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ORIGINAL RESEARCH

The Characterization of Sex Differences in Hypoglycemia-Induced Activation of HPA Axis on the Transcriptomic Level

Serpil Taheri[1,2] · Zuleyha Karaca[3] · Minoo Rassoulzadegan[4] · Ecmel Mehmetbeyoglu[5] · Gokmen Zararsiz[6] · Elif Funda Sener[1,2] · Kezban Korkmaz Bayram[1] · Esra Tufan[1] · Mustafa Caglar Sahin[1] · Mert Kahraman Marasli[1] · Mehmet Memis[1] · Halit Canatan[2] · Figen Ozturk[7] · Fatih Tanriverdi[3] · Kursad Unluhizarci[3] · Fahrettin Kelestimur[8]

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Abstract

Activation of the hypothalamic–pituitary–adrenal (HPA) axis using an insulin tolerance test (ITT) is a medical diagnostic procedure that is frequently used in humans to assess the HPA and growth-hormone (GH) axes. Whether sex differences exist in the response to ITT stress is unknown. Thus, investigations into the analysis of transcripts during activation of the HPA axis in response to hypoglycemia have revealed the underlying influences of sex in signaling pathways that stimulate the HPA axis. We assessed four time points of ITT application in *Balb/c* mice. After insulin injection, expression levels of 192 microRNAs and 41 mRNAs associated with the HPA, GH and hypothalamic-pituitary-gonadal (HPG) axes were determined by real-time RT-PCR in the hypothalamus, pituitary and adrenal tissues, as well as blood samples (Raw data accession:https://drive.google.com/drive/folders/10qI00NAtjxOepcNKxSJnQbJeBFa6zgHK?usp=sharing). Although the ITT is commonly used as a gold standard for evaluating the HPA axis, we found completely different responses between males and females with respect to activation of the HPA axis. While activation of several transcripts in the hypothalamus and pituitary was observed after performing the ITT in males within 10 min, females responded via the pituitary and adrenal immediately and durably over 40 min. Additionally, we found that microRNA alterations precede mRNA responses in the HPA axis. Furthermore, robust changes in the levels of several transcripts including *Avpr1b* and *Avpr2* observed at all time points strongly suggest that transcriptional control of these genes occurs mostly via differential signaling in pituitary and blood between males and females. Male and female HPA axis responses to ITT involve a number of sophisticated regulatory signaling pathways of miRNAs and mRNAs. Our results highlight the first robust markers in several layers of HPA, HPG and GH axis involved in ITT/hypoglycemia stress-induced dynamics.

Keywords Mice · Sex differences · Hypothalamic–pituitary–adrenal (HPA) axis · Hypothalamic–pituitary–gonadal (HPG) axis · Growth hormone (GH) axis · Insulin tolerance test (ITT) · Transcript profile · miRNAs

✉ Fahrettin Kelestimur
fktimur@erciyes.edu.tr; fahrettin.kelestemur@yeditepe.edu.tr

1 Betul Ziya Eren Genome and Stem Cell Center, Erciyes University, Kayseri, Turkey

2 Department of Medical Biology, Erciyes University Medical School, Kayseri, Turkey

3 Department of Endocrinology, Erciyes University Medical School, Kayseri, Turkey

4 Université Côte D'Azur, CNRS, Inserm, France

5 Department of Cancer and Genetics, Cardiff University, Cardiff, UK

6 Department of Biostatistics, Erciyes University Medical School, Kayseri, Turkey

7 Department of Pathology, Erciyes University Medical School, Kayseri, Turkey

8 Department of Endocrinology, Yeditepe University Medical School, Istanbul, Turkey

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Introduction

The Hypothalamic–Pituitary–Adrenal (HPA) axis is a dynamic structure comprising three main organs, hypothalamus, pituitary and adrenal glands, which are hormonally related and interact with each other to allow the organism to perform and maintain its vital functions and elicit appropriate responses of cortisol against stress (Papadimitriou and Priftis 2009; Herman and Tasker 2016). When an organism faces any stress, such as in the case of hypoglycemia, corticotrophin-releasing hormone (CRH) and arginine vasopressin hormone (AVP) secretion is thought to be induced from the hypothalamic paraventricular nucleus (PVN), which stimulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. In turn, ACTH stimulates the release of cortisol from the adrenal cortex. The HPA axis is inhibited by the binding of ACTH and cortisol to glucocorticoid receptors (GRs) in the hippocampus, hypothalamus and pituitary and by binding to mineralocorticoid receptors (MRs) in the hippocampus (Smith and Vale 2006; Papadimitriou and Priftis 2009; Wood 2013).

Sex differences in stress responses are thought to be mediated by hormonal changes, as well as the effects of gonadal hormones (e.g., testosterone, estradiol and progesterone), and all responses are dependent on the period of life during which the stress is experienced (Oyola and Handa 2017; Heck and Handa 2019). The mechanisms for highlighting responses to many stressors are complex and multifaceted (for review see (Heck and Handa 2019)). The different stress responses of males and females have not been examined in detail, although there are strong hypotheses that this occurs in animals and humans. In most previous studies of the HPA axis after application of a stressor factor, hormones and proteins were measured (Gerra et al. 2001; Smith and Vale 2006; Stephens et al. 2016; Heck and Handa 2019). For example, Trier Social Stress Test (TSST) reveals gender-related differences in the HPA axis stress response. As a result, testosterone in men and progesterone in women have an inhibitory effect on the HPA axis reactivity; men have greater ACTH and cortisol response to the TSST than do women during the follicular phase of the menstrual cycle (Stephens et al. 2016). Although multiple studies have reported sex differences (Koenig and Cho 2005; Chintamaneni et al. 2014), the molecular aspects of RNA signaling involved in the responses of the HPA axis to hypoglycemia have not been investigated.

The insulin tolerance test (ITT) is routinely used in the evaluation of HPA and growth-hormone (GH) axes (Erturk et al. 1998; Abdu et al. 1999; Dökmetaş et al. 2000; Karaca et al. 2010), but the mechanisms responsible for the activation of the HPA axis by hypoglycemia in males and females are not yet known. This test is used as a standard in the evaluation of growth-hormone and adrenal insufficiency in adults, but it is contraindicated in some people and has potential serious risks, making close monitoring necessary (Schmiegelow et al. 2003; Simsek et al. 2015; Kosak et al. 2017). The ITT is applied and interpreted in the same way for all patients, without considering gender differences. Its reproducibility is not sufficient compared with other tests used to evaluate the HPA axis, such as glucagon stimulation and low-dose ACTH stimulation tests (Simsek et al. 2015). Hypoglycemia is likely to cause activation of upper brain centers rather than in the hypothalamus, subsequently stimulating CRH secretion in the hypothalamus (Rao 2015).

However, hormones and proteins are unlikely to have been unique in initiating and sustaining their consequences. In contrast, stress responses in the form of circulating RNAs (non-coding RNA) are more satisfying in case that could be specific to the type of stress. ITT mediates with hypoglycemia activation of the HPA axis, but underlying process of transcription alteration with male and female distinction analysis has not been clearly reported in model animals.

Here, we tested the hypothesis in mice that transcript networks are activated by a single stressor (ITT) to the HPA axis and examined the individual strategies that adult males and females use to manage hypoglycemia in this context. To establish the importance of transcripts that influence these responses, we compared RNAs prepared from male and female mouse organs at different time points after application of ITT. Forty-one mRNAs are selected among hormones and proteins known to be involved in HPA, HPG and GH axis (see Fig. 1 and Oyola and Handa 2017; Heck and Handa 2019). Furthermore, by computational approaches with miRbase data, we tested 192 predicted miRNAs possibly engaged with these forty-one candidate transcripts as target.

The mechanisms by which miRNAs and mRNAs influence activity of the HPA axis are still obscure. Therefore, in this study, we identify several transcripts (mRNAs and miRNAs) involved in the HPA axis during ITT specific to male and/or to female mice.

Materials and Methods

Animals

Male ($n=48$) and female ($n=48$) 8-week-old *Balb/c* mice were used to investigate molecular mechanism of HPA axis activation to determine possible difference between the sexes. Animals were cared for and treated according to the Principles of Laboratory Animal Care (European rules). All

