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Gene expression profiling in the rhesus macaque: Experimental design considerations

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ABSTRACT

The development of species-specific gene microarrays has greatly facilitated gene expression profiling in nonhuman primates. However, to obtain accurate and physiologically meaningful data from these microarrays, one needs to consider several factors when designing the studies. This article focuses on effective experimental design while the companion article focuses on methodology and data analysis. Biological cycles have a major influence on gene expression, and at least 10% of the expressed genes are likely to show a 24-h expression pattern. Consequently, the time of day when RNA samples are collected can influence detection of significant changes in gene expression levels. Similarly, when photoperiodic species such as the rhesus macaque are housed outdoors, some of their genes show differential expression according to the time of year. In addition, the sex-steroid environment of humans and many nonhuman primates changes markedly across the menstrual cycle, and so phase of the cycle needs to be considered when studying gene expression in adult females.

1. Introduction

Humans and rhesus macaques (*Macaca mulatta*) are both long-lived primates, and they show many similarities in their anatomy, physiology, and genetics [1]. Consequently, macaques are regarded as pragmatic animal models for studying mechanisms that underlie normal and pathological human development and aging. Their use as translational animal models has many advantages. For example, rhesus monkeys can be maintained under carefully controlled environmental conditions (e.g., photoperiod, temperature, diet, and medication). In addition, animals of a specific age, size, sex, and genetic characteristic can be selected, thereby eliminating extraneous variables and self-selection bias that are typically associated with human clinical trials. Moreover, because the timing of necropsies can be carefully controlled in rhesus macaques, high quality postmortem RNA samples can be collected for gene expression profiling. Such studies require measurement of changes in the expression of individual genes, and traditionally have depended on techniques such as *in situ* hybridization histochemistry or RNase protection assays. More recently, however, the availability of rhesus-specific gene microarrays, has opened up the possibility of screening thousands of differentially-expressed genes all at once.

The present article addresses important points of primate physiology that need to be taken into consideration when designing gene microarray studies, while the companion article [2] focuses on methodological considerations associated with gene annotation and data analysis. By carefully addressing each of these issues one can optimize the physiological relevance of the differential gene expression results, and gain more meaningful insights into the mechanisms that underlie normal and pathological human physiology.

2. Experimental design considerations

2.1. Circadian rhythms

Most animals live in a changing environment, comprising day and night, and show corresponding adaptations in their physiology and behavior [3]. Underlying these adaptations are many hormones, such as cortisol, dehydroepiandrosterone sulfate, and leptin, which in rhesus macaques have pronounced circadian release patterns [4–6]. In primates, as in other mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus contains a master circadian oscillator that synchronizes and sustains these circadian rhythms. More recently, genetic components of the circadian clock mechanism have also been detected in various peripheral primate tissues [7,8], adding support to the view that circadian physiology is ultimately controlled by a network of coordinated circadian

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oscillators, rather than by a single master clock [9–11]. Importantly, the findings suggest that gene profiling from many peripheral tissues could yield results specific to the time of day when the RNA is collected. The importance of this consideration is underscored by a database of circadian gene expression, which has been compiled by the Genomic Institute of the Novartis Research Foundation, at http://expression.gnf.org/cgi-bin/circadian/index.cgi.

We recently examined circadian gene expression in adult female rhesus macaques [7]. Adrenal gland RNA samples were obtained at six time points across a 24-h period, with 4-h intervals: 3:00, 7:00, 11:00, 15:00, 19:00, and 23:00 h. Total RNA was isolated from the whole left adrenal gland, and hybridizations were performed using the Affymetrix human HG_U133A GeneChip® [2]. Raw data from the scanner images were filtered for significant amplitude and for 24-h rhythmicity, which enabled us to identify 335 genes with robust circadian oscillation patterns. These transcripts were then organized by hierarchical clustering, using a cosine correlation for distance measure, which yielded a temporal profile of rhythmic gene expression in the adrenal gland with peaks of expression distributed throughout the 24-h cycle (Fig. 1A). Next, the phase distributions were analyzed using Cosinor analysis (available at http://www.circadian.org/softwar.html). The expression profiles were grouped according to one of the six sampling times (Fig. 1B). Interestingly, the phase distributions of the various genes were not homogeneous: 72 transcripts peaked at 3:00 h, 78 transcripts at 7:00 h, 42 transcripts at 11:00 h, 32 transcripts at 15:00 h, 45 transcripts at 19:00 h, 66 transcripts at 23:00 h (Fig. 1C). Taken together these data indicate that the number of cycling transcripts increased during the dark phase, starting at around 19:00 h, and reached a maximum at 7:00 h, when the lights came on in the morning (Fig. 1C). The number of cycling transcripts reduced abruptly between 7:00 h and 11:00 h, and reached a minimum at 15:00 h. Details of the data analysis from this study were reported in Lemos et al. [6], and all of the expression data, and other pertinent biological information were deposited in the gene expression omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), with accession number: GSE2703. (ID: lemosd_rev_1; password: 720865595).

