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Identification of differentially expressed genes in pathways of cerebral neurotransmission of anovulatory mice

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ABSTRACT. Polycystic ovary syndrome is the classic example of loss of functional cyclicity and anomalous feedback. In this case, the excessive extra-glandular production and conversion of androgens to estrogens are the pathophysiological basis of the chronic anovulation. The literature describes an experimental model of the polymicrocystic ovary in obese diabetic mice with insulin resistance. The fact that these animals exhibit obesity, insulin resistance, and infertility demonstrates their skill as an experimental model for polycystic ovary. A recent study using long protocol for up to 40 weeks showed that anovulatory and obese mice transplanted with adipose tissue from animals with normal weight have multiple changes in their phenotype. These changes include reduction of body weight, prevention of obesity, insulin level normalization, and insulin tolerance tests, preventing the elevation of steroids and especially the reversal of fertility restoration with anovulation. Considering that there are close relationships between the ovulation process and the central nervous system, we propose to evaluate the gene expression levels of 84 different genes involved in neurotransmission and insulin pathways in addition to examining the

neurolipidosis differential murine brain before and after reversal of anovulation. The present study showed changes in gene expression of molecular markers in brain tissue of animals for brain neurotransmission pathways as well as pathways for insulin. GABAergic genes, muscarinic, serotonin receptors, receptor tyrosine kinase, and genes of interleukin 6 showed overexpression profile. There was also a change in the lipid content in anovulatory brain, obesity, and insulin resistant mice (Ob-/Ob-) compared with controls. The re-introduction of leptin in these animals appears to reverse, at least in part, this profile.

Key words: Differentially expressed genes; Neurotransmitter; Anovulation

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most frequent observed endocrinopathies of women during their reproductive age, and it is estimated to affect 6.8% of them (Diamanti-Kandarakis et al., 1999). Different from the previous hypothesis, PCOS seems now to be more related to insulin resistance rather than to the initially regarded as hyperandrogenism in reproductive age. Indeed, the insulin resistance and hyperinsulinemia participate in reproductive as well as metabolic disturbances associated with PCOS (Diamanti-Kandarakis, 2008). Implications may even extend beyond the reproductive axis, creating a need for a modified therapeutic management of affected women, especially from the cardiovascular point-of-view.

Another hallmark of the syndrome is a high GnRH pulse frequency resulting in elevated LH levels and LH/FSH ratio stimulating ovarian androgen production. It has been demonstrated that these women have decreased hypothalamic sensitivity to progesterone-mediated feedback, in addition to reduced luteal phase progesterone production resulting from frequent anovulatory cycles. This effect is mediated by androgens and aggravated by insulin resistance (Blanksby and Mitchell, 2010).

In animal models, a similar reproductive endocrine phenotype occurs when the Leptin gene is knockdown. Despite the well-known fact that the central nervous system plays a major role in PCOS, very little is known about the specific molecular events that take place inside the brain during the anovulatory process and metabolic syndrome.

Therefore, in the present article, our aim was to analyze the expression of 84 genes involved in modulating the biological processes of neurotransmitter biosynthesis, uptake, transport, and signaling through brain neurotransmitter receptors of female obese, insulin-resistant, non-ovulating, leptin-deficient (Ob/Ob) mice. Neurotransmission gene expression was further analyzed in the brain after the re-introduction of leptin in these animals.

MATERIAL AND METHODS

In vivo murine studies

Six-week-old female obese *ob*/*ob* mice together with their lean littermate controls were used. All procedures were approved by the Federal University of São Paulo Institutional Animal Care and Use Committee. Animals were housed with *ad libitum* access to food and water.

