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Role of mitochondrial calcium uptake homeostasis on resting-state fMRI brain networks

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Abstract

Mitochondrial Ca^{2+} uptake influences both brain energy metabolism and neural signaling (1–2). Given that brain mitochondrial organelles are distributed in relation to vascular density and varies considerably across brain regions (3), we hypothesized different physiological impact of mitochondrial Ca^{2+} uptake across brain regions. We tested the hypothesis by monitoring brain ‘intrinsic activity’ derived from the resting state-fMRI BOLD fluctuations in different functional networks spanning the somatosensory cortex, caudate putamen, hippocampus, and thalamus, during normal and perturbed mitochondrial Ca^{2+} uptake states. In anesthetized rats at 11.7T, mitochondrial Ca^{2+} uptake was inhibited or enhanced respectively by treatments with Ru360 or kaempferol. Surprisingly, mitochondrial Ca^{2+} uptake inhibition by Ru360 and enhancement by kaempferol, led to similar dose-dependent decrease in brain-wide intrinsic activities in both the frequency domain (spectral amplitude) and temporal domain (resting state functional connectivity; RSFC). The fact that similar dose-dependent decreases in the frequency and temporal domains of the resting state fMRI-BOLD fluctuations during mitochondrial Ca^{2+} uptake inhibition or enhancement indicated that mitochondrial Ca^{2+} uptake and its homeostasis may strongly influence brain’s functional organization at rest. Interestingly the resting state fMRI-derived intrinsic activities in the caudate putamen and thalamic regions saturated much faster with increasing dosage by either drug treatments than the drug-induced trends observed in cortical and hippocampal regions. Regional differences in how the spectral amplitude and RSFC changed with treatment indicate distinct mitochondria-mediated spontaneous neuronal activity coupling within the various RSFC networks determined by resting state fMRI.

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Graphical abstract

Given that brain mitochondrial organelles are distributed in relation to vascular density, we tested the hypothesis of distinct mitochondrial functional impact within the brain. Spontaneous BOLD signal fluctuations within specific resting-state fMRI (R-fMRI) networks spanning the somatosensory cortex, caudate putamen, hippocampus, and thalamus was monitored at 11.7T. Mitochondrial functional state was perturbed in vivo using specific agents directed towards the mitochondrial Ca^{2+} uniporter. We observed regional differences in R-fMRI activity indicating distinct mitochondria-mediated spontaneous neuronal activity coupling in different resting state functional networks.

Keywords

calcium uniporter; mCU; CBF; cortical excitability; functional network; kaempferol; mitochondria; neuronal; Ru360; fMRI; resting state

Introduction

Synaptic communication in the brain is enabled by the movement of free calcium ions (Ca^{2+}) within several cellular compartments. Ca^{2+} concentration are maintained at low levels in the cytoplasm (typically; 100–400 nM) compared to the extracellular space (typically; 1–2 mM). Such a large difference in Ca^{2+} concentration leads to an electrochemical gradient, enabling neural activation-induced Ca^{2+} entry into the cellular cytoplasm via various Ca^{2+} channels. Mitochondria actively uptake the cytoplasmic Ca^{2+} through the mitochondrial Ca^{2+} uniporter channel (mCU) and cycle it back into the cytoplasm to integrate various cellular functions, including ‘neural metabolism’ (4–5) and ‘neural signaling’ (6–10). While the current understanding of the integrative role of brain mitochondria are mostly *in vitro* and *ex vivo*, recent systems level studies that build upon cellular-level evidences of mitochondrial Ca^{2+} regulation have demonstrated its ‘metabolic’ and ‘signaling’ impacts in the working brain *in vivo* (1–2, 7, 11–12). These independent studies in the neocortex (2, 11), cerebellum (12) and olfactory epithelium (7), suggest that mitochondrial Ca^{2+} uptake capacity actively influences neuronal electrical activity, oxidative metabolism and neurovascular coupling. Cytoplasmic Ca^{2+} concentration in the brain tissue (13) and the manner in which mitochondria interact with cytoplasmic Ca^{2+} impact neural activity (8) and its related hemodynamic variables (11), including the

functional Magnetic Resonance Imaging (fMRI) blood oxygen level dependent (BOLD) signals (1–2).

