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Mitochondrial calcium, a potential sensor for homeostatic synaptic plasticity

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Abstract

Neural function is maintained through homeostatic mechanisms that are engaged following perturbations to the nervous system. Homeostatic plasticity is thought to be critical for establishing and stabilizing appropriate levels of network function. Neurons are proposed to detect deviations in activity through intracellular calcium signaling, such that changes in calcium levels initiate compensatory mechanisms that restore activity and calcium to baseline. This sensing process is generally assumed to occur in the cytoplasm, however, recent work suggests that it may reside inside mitochondria. We test this in the chick embryo (either sex) spinal cord. We show that perturbations known to induce homeostatic plasticity preferentially alter the mitochondrial proteome, including components of the tricarboxylic acid (TCA) cycle, a pathway sensitive to calcium entry into mitochondria. We then tested whether calcium influx into the mitochondrial matrix contributes to the induction of homeostatic plasticity in motoneurons. Pharmacological blockade of the mitochondrial calcium uniporter (MCU), which mediates calcium entry into the matrix, produced a robust and sustained increase in spontaneous network activity (SNA). Using Ru265 to inhibit MCU function, we confirmed a reduction in mitochondrial calcium, while cytoplasmic calcium levels were largely unchanged or slightly elevated. MCU blockade was accompanied by an increase in excitatory synaptic strength consistent with homeostatic synaptic plasticity. The underlying mechanisms overlapped with those previously described in this preparation following activity or neurotransmitter blockade. Together, these findings support a model in which mitochondria contribute to the initiation of homeostatic synaptic plasticity, potentially by sensing changes in calcium transients within the mitochondrial matrix.

Significance

Homeostatic plasticity is thought to play a critical role in maintaining circuit function. Although substantial progress has been made in identifying the mechanisms underlying the expression of homeostatic plasticity, the upstream triggers remain poorly understood. Cytoplasmic calcium has been proposed as a key signal in the detection of perturbations in neural circuit activity and in initiating compensatory responses. Here, we present findings consistent with the idea that the sensor for network activity and homeostatic synaptic plasticity tracks mitochondrial calcium. Identifying the sensor that initiates homeostatic mechanisms will be essential for understanding the functional objectives of this form of plasticity and may provide a foundation for pharmacologically targeting this pathway in conditions characterized by altered network activity.

Introduction

Homeostatic plasticity comprises a set of mechanisms that are engaged when neural activity is perturbed and act to restore function toward a stable operating range (Tien and Kerschensteiner, 2018; Wen and Turrigiano, 2024). For example, prolonged reductions in spiking or glutamatergic transmission increase excitatory AMPAergic miniature postsynaptic current (mPSC) amplitude and decrease inhibitory GABAergic mPSC amplitude throughout their distributions. This compensatory adjustment of the basic unit of synaptic strength is known as synaptic scaling (Turrigiano et al., 1998). In addition, a presynaptic form of homeostatic plasticity exists where compensatory changes in the probability of vesicle release are triggered by postsynaptic neurotransmitter blockade (Goel and Dickman, 2021). Finally, neurons also compensate through changes in membrane excitability, referred to as homeostatic intrinsic plasticity (Wen and Turrigiano, 2024).

Understanding how neurons detect that activity has deviated from a physiologically appropriate range remains unclear. Calcium signaling has long been proposed as a key activity sensor (Turrigiano, 2008; Turrigiano, 2012; O'Leary et al., 2015; Wen and Turrigiano, 2024). Reduced spiking diminishes calcium influx through voltage-gated calcium channels, consistent with the idea that intracellular calcium could serve as a proxy for firing rate. NMDAergic mPSCs are also involved in triggering homeostatic synaptic scaling (Sutton et al., 2006) through small calcium transients associated with these mPSCs, which could allow cells to homeostatically monitor synaptic transmission (Reese and Kavalali, 2015). Calcium-sensitive effectors, such as kinases, could initiate compensatory responses that restore calcium signaling following changes in calcium transient amplitude or frequency. Computational/theoretical studies have long supported the plausibility of calcium-based sensing mechanisms (Liu et al., 1998; Frank, 2014), and experimental work implicates specific calcium-dependent kinases (Thiagarajan et al., 2002; Ibata et al., 2008).

A straightforward model posits that calcium sensors reside in the cytoplasm. However, more recent work has suggested the possibility that a targeted level of spiking activity (setpoint) could be controlled by mitochondria (Styr et al., 2019; Ruggiero et al., 2021; Katsenelson et al., 2022). The mitochondrial matrix maintains a highly negative membrane potential, enabling efficient buffering of cytoplasmic calcium and positioning mitochondria as a potential calcium-sensing organelle. Mitochondria are enriched at synapses (Garcia et al., 2019) and are well known for supplying ATP and regulating cytoplasmic calcium, both essential for synaptic transmission (Cserep et al., 2018; Rangaraju et al., 2019; Duarte et al., 2023). These properties raise the possibility that mitochondria contribute not only to metabolic/synaptic support but also to the detection of activity deviations that engage homeostatic plasticity mechanisms.

Here, we tested whether mitochondria could serve as a calcium sensor for triggering homeostatic plasticity in the developing chick embryo spinal cord. In this system we have observed the recovery of embryonic movements in vivo following neurotransmitter blockade, and have identified multiple forms of homeostatic synaptic and intrinsic plasticity (Gonzalez-Islas and Wenner, 2006; Wilhelm and Wenner, 2008; Wilhelm et al., 2009; Wenner, 2020; Pekala and Wenner, 2022).

Mitochondria regulate synaptic function through ATP production and cytoplasmic calcium buffering (Duarte et al., 2023). Here, we specifically examined the potential role of mitochondria in sensing reduced neural activity/synaptic transmission to initiate homeostatic plasticity. Using an unbiased proteomic analysis of spinal cord tissue subjected to different activity perturbations known to induce homeostatic plasticity, we found significant alterations in the mitochondrial

proteome. To further test a mitochondrial role, we reduced calcium entry into the matrix (with cytoplasmic calcium levels largely preserved) and assessed spinal network excitability. Diminishing mitochondrial calcium influx produced a marked increase in spinal excitability, reflected by a robust rise in the frequency of spontaneous network activity (SNA). Although we did not detect clear homeostatic changes in intrinsic excitability, we did observe compensatory changes in synaptic strength (synaptic scaling). These changes were mediated by similar mechanisms to that observed previously with the blockade of activity or synaptic transmission. Our results support a potential model where reduced calcium influx could be sensed by mitochondria and trigger compensatory changes in synaptic strength and spinal activity to restore functional output.

Materials and Methods

Dissection

Experiments were performed on White Leghorn chicken embryos of either sex (Hy-Line North America) from embryonic day 8-10 (E8-E10 or Stage 34-36 (Hamburger and Hamilton, 1951). Spinal cords were isolated at E10, with attached spinal nerves, in cooled (14°C) oxygenated (95% O_2/5% CO_2) Tyrode's solution containing the following (in mM): 139 NaCl, 12 D-glucose, 17 $NaHCO_3$, 3 KCl, 1 $MgCl_2$, and 3 $CaCl_2$. After dissection, the cord was left overnight to recover in Tyrode's solution at 18°C. The cord was then transferred to a recording chamber and continuously oxygenated with Tyrode's solution (5 mM KCl) that was warmed to 27 ± 1°C.

