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**Insulin regulates activity-dependent mitochondrial depolarization and calcium dynamics
in dentate granule cells**

Abbreviated title: *Insulin limits DGC's mitochondrial depolarization*

Beatriz Ferrán^{1,2,3}, Hadyn M Rose^{1,2,3}, Jenna Gray^{1,2,3}, Traci L Redwine⁴, Sophia Pham^{1,2,3}, Oliver Rubin⁴, Heather C Rice^{1,2,5}, William E Sonntag^{1,2}, Carlos Manlio Díaz-García^{1,2,3}.

¹Department of Biochemistry and Physiology, University of Oklahoma Health Campus, Oklahoma City, OK 73104, USA

²Center for Geroscience and Healthy Brain Aging, University of Oklahoma Health Campus, Oklahoma City, OK 73104, USA

³ Harold Hamm Diabetes Center, University of Oklahoma Health Campus, Oklahoma City, OK 73104, USA

⁴ Graduate Program in Biomedical Sciences, University of Oklahoma Health Campus, Oklahoma City, OK 73104, USA

⁵ Aging & Metabolism Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA

Corresponding Author:

22 Carlos Manlio Díaz-García, PhD

23 ORCID: 0000-0002-4352-2496

24 Email: carlosmanlio-diazgarcia@ou.edu

25 Phone: 405-271-9021 x47817

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Abstract

Systemic insulin resistance is associated with reduced hippocampal energy metabolism and cognitive impairment both in humans and animal models. However, whether insulin rapidly regulates neuronal metabolic stability during activity remains unclear. Here, we examined insulin signaling and queried the corresponding mitochondrial responses in dentate granule cells (DGCs) of adult female mice. This layer of excitatory neurons within the hippocampus plays an essential role in learning and memory. Insulin robustly increased Akt phosphorylation in dentate gyrus tissue when insulin degradation was inhibited, and this response was attenuated by genetic deletion of the insulin receptor in DGCs, consistent with cell-autonomous signaling. We found that insulin did not alter mitochondrial membrane potential at rest, but significantly limited activity-evoked mitochondrial depolarization induced by neuronal stimulation. However, this change had no measurable impact on cytosolic ATP availability and was instead associated with Ca^{2+} dynamics. Removing Ca^{2+} ions from the extracellular solution, or disrupting mitochondrial Ca^{2+} uptake, prevented insulin modulation of the mitochondrial membrane potential. Furthermore, insulin limited cytosolic Ca^{2+} accumulation through an insulin receptor-dependent mechanism that persisted despite Akt inhibition. Insulin also accelerated the clearance of mitochondrial Ca^{2+} transients in active neurons, which occurred without changes in either neuronal excitability or pyruvate dehydrogenase phosphorylation. Together, these findings identify endogenous insulin degradation as a key regulator of hippocampal insulin signaling, and reveal a previously unrecognized interaction between insulin and Ca^{2+} signaling in stimulated DGCs. This work also

establishes a direct link between central insulin signaling and the maintenance of mitochondrial electrochemical homeostasis in the adult brain.

Significance Statement

Insulin signaling is known to influence synaptic plasticity, yet its direct effects on neuronal metabolism remain poorly defined. Here, we show that endogenous insulin degradation limits insulin signaling in adult hippocampal tissue. This finding suggests that inhibitors of the insulin degrading enzyme may hold the potential to improve the efficacy of insulin-based interventions aimed at improving learning and memory. Acute insulin signaling does not modify neuronal excitability, or cytosolic ATP availability. Instead, it stabilizes mitochondrial membrane potential and reduces calcium accumulation in active dentate granule cells. By identifying an insulin-dependent mechanism that regulates the electrochemical homeostasis of mitochondria in active neurons, this work provides insight into how brain insulin resistance may contribute to cognitive dysfunction in aging and neurometabolic disorders.

Keywords

Brain metabolism, Calcium, Hippocampus, Insulin, Insulin degrading enzyme, Mitochondria

Introduction

Reduced energy metabolism in both the central nervous system and peripheral tissues contributes to age-related declines in health span (Cholerton et al., 2011; Kullmann et al., 2016; Lieberman, 2003). Although the brain accounts for approximately 20% of total body energy consumption (Rolfe & Brown, 1997), it possesses only limited intrinsic energy reserves, with glycogen stored predominantly in astrocytes (Hirase et al., 2019). Consequently, neurons are highly susceptible to energetic stress, particularly during hypoglycemia (Cryer, 2007) and hypometabolic states marked by impaired glucose or pyruvate oxidation (Dowling et al., 2015; Rossi et al., 2020).

Hypometabolic states are closely associated with systemic insulin resistance and have been proposed as an early metabolic signature of cognitive decline (Willette et al., 2015). Similar reductions in glucose utilization emerge within the hippocampus as age-related cognitive impairment progresses toward Alzheimer's disease (Arnold et al., 2018; Mosconi et al., 2008). Supporting a causal role for insulin signaling in hippocampal function, animal studies have shown that neuronal overexpression of a constitutively active insulin receptor in the hippocampus delays age-associated cognitive decline (Frazier et al., 2020). Conversely, downregulation of neuronal insulin and insulin-like growth factor-1 receptors disrupts spatial learning and memory (Soto et al., 2019). These studies provide *prima facie* evidence that insulin has an important role in regulating brain metabolism.

Despite this compelling experimental evidence, attempts to enhance brain insulin signaling in humans have yielded mixed results, as intranasal insulin administration has shown inconsistent cognitive benefits across clinical studies (Craft et al., 2012, 2020). In contrast, both intranasal and intra-hippocampal insulin delivery have consistently improved learning and memory in rodent models (Brabazon et al., 2017; Gabbouj et al., 2019; McNay et al., 2010). Despite the potential

species differences, the studies underscore that a critical gap exists in our understanding of how insulin signaling is regulated within the adult hippocampus.

Previous studies indicate that local insulin delivery into the hippocampus increases extracellular lactate while concomitantly reducing glucose levels, consistent with enhanced glycolytic flux (McNay et al., 2010). However, it is uncertain whether neurons contribute to this metabolic response, as they contain vesicles with GLUT4 transporters that can be translocated to the plasma membrane of synaptic terminals independently of insulin (Ashrafi et al., 2017). Moreover, the constitutive degradation of PFKFB3 (Herrero-Mendez et al., 2009), which is a potential target for the insulin signaling pathway (Trefely et al., 2015), further challenges the concept that insulin has a direct role on the rapid regulation of glucose oxidation in neurons.

Alternatively, recent evidence suggests that insulin may modulate neuronal metabolism through mitochondrial pathways. Proposed mechanisms include the regulation of mitophagy (Hees et al., 2024) or the enhancement of pyruvate dehydrogenase activity, a response that has been reported to occur downstream of Akt activation in cardiac tissue (Karwi et al., 2020). Notably, studies examining insulin regulation of neuronal metabolism and function frequently employ insulin concentrations in the range of 0.1–10 μ M (De Felice et al., 2009; A. I. Duarte et al., 2006; Hees et al., 2024; Van Der Heide et al., 2005), far exceeding circulating physiological levels. One potential explanation for the increased insulin requirements in brain is the rapid degradation of insulin by the insulin-degrading enzyme (IDE), a peptidase abundantly expressed in the hippocampus (Cook et al., 2003), which may limit insulin bioavailability in ex vivo preparations.

Here, we demonstrate the activation of insulin signaling in dentate granule cells of the mouse hippocampus, a structure essential for learning and memory (Burgess et al., 2002; Neves et al., 2008), and characterized by robust insulin receptor expression (Dixon-Salazar et al., 2014; Lein et al., 2007). We identify insulin degradation as a major constraint on insulin signaling in the dentate gyrus and show that insulin preferentially enhances Akt, rather than Erk2, phosphorylation

in this region. Furthermore, we provide evidence that neuronal insulin signaling acutely regulates mitochondrial membrane potential and cytosolic Ca^{2+} dynamics in active neurons, without overt effects on neuronal excitability and cytosolic ATP dynamics. These findings support a new model for insulin action in the hippocampus, where insulin appears to preserve neuronal excitability while reducing the risk of Ca^{2+} overload during depolarizing stimuli.

Materials and Methods

Reagents

All reagents were purchased from Fisher Scientific (Suwanee, GA), unless otherwise specified. A zinc-free insulin solution from bovine pancreas (1.72 mM in 25 mM HEPES, pH 8.2) was obtained from Sigma (cat. # I0516-5ML). The insulin degrading enzyme inhibitor 6bK TFA was obtained from MedChemExpress (cat. # HY-110197) or Tocris (cat. # 5402), and a stock solution was prepared in ddH₂O at a concentration of 2.87 mM. The Akt inhibitor Akti-1/2 was obtained from Sigma (cat. # A6730-5MG) and a stock solution was prepared in DMSO at a concentration of 50 mM. Stock solutions were stored at -20°C until used for experiments.

Animals

Because previous in vivo observations demonstrate heightened hippocampal insulin sensitivity in female mice (Baehring et al., 2026; Jayarathne et al., 2022), we used mice of this sex for our studies. Conditional knockout mice for the insulin receptor (*IR^{fl/fl}*), with loxP sites flanking exon 4 [B6.129S4(FVB)-Insrtm1Khn/J; Strain #:006955, RRID:IMSR_JAX:006955] (Brüning et al., 1998), were used for studying the insulin signaling cascade in dissected dentate gyrus from the hippocampus. These animals were bred with *Dock10-Cre* mice (Kohara et al., 2014) and the resulting offspring were backcrossed with *IR^{fl/fl}* mice to generate animals with Cre-dependent downregulation of insulin receptors in dentate granule cells (DGCs) of the hippocampus (*IR^{fl/fl}::Dock10-Cre*, or *IR^{fl/fl}Cre* for simplicity). *Dock10-Cre* mice were also used for the expression of loxP-flanked biosensor constructs in dentate granule cells.