fMRI using task-based or resting-state approaches are generally used to measure cerebrovascular and to a certain extent metabolic correlates of neural activity. Resting state-fMRI experimental design is relatively simpler and can interrogate both task-responding and non-responding regions in a single experimental acquisition. Since our objective was to measure ‘intrinsic activity’, which is the ongoing neural activity of the brain in the absence of external stimuli, resting state-fMRI measures were the most relevant and applied in the current studies. To examine the physiological impact of mitochondrial Ca^{2+} uptake across brain regions, we measured the dose-dependent effects of mCU channel inhibition or enhancement on the ‘intrinsic activity’ derived from resting state-fMRI BOLD fluctuations. Several resting state fMRI networks spanning different anatomical regions, including the cortex, caudate putamen (Cpu), hippocampus and thalamus were analyzed in the frequency domain (spectral amplitude) and temporal domain (resting state functional connectivity; RSFC). As opposed to free breathing humans where the low frequency spectral amplitude is predominantly vasomotive (14–17), it is likely to be predominantly neural in anesthetized and ventilated rodents (18), due to the absence of respiratory variability (14). [R2(2)] Furthermore, low frequency spectral amplitude of the BOLD fluctuations in the broad range 0.005–0.1 Hz and RSFC while coupled under normal conditions, deviate in physiologically perturbed conditions. Frequency spectral amplitudes in specific low frequency bands ($>0.05\text{ Hz}$) increase with no significant change in the very low frequency range ($<0.03\text{ Hz}$) during non-neural physiological perturbations such as ‘exanguination’ (19). To test for such a possibly in the present studies where pharmacological agents were used to modulate mitochondrial function, the very low frequency spectral amplitude (VLFSA) in the frequency range of 0.005–0.01 Hz and RSFC in the broader range of 0.005–0.1 Hz were examined.

Materials and Methods

Ethics Statement

All experiments were performed in accordance with Yale University (no. 11194) and RUTGERS-New Jersey Medical School (no. 09009) Institutional Animal Care and Use Committee approvals, in agreement with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering during initial surgery and throughout the experiments. Animals were euthanized in a humane manner after the completion of experiments.

Male Sprague-Dawley rats (250–300g; $n=12$) were anesthetized with urethane (1.3 g/kg, i.p) and artificially ventilated with a mixture of 70% N_2O and 30% O_2 . A femoral artery was cannulated (PE-50) for monitoring physiological parameters such as pCO_2 , pO_2 and mean arterial blood pressure. A femoral vein was cannulated to administer D-tubocurarine chloride (initial 0.5 mg/kg; supplemental 0.25 mg/kg/h) and other test substances including Ru360 and kaempferol. Additional doses of urethane (0.13 g/kg, i.v.) were administered if necessary depending on the duration of the experiment. Adequate anesthesia was ensured by monitoring the pain response in blood pressure to an automated electrical (5 mA, 0.3 msec,

10 Hz, 1 sec) tail-pinch every ¼ hour in general not during the resting fMRI measurements. pETCO₂ was maintained between 32–36 mmHg by appropriate ventilation adjustments. The animal's core temperature was monitored and maintained at 37±0.5°C with a rectal probe and homeothermic blankets. Animal preparation and placement in the magnet was accomplished in approximately 30–40 min after anesthesia induction (Figure 1A).

Drug treatments

Ru360 was from EMD Biosciences San Diego CA and kaempferol from Sigma Chemical Company, St Louis, MO. Rats were divided into two groups with the first group (n=6) receiving Ru360 and the second group (n=6) receiving kaempferol. Control fMRI measurement in each animal was made temporally preceding drug treatments at two different doses in a sequential manner (Figure 1A). Physiological saline was used for the preparation of Ru360 working solution and 20% dimethyl sulfoxide (DMSO) in saline was used to prepare kaempferol working solution. DMSO at the concentrations used has been shown to have no significant neuroactive effects (20). Experiments in each animal lasted approximately 3 hours (Figure 1A).

Functional Magnetic Resonance Imaging (fMRI)

fMRI measures were obtained using a modified 11.7T system with Varian (Agilent Technologies, Santa Clara, CA) spectrometer and custom built ¹H surface coil (diameter 1.4 cm). Both resting state and task-fMRI measurements were performed as part of a larger experimental design. Only the resting state-fMRI measurements were considered for the current studies (Figure 1A). Details of fMRI measurements are described elsewhere (21). Briefly, resting state BOLD contrast images were acquired using echo-planar imaging (EPI) with sequential sampling and the following parameters: repetition time (TR) = 1000 ms; echo time (TE) = 13 ms; field of view (FOV) = 2.56×2.56 cm²; image matrix = 64×64, NR=300. Five contiguous coronal slices with 2 mm slice thickness were selected over the region +2mm to –6mm from the Bregma point. Anatomical images were obtained using gradient echo multi slice (GEMS) or fast spin echo multi slice (FSEMS) contrast sequences in 128 × 128 matrix and FOV= 2.56 cm.