Electrophysiology

Whole-cell patch-clamp recordings were obtained from chick embryo motoneurons, localized in lumbosacral segments 1 and 3 (LS1-LS3), identified by their lateral position in the

ventral cord. Motoneurons were held at −70 mV to acquire mPSCs in the presence of tetrodotoxin (TTX, 1μM). Tyrode's solution was used as the extracellular recording solution, and the intracellular patch solution contained the following (in mM): 5 NaCl, 100 K-gluconate, 36 KCl, 10 HEPES, 1.1 EGTA, 1 $MgCl_2$, 0.1 $CaCl_2$, 1 Na_2ATP, and 0.1 MgGTP, pH adjusted to 7.3 with KOH (280-300 mOsm). Patch-clamp tight seals (≫1 GΩ) were obtained using electrodes (5-10 MΩ) pulled from thin-walled borosilicate glass (World Precision Instruments) in two stages, with a P-87 Flaming/Brown micropipette puller (Sutter Instruments). Reported values were not corrected for a liquid junction potential of −12 mV, which was experimentally measured (Neher, 1992). Whole-cell currents were acquired using Axoclamp 200B (Molecular Devices) amplifier controlled by pClamp software (Molecular Devices). Currents were filtered online at 5 kHz and digitized at 10 kHz.

Recordings of mPSCs were performed in control cords and after acute treatment with a selective mitochondrial calcium uniporter (MCU) inhibitor Ru265 (5 μM) *in vitro* to assess rapid scaling. These experiments were performed in normal Tyrode's in the presence of TTX (1 μM). In order to follow spontaneous network activity, the whole caudal cord or rostral hemicord (3-5 segments) was drawn into a suction electrode. These recordings were performed without TTX in the bath (before and after Ru265, 5 μM). Electrical signals were measured using extracellular amplifiers (AC/DC differential amplifier model 3000, A.M. Systems, filtering parameters for capturing SNA: DC-1000Hz).

The mPSCs were acquired on an Axopatch 200B patch clamp amplifier (Molecular Devices), digitized (Digidata 1440, Molecular Devices) online using PClamp 10 (Molecular Devices) and analyzed manually using Minianalysis software (Synaptosoft) with a cutoff threshold of 5 pA. Detected mPSCs were accepted or rejected from a final data set following visual inspection of the waveform. AMPA and GABA mPSCs were isolated based on their decay kinetics

(AMPA mPSC τ ≤ 8 ms, GABA mPSC τ > 8 ms), as described previously (Gonzalez-Islas and Wenner, 2006). The mean values were obtained by determining the average mPSC amplitude and frequency for each cell by taking 30 currents/cell. Cumulative probability distributions were obtained by combining mPSC amplitudes (30 mPSCs per cell) in control or treated cords. The cumulative distributions of mPSCs (control vs drug treated) were compared with a Kolmogorov-Smirnov test with p value < 0.05 indicating that the treated and control cumulative distributions were different.

Imaging

Chick embryos were dissected to isolate spinal cords on embryonic day 10 (E10). For cytoplasmic calcium imaging ventral roots were left intact on the cord, and the cut end of the ventral root was drawn into a suction electrode (at LS 1 or LS 2) filled with Calcium Green-1 (dextran potassium salt, 10,000 MW anionic, C3713; Invitrogen) thus labeling MNs (O'Donovan et al., 2008; Ratliff et al., 2023). We used a ratio of indicator to vehicle (H_2O) of 1 mg : 10 µl.

In order to label neurons with mitochondrial calcium indicator mtGCamp6f, we cut a small window into the shell of white leghorn chicken eggs (Hy-line North America LLC) on embryonic day 3 (E3, stages 14-15 (Hamburger & Hamilton, 1951). Plasmids coding for the mtGCamp6f protein under control of a CMV promoter (Addgene) were injected into the central canal of the neural tube. Two electrodes spaced 4 mm apart were lowered onto the chorioallantoic membrane on either side of the embryo and 5 pulses (25 V, 50 msec, interval of 1 sec) were delivered using an ECM 830 electroporator (Blank et al., 2007).

All images were taken in the ventral view of the spinal cord. Regions of interest (ROIs) were drawn around labeled cell bodies to examine changes in fluorescent intensity during

episodes of spontaneous network activity, that can last for over 60 seconds (Landmesser and O'Donovan, 1984; O'Donovan et al., 2008). After a refractory period of several minutes, another episode of SNA occurred spontaneously, or was initiated with a brief stimulus pulse to the spinal nerves or spinal cord white matter. Both spontaneous and evoked episodes of SNA were recorded optically by measuring an increase in intensity of fluorescence due to calcium influx in these ROIs (5-15 frames/second). A 60-frame average of a non-labeled region of the cord was used for subtraction of background fluorescence from each ROI. Each of these background-subtracted ROIs were then normalized by dividing the intensity values in every frame by a 60-frame average of the pre-episode baseline fluorescence for that ROI. This calculation provided ΔF/F, which followed changes in cytoplasmic (calcium green) or mitochondrial (mtGCamp6f) calcium levels at baseline and during spontaneous or evoked episodes of SNA.

Drugs

TTX, D-(-)-2-Amino-5-phosphonopentanoic acid (APV), Dihydro-β-erythroidine (DHβE) and 1-N-naphthyl acetyl spermine (NASPM) were purchased from Tocris Bioscience. Ru265 and all other chemicals were purchased from Sigma-Aldrich.

Data analysis

Outliers for each parameter (defined as a value higher than the third quartile value + 2.2 times the interquartile range) and cells with <30 mPSCs were excluded from analysis. Comparisons of cumulative distributions were analyzed with a Kolmogorov–Smirnov test. We used estimation-based statistics with mean difference plots to estimate the effect sizes (Ho et al., 2019), unless stated otherwise. The effect sizes with 95% CI were reported in the figure legends. Where noted, we used a paired Student's t Test. Numerical values are given as mean ± SEM. Proteomics data were processed with Qlucore Omics Explorer Version 3.6(33). Data were

normalized to a variance of 1 and an average of 0 for statistical analysis and thresholding. For qRT-PCR data, ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli was done with Prism Version 10.5.0 (774). Gene ontology studies were performed with Metascape (Zhou et al., 2019) and Webgestalt (Elizarraras et al., 2024).

Membrane Preparation of Chicken Spinal Cord.