All animals were maintained in a temperature-controlled environment at ±24°C under a 12:12-h light-dark cycle and handled at least one time per week. Diet was a regular chow

(F6 Rodent Diet; Harlan Teklad, Madison, WI, USA) comprised of 52% carbohydrate, 18% fat, and 31% protein by calorie or KD (F-3666; Bio-Serv, Frenchtown, NJ, USA) comprised of 0.4% carbohydrate, 95% fat, and 4.5% protein by calorie. Animal groups were divided as follows: 1- Control group (CG): Composed of 5 normal weight, 2 to 3 months of age B6.V-*Lep*ob/J mice. After sacrifice brains were removed and kept in liquid nitrogen until use. 2- Obesity group (OG): Composed of 5 overweight, anovulatory, 2 to 3 months of age B6.V-*Lep*ob/J mice. After sacrifice brains were removed and kept in liquid nitrogen until use. 3- Obesity group transplanted (OGT): Composed of 5 overweight, anovulatory, 2 to 3 months of age B6.V-*Lep*ob/J mice that were recipient to fat transplantation from ovulating mice and sacrificed after 7 days of transplant. After sacrifice brains were removed and kept in liquid nitrogen until use.

Fat tissue transplant

Five female leptin-deficient (B6.V-Lep^{ob}/J, referred to as *ob/ob*) mice and their lean littermates were obtained from the animal facility of our University. A group of *ob/ob* mice received fat tissue transplantation as described by Gavrilova et al. (2000). Briefly, gonadal fat from wild-type littermates euthanized by cervical dislocation was placed in sterile PBS and cut into small pieces. The grafts were implanted subcutaneously through small incisions in the shaved skin of the back of recipient *ob/ob* mice anesthetized with isoflurane.

Approximately 1 g tissue was transplanted per mouse. Incisions were closed using wound clips and mice observed for wound closure. The second group of *ob/ob* mice was sham-operated. Mice were observed for 7 days after transplant, at which point they were sacrificed by cervical dislocation under isoflurane anesthesia.

RT profiler PCR-array mouse neurotransmitter receptors and regulators

The Mouse Neurotransmitter Receptors and Regulators RT^2 Profiler™ PCR Array, containing 84 genes involved in neurotransmission, was applied and run in triplicates together with five housekeeping genes (*18srRNA*, *HPRT1*, *RPL13A*, *GAPD*, and *ACTB*). Whole mouse brain was subjected to RNA extraction, cDNA synthesis, and PCR array as recommended by the manufacturer.

The amount of total RNA was quantified in ng/µL using the Nanodrop device. Gel electrophoresis of selected samples was performed on denaturating 2.0% agarose gel containing ethidium bromide and revealed the two rRNA bands corresponding to 28S and 18S. Sample purity was determined by calculating the 260/280 ratios and found within the 1.6 to 2.2 range in all samples.

The samples were stored at -80°C and cDNA was obtained by using the Reaction Ready™ First-Strand cDNA Synthesis Kit (SuperArray), and the procedures were performed following the manufacturer's instructions.

Negative control for genomic DNA and contaminating RNA were also conducted in each sample. Amplification, data acquisition, and the melting curve were carried out by iCycler iQ (Bio-Rad Laboratories, Hercules, CA, USA). The Ct and the melting curve of every gene were observed for every animal.

The threshold to determine the Ct value for each gene was automatically established by the iCycler iQ version 3 software that determined the SD of the points forming the baseline. Results are reported as Ct and normalized with the housekeeping gene 18SrRNA. The fold-change ratio for each gene was compared between the brain anovulatory groups and healthy mice counterparts. Analysis of variance followed by the Dunnett *post hoc* multiple comparison

tests was used to compare the Ct results generated by the RT Profiler PCR Array between anovulatory groups against the control group. Genes are reported as differently expressed between groups when the fold-change was at least three-fold up-regulated or down-regulated, and the P value (analysis of variance and post-test) was lower than or equal to 0.05.