In addition, transgenic mice expressing the Ca²⁺ biosensor GCaMP6f (Chen et al., 2013) under the Thy-1 promoter [C57BL/6J-Tg(Thy1-GCaMP6f)GP5.17Dkim/J; Strain #: 025393, RRID:IMSR_JAX:025393] (Dana et al., 2014) were used for measuring Ca²⁺ dynamics, local field potentials, and NAD(P)H responses in DGCs. All transgenic mouse lines were maintained in a

C57BL/6J background. Female *C57BL/6J* mice were used for immunoassays and live-cell imaging in brain slices, including the assessment of the mitochondrial membrane potential ($\Delta\psi_m$). We also used mice with selective downregulation of the mitochondrial Ca^{2+} uniporter in dentate granule cells (*Mcu^{fl/Δ}::Dock10-Cre*, or *Mcu^{fl/Δ}Cre* for simplicity) for the latter experiments. These animals were generated using a breeding strategy that included conditional *Mcu* knock-out mice (Kwong et al., 2015), *Sox2-Cre^{+/-}* mice (Hayashi et al., 2002), and *Dock10-Cre* mice (Kohara et al., 2014), as previously described (Díaz-García et al., 2021).

Mice were housed in groups of 3-5 animals in static disposable cages (Innovive IVC Rodent Caging System, San Diego, CA), located at a conventional facility with *ad libitum* access to standard chow diet (5053 Pico Lab, Purina Mills, Richmond, IN) and water. All experiments were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. All procedures were approved by and followed the guidelines of the Institutional Animal Care and Use Committee of the University of Oklahoma Health Campus (reference number 22-010-SEAI and 25-009-SEFAHIL). Mice were used for experiments at ages of 3-10 months old.

Acute hippocampal slice preparation

Brain slices from adult mice were obtained as previously described by our group (J. Gray et al., 2025; Rosen et al., 2025). For electrophysiology and imaging experiments, slices were separated into hemispheres. For immunoassays, the dentate gyrus was further dissected as previously described (Logan et al., 2024; Rose et al., 2024), using an NMDG-based slicing solution for microdissections (J. Gray et al., 2025; Rosen et al., 2025).

Acute brain slices and dissected dentate gyri were transferred to a chamber filled with artificial cerebrospinal fluid (ACSF) at 37°C, containing (in mM): 125 NaCl, 2.5 KCl, 1 NaH_2PO_4 , 26 NaHCO_3 , 2.5 CaCl_2 , 1.2 MgCl_2 , and 5 D-Glucose. The ACSF was constantly bubbled with a mix

of 95% O₂ and 5% CO₂, for adequate oxygenation and pH ~7.4. After a 35 min recovery period in these conditions, the chamber was placed at room temperature and the tissues were used for experiments within the following ~5 hours.

Jess capillary electrophoresis using protein extracts from dissected dentate gyri

Dissected dentate gyri were transferred to incubation chambers containing ACSF at 37°C, and equilibrated with 95% O₂ and 5% CO₂. These tissues were incubated for 10 or 30 min in ACSF supplemented with vehicle (similar solution from insulin stock), 6bK, insulin, or a combination of the latter. Then, dissected dentate gyri were washed in ice-cold PBS and immediately transferred into cryovials. Tissues were quickly spun down in a microfuge, and the excess of PBS was quickly removed. Two slices of dissected dentate gyrus per cryovial were then frozen on dry ice and stored at -80°C until use.

Frozen dentate gyri were homogenized in ice-cold modified RIPA buffer as previously described (J. Gray et al., 2025). Homogenates were then centrifuged at 14000×g for 15 min at 4°C, and the supernatant collected. Protein concentrations were determined using the detergent-resistant Bradford assay (Pierce).

Capillary electrophoresis immunoblots were performed using the 12-230 kDa separation module with the Jess capillary electrophoresis system, as previously reported (J. Gray et al., 2025; Rose et al., 2024). Primary antibodies and experimental conditions are summarized in **Table 1.**

Data were acquired and analyzed using the software Compass for Simple Western version 6.2.0 (Protein Simple, Minneapolis, MN). Antibody-based detection was multiplexed using the RePlex™ protocol and the protein determination module from Protein Simple. The phosphorylated to total protein ratio was determined for Akt, Erk, and PDH. The abundance of individual proteins was calculated as the area under the curve of the chemiluminescent signal

and normalized by total protein. In experiments that required protein loads below the detection limit of the total protein kit, readouts were normalized by the $\alpha\beta$ -Tubulin signal. For each protein of interest, data were divided by the average ratio of control experiments and reported as fold-change over this group.

Immunofluorescence staining

Mouse hemibrains were frozen on dry ice and kept at -80 °C. Prior to sectioning, brain tissue was embedded in Optimal Cutting Temperature (OCT) compound, and 10 μ m-cryosections were obtained using a Leica CM3050 S Cryostat (Leica Biosystems, Deer Park, IL); cryosections were kept at -80 °C until use. Frozen slides were air-dried for 30 min at RT and washed three times with PBS. Brain sections were fixed during 20 min with 4% PFA, washed three times with PBS to remove the fixative solution, permeabilized with 0.3% Triton X-100 for 15 min, and blocked with 5% goat serum / 5% BSA / 0.1% Triton X-100 for 1 hour at RT. Sections were then incubated overnight at 4 °C with anti-IR β antibody (Cell Signaling Technology Cat# 23413, RRID:AB_2924796) diluted 1:50 in blocking solution. After three washes with PBS to remove the primary antibody solution, brain sections were incubated with Alexa Fluor™ 594 conjugated anti-rabbit antibody (Thermo Fisher Scientific Cat# A-11037, RRID:AB_2534095) and Hoechst 33342 (Thermo Fisher Scientific Cat# H3570, RRID:AB_3675235) diluted 1:500 and 1:1000, respectively, in PBS. Sections were washed three times with PBS, and the fluorescence signal was protected with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific Cat# P36934).

Images were acquired using an inverted Leica DMI8 epifluorescence microscope equipped with DAPI and Texas Red (TXR) filter cubes, as previously described (Deepa et al., 2024). Images were collected without pixel binning using HI PLAN 4x/0.10 and HC PL FLUOTAR 40X/0.60 Corr PH2 objectives at a resolution of 2048 $\times$ 2048 pixels. Image analysis was performed using ImageJ v1.53k (NIH; <http://imagej.nih.gov/ij>). Regions of interest (ROIs) were defined over the dentate

granule cell layer of the hippocampus, identified by Hoechst staining, and the adjacent cortex. Background fluorescence was determined from tissue sections obtained from mice with intact insulin receptor expression that were processed in parallel, except that the primary antibody against the insulin receptor was omitted during immunofluorescence staining. Background intensity was quantified independently for each ROI (dentate granule cell layer and cortex) and subsequently subtracted from the corresponding measurements obtained in the experimental groups.

Biosensor expression in the hippocampus

Adeno-associated viruses encoding Ca^{2+} biosensors were stereotactically injected in the dentate gyrus of the hippocampus, as previously reported (Hayes et al., 2021). *Dock10-Cre* and *IR^{fl/fl}::Dock10-Cre* mice were injected with pAAV.Syn.Flex.GCaMP6f.WPRE.SV40 (Chen et al., 2013) (Addgene viral prep #100833-AAV9; <http://n2t.net/addgene:100833>; RRID:Addgene_100833), or pAAV.CAG.Flex.NES-jRCaMP1b.WPRE.SV40 (Dana et al., 2016) (Addgene viral prep #100849-AAV1; <http://n2t.net/addgene:100849>; RRID:Addgene_100849). *C57BL/6J* mice were injected with pAAV.Syn.GCaMP6f.WPRE.SV40 (Addgene viral prep #100837-AAV9; <http://n2t.net/addgene:100837>; RRID:Addgene_100837), as previously described (J. Gray et al., 2025). Addgene plasmids #100833, #100849, and #100837, were gifts from Dr. Douglas Kim & GENIE Project.

AAV9.hSyn.ATeam1.03-nD/nA was delivered into the dentate gyrus of female *C57BL6J* mice. This virus encoded a modified version of the ATP-sensitive biosensor ATeam1.03 (Imamura et al., 2009) with native dimerization domains in both the donor and acceptor species (nD/nA), which increase the dynamic range of the sensor (Kotera et al., 2010). *Dock10-Cre* mice were also injected with AAV9.hSyn.flex.4xCox8-RCaMP1h. This biosensor is a modified version of the Ca^{2+} biosensor RCaMP1h (Akerboom et al., 2013). Unlike a previously reported mitochondrially

targeted version that used a single N-terminal Cox8 targeting sequence (Díaz-García et al., 2021), this construct contains four tandem repeats of the Cox8 targeting sequence to enhance mitochondrial localization. In addition, the biosensor cassette is flanked by loxP sites, enabling Cre recombinase-dependent control of its expression.

Plasmids encoding ATeam1.03-nD/nA and 4xCox-RCaMP1h were obtained from Dr. Gary Yellen at Harvard Medical School, and packaged into AAV9 particles in 293T cells (ATCC, # CRL-3216), following previously described protocols (Kimura et al., 2019). For the packaging protocol, we also utilized plasmids pAAV2/9n (Addgene plasmid # 112865 ; <http://n2t.net/addgene:112865>; RRID:Addgene_112865) and pAdDeltaF6 (Addgene plasmid # 112867; <http://n2t.net/addgene:112867> ; RRID:Addgene_112867), which were gifts from James M. Wilson.

