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Article

Long-Term Effects of Stress During Adolescence on the Sex-Dependent Responses of Thyroid Axis and Target Tissues to Exercise

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Abstract

The response of the hypothalamic-pituitary-thyroid (HPT) to energy demands is perturbed by previous chronic stress perceived during the neonatal or the adult periods. We examined sex-specific effects of chronic variable stress (CVS) during adolescence on the responses of these axes and target peripheral tissues to voluntary wheel-running (Ex) in adult rats. Two weeks of Ex stimulated mediobasal hypothalamus (MBH)-*Pomc* expression in both sexes, but this response was blunted by CVS. CVS altered basal HPA activity only of adult males increasing paraventricular nucleus (PVN) *Gr* and arcuate-*Npy* expression, and serum Cort levels. Ex reduced serum Cort concentration in controls. CVS decreased MBH-*Dio2* and PVN-*Trh* expression, raising total and free T3 levels in females. In males, CVS reduced MBH-*Dio2*, *-Trhde*, and PVN-*Trh* expression, impairing exercise-induced expression and reducing serum T3/T4 ratio. The exercise-induced gene-expression of *Dio2* and *Pgc1a* in skeletal muscle, of *Adrb3*, *Dio2*, *Pparg*, *Hsl* in inguinal and gonadal white-adipose tissues, and of *Adrb3* and *Ucp1* brown adipose tissue was blunted by CVS in both sexes except *Hsl* in inguinal of both and BAT-*Dio2* in brown adipose tissue of females that were not affected by stress. These results support that adolescent stress impacts adult metabolic and neuroendocrine responses to exercise in a sex-specific manner.

Keywords: HPT axis; HPA axis; chronic variable stress; exercise; adolescence; sex differences; skeletal muscle; white adipose tissue; brown adipose tissue

1. Introduction

Modern societies bear the burden of a wide range of metabolic, cardiovascular, and psychological pathologies, many of which stem from a sedentary lifestyle and/or chronic stress, even in the early stages of development [1–3]. Exercise not only activates metabolism but also reduces stress and its detrimental effects, such as depression, preventing or ameliorating many of these diseases [4,5]. Exercise performance requires an immediate and adequate supply of energy, which depends on the concerted and efficient responses of the somatic, sympathetic, and neuroendocrine systems, which communicate with participating organs through neurotransmitters, hormones, and exerkines [5–8]. The hypothalamus is the center of energy homeostasis, as its diverse nuclei decode environmental, nutritional, and psychological signals, integrate them, and send neuronal signals as appropriate commands to the behavioral, sympathetic, and endocrine systems [8]. Neurons of the arcuate nucleus (ARC) expressing POMC or AgRP/NPY sense the energetic status of the organism due to a “loose” brain barrier in this area [8,9]; many of these neurons send afferents to the paraventricular nucleus (PVN) that, through multisynaptic contacts, project to the brainstem, to

sympathetic noradrenergic neurons that innervate peripheral organs; in turn, sensory neurons send information back to the brain, establishing bidirectional communication [9–13].

At the PVN level, neuroendocrine control relies on hypophysiotropic neurons that synthesize thyrotropin-releasing hormone (TRH) or corticotropin-releasing hormone (CRH) and release them from nerve terminals at the median eminence, triggering the activity of the hypothalamus-pituitary-thyroid (HPT: TRH: thyrotropin (TSH): T₄/T₃) [14,15] or hypothalamus-pituitary-adrenal (HPA: corticotrophin-releasing hormone (CRH): corticotrophin (ACTH): cortisol/corticosterone (Cort) axes [16]. Peripheral hormones, such as T₃/T₄ and glucocorticoids, exert feedback control at the hypothalamic and pituitary levels, maintaining homeostasis [14–17] (Supplementary Figure 1). T₃/T₄ and glucocorticoids are pleiotropic hormones and crucial endocrine regulators of energy homeostasis, as they regulate carbohydrate and lipid metabolism in multiple tissues and mobilize energy substrates to oxidizing tissues [17,18]. T₃, the transcriptionally active hormone, is formed from T₄ released from the thyroid and transformed to T₃ by deiodinase 1 (DIO1) in the liver or DIO2 in the median eminence, thyroid, pituitary, and many target tissues, while T₃ may be inactivated by DIO3; DIO2 is exquisitely regulated in a tissue-specific manner, setting the concentration of active T₃ in situ [19,20]. T₃ involved in the feedback of *Trh* transcription is probably formed mainly by DIO2 present in tanycytes, which are special glial cells lining the third ventricle whose prolongations are near TRH nerve terminals and portal vessels in the median eminence, and express the TRH-degrading ectoenzyme (TRH-DE) that inactivates released TRH into the median eminence before entering the portal vessels, thus regulating the concentration of TRH that reaches the thyrotrophs [21–23]. Thus, in “basal conditions” rigorous homeostatic mechanisms keep tissue levels of thyroid hormones with minor alterations mainly due to circadian cycle variations, to feedback inhibition by T₃ and to the regulated activity of tissue deiodinases [14,15,19,23].

The elements involved in HPT axis activity are stimulated in a fast and transient manner by energy demands such as acute cold exposure or increased physical activity, which increase *Trh* expression in hypophysiotropic neurons of the PVN and TSH serum concentration in male rats within the first hour, while serum T₄ or T₃ concentration increases may be detected later [24–28]. In contrast, situations of negative energy balance such as fasting, food restriction, exhaustive exercise, disease as well as various forms of either acute or chronic stress inhibit HPT axis activity [17,20,21,24,26,29,30]. However, the exercise-induced activation of the HPT axis has been questioned due to controversial reports on circulating levels of TSH or thyroid hormones in either humans or animals, which could be due to the type of exercise or energy status at the time of sampling [31]. Involvement of thyroid hormones in exercise is, however, supported by the diminished exercise performance of hypothyroid animals or humans [32].

After cortical motor command generation, exercise initiates with the contraction of skeletal muscles (SKM) that utilize carbohydrates and lipids as their main energy sources in a proportion that depends on intensity and duration of exercise, nutritional status and sex [8,33]. Muscle contraction provokes the release of myokines and metabolites (such as lactate) that signal the brain and various peripheral tissues to activate key metabolic pathways, such as lipolysis in adipose tissue, followed by adaptive changes in the participating organs [8,34]. Fatty acid oxidation and tissue adaptations continue during early recovery after exercise (up to 4h) [35]. Thyroid hormones (TH) are essential in the maintenance and regeneration of muscle cells, as well as for mitochondrial biogenesis by activating peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1- α) [36,37]. TH interact with the adrenergic signaling in a coordinated or synergistic fashion in white and brown adipose tissues (WAT, BAT) where they activate lipolysis, *de novo* lipogenesis, or thermogenesis [38–40]. Furthermore, TH participate in the adequate functioning of the cardiorespiratory system [41].

A diminished activity of the HPT axis may cause effects of hypothyroidism, as demonstrated in adult male rats exposed to a Cort injection or, to two weeks of chronic stress prior to one hour of cold exposure; stressed rats fail to show increased PVN *Trh* expression or serum TSH concentration, their body temperature decreases, and cold-induced BAT thermogenesis is absent [42,43]. Chronic stress also inhibits the HPT response to voluntary exercise: neither the expected increase in PVN *Trh* or

Pomc expression nor the loss of fat mass is detected [44]. Long-term effects caused by stress perceived during critical developmental periods like that imposed by maternal separation, which causes altered HPA axis reactivity in adults [45], blunt the cold-induced activation of PVN TRH neurons and the expression of BAT thermogenic proteins, as well as the response to exercise in adult rats [46,47]. The effects of stress on HPT responses to food deprivation or exercise are sex dimorphic [44,46,47].

Results obtained when combining stress exposure before cold or voluntary exercise support the interference of stress with the fast and efficient responses of the HPT axis required for adequate energy availability, which led us to propose that it may be one of the causes of subclinical hypothyroidism (SCH) [47,48]. Patients with SCH have high serum TSH concentrations but T4 or T3 within the normal range, hypothermia, exercise intolerance, and complaints of fatigue [32,49]. Women have higher incidence of SCH than men, obesity, thyroid diseases and many of stress-related disorders as anxiety and depression; they are also very susceptible to the long-term effects caused by stress during adolescence [17,48–53]. It is during adolescence that circuits between the prefrontal cortex and limbic regions are formed and consolidated, and the HPA axis matures. Stress-disrupting effects may last until adulthood, leading to increased stress reactivity and impulsivity, altered metabolic and neuroendocrine functions, and a high risk of developing psychiatric pathologies [54–58].

To demonstrate that stress-induced HPT axis dysfunction alters the expression of T3 targets in tissues involved in exercise, such as SKM, WAT, and BAT, we studied the long-term effects of chronic variable stress (CVS) during adolescence, a developmental period in which females show higher susceptibility to stress than males. Hence, male and female rats (control or CVS) were exposed to voluntary exercise for two weeks in adulthood; a pair-fed group was included to account for exercise-induced diminished food intake [27,44]. Elements involved in HPT and HPA axes activities (Supplementary Figure 1) were measured to evaluate responses to stress, exercise and food intake; *Pomc* and *Npy* expression in the ARC and serum concentration of leptin as sensors of energy status [9,59], and serum triglyceride concentration as an indirect measure of lipid mobilization [60]. We also measured in SKM and adipose tissues the expression profiles of *Dio2* that is regulated in some tissues by either stress, glucocorticoids or exercise [37,61,62], of *Pgc-1 α* that mediates biogenesis of mitochondria and oxidative capacity, fatty acid utilization and fiber-type switching [39,40], of adrenergic receptor $\beta 3$ (*Adrb3*) responsible of sympathetic-induced activation in all adipose tissues [63], of peroxisome proliferator-activated receptor γ (*Pparg*), a transcription factor that acts as lipid sensor and is involved in lipolytic and lipogenic actions as well as in exercise-induced browning of inguinal WAT (iWAT), and in glucose homeostasis in perigonadal WAT (pgWAT) [64], of hormone sensitive lipase (*HSL*, *Lipe*) that is the rate limiting step of lipolysis [65], and finally of uncoupling protein-1 (*Ucp1*) responsible for thermogenesis in BAT [66].

2. Results

2.1. Behavioral and Ponderal Variables During Adolescence

Behavioral tests were conducted throughout the CVS procedure (Figure 1A) to evaluate the state of animals during treatment, compared to rats from the control group. At day 7 of CVS (at PND 37), spontaneous physical activity was measured in PAS, the least invasive procedure that quantifies spontaneous locomotor activity; compared to controls (C), only CVS females had reduced ambulatory movements in the center and in the periphery (Figure 1B), whereas the total number of movements was similar to that of males (not shown). At PND 44, rats were subjected to the open field test (OFT), in which CVS females spent less time and covered less distance in the center than C females, while males showed no difference, except that total distance values were lower than those of C females (Figure 1C).

The EPM shows an anxious state more clearly, as the animal fears exploring the open arms of the maze; at PND 51, CVS females spent less time and traveled less distance in the open arms than C females (Figure 1D), whilst C and CVS males had similar values (not shown). OFT was repeated at

PND58 and again, only CVS females differed from controls with diminished time and distance in the center (Figure 1E). Males were tested in the EPM at PND 65, and at this time, CVS males showed more anxious behavior than unstressed controls (Figure 1F). Thus, when behavior was evaluated within the time frame of the CVS procedure, the results agreed with various reports *on the higher susceptibility of females to stress during adolescence*, as they showed increased emotional reactivity in the OFT and EPM, compared to males [76–79].

Food intake was higher in males than in females during adolescence (Figure 1G), but if calculated by body weight (relative food intake, RFI), it was higher in females [44] (Supplementary Table 3). Stressed females increased their food intake during the last two weeks of the CVS procedure (Figure 1G) with no difference in body weight (BW) compared to unstressed rats (Figure 1H). CVS males ate as controls (Figure 1G) but had a lower BW by PND 70 (Figure 1H), resulting thus in a higher RFI and a lower food efficiency (FE) than controls as reported [78] (Supplementary Table 3).

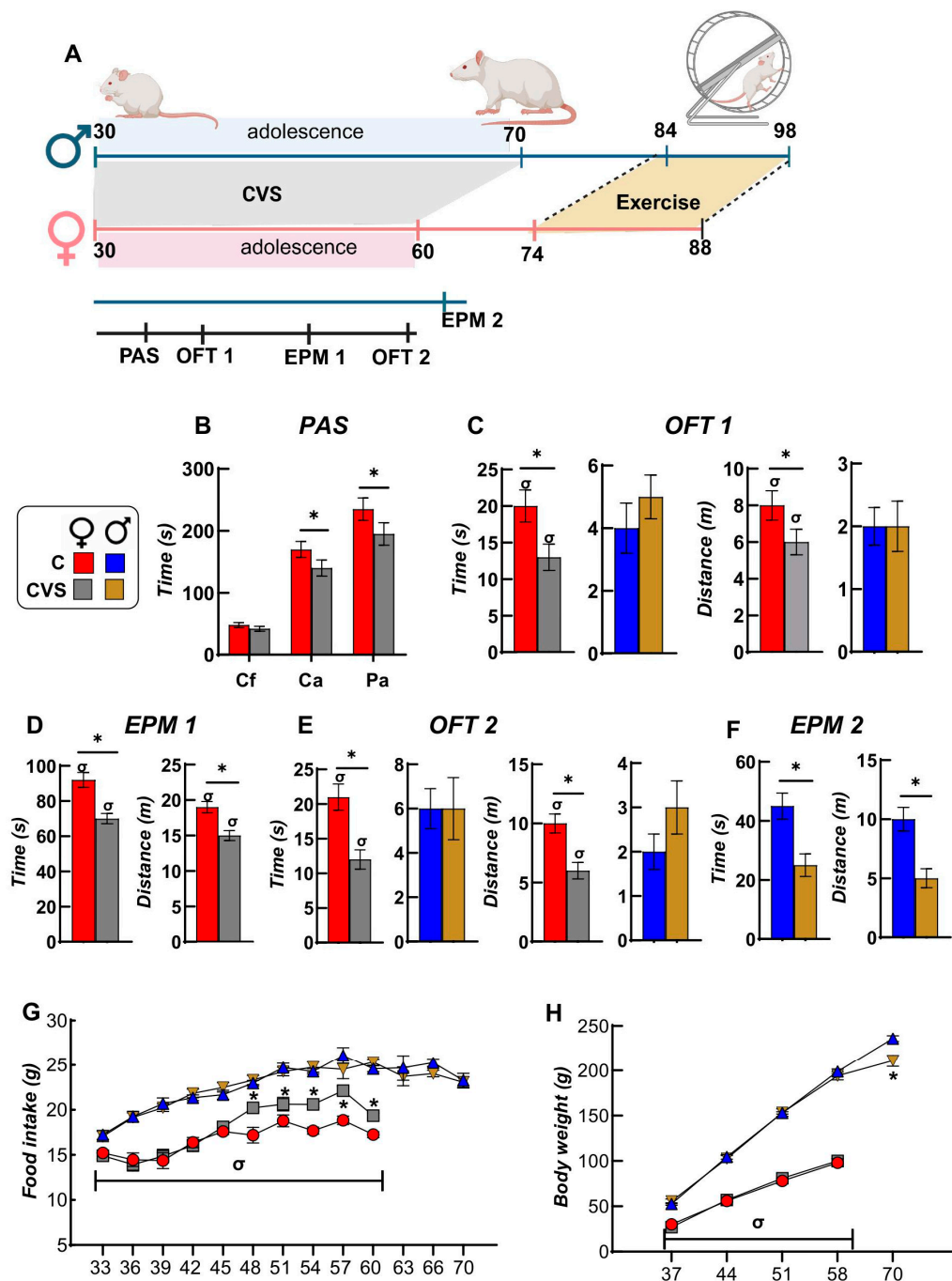


Figure 1. Chronic variable stress (CVS) during adolescence affects anxiety-like behavior, food intake, and body weight more in female rats than in males. (A) Male and female rats underwent the CVS paradigm during adolescence (PND30–70 for males and PND30–60 for females). Behavioral tests were conducted throughout the CVS procedure to assess changes during this period. Exercise was initiated 39 days after the onset of puberty (PND45 for males and PND35 for females). Figure created using BioRender. (B) Spontaneous locomotor activity was assessed using a photobeam activity system (PAS) on PND37 in female rats. The measurements included fine and ambulatory movements in the center of the cage (Cf and Ca) and ambulatory activity in the periphery (Pa). Time spent in the center and distance traveled in the open field test (OFT) were recorded at PND44 (OFT1; C) and PND51 (OFT2; E) for both sexes. Time and distance traveled in the open arms of the elevated plus maze test (EPM) were evaluated at PND58 in females (D) and PND65 in males (EPM2; F) Food intake (G) and

