

Asymmetric Opening of Mitochondrial Permeability Transition Pore in Mouse Brain Hemispheres: A Link to the Mitochondrial Calcium Uniporter Complex

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The prolonged entry of large amounts of calcium into the mitochondria through the mitochondrial calcium uniporter complex (MCUC) may cause the permeability transition pore (mPTP) to open, which contributes to the pathogenesis of several diseases. Tissue-specific differences in mPTP opening due to variable expression of MCUC components may contribute to disease outcomes. We designed this study to determine differential mPTP opening in mitochondria isolated from different regions of mouse brain and kidney and to compare it with the expression of MCUC components. mPTP opening was measured using mitochondria isolated from the left/right brain hemispheres (LH/RH, respectively) and from kidney cortex/medulla, while the expression level of MCUC components was assessed from total cellular RNA. Interestingly, LH mitochondria showed less calcium-induced mPTP opening as compared to RH mitochondria at two different calcium concentrations. Conversely, mPTP opening was similar in the renal cortex and renal medulla mitochondria. However, the kidney mitochondria demonstrated bigger and faster mPTP opening as compared to the brain mitochondria. Furthermore, asymmetric mPTP opening in the LH and RH mitochondria was not associated with the expression of MCUC components. In brief, this study demonstrates thus far unreported asymmetric mPTP opening in mouse brain hemispheres that is not associated with the mRNA levels of MCUC components.

Keywords: Mitochondrial Permeability Transition Pore (mPTP). Mitochondrial Ca²⁺ Uniporter Complex (MCUC). Brain Hemispheres. Kidney Cortex. Medulla.

INTRODUCTION

The transport of calcium ions (Ca²⁺) into the mitochondrial matrix via the mitochondrial Ca²⁺ uniporter complex (MCUC) is pivotal in the regulation of cellular bioenergetics and various signaling pathways (Giorgi, Marchi, Pinton, 2018). However, excessive accumulation of Ca²⁺ in the mitochondria has been associated with several diseases (Mammucari, Gherardi, Rizzuto, 2017). The abnormal increase of matrix Ca²⁺ can trigger the opening of a mega-channel in the inner mitochondrial membrane (IMM), referred to as the mitochondrial permeability transition pore (mPTP) (Giorgio *et al.*, 2018). This may cause bioenergetic collapse and swelling of the

mitochondria, ultimately leading to cell death by apoptosis and/or necrosis (Giorgi *et al.*, 2012). mPTP opening has been linked with the pathogenesis of many diseases, including stroke and acute kidney injury, and it remains an important target for therapeutic intervention (Eirin, Lerman, Lerman, 2017; Giorgi, Marchi, Pinton, 2018; Halestrap, Richardson, 2015).

mPTP opening requires a pathological influx of Ca²⁺ across the IMM, which is mainly driven by an extremely negative mitochondrial membrane potential. The molecular identity of this Ca²⁺ transport process was recently revealed with the discovery of a large protein complex known as the mitochondrial calcium uniporter complex (MCUC). The MCU is the main Ca²⁺ channeling subunit of this complex and is regulated by mitochondrial calcium uptake 1 and 2 (MICU1/2) in the intermembrane space (Oxenoid *et al.*, 2016). It has been proposed that MICU1/2 act as gatekeepers by participating

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in MCU closing/opening based on changes in cytosolic Ca^{2+} concentrations (Csordas *et al.*, 2013). Essential MCU regulator (EMRE) is another MCUC component that plays a major role in the formation and activity of the channel (Sancak *et al.*, 2013; Tsai *et al.*, 2016). In addition, a dominant-negative version of the MCU, i.e. MCUB, negatively regulates Ca^{2+} transport (Lambert *et al.*, 2019). Given the pathophysiological importance of mitochondrial Ca^{2+} in mPTP opening, the MCUC remains under intense investigation for therapeutic targeting in different diseases particularly those related to the brain, kidneys, and heart (Woods, Wilson, 2019).

The brain hemispheres bear anatomical and molecular asymmetries that are associated with different neurological functions, especially those related to cognition. Although initially reported in human brains, hemispheric asymmetry is conserved in other vertebrates as well (Duboc *et al.*, 2015; Ocklenburg, Gunturkun, 2012). The pathophysiological relevance of brain asymmetry is substantiated by the fact that various human brain disorders such as autism, dyslexia, and schizophrenia have been linked to abnormal patterns of hemispheric asymmetry at both structural and functional level (Giraldo-Chica, Schneider, 2018; Sun *et al.*, 2017; Wei *et al.*, 2018). Likewise, studies in rodent models of Alzheimer's disease have highlighted important roles of hemispheric asymmetry in the context of protein expression and response to pharmacological treatments (Manousopoulou *et al.*, 2016; Tsai *et al.*, 2009). At cellular level, metabolic asymmetry in the hypothalamus has been linked to reproductive and satiety states in male rats (Kiss *et al.*, 2020). Similarly, sidedness of glucose metabolism has also been reported in the cerebral hemispheres of human infants (Park *et al.*, 2017). Correspondingly, functional differences related to cellular bioenergetics, protein expression, and mitochondrial activity have also been reported in the cortex and medulla of the kidney (Arthur *et al.*, 2002; Schiffer, Gustafsson, Palm, 2018). However, whether there is disparate mPTP opening in mitochondria obtained from different regions of the brain and the kidney remains unknown. Likewise, the correlation of the expression level of MCUC components with the sensitivity of tissues towards mPTP opening remains an open question.

