malate +ADP + CoA + Carnitine vs AcAc vs  $\alpha$ KG +AcAc vs  $\alpha$ KG +AcAc +Mna vs  $\alpha$ KG +AcAc+ Rot (B) Final respiration status prior to metabolite analysis (C). Acetyl-CoA level for all five conditions (D). Energy charge for all five conditions calculated using AMP, ADP, and ATP. N=8 for all groups. Data are represented as  $\pm$  SEM. Mna, Malonic acid, Rot, Rotenone. All data are analyzed via unpaired T test to compare the control and SCOT KO in each condition. \* $P<0.05$ .