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Supplemental Information

New Insights into the Lactate Shuttle: Role of MCT4 in the Modulation of the Exercise Capacity

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Figure S1

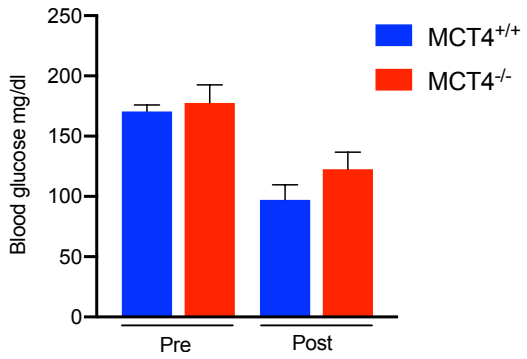
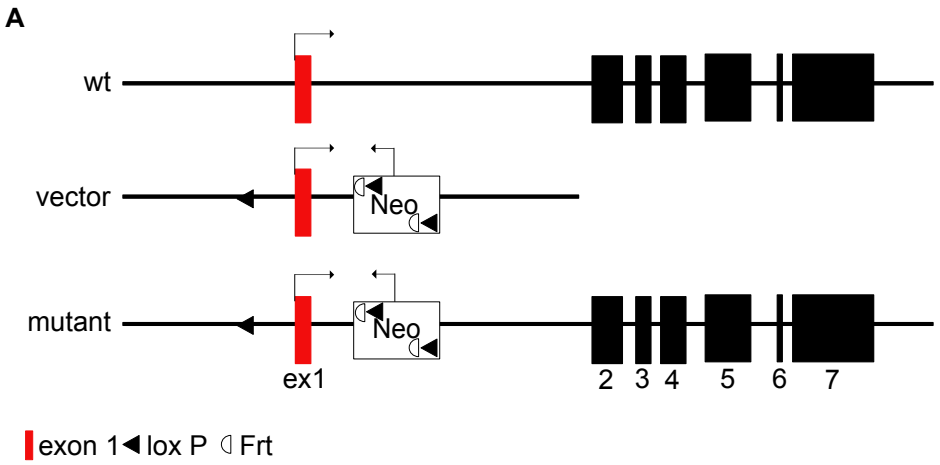


Figure S1, related to figure 2: Blood glucose measurement before and after treadmill test: (A) Venus blood glucose measured in 6 months old $MCT4^{+/+}$ and $MCT4^{-/-}$ before and after treadmill test, (n = 5 each group). Error bars = SEM.

Figure S2



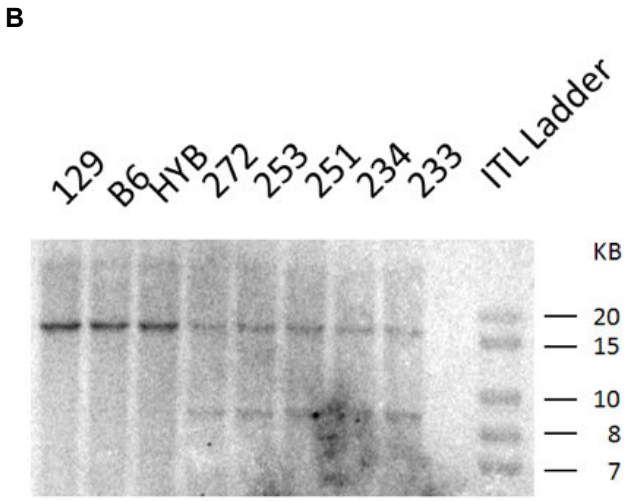


Figure S2, related to figure 5: Generation of the Bsg conditional mice. (A) Design of targeting vector to produce floxed Bsg mouse with placement of LoxP sites to target exon 1 of Bsg gene for deletion. (B) Southern blot confirmation of five ES clones (233, 234, 251, 253 and 272) as correctly targeted and used for injection.