MATERIAL AND METHODS

Animals, chemicals, and reagents

BALB/c Swiss albino mice (7–12 weeks old, 30–40 g) purchased from the National Institute of Health (Islamabad, Pakistan), were used for the experiments. The animals were maintained in the animal facility of Quaid-i-Azam University according to guidelines of the National Institute of Health (USA) and the Quaid-i-Azam University bioethics committee. All animal handling and experimentation protocols were approved by the Institutional Review Board. All chemicals and reagents used in this study were purchased from SERVA Electrophoresis (Germany), Carl Roth (Germany), and Thermo Fisher Scientific (USA) unless otherwise indicated.

Mouse surgery and organ isolation

Before dissection, the mice were physically examined, and only healthy mice were selected for organ isolation. The mice were anesthetized with chloroform before the surgery was performed. The brain and kidneys were removed and maintained in ice-cold wash buffer (phosphate-buffered saline [PBS]). Under a stereomicroscope, entire brain hemispheres were dissected from each other after the cerebellum and brain stem had been removed. Similarly, the cortical and medullary regions of the kidney were separated. All tissue parts were kept in ice-cold PBS until further processing.

Isolation, quantification, and viability testing of mitochondria

The mitochondria were isolated by conventional differential centrifugation in homogenization buffer (HB) containing 250 mM sucrose, 10 mM KCl, 0.1 mM EDTA, 250 mM mannitol, 25 mM HEPES, and 100 mM PMSF (phenylmethylsulfonyl fluoride). Using a Dounce homogenizer, ~50 mg brain and kidney tissues were homogenized by applying four and six passes, respectively. The tissue homogenates were centrifuged at 500 $\times g$ at 4°C for 10 min. The supernatant was collected and centrifuged again at 10,300 $\times g$ at 4°C for 10 min to

obtain crude mitochondria. The mitochondrial pellets were resuspended in HB and centrifuged at $10,300 \times g$ at 4°C for 10 min to purify the mitochondria. After final pelleting of the mitochondria, the pellets were dissolved in HB. Mitochondrial protein was quantified using the spectrophotometric Bradford assay and viability was determined using the MTT assay.

Mitochondrial swelling assay

The kinetics of mPTP opening in the mitochondria isolated from the mouse kidney and brain were measured in a time-lapse spectrophotometric assay using a Multiskan GO™ microplate reader (Thermo Fisher Scientific). Mitochondrial suspensions diluted in swelling assay buffer (120 mM KCl, 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], 20 μM EDTA, 5 mM glutamate, 5 mM malate) were added to each well in a microplate. The Ca^{2+} -mediated opening of mPTP was observed by measuring the decrease in light scattering (A540nm) of the mitochondrial suspension. The addition of exogenous Ca^{2+} (500 μM or 10 mM) induced immediate swelling of the mitochondria, which was measured for 10–45 min depending on the tissue of origin or protocol. The data were acquired using SkanIt™ software (Thermo Fisher Scientific) and analyzed using

MS Excel (Microsoft, USA). Each curve was normalized with the highest absorbance measured after the addition of Ca^{2+} . The amplitude and rate of drop in absorbance (slope) were calculated from the normalized curves to compare the data.

RNA isolation, complementary DNA (cDNA) synthesis, and real-time PCR (RT-PCR)

RNA was extracted from the respective brain and kidney parts using standard TRIzol reagent using the company's protocol (Thermo Fisher Scientific). Total RNA was isolated from approximately 30–40 mg tissue. The isolated RNA (1 μg) was reverse-transcribed to cDNA using a high-capacity cDNA synthesis kit (Thermo Fisher Scientific). The relative abundance of mRNA levels was measured by EvaGreen®-based RT-PCR chemistry (Solis BioDyne, Estonia) using a StepOne™ Real-Time PCR System (Thermo Fisher Scientific). Table I lists the mouse-specific primers used for the RT-PCR experiments. Each cDNA sample was run in triplicate, and beta actin (*Actb*) was used as the housekeeping gene. The data from four independent experiments were analyzed by a modified comparative threshold cycle ($\Delta\Delta\text{Ct}$) method (Pfaffl, 2001) to calculate the mRNA fold change, using the right hemisphere as the control.