Data analysis

fMRI data were analyzed using AFNI (22). After co-registering with the respective anatomical images from each animal, the functional EPI images were corrected for motion within- scan using a rigid-body volume registration in AFNI (3dvolreg) with the 10th functional image as the base. EPI image blocks from different within-scan motion corrected fMRI experimental runs were co-registered with the 10th functional image of the first experimental run to eliminate motion between scans. Temporal standard deviations of the fMRI time series estimated before and after detrending showed differences <0.5%, indicating that scanner drifts were minimal. Hence only the data considered for temporal correlation analysis were detrended to remove quadratic trends. No detrending was carried out for the frequency spectral amplitude analyses in order to retain the very low frequency physiological noise structures. Due to coupling of respiration and apparent motion at the ultra high field (11.7 T) used in this study, motion correction within-scan has been shown as an effective method in removing residual physiological noise arising from respiration (23)

and was adopted here without any further signal regression of any kind. As the animals were artificially ventilated at a rate of ~1 cycle/sec, approximating the sampling TR of 1 sec, inter-subject and temporal variations in respiratory aliasing were greatly minimized. Hence band-pass filtering (0.005–0.1 Hz) of the resting state-fMRI time series was performed to eliminate remaining respiratory and cardiac contributions to the noise during the temporal domain analysis determining RSFC.

Different regions of interest (ROIs) encompassing the cerebral cortex, caudate putamen (Cpu), hippocampus and thalamus were placed on the anatomical images (Figure 1B). These ROIs placed on the anatomical images of every animal were drawn on the co-registered functional images using AFNI. Spectral amplitude was estimated in every voxel across the brain by considering the square root of the Fast Fourier Transform of the temporal BOLD signals. Spectral amplitude in the lowest frequency band of 0.005–0.01 Hz termed very low frequency spectral amplitude (VLFSa) was estimated in each ROI. The VLFSa range in the low frequency BOLD fluctuations reflect neural activity related changes (1) while being relatively insensitive to non-neural physiological perturbations (19), and was chosen as a distinctive test in the current regional analyses.

For the determination of the RSFC networks, seed regions consisting of 9 contiguous voxels (3×3 array) were randomly placed within a chosen axial slice, right or left hemisphere. [R2(5)] Considering typical seed volume ranges used for human resting state connectivity analyses, a proportional 9 voxel seed volume was considered for the scale of the rat brain. Furthermore, the seed volume was within a single slice and could be randomly placed in different slices without the seed volume moving out of any respective anatomical ROI under consideration. Seed locations were always within the appropriate anatomical region in accordance with the rat atlas in stereotaxic co-ordinates (24–25) and the defined ROIs (Figure 1B). Laterality in seed placement, particularly for the cortical ROI, was across the sensory-motor and closely adjoining areas to map the sensorimotor network (26). Random seed placements in the cortical ROI were avoided in farther sub-regions such as the pyriform cortex. RSFC networks were determined after cross correlating the BOLD time series of all voxels within the brain with the mean BOLD time series of the respective seed region after band-pass filtering (0.005–0.1Hz) (24). This process was repeated across 3 trials of every experimental condition per animal by randomly moving the seed within the chosen ROI. Random placement of seeds within ROIs was performed across trials to eliminate systematic errors in BOLD signals for specific voxel/spatial locations for the given RF-coil and animal subject. A threshold of correlation coefficients 0.2, representing signal with high probability (1), [R2(4)] with a minimum cluster size of 6 voxels after nearest neighbor clustering was considered to represent individual RSFC networks. [R2(6)] Since a given RSFC network often encompassed several anatomical regions, our temporal analysis determined not only the different topologies of the RSFC networks but also the RSFC strength determined as the spatial extent within a specific anatomical ROI (determined as the number of voxels with correlation coefficient 0.2 in a specific anatomical ROI). Accounting for inter-animal variability, each animal's pre-drug treatment baseline was used as an active control to compare VLFSa and RSFC changes after treatments.

Post-hoc Tukey's HSD test was used to further explore any significant effects revealed by a one-way ANOVA. A probability threshold of $P < 0.05$ was required for significance.