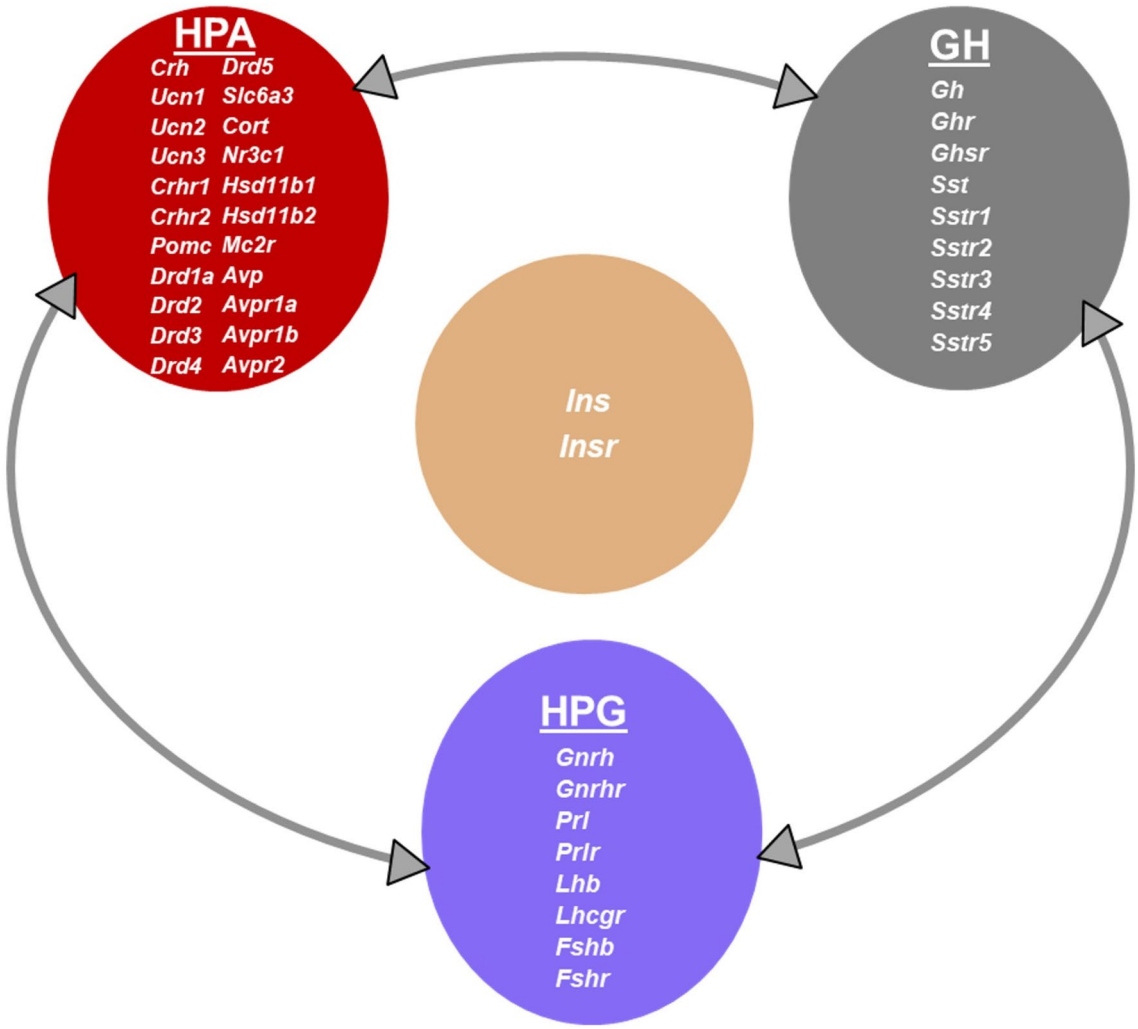


Fig. 1 List of studied transcripts related to HPA, HPG and GH axes

animal experimental protocols were approved by the Local Ethics Committee for Animal Experiments of Erciyes University (No: 2014/31).

Performing ITT on Mice

Mice were maintained in Erciyes University, Genome and Stem Cell Center, Transgenic Animals Department, Kayseri, Turkey equipped with controlled conditions (lights 06:00 to 18:00, temperature 23 °C and humidity 55%). All experiments were performed on a homogeneous group of animals of the same age maintained in the same facility. *Balb/c* mice were fasted for 12 h and randomized into five groups prior to the ITT. Mice were fasted in the evening from 21:00 with no water restriction. After fasting 12 h, experiments were started at 09:00. Two units of intraperitoneal insulin/kg (Cat No: HI0210, Humulin, USA) were injected into mice after a 12 h fast (Lolait et al. 2007). All tests were performed between 09:00 and 10:00 in isolated rooms. Concerning the estrus cycle, females were caged together from birth since it is known that females caged together have a tendency to become hormonally synchronized (Marchlewska-Koj et al. 1983; Jackson and Wordinger 1990; Dalal et al. 2001). In addition, each female estrous cycle is determined by vaginal examination, and females at the same stage of hormonal cycle (metestrus–diestrus) are selected, the period that the level of FSH and LH is low (Marchlewska-Koj et al. 1983). Glucose levels in these mice were measured from the tail vein before insulin injection (Basal, 0 min) and at 1, 10, 25 and 40 min after insulin injection. In this study, eight male and eight female mice were used as the control group. Control mice were completely comparable to the experimental group because all mice were from the same room, cage and age group of animals produced in our animal facility. Blood glucose levels (Supp Table S1.) were determined by plasma glucose test strips in all mice (On Call plus Blood Glucose

Monitoring System, USA). The limit of hypoglycemia was accepted to be between 20 and 50 mg/dl for mice (Lolait et al. 2007). Mice were immediately sacrificed by cervical dislocation at 1, 10, 25 or 40 min after insulin injection for the experimental group and saline buffer injection for the control group, and tissues were collected (hypothalamus, pituitary, adrenal glands and blood samples). All results are derived from whole extracts of the hypothalamus, pituitary and adrenal glands. Samples were stored at − 80° C in TRIzol until use.

RNA isolation and Real-Time PCR

Total RNA was isolated from tissue and blood samples using TRIzol (Cat No: 11667165001 Roche, Germany) (Ozkul et al. 2020), and cDNA was synthesized from obtained RNA samples using a cDNA synthesis kit (Cat no: 18091200, Thermo Fisher Scientific, USA). The cDNA samples were quantified using the high-throughput BioMark Real-Time PCR system (Fluidigm, South San Francisco, CA) for analysis by Q-PCR at the transcript level (Taheri et al. 2016; Karaca et al. 2020). For mRNAs and miRNAs of stress-mediated alterations of ITT, we selected forty-one mRNAs known to be involved in the HPA, Hypothalamic–Pituitary–Gonadal (HPG) and GH axes (Goel et al. 2014; Herman et al. 2016; Oyola and Handa 2017) (Fig. 1, Supp Table S2) and one hundred ninety-two miRNAs (Betel et al. 2008; MicroRNA.org 2010; Vlachos et al. 2015) in a test group from Exigon (Canada, USA).

In case of control transcript variations, we used two housekeeping transcripts (*Gapdh* and *Actb*) for validation of the results. All samples were run in duplicate, and values were normalized using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical Analysis

Data were collected using BioMark Real-Time PCR Analysis Software (Fluidigm, South San Francisco, CA), the linear derivative baseline correction method, and the auto global Cq threshold method. System-derived Cq values of 999 and values larger than 26 were considered non-specific and beyond the detection limits and were therefore removed. Median Limit of detection (LOD) Cq values were calculated across all arrays to impute missing values (Forootan et al. 2017). Data normalization was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). To detect the differentially expressed mRNA and miRNAs, linear models for the microarray and RNA-Seq data (limma) were applied. The Benjamini–Hochberg false discovery correction rate was used to adjust the p-values to account for multiple testing. Analysis was performed using R 3.1.1 software, and p- and adjusted p-values < 0.001 were considered statistically significant (Raw data accession:https://drive.google.com/drive/folders/10qI00NAtjxOepcNKxSJnQbJeBFa6zgHK?usp=sharing).

Results

A number of altered proteins and hormones have already been identified during activation of the hypothalamic–pituitary–adrenal (HPA) axis. We selected forty-one mRNAs of previously reported factors (Fig. 1) to be involved in the HPA, hypothalamic–pituitary–gonadal (HPG) and growth-hormone (GH) axes and their one hundred ninety-two computational predicted regulatory miRNAs for analysis during stress-mediated alteration (ITT). Insulin was injected (2 IU/kg human insulin, see "Materials and Methods") intraperitoneally into non-nourished 2-month old *Balb/c* mice (male or female) that came from the same batch of breeding. Estrous cycles of females maintained in the same cage are synchronized and maintained suppressed (Whitten effect) (Marchlewska-Koj et al. 1983; Jackson and Wordinger 1990; Dalal et al. 2001). Results of miRNA and mRNA are shown in Table 1 and Figs. 2, 3, 4, 5.

Distinct Responses of the HPA Axis in Male and Female Mice to ITT

Hypothalamic Responses to Hypoglycemia Trigger Expression of Multiple miRNAs and mRNAs in Males but are Very Rare in Females

Table 1 resumes all transcripts, tissues and time point analysis results. Figure 2 (heat map) visualizes hypothalamus results. Determination of a short list of selected transcript levels in the hypothalamus at various time points after performing ITT revealed that only one miRNA (*miR-184-3p*) was commonly up-regulated. *miR-184-3p* was detected within 1 min after insulin injection and was absent in other samples during the other time points in both sexes (Fig. 2a, c, d). Although expression levels of a few transcripts changed in the hypothalamus during the given time points after performing ITT, we observed a significant decrease in 41 miRNAs in the hypothalamus of male mice, particularly within the first minute after performing ITT. More variable results in miRNA levels were observed in male samples at 10, 25 and 40 min, while in the female hypothalamus, only one miRNA, *miR-7007-5p*, was consistently down-regulated at 1, 10 and 25 min, and *miR-6926-3p* was up-regulated 40 min after insulin injection (Fig. 2a). Transcript analysis of protein-coding genes showed parallel findings to the miRNA profiles, such as no responses in females and a significant response in males. In fact, transcripts for *Fshr, Drd4, Crhr2, Mc2r*, *Avpr2* and *Drd5* were increased exclusively

Table 1 miRNA and mRNA expression levels in the hypothalamus, pituitary, adrenal and blood of male and female mice after ITT

	1 min			10 min			25 min			40 min		
		Fold change	*P*		Fold change	*P*		Fold change	*P*		Fold change	*P*
Hypothalamus (male)	*miR-6929-5p*	−233.892,33	0.04	*miR-326-5p*	−0.83	0.01	*miR-29a-3p*	−1.576	0.005	*miR-6929-5p*	−555,342,37	0.00
	miR-124-3p	−176.24	0.05	*miR-15b-5p*	0.14	0.01	*miR-488-3p*	−32.890	0.006	*miR-326-5p*	−0.83	0.00
	miR-9-5p	−66.74	0.02	*miR-7038-3p*	−0.83	0.02	*miR-219a-2-3p*	−50.939	0.006	*miR-344 g-3p*	1.00	0.01
	miR-219a-2-3p	−63.70	0.00	*miR-452-3p*	−0.83	0.02	*miR-423-5p*	−0.759	0.010	*miR-149-3p*	1.92	0.04
	miR-488-3p	−46.27	0.00	*miR-6975-5p*	−0.83	0.02	*miR-128-3p*	−10.176	0.015			
	miR-222-3p	−21.88	0.01				*miR-22-3p*	−1.023	0.025			
	miR-150-5p	−19.91	0.02				*miR-181d-5p*	−0.965	0.028			
	miR-702-3p	−19.34	0.02				*miR-34b-5p*	−4.200	0.029			
	miR-128-3p	−15.56	0.00				*miR-23a-3p*	−0.165	0.030			
	miR-383-5p	−8.49	0.04				*miR-181b-5p*	−0.841	0.031			
	miR-19b-3p	−7.35	0.03				*let-7d-5p*	−0.355	0.032			
	miR-34a-5p	−6.35	0.04				*miR-344 g-3p*	0.611	0.032			
	miR-34a-5p-1	−5.37	0.04				*miR-222-3p*	−14.455	0.040			
	miR-34b-5p	−4.85	0.02				*miR-702-3p*	−15.493	0.042			
	miR-19a-3p	−4.52	0.03				*miR-150-5p*	−13.933	0.044			
	miR-206-3p	−4.27	0.03				*miR-125b-5p*	−0.623	0.044			
	miR-106a-5p	−2.45	0.05									
	miR-331-3p	−2.43	0.03									
	miR-181a-5p-1	−2.37	0.05									
	miR-329-5p	−2.18	0.05									
	miR-20a-5p	−2.13	0.04									
	miR-29a-3p	−2.05	0.00									
	miR-17-5p	−2.00	0.05									
	miR-204-5p	−1.99	0.02									
	miR-181b-5p-1	−1.88	0.03									
	miR-18a-5p	−1.83	0.04									
	miR-296-5p	−1.68	0.04									
	miR-106b-5p	−1.64	0.03									
	miR-22-3p	−1.45	0.01									
	miR-181d-5p	−1.31	0.01									
	miR-1a-3p	−1.15	0.02									
	miR-181b-5p	−1.12	0.01									
	miR-10a-5p	−1.04	0.02									
	miR-205-5p	2956,54	0.00									