Neurotransmitter receptors analyzed

Acetylcholine Receptors: *Anxa9*, *Chrm3*.
Muscarinic Acetylcholine Receptors: *Chrm1*, *Chrm2*, *Chrm3*, *Chrm4*, *Chrm5*.
Adrenocorticotropin Receptors: *Mc2r*.
Bombesin Receptors: *Brs3*, *Grpr*.
Cholecystokinin Receptors: *Cckar*, *Cckbr*.
Dopamine Receptors: *Drd1a*, *Drd2*, *Drd3*, *Drd4*, *Drd5*.
GABA-A Receptors: *Gabra1*, *Gabra2*, *Gabra3*, *Gabra4*, *Gabra5*, *Gabra6*, *Gabrb2*, *Gabrb3*, *Gabrd*, *Gabrg1*, *Gabrg2*, *Gabrp*, *Gabrq*, *Gabrr1*, *Gabrr2*, *Glra1*, *Glra2*, *Glra3*, *Glra4*.
Gastrin Receptors: *Cckar*, *Cckbr*, *Galr1*, *Galr2*, *Galr3*.
Neuromedin U Receptors: *Nmur1*, *Nmur2*.
Neuropeptide Receptors: *Npffr1* (*Gpr147*), *Npffr2* (*Gpr74*), *Ntsr1*.
Neuropeptide Y Receptors: *Gpr103*, *Prokr1* (*Gpr73*), *Prokr2* (*Gpr73l1*), *Gpr83*, *Npy1r*, *Npy2r*, *Npy5r*, *Npy6r*, *Pgr15l*, *Ppyr1*, *Prlhr* (*Gpr10*).
Peptide YY Receptors: *Npy1r*, *Npy2r*, *Npy5r*, *Npy6r*, *Ppyr1*.
Somatostatin Receptors: Sstr1, *Sstr2*, *Sstr3*, *Sstr4*, *Sstr5*.
Tachykinin Receptors: *Tacr1*, *Tacr2*, *Tacr3*.
Other Neurotransmitter Receptors: *Chrna1*, *Chrna2*, *Chrna3*, *Chrna4*, *Chrna5*, *Chrna6*, *Chrna7*, *Chrnb1*, *Chrnb2*, *Chrnb3*, *Chrnb4*, *Chrnd*, *Chrne*, *Chrng*, *Glrb*, *Htr3a*.

Regulation of neurotransmitter levels

Neurotransmitter Biosynthesis: *Chat*, *Gad1*, *Slc5a7*
Neurotransmitter Catabolism: *Ache*, *Comt*, *Maoa*.

Immunohistochemistry

To further confirm our results and also identify the localization of protein expression in the brain, immunohistochemistry was also performed for the most up-regulated genes, *Gabrb3* and *Gabrg2*.

RESULTS

Among the 84 genes studied between the brain of normal and obese mice, we identified 34 as differentially expressed after statistical analysis. Of that, 13 were considered down-regulated and 21 up-regulated (Table 1). Remarkably, after only 7 days of the fat tissue transplant, only one gene, Tacr3, remained up-regulated in the brain of the transplanted group.

A gene expression analysis was performed for all the genes described before and after transplantation, it was possible to notice that the great majority of the genes presented were hyperexpressed (Figure 1) at the moment before transplantation and after 7 days of the Transplantation, it was possible to identify a notable reversal of this characteristic (Figure 2).

Table 1. Gene expression level in of the brain analysis of anovulatory mice.