In summary, numerous genes in the rhesus macaque adrenal gland show a clear 24-h expression pattern. The same is probably true for other organs, as it has been estimated that at least 8–10% of the genes within an organ may show a 24-h expression pattern [12]. Consequently, gene expression profiles between experimental groups may show little difference if the RNA samples are collected at a time of day when the genes of interest are at the nadir of their circadian expression. Ideally, the optimal time of day for collecting RNA samples should be empirically determined for each gene of interest, and this information incorporated into the experimental design. This is particularly important when comparing gene expression profiles from a nocturnal rodent with that of a diurnal primate, such as the rhesus macaque, as data points obtained during an investigator's normal working hours would correspond to the rodent's subjective night but to the monkey's subjective day, and they so might not be directly comparable.

2.2. Seasonality

In humans, seasonal variations have been reported for blood pressure, immune response, birth rate and sleep duration, as well as for behavioral traits associated with seasonal affective disorders, bulimia nervosa, anorexia and suicide [13–15]. Consequently, the time of year when RNA samples are collected could also have a significant impact on gene profiling data. This is likely to be even greater in rhesus macaques, because in their native habitat, in

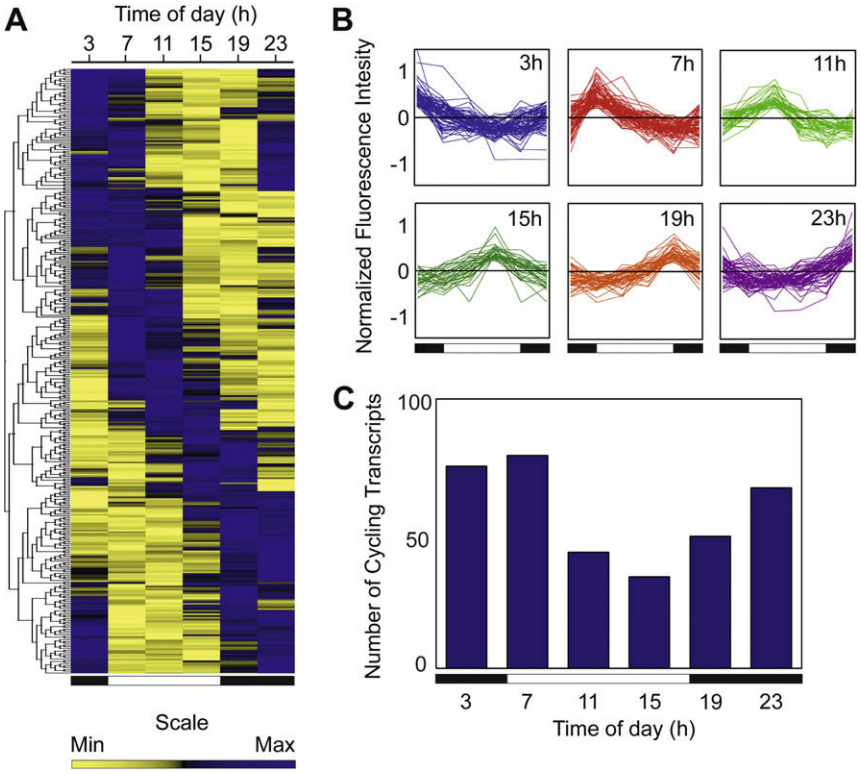

Fig. 1. Temporal gene expression profiles in the rhesus macaque adrenal gland, emphasizing marked 24-h differences. (A) Hierarchical clustering of the 335 oscillating transcripts. Each column represents a time point and each row represents a gene. Relationships between genes are depicted as a tree, with branch length reflecting the degree of similarity in time courses between the genes. (B) Gene expression profiles showing different phases across 24 h; the data have been normalized such that the medial signal intensity for each gene across all time points is 0. (C) Distribution of cycling transcripts across 24 h. In all panels the white and black bars represent day and nighttime, respectively (figure taken from Ref. [7], with permission, *Copyright 2006, The Endocrine Society*).