Table 1 (continued)

	1 min			10 min			25 min			40 min		
		Fold change	*P*		Fold change	*P*		Fold change	*P*		Fold change	*P*
	miR-184-3p	44,432,81	0.00									
	miR-27b-5p	83,573,10	0.00	*Fshr*	46,182	0.002						
	miR-199a-5p	99,481,38	0.00	*Drd4*	14,539	0.002						
	miR-196a-5p	217,496,96	0.00	*Crhr2*	34,802	0.004						
	miR-1251-3p	299,704,07	0.00	*Mc2r*	313	0.022						
	miR-146b-3p	830,638,42	0.00	*Avpr2*	2.494	0.025						
	miR-135b-3p	1.885,814,37	0.00	*Drd5*	72.911	0.047						
Hypothalamus (female)	*miR-184-3p*	80,877,57	0.00	*miR-7007-5p*	−239,960,07	0.01	*miR-7007-5p*	−239,960,13	0.006	*miR-6926-3p*	1,034,264,585	0.004
	miR-7007-5p	−239,960,24	0.01									
Pituitary (male)	*miR-1960*	3.04E+05	0.01	*miR-125b-5p*	0.581	0.031				*miR-125b-5p*	0,575,006,666	0.043
	miR-6481	4.61E+04	0.03	*miR-206-3p*	−1.52E+05	0.047						
	miR-190a-3p	1.04E+05	0.03	*miR-128-3p*	0.682	0.050						
	miR-702-3p	1.19E+05	0.03									
	miR-488-3p	1.19E+06	0.03									
	miR-223-3p	0.28	0.03									
	miR-487b-5p	6.84E+05	0.04									
	miR-1a-3p	0.41	0.05									
	Cort	−0.17	0.03	*Ins1*	−379.97	0.04	*Ins1*	−384,52	0.04	*Ins1*	−393.85	0.03
	Ins1	−395,37	0.03	*Ucn2*	−8.25	0.03	*Ucn2*	−7.69	0.05	*Ucn2*	−8.19	0.03
	Ucn2	−8.12	0.03	*Avpr2*	−0.80	0.03	*Drd5*	−6.04	0.05	*Drd5*	−6.02	0.05
	Avpr2	−0.76	0.04				*Sstr4*	−0.29	0.04			
	Drd5	−6.03	0.05				*Prlr*	0.91	0.05			
Pituitary (female)	*miR-5615-3p*	−5.80E+05	0.013	*miR-223-3p*	0.330	0.006	*miR-7038-3p*	−0.54	0.01	*miR-7038-3p*	−0.373	0.039
	miR-1982-5p	−5.18E+05	0.044	*miR-122-5p*	3.148	0.007	*miR-17-5p*	1.45	0.01			
	miR-6901-5p	−4.16E+05	0.013	*miR-18a-5p*	1.666	0.010	*miR-210-3p*	0.98	0.01			
	miR-6929-5p	−3.18E+05	0.046	*miR-17-5p*	1.437	0.011	*miR-5615-3p*	−5.80E+05	0.01			
	miR-423-5p	1.046	0.003	*miR-155-5p*	0.587	0.012	*miR-6901-5p*	−4.16E+05	0.01			
	miR-17-3p	1.072	0.024	*miR-1a-3p*	0.937	0.012	*miR-106b-5p*	1.93	0.02			
	miR-210-3p	1.093	0.006	*miR-25-3p*	0.828	0.012	*miR-184-3p*	1.56	0.02			
	miR-574-3p	1.100	0.007	*miR-93-5p*	2.002	0.013	*miR-141-3p*	0.63	0.03			
	miR-329-5p	1.149	0.008	*miR-5615-3p*	−5.80E+05	0.013	*miR-18a-5p*	1.38	0.03			
	miR-16-5p	1.181	0.008	*miR-6901-5p*	−4.16E+05	0.013	*miR-193a-3p*	2.91	0.03			
	miR-27a-3p	1.230	0.029	*miR-106a-5p*	1.678	0.018	*miR-106a-5p*	1.44	0.04			
	miR-423-5p	1.232	0.007	*miR-214-3p*	0.667	0.021						

Table 1 (continued)

	1 min			10 min			25 min			40 min		
		Fold change	*P*		Fold change	*P*		Fold change	*P*		Fold change	*P*
	miR-200a-3p	1.333	0.018	*miR-199a-5p*	1.534	0.021						
	miR-181b-5p	1.352	0.011	*miR-206-3p*	5.489	0.023						
	miR-30e-5p	1.361	0.013	*miR-20a-5p*	1.500	0.023						
	miR-143-3p	1.365	0.008	*miR-100-5p*	1.423	0.024						
	miR-106a-5p	1.440	0.036	*miR-106b-5p*	1.699	0.030						
	miR-134-5p	1.504	0.048	*let-7a-5p-1*	0.407	0.035						
	miR-17-5p	1.534	0.008	*miR-21a-5p*	0.310	0.036						
	miR-181a-5p	1.549	0.006	*miR-326-5p*	−0.336	0.040						
	miR-181c-5p	1.583	0.006	*miR-449c-5p*	−1.16E + 06	0.044						
	miR-181a-5p-1	1.676	0.016									
	miR-199a-5p	1.709	0.012									
	miR-15a-5p	1.752	0.012									
	miR-335-5p	1.808	0.015									
	miR-18a-5p	2.040	0.003									
	miR-205-5p	2.125	0.017									
	miR-106b-5p	2.339	0.006									
	miR-149-3p	3.345	0.036									
	miR-19a-3p	3.479	0.007									
	miR-96-5p	3.656	0.001									
	miR-193a-3p	4.105	0.004									
	miR-337-3p	4.821	0.012									
	miR-22-3p	4.859	0.003									
	miR-19b-3p	13.025	0.011									
	Avpr1b	139.76	0.00	*Avpr1b*	90.04	0.02	*Avpr1b*	81.17	0.03	*Sstr1*	−0.52	0.003
	Sstr1	−0.09	0.58							*Sstr1*	−0.52	0.003
	Gnrhr	1101	0.16							*Crhr2*	−0.24	0.001
	Crhr2	−0.10	0.12							*Drd2*	−3.40	0.04
	Drd2	−0.17	0.91							*Sstr4*	0.08	0.001
										Ucn2	1,00	0.01
										Sstr2	0.07	0.02
										Hsd11b2	0.24	0.02
										Lhcgr	−0.24	0.03
										Avpr2	0.11	0.03
										Ins1	95,92	0.03
										Ucn	0.69	0.03

Table 1 (continued)

	1 min			10 min			25 min			40 min		
		Fold change	*P*		Fold change	*P*		Fold change	*P*		Fold change	*P*
										Drd5	0.38	0.04
Adrenal (male)	*miR-296-5p*	0.183	0.024	*miR-451b*	9.04E + 05	0.01	*miR-296-5p*	0.237	0.009	*miR-7038-3p*	−1.4870	0.002
	miR-1a-3p	−0.463	0.030	*miR-1a-3p*	−0.42	0.03	*miR-128-3p*	0.149	0.023	*miR-1a-3p*	0.7561	0.010
	miR-675-3p	5.84E + 05	0.031	*miR-669 k-5p*	−0.09	0.04	*miR-34c-5p-1*	5.612	0.025	*miR-451b*	9.24E + 05	0.019
	miR-135b-3p	−2.41E + 06	0.031	*miR-1982-5p*	−1.70	0.04	*miR-7006-5p*	0.989	0.039			
	miR-429-5p	−0.617	0.031	*miR-191-5p*	−0.23	0.05	*miR-206-3p*	8.281	0.041			
	miR-429-3p	−0.617	0.031									
	miR-6919-3p	−0.617	0.031									
	miR-6967-5p	−0.617	0.031									
	miR-185-3p	−0.617	0.031									
	miR-344 g-3p	−0.617	0.031									
	miR-292b-3p	−0.617	0.031									
	miR-7038-3p	−0.617	0.031									
	miR-302c-5p	−0.617	0.031									
	miR-23b-5p	−0.617	0.031									
	miR-471-3p	−0.617	0.031									
	miR-665-5p	−0.617	0.031									
	miR-326-5p	−0.617	0.031									
	miR-6975-5p	−0.617	0.031									
	miR-149-5p	−0.617	0.031									
	miR-6953-5p	−0.617	0.031									
	miR-6904-5p	−1.134	0.036									
	miR-1982-5p	−1.858	0.041									
	Slc6a3	−0.05	0.02	*Mc2r*	−3019	0.01	*Slc6a3*	−0.04	0.04	*Sstr1*	−0.21	0.04
	Sstr1	−0.22	0.03	*Drd2*	−0.2	0.02	*Sstr1*	−0.23	0.03	*Sstr3*	−0.24	0.02
							Gnrhr	4.99	0.00			
							Sstr3	−0.23	0.03			
Adrenal (female)	*miR-6998-5p*	−0.859	0.003	*miR-6998-5p*	−1.021	0.001	*miR-6998-5p*	−1.165	0.001	*miR-6998-5p*	−1.242	0.001
	miR-5104	−2.490	0.005	*miR-5104*	−2.662	0.003	*miR-5104*	−3.243	0.001	*miR-5104*	−3.441	0.001
	miR-1982-5p	−4.693	0.013	*miR-1982-5p*	−5.467	0.007	*miR-1982-5p*	−6.521	0.003	*miR-1982-5p*	−6.808	0.003
	miR-214-3p	6.354	0.036	*miR-1943-5p*	-2.535	0.024	*miR-6901-5p*	−3.180	0.014	*miR-5615-3p*	8.94E + 05	0.010
				miR-7007-5p	−1.194	0.044	*miR-7007-5p*	−1.537	0.018	*miR-467f*	−1.140	0.011
				miR-6901-5p	−2.303	0.045	*miR-467f*	−0.857	0.032	*miR-6901-5p*	−2.879	0.020
				miR-1960	−1.091	0.047	*miR-1943-5p*	−2.299	0.034	*miR-25-5p*	−1.245	0.031
							miR-7018-5p	−1.755	0.047	*let-7a-5p*	−0.674	0.032

