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Impact of Restraint Stress on Mitochondrial Ion Transporter Activity in Mice Brain-Gut Regions and Gender Response to Aging

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Abstract

The ability to respond suitably and maintain a steady state after exposure to stressors is an essential dynamic element in maintaining ion homeostasis. Besides the factors linked to the stressor itself, there are aspects intrinsic to the organisms that are pertinent to shape the stress response, such as age, gender and genetics. This study in mice analyses the functional role of mitochondria, which may affect the integrated responses to psychological stress. Mitochondria depend on a series of ion transporters to interface the communication between the cytosol and the site of energy production, which is key to the survival of the organism. Ion transporters, like mCa^{2+}ATPase, F_1F_0ATPase and mH^+ATPase, are the functional components of the mitochondria involved in Ca^{2+}, H^+ homeostasis and energy production. Since the process of aging starts with the birth, and ends with the death of an organism, physiological and molecular processes tend to vary throughout aging. Moreover, males and females have qualitatively different mitochondria, and only a little is known about the mitochondrial responses to stressors. Therefore, we hypothesized that mitochondrial ion transporter functions would modulate the organism's multisystemic response to psychological stress in an age-, gender- and tissue-specific manner. In this study, BALB/c mice of different age groups (4 weeks-, 8 weeks-, 16 weeks- and 24 weeks-old mice) were subjected to restraint stress of 30 minutes for two consecutive days and the ion transporter activity was quantified in the different regions of the brain (cerebrum, cerebellum and hippocampus) and the gut (duodenum of the intestine, fundus and pyloric regions of the stomach). Overall, the data indicate that in mice both gender-specific and age-specific differential sensitivities to restraint stress exist in mitochondrial ion transporter function in the brain and gut regions. This further points to a decisive interactive role of stress and sex hormones in the energetics and ion transport performance of brain-gut axis in mice.

Keywords: Aging, Brain Gut Axis, Ion Transport, Mice, Mitochondria, Restraint Stress

1. Introduction

Organisms stay alive by preserving a complex dynamic and harmonic equilibrium called homeostasis which is threatened by stressors[1]. Stress is an acute threat to the steady-state and results in short- and long-term consequences. The reaction to a stress is diverse, resulting in a variety of discrete vagaries among individuals. Many causes result in stress, and the setting of distinct managing tactics will tip to specific differences on the susceptibility or resistance to stress[2]. The upkeep of homeostasis relies on the tight orchestration of elements involved in response to stress and recovery[3]. The regulation of the stress axis encompasses the organisation between multiple regions of the brain controlling the extent of the stress response and the negative feedback critical for its return to the start point[4]. The brain reacts to stress in a distinguished fashion

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depending on the inhibition and activation of neurons that are involved in various processes[5]. The gut and the brain are closely connected, and this partnership plays a crucial role in the functions of the gut, state of feeling and decision making in an organism[6]. This study elucidates the variances and mechanisms in stress-induced disturbances in functionally and structurally different tissues of the gut (fundus and pyloric regions of stomach and duodenum) and the brain (cerebral cortex, hippocampus and cerebellum) during chronological aging. The restraint stress model engaged in the present study combined both physical and psychological components of stress and has been linked with gut epithelial hyperpermeability, the primary route in many functional and organic bowel diseases[7].

This study in mice analyses an organelle that sustains life through energy transformation and intracellular signalling, the mitochondrion, which influences the integrated response to stress in mice. Gender and age influence the quality of mitochondria, but only a little is known about its link with stressors. Ion transporters like mCa^{2+}ATPase, F_1F_0ATPase and mH^+ATPase, are found to control the Ca^{2+} and H^+ homeostasis besides functioning in ATP synthesis in the mitochondria. This study, therefore, focuses on how aging would influence mitochondrial ion homeostasis and how age and gender difference can modify the ion transporter activity in the different regions of the brain (cortex, hippocampus and cerebellum) and gut (fundus and pyloric regions of the stomach and duodenum) after restraint stress. For this, we studied the impact of restraint stress on mice of different age groups (four, eight, sixteen and twenty four-week old mice); and a comparison was made in a gender-specific manner. We quantified the activity patterns of mCa^{2+}-ATPase, F_1F_0ATPase and mH^+-ATPase in stressed and non-stressed mice brain and gut of both males and females.

2. Materials and methods

2.1 Animal Holding Conditions

Swiss albino mice (*Mus musculus*) of BALB/c strain of different age groups (four-, eight-, sixteen- and twenty four week-old mice), born and maintained in the animal house of the University of Kerala, were used for the study. Mice were kept in groups having four mice each in polypropylene cages (Size: 29 × 22 × 14 cm) with stainless steel–wire mesh top. Mice were allowed to have a 12-hour light and 12-hour dark photoperiod at a temperature of 24 ± 4°C and relative humidity of 70 ± 10% with fewest noise levels and handling stress. Mice were given *ad libitum* access to the standard pelleted feed from Sri Sai Durga Feeds and Products, Bangalore, and purified tap water. Cage bedding was replaced once in two days. The experiment was carried out under the same conditions as in the animal house, and the animals were transported to the experimental location in their home cages.

2.2 Experimental Design

Male and female mice of four age groups i.e., four weeks (juveniles), eight weeks (adolescent), sixteen weeks (young adults) and twenty four weeks (mature adults) old mice were used for the experimental procedure. Animals were grouped a week before the experiment. Attention was given to reduce handling stress. The test groups were given restraint stress of thirty minutes for two days at a gap of twenty-four hours between restraining procedures, and suitable controls were also maintained. We followed the regulations of Institutional Animal Ethical Committee (IAEC) of the University of Kerala for setting the experimental protocol (IAEC-KU-25/2016-17-CEIB-SP (2)). In this work, an age-specific response in ion transporter function to restraint stress was analyzed in male and female BALB/c mice.

2.3 Sampling and Analysis

The test and control mice were euthanized after giving restraint stress. The tissues, cerebral cortex, hippocampus, cerebellum of the brain, fundus and pyloric regions of the stomach and duodenum of the intestine, were collected and stored in suitable buffers at -80°C for enzyme analysis.

2.4 Isolation of Mitochondria

Mitochondria were isolated from the various segments of the brain and gut adopting the method of Peter, *et al.*[8] with simple modifications. The tissue was prepared and homogenised (8-10 strokes) using a glass homogeniser. The collected homogenate was first centrifuged at 700 x g for three minutes at 4°C to separate the membrane constituents from mitochondria. The other portion was then centrifuged at 10,000 x g for ten minutes at 4°C. The pellets obtained were washed in the isolation buffer

containing BSA and centrifuged at 10,000 rpm for ten minutes. The pellets were then re-suspended in a sucrose solution (0.25M) and centrifuged again for ten minutes. The pellets were again re-suspended in sucrose medium, which served as the mitochondrial enzyme source. The protein content in the cytosolic and mitochondrial fractions of the tissues was measured using modified Biuret assay[9] with BSA as the standard.

2.5 Quantification of Ion-Specific ATPases

2.5.1 mCa^{2+}-ATPaseActivity (Mitochondrial Ca^{2+}-ATPase Activity)

In the mitochondrial fractions, the vanadate-dependent Ca^{2+}-ATPase activity was determined by following the method of Peter *et al.*[8] using vanadate as inhibitor. Samples were added to a 96-well microtiter plate containing either $CaCl_2$ or vanadate, in duplicate. The inorganic phosphate content released was determined in a microtiter plate reader (Biotek, Synergy HT). The change in absorbance at 700 nm between promoter and inhibitor assays was calculated using regression analysis and the rate of transport activity expressed in µM Pi h^{-1}mg $protein^{-1}$.