body weight changes (H) were monitored throughout the CVS procedure during adolescence in male and female rats. Results are expressed as mean $\pm$ S.E.M. and analyzed by Two-way ANOVA for each PND, followed by Tukey's post-hoc test. * $P < 0.05$ vs. C group; $\sigma P < 0.05$ vs. males of the same group. C: control group; CVS: chronic variable stress group.

2.2. Long-Term Effects of CVS on Voluntary Exercise

WR is a less stressful paradigm compared to other types of aerobic exercise, in which rats do not run continuously but in bouts, and more intensely during the first hours of dark period [69,80]. Rats were sacrificed three hours after lights were turned on, cage-changed and fresh food provided, at an unknown time after the last running bout; biochemical measurements correspond therefore to a recovery period from the last exercise bout and an unknown postprandial time.

Plots of individual distance covered per day revealed various peaks in C-Ex females, many separated by 4, some by 5 days, coinciding with the estrous cycle stage in rodents [81] (Figure 2A), which contrasts with the flat trend observed in C-Ex males (Figure 2C). Similar patterns were observed in the CVS-Ex groups (Figure 2B,D). Females gradually increased their daily running distance per day (Figure 2A,B). Females had a higher level of locomotion than males in the running wheel (Figure 2A,C), almost five times more (total distance run: females: C-Ex 23.27 km, CVS-Ex 25.74 km; males C-Ex 5.892 km, CVS-Ex 4.992 km) as reported [44,47,80] and observed with these rats in the OFT and EPM performed during adolescence (Figure 1C, E).

Vaginal smears were not taken before exercise (to avoid additional stress) but immediately after decapitation to verify the effect of experimental conditions on the cycle, as stress diminishes serum estradiol concentration or affects cyclicity [82]. At the end of the experiments, the four phases of the cycle were distributed similarly in control and stressed groups, with slight variations in the % of rats in diestrous or metaestrous (Figure 2E). Mean values of the serum concentrations of estradiol and prolactin were not different between experimental groups except for progesterone whose concentration increased in the CVS pair-fed rats (Figure 2F). These results support previous reports suggesting that female rats randomly cycling do not limit overall exercise performance [83].

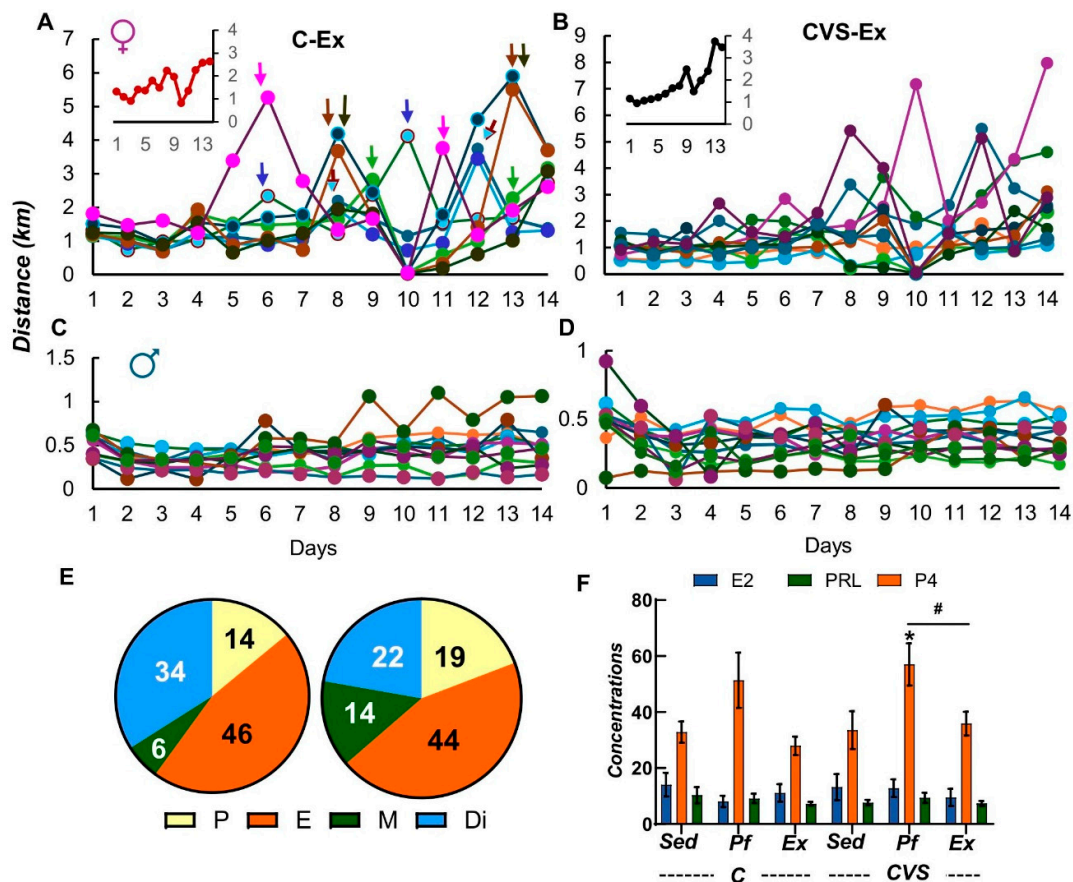


Figure 2. CVS does not affect the activity patterns of cycling female rats, or their hormonal profiles. Daily exercise patterns of female control (A) and rats submitted to CVS (B) reveal periodic peaks aligned with the 4–5-day estrous cycle (C-Ex, indicated by arrows), contrasting with the flat activity trend observed in control (C) and CVS (D) male rats. (E) Distribution of estrous cycle phases determined by cytology according to (Cora et al. Toxicol Pathol. 2015 Aug;43(6):776-93), of control and stressed groups were similar (figure shows all female rats). (F) Serum concentrations of estradiol (E2, pg/mL), prolactin (PRL, ng/mL), and progesterone (P4, ng/mL) in sedentary (Sed), pair-fed (PF), or exercised (Ex) female rats under control or CVS conditions. Stress had a significant effect on serum progesterone levels ($P=0.001$). Results are expressed as mean $\pm$ S.E.M. and analyzed by Two-way ANOVA followed by Tukey's post-hoc test. *: $P<0.05$ vs. C-SED; #: $P<0.05$ vs. CVS-Ex. P, proestrus; E, estrus; M, metestrus; Di, diestrus.

2.3. Voluntary Exercise Decreases Food Intake

Exercise modifies food intake in a manner that depends on its type and intensity, as well as the animal's sex and nutritional status [9,59]. As previously published, two weeks of WR diminished food intake in both sexes compared to sedentary rats [27,44,47], in both C and CVS rats, especially during the first 5 days, and this gradually normalized later in females (Figure 3A,B). Total food intake was lower in females than in males, either for C-Sed or C-Ex rats (Supplementary Table 4), even though females ran much more than males (Figure 2A-D); C-Ex females diminished food intake by 34% compared to that consumed by C-Sed, while in C-Ex males it was 20% lower (Supplementary Table 4) (females: C-Sed $100\pm2\%$, C-Ex $66\pm3\%$, CVS-Ex $72\pm3\%$; Males: C-Sed $100\pm13\%$, C-Ex $76\pm4\%$, CVS-Ex $82\pm5\%$) [44,47]; this level of reduced calorie intake (less than 40%) is considered mild and beneficial [84]. Sedentary rats gained (7-10%) BW from day 1 to 14th of the exercise trial; males more than females, and only CVS males gained less than controls (Supplementary Table 4). Food restriction (PF-rats) reduced body weight gain (BWg) to 2% of the control values in both sexes. Exercise also

decreased BWg, with notable reductions observed in CVS-Ex females and C-Ex males, while no significant loss was detected in CVS-Ex males (Figure 3C,E, Supplementary Table 4).

Lipolysis of WAT provides energy for muscle activity and other oxidizing tissues in situations of negative energy balance, such as food restriction or exercise; the utilization of different fat depots differs according to the task and sex [85]. Compared to C-Sed rats (= 100%), food restriction (C-PF) reduced only pgWAT mass in females (Figure 3D) probably due to their lower food intake, whereas exercise (C-Ex) decreased both visceral (gonadal and retroperitoneal) and subcutaneous (interscapular) WAT mass (Figure 3D). In contrast, CVS in females led to a similar reduction in WAT mass, including rWAT in CVS-PF and CVS-Ex (Figure 3D). Males of both C and CVS groups lost WAT weight only after exercise (Figure 3F). Variations in WAT mass relative to body weight showed similar trends (Supplementary Table 4). Females diminished food intake more than males, results not consistent with the reported higher susceptibility of female than male-pgWAT to mild calorie restriction, but reports are controversial [84,85]. The lack of correspondence between the reduction in BWg and WAT loss supports stress-induced loss of lean mass in males [86].

WAT secretes various hormones, such as leptin, which signals the energy status of the organism. Serum leptin levels correlated positively with iscWAT mass (Figure 3G,J), which agrees with the proportionality of fat mass to leptin blood concentration [87]. Leptin concentration was decreased by food restriction and by exercise in sera of females (Figure 3H), being lower in CVS-PF than C-PF due to the additional inhibitory effect of stress [88]; however, this decrease was counteracted in CVS-Ex (Figure 3H), confirming the effect of exercise in reducing stress [89]. Leptin serum concentration decreased to a similar extent in C-PF, CVS-PF or -Ex in males (Figure 3K) albeit WAT weight did not decrease in PF (Figure 3F) as BWg did (Figure 3E), in agreement with the determining role of general energy status on leptin release and not only of fat mass [87].

Serum triglycerides (Tg) and free fatty acids represent a major energy supply to active tissues; serum Tg concentration is proportional to WAT mass in basal situations, increases in the postprandial state and decreases by removal by active tissues in situations of chronic stress, cold or exercise [27,35,90,91]. There was a clear sex difference in the variations produced by food restriction or exercise. Female Tg serum concentration was lower than that of C-Sed in C-PF, CVS-PF and in C-Ex but not in CVS-Ex (Figure 3I), even though visceral WAT mass decreased in this group. In males, circulating levels of Tg were slightly reduced in C-PF while strongly in both exercised groups (Figure 3L) that did not show as high fat loss as females; these results suggest different dynamics in removal of Tg from blood and probably variations in their source (whether postprandial or from WAT lipolysis). Similar sexual dimorphisms in variation of serum Tg concentrations after exercise have been reported in humans and mice [90,91].

