Global identification of the genes and pathways differentially expressed in hypothalamus in early and established neurogenic hypertension

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Global identification of the genes and pathways differentially expressed in hypothalamus in early and established neurogenic hypertension. Physiol Genomics 43: 766–771, 2011. First published April 12, 2011; doi:10.1152/physiolgenomics.00009.2011.—The hypothalamus has an important etiological role in the onset and maintenance of hypertension and stress responses in the Schlager high blood pressure (BP) (BPH/2J) mouse, a genetic model of neurogenic hypertension. Using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays we identified 1,019 hypothalamic genes whose expression differed between 6 wk old BPH/2J and normal BP (BPN/3J) strains, and 466 for 26 wk old mice. Of these, 459 were in 21 mouse BP quantitative trait loci. We validated 46 genes by qPCR. Gene changes that would increase sympathetic outflow at both ages were: Dynll1 encoding dynemin light chain LC8-type 1, which physically destabilizes neuronal nitric oxide synthase, decreasing neuronal nitric oxide, and Hcrt encoding hypocretin and Npsr1 encoding neuropeptide S receptor 1, each involved in sympathetic response to stress. At both ages we identified genes for inflammation, such as CC-chemokine ligand 19 (Ccl19), and oxidative stress. Via reactive oxygen species generation, these could contribute to oxidative damage. Other genes identified could be responding to such perturbations. Atp2b1, the major gene from genome-wide association studies of BP variation, was underexpressed in the early phase. Comparison of profiles of young and adult BPH/2J mice, after adjusting for maturation genes, pointed to the proopiomelanocortin-α gene (Pomc) and neuropeptide Y gene (Npy), among others, as potentially causative. The present study has identified a diversity of genes and possible mechanisms involved in hyper-tension etiology and maintenance in the hypothalamus of BPH/2J mice, highlighting both common and divergent processes in each phase of the condition.

ESSENTIAL HYPERTENSION (EH) is a common polygenic trait involving differential expression of numerous, mostly unidentified, genes with small effects on blood pressure (BP) (11). Identification of which genes exhibit expression differences should help better understand EH etiology and pathophysiology. In both animal models and humans, there is increasing evidence of an important role for the sympathetic nervous system (SNS) in both development and progression of hypertension (17). A better understanding of the underlying cause of the sympathetic activation, together with its role in maintenance of elevated BP, is needed, however.

The Schlager BPH/2J mouse, a neurogenic model of genetic hypertension, exhibits dysregulation of catecholamine neurons in the hypothalamus (12, 37, 38). Acute sympathetic blockade decreases BP in BPH/2J mice (9), consistent with SNS involvement. Neuronal activation in the paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), and median preoptic nucleus of the hypothalamus of BPH/2J mice is higher than in normotensive BPN/3J mice during the active diurnal phase (7, 9). BPH/2J mice have a 67–88% higher cardiovascular response to aversive stressful stimuli, and response to restraint stress is abolished by pentolinium, a sympathetic blocker (8). The increased stress response involves neuronal activation in the DMH (7). The importance of the hypothalamus in BPH/2J hypertension and stress responses led us to attempt to uncover the underlying pathways and mechanisms by a genome-wide gene expression profiling approach. Therefore the aims of the present study were to 1) identify, at the genome-wide level, the genes that are differentially expressed in between BPH/2J and BPN/3J hypothalamus in early and established hypertension, and 2) conduct bioinformatic analyses to identify the pathways and possible mechanisms involved.

MATERIALS AND METHODS

Samples and tissue collection. The study involved Schlager BPH/2J and BPN/3J mice. Radiotelemetry studies by ourselves and others (8, 9), as well as tail-cuff measurements (39), have shown that BP of BPH/2J hypertensive mice is consistently high, mean arterial pressure (MAP) being 127 ± 2 mmHg (9). Similarly BPN/3J mice have consistently normal BP, MAP being 110 ± 1 mmHg (9). In mice younger than 10 wk, however, not only is it difficult technically to measure BP, but the BPH/2J strain is highly sensitive to stress, which would increase BP during the measurement process. Although tail-cuff data have been reported for BPH/2J mice as young as 6 wk of age, revealing a 35 mmHg higher systolic BP than in BPN/3J mice (39), the difference is likely to have been overestimated, emanating from the exaggeration of response to stress that is a feature of the BPH/2J strain (7, 8) during the tail-cuff procedure.