2.5.2 F_1F_0-ATPase Activity

The specific activity of F_1F_0-ATPase in the mitochondrial fractions was determined following the previous method[8] and oligomycin was used as inhibitor. Mitochondrial samples were added in duplicate to a 96-well microtiter plate, and the inorganic phosphate released was measured spectroscopically and expressed in µM Pi h^{-1}mg $protein^{-1}$.

2.5.3 mH$^+$-ATPase Activity (Mitochondrial H$^+$-ATPase Activity)

The H^+-ATPase activity in the mitochondrial fraction was measured adopting the method of Peter, *et al.*[8] using bafilomycin as inhibitor. The samples were added in duplicate to a 96-well microtiter plate havingbafilomycin. The addition of ATP initiated the reaction which was subsequently terminated by adding TCA (8.6%), and the inorganic phosphate content was determined in a microtiter plate reader (Biotek, Synergy HT). The difference in absorbance at 700 nm between promoter and inhibitor assays was calculated using regression analysis and the transporter activity was expressed in µM Pi h^{-1}mg $protein^{-1}$.

2.6 Statistical Analysis

The data generated were checked for normal distribution and variance homogeneity. The statistical significance between test and control groups for both the genders was tested using Two-way ANOVA followed by post-hoc Turkey test with the help of SigmaPlot11 (Systat Software Inc.) and the level of significance was accepted if $p< 0.05$.

3. Results

In the cortex of both male (Figure 1A) and female (Figure 1B) mice, a significant increase in mCa^{2+}-ATPase activity was noted in eight week ($p<0.001$), sixteen week ($p<0.001$) and twenty four week-old mice ($p<0.001$), and the highest activity was seen in eight week-old males and females when compared to four week old mice. In the cortical region of male mice, a significant increase in mCa^{2+}-ATPase activity was observed in four week ($p<0.001$) and eight week old mice ($p<0.01$) in response to restraint stress. However, a decline in activity was found in sixteen week ($p<0.01$) and twenty four week-old males ($p<0.001$) during stressed condition (Figure 1A). Restraint stress in females resulted in a remarkable decrease in mCa^{2+}-ATPase activity in eight week ($p<0.001$), sixteen week ($p<0.001$) and twenty four-week ($p<0.001$) even though an increase was found in four week old mice ($p<0.001$) (Figure 1B).

In the hippocampus of male mice, a significant increase in mCa^{2+}-ATPase ion transporter activity was observed in eight week ($p<0.001$), sixteen week ($p<0.001$) and twenty four-week old ($p<0.01$) (Figure 1C). In females, no significant variation was observed except in twenty four-week olds where it showed a significant increase ($p<0.01$) (Figure 1D). In the hippocampus of male mice an increase in mCa^{2+}-ATPase activity was noted during restraint condition in four week ($p<0.001$), eight week ($p<0.001$) and sixteen week-old mice ($p<0.001$) whereas no significant variation was noticed in twenty four-week old mice (Figure 1C). In females, a substantial increase in mCa^{2+}-ATPase activity was found in sixteen week ($p<0.001$) and twenty four-week old mice ($p<0.001$) but no significant change in response to stress was observed in four week and eight week-old females (Figure 1D).

In the cerebellum of males, a significant decrease in mCa^{2+}-ATPase activity was observed in eight weeks old mice ($p<0.001$) and the activity increased significantly in sixteen week ($p<0.001$) and twenty four-week old mice

(p<0.001) (Figure 1E). In females, a significant increase in mCa^{2+}-ATPase activity was found in eight week (p<0.001) and twenty four-week old mice (p<0.001) but no significant variation was observed in sixteen week-old mice (Figure 1F). In male mice, an increase in mCa^{2+}-ATPase activity during stressed condition was noticed in eight week (p<0.001) and sixteen week-old mice (p<0.001) and a decline was observed in four week (p<0.001) and twenty-four week old mice (p<0.001) (Figure 1E). However, in females, an upsurge in mCa^{2+}-ATPase activity was found in four week (p<0.001) and eight week-old mice (p<0.001). Sixteen week and twenty four-week-old female mice showed no significant difference in ATPase activity in response to stress (Figure 1F).