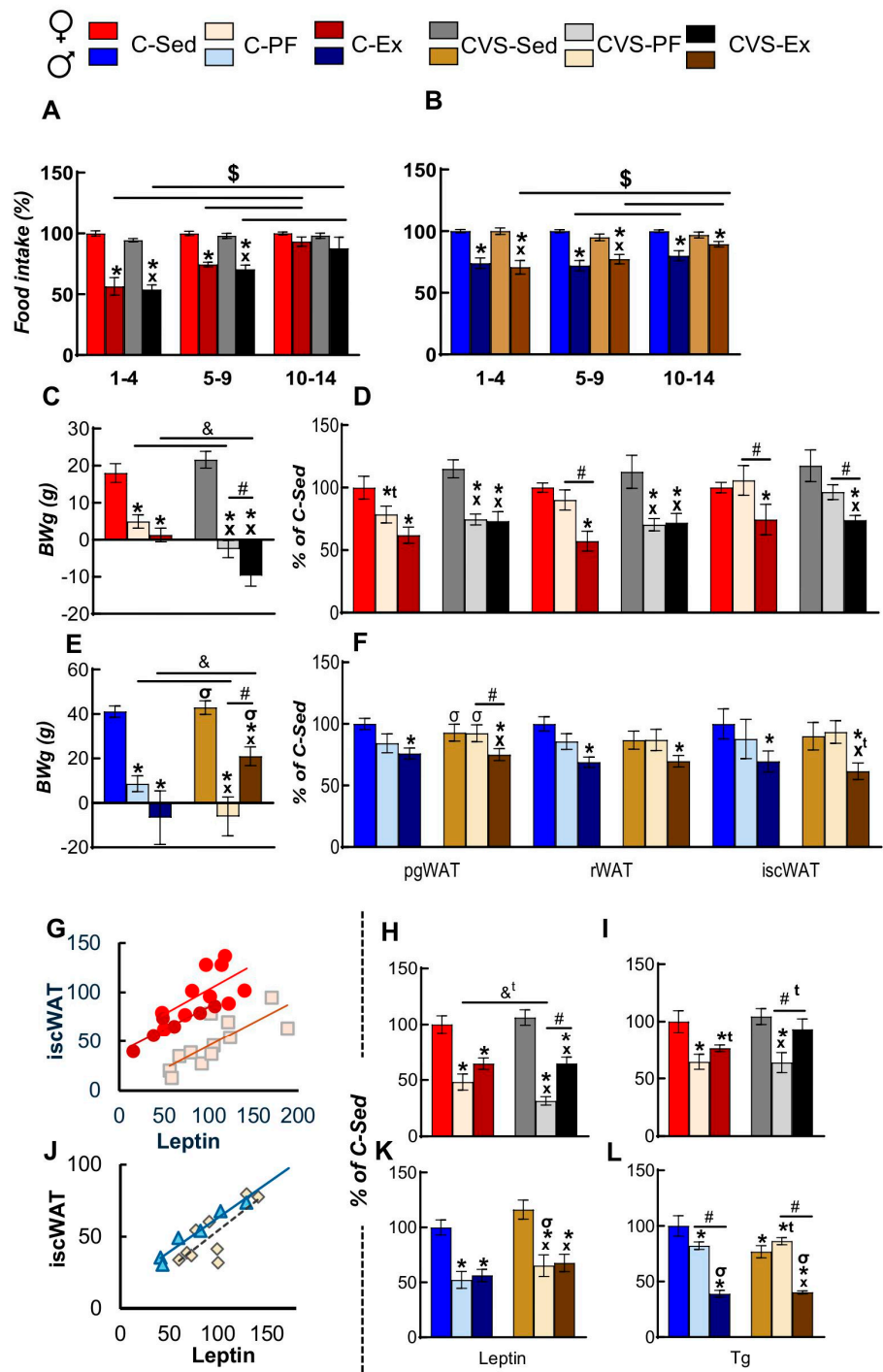


Figure 3. CVS affects the voluntary exercise response of ponderal variables and metabolic hormones in a sex-dependent manner. Female rats began voluntary exercise at PND74, and males at PND84 (considering the adulthood of each sex). Average food consumption over the 14-day exercise protocol in females (A) and males (B). Body weight gain (BWg) and weights of perigonadal (pg), retroperitoneal (r), and interscapular (isc) white adipose tissue (WAT) are shown for females (C, D) and males (E, F). Correlation between iscWAT mass and serum leptin concentration in female control (G) and male PF groups (J). Serum leptin and triglyceride (Tg) concentrations in female (H, I) and male (K, L) rats. Results are expressed as mean $\pm$ SEM and analyzed using three-way ANOVA, followed by Tukey's post-hoc test. A main effect of exercise was found for adipose tissue weights ($P < 0.0001$) and for leptin and triglyceride concentrations ($P < 0.0001$). A significant interaction between

exercise and sex was observed for triglyceride levels ($P < 0.0001$). \$: $P < 0.05$ vs. different day periods within the same group; *: $P < 0.05$ vs. C-Sed; x: $P < 0.05$ vs. CVS-Sed; #: $P < 0.05$ vs. CVS-Ex; &: $P < 0.05$ vs. CVS-PF or CVS-Ex; σ: $P < 0.05$ vs. females of the same group; t: $P < 0.05$ by t-test. C-: control; CVS-: chronic variable stress; Sed: sedentary; PF: pair-fed; Ex: exercise.

2.4. Long Term Effects of CVS on ARC and Serum Markers of Energy Homeostasis in Mild Food Restriction and Exercise

POMC and NPY expressing neurons of the ARC are key players in energy homeostasis as they sense the energy and nutritional status of the organism, inhibiting or stimulating their activity (respectively), as in situations of negative energy balance like food restriction that decreased ARC *Pomc* expression and increased that of *Npy* in PF groups of both females (Figure 4A,B) and males (Figure 4D,E) [87]. As reported for males, exercise increased *Pomc* expression considerably in C-Ex rats of both sexes (Figure 4A,D) [44,92] despite the decrease in serum leptin concentration (Figure 3H,K), but consistent with the role of ARC POMC neurons in physical activity control, and the existence of different subpopulations of POMC neurons containing particular receptors that respond specifically to different stimuli [9,59]. ARC *Pomc* expression was reduced in CVS-PF more than in C-PF, particularly in males (Figure 4A,D). Moreover, the exercise-induced increase in *Pomc* expression observed in controls was abolished in the CVS group of both sexes (Figure 4A,D).

Although leptin decreased similarly after food restriction or exercise suggesting negative energy status in both cases, *Npy* expression increased in PF rats as published [92,93] but not in exercised rats (Figure 4B,E); both groups ate equal amounts but while in PF reduced food intake was forced, in exercise, it was voluntary. A plausible explanation is that NPY is expressed in distinct populations of neurons within the ARC, such that some of these neuronal populations are activated by food restriction, whereas others are inhibited by exercise training. In the present study, ARC *Npy* mRNA levels were elevated in CVS-Sed males, consistent with previous reports in chronically stressed animals. Furthermore, *Npy* expression was further increased in CVS-Ex males (Figure 4E). Females in contrast responded as controls, increasing *Npy* levels in CVS-PF with no change in CVS-Ex (Figure 4B), with levels correlating negatively with ARC *Pomc* values in these latter groups (Figure 4C). Tg correlated negatively with MBH-*Pomc* expression in C-Ex and CVS-PF male rats (Figure 4F). The stress-induced increased expression of ARC *Npy* in males but not in females may be attributable to Cort and testosterone stimulatory transcriptional effects, absent in females probably due to estrogen inhibitory effects [96,97].

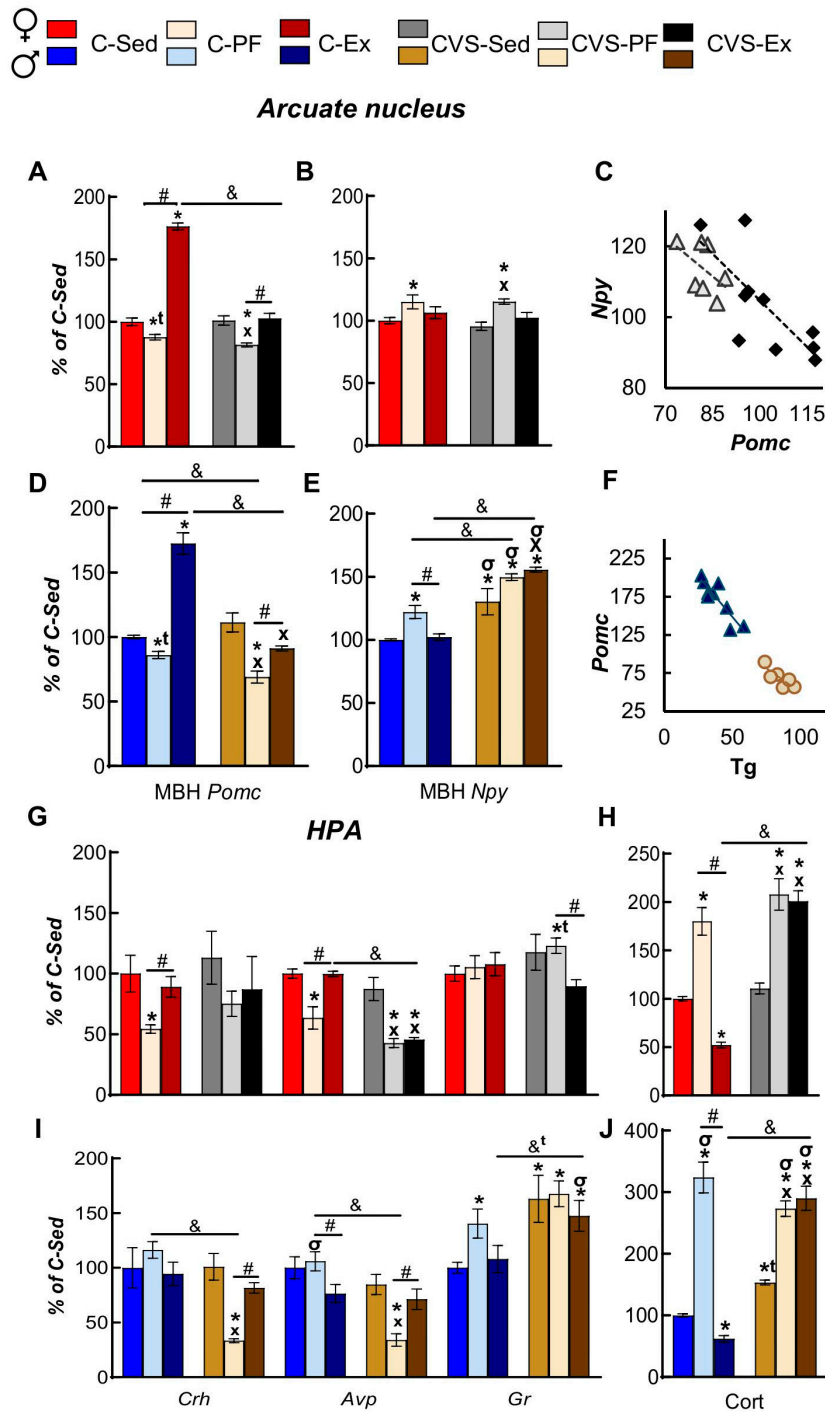


Figure 4. Impact of CVS and voluntary exercise on mRNA levels of neuropeptides in the medial-basal hypothalamus (MBH) and hypothalamic-pituitary-adrenal (HPA) axis activity. mRNA levels of pro-opiomelanocortin (Pomc) (A) and neuropeptide Y (Npy) (B) in the medial-basal hypothalamus (MBH) of female rats. (C) MBH Npy expression was negatively correlated with Pomc expression in the CVS-PF and CVS-Ex female groups. Expression levels of Pomc (D) and Npy (E) in the MBH of male rats. (F) Pomc mRNA levels were negatively correlated with serum Tg concentrations in the C-Ex and CVS-Sed male groups. Expression of mRNA levels of Crh, Avp, and Gr in the medial caudal paraventricular nucleus (PVN) of female (G) and male (I) rats. Serum corticosterone (Cort) concentrations in females (H) and males (J). Results are expressed as mean $\pm$ SEM and analyzed using three-way ANOVA, followed by Tukey's post-hoc test. Pomc and Npy were significantly

affected by activity and stress ($P < 0.000$). Furthermore, Npy expression was influenced by the main effect of sex ($P < 0.0001$). Exercise was a significant factor for Crh ($P = 0.004$) and Avp ($P < 0.0001$) expression levels. Stress also affected Avp ($P < 0.0001$) and Gr expression ($P = 0.0002$), whereas sex had a significant effect on Gr alone ($P < 0.0001$). Analysis of corticosterone (Cort) levels revealed a significant interaction between activity, stress, and sex ($P = 0.0001$), and each factor independently showed a significant main effect ($P < 0.0001$). *: $P < 0.05$ vs. C-Sed; x: $P < 0.05$ vs. CVS-Sed; #: $P < 0.05$ vs. -PF; &: $P < 0.05$ vs. CVS-PF or CVS-Ex; σ : $P < 0.05$ vs. females of the same group; t: $P < 0.05$ by t-test. C-: control; CVS-: chronic variable stress; Sed: sedentary; PF: pair-fed; Ex: exercise.

2.5. CVS Alters HPA Responses to Mild Food Restriction and Exercise

Long-term effects of CVS during adolescence on HPA responsivity to acute psychological stressors show sexual dimorphism [76–79]. We thus evaluated the long-term effects in basal HPA axis activity and in the stress response to food restriction or exercise at adulthood to evaluate changes in HPA axis responses. Basal HPA axis activity was not affected by CVS in adult females, PVN *Crh*, *Avp* or *Gr* expression as well as Cort serum concentration were similar to those of C-Sed (Figure 4G,H). *Crh* or *Avp* expression did not change either in CVS-Sed males, but *Gr* expression and serum Cort concentration increased (Figure 4I,J). The response to calorie restriction differed; in females, PVN *Crh* and *Avp* expression diminished in C-PF and CVS-PF and, *Gr* expression increased in CVS-PF compared to C-Sed; albeit due to the high dispersion of results *Crh* data did not achieve significance in the CVS-PF group (Figure 4G). In males, expression of *Crh* and *Avp* decreased only CVS-PF rats (Figure 4I), as reported in calorie-restricted male rats previously stressed by individual housing [92] or in animals under higher levels of calorie restriction or longer periods [98]. However, all pair-fed groups increased serum Cort concentration as reported [99] (Figure 4H,J); even though females had higher basal Cort concentration than males (C-Sed females: 275 ± 26 ng/mL vs males: 49 ± 3 ng/mL) as reported [47,51], the extent of increase was higher in males (Figure 4H,J).

Exercise prevented the inhibition of PVN-*Crh* and -*Avp* expression caused by food restriction in C-PF as levels in C-Ex or CVS-Ex normalized to those of female C-Sed or male CVS-Sed, except for *Avp* expression in CVS-Ex females which values remained as low as those of CVS-PF (Figure 4G,I). *Gr* expression increased only in CVS-ex males. In both sexes, serum Cort concentration diminished in C-Ex to lower levels than in C-Sed, consistent with the stress-reducing effect of exercise [80]; even though increased serum Cort concentration by WR has been reported [44,100], this did not occur after CVS as values in CVS-Ex remained as high as in CVS-PF (Figure 4H,J).

2.6. Long Term Effects of CVS on HPT Axis Activity in Mild Food Restriction and Exercise

As we have demonstrated that the small food restriction provoked by exercise inhibits the HPT axis in males [27] we included the pair-fed group to evaluate to distinguish the effects of exercise from those caused by the negative energy balance in the neuroendocrine responses of males and females and, to determine if changes could relate to effects on target organs. At the central level, TRH hypophysiotropic activity is regulated by two important enzymes synthesized in tanycytes, present in the MBH fragment dissected: DIO2, which provides T3 for feedback effects on *Trh* transcription [21] and TRH-DE, which inactivates released TRH at the median eminence [22,23]; both enzymes are modulated by fasting [21,101,102].

In females, MBH *Dio2* expression decreased by stress (CVS-Sed) and as reported, increased by exercise (Figure 5A) [27,44]; expression was lower than in C-Sed in all CVS-groups, slightly less in CVS-PF and CVS-Ex groups (Figure 5A). MBH *Trhde* expression was not modified in either group. PVN-*Trh* expression diminished by chronic stress (CVS-Sed) and food restriction (C-PF, CVS-PF) compared to C-Sed (Figure 5A). The inhibitory effect of NPY on PVN *Trh* expression [21] supports the negative relationship between the expression of both genes that extends to other elements of the axis such as TSH and T4 serum concentrations (Figure 5B).

There are controversial reports on the effects of calorie restriction on male and female rodents, but conditions vary considerably between groups [84]. In our conditions, reduction of circulating

TSH concentration by calorie restriction was detected only in CVS-PF, as reported in young rats kept in individual cages and under 33% food restriction for 3 weeks [102]. Serum TSH concentration also diminished in C-Ex but not in CVS-Ex, unlike the pattern detected in PVN *Trh* expression, supporting other factors and dynamics involved at hypothalamic and pituitary levels. No significant effects were observed in the serum concentration of T4 (Figure 5C) while that of T3 increased in C-PF and CVS groups; the serum T3/T4 ratio increased in C-PF, C-Ex and CVS-Sed females but not in CVS-PF or CVS-Ex due to the increased T4 concentration (Figure 5C).