Table 1 (continued)

	1 min			10 min			25 min			40 min		
		Fold change	*P*		Fold change	*P*		Fold change	*P*		Fold change	*P*
							miR-1960	−1.092	0.047	*miR-1943-5p*	−2.303	0.034
										miR-6985-3p	−0.829	0.045
										miR-7007-5p	−1,157	0.048
	Fshr	1,6	0.01	*Ucn2*	−12.25	0.004	*Sstr3*	−0.094	0.001	*Cort*	−0.279	0.030
	Hsd11b2	−1.343	0.02	*Nr3c1*	0.035	0.005	*Ghr*	0.306	0.003	*Insr*	0.756	3.55E+09
	Fshb	0.141	0.02	*Sstr4*	−0.258	0,006	*Avpr2*	−1.471	0,003	*Nr3c1*	0.055	4.08E+09
	Ins1	−407,083	0.02	*Ucn3*	−0.546	0.008	*Nr3c1*	0.036	0.004	*Sstr3*	−0.113	0.0001
	Drd3	0.011	0.03	*Insr*	0.438	0.010	*Ucn2*	−1.21	0.004	*Prlr*	2.153	0.0002
	Drd1a	0.106	0.03	*Avpr2*	−1.3	0.010	*Sstr4*	−0.265	0.004	*Avpr2*	−1.829	0.001
	Sstr2	−0.221	0.05	*Sstr3*	−0.070	0.010	*Sstr5*	−2.629	0.006	*Drd5*	−4.846	0.001
				Drd5	−3.546	0.011	*Drd5*	−3.656	0.008	*Ucn2*	−14,555	0.001
				Sstr5	−2.4	0.012	*Gnrh1*	0.043	0.011	*Sstr5*	−3.312	0.001
				Ins1	−423.303	0.018	*Hsd11b1*	0.665	0.013	*Sstr4*	−0.315	0.001
				Gnrhr	9.95	0.021	*Sstr2*	−0.262	0.017	*Hsd11b1*	0.918	0.001
				Gnrh1	0.040	0.022	*Ins1*	−4.13	0.017	*Gnrhr*	13.698	0.002
				Sstr2	−0.254	0.024	*Ucn3*	−0.467	0.019	*Gnrh1*	0.054	0.003
				Prlr	1.18	0.026	*Hsd11b2*	−1.232	0.023	*Ucn3*	−0.627	0.003
				Drd4	13.411	0.028	*Avpr1a*	0.019	0.025	*Drd4*	18.345	0.003
				Hsd11b2	−1.208	0.030	*Insr*	0.328	0.043	*Sstr1*	−0.027	0.004
				Sstr1	−0.018	0.044				*Ghr*	0.300	0.004
										Ins1	−514	0.005
										Hsd11b2	−1.541	0.007
										Ucn	−1.261	0.008
										Avpr1b	−25.096	0.011
Blood(male)	*miR-191-5p*	−2.76	0.04	*miR-1960*	−2.35.948	0.029	*miR-7070-3p*	1.27E+05	0.000	*miR-5615-3p*	852,642,9	0.02
	let-7a-5p-1	−1.83	0.05				*miR-6418-5p*	8.69E+05	0.000	*miR-449b*	0,803,237	0.02
	miR-452-3p	−0.97	0.00				*miR-96-5p*	-4.1E+04	0.004	*miR-191-5p*	−2,415,286	0.03
	miR-344 g-3p	−0.97	0.00				*miR-429-5p*	−0.61	0.004	*miR-1960*	−2,364,369	0.03
	miR-326-5p	−0.97	0.00				*miR-429-3p*	−0.61	0.004	*let-7a-5p-1*	−1.60,076	0.03
	miR-145a-5p	−0,63	0.04				*miR-6919-3p*	−0.61	0.004	*miR-23a-3p*	−0.241,724	0.04
	miR-23a-3p	−0.31	0.04				*miR-6967-5p*	−0.61	0.004	*miR-145a-5p*	−0.478,674	0.04
	miR-429-5p	1.54	0.00				*miR-185-3p*	−0.61	0.004			
	miR-429-3p	1.54	0.00				*miR-344 g-3p*	−0.61	0.004			
	miR-6919-3p	1.54	0.00				*miR-23b-5p*	−0.61	0.004			
	miR-6967-5p	1.54	0.00				*miR-471-3p*	−0.61	0.004			

Table 1 (continued)

1 min			10 min			25 min			40 min		
	Fold change	*P*		Fold change	*P*		Fold change	*P*		Fold change	*P*
miR-185-3p	1.54	0.00				*miR-665-5p*	−0.61	0.004			
miR-23b-5p	1.54	0.00				*miR-326-5p*	−0.61	0.004			
miR-471-3p	1.54	0.00				*miR-6975-5p*	−0.61	0.004			
miR-665-5p	1.54	0.00				*miR-452-3p*	−0.61	0.004			
miR-6975-5p	1.54	0.00				*miR-1960*	−2.68	0.018			
miR-6904-5p	1.54	0.01				*miR-23a-3p*	−0.27	0.026			
miR-467f	1.75	0.00				*miR-675-3p*	−5.3E + 05	0.040			
miR-302c-5p	1.85	0.01				*miR-3065-5p*	−0.26	0.045			
miR-126b-5p	1.85	0.01									
miR-292b-3p	1.91	0.00									
miR-7038-3p	2.23	0.01									
miR-449b	2.51	0.00									
miR-1943-5p	6.38	0.00									
miR-7007-5p	7.68	0.00									
miR-7118-5p	12.46	0.02									
miR-1982-5p	14.84	0.01									
miR-7018-5p	20.19	0.00									
miR-10a-5p	1394.96	0.00									
miR-204-5p	1579.56	0.00									
miR-376c-3p	2346.34	0.00									
miR-128-3p	6041.79	0.00									
miR-325-3p	7974.08	0.01									
miR-181a-5p	1.1E + 04	0.00									
miR-337-3p	1.1E + 04	0.01									
miR-143-3p	1.1E + 04	0.00									
miR-181c-5p	1.2E + 04	0.00									
miR-203-3p	1.5E + 04	0.00									
miR-205-5p	1.5E + 04	0.00									
miR-34c-5p	4.2E + 04	0.00									
miR-34b-5p	5.1E + 04	0.00									
miR-96-5p	8.0E + 04	0.00									
miR-34c-5p-1	8.8E + 04	0.00									
miR-184-3p	1.7E + 05	0.02									
miR-6481	1.9E + 05	0.00									
miR-669 k-5p	3.0E + 05	0.00									

Table 1 (continued)

	1 min			10 min			25 min			40 min		
		Fold change	*P*		Fold change	*P*		Fold change	*P*		Fold change	*P*
	miR-190a-3p	4.3E+05	0.00									
	miR-702-3p	5.5E+05	0.00									
	miR-675-3p	1.0E+06	0.01									
	miR-5625-3p	1.4E+06	0.00									
	miR-466n-5p	1.8E+06	0.01									
	miR-133b-3p	2.3E+06	0.00									
	miR-487b-5p	2.3E+06	0.01									
	miR-135b-3p	8.6E+06	0.00									
	Ucn	−34.440	0.00	*Ucn*	−32.8	0.001	*Sstr1*	0.5	0.001	*Slc6a3*	−0.236	0.038
	Slc6a3	−0.444	0.00	*Slc6a3*	−0.4	0.002	*Prl*	−52,3	0.002	*Prl*	−38.854	0.03
	Sstr1	−0.700	0.00	*Sstr1*	−0.7	0.01	*Prlr*	−0.10	0.01	*Crh*	−0.219	0.033
	Sstr3	−0.853	0.001	*Sstr3*	−0.9	0.001	*Avpr1b*	−117,9	0.001			
	Prl	−63.719	0.001	*Prl*	−60.04	0.004	*Slc6a3*	−0.4	0.004			
	Prlr	−0.140	0.001	*Prlr*	−0.1	0.01	*Crh*	−0.3	0.004			
	Crh	−0.356	0.001	*Crh*	−0.33	0.002						
	Avpr1b	−167,983	0.001	*Avpr1b*	−127,7	0.02						
	Fshb	−0.032	0.016	*Ucn3*	−0.96	0.02						
Blood (female)	*miR-221-3p*	−52.432	0.03	*miR-221-3p*	−51.756	0.036	*miR-221-3p*	−52.2697	0.035	*miR-221-3p*	−42.297	0.05
							miR-122-5p	68.6911	0.028			
				Sstr1	0.59	0.01	*Sstr1*	0.73	0.001	*Sstr1*	0.70	0.001
				Prl	33.9	0.01	*Prl*	40.1	0.002	*Prl*	45.4	0.001
				Mc2r	0.24	0,01	*Ucn*	84,9	0.01	*Prlr*	0.10	0.001
				Pomc	0.01	0.01	*Prlr*	0.06	0.01	*Avpr1b*	134.9	0.001
				Prlr	0.05	0.02	*Slc6a3*	0.21	0.01	*Slc6a3*	0.36	0.001
				Avpr1b	100	0.02	*Insr*	−0.01	0.006	*Crh*	0.20	0.001
				Ghsr	0.002	0.03	*Avpr1b*	115.3	0.02			
				Slc6a3	0.16	0.03	*Hsd11b2*	29.6	0.02			
				Gnrhr	0.12	0.04	*Sstr3*	2,98	0.02			