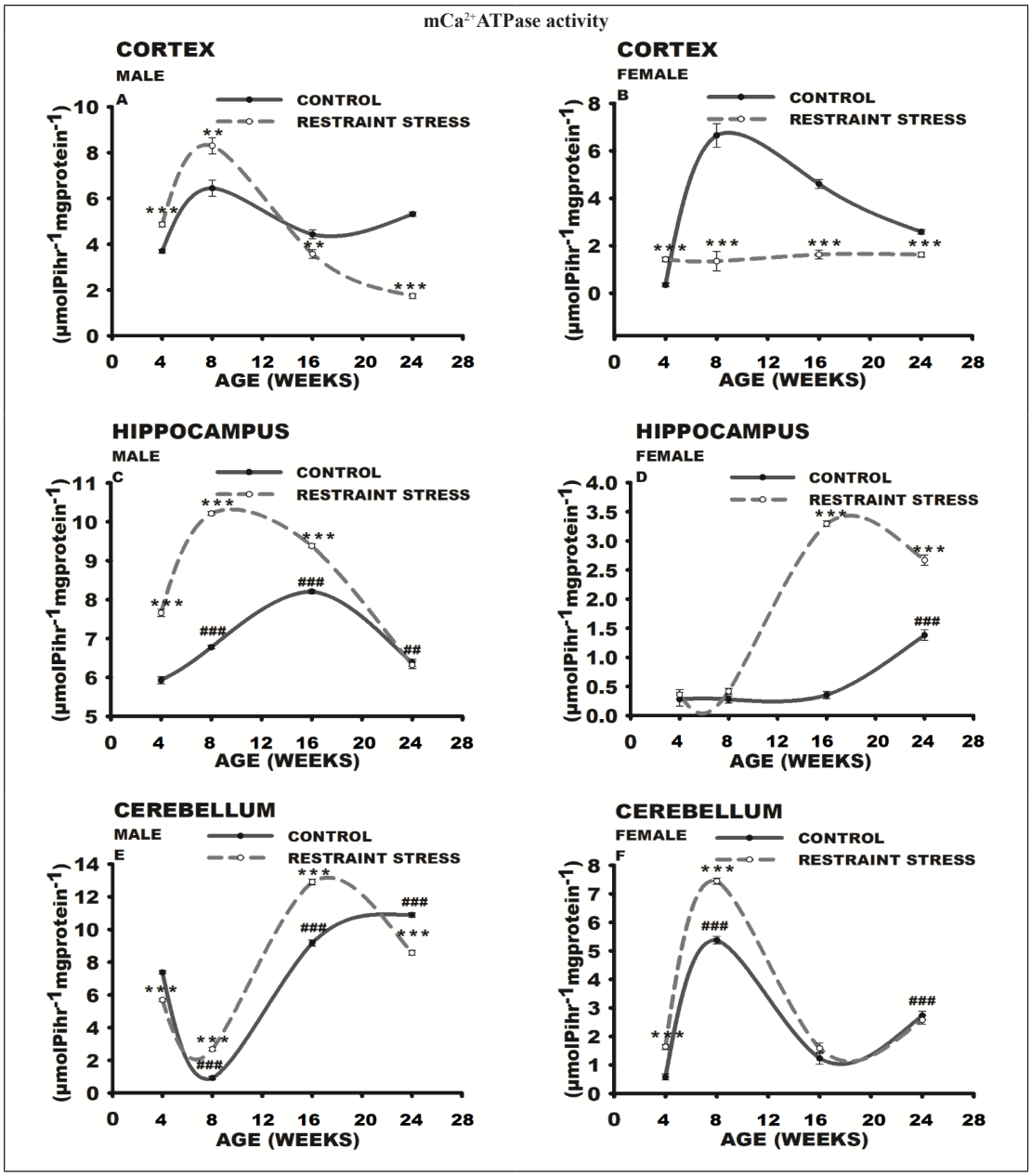

Figure 1. The activity pattern of mCa^{2+}ATPase during aging and restraint stress in the cortex, hippocampus and cerebellum of male and female mice. Each point is mean ±SE for four mice. The significance between control mice (4 weeks old) and aging mice groups (8,16 and 24 weeks old) was analysed using two-way ANOVA and represented as #(p< 0.05), ##(p<0.01) and ### (p< 0 001). Likewise, significance between similar aging mice groups with or without restraint stress was analysed and represented as * (p< 0.05), ** (p<0.01) and *** (p< 0 001).

In male fundus, an increase in mCa^{2+}-ATPase activity was noticed in sixteen week-old mice ($p<0.001$), whereas a significant decrease in activity was established in twenty four week-old mice ($p<0.05$) and no significant variation was found in eight week-old mice compared to four week old mice (Figure 2A). In female fundus, a significant increase in mCa^{2+}-ATPase activity was established in sixteen week ($p<0.001$) and twenty-four week-old mice ($p<0.001$) and no significant change was noticed in eight week-old mice compared to four week-old mice (Figure 2B).

In male pyloric stomach, a higher mCa^{2+}-ATPase activity was found in eight week ($p<0.01$) and twenty four week-old mice ($p<0.001$). Four week-olds showed no considerable variation in activity in response to stress (Figure 2A). In females, stress caused an increase in mCa^{2+}-ATPase activity in eight weeks ($p<0.001$) and sixteen weeks-old mice ($p<0.001$) but a significant decline was noted in twenty four weeks-old mice ($p<0.001$). No statistically significant variation in response to stress was found in the activity of four weeks-old mice (Figure 2B). In male pyloric stomach, a drop in mCa^{2+}-ATPase activity was seen in response to stress in eight week ($p<0.01$) and twenty four week-old mice ($p<0.001$) while a statistically significant increase was noticed in sixteen week-old mice ($p<0.01$). No significant variation was seen in response to stress in four week-old mice (Figure 2C). In female pyloric stomach, stress caused a surge in mCa^{2+}-ATPase activity in four week ($p<0.001$) and twenty four week-old mice ($p<0.001$) but a decline in mCa^{2+}-ATPase activity was found in eight week ($p<0.001$) and sixteen week-old mice ($p<0.001$) (Figure 2D). In male pyloric stomach, an increase in mCa^{2+}-ATPase activity was seen in eight week ($p<0.05$) and twenty four week-old mice ($p<0.001$) whereas no significant variation was detected in sixteen week-old mice compared to four week olds (Figure 2C). In female pyloric stomach, a substantial increase in mCa^{2+}-ATPase activity was noticed in eight week ($p<0.001$) and sixteen week-old mice ($p<0.001$) whereas no significant change was observed in twenty four week-old mice compared to four week old (Figure 2D).

In male duodenum a decrease in mCa^{2+}-ATPase activity was noticed in eight week ($p<0.05$), sixteen week ($p<0.001$) and twenty four week-old mice ($p<0.001$) compared to four week-old (Figure 2E). In female duodenum, an increase in mCa^{2+}-ATPase activity was noticed in eight week ($p<0.01$), sixteen week ($p<0.001$) and twenty four week-old mice ($p<0.001$) compared to four week-old mice (Figure 2F). In male duodenum, a significant upsurge in mCa^{2+}-ATPase activity was noticed in four week ($p<0.001$) and sixteen week-old mice ($p<0.001$), however a significant decline was observed in twenty four week-old mice ($p<0.001$) subjected to stress. No significant variation was observed in eight week-old mice due to stress (Figure 2E). In female duodenum, an increase in mCa^{2+}-ATPase activity was noticed during stress condition in eight week ($p<0.001$) and sixteen week–old mice ($p<0.001$) but a decrease in mCa^{2+}-ATPase activity was found in twenty four week-old mice ($p<0.001$) during stress condition. In four week-old mice, no change was noticed (Figure 2F).

The F_1F_0-ATPase activity in the cortex of male mice showed a significant increase ($p<0.001$) during aging, i.e., in eight week ($p<0.01$), sixteen week ($p<0.001$) and twenty four week old mice ($p<0.001$) (Figure 3A). The highest activity was found in twenty four week-old mice. In the cortical region of female mice, the F_1F_0-ATPase activity showed a significant increase in eight week ($p<0.001$) old mice but no significant change was found in sixteen week and twenty four week-old female mice (Figure 3B). In the cortical region of male mice an increase in F_1F_0-ATPase activity was observed in four week ($p<0.001$) and eight week-old mice ($p<0.001$) during stressed condition but a decline was noted in sixteen week ($p<0.05$) and twenty four week -old mice ($p<0.001$) (Figure 3A). In female cortex, an increase was observed in four week-old mice ($p<0.001$) and a decrease was found in eight week-old mice ($p<0.001$) but sixteen week and twenty four week-old females showed no significant variation in F_1F_0-ATPase activity during stressed condition (Figure 3B).

In the hippocampus of male mice, a substantial increase in F_1F_0-ATPase activity was noticed at eight week ($p<0.001$) and sixteen week-old mice ($p<0.001$) but no significant change was observed in twenty four week-old mice (Figure 3C). In female hippocampus, no significant change in F_1F_0-ATPase activity was found at the eight week-old stage whereas a significant increase was observed in sixteen week ($p<0.001$) and twenty four week-old mice ($p<0.001$) (Figure 3D). In male hippocampus, F_1F_0-ATPase was found to decline in the hippocampus of sixteen week ($p<0.001$) and twenty four week-old mice ($p<0.001$) during stressed condition whereas no statistically significant change was found in four week and eight week-old mice (Figure 3C). In female hippocampus, a decline in ATPase activity in response to stress was found in eight week ($p<0.001$) and sixteen