Functional gene groupings	Obese mice fold-change	P value	7 days of transplant	P value
Neurotransmitter Receptors				
Acetylcholine Receptors				
Anxa9	-4.11	0.020670	-9.32	0.000062
Chrm3	-1.79	0.974012	-7.57	0.000000
Muscarinic Acetylcholine Receptors				
Chrm1	48.84	0.091955	-3.07	0.000211
Chrm2	2.66	0.150482	-2.10	0.001728
Chrm3	-1.79	0.974012	-7.57	0.000000
Chrm4	1.69	0.214442	-2.58	0.001921
Chrm5	1.08	0.370781	-2.17	0.006942
Adrenocorticotropin Receptors				
Mc2r	-3.34	0.068337	-3.07	0.000145
Bombesin Receptors				
Brs3	-6.92	0.023793	-3.07	0.016254
Grpr	-2.53	0.708941	-2.50	0.000156
Cholecystokinin Receptors				
Cckar	-4.56	0.051037	-5.74	0.007709
Cckbr	-1.03	0.427239	-4.66	0.003998
Dopamine Receptors				
Drd1a	-1.31	0.688211	-6.59	0.000385
Drd2	-1.31	0.712160	-5.17	0.002160
Drd3	-1.18	0.575474	-1.48	0.225068
Drd4	-2.53	0.394967	-10.34	0.000607
Drd5	-4.11	0.116153	-6.36	0.000010
GABA-A Receptors				
Gabra1	58.08	0.079586	-4.20	0.000085
Gabra2	3.05	0.142180	-2.68	0.003229
Gabra3	17.27	0.102259	1.44	0.750036
Gabra4	20.53	0.121194	-1.54	0.255686
Gabra5	8.06	0.100040	-5.94	0.005289
Gabra6	5.13	0.120686	-14.12	0.000000
Gabrb2	22.78	0.095446	-5.54	0.003224
Gabrb3	232.32	0.070995	-2.97	0.000424
Gabrd	2.16	0.170917	-5.74	0.000006
Gabrg1	3.76	0.267210	1.26	0.403649
Gabrg2	164.28	0.071247	-1.09	0.443929
Gabrp	1.16	0.333973	-3.41	0.000438
Gabrq	-1.79	0.967364	-3.18	0.005660
Gabrr1	-2.62	0.444059	-2.58	0.100276
Gabrr2	-1.56	0.651516	-2.97	0.006893
Glra1	-2.53	0.479330	-2.50	0.117328
Glra2	-2.13	0.602807	-2.87	0.006055
Glra3	3.89	0.115340	-1.09	0.700959
Glra4	-2.62	0.665324	-3.41	0.022184
Gastrin Receptors				
Cckar	-4.56	0.051037	-5.74	0.007709
Cckbr	-1.03	0.427239	-4.66	0.003998
Galr1	-4.72	0.016241	-5.74	0.000000
Galr2	-2.81	0.304981	-2.87	0.148685
Galr3	-2.71	0.260494	-1.13	0.301424
Neuromedin U Receptors				
Nmur1	-1.36	0.487064	-3.41	0.005531
Nmur2	-1.79	0.953509	-3.07	0.000493
Neuropeptide Receptors				
Npffr1 (Gpr147)	-2.13	0.840610	-8.69	0.000004
Npffr2 (Gpr74)	1.47	0.230477	-1.96	0.002482
Ntsr1	1.16	0.292900	-2.03	0.019582
Neuropetide Y Receptors				
Qrfpr	4.32	0.067335	-1.16	0.357521
Prokr1 (Gpr73)	-3.84	0.170310	-2.41	0.095515
Prokr2 (Gpr7311)	-1.99	0.833335	-1.09	0.468912
Gpr83	1.88	0.190744	-7.06	0.000002
Npy1r	1.64	0.239341	-1.83	0.046601
Npy2r	1.53	0.251263	-1.54	0.054869

Continued on next page

Table 1. Continued.