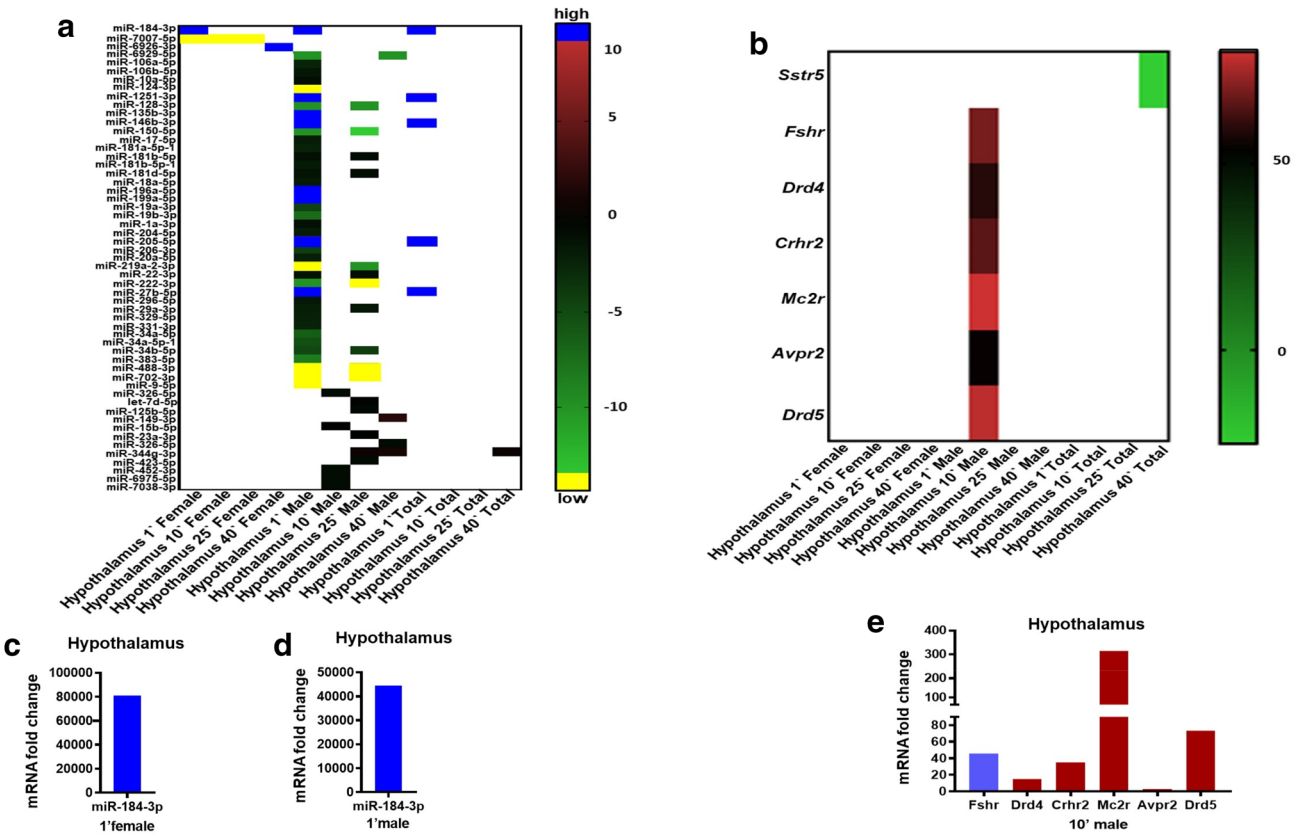


Fig. 2 Heat map displaying the expression value of transcripts associated with functional groups from hypothalamus tissues after ITT. All presented data are statistically significant according to saline buffer-injected control groups ($p < 0.05$). **a** Patterns of 55 differentially expressed miRNA, **b** Profiles of 7 differentially expressed mRNAs associated with functional groups from hypothalamus tissues. In the figure, columns represent groups, and rows represent statistically significant genes (Colors correspond to: white unchanged, black up-regulated, red intermediate up-regulated, blue highly up-regulated, green down-regulated and yellow highly down-regulated). A few miRNA and mRNA profiles are presented in the diagram as examples from data shown in the heat map. **c** Profile of *miR-184-3p*, the only miRNA up-regulated in female hypothalamus. **d** The same miRNAs are found as well up-regulated among other miRNAs expressed in the male hypothalamus. While non-changes were found for the mRNA transcripts under investigation here in the female, in contrast several mRNAs are found up-regulated expressed in the male hypothalamus; in **e** is shown a few examples found up-regulated at 10 min: *Fshr, Drd4, Crhr2, Mc2r, Avpr2* and *Drd5*). Red, purple, gray and yellow bars correspond to HPA, HPG, GH axes genes, respectively

in males 10 min after performing ITT (Fig. 2b, e); however, no changes were detected at other time points examined in male or female hypothalamus (Fig. 2b). Levels of *Mc2r* transcript were remarkably higher in males following ITT, especially 10 min only after developing hypoglycemia. The female hypothalamus showed a continuous absence of any changes in mRNAs and variation in only three miRNAs, while miRNA and mRNA variations were more numerous in males (Fig. 2a, b) (Table 1).

Hypoglycemia Induces Opposite Responses in the Pituitary of Males and Females

In the pituitary, in contrast to the hypothalamus, miRNA responses of female mice were numerous and occurred quickly at 1 and 10 min (Table 1 and Fig. 3). Rapid response was also observed in males, although altered factors were less numerous than in female mice. Again, important differences in the pituitary were observed between males and females; for example, eight miRNAs (miR-1960, miR-6481, miR-190a-3p, miR-702-3p, miR-488-3p, miR-223-3p, miR-487b-5p and miR-1a-3p) were up-regulated at 1 min, only miR-206-3p was extremely down-regulated at 10 min in males, while greater numbers of different miRNAs in females were altered at 1, 10 and 25 min (Table 1 and Fig. 3a). Pituitary responses to hypoglycemia in females were associated with modulation of several miRNAs. In males, only a few miRNAs were changed, and none of these miRNA responses were sustained during activation of the HPA axis. Only miR-206-3p in males was excessively decreased 10 min after performing the ITT. In contrast, many miRNAs were significantly up- or down-regulated in the pituitary of female Balb/c mice at all time points. Notably, significantly reduced

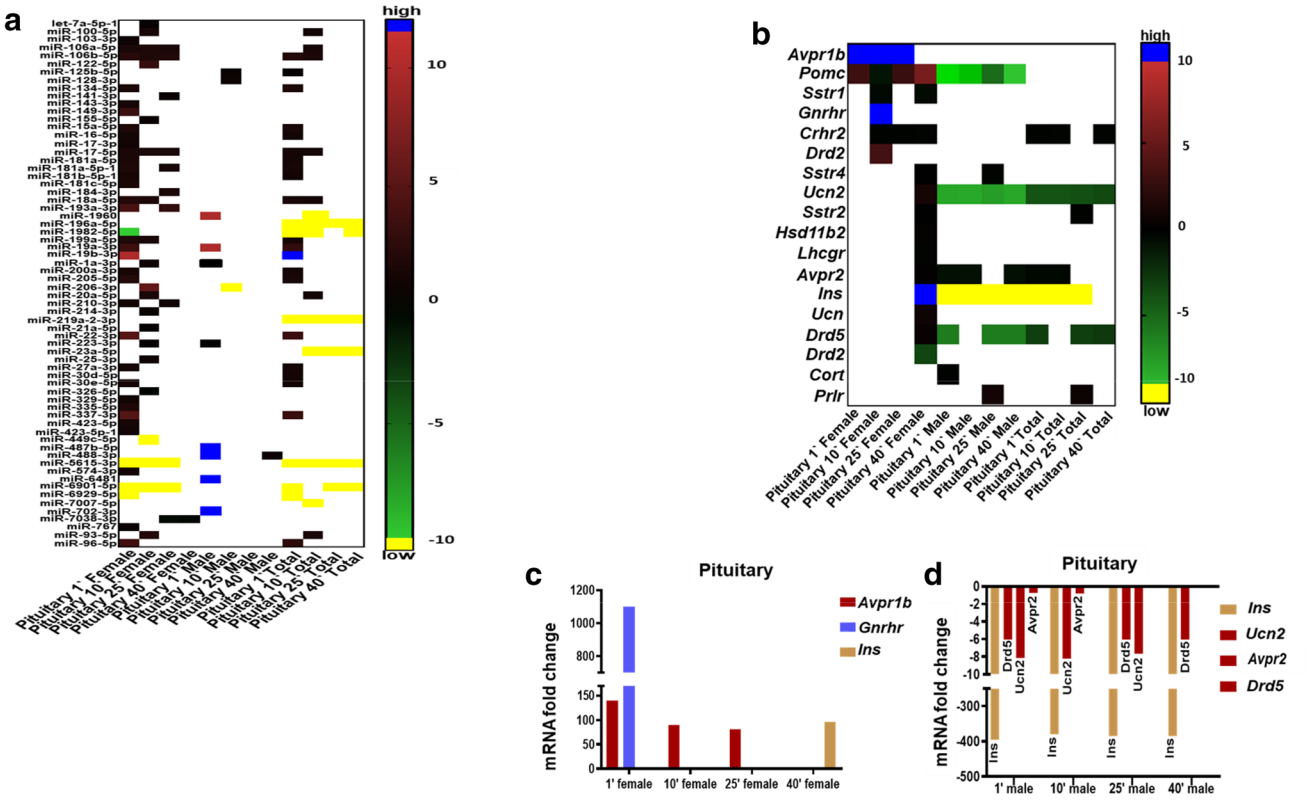


Fig. 3 Heatmap displaying the expression value of transcripts associated with functional groups from pituitary tissues after ITT. All presented data are statistically significant according to saline buffer-injected control groups ($p < 0.05$). **a** Patterns of the 68 differentially expressed miRNA. **b** Profiles of 18 differentially expressed mRNAs associated with functional groups from pituitary tissues. In the figure, columns represent groups, and rows represent statistically significant transcripts (Colors correspond to: white unchanged, black up-regulated, red intermediate up-regulated, blue highly up-regulated, green down-regulated and yellow highly down-regulated). A few mRNA profiles are presented in the diagram as examples from data shown in the heat map. **c** mRNA profiles of three (*Gnrhr, Avpr1b* and *Ins*) up-regulated transcripts expressed in the female pituitary. **d** mRNA profiles of four (*Ins, Ucn2, Avp2r* and *Drd5*) all down-regulated transcripts in most points expressed in the male pituitary. Red, purple, gray and yellow bars correspond to HPA, HPG, GH axes and Ins gene, respectively)