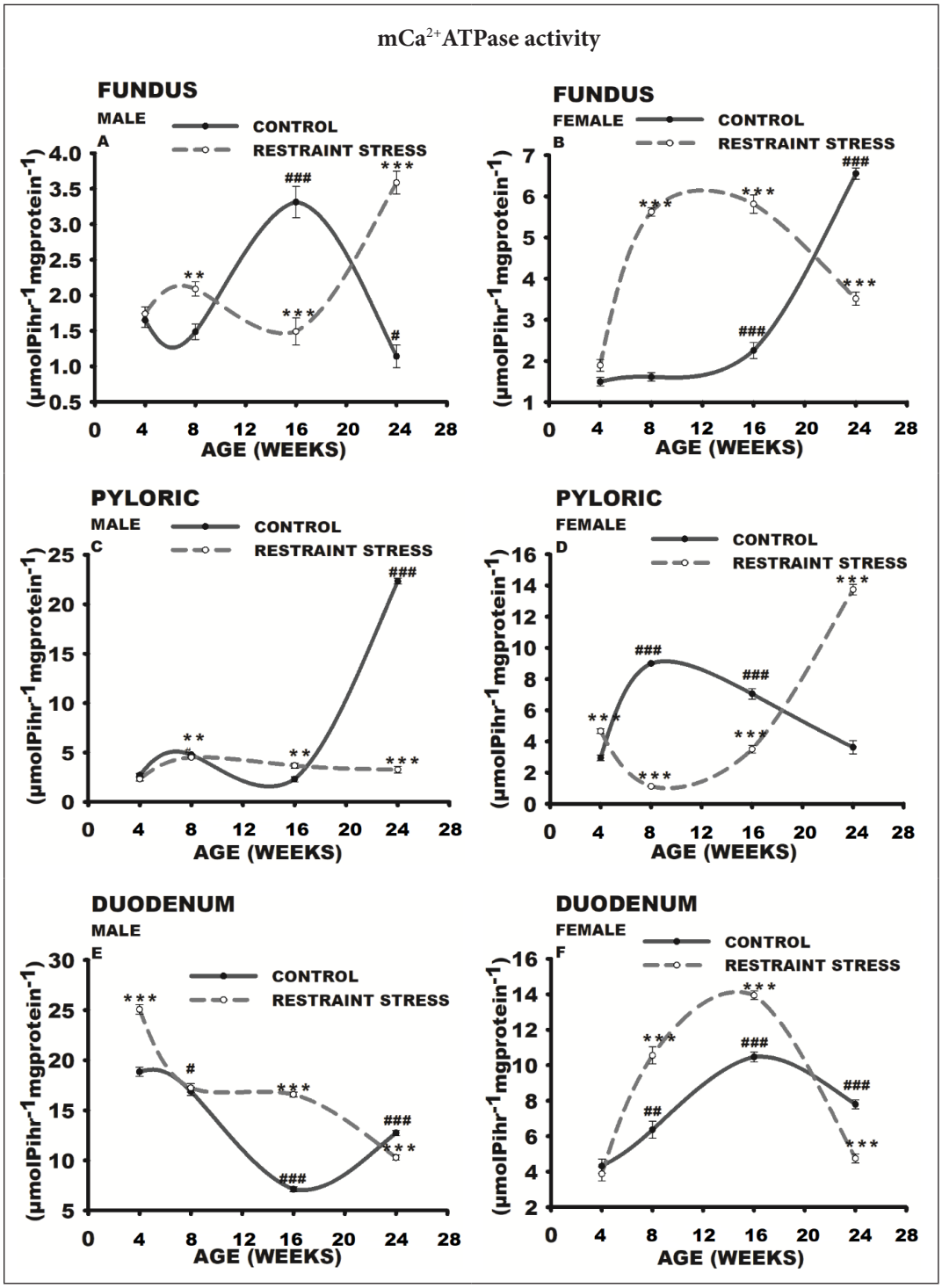

Figure 2. The activity pattern of mCa^{2+}ATPase during aging and restraint stress in the fundus, pyloric and duodenum of male and female mice. Other details, as in Figure 1.

week-old mice (p<0.001) but no significant change was found in four week and twenty four week-old mice (Figure 3D).

The cerebellum of male mice showed a significant increase in F_1F_0-ATPase activity in eight week (p<0.001) and sixteen week (p<0.001) old mice whereas a significant decline occurred in twenty four week-old mice (p<0.001) (Figure 3E). In females, a decrease in activity was found in eight week (p<0.001), sixteen week (p<0.001) and twenty four week-old mice (p<0.001) (Figure 3F). In male cerebellum, an increase was observed in four week (p<0.001) and twenty four week-old mice (p<0.001), however, a decrease in activity in response to stress in eight week (p<0.001) and sixteen week-old mice (p<0.001) (Figure 3E). In female cerebellum, an upsurge in activity was observed only in twenty four week-old mice (p<0.001). Stress resulted in a decreased activity in four week (p<0.001) and eight week-old females (p<0.001), however, no significant change was found in sixteen week-old mice (Figure 3F).

In male cortex, a decrease in F_1F_0-ATPase activity was found in eight week (p<0.001), and an increase in activity was found in sixteen week-old mice (p<0.001). However, no significant difference was found in twenty-four week-old males compared to four week-old mice (Figure 4A). In female cortex, an increase in F_1F_0-ATPase activity was found in sixteen week (p<0.001) and twenty four week-old mice (p<0.001) whereas, no significant difference was found in eight week-old mice compared to four week-old females (Figure 4B).

In male cortex, stress caused an augmented F_1F_0-ATPase activity in eight week (p<0.001) and twenty-four week-old mice (p<0.001) whereas a drop in F_1F_0-ATPase activity was noticed in sixteen week-old mice (p<0.001) in response to stress. No statistically significant change was evident in the activity of four week-old stressed mice compared to non-stressed mice (Figure 4A). In female cortex, a stress-dependent increase in F_1F_0-ATPase activity was found in four week (p<0.001), eight week (p<0.001) and sixteen week-old mice (p<0.001) but a decline in F_1F_0-ATPase activity was found in twenty four week-old stressed mice (p<0.001) (Figure 4B).

In male hippocampus, an increase in F_1F_0-ATPase activity was found in eight week (p<0.001), and sixteen week-old mice (p<0.001) whereas no significant change was found in twenty-four week-old mice compared to four week old (Figure 4C). In female hippocampus, an increase in F_1F_0-ATPase activity was noticed in eight week-old mice (p<0.01) and a decrease in sixteen weeks (p<0.001), and twenty four week-old females (p<0.001) were observed compared to four week (Figure 4D). In male hippocampus, an upturn in F_1F_0-ATPase activity in response to stress was found in four week-old mice (p<0.001) whereas a significant decline was detected in F_1F_0-ATPase activity of eight week (p<0.001), sixteen week (p<0.001) and twenty four week-old mice (p<0.05) (Figure 4C). In female hippocampus, a drop in F_1F_0-ATPase activity was noted in eight week (p<0.001) and sixteen week-old mice (p<0.001) in response to stress. Twenty four week-old mice (p<0.001) showed increased F_1F_0-ATPase activity and no significant change in F_1F_0-ATPase activity was noticed in four week-old mice after stress induction (Figure 4D).

In male cerebellum, an increase in F_1F_0-ATPase activity was noted in eight week (p<0.001) and twenty-four week-old mice (p<0.001) whereas no significant change was found in sixteen week-old males compared to four week old (Figure 4E). In female cerebellum, an increase in activity was noticed in eight week (p<0.001) and sixteen week-old mice (p<0.05); however, in twenty-four week-old mice, no significant variation was found (Figure 4F). In male cerebellum stress produced increase of F_1F_0-ATPase activity in four week (p<0.001) and sixteen week-old mice (p<0.001) whereas a significant decrease was found in twenty four week-old mice (p<0.001). Eight week-old mice showed no significant changes in activity due to stress (Figure 4E). In female cerebellum, a decrease in F_1F_0-ATPase activity was detected in eight week (p<0.001) and twenty four week-old mice (p<0.05) whereas an increase in F_1F_0-ATPase activity was found as a result of stress in sixteen week-old mice (p<0.01). No significant difference was perceived in the activity of four week-old mice after a stressed condition (Figure 4F).