In the present study, early (6 wk old) and established hypertensive (26 wk old) BPH/2J and age-matched normotensive BPN/3J mice (n = 6/group, total of 24 samples) were killed with an overdose of pentobarbital (Lethobarb) at 8 AM corresponding to the peak of the circadian variation in BP (9). The PVN and DMH hypothalamic nuclei, as defined by known anatomical boundaries (7), were removed immediately after death by P. J. Davern, who has extensive experience in dissecting PVN and DMH regions of the hypothalamus. The tissue was first preserved in dry ice and later transferred to a −80°C freezer, before being used for microarray experiments within 7 days. Each animal was considered an individual sample, and no pooling was
performed. The study was approved by the Alfred Hospital and University of Sydney Animal Ethical Review committees.

**RNA extraction.** RNA extraction used a commercially available kit (RNeasy, Qiagen) and was performed according to the manufacturer’s recommendations. RNA quality was confirmed based on a RNA integrity number >8 by use of an electrophoresis bioanalyzer (2100 Agilent Bioanalyzer). This experiment was performed by the Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Sydney, Australia. Quantification involved spectrophotometry (NanoDrop ND-100 spectrophotometer, Thermo Scientific) at the University of Sydney Laboratory.

**Microarray experiments and analyses.** mRNA was converted to ssDNA, labeled and hybridized to GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix), which analyze 28,869 gene transcripts using 764,885 probe sets (on average 27 probes per gene), according to the manufacturer’s instructions, and with the assistance of the Ramaciotti Centre. One microarray was performed for each of the 24 samples.

Results from 6 and 26 wk old mice were normalized using robust multiarray analysis (RMA) (19). Differentially expressed genes at each age were identified using a robust moderated two-sample t-test in the Limma package (44). Such genes were selected based on their adjusted for maturation effects, while avoiding direct nonage-matched comparisons, as we will now explain. Direct comparison to identify differentially expressed genes between early and established phases of hypertension we used a method that adjusted for maturation effects, while avoiding direct nonage-matched comparisons, as we will now explain. Direct comparison to identify differentially expressed genes between early and established hypertension samples is not advisable as such an analysis may identify many aging-related differentially expressed genes of limited interest. This analysis was performed using the adjusted fold difference (aFD) statistic we described previously (29). Differentially expressed genes were selected based on an absolute aFD value exceeding 2.0, where positive aFD values indicate higher expression in the adult hypertensive group and negative aFD values indicate higher expression in the early hypertensive group.

Hierarchical clustering used Euclidean distance and was performed with TMeV 4.5 (36). The Gene Ontology (GO) Database (2) was used to further interpret the differentially expressed gene data set and to identify overrepresented functional groups of genes. A hypergeometric test using GOstats (4) was used to determine if particular GO terms were more significant in the differentially expressed gene list than the gene list of the entire array. Upregulated and downregulated genes were examined separately. A gene set test (GST), implemented via the Limma package (44), was used to highlight pathways over- or underrepresented as a set, for all genes ranked via the “Core Analysis” function. Briefly, a data set containing differentially expressed genes and respective fold differences were first uploaded into the application. The genes were then correlated based on previous association between genes or proteins and known functional roles of genes. The biological relationship between two genes, represented as nodes, is shown as a line. Nodes with different shapes indicate different functional class.

The data set obtained has been deposited in the NCBI Gene Expression Omnibus (GEO) database according to MIAME guidelines with series accession number GSE25076.

**Semiquantitative real-time PCR.** Semiquantitative real-time PCR (qPCR) was conducted to confirm the results for genes whose functions were considered of interest to hypertension. The first-strand complementary synthesis reaction was performed using the SuperScript VILO CDNA Synthesis Kit (Invitrogen). Amplification reactions used the EXPRESS SYBR GreenER qPCR reagent system (Invitrogen) in a Light Cycler 480 qPCR machine (Roche). Primers were specifically designed around the most differentially expressed probe in the transcript cluster of each gene using Primer3 (35). When possible, primers were designed to flank an exon-exon junction. Primers and conditions used are shown in Supplemental Tables S1–S3 (please see online supplemental material). Samples were run in duplicate. The specificity of the qPCR was ensured by melting curve analysis and electrophoresis in agarose gels (data not shown). The β-actin mRNA (Actb) was used as reference transcript. The comparative \( C_T \) statistical method was used to assess significance (40).

The SPSS statistical package (SPSS for Windows, Release 17.0, 2008) was used for statistical analyses. qPCR results were tested for normal distribution using the skewness and kurtosis test. Independent sample t-tests were used to compare hypertensive and normotensive groups. Significance was set at \( P < 0.05 \).