Northern India and China, these monkeys are short-day seasonal breeders. They restrict their breeding activity to the fall and winter, thereby ensuring that their offspring are born when environmental conditions are more favorable for survival, in the late spring and summer. Not only are their circulating sex-steroid hormone levels affected by changes in day length (Fig. 2, left and right panels), but so also are their gene expression profiles in target organs such as the adrenal gland.

We recently examined this phenomenon in ovariectomized rhesus macaques [16], which were maintained for 10 weeks under either short winter photoperiods (8L:16D), medium spring/fall photoperiods (12L:12D), or long summer photoperiods (16L:8D). As in the circadian rhythm study described in Section 2.1, total RNA was isolated from whole adrenal glands, and hybridizations were performed using the Affymetrix human HG_U133A GeneChip® [2]. Data from individual microarrays were normalized using MAS 5.0 global scaling. Expression analysis was performed using GeneSifter software (Geospiza, Inc., Seattle, WA, USA) and showed that the expression of many functionally clustered genes was differentially influenced by the different photoperiods (Fig. 3). In particular, we observed significant differences in the expression of genes involved in development, lipid synthesis and metabolism, and immune response; these differences were corroborated using real-time PCR [2]. These data suggest that the primate adrenal gland undergoes both structural and functional changes as an adaptive response to long-term exposure to both short and long photoperiods. On the one hand, the findings provide a new perspective on the effects of seasonal variations in photoperiod on primate behavior, physiology and gene expression, which may have clinical implications for the treatment of seasonal disorders in humans. On the other hand, they highlight a potential environmental variable that could bias primate gene profiling studies, unless appropriate measures are taken to control for them.

2.3. Hormone effects in the brain

Microarray analyses indicate that ovarian steroid hormones have dramatic effects on gene regulation in peripheral target tissues [17,18], as well as in the brain [19–23]. However, applying such studies to the brain of primates is challenging because of several factors. Not only is the level of gene expression in the primate brain relatively low compared to peripheral tissues [24], the magnitude of the changes is relatively small in heterogeneous neuronal populations [25]. Other significant considerations are the inherent genetic and physiological variability associated with an out-bred animal model, as well as limited access and high costs of requirements for primate studies. Despite these obstacles, some attempts with microarray studies have recently been made to elucidate the influence of adrenal [26] and ovarian steroids [27,28] on gene expression in the nonhuman primate brain. Note, these earlier studies were conducted before monkey-specific microarrays had been developed and, therefore, they had to rely on the use of human arrays; these are inherently less sensitive when used in nonhuman primates because of the inter-species variation in gene sequences [29,30]. Nevertheless, these same human microarrays form the annotation base for the current version of the Rhesus macaque genome array, which is discussed in more detail in the associated article [2].