Figure S3

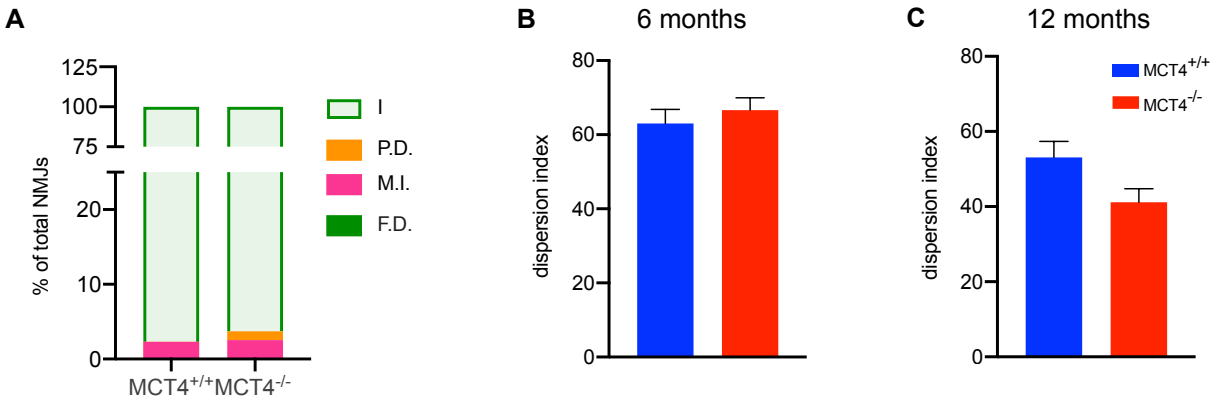


Fig S3, related to figure 7: Morphological analysis of NMJs in soleus muscles and postsynaptic endplate: (A) Quantification of intact and disrupted NMJs in soleus muscles of 12 months old $MCT4^{-/-}$ and $MCT4^{+/+}$ (n = 3 each group). Quantification of the density of Acetylcholine receptors (AChR) at the postsynaptic endplate from EDLs cross sections from (B) 6 months old (n = 4 each group, 6 NMJs per animal) and (C)12 months old (n = 3 each group, 3 NMJs per animal) $MCT4^{+/+}$ and $MCT4^{-/-}$ mice. Error bars = SEM.

Table S1 related to Figure 4: Contractile and morphometric measurements of EDL muscles.

	MCT4$^{+/+}$	**MCT4$^{-/-}$**	***P***
EDL contractility	N=4	N=5	
Twitch force (mN)	94.68±13.90	118.22±4.71	0.121
Twitch force (N/cm^2)	5.36±0.62	6.04±0.13	0.265
Tetanic force (mN)	345.08±53.87	447.76±7.18	0.070
Tetanic force (N/cm^2)	19.42±2.10	22.95±0.57	0.115
Twitch - TTP (ms)	20±0.42	20.10±0.27	0.841
Twitch - RFD (mN/sec)	12747.25±1909.27	14428.00±892.54	0.419
Fatigue AUC	956.90±83.52	981.38±82.61	0.843
EDL morphometry	N=4	N=5	
L_0 (mm)	11.93±0.37	12.56±0.10	0.106
Mass (mg)	9.98±0.63	11.74±0.52	0.066
CSA (mm^2)	1.76±0.12	1.96±0.08	0.191

Values are mean ± SEM. TTP, time to peak; RFD, rate of force development; CSA, cross sectional area; L_0, muscle length.

TRANSPARENT METHODS

Animals.

All animal procedures were performed in accordance with the Thomas Jefferson University Institutional Animal Care and Use Committee. MCT4 (*slc16a3)* knock out mice, stock no. TF3223A, were obtained from Taconic Farms. Mice were backcrossed for 10 generations to C57BL/6NTac to obtain a 99% C57BL/6 background. $Bsg^{flox/flox}$ mice were obtained from Dr. Nowak at University of Illinois. The mice were generated by inGenious Targeting Laboratory (www. Genetargeting.com). The construct was designed to target exon 1 for conditional deletion as shown in the supplementary material (**Fig. S1A**). Briefly, ten micrograms of the targeting vector were linearized by NotI and then transfected by electroporation of BA1(C57Bl/6 x 129/SvEv) (Hybrid) embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis and southern blotting to identify recombinant ES clones. Sequencing was also performed on purified PCR DNA to confirm the presence of the LoxP cassette using the SDL2 primer. Five positive clones were confirmed by southern blotting (**Fig. S1B**) and used for blastocyst injection, implantation and generation of chimeras. Germline transmission was confirmed in four of the clones. $Bsg^{flox/flox}$ were crossed with tamoxifen inducible Acta1-cre/Esr1 mice (Jackson Lab, stock no 025750 HSA-MCM). Induction of the cre-recombinase was obtained by daily injection of tamoxifen (2mg/kg), for 5 consecutive days. $Bsg^{flox/flox}$:$Cre^{+/-}$ mice injected with vehicle (15% ethanol in sunflower oil) were used as control. Mice were housed at 22ºC and under 12:12h light-dark cycle. Male mice were used in all the reported experiments except for the reported wheels running test and NMJs morphology data.