Free thyroid hormones were quantified only in experiment 2 (n=5-6/group); serum ft3 concentration of all CVS groups was higher than in sedentary controls supporting a main effect of chronic stress; the serum ft3/ft4 ratio, an index of metabolic conversion of T4 to T3 [103], followed the trend of ft3 changes but, was significantly different only in CVS-Ex (Figure 5D). Changes in serum ft4 concentration correlated positively with progesterone serum concentration in C-Sed whereas PVN *Trh* expression and serum ft3/ft4 concentrations did in the CVS-Sed and CVS-EX group, respectively (Figure 5E).

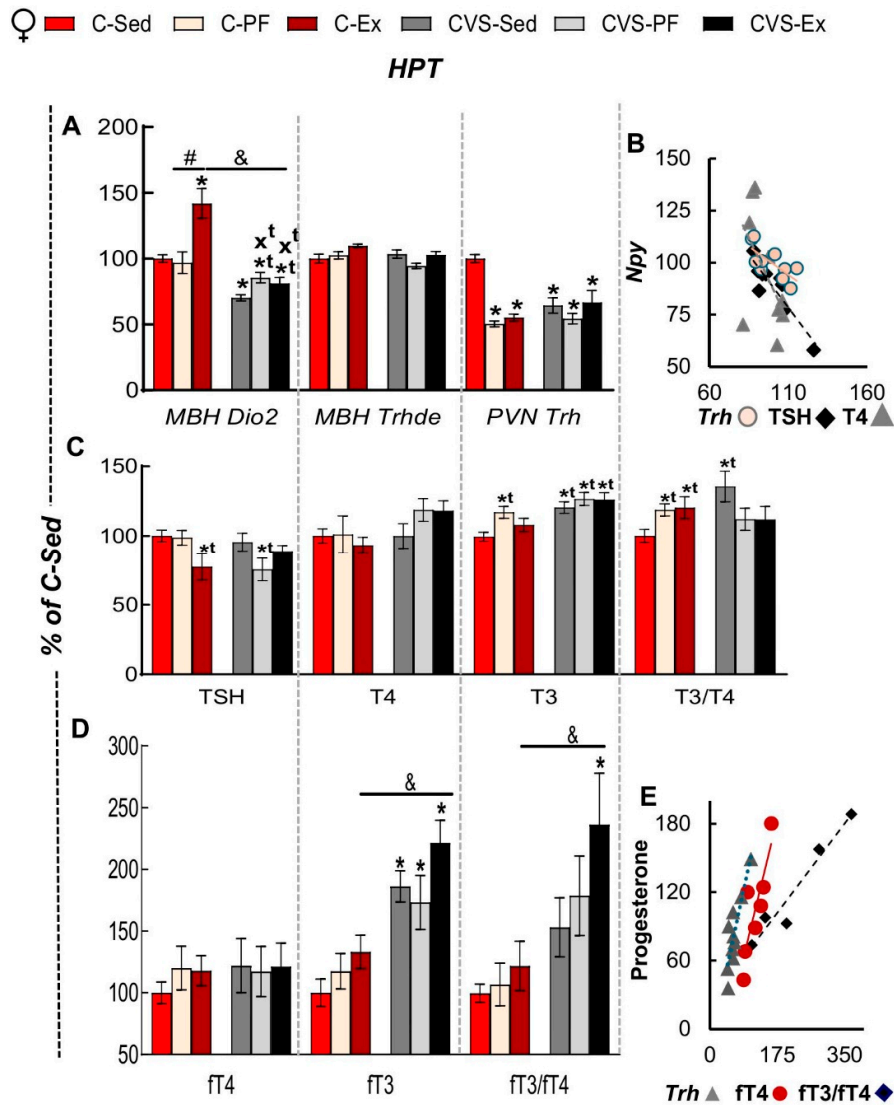


Figure 5. CVS affects the basal activity of the hypothalamus-pituitary-thyroid (HPT) axis and its response to voluntary exercise in female rats. (A) mRNA expression levels of deiodinase type 2 (Dio2) and TRH-degrading

enzyme (*Trhde*) in the medio-basal hypothalamus (MBH) and *Trh* in the medial-caudal paraventricular nucleus (PVN). (B) Correlation between MBH *Npy* expression levels and PVN *Trh* expression (C-PF), TSH (CVS-Ex), and T4 (CVS-Sed) serum concentrations. (C) Serum concentrations of TSH, total T4, and T3 and T3/T4 ratios. (D) Serum concentrations of free T4 and T3 (fT4 and fT3) and the fT3/fT4 ratio. (E) Correlation between serum progesterone and PVN *Trh* expression levels (CVS-Sed), serum fT4 concentration (C-Ex), and fT3/fT4 ratio (CVS-Ex). Results are expressed as mean $\pm$ SEM and analyzed using three-way ANOVA, followed by Tukey's post-hoc test. Exercise and stress impacted MBH *Dio2* and *Trh* in the MBH and PVN, respectively ($P < 0.0001$). Sex was also a significant factor in MBH *Trhde* and PVN *Trh* ($P = 0.001$). This pattern extended to circulating hormones, where activity was the main effect for TSH ($P < 0.0001$) and T4 ($P = 0.03$) levels. The effect of stress was significant for T4 ($P = 0.03$), T3 ($P = 0.0006$), and fT3 ($P < 0.0001$), whereas sex influenced the concentrations of T3, fT4, and fT3 ($P < 0.001$). *: $P < 0.05$ vs. C-Sed; x: $P < 0.05$ vs. CVS-Sed; #: $P < 0.05$ vs. -PF; &: $P < 0.05$ vs. CVS-PF or CVS-Ex; t: $P < 0.05$ by t-test. C-: control; CVS-: chronic variable stress; Sed: sedentary; PF: pair-fed; Ex: exercise.

In males, unlike the reported upregulation of hypothalamic *Dio2* after 25 days of 40% food restriction in male rats [104], it was not affected after of 14 days of 25% restriction (C-PF, Figure 6A). Similar to females, and as reported [27,44], the expression of *Dio2* increased in C-Ex but diminished in all CVS groups (Figure 6A). Expression of *Trhde* decreased by calorie restriction in control animals. CVS-PF and CVS-Ex *Trhde* expressions were higher than CVS-Sed but similar to C-Sed (Figure 6A). MBH *Trhde* expression correlated with that of *Dio2*, explained by the stimulatory effect of T3 on *Trhde* expression and activity (Figure 6B) [22,23]. *Trh* mRNA expression increased in C-Ex while as in females, decreased in C-PF [47] and in all CVS groups (Figure 6A). Circulating TSH concentration decreased by calorie restriction and exercise in controls (C-PF, C-EX) and to a similar extent in all stressed rats (CVS-Sed, -PF, -Ex) (Figure 6C). Serum T4 concentration tended to increase but only C-PF values were significantly higher than C-Sed, while those of C-Ex ($p=0.053$) or CVS- achieved no significance (Figure 6C). No differences were detected on serum T3 concentration (Figure 6C) even though levels in C-Ex correlated positively with distance ran (Figure 6E). However, the ratio of T3/T4 tended to diminish by stress, significantly only in the CVS-Ex group (Figure 6C).

Serum concentration of fT4 decreased by stress, in CVS-Sed and CVS-PF but not in CVS-Ex whose concentration tended to increase (Figure 6D). fT3 concentration was reduced in C-PF and in CVS-PF or CVS-Ex; thus, in consequence, fT3/fT4 was similar and modified only in stressed groups albeit with high variability; statistical differences were detected between CVS-Sed and CVS-PF and CVS-Ex (Figure 6D). Serum concentrations of C-Ex T4 and T3 correlated positively with distance [27,47]; while fT4/fT3 negatively (Figure 6E).

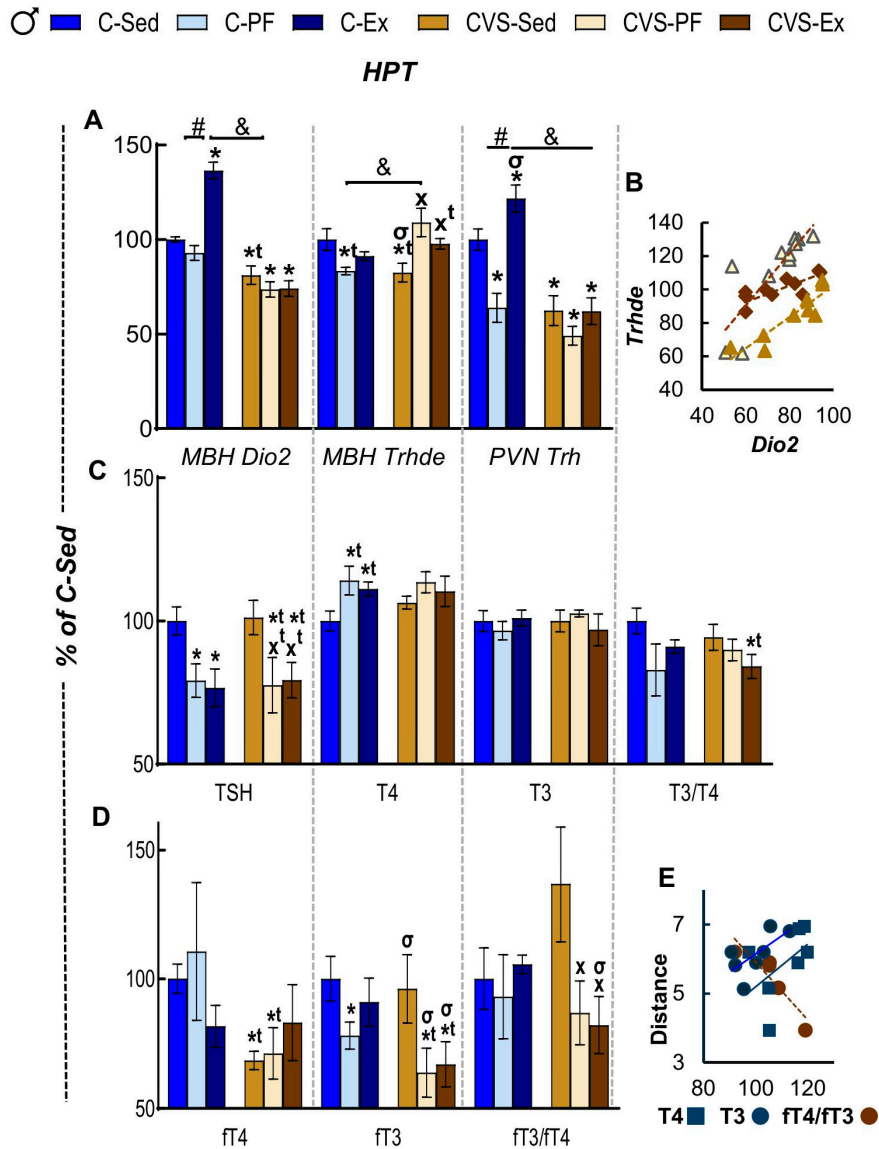


Figure 6. Impact of CVS on the basal activity of the hypothalamus-pituitary-thyroid axis (HPT) and its response to voluntary exercise in male rats. (A) mRNA expression levels of deiodinase type 2 (Dio2) and TRH-degrading enzyme (Trhde) in the medio-basal hypothalamus (MBH) and Trh in the medial-caudal paraventricular nucleus (PVN). (B) Correlation between MBH Trhde and MBH Dio2 expression levels in the CVS groups. (C) Serum concentrations of TSH, total T4, and T3 and T3/T4 ratios. (D) Serum concentrations of free T4 and T3 (fT4 and fT3) and the fT3/fT4 ratio. (E) Correlation between distance ran and serum concentrations of T4 (C-Ex, $P=0.08$), T3 (C-Ex), and fT3/fT4 ratio (CVS-Ex). Results are expressed as mean $\pm$ SEM and analyzed using three-way ANOVA, followed by Tukey's post-hoc test. *: $P < 0.05$ vs. C-Sed; x: $P < 0.05$ vs. CVS-Sed; #: $P < 0.05$ vs. -PF; &: $P < 0.05$ vs. CVS-PF or CVS-Ex; σ : $P < 0.05$ vs. females of the same group; t: $P < 0.05$ by t-test. C-: control; CVS-: chronic variable stress; Sed: sedentary; PF: pair-fed; Ex: exercise.

2.7. Long Term Effects of CVS on Gene Expression in SKM, WAT and BAT in Mild Food Restriction and Exercise

Although changes in the expression of the elements of HPT axis modified by either calorie restriction, exercise or stress are small, the importance of thyroid hormones is evident in cases of thyroid dysfunction that diminish exercise performance [32]. The expression of genes known to be

regulated by either exercise and/or thyroid hormones was thus evaluated in tissues involved in exercise such as muscle, white and brown adipose tissues.

T3 modulates multiple processes involved in muscle function and metabolism, including activation of PGC-1 α , a key regulator in mitochondrial biogenesis [105,106]. Considering that glucocorticoids decrease *Dio2* expression in muscle [107] and exercise stimulates the expression of *Dio2* and *Pgc1a* in muscle of mice and rats after treadmill running, and that *Dio2* inhibition attenuates the exercise-induced stimulation of *Pgc1a* in soleus and gastrocnemius SKM [37], we evaluated if chronic stress would alter their expression or the effect of voluntary exercise. *Dio2* and *Pgc1a* expression levels indeed increased after WR in C-Ex, the latter more in females than males, while pair-feeding caused no effect in either sex, except for a small increase in *Dio2* expression of female rats (Figure 7A,B). Stress decreased *Dio2* expression in CVS-Sed males, and of *Pgc1a* in CVS-PF females, while preventing the exercise-induced increase of both genes detected in C-Ex, though only partially in *Pgc1a* of CVS-Ex males (Figure 7A,B). TSH serum concentration correlated with *Dio2* expression in C-Sed female and C-Ex male rats coincident with the TSH stimulatory effect reported in BAT of cold exposed male rats and in cultured brown adipocytes [108,109].