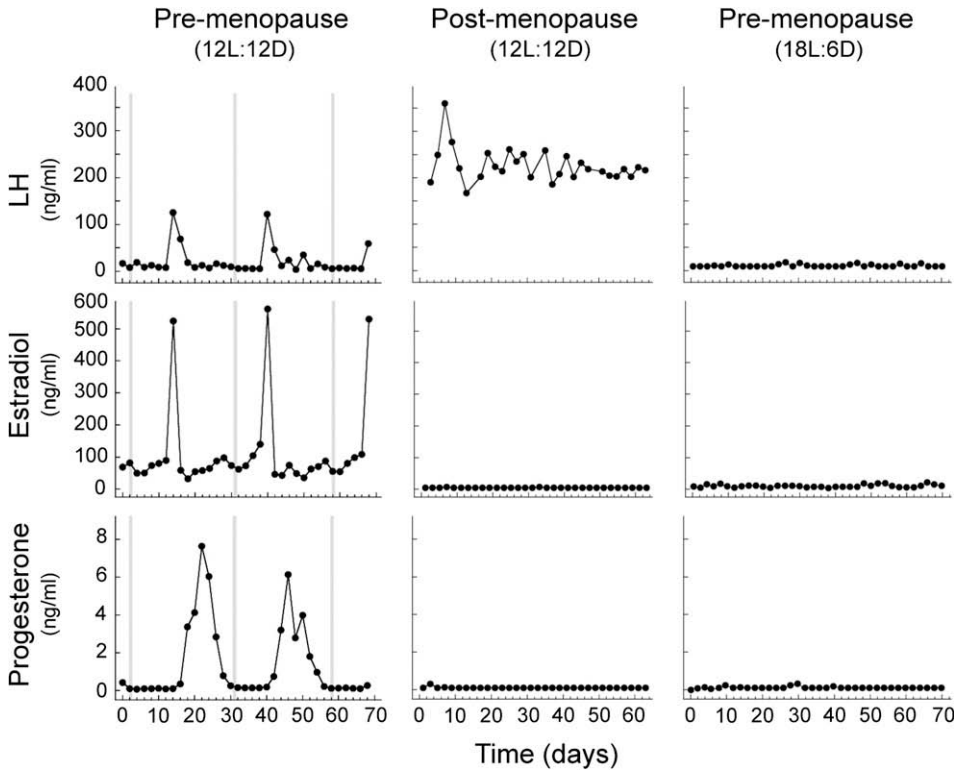

Fig. 2. Representative circulating reproductive hormone profiles of three adult female rhesus macaques, collected at least once every 2 days for 60–70 days. The animals were either maintained under fixed photoperiods comprising 12 h of light per day (12L:12D), or were exposed to long summer day lengths comprising 18 h of light per day (18L:6D, for 50 days prior to measurement of the hormone concentrations). The plasma/serum samples were assayed for luteinizing hormone (LH), estradiol and progesterone concentrations, and periods of menstruation were recorded (shaded vertical bars). Note the marked changes in sex-steroid concentrations that occur across the menstrual cycle (left panels), with estradiol peaking during the late follicular phase and progesterone peaking during the mid-luteal phase. After menopause, the animals stop cycling and their sex-steroid concentrations become basal while LH concentrations become markedly elevated (middle panels). Although long-term exposure of rhesus macaques to long photoperiods also causes their sex-steroid concentrations to become basal, LH concentrations do not become hyper-elevated; instead they remain very low (right panels) (data adapted from Refs. [32] and [33]).

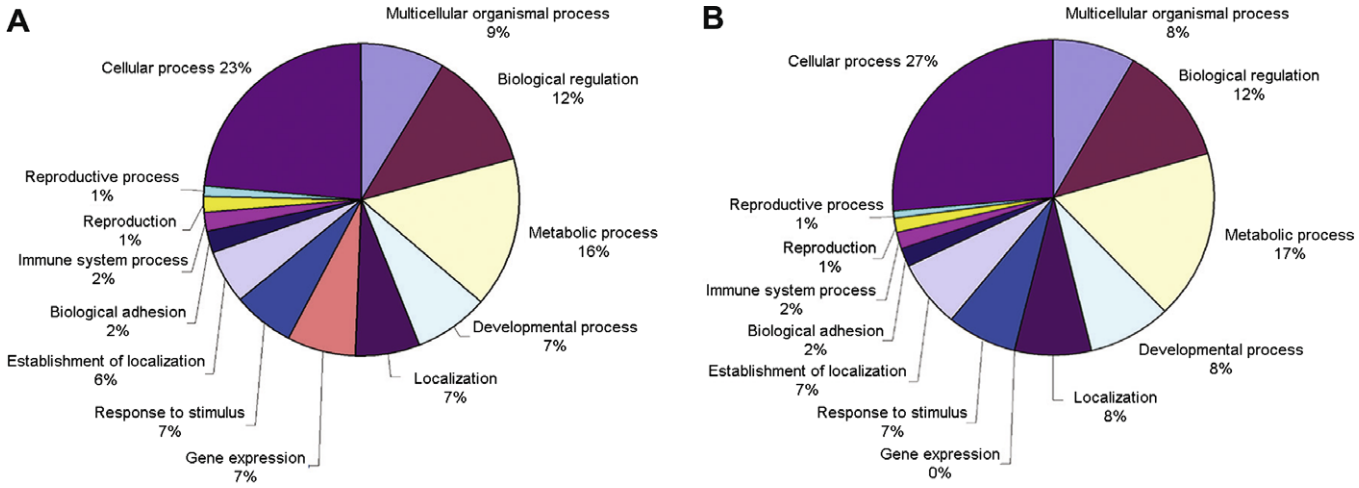

Fig. 3. Effect of day length on adrenal gland gene expression in the rhesus macaque. Ovariectomized animals were maintained under short winter photoperiods (8L:16D), spring/summer photoperiods (12L:12D), or long summer photoperiods (16L:8D), for 10 weeks. Adrenal gland RNA was hybridized to the Affymmetrix human HG_U133A GeneChip®, and the data were analyzed using the algorithm MAS 5.0. (A) Functional clustering of genes found to be differentially expressed between 8L:16D and 12L:12D photoperiodic exposures. (B) Functional clustering of genes found to be differentially expressed between 12L:12D and 16L:8D photoperiodic exposures. These data emphasize that photoperiod or season may significantly affect the level of gene expression in rhesus macaques (data adapted from Ref. [16]).