Rhodamine-123 loading in hippocampal slices

The cell-permeable $\Delta\psi_m$ indicator Rhodamine-123 (Rh123) was dissolved in absolute ethanol at a concentration of 10 mM, then sonicated using a Branson 1800-MTH Ultra Sonic Bath for 10-15 min, aliquoted, protected from light, and stored at -20°C until use. On the day of the experiments, Rh123 stock solution was dissolved in ACSF for a working concentration of 25 μM , and sonicated again. Slices were incubated in this solution at room temperature for 30 min while bubbling with a mix of 95% O_2 and 5% CO_2 . We imaged Rhodamine-123 in quenching mode to monitor its fluorescence lifetime, as previously reported in cell lines (Schneckenburger et al., 2004). By pursuing this imaging modality, we minimized drifting baseline measurements due to photobleaching.

Two-photon fluorescence lifetime imaging

Rh123-loaded brain slices were imaged with a Bergamo II multiphoton microscope equipped with digiFLIM (all digital time-correlated single-photon counting) coupled with a Tiberius tunable femtosecond Ti:Sapphire laser (both from Thorlabs Inc., Newton, NJ), as previously reported (Rosen et al., 2025). Cells were imaged with a 40X (NA 0.8, Olympus LUMPLFLN40XW), or 60X (NA 1.0, Olympus LUMPLFLN) water-immersion objectives. Excitation was induced with 920 nm light, the fluorescence emission light was split with an FF562-Di03 dichroic mirror and bandpass filtered with a FF01-525/50 filter (all filter optics from Semrock, Rochester, NY). Photons were detected with photomultiplier modules (Thorlabs).

The fluorescence lifetime of jRCaMP1b was monitored for proof-of-concept experiments using KCl stimulation, as previously described (Díaz-García et al., 2017). The fluorescence lifetime of CFP (the donor species in ATeam1.03-nD/nA) was monitored using settings previously reported (Díaz-García et al., 2021). Data acquisition was performed with the ThorImage®LS software, version 4.4.2025.4151 (Thorlabs). Each acquisition comprised the cumulative signal of three consecutive frames obtained at a resolution of 256 x 256 pixels, with an inter-acquisition interval of 3-10 s, depending on the experiment.

Lifetime histograms were fitted using nonlinear least-squares fitting in MATLAB (Mathworks, Natick, MA), with a two-exponential decay convolved with a Gaussian for the instrument response function (Yasuda et al., 2006). Fluorescence lifetime was reported as a standardized “Tau8” value, which restricts the time window for the bi-exponential fit of the photon count histograms to 8 ns, minimizing the variability due to random histogram shifts (Díaz-García et al., 2019).

Acute hippocampal slices were attached to a poly-L-lysine-coated coverslip and placed in a chamber with a continuous supply of oxygenated ACSF at a flow rate of 5 ml/min, delivered through a Dynamax RP-1 peristaltic pump (Rainin). The solution’s reservoir was preheated at 38°C in a water bath (VWR, model 1225) and the solution in the recording chamber was

maintained at 32–34°C using inline TC-324C heaters (Warner Instruments, Hamden, CT). DGCs were stimulated by perfusing a modified ACSF solution containing 50 mM KCl for 10 seconds (KCl was increased by replacing an equimolar amount of NaCl to preserve osmolality in the ACSF). Kynurenic acid (1 mM) and picrotoxin (100 μ M) were added to the ACSF to minimize ionotropic receptor signaling in the area. Alternatively, zero- Ca^{2+} solutions containing 0.1 mM EGTA (with regular or high KCl) were used to record changes in Rh123 lifetime in response to stimulation. The latter solutions were not supplemented with synaptic blockers, instead, additional 3.92 mM MgCl_2 was added to compensate for the loss in charge screening capacity after the removal of Ca^{2+} ions (Hille et al., 1975).

Electrophysiology and widefield fluorescence microscopy

Local field potentials and Ca^{2+} dynamics in response to antidromic stimulation were obtained as described elsewhere (J. Gray et al., 2025). The perfusion system used for these measurements was similar to the one used for digiFLIM experiments. A concentric bipolar electrode was placed in the hilus to directly stimulate the axonal tract that emanate from DGCs. Stimulation consisted of trains of 100 depolarizing pulses (0.1 ms duration, 1 mA intensity) delivered at 50 Hz. Stimulation commands, data visualization, and acquisition were performed using Wavesurfer 1.0.5 software (HHMI Janelia Research Campus), which was ran in MATLAB 2021a (Mathworks, Natick, MA).

GCaMP6f fluorescence was acquired every 50 ms using a Nikon 96170M HQ:F 712 filter cube with a 480/30x excitation filter, a 505LP dichroic mirror, and a 535/40m emission filter. NAD(P)H signals were acquired every 500 ms using a customized Nikon filter cube with a 355/50x excitation filter, a 400LP dichroic mirror, and a 460/50m emission filter. In selected experiments to test the efficacy of synaptic blockers, jRCaMP1b fluorescence was monitored using a Nikon 96157M 805 31002A filter cube with a 560/40x excitation filter, a 600LP dichroic mirror, and a 635/60m

emission filter. Image analysis was performed as previously described (Díaz-García et al., 2021; J. Gray et al., 2025).

Changes in fluorescence intensity (ΔF) were expressed as percentage change relative to the baseline level (F_0) according to the equation: $\Delta F/F_0 = 100\% \times (F_i - F_0)/F_0$, where F_i is the fluorescence in the i -th frame, and F_0 corresponds to the average fluorescence value of 3-4 datapoints prior to the stimulation, as previously reported (J. Gray et al., 2025).

In-cell calibration of ATeam1.03-nD/nA

Neuro-2A cells were plated onto protamine-coated coverslips in 35-mm Petri dishes and transfected with pAAV.hSyn.ATeam1.03-nD/nA plasmid using the Effectene transfection kit (Qiagen). Two to three days after transfection, cells were washed with PBS and transferred to a Petri dish containing a zero-ATP intracellular-like solution composed of (in mM): 137.5 K-Gluconate, 9 NaCl, 10 HEPES, 1 EGTA, 1.324 $MgCl_2$, 0.346 $CaCl_2$, 1 Na-Iodoacetate (to inhibit the glycolytic enzyme GAPDH), 5 ouabain (to inhibit the Na^+/K^+ ATPase), and 0.0025 oligomycin (to inhibit the mitochondrial ATP synthase). The solution was supplemented with 45 μM β -Escin to permeabilize the cells. Permeabilization was performed for 7 min at room temperature ($\sim 23^\circ C$) with the solution adjusted to pH 7.44.

For all subsequent steps, β -Escin was omitted from the extracellular solution. Cells were washed in a separate Petri dish and then transferred to a recording chamber containing solutions with defined ATP concentrations (0, 0.1, 0.3, 1, 3, 10, or 30 mM). These solutions were generated by mixing the zero-ATP solution with a high-ATP solution containing (in mM): 30 Mg-ATP, 107.5 K-Gluconate, 9 NaCl, 10 HEPES, 1 EGTA, 1.324 $MgCl_2$, 0.346 $CaCl_2$, 1 Na-Iodoacetate, 5 ouabain, and 0.0025 oligomycin, adjusted to pH 7.44 at room temperature. Fluorescence lifetime measurements of the donor fluorophore (CFP) within the ATeam1.03-nD/nA biosensor were acquired while perfusing the recording chamber at ~ 5 mL/min and maintaining the temperature

at 33°C, conditions that lowered the solution pH to approximately 7.3. Cells were equilibrated under these conditions for 10 min before image acquisition.

The calibration curve was constructed using the calculated concentrations of Mg-ATP rather than the nominal ATP concentrations. Mg-ATP concentrations were determined using the equations and dissociation constants implemented in Chelator (Schoenmakers et al., 1992), a publicly available software package (<https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaMgATPEGTA-TS.htm>). Calculations incorporated the ionic strength, concentrations of metals and chelators, temperature, and pH of each solution under recording conditions. The resulting data were fitted to a Hill equation as previously described (Díaz-García et al., 2019), constraining the upper asymptote to the fluorescence lifetime measured under zero-ATP conditions and leaving all remaining parameters unconstrained.

Experimental Design and Statistical Analysis

Statistical analyses and data visualization were performed using GraphPad Prism version 10.6.1 (GraphPad Software, San Diego, CA). Data were tested for normality and log-normality using a Kolmogorov-Smirnov test. Semiquantitative methods, i.e., relative protein abundance and fluorescence intensity-based measurements, were subjected to robust regression and outlier removal (ROUT) using a coefficient Q=1% (Motulsky & Brown, 2006).

Normal or log-normal datasets were compared using the parametric Student's t-test, and a Welch's correction was applied in cases of heteroscedasticity. The non-parametric Mann-Whitney U-test was used if at least one group did not fulfill normality or log-normality. For multiple comparisons of normal or log-normal distributions, a one-way ANOVA was applied. When strictly comparing three groups, a Fisher's LSD test was conducted (Meier, 2006); otherwise, a Holm-

Šídák post hoc test was selected. For non-gaussian distributions, a Kruskal-Wallis test with a post hoc Dunn's test was used.

Linear regression equations were compared using a method equivalent to analysis of covariance (ANCOVA) (Zar, 1984). Correlations were assessed using the Pearson's test. In all cases, a two-tailed p-value was reported with $\alpha = 0.05$. Unless otherwise indicated, data are presented as mean $\pm$ SEM. Individual data points represent one mouse for immunoassays and one acute brain slice for imaging experiments.