Chicken embryos were incubated with DHβE (5 μM) or vehicle control for 48 hours prior to dissection of the spinal cord at E10. Spinal cords were dissected from LS1 to LS8 in cooled and oxygenated Tyrode's solution and immediately flash frozen in liquid nitrogen. Frozen spinal cords were lysed by sonication in 0.1M sodium carbonate, pH 11 containing Complete anti-protease (Roche Cat# 11697498001) and incubated at 4°C for 30 minutes. Lysate was centrifuged in Beckman polycarbonate ultra-centrifuge tubes (Beckman Coulter Cat# 343778) for 30 minutes, 4°C, 68,000 RPM in a Beckman TLA 120.2 rotor. Supernatant was removed and the pellet was sonicated in fresh lysis buffer containing Complete, incubated at 4°C for 30minutes, and centrifuged for 30 minutes at 4°C, 68,000 RPM in the Beckman TLA 120.2 rotor. Supernatant was removed and the pellet was gently rinsed in ice cold phosphate buffered saline then suspended in Buffer A (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGTA, and 0.1 mM MgCl2), containing 0.5% Triton X-100 and Complete anti-protease, with sonication and incubated at 4°C for 30 minutes.

qRT-PCR from Chicken Spinal Cord.

Chicken embryos were incubated with DHβE (5 μM), gabazine (10 μM), or vehicle control for 48 hours prior to dissection of the spinal cord at E10. Spinal cords were dissected from LS1 to LS8 in cooled and oxygenated Tyrode's solution and immediately flash frozen in liquid nitrogen. RNA was isolated from frozen cords using Trizol (Invitrogen Cat# 15596026) following the

manufacturer's protocol for tissues. RNA concentration and purity were determined on a NanoDrop OneC (Thermo Scientific). Isolated RNA was reverse transcribed using Superscript III First-Strand System Kit (Thermo Cat# 18080051) according to manufacturer's protocol with 5µg of RNA per reaction and random hexamer primers. The resulting cDNA was used for qPCR on either a Roche LightCycler 480 or an Applied Biosystems Quantstudio 6 Flex with LightCycler 480 SYBR Green Master Mix (Roche Cat# 04707516001) and selected primers (Supplemental Table 1). Primers were designed with the IDT DNA Realtime PCR Tool (Integrated DNA Technologies). qRT-PCR was analyzed with standard curves for each primer pair generated from control treated chicken spinal cord RT-PCR.

Protein digestion

Samples were homogenized in urea lysis buffer (8 M urea, 100 mM Na_2HPO_4, pH 8.5) with HALT protease and phosphatase inhibitor cocktail (ThermoFisher) using a Bullet Blender (NextAdvance) and probe sonicated. Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce) and 100ug was aliquoted for digestion. Samples were reduced with 1 mM dithiothreitol (DTT) for 30 min, followed by 5 mM iodoacetamide (IAA) alkylation in the dark for another 30 min. Samples were diluted 4-fold with 50 mM triethylammonium bicarbonate (TEAB) before incubating with Lysyl endopeptidase (Wako) at 1:100 (w/w) for 12 hr. Trypsin (Promega) was then added at a 1:50 (w/w) ratio and digestion was carried out for another 12 hr after the urea concentration was diluted to 1 M with 50 mM TEAB. The peptide solutions were desalted with a C18 Sep-Pak column (Waters). Briefly, the Sep-Pak columns were activated with 1 ml of methanol, then equilibrated with 2 × 1 ml 0.1% triflouroacetic acid (TFA). The samples were loaded after acidification to a final concentration of 1% formic acid (FA) and 0.1% TFA. Each column was washed with 2 × 1 ml 0.1% TFA. Elution was performed with 1 ml 50% acetonitrile.

278

279 **Tandem mass tag (TMT) peptide labeling**

280 Briefly, each of the TMT reagents were dissolved in 246 µl anhydrous acetonitrile. The
281 samples were reconstituted in 100 µl of 100 mM TEAB buffer and mixed with 0.8 mg (41 µl) of the
282 corresponding labeling reagent channel. The reaction was incubated for 1 hr and subsequently
283 quenched with 8 µl of 5% hydroxylamine (Pierce). For each TMT plex, labeled peptides from all
284 10 channels were mixed and desalted with a 100 mg Sep-Pak column (Waters). The labeled
285 peptide mixture was eluted in 1 ml of 50% acetonitrile and dried by vacuum.

286

287 **High-pH off-line fractionation**

288 Dried samples were re-suspended in high pH loading buffer (1 mM ammonium formate,
289 2% (v/v) acetonitrile) and loaded onto ZORBAX Extend 300 C18 columns (4.6 mm × 250 mm with
290 5 µm beads) from Agilent. An Agilent 1100 HPLC system was used to carry out the fractionation
291 at room temperature with UV absorbance monitored at wavelength 280 nm. A total of 24 fractions
292 were acidified to a final concentration of 1% FA, and 5% of sample volumes were dried and
293 reserved for proteome analysis.

294

295 **LC-MS/MS and TMT data acquisition on an Orbitrap Lumos mass spectrometer**

296 The samples were run on a Fusion Lumos equipped with a NanoFlex nano-electrospray
297 source (ThermoFisher). The same volume of loading buffer (80 µl of 0.1% TFA) was added to
298 each of the fractions assuming equal distribution of peptide concentration across all 24 proteomic
299 subfractions. Therefore, an equal 2 µl (1 µg equivalent) of each fraction was loaded for proteomic
300 analysis. All fractions were separated on 25 cm long (75 µm ID) fused silica columns (New

Objective, Woburn, MA) packed in-house with 1.9 µm Reprosil-Pur C18-AQ resin (Dr Maisch). All fractions were eluted over a 140 minute gradient using an Easy nLC 1200 (Thermofisher). The mass spectrometer was operated in top speed mode with 3 second cycles. Both the MS and MS/MS scans were collected in the Orbitrap. The full scan was performed with a range of 375–1500 m/z, a nominal resolution of 120,000 at 200 m/z, automatic gain control (AGC) at 4×10^5, a 50 ms max injection time and a radio frequency (RF) lens setting of 30%. Higher-energy collision dissociation (HCD) MS/MS scans settings were the following: resolution of 50,000, AGC at 1×10^5, isolation width of 1.2 m/z, max injection time of 86 ms, and a collision energy of 36%. Only charge states from 2 + to 7 + were chosen for tandem MS/MS.

Protein identification and quantification

Raw data files were processed using Proteome Discoverer™ (version 2.1.1.21). MS/MS spectra were searched against the UniProt Knowledgebase (UniProtKB) chicken proteome database (downloaded in 2018). The SEQUEST HT search engine was used and parameters were as follows: fully-tryptic specificity; maximum of two missed cleavages; minimum peptide length of 6; fixed modifications for TMT tags on lysine residues and peptide N-termini (+ 229.162932 Da) and carbamidomethylation of cysteine residues (+ 57.02146 Da); variable modifications for oxidation of methionine residues (+ 15.99492 Da); deamidation of asparagine and glutamine (+ 0.984 Da); phosphorylation of serine, threonine and tyrosine (+ 79.9663 Da); precursor mass tolerance of 20 ppm; fragment mass tolerance of 0.05 daltons. Percolator was used to filter peptide spectral matches (PSM) and peptides to a false discovery rate (FDR) of less than 1% using target-decoy strategy Reporter ions were quantified from MS2 scans using an integration tolerance of 20 ppm with the most confident centroid setting. The search results and TMT quantification are included (Dataset S1).