Although a comparison of gene regulation between cortical regions has been reported in the primate [31], to the best of our knowledge no previous studies have comprehensively examined ovarian hormone effects on gene regulation in both classical neuroendocrine targets (the arcuate nucleus of the hypothalamus) and extra-hypothalamic brain regions (amygdala and hippocampus) of the primate. Our study examined hormone effects under normative physiological conditions: i.e., across the different phases of the menstrual cycle in adult rhesus macaques. As illustrated in Fig. 2 [32,33], there are three distinct phases of the primate menstrual cycle: (1) The early follicular phase (EF), characterized by low levels of estrogen (E) and progesterone (P); (2) The late follicular (LF) periovulatory surge, which is associated with the rapid rise and fall of E; and (3) The mid-luteal phase (ML), which is characterized by elevated levels of P. However, because of the within-animal variation and the fluctuation of hormone levels during the cycle, capturing animals in exactly the same phase can be challenging. Even with the aid of daily menses checks and hormonal assays, it was not possible to sample animals at exactly the same phase of their menstrual cycle. Finally, when this study was conducted the rhesus macaque-specific GeneChips® were commercially unavailable, and so the Affymetrix human HU133-plus 2.0 microarray platform was used instead [34].

Web-based software (GeneSifter) was used to perform 2-way ANOVA, in order to define a significant ($p < 0.05$) effect of brain region, the menstrual cycle phase, or any region by phase interaction. Threshold levels of 1.2-, 1.5-, 1.8-, 2-, 3-, and 4-fold change identified 24,636, 7776, 3519, 2341, 646, and 269 candidate genes, respectively, that could be reasonably organized for further analysis. We also found that without fold-change criteria increasing the p-value cutoff from significant ($p < 0.05$), to highly significant ($p < 0.01$), to very highly significant ($p < 0.001$), did not greatly diminish the number of candidate genes.

The primary finding was that the subtle effects of steroid hormone changes across the menstrual cycle were outweighed by the larger, significant differences in gene expression due to tissue-specific effects in the three experimental brain regions. Another way to examine the complexities of the multivariate data is to use Principal Components Analysis (PCA), which is another program offered in the GeneSifter suite. PCA reduces the complexities of the dataset, with the first principal components axis accounting for the majority of the variance in the data, the second principal component, which is arranged orthogonally to the first axis, accounts for as much of the remaining variation as is possible, and the process is repeated for each successive principal component. While PCA can generate several principal axes to better define the data, GeneSifter focuses on the first and second axis only. For example, in the menstrual cycle study the first component axis revealed a clear separation of the arcuate from both the amygdala and hippocampus, whereas the latter two regions were defined by the second principal components axis. The effect of the phase of the cycle (EF, LF, ML) remained tightly grouped within each brain region, with no overlap (Fig. 4).

A pattern of tissue-specific gene regulation was observed for many genes in the arcuate nucleus, when compared to the amygdala and hippocampus. Therefore, it should not be surprising that with well-known phenotypically-characterized neuronal populations in the arcuate nucleus, such as the dopaminergic and opioidergic neurons, there is a higher level of tyrosine hydroxylase and

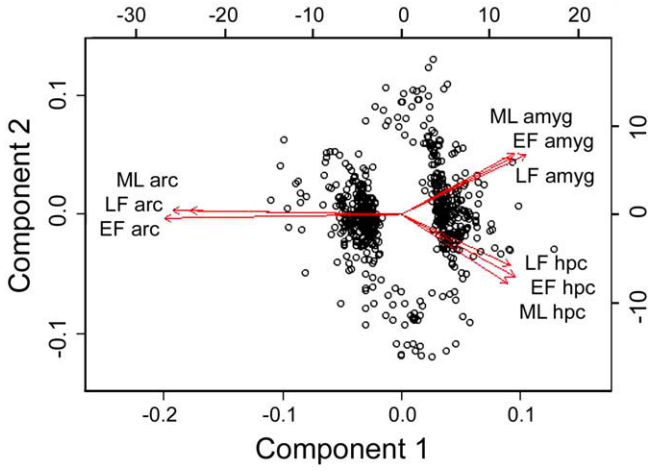

Fig. 4. Principal components analysis of the effect of menstrual cycle phase on gene regulation in the amygdala (amyg), arcuate nucleus (arc) and hippocampus (hpc). Expression between hypothalamic (arc) and extra-hypothalamic (amyg, hpc) regions was distinguishable along the first principal components axis, whereas the amyg and hpc were clearly separated along the second axis. Thus, the effect of brain region dominated gene expression patterns. EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