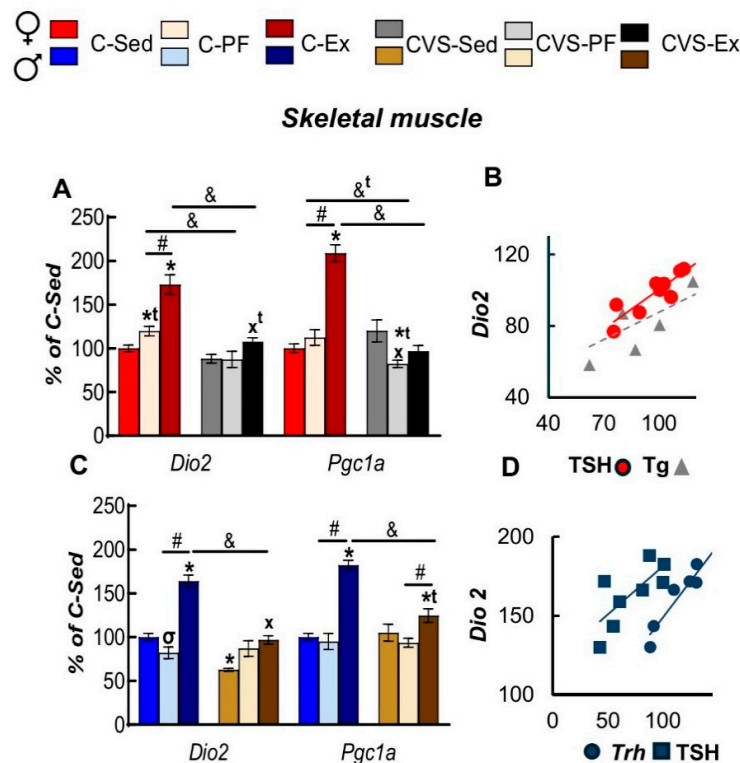


Figure 7. CVS alters the response to voluntary exercise on mRNA expression of genes that regulate the response of thyroid hormones in skeletal muscles in a sex-dependent manner. mRNA levels of deiodinase type 2 (*Dio2*) and peroxisome proliferator-activated receptor γ coactivator 1 α (*Pgc1a*) in the skeletal muscle of female (A) and male (B) rats. (C) Correlation between muscle *Dio2* expression levels and serum concentrations of TSH and triglycerides (Tg) in C-Sed and CVS-PF female rats. (D) Correlation between serum corticosterone (Cort) concentration and expression levels of muscle *Dio2* and *Pgc1a* in CVS male groups. (E) Correlation between muscle *Dio2* expression and PVN Trh expression (C-Ex), serum TSH (C-Ex), and T3 (CVS-SED) concentrations. Results are expressed as mean $\pm$ SEM and analyzed using three-way ANOVA, followed by Tukey's post-hoc test. The expression of *Dio2* and *Pgc1a* was significantly affected by exercise ($P < 0.0001$) and stress ($P < 0.0001$). Furthermore, a main effect of sex ($P = 0.0003$) was observed on *Dio2* expression. *: $P < 0.05$ vs. C-Sed; x: $P < 0.05$ vs. CVS-Sed; #: $P < 0.05$ vs. -PF; &: $P < 0.05$ vs. CVS-PF or CVS-Ex; σ : $P < 0.05$ vs. females of the same group; t: $P < 0.05$ by t-test. C-: control; CVS-: chronic variable stress; Sed: sedentary; PF: pair-fed; Ex: exercise.

2.7.1. Inguinal and Perigonadal WAT (iWAT, pgWAT)

Lipolysis is activated in adipose tissues by the stimulated sympathetic nervous system through the adrenergic receptor β_3 (*Adrb3*) [60] which stimulates PKA-induced phosphorylation of deiodinase 2 and of hormone-sensitive lipase, as well as the synthesis of *Dio2* and of *PPAR γ* , a transcription factor involved in many aspects of fatty acid metabolism and glucose homeostasis [110]. We thus evaluated the expression of these genes in subcutaneous inguinal WAT that undergoes several adaptations to exercise, and in visceral WAT that provides fuel in negative energy situations whose utilization differs according to the task and sex [85,111].

Expression of the adrenergic receptor β_3 (*Adrb3*) decreased by chronic stress in both WAT depots of female and male CVS-Sed rats (Figure 8A-D) which could relate to the inhibitory effect of dexamethasone on cultured adipocytes [112,113]. This receptor synthesis was also diminished by food restriction in either C- or CVS-PF rats of both sexes (Figure 8A-D) as reported in male rats submitted to conditions of higher calorie restriction [114,115]. A similar expression profile was detected for *Dio2* after food restriction, yet inhibited by stress only in CVS-Sed males (Figure 8B,D). *Pparg* expression differed among WAT depots, it was slightly decreased in iWAT of C-PF females but more in pgWAT, whose levels were lower than C-Sed in C-PF of both sexes and lowest in CVS-PF of males (Figure 8A,D); the higher reduction in CVS-PF is consistent with the inhibitory effect of Cort [116] and of stress-induced inhibition of this transcription factor in visceral WAT proposed as one of the mechanisms responsible in stress-induced diminished glucose tolerance [64].

Exercise stimulated the expression of *Adrb3* as expected [111,117,118] so as that of *Dio2* and *Pparg* [119] in both WAT depots (Figure 8A-D), increasing similarly in pgWAT and iWAT of females (Figure 8A,C) and pgWAT of males (Figure 8D) while only barely in males-iWAT (Figure 8B). Stress prevented the exercise-induced stimulation of this receptor in iWAT, levels of CVS-Ex remained as low as CVS-PF compared to a lesser inhibition in pgWAT since levels were higher than CVS-Sed though still lower than those of C-Ex (Figure 8A-D). *Dio2* expression followed that of *Adrb3* in all groups (Figure 8D) except in CVS-Ex of female iWAT whose levels were higher than C-Sed or CVS-Sed but lower than C-Ex (Figure 8A) consistent with the sex differences in WAT response to activation [63]. Exercise induced stimulation of *Pparg* similarly in all groups and this was curtailed by CVS.

In contrast to the general inhibition caused by food restriction on the expression of the analyzed genes, (Figure 8, Supplementary Figure 2), that of hormone sensitive lipase differed. HSL is the rate limiting enzyme in triglyceride hydrolysis [65], whose activity is regulated by β -adrenergic receptor-stimulated cAMP-PKA induced phosphorylation [65] and its expression is increased by glucocorticoids, exercise and calorie restriction [65,119–121]. *Hsl* (*Lipe*) expression increased by exercise and also by food restriction in both fat depots of C-Ex and C-PF, more in males than females even though females ran much more than males (Figure 8A-D). CVS increased *Hsl* expression in iWAT of CVS-Sed males, and more in CVS-PF and CVS-Ex than CVS-Sed in both sexes but higher stimulation in males than females (Figure 8 A,B). In contrast, *Hsl* expression of pgWAT was inhibited in CVS-Sed more in females than males, values remained low in CVS-PF or CVS-Ex except for a small increase in CVS-PF compared to CVS-Sed of males (Figure 8C,D).

Various parameters of the HPT axis correlated positively with the expression of *Adrb3* in pgWAT of both sexes (Figure 8E,G) similarly with iWAT (Supplementary Figure 5,6) and with *Hsl* in iWAT (Figure 8F,H). A positive effect of T4 on the expression of *Adrb3* has been reported [38]. Positive correlation between HPT and *Hsl* coincides with lipolytic actions [39].

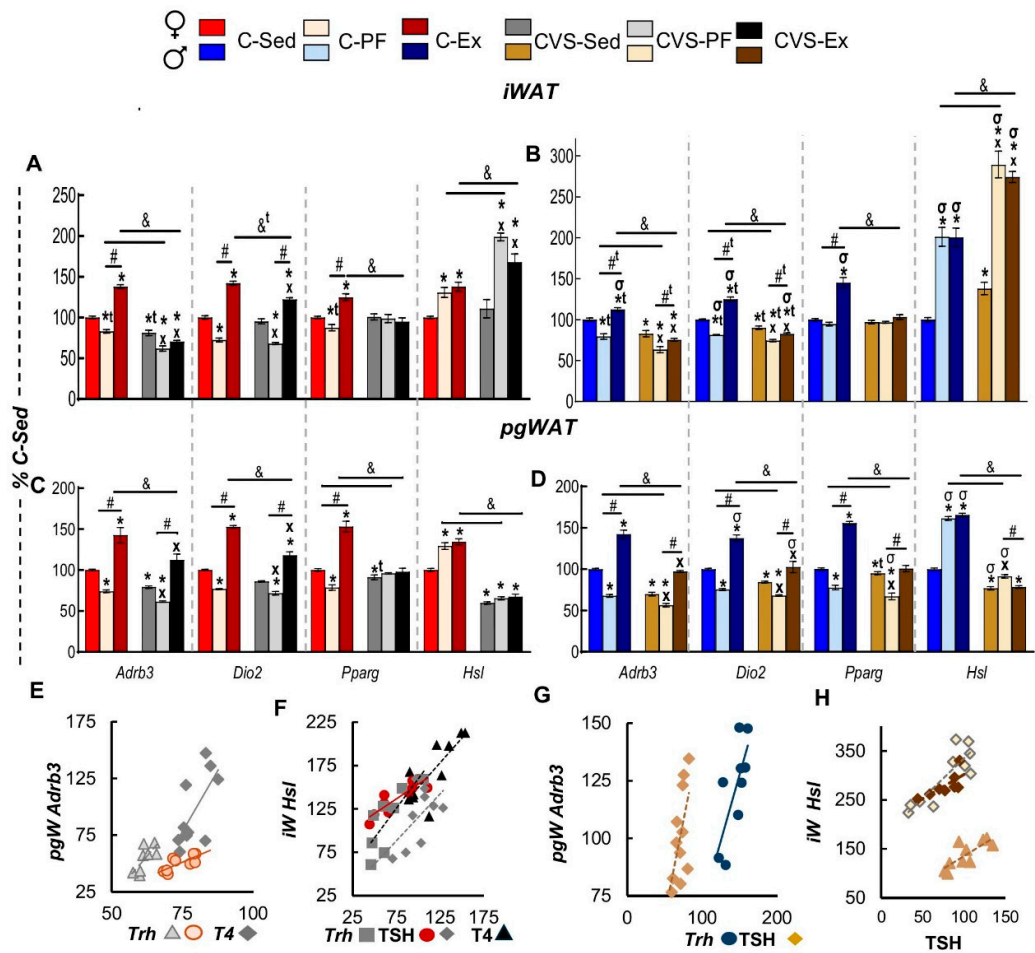


Figure 8. Sex differences in mRNA expression of genes that regulate lipid metabolism in inguinal (iWAT) and perigonadal (pgWAT) white adipose tissues. mRNA levels of adrenergic beta 3 receptor (Adrb3), diiodinase type 2 (Dio2), peroxisome proliferator-activated receptor gamma (Pparg), and hormone-sensitive lipase (Hsl) in iWAT of female (A) and male (B) rats. mRNA levels of Adrb3, Dio2, Pparg, and Hsl in pgWAT of female (C) and male (D) rats. (E) Correlation between pgWAT Adrb3 expression levels and PVN-Trh expression (C- and CVS-Pf) and serum T4 (CVS-Sed) concentrations. (F) Correlation between iWAT Hsl expression and PVN Trh expression (CVS-Sed), serum TSH (C-Ex, CVS-Sed), and T4 (CVS-Ex) concentrations. (G) Correlation between pgWAT Hsl expression and serum corticosterone (Cort) levels in females (CVS-Ex) and males (CVS-Ex). (H) Correlation between pgWAT Adrb3 expression and PVN Trh expression (C-EX) and serum TSH (CVS-Sed) levels in male rats. (I) Correlation between iWAT Hsl expression and serum TSH levels in male CVS- groups. Results are expressed as mean $\pm$ SEM and analyzed using three-way ANOVA, followed by Tukey's post-hoc test. Exercise ($P < 0.0001$) and stress ($P < 0.0001$) had significant effects on all genes measured (Adrb3, Dio2, Pparg, and Hsl) across both fat depots. The main effect of sex was also significant for nearly all genes, including Adrb3, Dio2, and Hsl, in both tissues ($P < 0.001$). The exception was Pparg, which was significantly affected by sex only in the iWAT ($P = 0.001$) but not in the pgWAT. *: $P < 0.05$ vs. C-Sed; x: $P < 0.05$ vs. CVS-Sed; #: $P < 0.05$ vs. -PF; &: $P < 0.05$ vs. CVS-PF or CVS-Ex; σ : $P < 0.05$ vs. females of the same group; t: $P < 0.05$ by t-test. C-: control; CVS-: chronic variable stress; Sed: sedentary; PF: pair-fed; Ex: exercise.

2.7.2. Brown Adipose Tissue

BAT is a highly metabolically active adipose tissue activated by cold exposure where norepinephrine and T3 act in synergy, enhancing thermogenesis by the energy released from mitochondrial membranes due to the uncoupling protein [66]. Involvement of BAT in exercise has been questioned due to conflicting results, but its participation in lipid and glucose homeostasis, as

well as its activation during exercise, is now recognized [84,119,123]. BAT response to either stress, food restriction or exercise varied in a sex dependent manner; mRNA levels of *Adrb3* and of *Ucp1* decreased by stress in CVS-Sed males, and by calorie restriction in C-PF males (C-Sed) (Figure 9C). In contrast, *Adrb3* expression increased in stressed males and females under calorie restriction (CVS-PF) (Figure 9A,C). Exercise enhanced mRNA levels of *Adrb3*, *Dio2* and *Ucp1* in C-Ex males and females (Figure 9A,C) whereas stress prevented the exercise-induced stimulation of *Adrb3* and *Ucp1* in CVS-Ex females and of *Dio2* and *Ucp1* in CVS-Ex males (Figure 9C) [62]. *Adrb3* levels of CVS-Ex males were as low as those of CVS-PF (Figure 9C) while *Dio2* expression in female increased to the same level as in C-Ex (Figure 9C). *Adrb3* expression varied proportionally to Cort concentrations only in females where it was stimulated in CVS-Sed, while decreased in males, consistent with the dual effect of Cort on the expression of this receptor [124].