levels of miR-5615-3p and miR-6901-5p were observed at 1, 10 and 25 min, in miR-449c-5p at 10 min and miR-7038-3p at 40 min of ITT (Fig. 3a). These opposing responses in miRNA levels consequently led to reduced transcript levels only in males and, accordingly, due to high levels of several miRNAs, several transcripts (*Ins, Ucn2, Avpr2* and *Drd5*) were continuously expressed at lower levels in the pituitary of male mice at all time points after performing ITT. In particular, *Ins* transcripts were excessively reduced (Fig. 3b and d). In contrast, in the pituitary gland of female mice, mostly transcripts were up-regulated, with several important transcripts expressing at very high levels. Notably, *Avpr1b* transcripts were significantly increased at 1, 10 and 25 min, and *Gnrhr* transcripts were significantly increased at 1 min in the pituitary of female mice. In addition, the highest number of transcript alterations was observed at 1 (miRNAs) and 40 (mRNAs) minutes in the pituitary of female mice. On the other hand, none of the mRNAs were increased in the pituitary of male mice at all time points, but we observed that levels of the *Ins* transcript were excessively reduced at all time points following the ITT in male mice, whereas levels of *Ins* transcript were significantly increased at 40 min in the female pituitary. However, the same observation for *Avpr1b* did not occur in the male pituitary. Additionally, *Avpr2* transcripts at 1, 10 and 40 min were significantly decreased in the pituitary of male mice. Robust changes in levels of *Avpr1b* and *Avpr2* transcripts at all time points strongly suggest that these molecules are involved with differential activation signals between female and male pituitary tissues (Table 1, Fig. 3a–d).

In male mice, most transcripts were unchanged or reduced at all time points following ITT. In particular, transcripts for *Cort, Ucn2, Avpr2, Drd5* and *Ins* were decreased, and expression levels of *Ins* transcript were excessively decreased at all time points after performing ITT compared to the control group. In female mice, transcripts were unchanged or increased at all time points following ITT.

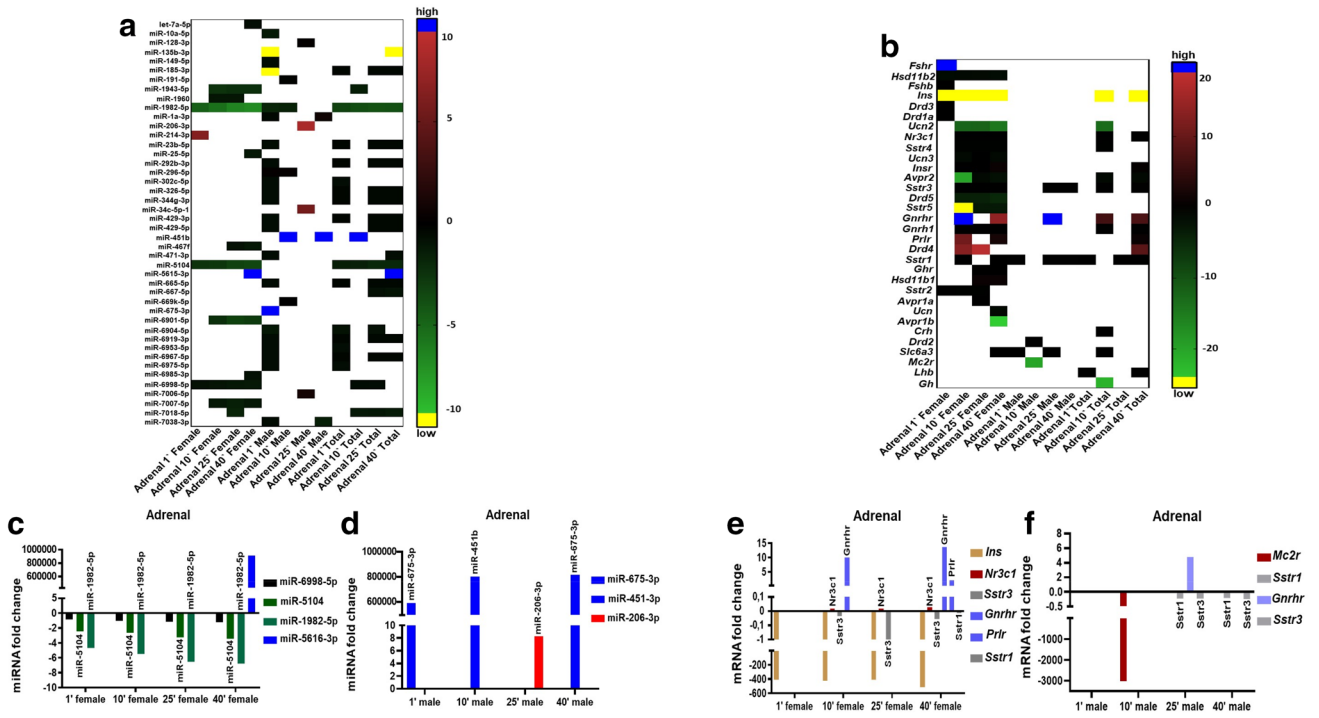


Fig. 4 Heatmap displaying the expression value of transcripts associated with functional groups from adrenal tissues after ITT. All presented data are statistically significant according to saline buffer-injected control groups ($p < 0.05$). **a** Patterns of 44 differentially expressed miRNA. **b** Patterns of 32 differentially expressed mRNAs. In the figure, columns represent groups, and rows represent statistically significant genes (Colors correspond to: white unchanged, black up-regulated, red intermediate up-regulated, blue highly up-regulated, green down-regulated and yellow highly down-regulated). A few miRNA and mRNA profiles are presented in the diagram as examples from data shown in the heatmap. **c** Profile of three miRNAs (*miR-6998-5p, miR-5104* and *miR-1982-5p*) down-regulated in all time points and one (*miR-5615-3p*) up-regulated only after 40 min in the female adrenal. **d** Example of two miRNA (*miR-675-3p* and *miR-451b*) expression profiles down-regulated in all points and one *miR-206-3p* up-regulated only after 40 min in the male adrenal. **e** mRNA profiles of two (*Gnrhr* and *Prlr*) up-regulated and four (*Ins, Nr3c1, Sstr1* and *Sstr3*) down-regulated transcripts expressed in the female adrenal. **f** mRNA profiles of three (*Mc2r, Sstr1* and *Sstr3*) down-regulated and one (*Gnrhr*) up-regulated transcripts expressed in the male adrenal. Red, purple, gray and yellow bars correspond to HPA, HPG, GH axes and Ins gene, respectively

Avpr1b transcripts were significantly increased in the pituitary of female mice at 1, 10 and 25 min of ITT (Fig. 3b–d).

Male and Female Adrenal Glands Exhibit Differential Responses to Insulin-Induced Hypoglycemia

The adrenal glands of male and female mice also reacted quite differently in terms of the number and profile of transcripts involved in HPA axis activation (Table 1, Fig. 4a and b). Furthermore, examination of adrenal glands after inducing hypoglycemia indicates that reduced numbers of miRNAs were affected in females (Fig. 4a). At all time points, in all samples from the adrenal glands, two miRNAs (*miR-214-3p* and *miR-5615-3p*) in females and five miRNAs (*miR-675-3p, miR-451b, miR-206-3p, miR-34c-5p-1* and *miR-451-b*) in males were up-regulated (Table 1 and Fig. 4a, c and d). In particular, *miR-451b* was excessively increased at 10 and 40 min (Fig. 4d). In females, *miR-6998-5p, miR-1982-5p* and *miR-5104* were down-regulated in a sustained pattern, while *miR-7007-5p, miR-1943-5p* and *miR-6901-5p* were down-regulated after 10 min (Fig. 4a and c). Higher signals were observed for *miR-5615-3p* at 40 min in females and at 1 (*miR-657-3p*), 10 (*miR-451b*), 25 (*miR-206-3p*) and 40 (*miR-451-3p*) minutes in males. The association of miR-451b and miR-5615-3p in stress-induced signaling pathways has not been previously reported, and further studies are needed to determine the function of these miRNAs in sex differences and HPA axis regulation (Fig. 4c, d). We report here that the most intense response of the HPA axis to hypoglycemia was observed in the adrenal glands of female mice (Fig. 4b). Among the other mRNAs tested, male adrenal responses differed remarkably, with reductions in levels of several molecules, especially at the 1 min time point. *Ins* transcripts were significantly down-regulated at all time points in the female adrenal glands (Table 1, Fig. 4b, e). *Fshr* transcripts were found at high levels immediately after insulin injection only in the female adrenal glands, while *Gnrhr* was increased at 10 and 40 min in females and 25 min in males (Fig. 4e). In the male adrenal glands, levels of *Mc2r* transcript were excessively decreased at 10 min, and