**RESULTS**

In early hypertension we identified 1,019 well-annotated genes whose expression differed between the 6 wk old mice of each strain (FDR < 0.001). Table 1A summarizes the data for selected genes that we validated by qPCR. For complete information see Supplemental Table S4 and Supplemental Figs. S1 and S2 in the online supplement. In silico interaction networks among the genes are shown in Supplemental Fig. S3. Hierarchical clustering showed that hypertensive and normotensive samples have distinctive patterns of gene expression (Supplemental Fig. S4). Among the differentially expressed genes, there was a significant overrepresentation of genes involved in G protein-coupled receptor protein signaling pathway and GABA receptor activity and a downrepresentation of calcium ion transport, nitric oxide (NO) biosynthetic and metabolic processes, among others. GST showed an overrepresentation of terms for neuuropeptide signaling pathway, and a downrepresentation of terms for blood circulation, ATP synthesis coupled proton transport, cytochrome-c oxidase activity, and respiratory and electron transport chain (ETC). Details of GO and GST analyses are shown in Supplemental Tables S5 and S6, respectively.

In established hypertension, 466 well-annotated genes differed between the 26 wk old hypertensive and normotensive mice (FDR < 0.05). Summarized data, showing only validated genes, appear in Table 1B. For complete information see Supplemental Table S7 and Supplemental Figs. S5 and S6. Of these 466 genes, 47% were in common with the set of genes identified in early hypertension (Supplemental Table S8). In silico interaction networks among the genes are shown in Supplemental Fig. S7. Hierarchical clustering indicated that BPH/2J and BPN/3J have distinctive patterns of gene expression (Supplemental Fig. S8). GST showed a significant overrepresentation of terms for G protein-coupled receptor protein signaling pathway, immune response, apoptosis and respiratory and ETC, and a downrepresentation of terms for blood circulation, blood vessel development, and others. For details see Supplemental Tables S9 and S10.

In the early phase of hypertension, using an aFD value of \( \geq 2 \), we identified 51 well-annotated genes (Supplemental Table S11, Supplemental Figs. S9–S11). Table 2 summarizes genes we validated. The fold-change in Pomc by qPCR was considerably larger than the microarray value. Such findings are not uncommon (48) and most likely represent differences between

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1 The online version of this article contains supplemental material.
the hypothalamus of the Schlager BPH/2J hypertensive mouse compared with normotensive BPN/3J mice and validated by qPCR

<table>
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<th>Official Gene Symbol</th>
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<th>qPCR P Value</th>
<th>Fold Difference (array)</th>
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Values represent mean of the fold difference between hypertensive and normotensive samples. Positive fold difference values indicate higher expression in hypertensive and negative fold difference values indicate higher expression in normotenom.

solid-state and solution hybridization, coupled with use of the RMA algorithm, which gains in providing greater specificity and sensitivity but loses by blunting the magnitude of the fold change (19). In silico interaction networks among the genes are shown in Supplemental Fig. S12. In early hypertension, GO analysis (Supplemental Table S12) found an overrepresentation of terms such as regulation of BP, inflammatory response, circulatory system process, and blood circulation, while GST indicated a downrepresentation of respiratory and ETC, electron carrier activity, and lipid biosynthetic process (Supplemental Table S13).

Of the genes we identified in the early phase of hypertension in the BPH/2J mice, 330 of them (32%) resided at 21 BP quantitative trait loci (QTL) that have been described in mice, and 129 genes (28%) in the established phase were in 19 QTLs for mouse BP (Supplemental Tables S14 and S15) (14, 47). Genes for which polymorphisms have been associated with EH are shown in Supplemental Table S16. These included ATP2B1, the most prominent gene to emerge from large genome-wide association (GWA) studies of human BP and EH (25). Supplemental Tables S1–S3 show all of the qPCR results. Of the 66 genes whose altered expression we attempted to validate by qPCR, 70% were validated. Most of the genes that could not be validated exhibited smaller differences in expression by qPCR, as was also the case for their microarray results. The possible function of the genes validated and their chromosomal location is shown in Supplemental Table S17.

**DISCUSSION**

This study is, to our knowledge, the first to evaluate genome-wide gene expression signatures in the hypothalamus of any species in hypertension. The number of potentially relevant genes was considerable, and not all will be discussed here. The effect on BP of particular promising candidate proteins encoded by the differentially expressed genes we identified will require further extensive experimentation, such as gene targeting and in vivo silencing, all of which is well beyond the scope of the present study. The genes we have identified point to processes altered in the early and established phases of hypertension. Some may have an etiological role in BP regulation and stress response. In this regard it is particularly noteworthy that our gene list included genes coinciding with the limited number of human genes that have to date emerged from large GWA studies, namely Atp2b1 (25), Pmsf1 (1), and Rsgr2 (1) in early and Edn3 (25) in established Schlager hypertension.