Histology.

Tibialis anterioris (TA) muscle and extensor digitorum longus (EDL) muscles were frozen in Tissue-Tek OCT compound (WVR, Atlanta GA) and stored at -80ºC. 10µm cryosections were cut and used for subsequent histological and immunofluorescence stainings. For H&E staining, a standard procedure was followed. For fiber typing analysis, sections were stained with monoclonal antibodies against myosin heavy chains produced by Dr Schiaffino's lab at University of Padova and distributed by the Developmental Studies Hybridoma Bank (DHSB, University of Iowa). Ba-D5 (supernatant), specific for MyHC-1 was used at 1:100 dilution, Bf-F3 (purified antibody), specific for MyHC-IIB was used at 1:100 and Sc-71 (supernatant), specific for MyHC-IIA, was used at 1:50. The secondary antibodies: donkey anti-mouse IgG2b AlexaFluor647 to bind Ba-D5; donkey anti-mouse IgM AlexaFluor 555 to bind Bf-F3 and donkey anti-mouse IgG1 AlexaFluor 488 to bind Sc-71 (Life Technologies, New York) were diluted 1:500. Membranes were counterstained with anti-laminin antibody (L-9393, Sigma) or wheat germ agglutinin (WGA) conjugated with AlexaFluor 488 or AlexaFluor 647 (Thermo Fisher). Images were collected using the epifluorescence microscope Eclipse 800 (Nikon) and analysis of the distribution of the different fiber types were performed with the ImageJ software. For the analysis of MCT1 and MCT4 distribution, anti-MCT1 and anti-MCT4 antibodies previously characterized by our laboratory (Philp et al., 2003) were used. For spinal cord histological analysis, samples were collected and fixed overnight in 4% paraformaldehyde. After cryopreservation in 30% sucrose solution the samples were frozen in Tissue-tek OCT compound and stored at -80. For motoneurons analysis, 25um thick transversal sections from the lumbar portion of the spinal cord were cut and stained with 0.5% cresyl violet acetate solution and images were captured using cellSens Entry software and the Olympus DP22 camera attached to an Olympus CX41 microscope (Olympus Scientific Solutions Americas). Motoneurons were identified as larger polygonal cells, with diameter larger than 100µm in the ventral horn. For every subject 3 different sections, 150 µm apart were evaluated.

Exercise tolerance test.

Exercise tolerance was assessed using a motorized Exer3-6 treadmill system with shock counter (Columbus Instruments, Columbus OH). Mice were acclimated to the treadmill for 3 consecutive days with a 10 minutes session at 0 incline (1- zero shock, 5m/min speed, 2- 1hz 0.5mA shock, 5m/min speed for 5 min, 10m/min speed 5min, 3- 1hz 0.5mA shock, 10m/min speed). Between acclimation and test the mice rested for 48h. The test was performed at 5º incline, it started at 10m/min and the speed was increased by 2m/min every 3 minutes, until a maximum speed of 26m/min was reached. Maximum speed, time on belt and distance run, as well as the number of the shocks received during the test, were recorded. The mice were considered exhausted when they sat on the shock grid for 5 consecutive seconds despite the shocks.

Voluntary wheel running test.
Mice were single housed in cages equipped with wheels (Columbus Instrument) for 7 days. Wheel revolutions during the last 60 hours were acquired and analyzed.

RNA analysis.
RNA was extracted with TRIzol Reagent (Thermo Fisher) and cDNA synthesis was performed using the RNA to cDNA EcoDry™ Premix kit (Clontech). SYBR green qPCR analysis was performed on the QuantStudio 5 real time system (Thermo Fisher). Relative expression was calculated using *Rplp0* as housekeeping. The primers used were:
Car2: Fw CAGCGAGCAGATGTCTCATTTC, Rev TTCTTTAGCGGCTGAGCTGG,
Car3: Fw TTAAGACGAAGGGCAAGGAGG, Rev AGGAGCCGTGATAGGTCCAA,
Car4: Fw GAGCCAGCTATCCACGTTCC, Rev TTCCGGTCTGCTCTGCCTAT,
NEH1: Fw GCCTCATGAAGATAGGTTTCCA, Rev ACGTCTGATTGCAGGAAGGG,
MCT1: Fw TGTTAGTCGGAGCCTTCATT, Rev CACTGGTCGTTGCACTGAATA,
Rplp0: Fw: AGATTCGGGATATGCTGTTGGC, Rev TCGGGTCCTAGACCAGTGTTC.