pro-opiomelanocortin mRNA expression, respectively. Similarly, as hypothesized because the steroid-regulated hypothalamus is a component of the hypothalamo–pituitary–gonadal axis, relatively high levels of estrogen receptor 1 and androgen receptor were also observed, as well as fibronectin, an estrogen-regulated extracellular matrix protein [35]. Conversely, genes associated with the mechanisms of learning and memory, such as CAM Kinase II and several genes of the glutamatergic system (which regulates transmission mediated by the excitatory amino acid glutamate), were found to be highly expressed in the hippocampus [36].

Because of the individual variability in macaque cycle profiles, it was difficult to obtain animals during exactly the same phases of the menstrual cycle. To overcome this problem we have also employed an alternate strategy for studying the influence of sex steroids on gene expression, namely, bilateral ovariectomy followed by hormone replacement therapy (HRT) [37]. Ovariectomized, adult rhesus macaques were either given: (1) no HRT, or (2) received supplemental estrogen for 4 weeks, or (3) estrogen for 4 weeks and estrogen plus progesterone during the last 2 weeks of treatment. This artificial cycle maintained physiological levels of steroids, which were less variable than in normal menstrual cycles, providing a more stable hormonal treatment milieu within each treatment group. Gene regulation was examined in the same brain regions as those in the menstrual cycle study. However, this study was performed using the recently-developed Affymetrix GeneChip® Rhesus macaque genome array. As in the menstrual cycle study described above, the goal of this study was to examine the effects of HRT on gene regulation in the hypothalamus, amygdala and hippocampus.

Employing the GeneSifter software, we again tested with a 2-way ANOVA, for the effect of tissue region by hormone treatment. At the $p < 0.05$ level, and invoking the Benjamini–Hochberg correction for false discovery rate, threshold levels of 1.2-, 1.5-, 1.8-, 2-, 3-, and 4-fold change identified 21,317, 6609, 2806, 1809, 427 and 160 candidate genes, respectively. PCA (data not shown) revealed that the hypothalamus separated from the other regions along the first principal component. Interestingly, only the amygdala in the ovariectomized untreated group was markedly separated from the rest of the treatment groups in the amygdala and hippocampus, along the second component axis.

Once candidate genes have been identified, bioinformatic approaches can be used to organize the data by genes that share common biological or mechanistic processes. To this end the GeneSifter program offers Gene Ontology or KEGG (Kyoto Encyclopedia of Genes and Genomes), which graphically organizes the data into pathways and biological processes. The significance of these pathways is determined using a z-score, which identifies ontology terms or pathways that are significantly over-represented or under-represented on the microarray [38]. For this particular study, significant z-scores were determined primarily for the neuroactive ligand–receptor interaction, GnRH signaling pathway, MAPK signaling pathway, calcium signaling pathway, axon guidance and gap junction (Table 1). This reduction of the candidate gene list into more manageable functional groups facilitates interpretation of the data in mechanistic terms.

Table 1
Examples of a few of the KEGG pathways (25 in total), in which genes that were affected by HRT treatment, brain region or the interaction (2-way ANOVA, $p < 0.05$, 3× change) are significantly over-represented. "Array" indicates the number of specific pathway genes that were represented on the genome array.

KEGG pathway	Genes	Array	z-Score
Neuroactive ligand–receptor interaction	16	198	6.27
GnRH signaling	7	69	4.84
MAPK signaling	7	150	2.35
Calcium signaling	6	133	2.08
Axon guidance	5	79	2.75
Gap junction	5	69	3.11
Adherens junction	4	58	2.65
ERB signaling	4	62	2.49

3. Closing remarks

Because of their close genetic similarity to humans, rhesus macaques represent excellent animal models for gene profiling studies, using comprehensive species-specific microarrays. However, there are potential pitfalls with this new technology, which can affect validity of the results. Data from our rhesus macaque gene expression studies emphasize the importance of experimental designs that control for changes in hormonal milieu. This is especially pertinent when studying females, as many of their hormones are influenced by the phase of the menstrual cycle and menopausal status, and also vary seasonally and across the 24-h day.

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