Npy5r	10.27	0.097446	-2.03	0.000747
Npy6r	-3.23	0.276981	-4.66	0.015249
Pgr15l	1.58	0.225764	1.21	0.114330
Ppyr1	-2.20	0.609688	-3.41	0.001583
Prlhr (Gpr10)	-1.79	0.945939	-3.18	0.004313
Peptide YY Receptors				
Npy1r	1.64	0.239341	-1.83	0.046601
Npy2r	1.53	0.251263	-1.54	0.054869
Npy5r	10.27	0.097446	-2.03	0.000747
Npy6r	-3.23	0.276981	-4.66	0.015249
Ppyr1	-2.20	0.609688	-3.41	0.001583
Somatostatin Receptors				
Sstr1	-1.31	0.888664	-1.09	0.499841
Sstr2	11.39	0.120448	-1.83	0.182661
Sstr3	-3.01	0.629782	-5.35	0.000059
Sstr4	2.08	0.889827	-1.43	0.214445
Sstr5	2.16	0.174080	-1.16	0.357521
Tachykinin Receptors				
Tacr1	-2.36	0.844945	-2.41	0.002405
Tacr2	-6.23	0.010367	-11.47	0.001098
Tacr3	14.03	0.109652	4.53	0.026174
Other Neurotransmitter Receptors				
Chrna1	-6.92	0.001124	-7.06	0.000001
Chrna2	2.01	0.436780	-2.33	0.170204
Chrna3	-1.27	0.579056	-9.65	0.000013
Chrna4	2.23	0.182199	-4.50	0.022366
Chrna5	-4.89	0.066526	-2.41	0.078253
Chrna6	-2.36	0.552564	-2.97	0.000157
Chrna7	-3.23	0.328895	-3.18	0.047374
Chrnb1	-3.58	0.162537	-1.96	0.113367
Chrnb2	8.34	0.173478	1.26	0.326259
Chrnb3	-2.81	0.310689	-1.89	0.101501
Chrnb4	-3.97	0.123375	-7.84	0.000201
Chrnd	4.96	0.124909	-1.25	0.166996
Chrne	-15.35	0.015633	-10.70	0.013186
Chrng	-1.99	0.823609	-2.25	0.028641
Glrb	94.35	0.066486	1.49	0.538925
Htr3a	-4.26	0.175870	-2.87	0.089983
Regulation of Neurotransmitter Levels				
Neurotransmitter Biosynthesis				
Chat	-1.14	0.656465	-2.87	0.040200
Gad1	27.10	0.086453	-2.33	0.093293
Slc5a7	4.63	0.167870	1.26	0.493606
Neurotransmitter Catabolism				
Ache	2.08	0.181419	-2.97	0.000575
Comt	-1.40	0.979578	-6.36	0.000836
Maoa	3.51	0.108982	-2.68	0.002493

Immunohistochemistry analysis

The results obtained in the immunohistochemical tests confirmed the molecular findings by using antibodies specific for the differentially expressed genes found in brain tissue of mice (Figures 3 and 4); it is possible to verify the presence of specific large-scale brain tissue antibodies.

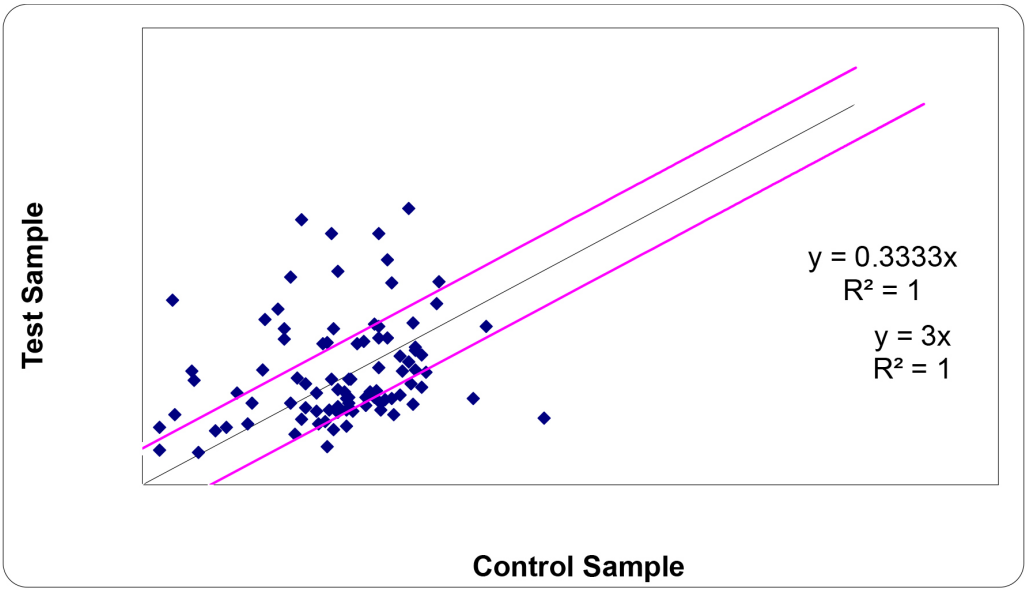

Figure 1. Gene expression in brain before transplantation.