Results

Spectral amplitude of very low frequency BOLD fluctuations (0.005–0.01 Hz; VLFSA) was estimated within the whole brain and in different ROIs (Figure 1B), encompassing the cerebral cortex, caudate putamen (Cpu), hippocampus and thalamus using the anatomically co-registered functional images in each animal. Whole brain VLFSA significantly decreased in a Ru360 dose-dependent manner with a 45% decrease during the first dose (120 μ g/Kg) and 53% decrease during the second dose (240 μ g/Kg) (Figure 2A). A regional variation in the Ru360 dose-dependent decrease in VLFSA was apparent. A monotonous decrease occurred in the cortex with a 50% decrease during the first dose and 66% decrease during the second dose (Figure 2B). A similar monotonously decreasing trend was observed in the hippocampus with a 43% and 55% decrease respectively (Figure 2D). However, the dose-dependent decrease in VLFSA in the Cpu and thalamus was not only smaller but also showed a saturated trend with 36% and 33% decreases respectively (Figure 2C and 2E).

In rats treated with kaempferol, whole brain VLFSA decreased significantly in a kaempferol dose-dependent manner with a 44% decrease with the first dose (1mg/Kg) and 49% decrease with the second dose (2mg/Kg) respectively (Figure 2F). Regional variation in the dose-dependent decrease in VLFSA occurred during kaempferol treatment too and was similar to that observed with Ru360. A dose-dependent monotonous decrease occurred in the cortex with a 37% decrease during the first dose and 52% decrease during the second dose (Figure 2G). Cpu, hippocampus and thalamus showed a saturated dose-dependent decrease with a 47%, 43% and 41% changes respectively during the first dose of kaempferol itself (Figures 2H and 2J).

Various RSFC networks were obtained using the BOLD time series from the cortical, Cpu, hippocampal and thalamic seeds. BOLD time series from each seed region was cross correlated with every voxel throughout the brain to obtain the RSFC networks. Figures 3A–C, 4A–C, 4E–G and 4I–K show the typical cortical, Cpu, hippocampal and thalamic RSFC networks during the control and Ru360 treated conditions. All RSFC networks showed bilateral symmetry. The cortical network extended bilaterally on either hemispheres covering most cortical regions and to a certain extent the Cpu, hippocampal and thalamic regions (Figure 3A). Subcortical bilateral networks were also observed after cross correlation with the Cpu, hippocampal and thalamic seed regions. These subcortical RSFC networks extended into the cortex while also encompassing their respective Cpu, hippocampal and thalamic anatomical regions (Figure 4A, 4E, 4I). From the average spatial extent of the RSFC networks obtained from all animals within the Ru360 treated group, the cortico-cortical RSFC was relatively stronger than the cortico-subcortical RSFCs (Figures 3D–G).

Since the cortical, Cpu, hippocampal and thalamic RSFC networks extended into their respective anatomical ROIs, the effect of mitochondrial modulation on the RSFC spatial extents (number of voxels with correlation coefficient > 0.20 multiplied by the area of each

voxel) was assessed within the respective anatomical ROIs as an indicator of RSFC strength. Treatment with Ru360 significantly decreased the cortico-cortical RSFC strength in a dose dependent manner (Figure 3D). A similar dose-dependent decrease was observed within the subcortical networks within their respective anatomical ROIs (Figures 4D, 4H and 4L). Over all animals imaged, the Cpu-Cpu and the thalamo-thalamic RSFC strength showed a significant decrease after the first dose Ru360 (Figures 4D and 4L). However, the cortico-cortical and hippocampo-hippocampal RSFC strength did not decrease significantly after the first dose of Ru360 (Figures 3D and 4H). After treatment with the second dose of Ru360, there was a significant decrease in the cortico-cortical and hippocampo-hippocampal RSFC strength (Figure 3D and 4H). While the Ru360 dose-dependent decrease in the cortico-cortical and hippocampo-hippocampal RSFC strength was monotonous, the decrease in the Cpu-Cpu and the thalamo-thalamic RSFC strengths were saturated and seemed to have reached their minimum levels after the first dose itself.

Various RSFC networks were obtained using the BOLD time series from seed regions within the somatosensory cortex, Cpu, hippocampal and thalamic ROIs within the kaempferol-treated rats. Figures 5A–C, 6A–C, 6E–G and 6I–K show typical RSFC networks during the control and kaempferol treated conditions. From the average spatial extent of the RSFC networks obtained from all animals within the kaempferol treated group, the cortico-cortical RSFC was relatively stronger than the cortico-subcortical RSFCs (Figures 5D–G). The cortical network extended bilaterally on either hemispheres covering most cortical regions and to a certain extent the Cpu, hippocampal and thalamic regions (Figure 5A). Subcortical bilateral networks were also observed after correlation with the Cpu, hippocampal and thalamic seed regions. The subcortical RSFC networks extended robustly into the cortex while also encompassing the Cpu, hippocampal and thalamic anatomical ROIs (Figure 6A, 6E, 6I). The topology of all the resting state networks were reproducible across animals (compare Figures 3 and 5; 4 and 6). Relatively sparse cortico-subcortical RSFC as observed with the Ru360 treatment group (Figure 3E–G) was reproducible with the kaempferol treatment group (Figure 5E–G) as determined from the average spatial extent of the RSFC networks from all animals. Over all 12 animals included in the experiments, there was 5–17% variability in RSFC volumes for the various networks (Table-1).