Results

Inhibition of the insulin-degrading enzyme unmasks insulin signaling in the dentate gyrus

We first sought evidence that insulin activates canonical signaling pathways in the dentate gyrus, a hippocampal region with high neuronal density (Richards et al., 2013; Rogers Flattery et al., 2020; West & Gundersen, 1990). Dentate gyri were incubated in ACSF containing insulin (100 nM) or vehicle for 30 min, and Akt phosphorylation was quantified using Jess capillary immunoblotting. Detectable signals for total Akt and its phosphorylated form (pAkt) were observed at a molecular weight of ~64 kDa.

Insulin alone did not alter the pAkt/Akt ratio in dentate gyrus tissue (**Figure 1A-B**). Because insulin can be rapidly degraded in brain tissue, we next tested whether inhibiting the insulin-degrading enzyme (IDE) would enhance signaling. Co-application of insulin with the IDE inhibitor 6bK (2.2 μ M) produced a ~2.5-fold increase in the pAkt/Akt ratio compared with 6bK alone in dentate gyri from female mice (**Figure 1C**). This change was specifically driven by Akt phosphorylation (**Figure S1A**), considering that the total abundance of Akt remained unaltered (**Figure S1B**). Akt phosphorylation was not affected by 6bK alone, and failed to respond to insulin application when the Akt inhibitor Akti-1/2 (5 μ M) was co-applied (**Figure S1C-D**). Furthermore, lowering the insulin concentration to 10 nM failed to increase the pAkt/Akt ratio even in the presence of the IDE inhibitor (**Figure S2**).

To determine whether insulin also activated alternative pathways, we examined Erk phosphorylation (A. I. Duarte et al., 2012). We detected bands for total Erk1 and Erk2 at ~48 and 43 kDa, respectively, but mainly for Erk2 in its phosphorylated form. The pErk2/Erk2 ratio was unchanged by insulin treatment (**Figure 1D**), indicating selective engagement of the Akt pathway.

The insulin-mediated phosphorylation of Akt was observed regardless of whether 6bK was applied simultaneously or prior to insulin treatment (**Figure S3**). However, additional experiments confirmed that Akt activation required prolonged exposure to insulin, as a shorter application was ineffective (**Figure S4**). Based on these findings, subsequent experiments used co-application of insulin (100 nM) and 6bK (2.2 μ M) for 30 min.

Insulin attenuates activity-induced mitochondrial depolarization

We next investigated whether insulin signaling influences neuronal bioenergetics in acute hippocampal slices using fluorescence lifetime imaging of rhodamine-123 (Rh123) to monitor the mitochondrial membrane potential ($\Delta\psi_m$) (**Figure 2A–C**). Rh123 lifetime measurements were stable at baseline and responded robustly to the mitochondrial uncoupler FCCP, confirming sensitivity to mitochondrial depolarization (**Figure 2D–F**).

Brief exposure of dentate granule cells (DGCs) to high extracellular K^+ (50 mM, 10 s) produced transient increases in cytosolic Ca^{2+} (**Figure S5**) and a delayed but reversible increase in Rh123 lifetime, indicating mitochondrial depolarization during neuronal activation (**Figure 2G**). Baseline Rh123 lifetime and the overall temporal profile of responses were similar between insulin treated and control DGCs (**Figure 2H–I**). However, insulin significantly reduced the magnitude of activity-induced mitochondrial depolarization by approximately 30% compared with control conditions (**Figure 2J**). Importantly, baseline Rh123 lifetime was not correlated with the stimulus-induced response (**Figure S6**), suggesting that any variation in baseline levels is unlikely to have biased the observed effect. Although statistical significance was achieved only in the dentate gyrus granule cell layer, similar trends were observed in the molecular layer and hilus (**Figure S7**).

Together, these findings indicate that insulin does not alter mitochondrial polarization at rest but stabilizes mitochondrial function during periods of neuronal activity.

Neuronal insulin receptors are required for insulin modulation of mitochondrial membrane potential

Importantly, it is rarely established whether the reported insulin effects on neural tissue are mediated specifically through insulin receptors (IRs). This distinction is critical, as high insulin concentrations can activate insulin-like growth factor-1 receptors (IGF-1Rs), which are also expressed in the brain and share overlapping downstream signaling pathways with IRs (Milstein & Ferris, 2021). Based on these considerations, we tested the hypothesis that acute insulin signaling through neuronal IRs activates Akt in the adult hippocampus and promotes mitochondrial energization.

To determine whether insulin signaling occurs in DGCs, we generated mice with reduced insulin receptor expression in this neuronal population ($IR^{fl/fl}Cre$). As expected, immunofluorescence staining for the insulin receptor beta subunit (IR β) revealed attenuated signal in the DGC somata and the molecular layer, but strong signal elsewhere in the brain (**Figure 3A**). IR β staining in the DGC layer from $IR^{fl/fl}Cre$ mice decreased by ~52% when compared to $IR^{fl/fl}$ mice (**Figure 3B**). This surprising persistence in IR β immunostaining may result from signals in the neuropil within the DGC layer, e.g. neurites from hilar interneurons (Amaral et al., 2007), incomplete Cre-mediated recombination (Bao et al., 2013) and/or delayed Dock10 expression in adult-born DGCs (Castillon et al., 2026).

Dentate gyrus extracts from $IR^{fl/fl}Cre$ mice only exhibited ~30% reduction in IR protein levels (**Figure 3C**). This is expected, considering that, in addition to DGCs, the dentate gyrus contains astrocytes and other neuronal types (Amaral et al., 2007; Chalmers et al., 2024) where IR expression should remain unaffected in our knockout model. Insulin still increased Akt phosphorylation in these tissues, but the magnitude of activation was reduced by ~66% compared with controls (**Figure 3D–E**), consistent with partial neuronal deletion and residual signaling in other cell types.

We next asked whether neuronal IR signaling was required for insulin regulation of mitochondrial function. Insulin failed to modify the mitochondrial responses measured with Rh123 in DGCs from IR-deficient mice (**Figure 3F–G**), indicating that neuronal insulin receptors are necessary for insulin modulation of $\Delta\psi_m$.

Acute insulin signaling does not affect cytosolic ATP dynamics in DGCs

Insulin signaling appears well positioned to preserve ATP availability in active neurons (J. M. N. Duarte, 2023). Indeed, the attenuation of mitochondrial depolarization during neuronal stimulation may be indicative of enhanced bioenergetic capacity. However, whether such metabolic adaptations occur on the timescale of the acute insulin treatments used in the present study remains unclear.

To test this possibility, we first calibrated the biosensor ATeam1.03-nD/nA (ATeam for simplicity) in permeabilized Neuro-2A cells. Because ATP binding causes Förster Resonance Energy Transfer (FRET) from a CFP donor to a YFP acceptor in this biosensor (Imamura et al., 2009), we expected decreases in the CFP fluorescence lifetime upon the addition of ATP. These changes were detected in the range of ATP concentrations from 0.1 to 30 mM (**Figure 4A**). The calibration of the biosensor at the near-physiologic temperature used in slice experiments ($\sim 33^\circ\text{C}$) resulted in upper and lower asymptotes of 1.77 and 1.13 ns, respectively, a midpoint of 2.01 mM, and a Hill coefficient of -1.51 (**Figure 4B**). Overall, the dynamic range, affinity, and sensitivity of ATeam1.03-nD/nA fluorescence lifetime appear well suited for measuring cytosolic ATP levels in a wide range of mammalian cells, which have estimated ATP concentrations of 1–12 mM (Greiner & Glonek, 2021).

Experiments conducted in DGCs, treated with 6bK only, show that ATeam's CFP lifetime at rest was 1.21 ± 0.02 ns (mean $\pm$ SEM; $N_{\text{slices}} = 11$; $N_{\text{mice}} = 6$), corresponding to an average ATP concentration of ~ 7.5 mM. Stimulating with a high KCl solution for 10s only caused a marginal

increase in lifetime, but extending the stimulation to 30s exacerbated this response, causing an increase in lifetime of 0.020 ± 0.004 ns and 0.092 ± 0.017 ns, respectively (**Figure 4C-D**). DGCs that were incubated with insulin and 6bK exhibit an ATeam's CFP lifetime of 1.20 ± 0.02 ns at baseline (mean $\pm$ SEM; $N_{\text{slices}} = 11$; $N_{\text{mice}} = 7$), which was similar to the non-stimulated values of 6bK-treated controls (**Figure 4E**). Finally, comparisons of the increase in CFP lifetime (decrease in ATP concentration) yielded indistinguishable results between insulin-treated and control DGCs, both for the 10s (**Figure 4F**) and the 30s stimulation (**Figure 4G**).

Insulin could also stabilize the membrane potential of the mitochondrial network by promoting the elimination of damaged, depolarization-prone mitochondria (**Figure 5A**). Indeed, typically fragmented, depolarized mitochondria undergo mitochondrial fission and mitophagy (Twig & Shirihai, 2011). First, we found similar abundance of TOM20, MDH2, and SOD2, in our experimental groups (**Figure 5B**), suggesting preserved mitochondrial content. Next, we queried the phosphorylation state of residues S616 and S637 in the fission protein Drp1, a potential target for Akt (Kim et al., 2016; Lee et al., 2022). Phosphorylation of S616 in Drp-1 promotes mitochondrial fission, while phosphorylation at S637 antagonizes it (Ko et al., 2016). However, we did not detect any change in Drp1 abundance or phosphorylation state due to insulin treatment (**Figure 5C**). Furthermore, the fusion protein Opa1 (**Figure 5D**), and proteins involved in autophagy/mitophagy, i.e., ATG7 and Beclin-1 (Choubey et al., 2014; Zhang et al., 2009), remained unchanged as well (**Figure 5E**).