Intramuscular lactate measurement.
After stimulation, muscles were rapidly frozen in liquid nitrogen. 30 to 50 mg of gastrocnemius muscle were homogenized in ice cold 0.6M perchloric acid with a handheld homogenizer and then centrifuged at 14000 rpm for 15 minutes. The supernatant pH was neutralized with 1.8M potassium carbonate. After further centrifugation the supernatants were collected and used for lactate assay using a commercial kit (Trinity Biotech).

Blood Glucose measurement.
Blood glucose was measured before and right after treadmill test from tail blood using a commercialy available glucose meter (Precision Xtra Glucose Meter, Abbot)

Western Blot analysis.
Skeletal muscle tissue was homogenized in a buffer containing 50mM Tris, pH8, 150mM NaCl, 5mM EDTA, 0.1% SDS, 1% Triton X-100 and 0.5% Deoxycholic acid. Samples were separated in a 4-12% Bis-Tris precast Nupage gels (Thermo fisher) and blotted onto PVDF membranes. Membranes were blocked in 5% no-fat milk in TBS-tween buffer and incubated overnight with primary antibodies. After washing the membranes in TBS-tween buffer they were incubated in HRP conjugated secondary antibodies for 1h at room temperature. We developed the blots using SuperSignal West Dura ECL substrate (ThermoFisher Scientific) and imaged using FluorChem M ProteinSimple imager.

CMAP and ex vivo EDL contractility analysis.
During all ex vivo and in vivo procedures, the genotype of the mice was not disclosed to the operator. For the analysis of Compound Muscle Action Potential (CMAP), mice were anesthetized with 1% isoflurane. Depth of anesthesia was confirmed by toe pinch and by monitoring heart rate and SpO_2 with a pulse oximeter (PhysioSuite, Kent Scientific). The sciatic nerve was exposed, crushed distally to the muscle to prevent retrograde propagation of the stimuli, and connected to a custom-made bipolar stimulating electrode driven by a Grass S48 stimulator. Two recording silver EMG needle-electrodes were applied at fixed distance (5 mm), one in the gastrocnemius and the other near the Achilles tendon. The ground electrode was connected to the skin of the back of the mouse. The recording electrodes were then connected to a Warner Instruments DP-311 differential amplifier, band-pass filtered (0.1-10k Hz) and finally fed into a PowerLab/8Sp A/D converter (AD Instruments). One leg of each mouse was electrically stimulated, while the other was left unstimulated and used to dissect the EDL muscle for *ex vivo* contractility testing after completion of the CMAP protocol. Three trains of ten stimulating pulses (5V, 0.1ms duration) were applied for each frequency tested (1, 3, 5, 10, 20, 40Hz), allowing 2 minutes for recovery between each train. Data were acquired and analyzed with LabChart8 (AD Instruments), calculating EMG amplitude and root mean square (RMS) of the signal.
Muscle physiological analysis was performed on isolated EDL muscles using an Aurora Mouse 1200A System equipped with Dynamic Muscle Control v.5.415 software. EDL muscles were dissected and analyzed in constantly oxygenated Ringer's solution (100mM NaCl, 4.7mM KCl, 3.4mM $CaCl_2$, 1.2mM KH_2PO_4, 1.mM $MgSO_4$, 25mM HEPES, 5.5mM D-glucose) at 24°C. The twitch stimulation protocol applied was a single stimulus with a duration of 0.2 ms. Muscle length was adjusted to obtain the maximal twitch response and this length was measured and recorded as optimal length (L_0). For measuring tetanic maximal force generation, the stimulus was repeated at a frequency of 120Hz for 500ms. Five minutes were allowed between two tetanic contractions to ensure muscle recovery. For

induction of fatigue, 5 min after the last maximal tetanic contraction, muscles were stimulated every second for 8 min using 40-Hz pulses lasting 330 ms. Muscle cross-sectional area (CSA) of EDL muscles was calculated by dividing the muscle mass by the product of the muscle density coefficient (1.06 g/cm^3), muscle L_0, and the fiber length coefficient (0.45 for EDL). Specific force was determined by normalizing maximum isometric tetanic force to CSA.