Results

Mitochondrial proteome is modified by perturbations that trigger synaptic scaling.

Previous work implicated mitochondrial function in homeostatic plasticity, including synaptic scaling (Ruggiero et al., 2021). In order to test the possibility that mitochondria are affected by or participate in synaptic scaling we carried out a proteomic analysis of differentially expressed proteins following distinct perturbations that trigger scaling in different systems. We triggered GABAergic and AMPAergic scaling in the E8-10 chick embryo as we had done previously by reducing $GABA_A$ receptor (GABAR) activation either through direct blockade of GABARs (equivalent of 10 μM gabazine, (Wilhelm and Wenner, 2008)) or by reducing GABA vesicle release with a nicotinic antagonist (equivalent of 5 μM DHβE, (Garcia-Bereguiain et al., 2016; Gonzalez-Islas et al., 2016)). GABA is depolarizing and excitatory at this stage in the chick embryo spinal cord. We also compared these data with our previous study where homeostatic plasticity was elicited by APV/TTX-treatment in mouse primary cultured neurons (Bulow et al., 2021). Gabazine or DHβE were injected in vivo at embryonic day 8 (E8) and we then isolated the lumbosacral spinal cords from embryos two days later to analyze the whole proteome (gabazine) or the proteome of a membrane-enriched fraction at E10 (DHβE). The entire proteome was analyzed in treated and control embryos and selected as differentially up-or down-regulated proteins compared to those with a cut-off below $q < 0.05$ or $p < 0.00037$ (t-test corrected by Benjamini-Hochberg multiple corrections). We identified 45 proteins in the membrane-enriched fraction whose expression was sensitive to DHβE in one experiment, and 587 proteins in the whole proteome from cords exposed to gabazine in two other experiments (Figure 1A, Dataset S1). There are several possible reasons for the smaller number of protein changes observed in

the membrane fraction treated with DHβE. Gabazine completely blocks GABAR activation due to evoked or spontaneous GABA release, whereas DHβE only partially reduces GABAR activation due to spontaneous GABA release. The membrane fraction is necessarily only one part of the entire proteome. Finally, technical features of the mass spectrometry experiments (performed using data-dependent acquisition) were inherently subject to run-to-run sampling variability. Collectively, gabazine and DHβE identified 632 non-redundant proteins modified by these treatments. To further explore potential mitochondrial mechanisms, we performed an additional analysis based on the mitochondrial complexes to which the altered proteins belong. This analysis revealed increased representation of proteins from the mitochondrial ribosome (e.g., MRPS and MRPL family members; Figure 1A, Dataset S1) as well as components of respiratory chain Complex I (NDUF proteins; Figure 1A, Dataset S1). These changes are consistent with either increased respiratory activity or an increase in mitochondrial content following treatment. Due to the paucity of validated antibodies for chicken antigens, we selected a group of proteins to assess changes in expression by qRT-PCR (Figure 1B). Among the 11 proteins selected for confirmation, we found that the levels of transcripts encoding TFRC, MAGI2, and SMN were modified in a manner concordant with the proteome (Figure 1B).

To identify shared ontologies across pharmacological strategies for induction of homeostatic plasticity, we also compared DHβE and gabazine to a different system where we had previously triggered scaling. We induced scaling in rodent cortical cultures by blocking NMDAergic mPSCs with APV and TTX (Bulow et al., 2019). APV/TTX-treatment changed the levels of 113 proteins (Bulow et al., 2021). We subjected the DHβE, gabazine, and APV datasets to ontological analysis using Metascape and identified Metabolic Process as a priority parental ontology shared by all these datasets (Zhou et al., 2019)(Figure 1C, Dataset S1, -log10 p DHβE = -3.09, gabazine = -5.8 and -18.1, and APV/TTX = -4.13). We then used the Webgestalt

bioinformatic tool to identify specific ontological enrichment across all proteins sensitive to these drugs (Figure 1D, Dataset S1). We found that the mitochondrial pathway WP2453 TCA Cycle and Deficiency of Pyruvate Dehydrogenase complex was the most enriched pathway, 8.97-fold, among all the proteins sensitive to drugs (Figure 1D, Dataset S1, Benjamini-Hochberg FDR 0.0207). This result was further supported by the 63 MitoCarta 3.0 mitochondria-annotated proteins present in all these datasets (Figure 1E, OR = 1.62, 95% CI [1.25–2.12]; Fisher's exact test, $p = 4.4 \times 10^{-4}$), corresponding to a 1.54-fold enrichment over random expectation). These results show that three diverse drug treatments/conditions, share in common the ability to trigger synaptic upscaling and prominently modify the mitochondrial proteome. These results align with previous work in cultured hippocampal neurons (Ruggiero et al., 2021) and further strengthened the argument that mitochondria could be a target for, or modulators of homeostatic plasticity.

Manipulation of mitochondrial calcium triggers changes in SNA

The proteomic results were consistent with the idea that mitochondria were involved in triggering homeostatic synaptic upscaling. Previous experimental and computational studies suggested that cells could assess intracellular calcium as a sensor for changes in spiking activity (Turrigiano et al., 1994; Liu et al., 1998; Wen and Turrigiano, 2024). Reduced spiking activity should reduce intracellular calcium signaling in the cytoplasm that could trigger the expression of homeostatic mechanisms like scaling, which could act to restore activity levels - cytoplasmic calcium hypothesis. However, our proteomic results (Figure 1) suggested that mitochondria could be intimately involved in the homeostatic response and therefore raised the possibility that the homeostatic sensors of calcium could be within the mitochondrial matrix - mitochondrial calcium hypothesis.

Thus, we considered the possibility that the reduced calcium in the mitochondrial matrix could trigger compensatory increases in cellular or network activity through the recruitment of homeostatic plasticity mechanisms. To test the mitochondrial calcium hypothesis, we blocked calcium entry into the mitochondria by inhibiting the mitochondrial calcium uniporter (De Stefani et al., 2011) with Ru265 (5 μM, (Woods et al., 2019)). If reduced matrix calcium triggers homeostatic increases in cellular excitability, then we should see increases in some feature of excitability in the isolated cords following Ru265 treatment. If instead, the cytoplasmic calcium hypothesis is correct then we may observe reduced or unaltered excitability as mitochondrial calcium block could either leave the cytoplasmic calcium signaling unperturbed or increased (by reducing mitochondrial buffering). Remarkably, when we added Ru265 to the isolated cord we saw a dramatic 5.5-fold increase in SNA frequency (Figure 2, from 10.5 minute intervals before Ru265 to ~1.9 minute intervals 3 hours after Ru265, ($p < 0.0001$ paired Student's t Test, $n = 4$)). The observed increase in SNA frequency was rapid, occurring within 1 hr of drug application. This result was consistent with the possibility that a calcium sensor was in the matrix of the mitochondria, and when matrix calcium was reduced an excitatory compensation was triggered.