Altogether, our results indicate that acute insulin regulation of $\Delta\psi_m$ is unrelated to ATP availability in the cytosol or the bioenergetic quality control of mitochondria.

Insulin limits cytosolic Ca^{2+} elevations without altering DGC excitability

A reduction in mitochondrial depolarization could reflect decreased neuronal activation. To test this possibility, we measured antidromically evoked population spikes in DGCs (**Figure S8**).

These local field potentials were indistinguishable between insulin-treated and control slices, with similar amplitudes, slopes, and time courses (**Figure 6A–B**), indicating preserved neuronal excitability.

We then examined cytosolic Ca^{2+} dynamics using GCaMP6f imaging (**Figure 6C–D**). Antidromic stimulation produced transient increases in fluorescence that returned to baseline in both groups. Despite substantial variability in response amplitudes, insulin treatment reduced the average peak GCaMP6f fluorescence by approximately 34% compared with controls (**Figure 6E–F**), without altering the timing of peak or decay kinetics (**Figure 6G**).

To test whether this effect required insulin receptor signaling, *IR^{fl/fl}* Cre mice were injected with AAVs encoding a Cre-dependent GCaMP6f construct, and Ca^{2+} dynamics were monitored in DGCs (**Figure 6H**). Because GCaMP6f expression was restricted to DGCs in which Cre-mediated recombination had occurred, the measurements selectively reflected insulin receptor-deleted neurons. As expected, insulin failed to attenuate cytosolic Ca^{2+} elevations in these DGCs (**Figure 6I**).

To determine whether this effect also required Akt activation, similar experiments were performed in female wild-type mice, with an additional group of slices treated with 6bK, insulin, and the Akt inhibitor Akti-1/2 (**Figure 6J**). Consistent with our previous experiments in slices with intact insulin receptor expression, insulin reduced the peak Ca^{2+} response to antidromic stimulation in DGCs from wild-type mice. Strikingly, Akti-1/2, applied under conditions previously shown to prevent Akt phosphorylation, produced an intermediate Ca^{2+} response that was not statistically different from that of 6bK alone (**Figure 6K**).

These results indicate that insulin reduces activity-evoked cytosolic Ca^{2+} accumulation while preserving the ability of neurons to fire action potentials synchronously. Insulin's effect on

cytosolic Ca^{2+} dynamics requires the insulin receptor but is not entirely dependent on Akt activation.

Insulin modulation of mitochondrial depolarization requires Ca^{2+} influx but does not involve PDH activation

We sought to determine whether insulin also regulates mitochondrial Ca^{2+} dynamics in active neurons. For these experiments, selective expression of a mitochondrially targeted variant of the biosensor RCaMP1h (mt-RCaMP1h) was achieved in DGCs of *Dock10-Cre* mice (**Figure 7A**). Direct electrical stimulation produced robust increases in mt-RCaMP1h fluorescence, which outlasted the observed cytosolic Ca^{2+} transients (**Figure 7B-C**). In contrast to the cytosolic Ca^{2+} transients, insulin did not modify the magnitude of the initial response in mt-RCaMP1h (**Figure 7D**), which peaked ~8 seconds after the cessation of the stimulus. In our experimental conditions, the time to reach these peak values was also similar between groups (**Figure 7E**). However, DGCs incubated with insulin and 6bK exhibited a decay time from maximum fluorescence to 75% of the peak of 36 ± 3 s (mean $\pm$ SEM), significantly shorter than the 47 ± 4 s observed in cells treated with 6bK alone (**Figure 7F**).

Notably, increasing stimulus duration produced only a nonsignificant trend toward higher mt-RCaMP1h fluorescence in insulin-treated DGCs (**Figure S9**). This stability of mt-RCaMP1h peak fluorescence observed at longer stimulus is unlikely to reflect biosensor saturation. Resting mitochondrial Ca^{2+} concentrations are estimated to be in the range of 100-200 nM (Mustaly-Kalimi et al., 2025), approximately five- to tenfold below the midpoint of the biosensor response curve (Akerboom et al., 2013). Moreover, RCaMP1h fluorescence can increase by approximately twofold across its dynamic range (Akerboom et al., 2013), whereas the responses measured here were only 13-72% above baseline. Together, these observations suggest that our stimulation paradigm recruits most of the mitochondrial Ca^{2+} uptake capacity available during the transient elevation of cytosolic Ca^{2+} . Consequently, the insulin-induced acceleration of mt-RCaMP1h decay

is more likely attributable to modulation of slow-acting local Ca^{2+} sources and/or Ca^{2+} extrusion mechanisms than to limitations in mitochondrial Ca^{2+} uptake capacity.

Considering that insulin modulates both cytosolic and mitochondrial Ca^{2+} dynamics in active DGCs, we next tested whether Ca^{2+} influx was necessary for insulin effects on $\Delta\psi_m$. In zero- Ca^{2+} extracellular solution, neuronal stimulation still produced mitochondrial depolarization (**Figure 7G**); however, insulin no longer reduced the magnitude of this response (**Figure 7H–I**). Thus, extracellular Ca^{2+} influx is required for insulin-mediated stabilization of $\Delta\psi_m$.

To determine whether mitochondrial Ca^{2+} uptake is also required, we utilized DGCs carrying biallelic inactivation of the mitochondrial Ca^{2+} uniporter (MCU), as previously described (Díaz-García et al., 2021; Rose et al., 2024; Rosen et al., 2025). Rh123 lifetime was monitored in the presence of extracellular Ca^{2+} (**Figure 7J**), yielding baseline values in insulin-treated cells comparable to those observed in controls (**Figure 7K**). Strikingly, MCU-deficient DGCs exhibited similar Rh123 responses to neuronal stimulation regardless of insulin application (**Figure 7L**). Thus, neuronal mitochondria must retain the capacity to take up Ca^{2+} for the effects of insulin on $\Delta\psi_m$ to be observed.

Because both insulin and Ca^{2+} signaling can enhance mitochondrial metabolism via activation of pyruvate dehydrogenase (PDH) (**Figure 8A**), we examined PDH phosphorylation (Denton, 2009; Karwi et al., 2020). Insulin treatment did not alter the p-PDH/PDH ratio in dentate gyrus extracts (**Figure 8B**), suggesting that PDH activation does not mediate the observed effects.

To further evaluate the Ca^{2+} -mediated regulation of mitochondrial metabolism, we measured NAD(P)H autofluorescence during neuronal stimulation (Brennan et al., 2006; Díaz-García et al., 2021). Upon antidromic stimulation, a rapid decrease in NAD(P)H autofluorescence (oxidative phase) is quickly followed by a reductive phase where the NAD(P)H signal surpasses the non-stimulated level (**Figure 8C–E**). Both oxidative (“dip”) and reductive (“overshoot”) phases of the

NAD(P)H signal scaled with the integrated cytosolic Ca^{2+} elevations, but the relationships between Ca^{2+} transients and NAD(P)H responses were unchanged by insulin treatment (**Figure 8F–G**). These findings do not support a role for acute insulin signaling in modulating Ca^{2+} -dependent regulation of PDH or other tricarboxylic acid cycle dehydrogenases during neuronal activation.

Collectively, our results demonstrate that endogenous insulin degradation limits hippocampal insulin signaling, and that insulin activation of neuronal insulin receptors stabilizes $\Delta\psi_m$ during neuronal activity in a Ca^{2+} -dependent manner.

Discussion

Insulin resistance has been linked to cognitive impairment and reduced hippocampal glucose utilization, nevertheless insulin signaling in the adult hippocampus has remained unclear. Here, we show that acute insulin signaling in dentate granule cells is regulated by insulin degradation rather than receptor availability. Specifically, inhibition of insulin degradation unmasked Akt activation in the dentate gyrus of adult female mice. These findings identify insulin degradation as a critical regulator of hippocampal insulin responsiveness and establish Akt activation as the dominant downstream signaling event in the dentate gyrus.

Contrary to expectations based on receptor affinity (reviewed by Belfiore et al., 2009), insulin application at 100 nM failed to activate Akt signaling in dissected hippocampal tissue unless insulin degradation was pharmacologically inhibited. This finding indicates that insulin bioavailability, in addition to receptor binding *per se*, is a major determinant of insulin responsiveness in the dentate gyrus. Although insulin concentrations used in this study exceed circulating levels in rodents and humans (0.04 – 1.7 nM) (Strubbe et al., 1988; Craft et al., 1998; Nelson et al., 2022), insulin concentrations within the brain parenchyma remain poorly defined. Our doses indicate that bovine insulin does not engage the insulin signaling pathway at these concentrations, which is consistent with studies showing that non-murine insulins are only effective in neural tissue from mice when increased by 10-100 fold, depending on the species (Qiu et al., 2014).

Cerebrospinal fluid contains only a fraction of circulating insulin levels (Craft et al., 1998; Strubbe et al., 1988), and while it is plausible that insulin is locally produced by some neural cells (S. M. Gray et al., 2014), the effective concentration experienced by hippocampal neurons in vivo

remains unknown (Rae et al., 2024). Importantly, supraphysiological insulin concentrations are routinely required to elicit measurable effects on neuronal excitability, synaptic transmission, and metabolism across *in vitro*, *ex vivo*, and *in vivo* paradigms (Barutçu et al., 2023; Van Der Heide et al., 2005; Wan et al., 1997). Our results provide a mechanistic explanation for this observation by identifying insulin degradation by IDE as a major factor regulating insulin response in neural tissue.