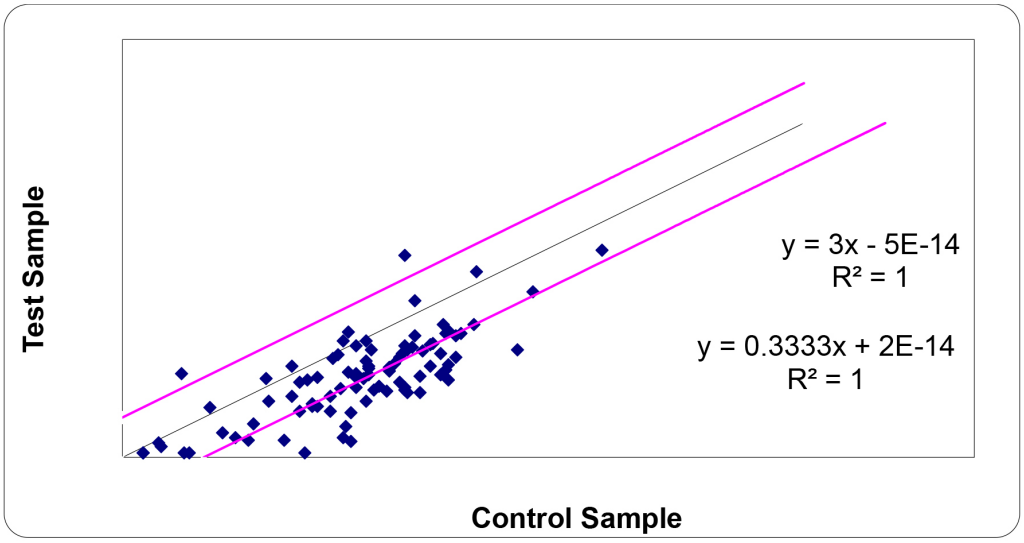

Figure 2. Gene expression in brain after 7 days of fat tissue transplantation.

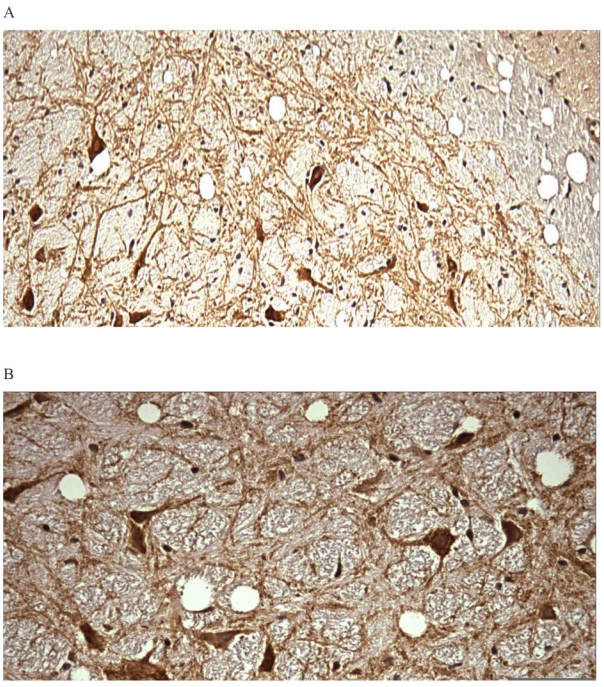

Figure 3. A. Anti- Gabrg2. **B.** Anti- Gabrg2 (Increased A: 200X; B: 400X).