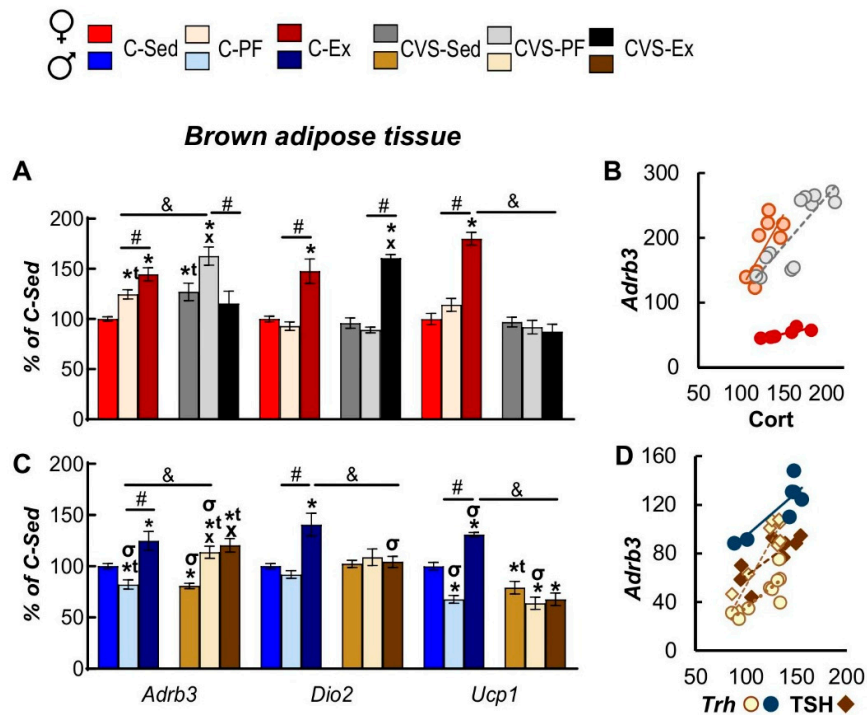


Figure 9. CVS alters the effects of voluntary exercise on mRNA expression of thermogenic genes in brown adipose tissue (BAT) in a sex-dimorphic manner. mRNA levels of adrenergic beta 3 receptor (*Adrb3*), deiodinase type 2 (*Dio2*), and uncoupling protein 1 (*Ucp1*) in the brown adipose tissue of female (A) and male (C) rats. (B) Correlation between BAT-*Adrb3* expression and serum corticosterone (Cort) levels in C- and CVS-PF and C-Ex female rats. (D) Correlation between BAT-*Adrb3* expression and PVN *Trh* expression (C-Ex, CVS-PF) and serum TSH levels (CVS-Ex). Results are expressed as mean $\pm$ SEM and analyzed using three-way ANOVA, followed by Tukey's post-hoc test. Exercise influenced the expression of all measured genes: *Adrb3*, *Dio2*, and *Ucp1* ($P < 0.0001$ for all). The main effect of sex was also significant for *Adrb3* and *Ucp1* expression ($P < 0.0001$). Finally, a significant effect of stress was observed only for *Ucp1* ($P < 0.0001$). *: $P < 0.05$ vs. C-Sed; x: $P < 0.05$ vs. CVS-Sed; #: $P < 0.05$ vs. -PF; &: $P < 0.05$ vs. CVS-PF or CVS-Ex; σ : $P < 0.05$ vs. females of the same group; t: $P < 0.05$ by t-test. C-: control; CVS-: chronic variable stress; Sed: sedentary; PF: pair-fed; Ex: exercise.

2.8. Correlation Analyses

Linear correlation analyses revealed several changes in either gene expression or serum hormone concentrations that were proportional to variations in several of the variables measured (some depicted in corresponding figures) and also among different tissues; although they do not represent a causal relationship, various correlations reflected known physiological functions

(mentioned in various figures) and thus supported the reliability of results. Representing the correlations among the different variables in Hive plots allowed us to recognize many associations in the exercised rats between components of the HPT axis and target molecules in peripheral tissues (Figure 10; *note: abbreviations of tissues and genes in this section correspond to those used in the hive plots*).

Exercise induced associations in with various elements of the HPT axis (Figure 10). For example, in females *Trh* related negatively with scW, FI consistent with the inhibitory action of TRH on food intake [125]; TSH correlation with gW *Pprg*, BAT D2, BAT *Ucp1*, scW mass, and FI, coincides with known effects of TSH [109,122]. Stress hindered most of C-Ex correlations but generated others, more negative than in C-Ex (Figure 10 and Supplementary Figure 5,6). Many more were related in C-Ex males than in C-Ex females as for example, in males, variations of *Trh* and TSH values associated positively with *M D2*, gW *Ab3* and BAT D2 and negatively with gW D2; *Trh* correlated positively with BAT Ab3, negatively with *T4*, *T3* and gW *Hsl*, while TSH positively with PRL, gW *Hsl*, negative with iW *Hsl* and *Crh*.

Compared to the exercised controls, less associations were found in the pair-fed groups and even less in sedentaries, many of them different from those registered in exercised groups (Supplementary Figure 3,4). Hive plots illustrated various “hubs” where one variable associated with several others, for example between the concentrations of *Npy* and with several variables in C-Pf CVS-Sed and C-Pf of both sexes and CVS-Ex of females supported by the NPY effects on lipid and carbohydrate metabolism, energy expenditure and body temperature regulation [126]. TSH showed over 3 different associations in CVS-PF of females as well as the C-PF, CVS-Pf, C-Ex and CVS-Ex of males (Supplementary Figure 3,4); *T4* in CVS-Sed and CVS-Ex females whereas *T3* in CVS-PF and CVS-Ex females; multiple associations are justified by the effects of TH and TSH including the presence of TSH-receptor in several tissues [108,109,122]. Correlation of *Trh* with elements of SkM and BAT is supported by the effect of TRHergic neurons of the PVN on sympathetic neurons that contact these tissues [127].

Importantly, Hive plots revealed several correlations among tissues that support the concerted responses of various tissues in response to energy demands. These include muscle *Dio2* in CVS-Sed females and of males to a lesser degree, so as in C-PF. Males displayed various hubs between tissues: gW *Pparg* in C-PF and CVS-Ex; iW *Pparg* in C-PF, iW *Hsl* in C-Ex, *UCP1* in C- and CVS-Sed (Figure 10, Supplementary Figure 4).

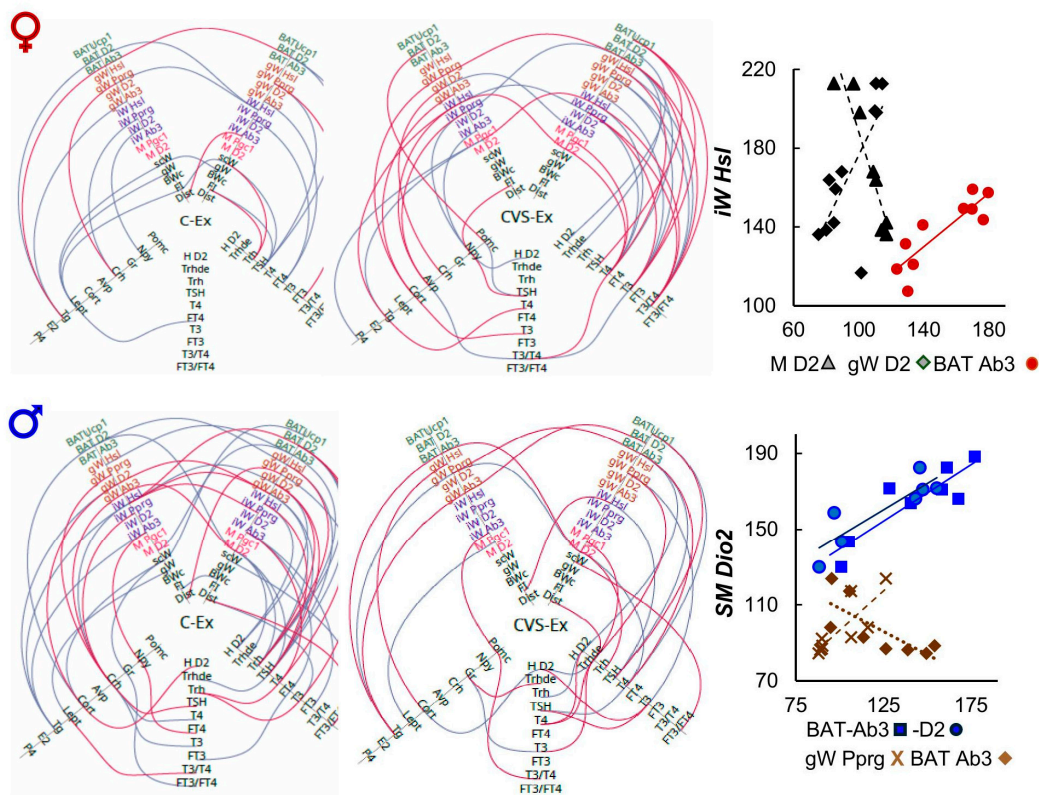


Figure 10. Hiv eplot of correlations between gene expression in the hypothalamus, peripheral tissues, and serum hormones of exercised rats. Hive plot diagrams illustrate significant ($P < 0.05$) Pearson correlations among gene expression levels in the hypothalamus and peripheral tissues and circulating hormone concentrations in exercised female and male rats. Blue edges denote positive correlations and red edges denote negative correlations. H, hypothalamus; gW, gonadal white adipose tissue (WAT); iW, inguinal WAT; M, skeletal muscle; scW, interscapular subcutaneous WAT; FI, food intake; BWc, body weight; Dist, total distance traveled (exercise performed). Hive plots created with software: https://codeberg.org/rgarcia-herrera/neuro_endocrine_correlations.

3. Discussion

Chronic stress has been implicated in various metabolic and psychiatric disorders, especially when experienced during critical developmental periods, such as the perinatal stage or adolescence, leading to long-lasting effects [3,54,128]. The ameliorative effects of exercise on stress-induced disruptions are actively investigated, with most research focusing on exercise performed before or concurrently with stress exposure [129]. Fewer studies address the immediate or long-term effects of chronic stress prior to exercise on performance and plasticity [130]. We previously reported that rats exposed to stress during the neonatal period or adulthood exhibit inhibited HPT axis responses to energy demands, such as cold exposure or exercise. Here, we examined how chronic stress during adolescence affects the neuroendocrine and metabolic responses to exercise in adult male and female rats. Adolescence was chosen to ensure detectable long-term effects in females, who are more resilient to stress at other stages of life [76,78,131].

Behavioral tests during adolescent chronic variable stress (CVS) confirmed that females exhibited stress effects from early puberty [77,131,132], whereas males showed effects lasting until late adolescence, possibly reflecting a near adult stage [57,133]. The results summarized in Table 1 reveal the differential stress effects on adult responses to negative energy challenges, such as mild calorie restriction (CR) and voluntary exercise. Mild CR inhibited several studied parameters, while

exercise stimulated them; prior chronic stress prevented these exercise-induced effects. Validation of the paradigm through the evaluation of HPA axis components in adulthood revealed persistent effects of adolescent CVS in both sexes. Although behavioral tests confirmed increased anxiety in CVS females, basal HPA axis alterations were only observed in males. CVS sedentary males showed activation characterized by elevated serum corticosterone, PVN glucocorticoid receptor, and ARC *Npy* expression [76,77]. Previous studies on adult male CVS effects have been inconsistent, reporting either no changes in PVN *Trh* or circulating TSH/thyroid hormones [43,134] or basal hormonal suppression [29,30,135,136].

Table 1. Summary of responses to 14 days of food restriction (pair-fed) or exercise in female or male adult control or previously stressed rats during adolescence (CVS). Arrows indicate significant changes compared to those in the control sedentary group (C-Sed). The direction of the arrow denotes the nature of the change (↑ increase; ↓ decrease). The size and boldness of the arrows represent differences in the level of change. Filled cell colors highlight sex-specific effects, either a significant change observed in only one sex or opposite responses between sexes.

	FEMALES						MALES					
	C			CVS			C			CVS		
	Sed	PF	Ex	Sed	PF	Ex	Sed	PF	Ex	Sed	PF	Ex
Food intake	--	↓	↓	--	↓	↓	--	↓	↓	--	↓	↓
ΔBW	--	↓	↓	--	↓	↓	--	↓	↓	--	↓	↓
pgWAT	--	↓	↓	--	↓	↓	--	↓	↓	--	↓	↓
rWAT	--	↓	↓	--	↓	↓	--	↓	↓	--	↓	↓
iscWAT	--	↓	↓	--	↓	↓	--	↓	↓	--	↓	↓
Leptin	--	↓	↓	--	↓	↓	--	↓	↓	--	↓	↓
TG	--	↓	↓	--	↓	↓	--	↓	↓	↓	↓	↓
Pomc	--	↓	↑	--	↓	↓	--	↓	↑	--	↓	↓
Npy	--	↑	--	--	↑	--	--	↑	--	↓	↑	↑
Crh	--	↓	--	--	--	--	--	--	--	--	↓	--
Avp	--	↓	--	--	↓	↓	--	--	--	--	↓	--
Gr	--	--	--	--	↑	--	--	↑	--	↑	↑	↑
Cort	--	↑	↓	--	↑	↑	--	↑	↓	↑	↑	↑
MBH Dio2	--	--	↑	↓	↓	↓	--	↑	↑	↓	↓	↓
Trhde	--	--	--	--	--	--	--	↓	--	↓	--	--
Trh	--	↓	↓	↓	↓	↓	--	↓	↑	↓	↓	↓
TSH	--	--	↓	--	↓	--	--	↓	↓	--	↓	↓
T4	--	--	--	--	--	--	--	↑	↑	--	--	--
fT4	--	--	--	--	--	--	--	--	--	↓	↓	--
T3	--	↑	--	↑	↑	↑	--	--	--	--	--	--
T3/T4	--	↑	↑	↑	↑	--	--	--	--	--	↓	↓
fT3	--	--	--	↑	↑	↑	--	↓	--	--	↓	↓
fT3/fT4	--	--	--	--	--	↑	--	--	--	--	--	--
SM Dio2	--	↑	↑	--	--	--	--	--	↑	↓	--	--
SM Pgc1a	--	--	↑	--	↓	--	--	--	↑	--	--	↑
iW Adrb3	--	↓	↑	↓	↓	↓	--	↓	↑	↓	↓	↓
iW Dio2	--	↓	↑	--	↓	↑	--	↓	↑	↓	↓	↓
iW Pparg	--	↓	↑	--	--	--	--	--	↑	--	--	--
iW Hsl	--	↑	↑	--	↑	↑	--	↑	↑	↑	↑	↑
pgW Adrb3	--	↓	↑	↓	↓	--	--	↓	↑	↓	↓	--
pgW Dio2	--	↓	↑	--	↓	↑	--	↓	↑	↓	↓	--
pgW Pparg	--	↓	↑	↓	--	--	--	↓	↑	↓	↓	--
pgW Hsl	--	↑	↑	↓	↓	↓	--	↑	↑	↓	--	↓
BAT Adrb3	--	↑	↑	↑	↑	--	--	↓	↑	↓	↑	↑
BAT Dio2	--	--	↑	--	--	↑	--	--	↑	--	--	--
BAT Ucp1	--	--	↑	--	--	--	--	↓	↑	↓	↓	↓

In our study, adolescent CVS reduced PVN-*Trh* expression and serum-free T4 levels in males, as well as MBH *Dio2* and *Trhde* expression. Such reductions could favor HPT axis activation by diminishing inhibitory T3 feedback and enhancing *Trh* transcription and TRH availability to the thyrotrope. However, clarification requires kinetic studies, as these changes may be transient. In females, PVN-*Trh* and MBH-*Dio2* also decreased slightly, whereas total and free T3 serum concentrations increased (Table 1; detailed T3 discussion follows).