TABLE I - Mouse specific primers for Real Time quantitative PCR

S.No.	Name	Primer Sequence	Tm (°C)	Amplicon size (bp)
1	MCU-F	TCGACCTAGAGAAATACAATCAGC	62.0	137
2	MCU-R	CACGGTCATCTCGGATCATTC	62.0	
3	MICU1-F	TTGACTTGAATGGAGACGGAG	61.8	138
4	MICU1-R	AACATAAGCCAGACTTGAGGG	62.0	
5	MICU2-F	GATCTTTGACCTGGACGGG	62.0	150
6	MICU2-R	ACTCCCTTGATGCTTTCCTTC	62.0	
7	EMRE-F	GTGATCCCCTTCTCTATGTCG	62.0	137
8	EMRE-R	TCAGTGCTTCTCCTGTGC	62.0	
9	MCUb-F	GGGATATCATGGAGCCAGTTAC	62.0	147
10	MCUb-R	GCTGCGATTCTTGTGGAAG	62.0	

TABLE I - Mouse specific primers for Real Time quantitative PCR

S.No.	Name	Primer Sequence	T _m (°C)	Amplicon size (bp)
11	Actin-F	GCACCACACCTTCTACAATGA	55.8	207
12	Actin-R	GAGTCCATCACAATGCCTGT	56	

Statistical analysis

The data are reported as the means \pm SEM. The differences between groups were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA) using the Bonferroni post-hoc test. A *p*-value $<$ 0.05 was considered statistically significant.

RESULTS

MTT assay confirms the presence of healthy mitochondria in brain and kidney preparations

The accurate measurement of mPTP opening requires healthy mitochondria, which are bioenergetically active. Therefore, to assess the suitability of our protocol for isolating viable

organelles, we measured mitochondrial dehydrogenase activity using the MTT assay. Our data demonstrated that incubating the mitochondria with MTT solution reduced the water-soluble yellow tetrazolium dye to purple formazan crystals, as indicated by the increased absorbance at 570 nm (Figure 1A&B). This finding corroborates the presence of functional dehydrogenase activity in the mitochondria isolated from the mouse brain and kidney. However, we did not observe any significant differences in the purple-color formation by mitochondria from either the left or right hemispheres (LH and RH, respectively) (Figure 1A). Similarly, there was no considerable variation in the dehydrogenase activity of mitochondria from the kidney cortex and medulla (Figure 1B). These data, therefore, confirm the reliability of our assay for isolating healthy mitochondria from two different organs in mice.

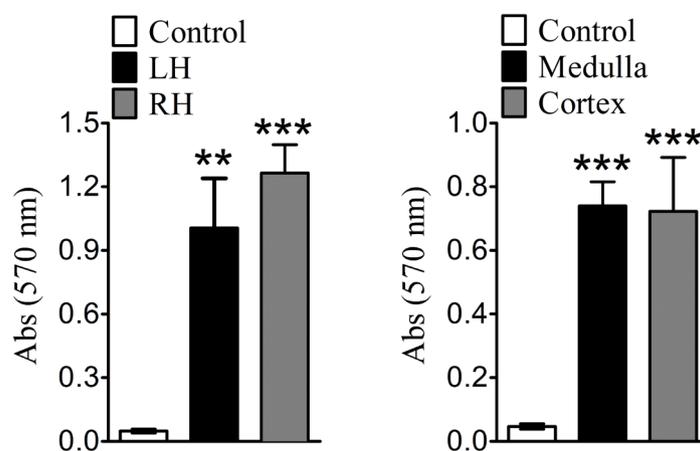


FIGURE 1 - Confirmation of mitochondrial viability with MTT assay. The effectiveness of the mitochondria isolation assay was tested using equal quantities of freshly isolated mitochondria from mouse brain LH and RH (A) and mouse kidney cortex/medulla (B). Mitochondrial HB was used as the control. The intensity of the purple color formed due to the formation of formazan crystals by the action of mitochondrial dehydrogenases was measured at 570 nm. Data represent the mean \pm SEM of 3–4 independent mitochondrial preparations. Statistical significance was calculated using one-way ANOVA with Bonferroni post-hoc testing; ****p* $<$ 0.0001, ***p* $<$ 0.001.

Mitochondria derived from mouse brain hemispheres have differential mPTP opening

mPTP opening due to diverse pathological insults may lead to an influx of water and the induction of mitochondrial swelling. In our experiments on isolated mitochondria, we monitored mitochondrial swelling by measuring a decrease in absorbance at 540 nm. This was achieved by the addition of external Ca^{2+} (500 μM), which triggered a slow but consistent decrease in absorbance, indicating mitochondrial swelling due to mPTP opening

(Figure 2A&B). Interestingly, the Ca^{2+} -dependent drop in absorbance, shown as amplitude in LH mitochondria, was significantly smaller as compared to RH mitochondria (Figure 2C). Similarly, the rate of decrease in absorbance, indicating the speed of mPTP opening, was also slower in the LH mitochondria (Figure 2D). The data validate our hypothesis that there is asymmetric mPTP opening in mitochondria from mouse brain hemispheres, with RH showing a rapid Ca^{2+} -induced swelling response in comparison to LH.