In the cortical region of male mice, a substantial increase in mH^+-ATPase activity was observed in eight week (p<0.001) and twenty four week-old mice (p<0.001), whereas in sixteen week-old mice, a significant decline (p<0.001) was noticed (Figure 5A). In female cortex, a significant reduction in mH^+-ATPase activity was observed in eight week (p<0.01), sixteen week (p<0.001) and twenty four week-old mice (p<0.001) (Figure 5B). In the cortical region of male mice, a decline in mH^+-ATPase activity was observed in response to stress in four week, eight week (p<0.001) and twenty four week-old mice (p<0.001). However, in sixteen week-old males, an increase (p<0.001) was noticed (Figure 5A). Similarly,

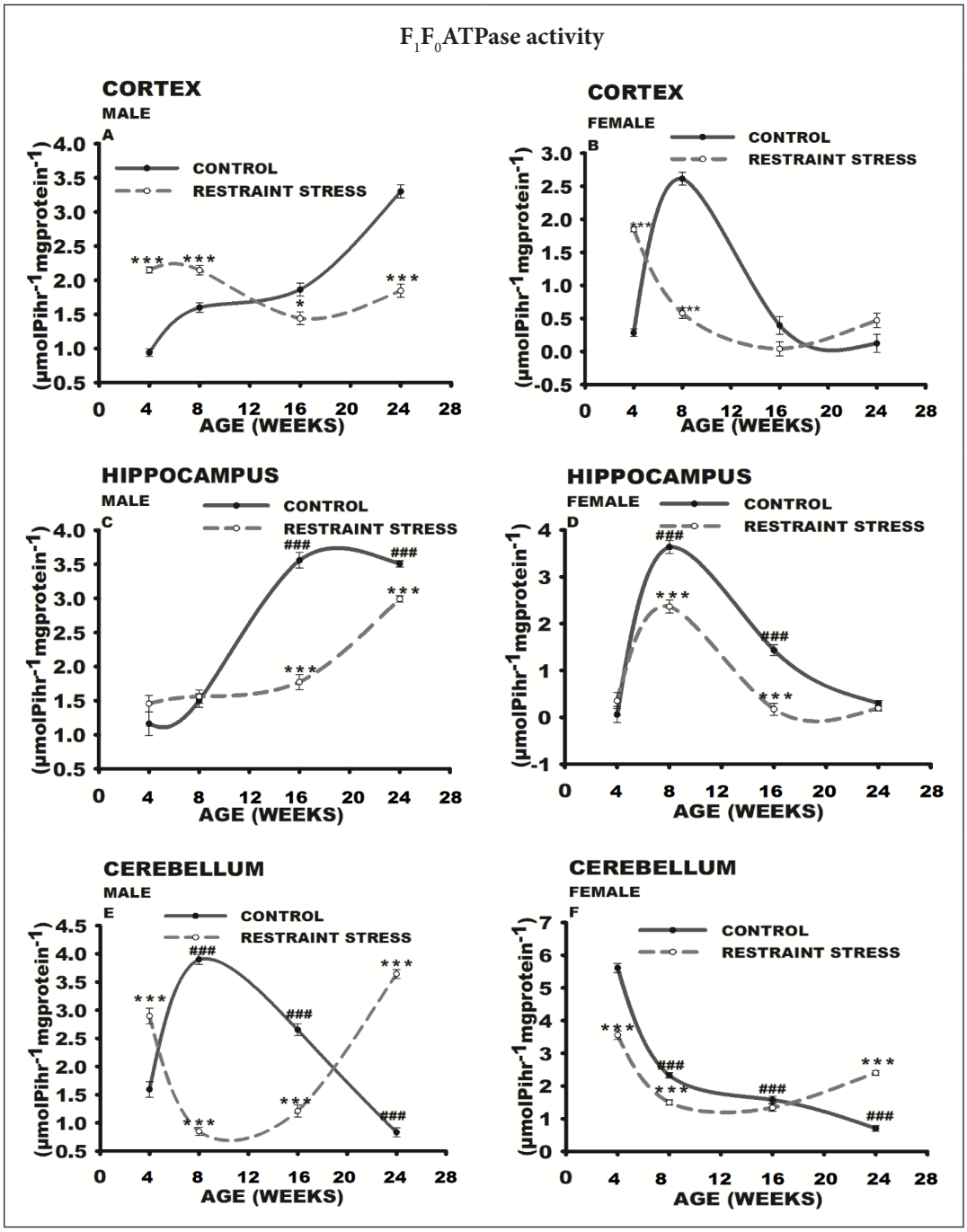

Figure 3. The activity pattern of F_1F_0ATPase during aging and restraint stress in the cortex hippocampus and cerebellum of male and female mice. Other details, as in Figure 1.

in female cortex, a decrease in activity was noted in four week (p<0.001), eight week (p<0.01) and twenty four week-old mice (p<0.01) and increased activity was observed in sixteen week olds (p<0.001) (Figure 5B).

In the hippocampus of male mice, a significant increase in activity was noticed in eight week (p<0.001) and sixteen week-old mice (p<0.001) whereas no significant difference was observed in twenty four weeks-old male (Figure 5C). In female hippocampus, a significant increase in ATPase activity was noticed in eight week (p<0.05), sixteen week (p<0.001) and twenty four week-old mice (p<0.001) (Figure 5D). In the male hippocampus, an increase in activity was noticed in four week (p<0.001), eight week (p<0.001) and twenty four week-old mice (p<0.001) and