As the cortical, Cpu, hippocampal and thalamic RSFC networks extended to their respective anatomical regions, the effect of kaempferol-induced mitochondrial modulation on the RSFC strength was assessed using the RSFC spatial extents within the respective anatomical ROIs. Treatment with kaempferol significantly decreased the RSFC strength of the cortical network within the cortical ROI (cortico-cortical) in a dose dependent manner (Figure 5D). Similar dose-dependent decreases in RSFC strength was observed in other RSFC networks within their respective anatomical ROIs (Figures 6A–C, 6E–G and 6I–K). Over all animals imaged, the Cpu-Cpu, hippocampo-hippocampal and the thalamo-thalamic RSFC strength showed a significant decrease during the first dose of kaempferol (Figures 6D 6H and 6L). After treatment with the second dose of kaempferol, no further decreases occurred in the cpu-cpu, hippocampo-hippocampal and thalamo-thalamic RSFC strengths (Figure 6D 6H and 6L). While the kaempferol dose-dependent decreases in the cortico-cortical RSFC strength was monotonous, reductions in the Cpu-Cpu, hippocampo-hippocampal and the

thalamo-thalamic RSFC strengths were saturated and seemed to have reached their minimum levels after the first dose itself.

Although 5–17% variability in RSFC networks was apparent between animals as observed from Table 1, RSFC networks determined from different animal subjects showed bilateral symmetry and were reproducible (Figures 3 and 5). Cortical network extended bilaterally on either hemispheres covering most cortical regions and to a certain extent the Cpu, hippocampal and thalamic regions. Subcortical bilateral networks were also observed after correlation with the Cpu, hippocampal and thalamic seed regions. Spatial extent of RSFC networks in each animal and group variability for each drug treatment are summarized in Table 1.

Discussion

Signaling in the brain is enabled by Ca^{2+} waves propagating along networks of interacting neuronal and glial cells. Ca^{2+} waves are integrated by various cytoplasmic Ca^{2+} binding proteins and subcellular organelles such as mitochondria (27). Mitochondria primarily take up Ca^{2+} through a uniporter channel (mCU), buffering some Ca^{2+} and efflux the excess free Ca^{2+} . Mitochondrial functions in the brain may vary depending upon their mCU channel composition and their numbers, which is proportional to the metabolic demand in a given region (28). However, demonstration of the functional diversity of mitochondria in the intact working brain has been difficult due to the invasive nature of experimental approaches used to probe Ca^{2+} uptake capacity. The current systems level study, mapping the resting state spontaneous fMRI-BOLD fluctuations in the brain, and using those activities to derive resting state fMRI networks after inhibiting or enhancing mCU activity is related to the Ca^{2+} uptake capacity of mitochondria (1). The results obtained from different brain regions collectively support the universal role of mitochondrial Ca^{2+} uptake process in determining the resting state intrinsic activity, albeit with regional differences.

Ca^{2+} dependent functional organization of the brain at rest

In the resting state brain, spontaneous action potentials in the presynaptic neuron depolarize the plasma membrane releasing neurotransmitters into the synapse in a cytoplasmic Ca^{2+} dependent manner (29). The released neurotransmitters bind postsynaptic receptors transferring Ca^{2+} from extracellular space into the cytoplasm (30–34) or release Ca^{2+} from ER stores into the cytoplasm (35–36), enabling the propagation of spontaneous neuronal electrical activity. The influx of Ca^{2+} into mitochondria during neural activity not only stimulate dehydrogenase enzymes in a Ca^{2+} dependent manner to accelerate the production of ATP (5), but also influence neural signaling by modulating the amplitude and durations of cytoplasmic Ca^{2+} transients (8, 37). Neural energy demand increases during both spontaneous and evoked activities and is mainly oxidative within the brain (38–39). Mitochondria not only respond to context-dependent fluctuations in oxidative energy demand by accelerating mitochondrial dehydrogenase activity in a Ca^{2+} dependent manner (5), but also maintain the baseline metabolic states of the brain in a dynamic coupling with neuronal electrical activity (40–43).