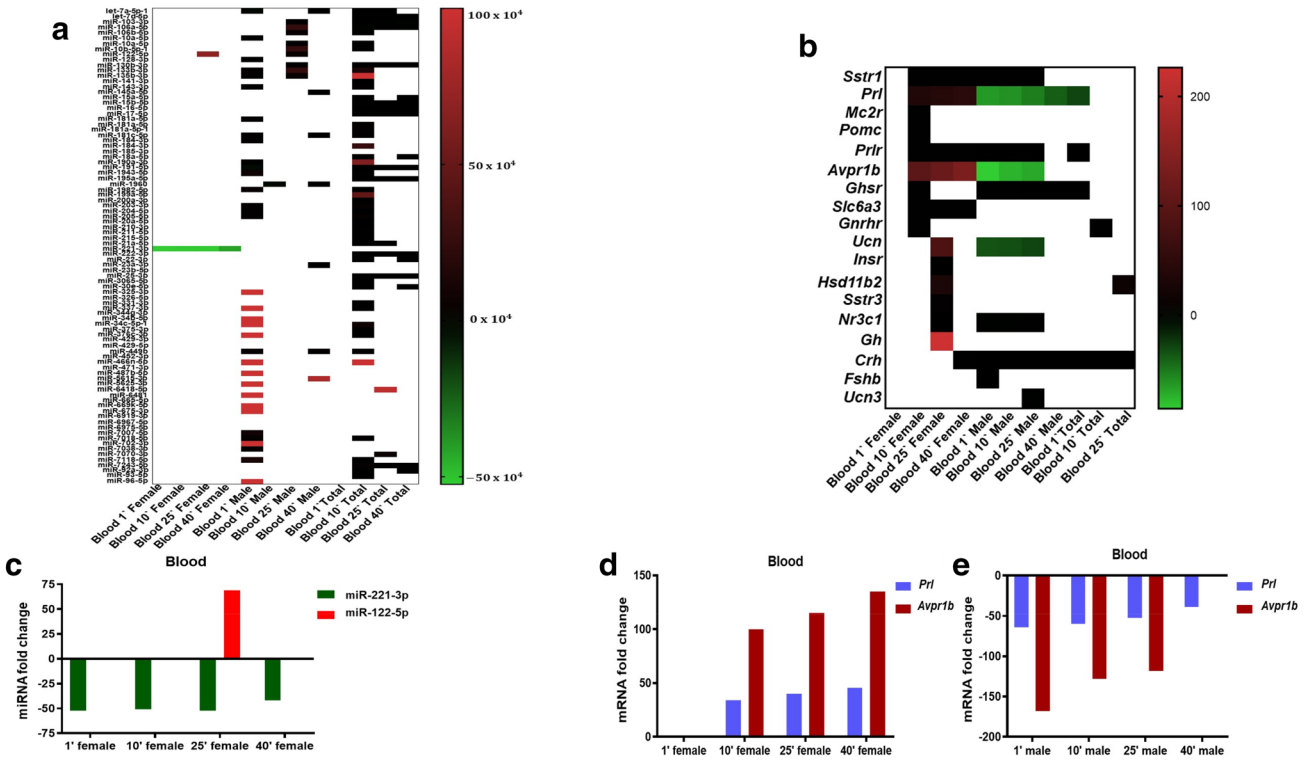


Fig. 5 Heatmap displaying the expression value of transcripts associated with functional groups from blood samples after ITT. All presented data are statistically significant according to saline buffer-injected control groups ($p < 0.05$). **a** Patterns of 88 differentially expressed miRNAs. **b** Patterns of 18 differentially expressed mRNA. In the figure, columns represent groups, and rows represent statistically significant genes (Colors correspond to: white unchanged, black up-regulated, red intermediate up-regulated, blue highly up-regulated, green down-regulated and yellow highly down-regulated). A few miRNA and mRNA profiles are presented in the diagram as examples from data shown in the heatmap. **c** Example of *miR-221-3p* transcripts stably down-regulated in all points in contrast to *miR-122-5p* induced only at 25 min in the female blood. **d** Two mRNAs (*Prl* and *Avpr1b*) stably up-regulated profiles observed in the female blood. **e** In contrast, the same mRNAs (*Prl* and *Avpr1b*) stably down-regulated in male blood. Red, purple, gray and yellow bars correspond to HPA, HPG, GH axes and Ins gene, respectively

transcripts of *Sstr1* and *Sstr3* were significantly lower at 25 and 40 min after performing ITT (Fig. 4f). Although only a few transcripts were found to be altered in the male adrenal glands, remarkably more changes were noted in transcript levels in the female adrenal glands at 1, 10, 25 and 40 min after ITT. Transcript levels of *Cortisol, Drd1a, Hsd11b2, Fshb, Drd3* and *Sstr2* were significantly decreased in female mice compared to the control group at 1 min after ITT. Other transcripts were also found at lower levels (see Fig. 4).

We propose that the reduction in *Mc2r* transcript levels in the adrenal glands of male mice at 10 min following ITT decreased ACTH responses from the pituitary by decreasing *Mc2r* levels following hormonal responses. We observed an increase in *Gnrhr* transcript levels at 10 (female) and 25 (male) minutes that revealed activation of both the HPG and HPA axes in response to hypoglycemia during the ITT (Fig. 4e and f).

Figure 3b, *Ins* transcripts were continuously decreased at all time points after performing the ITT in the pituitary of male mice while in agreement with Stanley et al. (Stanley et al. 2019) in adrenal glands of female mice (Fig. 4b). These results highlight that major responses to hypoglycemia occur in distinct tissues between males (primarily in the pituitary) and females (primarily in the adrenal glands) (Figs. 2 and 6).

Hypoglycemia Leads in the Blood to Immediate Transcript Alterations in the Male, While in the Females, take Longer

Evaluation of blood from male and female mice for miRNA products at the given time points after ITT indicates that, except after 1 min, levels of many miRNAs were significantly different after 10 min, in particular and continuing at 25 and 40 min. The miRNA profiles in the blood (Fig. 5a) and hypothalamus (Fig. 2a) of each sex were similar, that means in female signals were rare, only two miRNAs changed: *miR-122-5p* was increased at 25 min, and *miR-221-3p* continuously decreased (Table 1 and Fig. 5a), while immediate and multiple responses were observed in males (Table 1 and Fig. 5a). Male blood showed several important

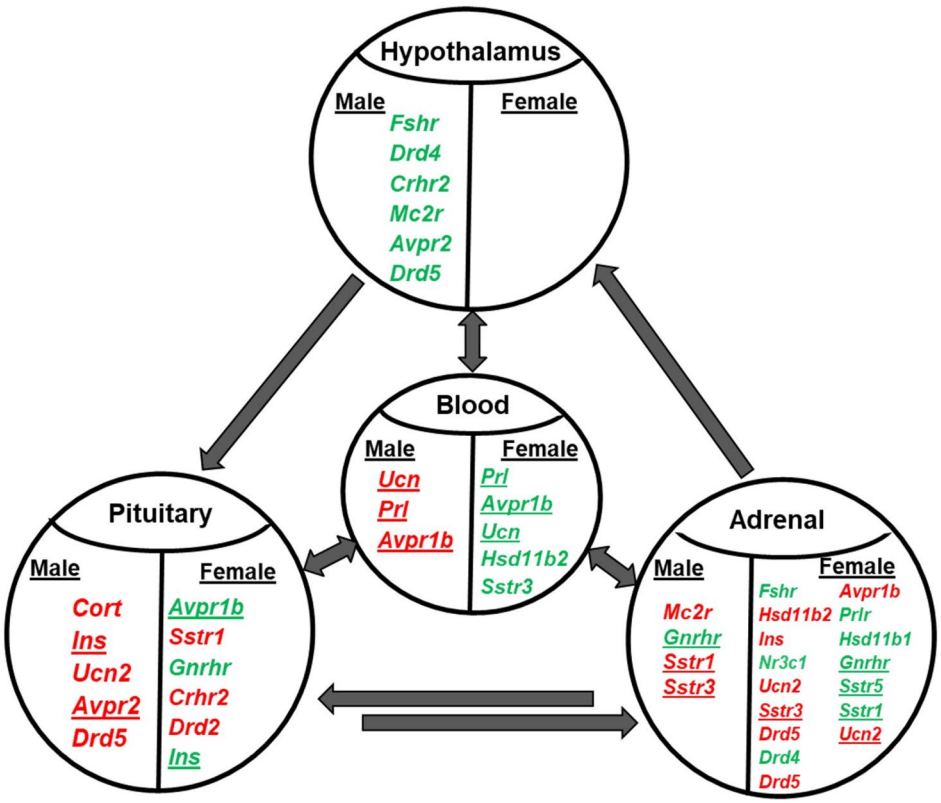


Fig. 6 Summary of the down and up-regulated transcripts in the hypothalamus, pituitary, adrenal, and blood of female and male mice at all time points after ITT (Green corresponds up-regulated, red corresponds down-regulated) (Underlined genes have expression in both males and females)

alterations. Starting at 1 min, immediate responses to insulin were detected, and expression levels of many miRNAs were changed in males (Table 1).

Recent studies have shown that *miR-221-3p* and *miR-122-5p* are associated with many diseases, such as hepatocellular carcinoma and cardiac arrest (Devaux et al. 2017; Wang et al. 2019). However, possible association of *miR-221-3p* and *miR-122-5p* with the HPA axis has not yet been investigated.

Sex differences are particularly noteworthy with *Avpr1b* and *Prl* transcripts which were excessively decreased in the blood of male mice at all time points after performing ITT (Fig. 5d and e), while these molecules were excessively increased in the blood of female mice. In addition, other transcripts (*Sstr1, Sstr3, Slc6a3, Prlr*) that showed decrease at all time points after ITT in male mice showed, in contrast, increases in the blood of female mice (Fig. 5b).

Discussion

Overall a series of selected transcript analysis involved in hypothalamic–pituitary–adrenal (HPA), hypothalamic–pituitary–gonadal (HPG) and growth-hormone (GH) axes revealed that ITT induced important alteration in a large number of miRNAs and mRNAs at the same time in hypothalamus, pituitary and adrenal tissues, as well as in the blood of male mice, while in female mice, alterations were mostly observed in the pituitary and adrenal glands. The male hypothalamus showed multiple responses by several miRNA and mRNA alterations at the very beginning of the first minute (miRNA) and 10 min (RNA) after insulin administration, suggesting that miRNAs are the first signals triggering the response to ITT followed by several mRNA transcript variations at later time points. On the other hand, the female hypothalamus did not react to hypoglycemia in the same manner as observed in males, with no apparent changes recorded in the candidate transcripts at any times, except for changes in three miRNAs. Only one miRNA (miR-184-3p) was increased in the hypothalamus in both sexes immediately (very start) after ITT and this miRNA has been shown to be involved in the development of the peripheral nervous system (PNS) in Drosophila (Peng et al. 2015). Apparently, here, induction of *miR-184-3p* at the same range in both sexes is not sufficient to induce transcriptional changes in the female hypothalamus. In contrast, in males, variations in several miRNA levels at the same time points are accompanied with variations in mRNA candidate levels. Transcripts for *Fshr, Drd4, Crhr2, Mc2r, Avpr2* and *Drd5* were increased exclusively in males 10 min after performing ITT; however, no changes were detected at other time points examined in male or female hypothalamus. Levels of *Mc2r* transcript were remarkably higher in males following ITT. At the protein level, it is reported that

melanocortin receptors (MCRs) are the primary receptors of the melanocyte-stimulating hormone (MSH) produced by *Pro-opiomelanocortin (Pomc)* together with ACTH, and these receptors play a role in the response to stress (Cai and Hruby 2016; Tooke et al. 2019). The MC2R protein plays a critical role in regulation of the HPA axis in mammals (Wingenfeld and Otte 2018). ACTH released from the anterior pituitary binds to MC2R on internal cells of the adrenal cortex, leading to the synthesis and release of glucocorticoids from steroid hormone-producing cells. The MC2R protein is activated by both ACTH and MSH (Dores et al. 2016). In the hypothalamus of male mice, *Mc2r* transcript may be increased to support the negative feedback mechanism 10 min after performing ITT because the activity of the HPA axis is inhibited by binding of cortisol to NR3C1 (GR) and by binding of ACTH to MC2R receptors in the hypothalamus and pituitary (Dores et al. 2016).