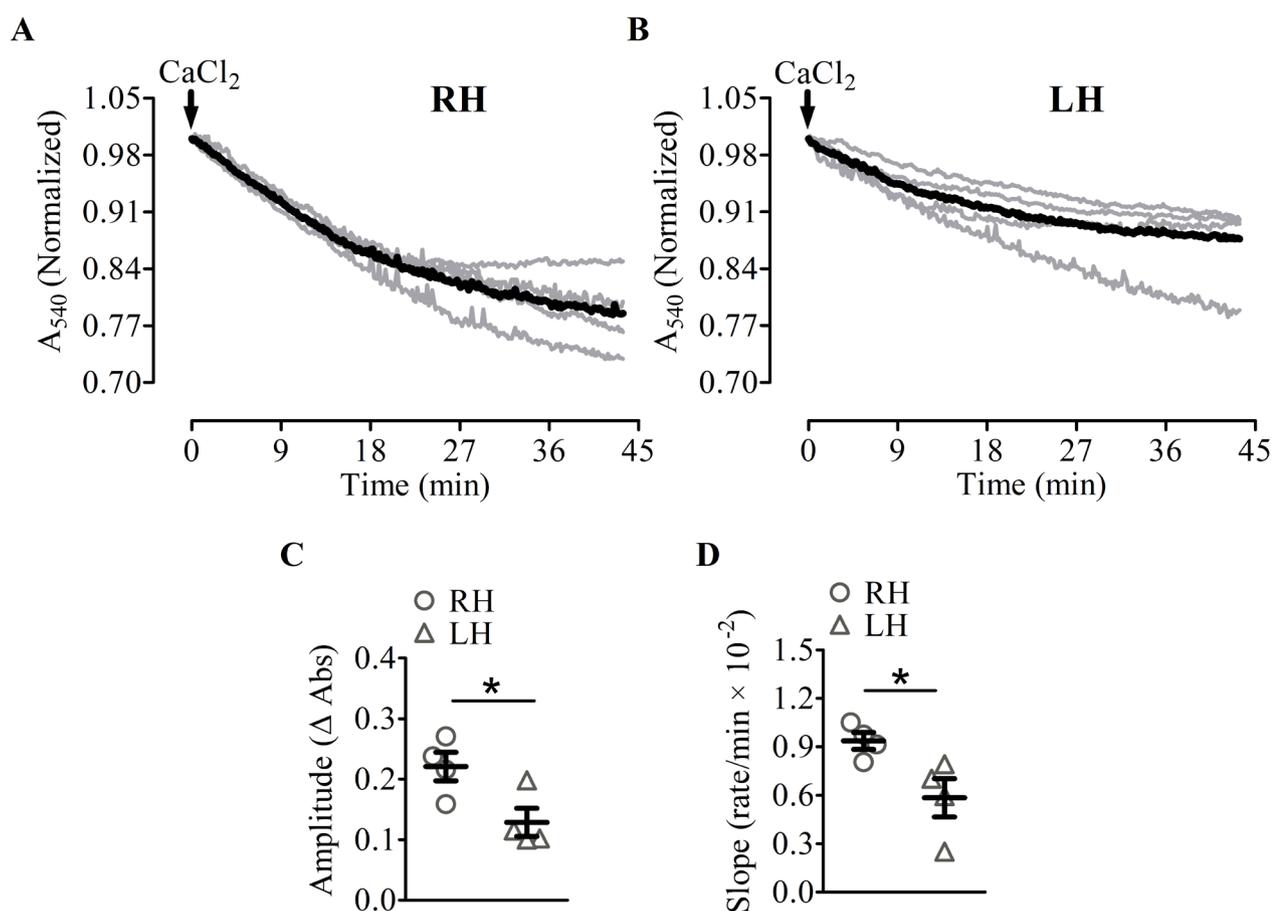


FIGURE 2 - Differential mPTP opening was seen between mouse brain hemispheres upon exogenous addition of 500 μM Ca^{2+} . (A, B) The swelling curve for the RH and LH; grey lines indicate the average of multiple traces from each mouse; black bolded lines show the mean from all mice. (C) The mean amplitude (ΔAbs : maximal difference) was calculated from grey curves, showing a significant difference in mPTP opening between LH and RH. The scatter plot represents the mean \pm SEM, $n = 4$ mice, $*p = 0.03$. (D) The plot demonstrates the slope of mPTP opening as calculated by rate/minute. The scatter plot represents the mean \pm SEM; $n = 4$ mice, $*p = 0.03$.

To confirm the variable mPTP opening in the mouse brain hemispheres, we also determined the mitochondrial swelling at a much higher Ca^{2+} concentration (10 mM) over a shorter period (10 min vs 45 min). Consistent with our findings at low Ca^{2+} , we observed diminished mitochondrial swelling in the LH as compared to the RH upon stimulation with 10 mM Ca^{2+} , indicating reduced

mPTP opening (Figure 3A&B). The maximum drop in mitochondrial swelling, as shown by amplitude, was significantly reduced (Figure 3C), along with a downwards tendency in the rate of swelling (Figure 3D) in the LH as compared to the RH. Collectively, these results confirm the assumption that mitochondria from mouse brain LH and RH demonstrate differential mPTP opening.