Consistent with previous reports [27,44], exercised rats consumed 24–28% less food than controls, paralleling CR levels known to extend lifespan. Responses to CR differ by sex: females lose less white adipose tissue (WAT) and lower energy expenditure more than males at equivalent intake (30 or 40% CR) [84,114]. Here, females ran more and consumed less than males, explaining findings such as gonadal WAT loss, which was evident only in pair-fed females. Most expected metabolic changes appeared in both sexes at varying degrees. Exceptions included similar leptin reductions and ARC *Pomc* decreases with ARC *Npy* increases in control pair-fed rats [87,88,98]. CR inhibited central HPA activity in pair-fed females (reduced PVN *Crh* and *Avp* expression), paralleling findings in CVS females post-stress [78]; both CVS pair-fed females and males showed diminished PVN *Crh* and *Avp* mRNA levels [92,98]. The CR-induced PVN *Crh* and *Avp* suppression in pair-fed females does not seem to be mediated by feedback since serum corticosterone was similarly elevated across groups, although PVN glucocorticoid receptor upregulation was observed only in CVS males, including sedentary animals. Despite elevated corticosterone, CR in pair-fed males did not alter HPA activity or PVN *Crh* expression in CVS males, potentially attributable to MBH AGRP neurons stimulating CRH release at the median eminence without affecting PVN *Crh* [137], supported by increased MBH *Npy* and its correlation with serum corticosterone in CVS pair-fed males, indicating the involvement of NPY/AGRP neurons.

HPT axis activity decreases at multiple levels during negative energy balance [138]. Even mild CR inhibited PVN *Trh* expression more in females than in males among pair-fed controls; levels in CVS pair-fed groups matched those in CVS sedentary rats, underscoring the stress effects. Fasting-induced tertiary hypothyroidism is attributed to increased tanycyte *Dio2* expression, which produces T3 that suppresses *Trh* transcription via leptin reduction and corticosterone elevation [21,139]. However, both pair-fed groups showed low leptin and high corticosterone levels, and *Dio2* expression was unaffected in pair-fed controls and decreased in CVS pair-fed rats. Moreover, *Dio2* was reduced in CVS sedentary animals despite elevated corticosterone levels but normal leptin levels, in contrast to increased *Dio2* levels in corticosterone-treated male mice [140]. MBH *Trhde* expression, T3-responsive, and paralleling *Dio2* during fasting [72] were also decreased in CVS sedentary and pair-fed controls. *Trhde* levels normalized in CVS pair-fed and CVS exercise groups, implying additional factors beyond *Dio2*-generated T3; POMC neurons may contribute given the positive correlations between *Pomc*, *Dio2*, and *Trhde* in CVS sedentary rats. Circulating TSH levels decreased in pair-fed groups, except in pair-fed females, possibly reflecting the stimulatory effects of estrogen [141]. Thyroid hormone concentrations differed by sex: females exhibited slight T3 increases in control and CVS pair-fed groups and elevated free T3 in the CVS exercise group; males showed decreased T4 (control pair-fed), free T4 (CVS pair-fed), and free T3 (control and CVS pair-fed) levels. The reduction in male T4 levels aligns with CR-induced axis suppression, whereas the elevation in female T3 levels suggests additional regulatory mechanisms.

Exercise imposes a negative energy balance, further enhanced by voluntary food restriction, necessitating careful differentiation between exercise- and intake-related effects. The functional heterogeneity of ARC POMC and NPY neurons is crucial [9,94]. Exercise elevates the firing of ARC POMC neurons and suppresses NPY neurons independently of leptin, despite leptin's serum decline during exercise [94]. Conversely, chronic restraint stress reduces excitatory input to ARC POMC neurons, thereby diminishing their responsiveness [142]. Exercise stimulated MBH-*Pomc* expression similarly in unstressed males and females, with females running more than males. Prior CVS exposure attenuated this response, although MBH *Pomc* expression did not correlate with the distance run, likely due to neuronal heterogeneity in receptors, co-transmitters, and projection targets [9,93,94].

Voluntary exercise does not induce exhaustion or stress; instead, it promotes neuroplasticity, increases Bdnf, and reduces glucocorticoid receptor expression in the hippocampus and frontal cortex, thereby enhancing cognition and reducing stress [100,129,143,144]. Correspondingly, exercise normalized PVN *Crh* and *Avp* expression suppressed by food restriction and lowered serum corticosterone below sedentary controls in both sexes, although these effects were absent in the CVS

exercise groups. Whether prolonged exercise can counteract CVS effects and sex differences therein remains to be elucidated [129].

Exercise also triggers mechanisms that restore energy balance, including transient HPT axis activation, as shown by the correlations between exercise distance and circulating thyroid hormones [27,47]. Previously, we used pair-fed controls to isolate exercise effects [27,44,47], finding numerous changes similar to those observed in CR or stress (Figure 11). Only PVN *Trh* levels were significantly elevated in control exercised males. These subtle and transient HPT axis changes depend on timing, with kinetic studies showing peak PVN *Trh* expression and serum TSH within one hour post-acute cold exposure [25] or physical activity [26], normalizing thereafter; similar transient serum TSH increases occur after swimming [145] or treadmill running [24,28]. The precise regulation of deiodinases, transporters, and clearance enzymes ensures rapid homeostatic recovery [146]. Except in pathological or extreme conditions, such as fasting [21], HPT axis dysfunction is more apparent during energetic challenges, such as cold or exercise.

Skeletal muscle, a primary thyroid hormone target that is critical for exercise performance, exhibits sex-dependent responses. Thyroid hormones are essential for muscle function and maintenance [17,36,37,105–107]. Exercise stimulates *Dio2*, increasing local T3 and *Pgc1a* expression [37], observed in control exercised but not in CVS rats. Females had similar exercise-induced *Dio2* expression but higher *Pgc1a* expression than males, potentially linked to elevated circulating T4. Among the analyzed tissues, skeletal muscle *Dio2* showed the greatest exercise response (Supplementary Figure 2). A recent study demonstrated increased gastrocnemius T3 and *Dio2* levels following repeated mild treadmill running [147]. CVS blunted the *Dio2* response, explaining the diminished *Pgc1a* upregulation. The lack of exercise response in stressed animals suggests impaired energy substrate utilization and muscle fiber maintenance, and elevated glucocorticoids contribute mainly to muscle atrophy [148].

White adipose tissue (WAT) depots supply energy substrates with sex-specific roles [63,85,149]. Exercise induces subcutaneous WAT adaptations, including sympathetic innervation, mitochondrial biogenesis, and metabolic gene expression [150]; visceral WAT regulates glucose homeostasis and inflammatory tone via cytokines [151]. We measured parameters reported as exercise-stimulated or CR-responsive in WAT and reproduced these findings: exercise enhanced, whereas CR reduced adrenergic receptor, *Dio2*, and *Pparg* expression. Tissue comparisons (Supplementary Figure 2) revealed that exercise-induced *Adrb3* expression was similar in subcutaneous and visceral WAT and BAT in females, while males exhibited higher *Adrb3* expression in visceral WAT. Inguinal WAT showed greater susceptibility to CVS inhibition than gonadal WAT or BAT in both sexes, whereas CR effects were uniform in WAT but milder in BAT, which remained at or above the control levels. Exercise increased *Dio2* more in perigonadal than inguinal WAT, more so in males, and CR and CVS effects were similarly inhibitory across tissues. *Pparg* responses in controls did not parallel *Dio2*, except for lower stimulation in female inguinal WAT compared to perigonadal WAT, blunted by CVS but not inhibited by CR, likely reflecting PPARG's multifaceted regulatory roles and correlation with TSH and TH in exercised groups, consistent with direct TSH effects and potential myokine-mediated WAT interactions [123,152]. In contrast, *Hsl* expression responses to exercise or CR differed markedly by adipose depot and sex; exercise stimulated *Hsl* more in males, particularly in inguinal WAT, but also in pgWAT. Reports on HSL distribution and CR effects are inconsistent [65,121], reflecting regulation via multiple factors: adrenergic agonists rapidly increase HSL enzymatic activity through PKA-induced phosphorylation without altering mRNA levels, while dexamethasone elevates *Hsl* mRNA over threefold in vitro [120]. CVS caused higher CR- or Ex-induced *Hsl* expression in iWAT while decreasing it in pgWAT in all groups. Pathological conditions such as metabolic syndrome show altered local GC metabolism [153]; in mice, reduced local GC production due to adipose-specific H6PDH inhibition leads to decreased expression of *Hsl* and *Atgl* in epididymal WAT [154], which may contribute to stress-induced obesity in humans.

Coordinated expression patterns across adipose tissues support their integrative functions. BAT, once questioned in exercise roles, is strongly activated by exercise with increases in *Adrb3*, *Dio2*, and

Ucp1, the latter of which mediates thermogenesis [66,118,119,123]. Recently, *Ucp1* induction in male mice has been linked to exercise-induced muscle-derived factors such as IL-16 [155]. Unlike other tissues where CR and stress blunted marker expression and suppressed exercise responses, female BAT was resistant to CVS effects; *Dio2* expression in CVS exercised females matched controls, and CVS pair-fed females even exceeded the controls. Beyond energy expenditure, increased BAT DIO2 may contribute to elevated circulating T3 levels [156,157].

T3 concentrations in the CVS pair-fed, CVS exercised, and control pair-fed groups were higher than those in the control sedentary or exercised rats, suggesting additional mechanisms, such as Dio3 inhibition or altered thyroid hormone clearance. DIO3 seems unlikely to explain these results since chronic stress does not affect Dio3 activity in the cerebral brain areas of male rats [61], and fasting induces WAT Dio3 mRNA [158], reducing serum and liver T3 and T4 levels. Furthermore, glucocorticoids suppress Dio3 in vitro and upregulate the expression of liver enzymes involved in thyroid hormone clearance in vivo [158–160]. Other players, such as transporters or binding proteins, warrant further investigation. In particular, because CVS-induced stimulation was more pronounced for free T3, this suggests the regulation of additional factors, such as thyroid hormone-binding proteins. Although stress generally inhibits HPT axis activity, elevated T3 levels have been reported in postwar PTSD veterans and other psychiatric disorders, including bipolar I disorder [161–163].

Chronic stress blunted nearly all exercise-induced increases in gene expression. Many effects may arise from corticosterone-mediated transcriptional interference via cross-talk mechanisms, including the inhibition of PKA pathways regulating *Trh* and *Crh* synthesis in the PVN [164,165] and *Dio2* expression in BAT [62]. However, these interactions occur within narrow temporal windows dictated by glucocorticoid receptor dynamics. It is increasingly recognized that chronic stress effects stem less from sustained corticosterone elevations and more from altered brain circuitry modulating neuronal activity and stress perception dynamically [166]. Similarly, exercise-induced hippocampal plasticity reduces stress reactivity and promotes resilience, which requires time—approximately three weeks in females and] in males [167]. Thus, the two-week exercise used here revealed early perturbations from prior stress that may influence subsequent processes.

The effects of chronic stress on HPT axis responses to exercise vary by stress paradigm and sex and are not fully dependent on HPA axis alterations. Unlike stress models previously studied, this study focuses on adolescent-restricted CVS, distinct from adult intermittent restraint or post-weaning social isolation [44] and neonatal maternal separation [47]. Adult restraint stress abolishes exercise-induced WAT loss in both sexes but not PVN- *Trh* induction in males [44], whereas adolescent CVS does not impair exercise-induced WAT loss but inhibits PVN *Trh* upregulation. Adolescent isolation induces stronger stress responses in males and reduces exercise-induced MBH *Pomc* and *Dio2* expression in both sexes, similar to our findings [44]. In contrast, exercise rescues fat loss in maternally separated males but not females and suppresses exercise-induced increases in MBH *Pomc* levels in females [47]. Our study confirms that adolescent CVS causes persistent HPA activation in males, elevated anxiety-like behavior in females, and disrupts exercise-induced gene expression regulating HPT axis activity and peripheral metabolic tissues. The effect of adolescent stress on increased circulating total and free T3 may involve effects on some of the brain circuits involved, as it is at this stage that adolescent circuits mature and females are more susceptible to stress effects; however, this awaits resolution. Future studies should explore whether prolonged exercise can overcome the effects of chronic stress and consider psychological history when evaluating exercise performance clinically.

Caveats: Most exercise studies have addressed dynamic and transient neuronal and molecular responses. Here, voluntary wheel running led to individual variability in the timing and duration of activity. Our analyses represent a snapshot taken three hours post-possible last running session, during the animals' inactive phase, recovery period, and fluctuating sleep and feeding bouts. Further studies exploring different times of sacrifice and different days of exercise are required.

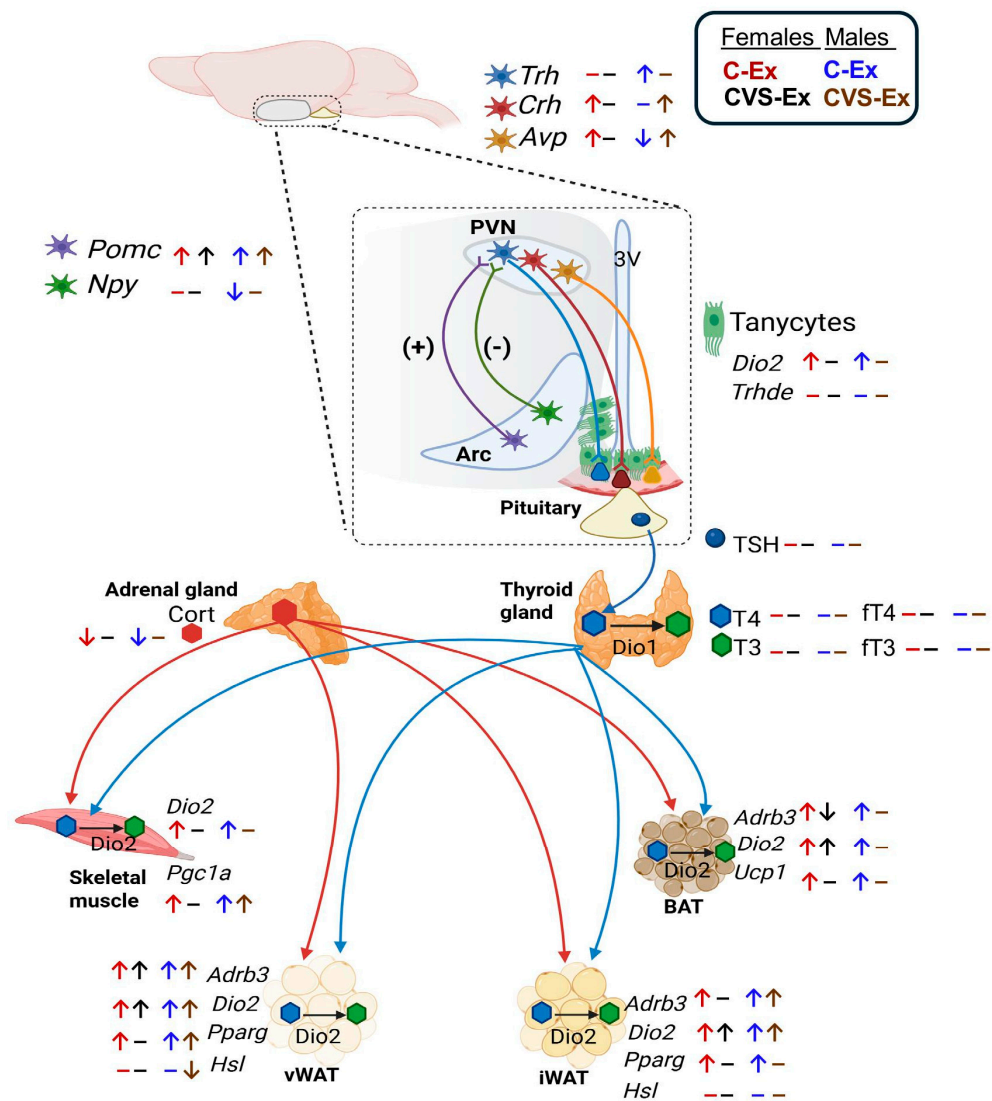


Figure 11. Effects of exercise on the HPT, HPA axes and target tissues of female and male controls and chronic variable stressed rats (CVS) compared to pair-fed groups. The diagram summarizes the sex-dependent long-term effects of adolescent chronic variable stress (CVS) and adult voluntary exercise (Ex) on key components of the hypothalamic-pituitary-thyroid (HPT) and adrenal (HPA) axes and thyroid hormone-responsive genes in peripheral tissues. Groups are represented by different colors for males and females; upward arrows (↑) indicate an increase, downward arrows (↓) a decrease, and dashed lines (-) indicate no significant change, relative to either the C-PF or CVS-PF group. C-: control; CVS-: chronic variable stress; PF: pair-fed; Ex: exercise. HPT axis image created using biorender.com.