CRH and their receptors (*Crhr1* and *Crhr2*) play a critical role in regulating the neuroendocrine stress response. In addition, it has repeatedly been shown that stress alters dopaminergic functions in the brain, including dopamine release and receptor (DRDs) binding (Kasahara et al. 2011). We have shown that *Crhr2, Drd4* and *Drd5* transcripts were increased at 10 min only in the male hypothalamus after ITT. This situation indicates that the dopaminergic system is also activated in the male hypothalamus together with hypoglycemia.

The present results indicate that in male mice, transcript variations begin in the blood and hypothalamus. The specific mechanisms for divergent activation of the HPA axis between males and females are unknown. Here, with a single stress (ITT), we clearly report that all tissues (hypothalamus, pituitary, adrenals and blood) responded differentially and that the range of activation signals was influenced by sex differences in terms of miRNA and mRNA transcripts. As expected, this could be of importance for appropriate diagnosis of the responses from the HPA axis insufficiency.

Stress with insulin could therefore plausibly affect several clinical outcomes, since HPA, HPG and GH–IGF axes are related to one other. Generally, activation of the HPA axis results in an immediate response from the hypothalamus during the first minutes after ITT. However, as mentioned above, this appears to be dependent on sex, underlying the influence of gonadal hormones estrogen and progesterone from the ovaries and androgens and testosterone from the testes. It is known that hypoglycemia reaching 50 mg/dl significantly increases cortisol and GH levels in men but not in women (Stanley et al. 2018). However, the effects of both are slow, on the order of hours, exerting physiologically opposite responses from neuroendocrine, cardiovascular and nervous systems that are reduced in women.

According to Stanley et al. during hypoglycemia, insulin gene expression decreases in the male pituitary and female adrenal glands, and this situation may be related to the sex differences observed here (Stanley et al. 2019). Another important difference noted here is in the *Avpr2* and *Avpr1b* transcripts (see summary in Table 1 and Fig. 6) shows transcript alteration in the hypothalamus, pituitary, adrenal and blood of female and male mice at different time points after insulin injection until hypoglycemia develops. Data from this study indicate that ITT induces transcripts of *Avpr1b* in the female blood and pituitary but reduces in the male blood. In contrast, *Avpr2* is only affected in males, increasing in the hypothalamus and decreasing in the pituitary. These results are in agreement with a previous study (Kacheva et al. 2015) that linked AVP to activation of the hypothalamic–pituitary–adrenal system, which is involved in the regulation of osmotic homeostasis and blood pressure. AVP is a hormone secreted from the hypothalamus that increases ACTH release during stress and has synergistic effects with CRH (Kacheva et al. 2015). Synthesized from central nervous system and released, AVP acts by binding to two different receptors localized in different brain regions (AVPR1A, AVPR1B) and kidneys (AVPR2). These receptors are G protein-coupled receptors stimulated by phospholipase C activation (Roper et al. 2011). Although the receptors are structurally similar and share common secondary messenger systems, they differ in their functional properties (Stewart et al. 2008; Roper et al. 2011; Mohan et al. 2019). Many studies have shown that AVP and AVP receptors play roles in regulating the HPA axis (Joseph and Whirledge 2017). However, CRH was later reported to be more important for regulation of the HPA axis (Kovács et al. 2000; Spiga et al. 2009, 2014). In this study, we report for the first time that the regulation of AVP transcripts in the activation of the HPA axis in response to hypoglycemia is distinct between the sexes. According to our results, *Avpr1b* and *Avpr2* transcripts seem to play a role in regulation of the HPA axis with sex-dependent mechanisms.

Furthermore, there were greater alterations in transcript levels not only in the mRNAs of known proteins involved in the HPA axis but also in several other mRNAs and miRNAs associated with the other axes. In addition, our results demonstrated that HPA axis signaling leads to important miRNA alterations at a given time, which precede subsequent and important changes in transcript levels at later time points in both males and females. According to these results, miRNA transcripts seem to be the first signals triggered in all organs. To date, our report is the first to examine transcript profiles with respect to sex differences induced by hypoglycemia in any species. These findings support previous research as well as the notion that hypoglycemia activates not only HPA axis-related transcripts but also affects the HPG axis in response to ITT, indicating that the HPA and HPG axes are interrelated, and activation of one affects the function of the

other. Both testosterone and estrogen regulate the response of the HPA axis (Briski et al. 2020).

The hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the synthesis and secretion of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) via gonadotropin-releasing hormone receptor (GnRHR). Studies show that stress inhibits the HPG axis by suppressing GnRH secretion, which results in decreased LH and testosterone levels. In this study, we observed that *Gnrhr* transcript levels were increased at 1 min in the pituitary of female mice, then at 10 (females) and 25 (males) minutes in the adrenal. In addition, *Fshr* transcripts were found at high levels immediately after insulin injection only in the female adrenal glands and *Prl* transcripts were excessively decreased in the blood of male mice at all time points after performing ITT. In the literature, it has been shown that elevated PRL levels can inhibit GnRH release from the hypothalamus. Furthermore, PRL-mediated inhibition of GnRH leads to decreased pituitary FSH/LH secretion (Zirkin and Papadopoulos 2018; Liu et al. 2019). According to our results, increased *Gnrhr*, *Fshr* and *Prl* transcript levels may have arisen as a negative response to the reduced amount of GnRH. Our results reveal activation of both the HPG and HPA axes in response to hypoglycemia during the ITT.

In our study, as expected, females showed more responsiveness than males in all tissues examined, except in the hypothalamus, in terms of mRNA and miRNA transcript variations after ITT. It is known that estrogen is a principal endocrine regulator of glucostasis and suppresses the negative feedback mechanism (Briski et al. 2020). These increased responses observed in mRNA and miRNA profiles in females could explain the suppression of the negative feedback mechanism by estrogen (Fig. 3, 4, 6).

Nevertheless, some of the factors involved in the stress-responsive constellation have been identified. These include estrogen treatment increasing ACTH and cortisol levels by suppressing their negative feedback mechanism (Heck and Handa 2019) and testosterone reducing ACTH and cortisol levels in response to stress. Activation of the HPA axis, as well as other stress systems, affects a range of hormonal and behavioral processes. In contrast, recurrent and chronic activation of the HPA axis has a negative effect on gonadal hormone secretion (Handa and Weiser 2014; Heck and Handa 2019). Nonetheless, this is a correlation and does not necessarily demonstrate causation (Hillerer et al. 2014). Here, different responses of the HPA axis to ITT between the sexes were caused by the maximum concentrations of stressors (ITT). Both groups of mice were the same age (adults, 12 weeks), but HPG-mediated fluctuations in the levels of estradiol that occur across a 4-day estrous cycle in female mice were less in the present females, as they are from a parallel pedigree and were grouped together, a factor that induces synchronization of female estrus cycle and suppression of estrus for a while (Marchlewska-Koj et al. 1983; Jackson and Wordinger 1990; Dalal et al. 2001). Importantly, our work excluded most factors that confuse this type of study, such as type of stressor, overall health and temperature. In addition, our data indicate that males respond with both short-term quick (hypothalamus) and long-term slow (pituitary) responses, in contrast to females, suggesting that the HPA axis in females will respond to stress only in the form of long-term slow (pituitary) responses, albeit to a higher level than males.

Although humans exhibit less pronounced sex differences than rodents, studies examining women in varying stages of the menstrual cycle (estradiol variations) compared to males have accordingly demonstrated gonadal hormone influences (Kirschbaum et al. 1999). In addition, sex differences in the regulation of the HPA axis have not yet been adequately investigated in humans. Although males have lower cortisol levels in response to various acute stresses, both men and women are generally evaluated using the same criteria (Goel et al. 2014; Oyola and Handa 2017; Heck and Handa 2019). Possible differences observed between the sexes in the regulation of the HPA axis after performing ITT may also be related to the amount of hormones retained in storage form. Hormones can be stored for rapid use in acute situations, such as during hypoglycemia and trauma (Gracia-Navarro et al. 2003). However, although the level of hormones in storage are different (Dannies 1999), both estrogen and testosterone affect regulation of the HPA axis.

Conclusion

In conclusion, the response of the HPA axis was maximized in female mice in the pituitary and adrenal glands but was much weaker in the hypothalamus. Furthermore, greater responses of the HPA axis to hypoglycemia arise primarily from the pituitary, but a more balanced response to hypoglycemia was observed in the HPA axis in male mice compared to females. Thus, under conditions where a stress response is followed at transcript levels, there are robust changes in Avpr1b and Avpr2 transcripts at all time points after ITT, strongly suggesting that these molecules are controlled via different signals in males and females (see Figs. 2–6). Here, the mechanisms shaping miRNA level, dynamics and subsequently transcript targets are unclear (Gebert and MacRae 2019; Treiber et al. 2019; Shi et al. 2020). New ways of processing miRNAs have recently been characterized. Additionally, ITT/hypoglycemia conditions may influence miRNA pairing and our mouse model could help elucidate the underlying in vivo mechanism of acceleration/deceleration of miRNA degradation.

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