To ensure that Ru265 was reducing matrix calcium we monitored the level of calcium after Ru265 treatment through the expression of a mitochondrial calcium indicator. We electroporated plasmids carrying the mitochondrial calcium indicator mt-GCamp6f at E3 as previously described (Lindsly et al., 2014). Plasmids predominantly labeled motoneurons in the ventro-lateral part of the spinal cord (Figure 3A). The labeled mitochondria could be observed in the soma and along dendrites of motoneurons (Figure 3A transverse slice). The imaging of episodes of SNA showed the occurrence of mitochondrial calcium transients ($\Delta F/F$) and these transients were reduced ~40 minutes after bath application of Ru265 (Figure 3A). To better compare these mitochondrial calcium transients to the population activity of the motoneurons we carried out simultaneous

extracellular motoneuron recordings from ventral roots (VR) with cytoplasmic and mitochondrial calcium imaging (Figure 3B). We electroporated embryos with the mtGCamp6f-containing plasmids at E3, and isolated the cord at E10, at which time we retrogradely-labeled a separate population of motoneurons overnight with the cytoplasmic calcium indicator Calcium Green-1 through a ventral root (see methods (Ratliff et al., 2023)). We then imaged calcium transients in motoneurons that were electroporated with mtGCamp6f or retrogradely-labeled with Calcium Green-1 while simultaneously recording motoneuron population signals from suction electrodes of the VR. We found that the timing of the signals from the extracellular VR recording and cytoplasmic calcium transient were similar (Figure 3B), consistent with previous results (O'Donovan et al., 1994). As expected, the mitochondrial calcium transient was slower to rise and fall. The result clearly shows mitochondrial and cytoplasmic calcium signals were different. Next we determined how blocking mitochondrial calcium uptake might influence cytoplasmic calcium levels by monitoring cytoplasmic calcium signals. We found that MCU blockade slightly increased baseline calcium fluorescence ~30 minutes after Ru265 (Figure 3C) but had no effect on peak fluorescence during episodes of SNA (Figure 3D). In addition, we found that Ru265 slowed down the cytoplasmic calcium clearance after a burst of SNA (Figure 3E). We measured the time at which the cytoplasmic calcium signal and ventral root signal returned to baseline after the episode of SNA. We found that they were nearly synchronous (no time difference) before Ru265, but after addition of the drug there was an approximate 5 second delay in the return to baseline of the cytoplasmic calcium signal compared to that of the VR (Figure 3E, n = 2 cords). Therefore, Ru265 reduced mitochondrial calcium entry, and transiently increased cytoplasmic calcium. The observations that Ru265 increased SNA frequency, reduced mitochondrial calcium, and slightly increased cytoplasmic calcium was more consistent with the mitochondrial calcium hypothesis.

MCU block with Ru265 did not change resting membrane potential

Previously, we have shown that SNA-driven embryonic movements in the living chick begin to recover within 2 hours of injecting either GABAergic or glutamatergic neurotransmitter receptor antagonists (Wilhelm and Wenner, 2008). This recovery was mediated at least in part by a rapid (within ~2 h) depolarization (~10 mV) of resting membrane potential (RMP) (Gonzalez-Islas et al., 2020). Therefore, we tested the possibility that blocking the MCU with Ru265 might also produce a depolarization that could lead to the higher frequency of SNA. We found that there was no difference in RMP between control and Ru265-treated motoneurons (control: - 48.3 $\pm$ 0.2 mV, n = 18 vs. MCU -48.6 $\pm$ 0.3 mV, n = 19, p = 0.84). The result suggested that the increase in excitability triggered by reduced mitochondrial calcium was not mediated by a rapid change in the RMP.

MCU blockade triggers rapid AMPAergic scaling

We have recently demonstrated a rapid form of homeostatic synaptic scaling in embryonic motoneurons (Pekala and Wenner, 2022). We found that AMPAergic and GABAergic mPSCs increase in amplitude following 1-2 hours of activity and NMDAR blockade (APV/TTX) compared to controls (treated only with TTX). We therefore tested the possibility that this rapid compensatory scaling could be triggered by reducing mitochondrial calcium and potentially contribute to the increase in SNA excitability. In order to evaluate whether changes in mitochondrial calcium could trigger rapid scaling, we isolated spinal cords from chick embryos and recorded mPSCs from motoneurons starting at least 1h after adding Ru265.

We found that Ru265 treatment triggered an increase in AMPA mPSC amplitude by 23% compared to control (control 11.8 $\pm$ 0.5 pA, n = 18 vs. Ru265 14.5 $\pm$ 0.8 pA, n = 19, p = 0.006, Figure 4A, Table 1). We also confirmed that following MCU block AMPAergic mPSC amplitudes

increased across the entire distribution compared to control, as presented by cumulative amplitude distribution plots ($p < 0.0001$, $D = 0.23$, Klomogorov-Smirnov (KS) test, Figure 4B). The increase in mPSC amplitudes was slightly smaller but similar to that produced by acute APV (50 μM) treatment (Figure 4A, Table 1 – Ru265/TTX vs APV/TTX not significantly different: 14.5 ± 0.8 pA, n = 19 vs 16.2 ± 1.0 pA n = 18, p = 0.19; Figure 4B – KS test p = 0.03, D = 0.09). The results demonstrated that blocking the MCU and reducing mitochondrial calcium entry was capable of triggering AMPAergic scaling.

We have previously shown that both rapid and slow AMPA mPSC upscaling induced by acute NMDA mPSC blockade (APV/TTX) or 2-day blockade of GABARs *in ovo*, was mediated by insertion of GluA2-lacking calcium permeable AMPARs (Garcia-Bereguian et al., 2013, Pekala and Wenner 2022). We therefore tested if Ru265-induced scaling occurs through a similar mechanism by blocking calcium-permeable AMPARs with bath applied NASPM (20 μM, added 30 minutes after Ru265, and incubated for at least 30 minutes). NASPM diminished AMPAergic scaling induced by MCU blockade, however mPSC amplitudes did not to go back to control levels (Figure 4A, Table 1 - decreased scaling by ~8%, Ru265 14.5 ± 0.8 pA, n = 19 vs. Ru265 + NASPM 13.4 ± 0.6 pA, n = 16, $p = 0.28$, Figure 4B – KS test p = 0.005 D = 0.11). GluA2-lacking AMPARs exhibit faster mPSC kinetics (Geiger et al., 1995). Therefore, we also measured mPSC rise and decay time and saw that both were significantly decreased (faster) in the Ru265/TTX and APV/TTX conditions (Table 1), consistent with the idea that there was an insertion of GluA2-lacking AMPARs in both conditions. When NASPM was added to block GluA2-lacking AMPARs, the kinetics were no different than control (Figure 4A, Table 1). The results suggested that GluA2-lacking AMPAR insertion is a common mechanism for rapid scaling induced by blockade of either NMDARs or the MCU, although additional mechanisms may be involved for MCU-induced scaling. To further determine whether rapid scaling triggered by Ru265 or APV treatment shared

similar pathways we treated cords with APV and Ru265 (in TTX) to see if there was an occlusion. We found that the combination of both treatments was not additive, and in fact produced a weaker scaling than either treatment alone suggesting some interaction (Figure 4A, Table 1, the amplitude of mPSCs after Ru265+APV increased by 9% compared to TTX alone, while after APV or Ru265 alone amplitudes increased by 38% and 23% respectively; Figure 4B – KS test comparing Ru265 + APV vs TTX alone was different p = 0.02, D = 0.09). These results suggested that rapid AMPAergic scaling induced by mitochondrial calcium inhibition shared similar mechanisms to previous studies that triggered scaling in different ways.