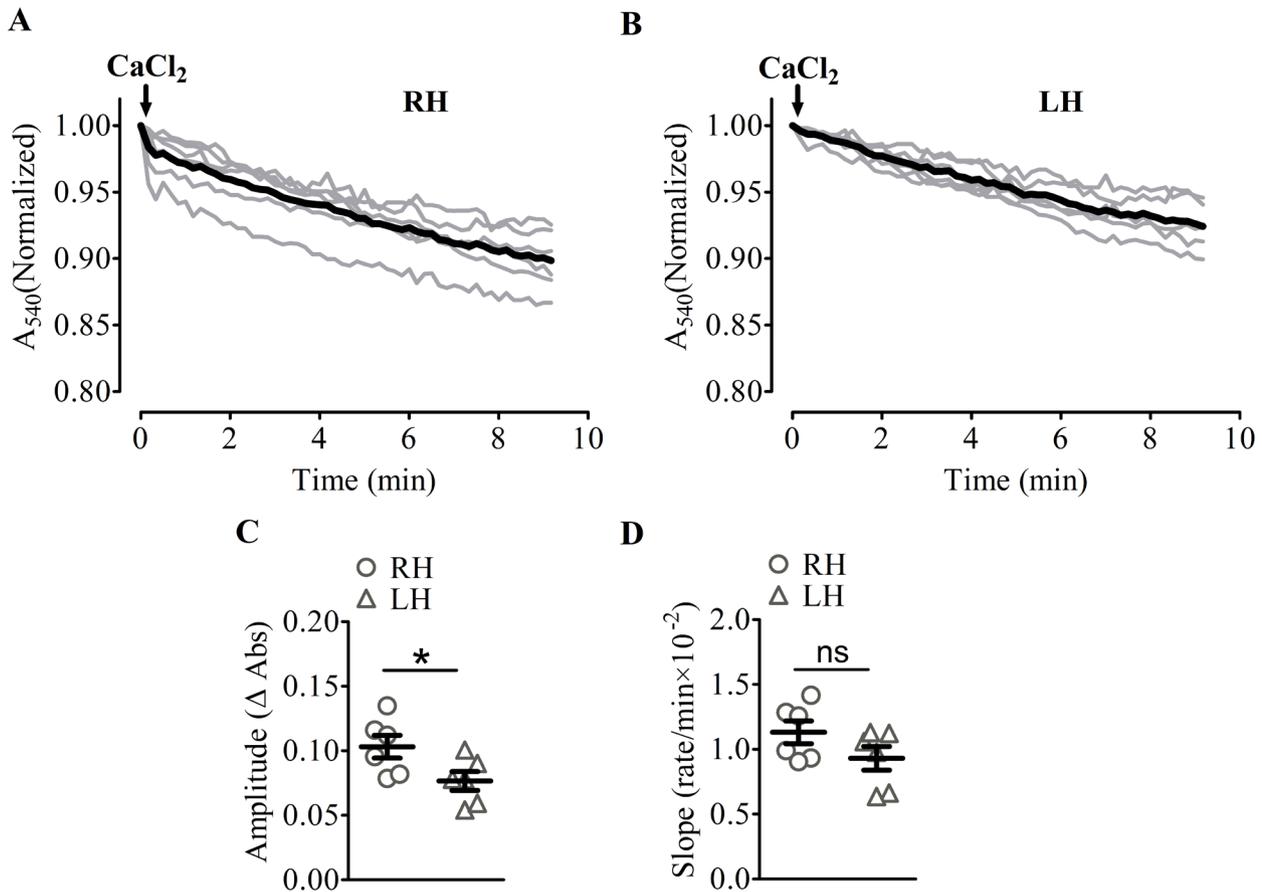


FIGURE 3 - The addition of 10 mM Ca^{2+} also triggered asymmetric mPTP opening in mouse brain hemispheres. (A, B) Curves show mitochondrial swelling upon the addition of 10 mM Ca^{2+} in the LH and RH. Grey lines indicate the average of multiple traces from each mouse; black bolded lines indicate the mean from all mice. The curves show a slow and consistent decrease in absorbance over 10 min. (C) The mean amplitude (ΔAbs : maximal difference) was calculated from grey curves, showing a significant difference in mPTP opening between LH and RH. (D) The plot represents the slope of mPTP opening in the LH and RH as rate/minute. Data in both scatter plots represent the mean \pm SEM; $n = 6$ mice, $*p = 0.04$, ns = non-significant.

Cortical and medullary mitochondria from mouse kidney are more sensitive to mPTP opening

To validate our protocol and to determine comparative mPTP opening in different parts of mouse

kidney, we investigated the swelling of mitochondria from mouse kidney cortex and medulla. Notably, mitochondria from kidney cortex and medulla demonstrated rapid and strong mPTP opening upon the addition of 500 μM Ca^{2+} (Figure 4A). However, there was no difference in swelling

of the mitochondria from the two parts of the kidney as determined by amplitude (Figure 4B) and the rate of decrease in the absorbance (Figure 4C). Interestingly, comparison of the data from the brain versus kidney mitochondria revealed significantly greater mPTP opening in cortical and medullary mitochondria as

compared to LH and RH mitochondria (Figure 4D). Similarly, the kinetics of mPTP opening in the kidney-derived mitochondria were considerably faster than that of the LH and RH mitochondria (Figure 4E). These findings confirm the greater sensitivity of kidney mitochondria towards Ca^{2+} -induced mPTP opening.