Consistent with this interpretation, inhibition of IDE elevated Akt phosphorylation in dentate gyri from female mice, whereas Erk2 phosphorylation remained unchanged. This selective activation supports prior work identifying Akt, rather than Erk, as the predominant signaling axis downstream of insulin receptors in the brain (Grillo et al., 2009; McNay et al., 2010; Zhao et al., 2019). Additionally, genetic ablation of insulin receptors in dentate granule cells prevented insulin actions on their $\Delta\psi_m$, demonstrating that the observed signaling response is mediated predominantly through insulin receptors rather than off-target activation of insulin-like growth factor-1 receptors (IGF-1Rs), as cautioned by previous studies (McNay et al., 2010; McNay & Recknagel, 2011). Although IGF-1Rs are widely expressed in the brain (Ashpole et al., 2015) and can bind insulin at high concentrations (Schumacher et al., 1991), deletion of both IR alleles attenuated Akt activation, effectively excluding IGF-1R homodimer signaling as the primary driver of Akt phosphorylation. While we cannot exclude a contribution from IR/IGF-1R hybrids to the original response (Belfiore et al., 2009; Milstein & Ferris, 2021), any such contribution would remain dependent on the presence of insulin receptors in DGCs.

Beyond signaling, our data revealed a functional consequence of insulin action on mitochondrial function. Insulin signaling stabilized the $\Delta\psi_m$ in dentate granule cells during periods of elevated activity. Notably, this rapid stabilization of $\Delta\psi_m$ was independent of changes in pyruvate dehydrogenase activity, mitochondrial NAD(P)H dynamics, or ATP availability in the cytosol, distinguishing neuronal insulin signaling from canonical mechanisms previously

described in skeletal and cardiac muscle (Feldhoff et al., 1993; Rao et al., 1998; Karwi et al., 2020).

Our results support a model in which insulin has a key role in limiting excessive cytosolic and mitochondrial Ca^{2+} accumulation, thus preventing activity-induced mitochondrial depolarization. This interpretation is consistent with prior studies showing that insulin reduces ionic currents through voltage-gated Ca^{2+} channels and attenuates Ca^{2+} release from intracellular stores in neurons and sensory cells across species (Stella et al., 2001; Maimaiti et al., 2017). Insulin-mediated regulation of Ca^{2+} transfer from the endoplasmic reticulum to mitochondria represents a particularly compelling avenue for future investigation, as this process can persist beyond the cytosolic Ca^{2+} transient and promote prolonged mitochondrial Ca^{2+} accumulation in stimulated neurons (Dhoundiyal et al., 2025; Hirabayashi et al., 2017).

Alternatively, insulin-dependent regulation of Ca^{2+} extrusion mechanisms should be considered. $\text{Na}^+/\text{Ca}^{2+}$ exchange is another important regulator of Ca^{2+} homeostasis in DGCs (Meyer et al., 2022) and insulin may enhance the activity of the underlying transporter proteins through palmitoylation (Gök et al., 2022). Furthermore, evidence from pancreatic acinar cells indicates that insulin can also support the activity of Ca^{2+} ATPases at the plasma membrane during mitochondrial depolarization, provided that glycolytic capacity remains intact (Mankad et al., 2012).

Overall, by constraining Ca^{2+} elevations and mitochondrial depolarization, insulin may help preserve neuronal viability during excitotoxic stress, as previously shown in cultured cortical neurons (Krasil'nikova et al., 2019). These neuroprotective effects are not confined to the central nervous system, as insulin-dependent stabilization of $\Delta\psi_m$ has likewise been reported in cultured peripheral neurons (Huang et al., 2005). Despite its effects on $\Delta\psi_m$, acute insulin signaling did not measurably alter the ability of neurons to meet the energetic demands imposed by transient KCl-induced depolarization in our studies. However, our studies focused on ATP dynamics, and

ATP levels are strongly buffered by multiple compensatory pathways that can mask rapid imbalances between energy production and consumption (Rosen et al., 2026).

Of note, our estimated resting cytosolic ATP concentration in dentate granule cells (~7.5 mM) approximates the reported values in cardiac and skeletal muscle rather than the ~2.6-fold smaller values expected in whole brain tissue (Greiner & Glonek, 2021). Our estimate is also substantially higher than the 0.5–3 mM range previously reported for cultured neurons (Ainscow et al., 2002) and presynaptic terminals (Rangaraju et al., 2014; Straub et al., 2025). These discrepancies may reflect differences in experimental preparations (acute brain slices versus primary neuronal cultures), the neuronal populations examined, the subcellular compartment in which ATP was measured, or the calibrations used to convert biosensor readout to ATP concentration.

It is also worth noting that our analyses were limited to measurements of ATP in the bulk cytosol, which may not fully reflect insulin-dependent changes in ATP availability within mitochondria. Indeed, cytosolic and mitochondrial ATP dynamics appear to be differentially regulated. Studies in primary neuronal cultures have shown that changes in mitochondrial membrane potential ($\Delta\psi_m$) and cytosolic ATP do not necessarily exhibit the same magnitude or time course following inhibition of glycolysis or oxidative phosphorylation (Surin et al., 2012). Likewise, metabolic inhibition and hypoxia produce distinct temporal profiles and amplitudes of cytosolic and mitochondrial ATP signals in HEK293T cells, despite continuous adenine nucleotide exchange through the adenine nucleotide translocator (White et al., 2023). Therefore, it is possible that insulin exerts effects on mitochondrial energy metabolism that were not detected by our measurements. Future experiments using mitochondrially targeted ATP biosensors will be necessary to directly test this hypothesis.

Moreover, metabolic adaptations associated with insulin signaling, including increases in ATP production and NAD(P)H availability, as well as protection against oxidative stress, typically require longer periods of exposure (A. I. Duarte et al., 2005; Huang et al., 2005). These

729 observations suggest that insulin exerts both rapid effects on mitochondrial stability through Ca^{2+} -
730 dependent mechanisms and slower metabolic adaptations that emerge during sustained
731 signaling, perhaps through transcriptional programs. Future investigations will determine whether
732 insulin also regulates the cellular antioxidant capacity of neurons, as well as their lipid and amino
733 acid metabolism, processes with important implications for metabolic signaling, cell polarity, and
734 survival.

735 Taken together, our findings identify insulin degradation as a previously underappreciated
736 regulator of hippocampal insulin signaling and reveal a novel role for insulin in stabilizing cytosolic
737 Ca^{2+} levels and mitochondrial membrane potential during neuronal activity. The immediate
738 proximal mediator of these effects remains unclear, as pharmacological inhibition of Akt failed to
739 fully reverse the insulin-dependent attenuation of neuronal Ca^{2+} transients.

740 One intriguing possibility is that insulin modulates ion channel activity through PI3K-
741 dependent phosphoinositide signaling (Ishij et al., 2014). Reductions in the PI3K substrate PIP2
742 have been shown to decrease the current through high-voltage activated Ca^{2+} channels (Suh et
743 al., 2012), which contribute substantially to activity-evoked Ca^{2+} transients in neurons (Díaz-
744 García et al., 2021; Hotka et al., 2020). Elucidating this and other candidate mechanisms will be
745 an important objective of future studies. Understanding how insulin availability, degradation, and
746 receptor signaling intersect in the adult and aging hippocampus may provide critical insight into
747 the metabolic vulnerabilities underlying neuronal dysfunction in metabolic disorders and age-
748 related diseases.

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1215 **Figure Legends**

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1217 **Figure 1. Insulin triggers Akt phosphorylation upon inhibition of the insulin degrading**
1218 **enzyme (IDE) in the dentate gyrus.** (A) *Left:* Representative digital lanes of Jess capillary
1219 electrophoresis for vehicle (Veh) and insulin (Ins) treated dentate gyri, dissected from adult *IR^{fl/fl}*
1220 female mice. Phosphorylation of Akt was used as a proxy for insulin signaling. Total protein was
1221 determined as a loading control in these immunoassays. *Right:* Quantification of protein levels.
1222 Incubation with insulin (100 nM) alone for 30 min was ineffective in driving Akt phosphorylation
1223 ($N_{\text{mice}}=6$). (B) Graphic representation of the insulin signaling cascade and the insulin degrading
1224 enzyme (IDE) as a regulator of hormone availability in the hippocampus. (C) IDE inhibition with
1225 the drug 6bK (2.2 μM) allowed insulin-mediated activation of Akt ($N_{\text{mice}}=13$), (D) but not of Erk2
1226 ($N_{\text{mice}}=7$).

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1228 **Figure 2: Acute insulin treatment attenuates mitochondrial depolarization in active dentate**
1229 **granule cells.** (A) Schematic representation of the method used to monitor $\Delta\psi\text{m}$. (B) Acute
1230 hippocampal slice imaged using Infrared Differential Interference Contrast microscopy (IR/DIC).
1231 Anatomic subregions within the dentate gyrus are identified: molecular layer (*m.l.*), dentate
1232 granule cell (*DGC*) layer, and the hilus (*H*). The insert shows a high magnification image of a
1233 region of interest. (C) Epi-fluorescence signal of the same slice, loaded with the $\Delta\psi\text{m}$ reporter
1234 Rhodamine-123 (Rh123). The insert represents a high magnification image captured with 2-
1235 photon microscopy. (D) Intensity image of the dentate gyrus, followed by pseudo-colored
1236 fluorescence lifetime images of Rh123 before (Baseline) and after the sequential addition of the
1237 mitochondrial complex-V (ATP synthase) inhibitor oligomycin (OGM), and the electron transport
1238 chain uncoupler FCCP. (E) Time course of the Rh123 fluorescence lifetime in the DGC layer,
1239 when exposed to these mitochondrial poisons (mean $\pm$ SEM; $N_{\text{slices}}=7$; $N_{\text{mice}}=3$). (F) Comparison
1240 of Rh123 lifetimes among these conditions. (G) Intensity image of the dentate gyrus, followed by

pseudo-colored fluorescence lifetime images of Rh123 before (Baseline) and after a 10s exposure to an extracellular solution containing 50 mM KCl. (H) Time course of Rh123 fluorescence lifetime in DGCs treated with 6bK (2.2 μ M), with or without insulin (100 nM) for 30 min, and transiently stimulated with high KCl (mean $\pm$ SEM $N_{\text{slices}}=17$; $N_{\text{mice}}=8$). (I) Acute insulin application does not affect the Rh123 lifetime in non-stimulated DGCs. (J) Insulin attenuates the KCl-induced increase in Rh123 lifetime (Δ Rh123) from the DGC layer. Experiments were conducted in female C57BL/6J mice.