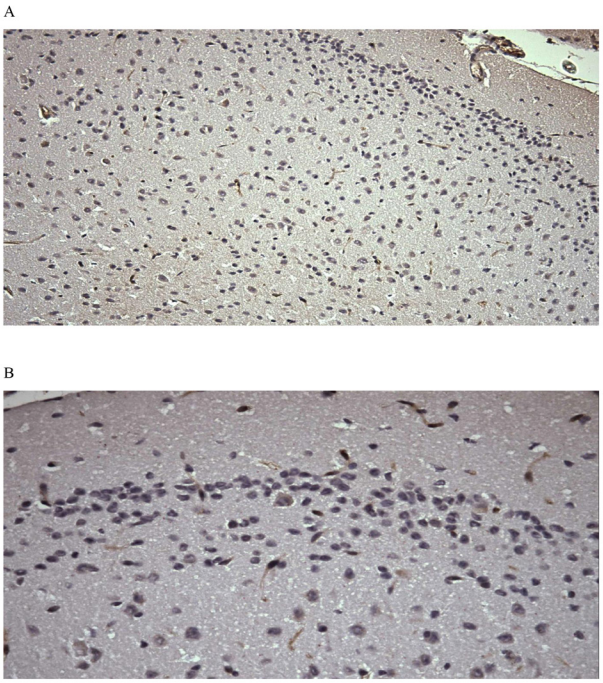

Figure 4. A. Anti-Gabrb3. **B.** Anti-Gabrb3 (Increased A: 200X; B: 400X).

DISCUSSION

In the present article, we were able to identify aberrant expression of genes in the brain of female obese, insulin-resistant, non-ovulating mice. Indeed, after analyzing 84 genes involved in modulating biological processes such as neurotransmitter biosynthesis, uptake, transport, and signaling we identified, after a triplicate real-time PCR confirmation, a total of 34 genes whose expression were found altered in the brain from anovulatory animals.

Among these genes, the *GABA-A* receptors were the most abundant up-regulated category responding for 32.2% (11 of 34 genes) of all differentially expressed genes. The second more abundantly affected category of neurotransmitter receptors, in anovulatory brains, was the Cholinergic Receptors Nicotinic (*CHRN*) accounting for 20.5% (7 of 34 genes) of all abnormally expressed genes. The third more commonly misbalanced group of genes was the one involved in coding the Neuropeptide Y Receptors responding for 11.7% (4 of 34 genes) of all differentially expressed genes. The other 12 misbalanced genes were scattered in other classes displayed in Table 1.

It is well known that the feedback regulation of GnRH neurons by steroid hormones is critical to normal reproductive function and occurs in part through altered GABAergic drive to these cells (Qublan and Malkawi, 2005; Zafar, 2005; Rouzi et al., 2006; Freret et al., 2006; Costello et al., 2007a,b). Altered sensitivity to steroid feedback is associated with forms of hypothalamic infertility such as the common reproductive disorder PCOS, a leading cause of infertility in women.

Indeed, in the present study, we were able to show that in our model of anovulation, obesity and insulin resistance, the lack of leptin was followed by a remarkable up-regulation of *GABA-A* mRNA receptors that was completely normalized after only 7 days of leptin introduction.

This finding suggests that the GABAergic system may play a role in forms of hypothalamic infertility; however, there is some controversy on whether the GABAergic activity, a known inhibitory pathway, can inhibit GnRH neurons.

GABA inhibits most mature neurons, but this is controversial for GnRH neurons. In fact, GnRH neurons maintain elevated chloride (Chehab et al., 1996), as do other neurons derived from olfactory placode (Mounzih et al., 1997), and may thus be excited by GABA-A receptor activation. Studies using functional approaches to examine GnRH neurons differ as to whether *GABA* excites or inhibits these neurons (Chehab et al., 1996; Mounzih et al., 1997; Farooqi et al., 2002; Oral et al., 2002), but direct correlations between reduced fertility and reduced GABAergic drive support the former (Zhang et al., 1994; Schubring et al., 1997). Regardless of the response of GnRH neurons to *GABAAR* activation, *GABAARs* clearly play a role in fertility regulation.

The different actions of acetylcholine (ACh) are mediated by activation of nicotinic (ionotropic) and muscarinic (metabotropic) classes of receptors. Nicotinic receptors (*nAChRs*) are ligand-gated receptor channels that are cation-specific but do not distinguish readily among cations (Edman and MacDonald, 1978). Both classes of cholinergic receptors are expressed throughout the brain and are abundant in the hypothalamus, where monoaminergic neurons that terminate on GnRH and other peptidergic neurons are located (Goldzieherand Green, 1962). The GnRH neurons of the hypothalamus are innervated by noradrenergic neurons located in the hindbrain, and catecholamines have been implicated in the regulation of GnRH release. In contrast, there is less evidence for a role of cholinergic innervation in the control of GnRH secretion.

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