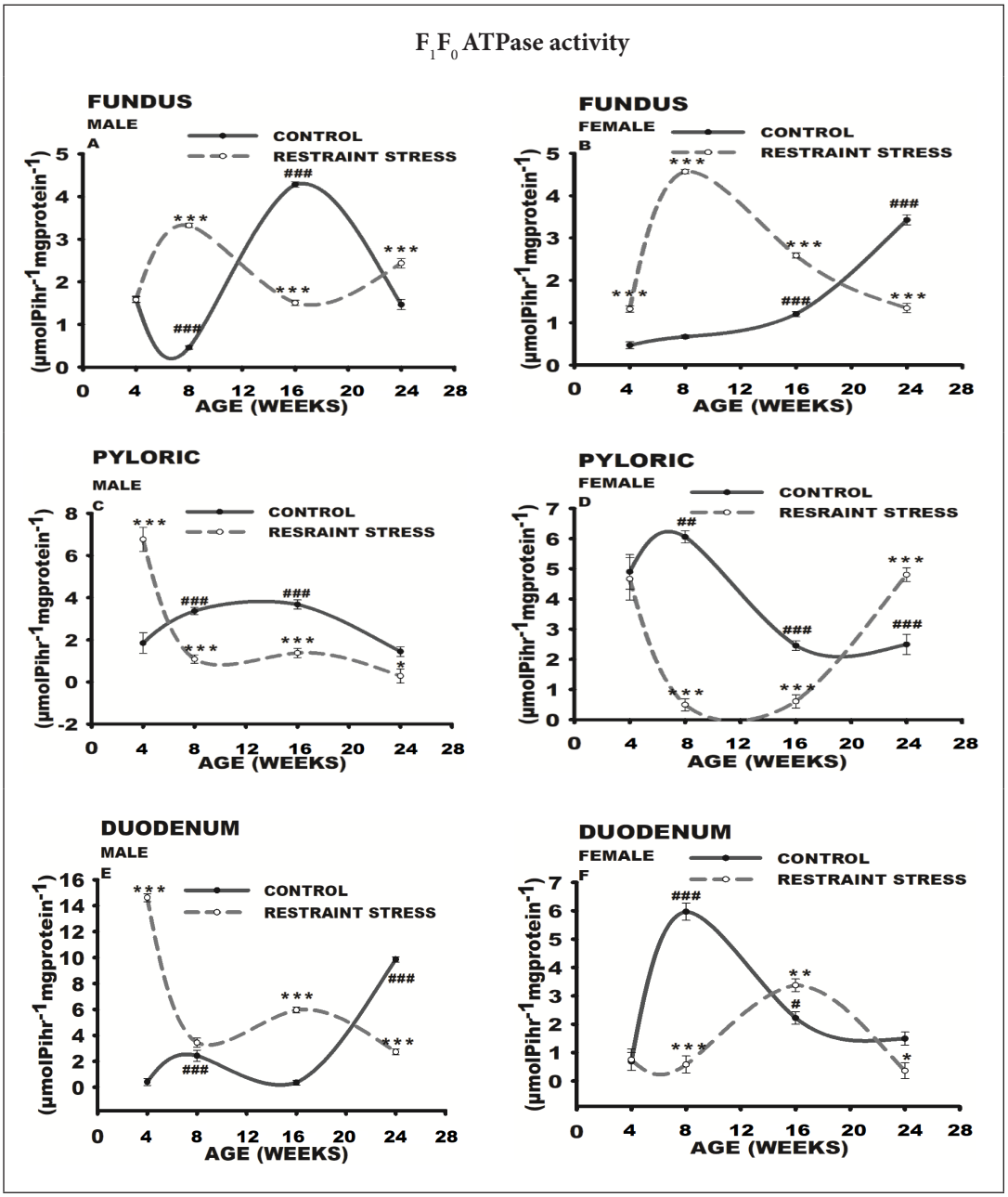

Figure 4. The activity pattern of F_1F_0ATPase during aging and restraint stress in the fundus, pyloric and duodenum of male and female mice. Other details, as in Figure 1.

no significant difference was found in sixteen week-old mice during stressed condition (Figure 5C). Similarly, in females, an increase in mH^+-ATPase activity in response to stress was noted in four week ($p<0.05$), eight week ($p<0.001$) and twenty-four week-old mice ($p<0.001$). On the other hand, a decrease in activity was noticed in sixteen week-old females ($p<0.001$) (Figure 5D).

In the cerebellum, a significant increase in mH^+-ATPase activity was noticed in eight week ($p<0.001$) and twenty four week-old male mice ($p<0.001$) but in sixteen week-old males a significant decrease ($p<0.01$) was observed compared to four week-old mice (Figure 5E). In female cerebellum a significant increase ($p<0.001$) was observed in all age groups compared to four week-old mice, and the highest activity was noticed in twenty four week-old mice (Figure 5F). In response to stress, an increase in mH^+-ATPase activity was observed in four week ($p<0.001$), eight week ($p<0.001$), sixteen week

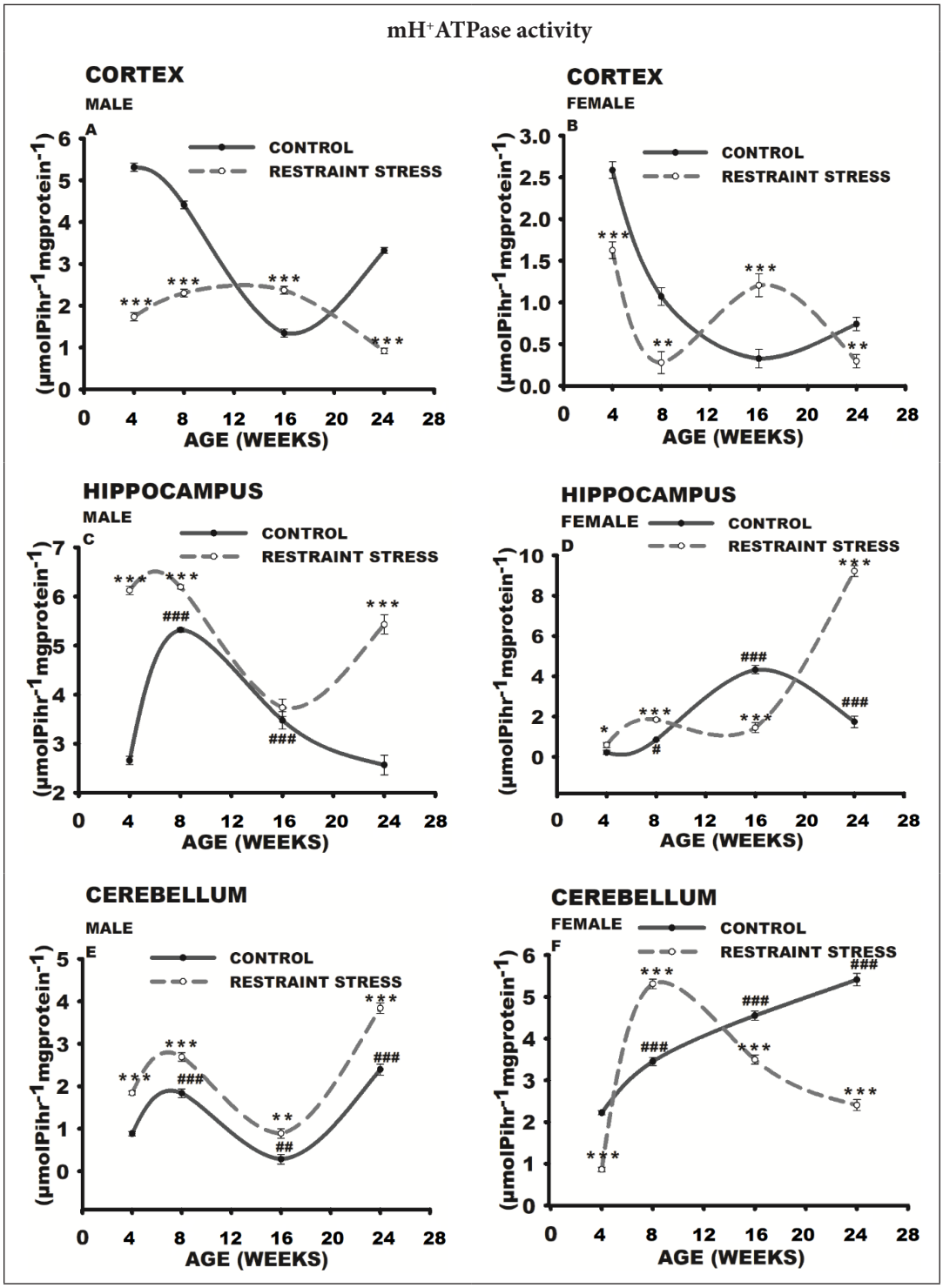

Figure 5. The activity pattern of mH⁺ATPase during aging and restraint stress in the cortex, hippocampus and cerebellum of male and female mice. Other details, as in Figure 1.

(p<0.01), and twenty four week-old male cerebellum (p<0.001) (Figure 5E). In female cerebellum, a decline in activity during stressed condition was noted in four week (p<0.001), sixteen week (p<0.001) and twenty four week-old mice (p<0.001) but an increase was noticed in eight week-old mice (p<0.001) (Figure 5F).