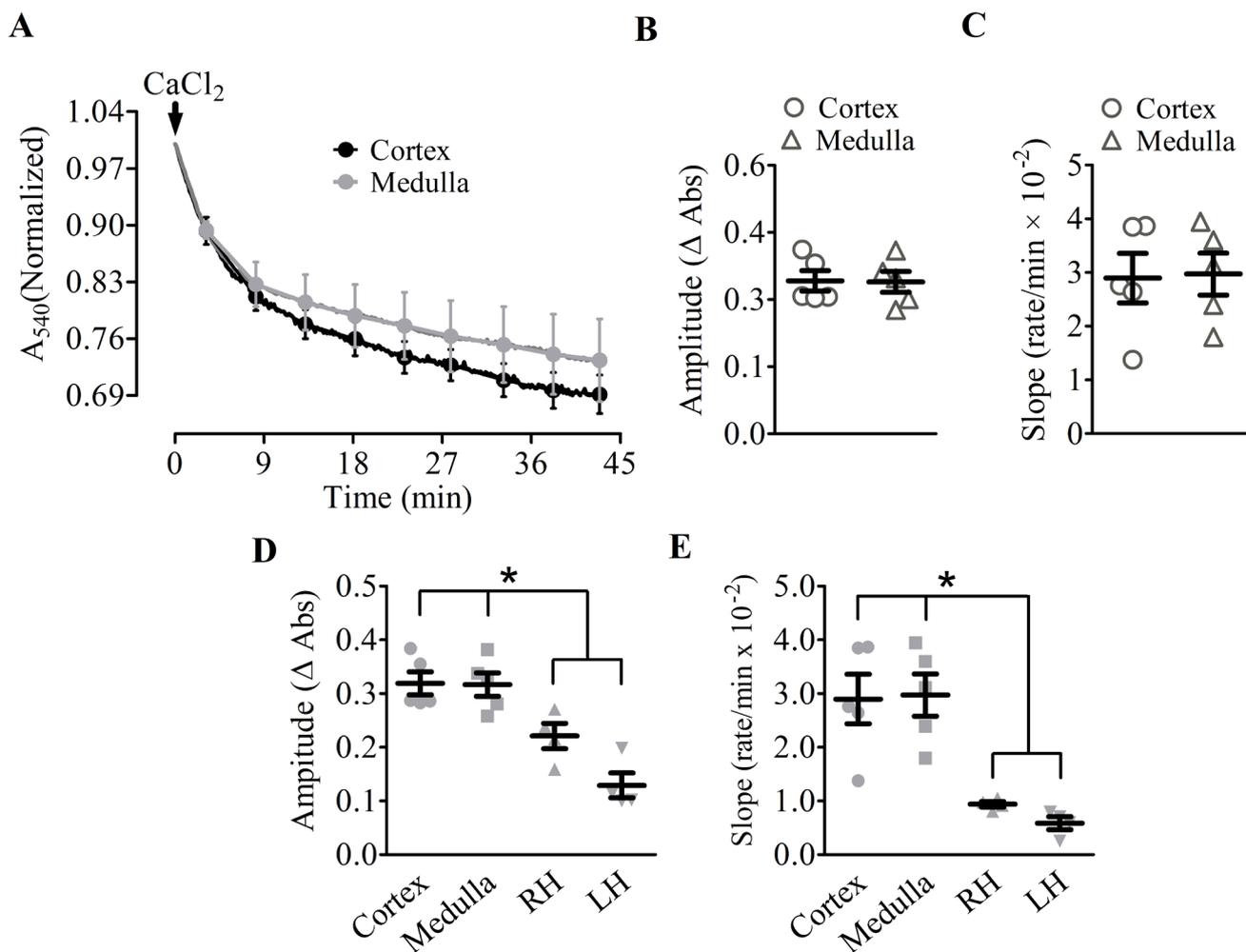


FIGURE 4 - Induction of mPTP opening in mouse kidney cortical and medullary mitochondria upon the addition of $500 \mu\text{M}$ Ca^{2+} . (A) Mitochondria were suspended in HB, and Ca^{2+} ($500 \mu\text{M}$) was added to stimulate mPTP opening. Curves represent the mean of traces from the cortex and medulla of multiple mice; error bars indicate the SEM. (B) The scatter plot indicates the mean amplitude (Δ Abs: maximal difference), showing no variation in mPTP opening between the cortex and medulla mitochondria. Data represent the mean \pm SEM; $n = 5$ mice. (C) The speed of mPTP opening, shown by the slope (rate/minute), was not significantly different between the cortex and medulla. Data represent the mean \pm SEM; $n = 5$ mice. (D) Comparison of curve amplitudes obtained from mouse kidney (cortex and medulla) and brain (LH and RH) mitochondria using one-way ANOVA. Bars represent the mean \pm SEM; * $p < 0.05$. (E) Comparison of the speed of mPTP opening (slope) between mouse kidney (cortex and medulla) and brain (LH and RH) using one-way ANOVA. Bars represent the mean \pm SEM; * $p < 0.05$.