We also tested for potential compensatory changes in AMPA mPSC frequency. While APV/TTX did lead to an increase in mPSC frequency compared to controls, the slight increase in frequency of AMPA mPSCs in Ru265/TTX was not significant (Table 1 - control 0.70 ± 0.12, n = 18 vs Ru265 1.07 ± 0.14, n = 19 p = 0.06; and control vs APV 1.22 ± 0.13, n = 18, p =0 .007).

Rapid GABAergic scaling was observed following MCU blockade

We have previously shown that acute NMDAR mPSC blockade triggered rapid GABAergic upscaling (Pekala and Wenner, 2022). Similar to this result, we found that acute block of MCU triggered rapid upscaling of GABA mPSCs by 16% compared to control (Figure 5A, Table 2 - control 22.1 ± 0.3 pA, n = 16 vs. Ru265 25.7 ± 0.3 pA n = 15, $p < 0.05$,). The GABAergic mPSC amplitudes increased across the entire distribution, as presented by cumulative amplitude distribution plots (Figure 5B, KS test D = 0.21, p < .0001). The change in GABAergic scaling was similar to what we observed following APV (Figure 5A, Table 2 - Ru265 vs APV was not significantly different, Ru265: 25.7 ± 0.3 pA, n = 15 vs APV/TTX 24.9 ± 0.2 pA, n = 15, p = 0.59). In order to see if there was an occlusion of Ru265- and APV-induced scaling we treated cords

521 with APV and Ru265 and recorded GABAergic mPSCs. We found that there was some level of
522 occlusion as the combined treatment was not simply additive - the amplitude of mPSCs after
523 Ru265/APV treatment was not significantly different than Ru265 alone (Figure 5A, Table 2 - p =
524 0.56). The results are consistent with the idea that scaling induced by either MCU or NMDAR
525 blockade are mediated by similar mechanisms.

526

527 Previously we demonstrated that the mechanism underlying GABAergic scaling triggered
528 by APV (rapid) or two-day in vivo GABAergic blockade (slow) was an accumulation of intracellular
529 chloride, thus increasing mPSC amplitude through increases in the driving force for Cl^- currents
530 (Gonzalez-Islas et al., 2010; Lindsly et al., 2014). We therefore tested whether Ru265-induced
531 scaling was also mediated by increases in intracellular chloride. We used the genetic chloride
532 indicator Clomeleon, which is a ratiometric fluorescent fusion protein (Kuner and Augustine,
533 2000). We electroporated plasmids containing the indicator at E3 and then assessed chloride
534 levels in the isolated cord one week later at E10 as described previously (Lindsly et al., 2014).
535 We found that chloride levels in motoneuron cell bodies increase by ~15 mM 2-4 hours after
536 adding Ru265 (in the presence of TTX, Figure 6). These results suggest that mechanism of
537 GABAergic scaling occurs through an increase in intracellular chloride as described previously
538 for rapid and slow scaling. Changes in driving force for GABAergic currents would not necessarily
539 be expected to result in altered kinetics of mPSCs, but if there were additional changes in the
540 kinds of GABAR subtypes, then this might be expressed in the kinetics of these currents. We
541 found that there were no changes in rise time or decay of GABAergic mPSCs following
542 Ru265/TTX, however both rise time and decay became faster following APV/TTX. These results
543 are consistent with the idea that MCU or NMDAR blockade trigger scaling through chloride
544 accumulation, but that APV/TTX may also alter the composition of the GABARs.

545

We also found that both Ru265/TTX and APV/TTX-treatment did increase GABAergic mPSC frequency, but observed changes were significant only with MCU blockade (Table 2 - control 0.12 ± 0.002, n = 14; Ru265 0.22 ± 0.01, n = 15, p = 0.01; APV 0.16 ± 0.01, p = 0.15).

Discussion

In the current study we showed several findings consistent with the idea that homeostatic responses were triggered by reduced calcium levels in the mitochondrial matrix. First, proteomic analysis following perturbations that trigger synaptic scaling highlight the importance of mitochondria. Second, blocking calcium entry to the matrix using a mitochondrial calcium uniporter antagonist led to a significant increase in spontaneous network activity as well as AMPAergic and GABAergic synaptic strengthening. The mechanisms of scaling following MCU blockade were similar to those previously demonstrated in this system (GluA2-lacking receptor insertion and chloride accumulation). On the other hand, we did not observe obvious changes in intrinsic membrane excitability.

It was clear that Ru265 rapidly (within an hour) increased the frequency of SNA in the isolated spinal cord preparation. Previous work showed that glutamate or $GABA_A$ receptor blockade led to a rapid depolarization of the RMP (within an hour) (Gonzalez-Islas et al., 2020), and after two-day GABAR blockade *in ovo* there was an alteration in voltage-gated conductances (Wilhelm et al., 2009). Both of these homeostatic intrinsic plasticity mechanisms increased motoneuron firing rate. However, here we did not find any change in RMP, suggesting that the mechanism of the MCU-induced increase of SNA frequency is distinct. We could not detect any obvious changes in spiking activity, however it was difficult to compare the FI curve in control and

570 Ru265 conditions; the RMP in the inter-episode interval never stabilized once Ru265 reduced the
571 interval to 2 minutes. Therefore, we cannot rule out the possibility that there were changes in the
572 activation of certain voltage-gated channels. Recent work in the presynaptic terminal of the Calyx
573 of Held has suggested that Ru265 can have off target effects. This study found that Ru265
574 reduced a presynaptic Ca^{2+} current (P/Q-type) inhibiting excitatory transmission, and induced a
575 less clear effect on a slowly activating K^+ current (KCNQ), although it did not influence overall I_K
576 (Xu et al., 2024). These observations were based on the application of 50 μM Ru265, 10 fold
577 higher than in our study. We did not see any clear reduction in mPSC frequency, but rather there
578 appeared to be a slight increase (Table 1, Table 2). Together, our results showing that the MCU
579 blocker did not trigger intrinsic homeostatic mechanisms (e.g. RMP), but did trigger scaling
580 suggests the possibility that there are different sensors for different homeostatic mechanisms.