In male fundus, a decrease in activity was found in eight week (p<0.001), sixteen week (p<0.05) and twenty four week-old males (p<0.001) compared to four week old mice (Figure 6A) but in female fundus, an increase in its activity was found in twenty four week old mice (p<0.001), and no significant change was noticed in eight week and sixteen week-old females compared to four week old mice (Figure 6B). In male fundus, a reduction in mH^+-ATPase activity was noticed in four week-old mice (p<0.001) whereas stress caused a surge in the mH^+-ATPase activity of eight week (p<0.001), and twenty four week-old mice (p<0.001). No significant change in response to stress was seen in sixteen week-old mice (Figure 6A). In female fundus, stress prompted an upsurge in mH^+-ATPase activity in four week (p<0.01), eight week (p<0.001) and sixteen week-old mice (p<0.001). Conversely, a decline in mH^+-ATPase activity was found in twenty four week-old stressed mice (p<0.05) (Figure 6B).

In male pyloric stomach (Figure C) and female pyloric stomach (Figure 6D), the activity of mH^+ATPase increased in sixteen week-old mice (p<0.001) although no significant difference was found in eight week and twenty-four week-old mice compared to four week old in both the sexes. In male pyloric stomach, a rise in mH^+-ATPase activity was found in response to stress in eight week-old mice (p<0.001) whereas a substantial reduction in mH^+-ATPase activity was noticed in sixteen week old (p<0.001). Four week-and twenty four week-old mice showed no significant difference in activity because of stress (Figure 6C). In female pyloric stomach, a decrease in mH^+-ATPase activity was found in sixteen week-old mice (p<0.001) whereas no significant change was noticed in response to stress in four week, eight week and twenty four week-old mice (Figure 6D).

In male duodenum, an increase in ATPase activity was observed in eight week-old mice (p<0.001) but no significant variation was found in sixteen week and twenty four week-old mice compared to four week old (Figure 6E). In female duodenum, a substantial increase in activity was found in eight weeks (p<0.001), sixteen weeks (p<0.001) and twenty four week-old mice (p<0.001) compared to four weeks old (Figure 6F). In stressed male duodenum, a decrease in mH^+-ATPase activity was imminent in eight weeks-old mice (p<0.001), and a significant increase was found in twenty four week-old mice (p<0.001). However, no significant change was noticed in the activity of four week and sixteen week-old stressed mice compared to controls (Figure 6E). In female duodenum, restraining stress caused an increase in the mH^+-ATPase activity of four week (p<0.05) and sixteen week-old mice (p<0.01) whereas a decrease in activity was detected in eight week (p<0.001) and twenty four week-old mice (p<0.01) compared to non-stressed mice (Figure 6F).

4. Discussion

Mitochondria perform unique roles in diverse tissues but together, by communicating among each other, safeguard the effective integration, sensing and signalling of metabolic information. This multi-site stress modulation of metabolism shows distributed control networks, renowned for improving the function of complex systems[10]. This fascinating set of mitochondrial functions, from cellular to behavioural, leads us to contemplate the possible association of these multifunctional organelles as a connection between metabolic dysregulation and psychopathology[11].

Management of calcium levels by mitochondria is significant in a cell's life. The Ca^{2+} ions are involved in energy production for cellular activity in buffering and shaping cytosolic calcium concentrations and in determining the fate of the cell by activating or inhibiting programmed cell death[12]. Mitochondria and the processes involved in the regulation of calcium homeostasis have been broadly studied, but they still provide investigators with enduring and new challenges[13]. The Ca^{2+}ion is the principal cellular signal, and variations in intracellular Ca^{2+} regulate cellular processes that are involved in normal functioning and the development of stress-related diseases. Here an investigation on the differences in spatial and temporal mitochondrial sensitivity to Ca^{2+} in the context of restraint stress and aging is carried out. Since, the Ca^{2+}homoeostasis is found to vary with aging, a tissue- and gender-specific discrepancy was detected in the ion transporter activity in all the age groups of mice. Mitochondrial ion channels show a crucial role in synaptic plasticity, through variations in the re-release or uptake of calcium into the nerve cells, which can

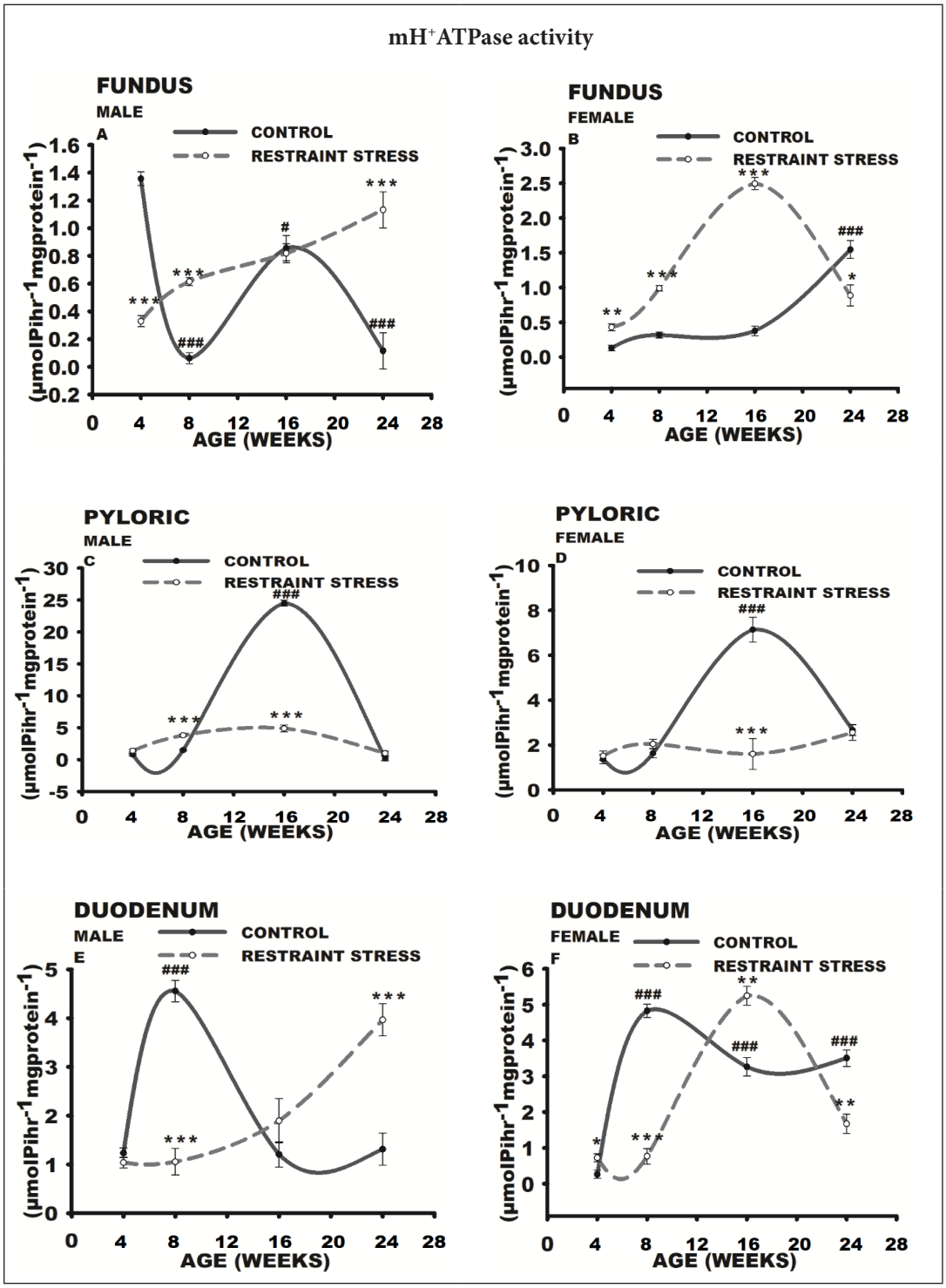

Figure 6. The activity pattern of mH⁺ATPase during aging and restraint stress in the fundus, pyloric and duodenum of male and female mice. Other details, as in Figure 1.

improve or quash neurotransmitter discharge into the synapse and the efficacy of energy production over time (mitochondrial plasticity). Since the Ca^{2+}ion enables the discharge of neurotransmitters from the nerve cells, it is hypothesised that a stress response is a personalised response intended at reinstating the milieu interior, where Ca^{2+} transporter activity also should take effect. In this study, a differential pattern was observed in the Ca^{2+} transporter activity of the cerebral cortex, hippocampus and cerebellum in response to restraint stress. The cut-down in the activity in the cortex of females point to the increased susceptibility of them to Ca^{2+} toxicity after stress exposure. Hippocampus as a target of stress and sex hormones has revealed a considerable degree of structural plasticity and remodelling in the adult brain that differs between the sexes. The absence of any notable change in mitCa^{2+}-ATPase activity in the cerebellum, especially in mature and adult females, tips towards reduced degree of stress-handling during this age in the cerebellar region.