Correlation of differential mPTP opening in brain hemispheres with the expression of MCUC components

The mitochondrial uptake of Ca^{2+} is primarily mediated by the MCUC, which controls both the physiological and pathological functions of the cell. Therefore, we investigated the expression of different MCUC components in the two brain hemispheres as a possible contributing factor to the differential mPTP opening in the LH and RH. Our data show that the relative abundance of *Mcu* mRNA, the pore-forming subunit of MCUC, was not different between the two hemispheres (Figure 4A). The pore opening of the MCU is regulated by other MCUC components, including MICU1, MICU2,

and MCUb. Thus, variations in their expression may also influence the Ca^{2+} -triggered asymmetric mPTP opening in the brain reported herein. Nevertheless, our results indicate that the relative abundance of these proteins was unaltered in the LH and RH (Figure 4B–D). Furthermore, the quantitative PCR data revealed a downwards tendency in the LH expression of Emre, another vital MCUC component that participates in the formation of MCUC higher-order structure in metazoans. However, our data did not achieve statistical significance, probably due to the higher standard deviation (Figure 5E, $p = 0.052$). We can, therefore, conclude that the mRNA expression levels of the pore-forming and regulatory components of the MCUC are not different in relation to variable mPTP opening in the mouse brain hemispheres.

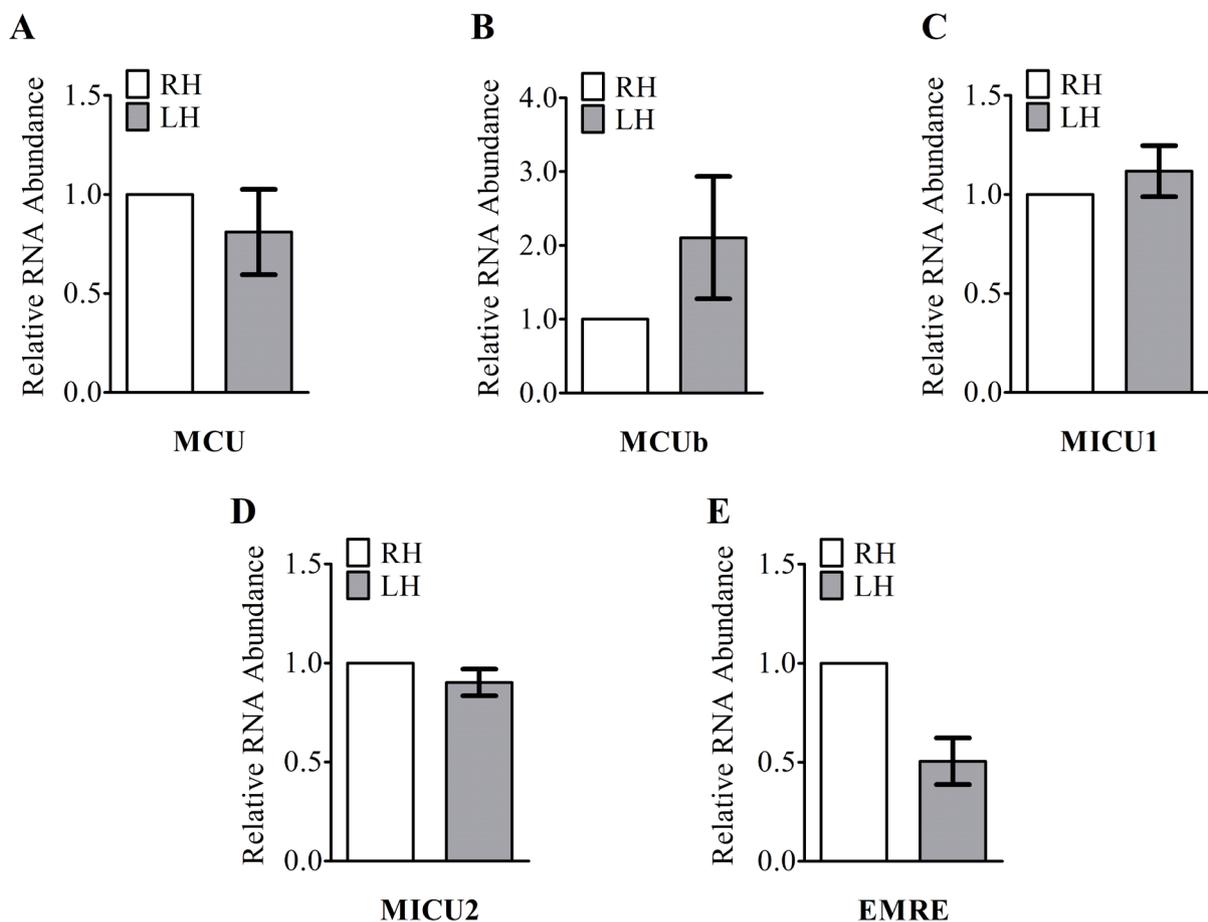


FIGURE 5 - Expression profile of MCUC subunits in mouse brain LH and RH reveal no significant difference. (A–E) The bar graphs show the relative abundance of *Mcu*, *MCub*, *Micu1*, *Micu2*, and *Emre* mRNA, presented as fold change, using RH as the reference sample. $n = 3$ –4 mice.