581

582 Our results showed that both GABAergic and AMPAergic synaptic strength was rapidly
583 increased following Ru265. A similar form of rapid scaling was first described in cultured neurons
584 and slices from hippocampus when NMDAR mEPSCs were blocked (Sutton et al., 2006; Aoto et
585 al., 2008). We carried out several tests to see if Ru265-mediated synaptic compensation was
586 mechanistically similar to previous work in the chick embryo spinal cord showing compensatory
587 synaptic strengthening following NMDAR mEPSC blockade, activity blockade, or GABAR
588 blockade (Gonzalez-Islas et al., 2010; Garcia-Bereguiain et al., 2013; Lindsly et al., 2014; Wenner
589 and Pekala, 2022). The GABAergic scaling in chick embryo motoneurons triggered by all three
590 perturbations was mediated by an increase in intracellular chloride concentration. This
591 mechanism clearly contributed to GABAergic synaptic strengthening following Ru265 treatment
592 (Figure 6). Further, our occlusion experiments (Figure 5A-B) suggest similar, but not identical,
593 mechanisms mediating GABAergic strengthening following the blockade of MCU or NMDAR-
594 mediated mPSCs. We have also discovered that inhibition of NMDAR mPSCs, but not MCU,

produce GABAergic mPSCs with faster kinetics, consistent with the possibility that APV-treatment leads to changes in GABAR subunits in addition to chloride accumulation. We have shown previously that compensatory AMPAergic synaptic strengthening was mediated through the insertion of GluA2-lacking AMPA receptors. We did see evidence that there was a component of the Ru265-dependent scaling that was mediated by GluA2-lacking AMPA receptors as the combination of NASPM + Ru265 did produce a significant leftward shift in the cumulative distribution compared to Ru265 alone (Figure 4B). Further, AMPAergic mPSC kinetics were faster in both Ru265- and APV-treated cords, consistent with the insertion of the faster GluA-2 lacking receptors. However, treatment with NASPM + Ru265 resulted in mPSCs that were still larger than in the control, leaving open the possibility that there is a transition from GluA2-lacking to GluA2-containing AMPA receptors as described previously for rapid scaling (Sutton et al., 2006). Regardless, the expression of AMPAergic and GABAergic scaling was not mechanistically identical following NMDAR and MCU blockade, and it will be important going forward to better understand how reductions in mitochondrial calcium entry alter synaptic strength.

We propose that blockade of postsynaptic MCU would be the most likely trigger for scaling as the mechanisms are expressed postsynaptically (AMPAR insertion and chloride accumulation) and classically the sensor has been thought to be postsynaptic. Scaling could contribute to the increases in SNA frequency, but it is likely that there are other mechanisms that lead to the dramatic increases in SNA. Ru265 also blocks the presynaptic MCUs, which could produce homeostatic increases in probability of release. Recent work has demonstrated that inhibition of mitochondrial complex 1 specifically in the presynaptic neuron triggers an increase in transmitter release, and this is dependent on reactive oxygen species (Mallik et al., 2025).

Blockade of calcium entry into the mitochondria induced large increases in SNA and synaptic strengthening, which would be expected to require increases in energy consumption. Paradoxically, inhibiting mitochondrial calcium entry could reduce ATP production by slowing the TCA cycle, however this remains unknown in the embryonic chick spinal cord. In Drosophila, it appears that inhibition of mitochondrial complex 1 leads to a greater dependence on glycolysis for ATP production (Mallik et al., 2025). Further, several studies suggest that ATP production in early development occurs through aerobic glycolysis (Steiner, 2019; Rajan and Fame, 2024).

How does Ru265 trigger these changes in synaptic strength? While we do not rule out the possibility that changes in cytoplasmic calcium could contribute to compensatory synaptic strengthening, we did not observe a reduction in cytoplasmic calcium levels/transients with Ru265 (Figure 3C-D). In fact, there was a small increase in baseline cytoplasmic calcium levels and a delay in the clearance of cytoplasmic calcium following an episode of SNA (Figure 3C/E). In homeostatic terms an increase in cytoplasmic calcium (associated with greater activity) would be expected to lead to a compensatory weakening of excitatory synaptic strength. Previous studies have modeled cytoplasmic calcium as a sensor for triggering homeostatic mechanisms (O'Leary et al., 2015; Sun et al., 2024). However, any changes in cytoplasmic calcium could also impact mitochondrial calcium and act through signaling in this organelle. Recent work is consistent with the idea that reductions in mitochondrial calcium trigger homeostatic plasticity (Katsenelson et al., 2022). In the study mitochondrial-cytoplasmic calcium coupling was shown to be critical to homeostatic responses, including scaling, and this occurred through the MCU and it's association with insulin-like growth factor-1 receptor.

Previous work has suggested that rapid scaling of both AMPAergic and GABAergic synapses in the chick embryo spinal cord were due to reductions in the frequency of NMDA receptor activation from spontaneous release of glutamatergic vesicles (Pekala and Wenner, 2022). Further, a slow form of AMPAergic and GABAergic scaling (2 days) can be induced by decreasing the frequency of $GABA_A$ receptor activation (depolarizing in the embryo) due to spontaneous release of GABAergic vesicles. Blocking spontaneous neurotransmission (mPSCs) can also reduce calcium entry into the cytoplasm (Reese and Kavalali, 2015; Kavalali and Monteggia, 2020), which could be sensed by mitochondria. Consistent with the idea that rapid scaling is mediated by mitochondria located near the synapse is the observation that this form of plasticity appears to occur in local domains influenced by mPSC receptor activation (Sutton et al., 2006; Gonzalez-Islas et al., 2018; Pekala and Wenner, 2022). There is evidence that calcium entry through NMDARs can signal local mitochondria to undergo fission, increase mitochondrial calcium, and alter local translation at those synapses (Li et al., 2004; Divakaruni et al., 2018; Rangaraju et al., 2019). Interestingly, the mPSCs, and potentially their associated calcium transients, are far more significant in triggering scaling in chick embryo motoneurons than the large depolarizations and calcium transients associated with SNA (Garcia-Bereguiain et al., 2016). This has also been observed in cultured neurons (Sutton et al., 2006; Fong et al., 2015), although other studies show that alterations in spiking alone can trigger scaling (Ibata et al., 2008; Goold and Nicoll, 2010). It will be important going forward to understand how calcium is involved in mediating scaling in these different contexts.

Mitochondria have long been known to have homeostatic functions within a neuron (Duarte et al., 2023; Sayehmiri et al., 2024). Synaptic transmission and plasticity are ATP-dependent processes that rely on synaptic usage and this is homeostatically regulated through mitochondrial ATP generation (Vos et al., 2010; Rangaraju et al., 2019). Further, mitochondria

buffer excess intracellular calcium within the presynaptic terminal to maintain synaptic transmission and plasticity (Cserep et al., 2018; Datta and Jaiswal, 2021). Here we describe a potential new homeostatic role for mitochondria to act as a sensor for triggering homeostatic synaptic plasticity by assessing mitochondrial calcium transients.