In situ hybridization.
RNAScope technology was used for in situ hybridization following manufacturer's protocol. Briefly, 10um thick section were pretreated with hydrogen peroxide and protease. The slides were then incubated with RNAScope probe (mmSlc16a3) for 2 h at 40ºC followed by the amplification steps according to protocol. Signal was developed using the RNAscope 2.5 HD Manual Detection Kit Red. The slides were then blocked in 5% BSA and immunostained with anti GFAP (Sigma) over night. The next day donkey anti rabbit AlexaFluor 488 (Thermo Fisher) secondary antibody was applied for 1 hour and after washing slides were mounted in ProLong Diamond antifade mounting media with DAPI (Thermo Fisher). Images were captured on a Nikon Eclipse E800 with 4x/0.13 and 40x/0.75 DIC objectives.

Sciatic nerve morphological analysis.
Sciatic nerves were dissected and fixed in 4% glutaraldehyde in 0.1M Millonig buffer for 2 hours, rinsed in 0.54% glucose in Millonig buffer overnight and osmicated in 2% OsO_4 for 45 minutes. After dehydrations the samples were embedded in Durcupan resin. 500nm sections were cut with a glass knife using a Leica UCT ultramicrotome, and then stained with 1% Toluidine blue and images were captured with Olympus CX41 microscope (Olympus Scientific Solutions Americas). Morphometric analysis of the myelinated axons was done with the Halo software, Axon module (IndicaLab, Albuquerque NM, USA). Axons and fibers diameter were recorded, and g-ratio was calculated.

Neuromuscular junction staining.
EDLs and solei were dissected, cleaned from connective tissue and fixed in 4%PFA and processed for immunohistochemistry as previously described (Wright et al., 2007). They were then incubated with rhodamine-conjugated alpha-bungarotoxin (Thermo Fisher) to label acetylcholine receptors. After permeabilization in methanol, samples were blocked in 2% BSA and then incubated overnight with primaries antibodies, SMI-312R (Covance) and SV-2 (Hybridoma bank) for labeling motor axons and terminal vesicles, respectively. After washing in PBS, samples were incubated for 1hour with with FITC anti-mouse IgG secondary (Jackson ImmunoResearch). Samples were mounted with Vectashield mounting media and coverslipped. Samples were then analyzed for total numbers of intact, completely denervated, partially denervated and multiply innervated NMJs. Each junction was categorized as 1) intact if there was complete overlap between presynaptic nerve terminal and postsynaptic AChRs, 2) partially denervated with only partial occupation of postsynaptic AChRs by the overlying nerve terminal, or 3) completely denervated, with no occupation of postsynaptic AChRs by the nerve terminal. To ensure that a junction was completely denervated, and that the absence of the nerve terminal was not an artifact of the staining procedure, junctions were only counted as completely denervated if there were other nearby labeled junctions in the same viewing plane. Rapresentative images were captured using a Nikon A1R confocal microscope, with a 40X/1.3 oil objective. For analysis of the post synaptic endplate density, 25µm thick frozen cross sections of EDLs muscle were stained with rhodamine-conjugated alpha-bungarotoxin. Z-stack images of en-face NMJs were collected using the Nikon A1R confocal microscope and processed with ImageJ to obtain a maximum intensity projection from the Z-stack. Using the Cell Profiler software the images were batch processed and total area occupied by the staining and the perimeter were quantified. Dispersion index was calculated as ratio between the total perimeter and total area.

Statistical analyses.
Data are represented as mean ± SEM. Data were processed and analyzed with GraphPad Prism 8 (GraphPad software). Before any statistical analysis was applied, data were assessed for normality using the D'Agostino-Pearson's test. Normally distributed data were compared using the student's t test, for non-normlly distributed data, the Mann-Whitney test was applied. Data in **Fig.2A** were analyzed using a One-Way ANOVA test, followed by Tukey's test for multiple comparisons. Data in **Fig.3E-F and 4D** were analyzed with a 2-way ANOVA test followed by Tukey's test for multiple comparisons. For analysis of NMJs in **Fig.7B** a Chi-square test was applied. $P < 0.05$ was considered statistically significant.

REFERENCES:

Wright, M.C., Cho, W.-J., Son, Y.-J., 2007. Distinct patterns of motor nerve terminal sprouting induced by ciliary neurotrophic factor vs. botulinum toxin. J. Comp. Neurol. 504, 1–16.