Figure 3. Neuronal insulin receptors contribute to insulin-dependent Akt activation in the dentate gyrus. (A) Representative images of fluorescence immunostaining for the insulin receptor beta subunit (IR β). *IR^{fl/fl}::Dock10Cre* mice (*IR^{fl/fl}Cre* for simplicity) exhibited decreased IR β signal in DGCs but preserved signals elsewhere, including the cortex (Ctx). (B) Quantitation of the IR β signal in DGCs, relative to the signal in the cortex ($N_{\text{mice}}=6$ per group). (C) Representative digital lanes of Jess capillary immunoassays for IR β in protein extracts obtained from dentate gyri. Tissues with bi-allelic downregulation of the *Insr* gene in DGCs showed decreased IR β levels with respect to controls ($N_{\text{mice}}=7-8$ per group). Chemiluminescent signals were normalized by total protein (TP). (D) Dissected dentate gyri with IR β downregulation in DGCs show mildly increased Akt phosphorylation following insulin/6bK application ($N_{\text{mice}}=20$). (E) The effect-size of insulin treatment in tissues from *IR^{fl/fl}* mice ($N=13$, as in Figure 1C) was higher than those from *IR^{fl/fl}Cre* mice ($N=20$). The effect size on Akt activation was calculated as the ratio of pAkt/Akt in the Ins/6bK condition to that in the 6bK-alone condition from the same mouse. The discontinuous line represents the control level. (F) Time course of Rhodamine-123 (Rh123) fluorescence lifetime recorded in the DGC layer of *IR^{fl/fl}Cre* mice. Neurons were transiently depolarized (10s) with a high KCl (50 mM) extracellular solution. Traces represent the mean $\pm$ SEM of individual slices ($N_{\text{slices}}=13$, from $N_{\text{mice}}=6$). (G) Comparisons of resting Rh123 lifetimes,

and the magnitude of the response to stimulation (ΔR_{h123}), in $IR^{fl/fl}$ Cre slices incubated with 6bK, with or without insulin.

Figure 4. Acute insulin signaling does not modify ATP availability in dentate granule cells.

(A) Schematic representation of the operating principle of the ATP biosensor ATeam1.03-nD/nA. ATP binding promotes Förster Resonance Energy Transfer (FRET) from CFP to mVenus. The two-photon excitation and emission wavelengths (λ_{2p-ex} and λ_{em} , respectively) are represented. (B) Effects of ATP binding on the decay histograms of CFP fluorescence. In the absence of ATP, the average CFP fluorescence lifetime mainly depends on the rate of radiative (k_R , e.g., fluorescence) and non-radiative (k_{NR} , e.g., quenching) relaxation. The rate of FRET (k_{FRET}) elicited by ATP decreases the CFP fluorescence lifetime. (C) Permeabilized Neuro-2a cells expressing ATeam1.03-nD/nA were exposed to different concentrations of ATP in the bathing solution. (D) Calibration curve based on changes in the fluorescence lifetime of the donor species CFP in the ATeam1.03-nD/nA biosensor (datapoints represent the mean $\pm$ SD; N_{cells} = 59-92). (E) Representative images of ATeam1.03-nD/nA CFP fluorescence lifetime in DGCs during an experiment using a 50 mM KCl solution for stimulation. (F) Average traces of ATeam1.03-nD/nA CFP fluorescence lifetime in DGCs from *C57BL/6J* mice (data represent the mean $\pm$ SEM of individual slices; N_{slices} = 11, N_{mice} = 6-7). (G) Comparison of ATeam lifetime at baseline. (H) The increase in ATeam lifetime after a 10s long KCl stimulation was not modified by insulin application. (I) ATeam responses were also similar between insulin-treated and control DGCs after stimulating with KCl for 30s.

Figure 5. Acute insulin treatment does not alter protein markers of mitochondrial abundance, redox state, or dynamics in the dentate gyrus. (A) Schematic representation of

proteins that regulate mitochondrial fusion, fission, and turnover. (B) Comparison of mitochondrial protein levels ($N_{\text{mice}} = 6$ for TOM20 and MDH2; $N_{\text{mice}} = 13$ for SOD2). (C) Quantification of Drp-1 and its phosphorylated forms ($N_{\text{mice}} = 6$). (D) Comparison of Opa1 abundance ($N_{\text{mice}} = 6$). (E) Determination of mitophagy/autophagy proteins ($N_{\text{mice}} = 8$). Protein abundance was normalized by the signal of $\alpha\beta$ -tubulin ($\alpha\beta$ -tub), or total protein (TP).

Figure 6. Insulin decreases the accumulation of Ca^{2+} in the cytosol of active dentate granule cells. (A) Graphic representation of electrode placement in an acute hippocampal slice to record local field potentials (LFP) in the dentate granule cell (DGC) layer. Antidromic stimulation consisting of 100 electrical pulses was delivered at 50Hz in the hilus. The insert represents the average LFP elicited by individual depolarizing pulses in the stimulation train ($N_{\text{slices}} = 10-11$; $N_{\text{mice}} = 5$). Traces are expressed as mean $\pm$ SEM of individual slices. The initial oscillation in voltage (which has been trimmed and shaded) corresponds to the stimulus artifact. The negative deflection in the LFP (i.e., the population spike) results from the firing of action potentials by DGCs in the vicinity of the recording electrode. (B) Comparisons of the parameters derived from the LFPs show no overt effect of insulin treatment on neuronal excitability. The rate of voltage change in the descending phase of the LFP (slope), the magnitude of the response (Peak), and the kinetics of the LFP, i.e., the time to peak (from the beginning of the stimulation pulse) and the full width to half maximum (FWHM), were compared between DGCs from slices incubated with 6bK, with or without insulin treatment (100 nM for 30 min). (C) Micrograph of the dentate gyrus within an acute hippocampal slice, obtained using infrared illumination and differential interference contrast (IR/DIC). The molecular layer (m.l.), DGCs, and hilus were labeled for reference. (D) Filmstrip of GCaMP6f fluorescence before and after antidromic stimulation in the same slice. Relative changes in fluorescence (Flrsc.) were color-coded as indicated in the scale bar on the right. (E) Average traces of GCaMP6f fluorescence in the DGC layer of female *Thy1-GCaMP6f*

mice ($N_{\text{slices}} = 9-11$; $N_{\text{mice}} = 5$). (F) Insulin-treated DGCs show decreased GCaMP6f excursions in response to stimulation (ΔGCaMP). (G) The time course of GCaMP6f responses was unaffected by insulin treatment. (H) Traces of GCaMP6f fluorescence in DGCs from *IR^{fl/fl}Cre* mice ($N_{\text{slices}} = 13-14$; $N_{\text{mice}} = 5$), with selective downregulation of insulin receptors in DGCs. (I) Comparison of peak values in these recordings. (J) Traces of GCaMP6f fluorescence in DGCs from *C57BL/6J* mice ($N_{\text{slices}} = 12-14$; $N_{\text{mice}} = 5-6$). (K) Ca^{2+} transients of similar amplitude were observed in slices co-treated with insulin and 6bK, with or without Akt inhibition by Akti-1/2 (5 μM).

Figure 7. Insulin accelerates the decay of stimulation-induced mitochondrial Ca^{2+} transients, a process required for insulin-mediated stabilization of the mitochondrial membrane potential. (A) Selective expression of the mitochondria-targeted Ca^{2+} biosensor mt-RCaMP1h in DGCs. Cre-dependent AAV constructs were delivered into the dentate gyrus of *Dock10-Cre* mice. (B) Antidromic stimulation elicits long-lasting increases in mt-RCaMP1h fluorescence. (C) Time course of mt-RCaMP1h fluorescence in stimulated DGCs after a 30 min incubation with 2.2 μM 6bK alone or in combination with 100 nM insulin ($N_{\text{slices}} = 10$; $N_{\text{mice}} = 4$ per group). Traces represent the mean $\pm$ SEM of individual slices. (D) Peak mt-RCaMP1h fluorescence was undistinguishable between experimental conditions. (E) Comparison of the time to peak mt-RCaMP1h fluorescence. (F) Insulin modulates the decay kinetics of the mt-RCaMP1h response. (G) Rhodamine-123 (Rh123) fluorescence lifetime transiently increases upon KCl stimulation in the DGC layer while perfusing the slices with a Ca^{2+} free extracellular solution. Traces represent the mean $\pm$ SEM of individual slices ($N_{\text{slices}} = 10-11$; $N_{\text{mice}} = 4$ per group). These experiments were performed in tissues from female *C57BL/6J* mice. (H) Comparison of the Rh123 lifetime at baseline show no difference between groups. (I) The change in Rh123 lifetime due to KCl stimulation was not affected by insulin application when Ca^{2+} was omitted in the ACSF. (J) Time course of Rh123 lifetime in MCU-deficient DGCs. Traces represent the mean $\pm$ SEM of

individual slices ($N_{\text{slices}} = 13$; $N_{\text{mice}} = 6$ per group). (K) Comparison of Rh123 lifetime at baseline in these mice. (K) Insulin does not limit mitochondrial depolarization in MCU-deficient DGCs.