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Figure Legends:

Figure 1. Proteome modifications associated with perturbations that trigger synaptic scaling. **A)** Volcano plots of DHβE- and gabazine-treated spinal cords. Segmented line

depicts q < 0.05. Inserts show PCA analysis of proteome data thresholded at q < 0.05. Red and blue symbols depict proteins up- and down-regulated, respectively, after DHβE or gabazine treatment. **B)** qRT-PCR confirmation of selected hits in A. **C)** Metascape ontology analysis of all differentially expressed proteins after different treatments that induce synaptic scaling. **D)** Webgestat analysis of all non-redundant proteins belonging to the four datasets in C. **E**) UpsetR plot depicts overlap of proteins belonging to the four datasets in C with Mitocarta 3.0.

Figure 2. Blockade of MCU triggers an increase in SNA frequency. **A)** Schematic of the isolated spinal cord and extracellular suction electrode recording showing episodes of spontaneous population motoneuron activity before and after Ru265. B) SNA frequency is increased after addition of Ru265 in four different isolated spinal cords (color coded).

Figure 3. Mitochondrial and cytoplasmic calcium imaging in E10 spinal motoneurons. **A)** Mitochondrial calcium associated with an episode of SNA is reduced 40 minutes after adding Ru265, labeled with genetically-expressed mitochondrial calcium indicator mt-GCamp6f. Ventral view through the white matter shows mt-GCamp6f labeled motoneurons (left). The image is an average of 10 frames taken during the peak of the signal generated during an episode of SNA. Traces show mitochondrial calcium signal as a percentage change ($\Delta F/F$) for ROI's drawn over 3 different motoneuron cell bodies. **B)** Comparison of cytoplasmic and mitochondrial calcium transients in motoneurons with ventral root recordings from motoneurons showing the relative timing of the responses. **C)** Baseline cytoplasmic calcium fluorescence just before SNA onset was transiently increased 30 minutes after Ru265 compared to controls at equivalent times ((5 μM, *p ≤ 0.05) - two-sided permutation t-test)). Top, average values from 10 cells in each cord (filled circles), and mean values of 3 cords/30 cells (represented by the gap in the vertical bar) and SD

884 (vertical bars). Bottom, Mean differences between control and treated groups, as a bootstrap
885 sampling distribution after 30 and 90 minutes with Ru265. **D)** Peak fluorescence during episodes
886 of SNA was no different than controls after Ru265 (5μM, again 10 cells/cord across 3 cords - two-
887 sided permutation t-test). **E)** Following an episode of SNA the return to baseline of the
888 cytoplasmic calcium signal is delayed compared to that of the VR recording in the presence of
889 Ru265 (5μM) in two preparations.

890

891 **Figure 4.** MCU blockade triggers rapid AMPAergic scaling, similar to rapid scaling
892 triggered by NMDAR blockade. **A)** AMPA mPSC amplitudes in control motoneurons or in
893 the presence of APV, Ru265, NASPM or APV+Ru265. Top, representative traces of
894 mPSCs in different conditions (each trace is an average of 30 mPSCs/cell). Middle,
895 recordings from single cells (filled circles), mean values (represented by the gap in the
896 vertical bar) and SD (vertical bars) are shown. Bottom, filled circles represent mean
897 difference. Vertical error bars indicate 95% CIs. **B)** Cumulative distributions of AMPAergic
898 mPSC amplitudes in different conditions. All recordings were performed in the presence
899 of TTX in the bath. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.

900 **Figure 5**. MCU blockade triggers GABAergic scaling, similar to rapid scaling triggered by
901 NMDAR blockade. **A)** GABA mPSC amplitudes in control motoneurons or in the presence
902 of APV, Ru265, or both. Top, representative traces of mPSCs in different conditions (each
903 trace is an average of 30 mPSCs/cell). Middle, recordings from single cells (filled circles),
904 mean values (represented by the gap in the vertical bar) and SD (vertical bars) are shown.
905 Bottom, filled circles represent mean difference. Vertical error bars indicate 95% CIs. **B)**

Cumulative distributions of GABAergic mPSC amplitudes in the various conditions. All recordings were performed in the presence of TTX in the bath. *$p < 0.05$, **$p < 0.01$.

Figure 6. Ru265 treatment produces intracellular chloride accumulation at the cell body as expected for GABAergic scaling. Clomeleon-labeled motoneurons (10 motoneurons/ROI per cord, n = 2 cords) were imaged for YFP/CFP fluorescence ratios after adding Ru265 in the presence of TTX over time.

Tables:

Table 1. AMPA mPSC parameters. Average values for amplitude, frequency, rise time, decay time are provided, along with n's, SEM, percentage change (compared to control), and p values (compared to control). TTX is in bath for all conditions.

Table 2. GABA mPSC parameters. Average values for amplitude, frequency, rise time, decay time are provided, along with n's, SEM, percentage change (compared to control), and p values (compared to control). TTX is in bath for all conditions.

Table 1. AMPA mPSC parameters					
	control	**APV**	**Ru265**	**Ru265/NASPM**	**Ru265/APV**
Amplitude (pA)					
% change		38	23	14	9
n	18	18	19	16	19
SEM	0.45	1.03	0.80	0.59	0.79
Average	11.77	16.20	14.48	13.36	12.81
p value		0.002	0.006	0.034	0.265
Rise time (ms)					
% change		-15	-15	-3	-10
n	18	18	19	16	20
SEM	0.07	0.09	0.07	0.07	0.07
Average	1.72	1.46	1.46	1.67	1.54
p value		0.020	0.009	0.646	0.078
Decay time (ms)					
% change		-24	-15	-3	-11
n	18	17	19	16	19
SEM	0.12	0.15	0.16	0.19	0.12
Average	3.50	2.64	2.97	3.40	3.12
p value		< 0.0001	0.013	0.656	0.038
Frequency (Hz)					
% change		73	52	58	14
n	18	18	19	16	20
SEM	0.12	0.13	0.14	0.16	0.12
Average	0.70	1.22	1.07	1.11	0.80
p value		0.007	0.063	0.052	0.569

Table 2. GABA mPSC parameters				
	control	**APV**	**Ru265**	**Ru265/APV**
Amplitude (pA)				
% change		13	16	21
n	16	15	15	18
SEM	0.30	0.20	0.32	0.29
Average	22.11	24.89	25.68	26.73
p value		0.070	0.045	0.009
Rise time (ms)				
% change		-21	-3	-3
n	16	16	15	20
SEM	0.06	0.07	0.06	0.07
Average	5.52	4.39	5.37	5.34
p value		0.002	0.638	0.650
Decay time (ms)				
% change		-22	8	-4
n	16	16	15	20
SEM	0.38	0.24	0.44	0.31
Average	23.22	18.07	24.99	22.31
p value		0.068	0.440	0.679
Frequency (Hz)				
% change		34	85	313
n	14	14	15	20
SEM	0.00	0.01	0.01	0.01
Average	0.12	0.16	0.22	0.37
p value		0.146	0.006	0.000

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