DISCUSSION

Prolonged mPTP opening in mitochondria from the brain has been linked to cell death via necrosis and/or apoptosis (Gainutdinov *et al.*, 2015). This phenomenon has been proposed to be involved in various brain pathologies such as stroke-associated ischemia-reperfusion injury and neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and Huntington's disease (Gainutdinov *et al.*, 2015; Kalani, Yan, Yan, 2018). Asymmetry of brain functions, especially that originating from the cerebral cortex, has been reported in various physiological and disease processes in both humans and rodents (Gao, Zhang, 2008; Levy *et al.*, 2019; Minkova *et al.*, 2017; Shipton *et al.*, 2014; Sun, Walsh, 2006; Zhai, Feng, 2018). In the present study, we report a thus far unknown asymmetric opening of mPTP in mitochondria of the LH and RH of mouse brain. Our findings highlight the intriguing fact that the RH had greater and faster mPTP opening upon the exogenous addition of pathological Ca^{2+} as compared to the LH. However, there was no difference in the mitochondrial dehydrogenase activity; an indicator of mitochondrial metabolism. Asymmetries in mouse brain hemispheres in the context of protein expression and therapeutic treatment have been linked to the pathophysiology of Alzheimer's disease in model organisms (Manousopoulou *et al.*, 2016; Tsai *et al.*, 2009). Similarly, regional differences in molecular architecture (Sun, Walsh, 2006), gene expression (Muntane *et al.*, 2017), signal transduction (Grabrucker *et al.*, 2017), glucose metabolism (Park *et al.*, 2017), and mitochondrial function (Toth *et al.*, 2015) have also been demonstrated in mammalian brains.

The present study also shows that, unlike brain mitochondria, the cortical and medullary mitochondria from mouse kidney did not reflect any differential mPTP opening. However, comparative analysis of mPTP opening between the brain and kidney mitochondria revealed the interesting fact that the brain mitochondria were resistant to Ca^{2+} -induced mPTP opening as compared to the kidney mitochondria. Previous studies have demonstrated slow and lesser Ca^{2+} -induced mPTP opening in brain mitochondria when compared to heart

and liver mitochondria (Berman, Watkins, Hastings, 2000; Eliseev *et al.*, 2007). An exciting report by Eliseev *et al.* (2007) has proposed the low expression of cyclophilin D (a key mPTP regulator) as a plausible cause for the greater resistance of brain mitochondria to Ca^{2+} -induced mPTP opening in relation to liver and heart mitochondria.

MCUC-mediated mitochondrial Ca^{2+} homeostasis plays an important role in various brain functions such as bioenergetics, synaptic activity, and cognition (Kannurpatti, 2017). There is also consensus on the pathophysiological relevance of MCUC components, especially MCU, in mPTP-dependent damage of the brain (Giorgi, Marchi, Pinton, 2018). Interestingly, variable expression of MCU and its regulatory components has been demonstrated in different brain regions and cells (Markus *et al.*, 2016). However, whether there is variation in the expression of MCUC components in the LH and RH of mouse brain that contributes to asymmetric mPTP opening remains unknown. Our data demonstrate that *Mcu* mRNA levels were not considerably different between the two hemispheres, and we propose that the level of *Mcu* mRNA is likely not the reason for the asymmetric mPTP opening in the mouse brain hemispheres.

MCU activity is regulated by many regulatory proteins, including MICU1/2 (Kamer, Mootha, 2014) and MCUB (Raffaello *et al.*, 2013). Alteration of MICU1/2 expression and dysfunction, for example, cause dysregulation of mitochondrial Ca^{2+} homeostasis, leading to disease-associated cell damage (Mammucari, Gherardi, Rizzuto, 2017). Moreover, variable mitochondrial Ca^{2+} uptake mechanisms and mPTP opening have also been proposed to be a result of the differential stoichiometry of the MCUC subunits (Chapoy-Villanueva *et al.*, 2019; Paillard *et al.*, 2017). However, contrary to these studies, our data from the mouse brain hemispheres show no noticeable difference in the expression of *Micul1*, *Micul2*, and *Mcub* mRNA. EMRE is also a vital component of the MCUC in metazoans (Sancak *et al.*, 2013). Here, we observed a strong downwards tendency in EMRE expression in the LH, but statistical significance was not achieved, probably due to the higher biological variation in mice.

In summary, our data provide the first evidence that mouse brain hemispheres show asymmetry in Ca^{2+} -induced mPTP opening, which was not observed in mitochondria from mouse kidney cortex and medulla. We also reveal that the brain mitochondria have a higher Ca^{2+} threshold for mPTP opening when compared to the kidney mitochondria. Lastly, we report that the expression level of MCUC components is not a contributing factor to asymmetric mPTP opening in the mouse brain hemispheres.

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