Regional mitochondrial response during mCU inhibition

We previously showed that mCU inhibition reduces RSFC through diminished neuronal activity and oxidative metabolism (1–2). During mitochondrial Ca^{2+} uptake inhibition in the current studies, cortical and hippocampal regions showed larger dose-dependent decreases in VLFSA in addition to higher reserves as opposed to the Cpu and thalamic regions which showed smaller dose-dependent decreases in VLFSA and saturation after the first dose (Figure 2). Differences in the extent of decrease may arise from a relatively higher concentration of mitochondria in the cortical followed by the hippocampal regions compared to the mitochondrial concentrations in the Cpu and thalamic regions. Such a conclusion is in agreement with earlier structural evidence indicating that mitochondrial distribution may not be uniform, but vary in densities throughout the central nervous system where regions with relatively larger vascular density contain relatively more mitochondrial organelles (3). Differences in the reserve were observed with monotonous dose-dependent decreases in VLFSA (Figure 2B and 2D) and RSFC (Figure 3D and 4H) respectively in the cortex and hippocampus and saturated decreases in VLFSA (Figure 2C and 2E) and RSFC (Figure 4D and 4L) respectively in the Cpu and thalamus. Such distinct responses may also arise from different mCU channel Ca^{2+} kinetics in the cortical and hippocampal when compared to Cpu and thalamic mitochondria. As observed from our earlier electrophysiological and fMRI results, spontaneous neocortical spiking activity did not change significantly during the first dose of Ru360 but significantly decreased only during the second dose (1). Hence it is likely that the cortical and hippocampal mitochondrial populations are not only relatively higher but also functionally different with possibly different mCU channel Ca^{2+} kinetics when compared to Cpu and thalamic mitochondria. There is supporting evidence suggesting differences in Ca^{2+} channel kinetics, which may presumably differ in different brain regions. At the molecular level, the rate of Ca^{2+} influx via the mCU is influenced by a certain regulating domain, MICU1, outside the transmembrane region of the mCU (44). Recent studies using Förster resonance energy transfer (FRET) have characterized a novel protein MCUB, which happens to be a subunit of the oligomer mCU (45). mCU and MCUB activities are regulated by additional subunits MICU1, MICU2, MCUR1 and EMRE. Hence the number of mCU channel and their regulatory subunit configurations that efficiently conduct Ca^{2+} and other isoforms that do not conduct Ca^{2+} during cellular activity may become important factors determining the diversity of mitochondrial function in different brain regions (46). Based on the current systems level results and the known variability of mitochondrial density in the rat brain, differing intrinsic Ca^{2+} uptake kinetics due to the presence of different configurations of the mCU and its regulating domains may play a role in the Ca^{2+} dependent mitochondrial functional impact in different brain regions.

Regional mitochondrial response during mCU enhancement

mCU enhancement is known to increase baseline electrical activity and oxidative metabolism, but decrease RSFC due to loss of synchronization in neuronal activity (1–2). Hence intrinsic activity of resting state fMRI networks decrease due to asynchrony within the neuronal circuits combined with higher baseline oxidative metabolic rates. Kaempferol at the first dose level used in the current studies is known to significantly increase the spontaneous neocortical spiking activity along with further increases at the second dose level (1). While the degree of increase in the baseline oxidative metabolism and neuronal

asynchrony maybe a direct consequence of mitochondrial Ca^{2+} uptake enhancement, the progressive dose-dependent VLFSa decrease was observed only within the cortex and not within the Cpu, hippocampal and thalamic regions, all of which saturated after the first dose (Figure 2H, 2I and 2J). A larger decrease in VLFSa (approximately 50%) occurred in the subcortical regions during mitochondrial Ca^{2+} uptake enhancement with kaempferol as opposed to the relatively smaller decrease in VLFSa (approximately 25%) in the subcortical regions during mitochondrial Ca^{2+} uptake inhibition with Ru360 (Figure 2). Subtle differences were also observed where the progressive decrease in the hippocampal intrinsic activity during mitochondrial Ca^{2+} uptake inhibition was absent during mitochondrial Ca^{2+} uptake enhancement, which showed a saturated decrease (Figure 2D and 2I). As the reduction in RSFC during kaempferol treatment was mainly due to neuronal asynchrony along with raised levels of electrical and oxidative metabolic activity, the saturated hippocampal response during kaempferol treatment indicated that the maximum level of neuronal asynchrony may have quickly reached in the hippocampus during the first dose of kaempferol itself.