Previous genome-wide gene expression studies of Schlager mice (15, 16, 34) did not involve brain tissue (9). Despite this, some of the genes we identified in hypothalamic were also seen in studies of other tissues of Schlager mice. They included Atg4a, Diras2, Gpd3, Kenk1, and Plekhh1. Either common or divergent roles in each tissue are possible. The present hypothalamic gene expressions are more likely the primary drivers of elevated BP in Schlager mice. Neuropeptide S (NPS), for example, activates the NPS receptor (encoded by Npsr1; Table 1) in hypothalamic explants to increase vasopressin and corticotropin-releasing hormone release (43), which can each exert BP elevating effects.

Table 2. Genes whose expression differed between the early and established phases of hypertension in the Schlager BPH/2J mouse and that were validated by qPCR

<table>
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<th>GenBank Accession #</th>
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<th>aFD (array)</th>
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<td>Cck</td>
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<td>Hdc</td>
<td>NM_008230.5</td>
<td>−8.76</td>
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<td>Npy</td>
<td>NM_023456.2</td>
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<td>−2.46</td>
</tr>
<tr>
<td>Pomc</td>
<td>NM_008895.3</td>
<td>−138</td>
<td>−3.35</td>
</tr>
<tr>
<td>Ptk2a</td>
<td>NM_172498.2</td>
<td>1.69</td>
<td>2.04</td>
</tr>
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<td>Ramps3</td>
<td>NM_019511.2</td>
<td>5.10</td>
<td>2.92</td>
</tr>
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<td>Rora</td>
<td>NM_013646.1</td>
<td>2.18</td>
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Values represent mean of the adjusted fold difference (aFD) statistic between prehypertensive and hypertensive samples, based on an absolute aFD value exceeding −2.0. Positive aFD values indicate higher expression in the hypertensive group, and negative aFD values indicate higher expression in prehypertensive group.
Genes and mechanisms leading to an increase in SNS activity. Genes for catecholamine biosynthesis were not amongst those we identified. Nor were genes of the renin-angiotensin system. Our gene lists included, however, the adrenergic gene *Adra1B* and the gene for chromogranin (*Chga*) (Table 1). Chromogranin, which is increased in plasma of EH patients (30, 45), catalyzes the formation of catecholamine storage vesicles (22).

Several genes had potential downstream neuronal effects. One of these, *Hcrt*, encodes hypocretin, which activates catecholamine neurons by a stress-like effect (5). When given intracerebroventricularly, hypocretin acts on PVN neurons to increase BP, heart rate, and renal SNS activity (41, 42). It stimulates sympathetic outflow and the sympatho-adrenomedullary system (21, 32) and *Hcrt* knockout lowers BP (21). The elevation in *Dynll1* mRNA in both phases of hypertension would raise dynin light chain LC8-type 1, which interacts with and destabilizes the neuronal NO synthase (nNOS) dimer, resulting in inhibition of nNOS enzyme activity, reducing NO bioavailability (13, 20), and so increasing SNS activity (18, 23, 49). We also observed differential expression of other genes involved in NO availability and production, namely *Adm*, *Ddah1*, *Scn4b*, *P2rx4*, and *Rora* in early hypertension, and *Arg2* and *Pkd2* in established hypertension (Table 1). These too might increase SNS activity. In our young mice GO analysis revealed, moreover, downrepresentation of genes involved in NO biosynthetic and metabolic processes.

Similar mechanisms involving different genes in early and established hypertension. In both early and established hypertension we identified different genes whose products have similar neurogenic BP elevating effects, including stimulation of inflammation (32) and oxidative stress (33) (Fig. 1). The only inflammation gene in common between each age was CC-chemokine ligand 19 (*Ccl19*), which was overexpressed >4.5-fold. This protein attracts lymphocytes and dendritic cells, so contributing to chronic and autoimmune inflammation (24).

Oxidative stress can be increased by mitochondrial dysfunction, decreased NO production, and reduced protection against oxidative stress. Both GO and GST analyses highlighted genes for mitochondrial respiration and ETC in both phases of hypertension, and immune response in established hypertension.

The increase in reactive oxygen species (ROS) that accompanies oxidative stress and inflammation would damage mitochondrial and nuclear DNA, membranes and proteins, and increase SNS activity and thus BP. These mechanisms persist from the onset through to the maintenance phase. Some of the gene expression changes, such as for ribosomal proteins and transcription factors, might serve to counteract the increase in ROS and its effects.