4. Materials and Methods

4.1. Animals and Experimental Groups

Wistar rats were bred and raised at the Institute’s animal pathogen-free installation, with a 12h light/dark cycle (lights at 7:00h), independent filtered air and positive pressure in each room. The rat colony is renewed every five years with 10 non-related male and 15 female Wistar rats from Charles River Laboratories (MA, USA). Only two people took care of the animal husbandry keeping strict registry of crossbreeding to avoid family inter-crossing [43]. Rats were kept with food (Teklad 2018SX, Envigo, USA) and purified water (in glass bottles to limit endocrine disruptors) *ad libitum*.

All procedures followed the Guide for the care and use of laboratory animals [67], the Mexican norm NOM-062-ZOO-1999, and Institute Bioethics Committee authorizations (No. 273, 318), taking care to keep stress at a minimum [42,43].

Two independent experiments were performed for either males or females. For each experiment, 12 female virgin rats (3 months old) were mated with unrelated males, the day of birth registered, and the litter culled to 10 pups/mother; at weaning (post-natal day [PND] 21) pups of each mother were placed by sex in a cage. Each experiment required 6 groups of 5-6 rats each; 3 groups were submitted to CVS and the other were controls; groups were formed at PND 30 placing one pup of each mother in a different cage, ending with 2 pups/cage using 3 cages/group with no siblings; 9 cages were used as control [C] and the other nine for CVS. CVS rats were housed in a different room from C rats to avoid the stress of noise produced by taking cages out of the room every day for the CVS procedure, and of the noise of stressed rats. Eighteen rats were submitted, during the adolescent period, every day to the CVS paradigm applying one different stressor per day during the light period according to [43,68] modified as described in Supplementary Table 1.

Because the period of adolescence extends over puberty that is restricted to hormonal and physical changes, the period of adolescence was chosen according to [56], from PND 31-60 for females and PND 31-70 for males, to guarantee the inclusion of the sensible periods considering the existing controversies regarding the end of adolescence in rats [54–58] (Figure 1A). Behavioral tests were conducted throughout the CVS procedure to evaluate the state of animals during treatment.

4.2. Behavioral Tests

Rats were submitted to behavioral tests during the light period, starting at 10:00 h. Spontaneous locomotor activity At PND 37 locomotor activity was evaluated by placing rats individually for 5 minutes in a standard cage with a photobeam activity system (PAS-Home Cage, San Diego Instruments Co.) connected to a computer that registers: central and peripheral activities, ambulatory and fine movements, and rearing. Photobeams are placed around the cage and the program can distinguish fine movements from locomotion in the cage.

Open Field Test (OFT)

At PND 44 and 58, rats were placed for 5 minutes and left to explore freely in an enclosed squared arena of 100×100×60 cm made of opaque black acrylic (San Diego Instruments Co.), under dim instead of bright light to distinguish small changes [26]. Total distance traveled (measure of total locomotion), distance and time spent in the center of the area, and the number of entries to the center were registered and analyzed with the SMART 2.5 software (Panlab). Reduced time and distance spent in the center indicate anxiety behavior (inferred by the avoidance of crossing the center of the field).

Elevated Plus Maze (EPM)

At PND 51 male and female rats and only on PND 65 male rats were submitted to the EPM [26] (San Diego Instruments Co.). Rats were placed for 5 min at the center of the EPM, time spent in the open or closed arms, and distance traveled was registered, and analyzed with the software SMART 2.5 (Panlab).

4.3. Voluntary Exercise

Two weeks after the end of adolescence and of CVS or C treatment, rats were exposed to voluntary wheel running (WR), at PND 74 for females, PND 84 for males [55]. Control and CVS animals were divided into 3 groups (5-6 rats/group), avoiding siblings within groups: sedentary (Sed) fed *ad libitum*, pair-fed (PF) to food intake of exercised rats, and exercised (Ex) [27,44]. C-Sed and CVS-Sed rats were left undisturbed in a different room from Ex and PF groups. As rodents are nocturnal and run most of the time during the dark period [69], C-Ex and CVS-Ex rats were placed individually, only during the dark period (at light change, from 19:00 to 7:00h) for 14 days, in a cage with a running wheel (AccuScan Instruments INC) with fresh food and water; at 7:00h animals were

returned with their cage mate with food and water. Food intake was monitored after each cage change (at 7:00h and 19:00h), and distance registered at 7:00h. The average amount of food consumed by the Ex rats at each period was provided to the PF rats; during the dark period control PF rats were placed in individual cages to account for the 12h-stress of isolation [70] that exercised rats had in the cage with the running wheel; during the light period they were returned with their home cage mate.

4.4. Tissue Collection

After 2 weeks of voluntary WR, on day 15th rats were euthanized; each rat was introduced, starting at 9:00h, to the nearby laboratory to be decapitated with a sharp guillotine by an experienced technician. To avoid time differences between groups that may involve circadian variations in hormone or mRNA levels, rats were alternated between the different groups. Trunk blood was collected, serum was separated by centrifugation and stored in aliquots at -20 °C. Vaginal smears were taken immediately after decapitation to avoid stress and analyze by cytology the stage of estrous cycle. The brain was carefully excised from the skull, ensuring the optic nerve was severed beforehand to preserve the median eminence and placed in aluminum foil over dry ice. Peripheral tissues such as gastrocnemius muscle, WAT and BAT were rapidly removed; perigonadal (pg), retroperitoneal (r) and interscapular (isc) WAT depots were weighed fresh before freezing, and inguinal WAT (iWAT) were dissected and frozen without weighing (to avoid RNA degradation due to the dissection time). All tissues were wrapped in aluminum foil and kept at -70 °C. At least five people were involved in the dissection procedure to minimize time between sacrifices.

4.5. Brain Dissections

Frozen brains were placed in a cryostat (Thermo Scientific HM525) for coronal sectioning; after verifying alignment and recognizing the end of anterior PVN in 20 µm slices (toluidine blue staining), three consecutive 200 µm coronal slices were cut, which included the median-caudal PVN (Bregma -1.3 to -1.9 mm), and 5 slices for the medial-basal hypothalamus (MBH, Bregma -1.92 to -2.92 mm) [71]. Each section was placed horizontally on a glass cover of a petri dish over dry ice. For the 3 anterior slices, the PVN from each side of the 3rd ventricle was cut with a 0.5 mm internal diameter sample corer (Fine Science Tools), and stored in tubes at -70 °C. The following 5 slices included the ARC and the median eminence with the lower third of the 3rd ventricle containing the $\beta 2$ tanycytes that express *Dio2* and thyrotropin-releasing hormone-degrading ectoenzyme (*Trhde*) [22,72]; from each slice, a 0.5 mm internal diameter sample corer was placed at the center and most ventral end of hypothalamus.

4.6. Biochemical Measurements

Hormone quantification in serum

TSH (reagents and protocol from NIDDK, antibody ID AB_2891204) and Cort (Merck-Millipore, antibody ID AB_90543) were quantified by radioimmunoassay and other hormones with ELISA kits following manufacturer's instructions, except that for total T3 (cat. IIDE 2021, Diagnóstica Internacional, Zapopan, México) and T4 (cat. IIDE 2022, Diagnóstica Internacional), an aliquot of 25 µL of hypothyroid rat serum was added to the standard curves as reported [44]. Free T4 (fT4) (cat. E-EL-0122) and free T3 (fT3) (cat. E-EL-0079) were measured using kits from Elabscience only in sera of rats from experiment 2. Estradiol (cat. 80548), progesterone (cat. 80558), prolactin (cat. 80560) and leptin (cat. 90040) were quantified using kits from Crystal Chem. Samples were measured in duplicate, the mean was taken as n = 1; intra-assay and inter-assay coefficients of variation were <10%.

4.7. RNA Extraction and mRNA Quantification

Total RNA was isolated from PVN, MBH, and gastrocnemius muscle by the acid guanidinium thiocyanate-phenol-chloroform method [73]. For adipose tissues, modifications included a first centrifugation at 870g for 10 min and, an additional chloroform wash to remove fat. Purity and

integrity of RNA were verified by electrophoresis and S18 and 28 bands quantified; samples with a 28/18 ratio of less than 1.5 were discarded. One μg RNA was retro-transcribed (M-MLV, 200 U/ μL , Invitrogen), cDNA amplified by end-point PCR for MBH, gastrocnemius muscle, BAT and WATs, using recombinant Taq DNA polymerase (Biotecnologías Moleculares, México) and linearity verified against internal markers cyclophilin A (*Ppia*) or hypoxanthine phosphoribosyl transferase 1 (*Hprt*) [44,46]. Genes studied in the PVN were quantified by qPCR (Syngreen Mix, PCR Biosystems) on the RotorGene Q (Qiagen) with an initial denaturing stage at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, annealing temperature of 60 °C for 20 s, and a 72 °C extension period for 20 s. Melting curve analysis was performed to verify that only one PCR product was detected in each sample. The relative expression of the target genes with respect to the reference genes was calculated using the Pfaffl method [74]. The expression level of target genes was normalized against *Hprt* or *Ppia*, as indicated in figures. End-point PCR and qPCR primer sequences used are described in Supplementary Table 2.

4.8. Statistical Analyses

Two independent experiments were performed/sex, $n=5-6/\text{group}$ in each experiment; mean of C-Sed values of each experiment was taken as 100% and the value for each animal calculated as % of C-Sed mean; % values of the two experiments were pooled, calculated as the mean $\pm$ S.E.M and analyzed with GraphPad Prism 10. Two-way ANOVA was used to determine the significance of treatment and/or sex for variables measured before exercise; three-way ANOVA to determine the significance of stress, exercise, and sex; food intake and body weight gain were analyzed by two-way ANOVA of repeated measures. In some cases, a two-tailed t-test was used, as stated in figure legends. Statistical significance was set at $p \leq 0.05$. ANOVA data are presented in Supplementary Tables 5A-C. All comparisons were made against C-Sed unless specified otherwise in the text.

We evaluated the Pearson correlation coefficient and the significance of the interaction, between all variables of all tissues measured, using GraphPad Prism 10; for visual analysis of correlation networks we developed hive plots (Figure 10) [75] [created with software: https://codeberg.org/rgarcia-herrera/neuro_endocrine_correlations]. Only significant correlations showing linearity were included, discarding links between two separate sets of data.

Supplementary Materials: The following supporting information can be downloaded at website of this paper posted on Preprints.org, S1: Supplementary tables; S2: Supplementary figures.

Author Contributions: Conceptualization, PJB; methodology, MPMO and KG; hive plots, RGH; statistical analysis, LJH and MPMO; writing—original draft preparation, PJB; writing—review and editing, PJB, JLC and LJH; funding acquisition, PJB. All authors have read and agreed to the published version of the manuscript.”

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Data Availability Statement: The original data presented in the study are openly available in FigShare at DOI 10.6084/m9.figshare.29579270.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

ACTH:	corticotrophin
Adrb3:	adrenergic receptor β 3
AgRP:	Agouti-related peptide
ARC:	arcuate nucleus
Avp:	Arginine vasopressin
BAT:	brown adipose tissue
BW:	body weight
BWg:	body weight gain
C:	control
Cort:	corticosterone
CR:	calorie restriction
CRH:	corticotropin-releasing hormone
CVS:	chronic variable stress
Dio1:	deiodinase 1
Dio2:	deiodinase 2
Dio3:	deiodinase 3
EPM:	Elevated Plus Maze
Ex:	exercised (voluntary wheel-running)
FE:	food efficiency
FI:	Food Intake
fT3:	free T3
fT4:	free T4
Gr:	Glucocorticoid receptor
HPA:	hypothalamus-pituitary-adrenal
Hprt:	hypoxanthine phosphoribosyl transferase 1
HPT:	hypothalamic-pituitary-thyroid
HSL (Lipe):	hormone sensitive lipase
IL-16:	Interleukin-16
iWAT:	inguinal WAT
MBH:	mediobasal hypothalamus
NPY:	Neuropeptide Y
OFT:	Open Field Test
P4:	Progesterone
PAS:	photobeam activity system
PF:	pair-fed
Pgc-1 α :	peroxisome proliferator-activated receptor γ coactivator 1 α
pgWAT:	perigonadal WAT
PND:	post-natal day
POMC:	Pro-opiomelanocortin
Ppia:	cyclophilin A
Pparg:	peroxisome proliferator-activated receptor γ
PRL:	Prolactin
PVN:	paraventricular nucleus
RFI:	relative food intake
SCH:	subclinical hypothyroidism
Sed:	sedentary
SKM:	skeletal muscles
T3:	Triiodothyronine
T4:	Thyroxine
Tg:	triglycerides

TH:	Thyroid hormones
TRH:	thyrotropin-releasing hormone
TRH-DE (Trhde):	TRH-degrading ectoenzyme
TSH:	thyrotropin
Ucp1:	uncoupling protein-1
WAT:	white adipose tissues
WR:	wheel running

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