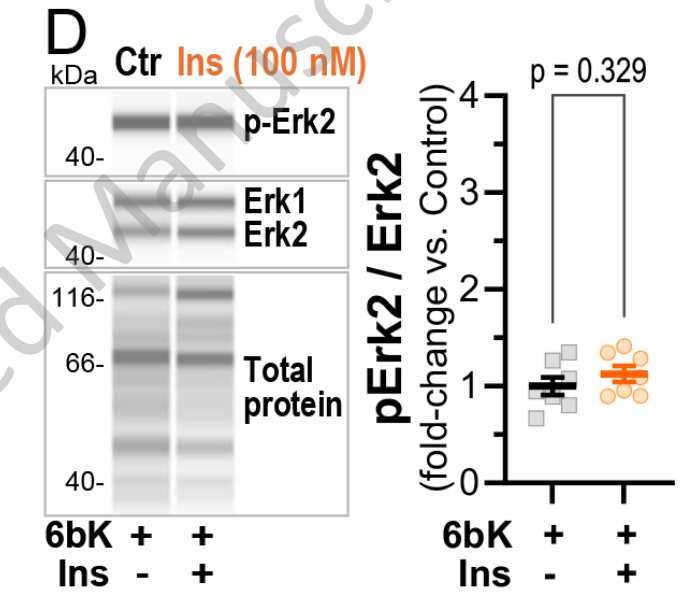
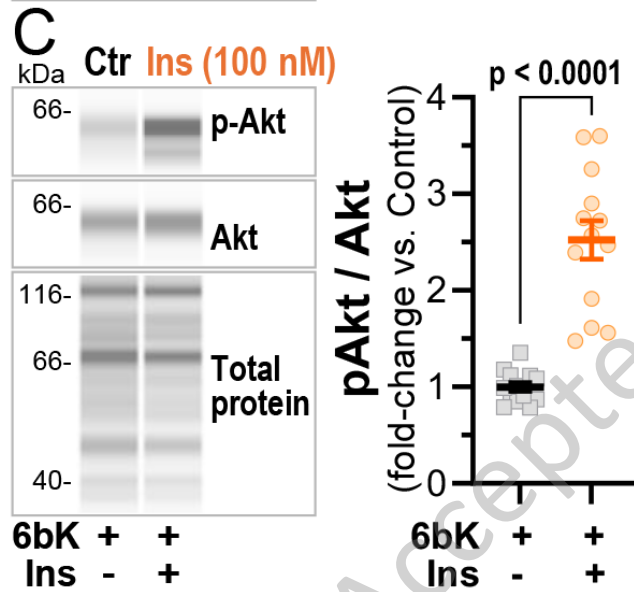
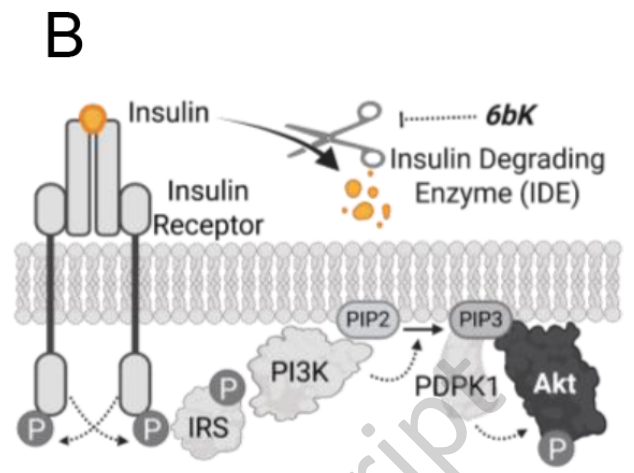
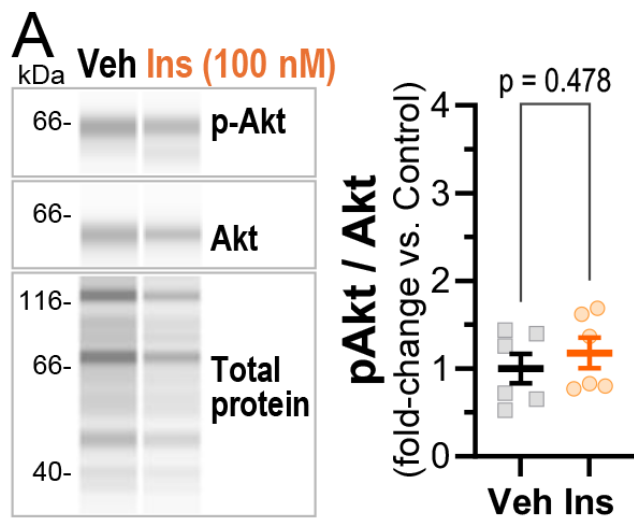
Figure 8. Acute insulin signaling neither activates pyruvate dehydrogenase (PDH) nor increases mitochondrial NAD(P)H production in neurons. (A) Graphic representation of the potential crosstalk between insulin and Ca^{2+} signaling cascades in the regulation of pyruvate dehydrogenase (PDH) activity in neurons. Akt- or Ca^{2+} -dependent relief of PDH inactivation by phosphorylation would allow more pyruvate oxidation by mitochondria, resulting in elevated intra-mitochondrial NADH levels. (B) Jess digital lanes of protein extracts from dentate gyri of female $IR^{fl/fl}$ mice incubated for 30 min in ACSF with 6bK alone (2.2 μM), or in combination with insulin (100 nM). Acute insulin treatment does not affect the pPDH/PDH ratio ($N_{\text{mice}} = 13$). (C) NAD(P)H autofluorescence measurements were performed in the same slices used for electrophysiology and Ca^{2+} imaging experiments. (D) Filmstrip of NAD(P)H autofluorescence before and after antidromic stimulation in the same slice. Relative changes in fluorescence (Flrsc.) were color-coded as indicated in the scale bar on the right. (E) NAD(P)H responses to stimulation in DGCs from female *Thy1-GCaMP6f* mice exhibit a brief dip in fluorescence (oxidative phase), followed by an increase in fluorescence that transiently overshoots the initial level (reductive phase) ($N_{\text{slices}} = 9-11$; $N_{\text{mice}} = 5$ per group). Traces are expressed as mean $\pm$ SEM of individual slices. (F) Linear regressions between the magnitude of the oxidative phase (NAD(P)H dip) and the total Ca^{2+} influx into cytosol, as determined by the area under the curve (AUC) of the signal from the Ca^{2+} biosensor GCaMP6f. (G) The reductive phase or NAD(P)H overshoot was also indistinguishable between these conditions after correcting for total Ca^{2+} accumulation in the cytosol during stimulation.

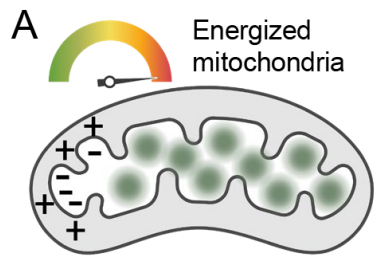
1364 **Table 1. Antibodies and sample concentrations for capillary electrophoresis immunoblots.**

Target Protein	Vendor	Catalog #	RRID	Dilution	[Sample] ($\mu\text{g}/\mu\text{l}$)
$\alpha\beta$ -Tubulin	Cell Signaling Technology	2148	AB_2288042	1:50	0.01
Akt (pan)	Cell Signaling Technology	4691	AB_915783	1:100	0.01
p-Akt (Ser473)	Cell Signaling Technology	4060	AB_2315049	1:50	0.7
Atg7	Cell Signaling Technology	8558	AB_10831194	1:50	0.7
Beclin-1	Cell Signaling Technology	3495	AB_1903911	1:50	0.7
Drp1	Cell Signaling Technology	8570	AB_10950498	1:50	0.01
p-Drp1 (S616)	Cell Signaling Technology	4494	AB_11178659	1:20	0.75
p-Drp1 (S637)	Cell Signaling Technology	6319	AB_10971640	1:20	0.75
p44/42 MAPK (Erk1/2)	Cell Signaling Technology	9102	AB_330744	1:100	0.01
p-Erk1(T202/Y204) / p-Erk2(T185/Y187)	Bio-Techne/R&D Systems	AF1018	AB_354539	1:50	0.1
IR β	Cell Signaling Technology	3025	AB_2280448	1:50	0.1
MDH2	Cell Signaling Technology	11908	AB_2797764	1:50	0.7
OPA1	Cell Signaling Technology	80471	AB_2734117	1:50	0.01
PDH-E1	Cell Signaling Technology	2784	AB_2162928	1:100	0.01
p-PDH-E1-A type I (Ser293)	Novus Biologicals	NB110-93479	AB_1237282	1:50	0.1
SOD2	Cell Signaling Technology	13141	AB_2636921	1:50	0.01
TOM20	Cell Signaling Technology	42406	AB_2687663	1:50	0.01

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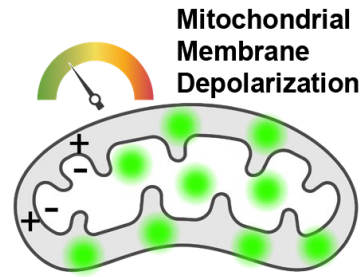
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ETC uncoupler

Neuronal activity



Cessation of the stimulus