A portion of the differentially expressed BPH/2J genes might include passenger genes, alleles of which became fixed in each strain after the original selective breeding for high and normal BP traits in Schlager mice, meaning that a portion of the differences between strains could reflect differences in genetic backgrounds that include not just genes that affect BP (34).

Changes during passage from early to established hypertension. Our comparison of data for young and adult mice, adjusted by their age-matched controls, enabled insight into the initial trigger(s) that raise BP. Young mice had higher levels of mRNA for histidine decarboxylase (*Hdc*), which produces histamine that can stimulate release of norepinephrine and vasopressin (10), neuropeptide Y (*Npy*), which affects catecholamine production (3) and pro-opiomelanocortin-α (*Pomc*), which regulates sympathetic outflow (3). The role of *Npy* in early hypertension only, sustained by higher receptor levels via *Npy2r* expression in this phase, is also seen in the spontaneously hypertensive rat (46).

In adult BPH/2J mice we observed higher levels of mRNA for cholecystokinin (*Cck*), which is able to stimulate vasopressin and oxytocin release (28), receptor activity modifying protein 3 (*Ramp3*), which is correlated with stress-induced hypertension (26), RAR-related orphan receptor alpha (*Rora*), a transcription factor that provides neuroprotection by increas-
ing the expression of antioxidant enzymes (6), and protein tyrosine kinase 2β (Ptk2b), which can be activated by ROS to inhibit nNOS (27).

The multiplicity and functions of genes exhibiting altered expression in young BPH/2J mice is consistent with a rich repertoire of mechanisms, some of which are similar between early and established hypertension.

Sclagler BPH/2J mice have an exaggerated response in BP to stress (7, 8). Our gene list included stress response genes such as Hcrt and Npsr1 whose elevated expression in both phases could be involved in Sclagler hypertension. Hypocretin neurons are active in the PVN during stress (5), and hypocretin exerts stress-like behavioral effects, ameliorated in Hcrt knockout mice (21). NPS, via its receptor, stimulates arousal and hyperactivity (31). It increases the release of CRF in hypothalamic explant cultures and, when given intracerebroventricularly, increases plasma corticotropin and corticosterone (43). Knockout of Npsr1 in mice has confirmed the essential role of Npsr1 in stress-induced corticosterone release (50).

Of genes we found that colocalized with BP QTLs in 37 inbred strains of mice (14, 47) (Supplemental Tables S14 and S15), we validated differential expression of Adra1b, Ccl19, Chga, collagen triple helix repeat containing 1 gene (Cthrc1), Dynll1, neuroglobin gene (Ngb), natriuretic peptide receptor 2 gene (Npr2), Npsr1, Npy2r, nucleoredoxin (Nxor), and vav 3 oncogene (Vav3) in early hypertension (Table 1A), and, in established hypertension, the arginine type II gene (Arg2), Ccl19, Chga, Cthrc1, Dynll1, Npsr1, polycystic kidney disease 2 gene (Pkd2) and succinate dehydrogenase complex subunit C integral membrane protein gene (Sdhc) (Table 1B). Thirteen of the 14 mouse BP QTLs identified in Supplemental Table S15 correspond to regions in the human genome linked to BP (14). A limitation of this comparison is that the total portion of the genome covered by the BP QTL regions described by Feng et al. (14) is 48%, so caution is advised in interpretation of these co-localizations until more precise interval mapping is performed.

In conclusion, the genome-wide hypothalamic gene signatures we identified in early and established neurogenic hypertension in the Sclagler BPH/2J mouse include genes potentially involved in the onset and maintenance of hypertension, or both, as well as counterregulatory responses. Most new genes identified have unknown roles, especially within the hypothalamus. Genes we identified by expression arrays were supported by qPCR measurements for most with relevant biological meaning, by colocalization with BP QTLs that have been identified in mice, by association studies of gene variants for human BP and EH, including large GWA studies, and by robust statistical analyses. Our data provide guidance for conducting an extensive series of functional experiments well beyond the scope of the present study. A future challenge will be to decipher the integration of the complex hypothalamic pathways that affect hypothalamic output and resulting SNS effects on other tissues. Our findings provide a basis for more rational design of experiments aimed at elucidation of the mechanisms involved in Sclagler BPH/2J neurogenic hypertension, its hyperreactivity to stress, and, ultimately, neurogenic components of EH in humans. Other eventual outcomes could include the identification of biomarkers, pharmacogenomic applications and novel therapies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

NOVEL GENES FOR NEUROGENIC HYPERTENSION