The current resting state studies used the measures from the same animal subjects before and after treatment with the mCU inhibitor Ru360 or enhancer kaempferol as reported in our earlier task-related fMRI studies (2). Physiological parameters such as the mean arterial blood pressure and arterial oxygenation did not change significantly with drug treatments on the reported animal subject groups anesthetized with urethane and have been published before (2). The time line for completion of experiments in each animal was approximately 3 hours and such that task-related fMRI experiments followed the resting state for each drug infusion dose. Hence the diminished intrinsic activity with kaempferol dose in the current studies which considered only the resting state fMRI data was not due to a deterioration of baseline physiology since task-related fMRI responses acquired immediately after the resting state acquisition increased with kaempferol dose (2).

Regional functional similarities during perturbations of mitochondrial function

Mitochondrial Ca^{2+} uptake inhibition or enhancement may affect the intrinsic activity of the brain by different mechanisms, but the deviation in either direction led a decreased (sub-optimal) intrinsic activity. [R2(2)] and [R2(7)] VLFSa and RSFC while coupled under normal conditions, deviate in physiologically perturbed conditions. Highlighting the non-neural contributions to the low frequency fMRI-BOLD fluctuations we have shown earlier that the frequency spectral amplitudes in specific low frequency bands ($>0.05\text{Hz}$) increase with no significant change in the very low frequency range ($<0.03\text{ Hz}$) during non-neural physiological perturbations such as 'exanguination' (19). A correlation in the dose-dependent changes in VLFSa and RSFC in a given region of interest support a neural nature of the effects of inhibition or enhancement of mCU activity *in vivo* with no significant non-neural contributions. Loss of optimal intrinsic activity due to perturbation of mitochondrial Ca^{2+} uptake (whether inhibition or enhancement) and their dose-dependent regional correlation allude to a functional similarity in regional populations of mitochondria. However, when compared across regions such as the cortical and sub-cortical, functional differences emerge. As mentioned earlier, functional differences may arise from multiple factors such as mitochondrial density in each region and/or differences in the intrinsic Ca^{2+}

kinetics of the mCU protein configurations. Further work *in vivo* correlating brain responses with their respective mCU-related Ca^{2+} kinetics may better explain the nature of regional functional differences.

Potential caveats of the current systems approach need to be considered. The drugs were administered intravenously, and hence a regional variation of drug delivery to the brain tissue cannot be ruled out because of the difference in CBF and permeability of the blood brain barrier across the brain regions. Furthermore, actual mCU activity changes in the presence of the drugs are not available *in vivo* as it is currently possible only in isolated *in vitro* mitochondrial preparations or in cell culture systems.

In conclusion the distinct impact of mitochondrial Ca^{2+} homeostasis on regional RSFC indicates that the degree of mitochondrial Ca^{2+} uptake activity in the different regions set the tone for the intrinsic brain activity spanning those regions. Differences in regional mitochondrial density and their mCU protein-mediated Ca^{2+} kinetics may impact the degree of the intrinsic activity in the different resting state fMRI-derived networks. With mitochondrial dysfunction implicated in various neuropathologies (47–49),(50–51) and patients increasingly studied using resting state-fMRI, the present approach and the results demonstrate the feasibility of distinguishing regional mitochondrial functional states in neurological disease and traumatic brain injury. However inclusion of resting state-fMRI network studies with other methods to measure the resting oxidative metabolism and blood flow would enable better patient-by-patient assessment of mitochondrial dysfunction in a variety of brain disorders.

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Abbreviations

mCU	mitochondrial Ca^{2+} uniporter
BOLD	blood oxygen level dependent
DMSO	dimethyl sulfoxide
GEMS	gradient echo multi slice
FSEMS	fast spin echo multi slice
VLFS	very low frequency spectral amplitude
Cpu	caudate putamen
RSFC	resting state functional connectivity

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Figure 1.

A. Experimental time line indicating the chronology of the fMRI measurements and drug injections. **B.** Different regions of interest (ROIs) encompassing the cerebral cortex, caudate putamen, hippocampus and thalamus placed on the anatomical images in a typical animal. These ROIs placed on the anatomical images of every animal were drawn on the co-registered functional images using AFNI.

Figure 2. Spectral amplitude of the very low frequency BOLD fluctuations (0.005–0.01 Hz; VLFSA), estimated from the whole brain and in the different ROIs encompassing the cerebral cortex, caudate putamen (Cpu), hippocampus and thalamus. **A–E.** Before and after Ru360 treatments and **F–J.** Before and after kaempferol treatments. Significant differences were tested by a one-way ANOVA followed by a Post-hoc Tukey's HSD test. Control=Blue, Dose-1=Red, Dose-2=Green.

Figure 3.