In this study, different regions of the gut presented different patterns in mCa^{2+}-ATPase activity in response to restraint stress, and this may be correlated with their differential potential of Ca^{2+}-absorption. Studies indicate that during aging several aspects of Ca^{2+} homeostasis such as Ca^{2+} influx, release of Ca^{2+} from intracellular spaces and Ca^{2+} uptake processes by the sarcoplasmic reticulum and mitochondria, might be affected[14]. The present study characterized the changes in the intracellular Ca^{2+} stores during stress response in the brain and gastrointestinal smooth muscle from juveniles to mature adults. The results showed that aging alters Ca^{2+} stores and a differential pattern with respect to age, gender and tissue was observed in response to stress. These changes might affect smooth muscle contractility after restraint stress and may be linked to stress-induced delayed gastric emptying.

The F_1F_0-ATPase (or ATP synthase) is an oligomycin-sensitive ATPase in mitochondrial inner membranes. It either enables the synthesis of ATP in a process driven by the Proton Motive Force (PMF) or uses energy from ATP hydrolysis to pump protons against concentration gradient across the membrane[15]. A region-specific differential pattern was noted in the F_1F_0-ATPase activity of mice subjected to restraint stress. The differences in enzyme activity between the cerebrum, hippocampus and cerebellum after stress indicate different requirements of ATP in the regions of the brain. The differential regulation in enzyme activity and ATP requirements may also be due to different responsiveness of these regions to catecholamines and glucocorticoids after restraint stress. These differences might be related to physiological and regionally different functions involved in stress-induced emergency responses such as emotion, arousal level, endocrine and autonomic nervous system responses[16]. We observed age- and gender-specific differences in this study. For instance, in the fundus of juveniles exposed to stress resulted in a decline in F_1F_0-ATPase activity in males, and an increase was noted in females. However, in the pyloric region of stomach and duodenum, juvenile males subjected to restraint stress showed an increase in F_1F_0-ATPase activity and a decline was noted in juvenile females. In adolescent mice, restraint stress resulted in an upsurge in F_1F_0-ATPase in fundus of both male and female mice, but the decline was found in the pyloric region. However, in duodenum of adolescent mice subjected to restraint stress, an increase in F_1F_0-ATPase activity was observed in males, and a decline was noted in adolescent females. Likewise, in young and mature adults, age-, gender- and region-dependent differences were found in the F_1F_0-ATPase activity in response to stress. The observations suggest that during the various phases of the life-cycle the expression, the proper membrane assembly and turn-over of the F_1 and F_0 moieties of the mitochondrial ATP-synthase complex are controlled explicitly by different factors[17]. The enzyme is regulated differently during restraint stress because of the diversity of physiological circumstances under which they function[18].

Modulation of intracellular pH plays a vital role in maintaining physiological processes in living cells since most enzymes function correctly only under a narrow window of pH. The cytoplasm is incessantly acidified by cellular metabolism and the negative resting membrane potential also favors the influx of protons; this poses a constant challenge to the maintenance of cytosolic pH. Homeostasis of intracellular pH depends on the buffering capacity of intracellular bicarbonate concentration, other weak acids or bases and macromolecules. A variety of membrane transporters respond instantaneously to the fluctuation of intracellular pH and extracellular pH[19]. Proton pumps are highly specialized H^+ transporters that utilise cytoplasmic ATP to drive acid equivalents across biological membranes. As such, these transporters exist in the gastric epithelium, membranes of mitochondria and individual cytoplasmic organelles[20].

The pH of intracellular partitions is carefully organised and is critical for several biological processes like coupled transport of small molecules, protein degradation and membrane trafficking[21]. The H^+ transport across the mitochondrial inner membrane is the key of the chemiosmotic theory where the H^+-ATPase produces an electrochemical gradient of H^+ ions. This bafilomycin sensitive ATP-dependent H^+ pump delivers energy in the form of proton-motive force while stopping excess acidification in the inner regions of the organelles[22]. In this work, mH^+-ATPase activity showed a differential response to stress with respect to age, gender and in various regions of the brain and the gut. The notable rise in the mH^+-ATPase activity in a few regions of the brain at different stages of life and its decrease in other regions indicate that stress has both specific and spatial actions on the brain ion transporters. Likewise, in the fundus of males, exposure to stress resulted in a decline in mH^+-ATPase activity of juveniles, but an increase was noted in adolescents, and mature adults and no noteworthychangewas found in young adults. However, in females, an upsurge was foundin the mH^+-ATPase activity of juveniles, adolescents, and young adults and a decrease in activity were found in mature females. In the pyloric region of both males and females, stress-induced alteration in mH^+-ATPase activity was more prominent in young adults, and no significant variation in response to stress was observed in juveniles and mature adults. It may point to the fact that the acidification pattern was more prominent in young adults of both sexes whereas juveniles and mature adults are more prone to stress-induced changes at least in mH^+-ATPase transporter function. In the duodenum of adolescent mice of both sexes, a decline in mH^+-ATPase activity in response to stress was found. However, in the duodenum of mature adults, stress resulted in increased activity in males and a decline in females. This differential pattern of mH^+-ATPase activity points to an ATP-dependent proton pump activity that establishes a proton-motive force which is crucial for the acid-base equivalents of the gut.

Overall, a differential response of mitochondrial ion transporters was observed in the various regions of gut and the brain after restraint stress. Interestingly, different ion transporters showed different stress responses in various stages of life. The results showed that mitochondria as the target of stressor, modifies its ion transport control as evident in the ion transporter responses to restraint stress. Consequently, we could find that the cellular modulation by stressors can be diverse multisystemic stress-responses because of diverse and crucial characteristics of mitochondrial functions. Furthermore, the gender and tissue-specific differences in ion transporter activities could indicate a gonadal hormonal interference, like estradiol-driven sex differences in stress response, as similar influence could affect the working of HPA axis, like the HPA axis sensitivity to glucocorticoid negative feedback mechanisms[23]. The study also showed that response to stressors is varied, underpinning a broad spectrum of distinct changes amongst stress-exposed individuals. Several factors can cause varied responses to stressors and the setting of distinct coping strategies that will lead to individual differences on the susceptibility or resistance to stress[2]. Collectively, we found gender- and age-specific differential patterns of mitochondrial ion transporter response to restraint stress and that point to the crucial interactive role of stress hormones and sex hormones on the energetics and ion transport of brain-gut axis in mice.

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