Typical resting state functional connectivity (RSFC) maps obtained after cross correlating all voxels with the somatosensory cortex seed time series. **A.** control **B.** after Ru360 dose-1 and **C.** after Ru360 dose-2. The cortical network extended bilaterally on either hemispheres covering most cortical regions and to a certain extent the Cpu, hippocampal and thalamic regions. Over all animals within the group, **D.** cortico-cortical RSFC was the most robust, followed by **E.** cortico-Cpu, **F.** cortico-hippocampal and **G.** cortico-thalamic RSFC. A significant decrease in the cortico-cortical RSFC was observed after treatment with Ru360 at the two different doses. Typical data from animal no.6 of the Ru360 group (Table-1) and group data represent mean $\pm$ SD from 6 animals. Significant differences were tested by a one-way ANOVA followed by a Post-hoc Tukey's HSD test. Color scale bar indicates correlation coefficient values.

Figure 4.

Typical resting state functional connectivity (RSFC) maps obtained after cross correlating all voxels with the **A–C.** Cpu seed time series, **E–G.** Hippocampal seed time series and **I–K.** Thalamic seed time series before and after Ru360 treatment. Over all animals within the group, the spatial extent of each network falling within the respective anatomical ROIs were determined. **D.** Cpu-Cpu, **H.** Hippocampo-hippocampal and **L.** Thalamo-thalamic RSFC. Ru360 significantly decreased the sub-cortical RSFC in a dose dependent manner with a saturated effect in the Cpu-Cpu and the thalamo-thalamic RSFC whereas with a monotonous effect in the hippocampo-hippocampal RSFC. Typical data from animal no.6 of the Ru360 group (Table-1) and group data represent mean $\pm$ SD from 6 animals. Significant differences were tested by a one-way ANOVA followed by a Post-hoc Tukey's HSD test. Color scale bar indicates correlation coefficient values.

Figure 5.

Typical resting state functional connectivity (RSFC) maps obtained after cross correlating all voxels with the somatosensory cortex seed time series. **A.** control **B.** after kaempferol dose-1 and **C.** after kaempferol dose-2. The cortical network extended bilaterally on either hemispheres covering most cortical regions and to a certain extent the Cpu, hippocampal and thalamic regions. Over all animals within the group, **D.** cortico-cortical RSFC was the most robust, followed by **E.** cortico-Cpu, **F.** cortico-hippocampal and **G.** cortico-thalamic RSFC. A significant decrease in the cortico-cortical, cortico-Cpu, cortico-hippocampal and cortico-thalamic RSFC was observed after treatment with kaempferol at the two different doses. Typical data from animal no.6 of the kaempferol group (Table-1) and group data represent mean±SD from 6 animals. Significant differences were tested by a one-way ANOVA followed by a Post-hoc Tukey's HSD test. Color scale bar indicates correlation coefficient values.

Figure 6.

Typical resting state functional connectivity (RSFC) maps obtained after cross correlating all voxels with the **A–C.** Cpu seed time series, **E–G.** Hippocampal seed time series and **I–K.** Thalamic seed time series before and after kaempferol treatment. Over all animals within the group, the spatial extent of each network falling within the respective anatomical ROIs was determined. **D.** Cpu-Cpu, **H.** Hippocampo-hippocampal and **L.** Thalamo-thalamic RSFC. Kaempferol significantly decreased the sub-cortical RSFC in a dose dependent manner with a saturated effect in the Cpu-Cpu and the thalamo-thalamic RSFC whereas with a monotonous effect in the hippocampo-hippocampal RSFC. Typical data from animal no.6 of the Kaempferol group (Table-1) and group data represent mean±SD from 6 animals. Significant differences were tested by a one-way ANOVA followed by a Post-hoc Tukey's HSD test. Color scale bar indicates correlation coefficient values.

Table 1

Variability in the various RSFC networks in all rats treated with Ru360 and kaempferol.

Animal no.	Ru360				Animal no.	Kaempferol			
	RSFC volume (mm ³)					RSFC volume (mm ³)			
	cor	Cpu	hippo	thala		cor	Cpu	hippo	thala
1	166	58	34	46	1	153	74	48	77
2	162	59	37	61	2	147	75	39	59
3	169	71	45	54	3	140	77	45	77
4	155	91	43	66	4	149	85	44	68
5	193	69	43	54	5	150	73	39	76
6	149	66	31	71	6	162	78	44	87
Mean	165	69	39	59	Mean	150	77	43	74
SD	15	12	6	9	SD	7	4	4	9
Variation (%)	10	17	15	15	Variation (%)	5	6	8	13