Supplemental Methods

NTS Identification, Microdissection, RNA Extraction. Animals were sacrificed and tissue was immediately collected from two littermate pairs at a time on four separate days for each time point: from each pair one animal experienced elevated blood pressure via infusion of phenylephrine and the other was given a sham infusion of saline. Brainstems were excised, and blocks containing the NTS were placed in ice-cold, artificial cerebral spinal fluid (ACSF) (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 24 mM D-Glucose). The blocks were cut into 250 µm coronal sections using a McIlwain Tissue Chopper (Campden Instruments, Lafayette, IN). Sections were floated in ice-cold ACSF and those containing the NTS were selected. The NTS was identified based on anatomical landmarks and was punched out using a 750 µm diameter micropunch (Stoelting, Chicago, IL). To preserve quality of RNA, the NTS was obtained within 10 minutes post mortem. Total RNA was extracted using Qiagen's RNeasy mini kit (Qiagen, Valencia, CA), yielding 200-900 ng of total RNA. RNA quality was assessed using a RT-PCR protocol for high and low copy number genes (β-actin and tyrosine hydroxylase respectively). Tyrosine hydroxylase was selected because it is specific to the NTS at the slice level, confirming that the punches contained NTS.

Microarray Manufacture. Microarrays were fabricated using a rat clone set (GF200; ResGen Huntsville, AL, and Invtrogen, Carlsbad, CA) for cDNA microarrays consisting of approximately 8,800 sequence-verified non-redundant

(as of Unigene build 150). The cDNA clones from all rat cDNA targets were prepared from freshly grown overnight bacterial cultures by PCR amplification using GF200 primers (Invitrogen, Carlsbad, California). PCR products were purified and verified by agarose gel electrophoresis, and the yield was determined spectrophotometrically (NanoDrop Inc., Wilmington, DE). cDNAs were mixed with an equal volume of DMSO (10-70 ng/µl) and printed onto Corning cDNA slides (Corning, Corning, NY) using a MicroGrid II arrayer (Genomic Solutions, Ann Arbor, MI). Microarrays were air dried for 30 min and cross-linked by UV irradiation. We printed arrays of 18,240 spots representing ~9000 clones in adjacent duplicate spots. The arrays used in the present study had approximately 5,300 annotated genes based on the Entrez Gene database.

RNA Amplification, Labeling. Total RNA (70-400 ng) was amplified using two rounds of the antisense RNA (aRNA) technique(29), yielding on average 180 µg aRNA (MessageAmp, Ambion, Austin, TX). aRNA (1.125 µg) was reverse transcribed (Superscript II, Invitrogen) using random primers to generate single stranded Cy5-amino-allyl labeled cDNA from the experimental samples. vCDNA reference was Cy3-amino-allyl labeled(15).

Hybridization. Microarrays were prehybridized in 1% bovine serum albumin, 5x SSC, 0.1% SDS for 45 min at 42°C, washed in H_2O and dried by centrifugation. Cy3-vDNA and Cy5- cDNA samples were mixed with 50 µl of DIG Easy Hybridization buffer (Roche, Indianapolis, IN) containing 25 µg each of yeast

tRNA and calf thymus DNA and applied to the microarrays for hybridization at 37°C for 16 hours in a hybridization chamber (Corning, Corning, NY) in the dark with gentle agitation. Slides were washed for 10 minutes at 50°C in 1x SSC and 0.1% SDS in shaking incubator, followed by a 1 minute wash in 1x SSC, three 1 minute washes in 0.1x SSC, and one rinse in H_2O, at room temperature. Slides were dried by centrifugation and scanned with the ScanArray 5000XL (PerkinElmer, Wellesley, MA).

Microarray Data: Microarray data is available at http://www.dbi.tju.edu/hypertension and at Gene Expression Omnibus (GEO) with accession GSE8585.

qRT-PCR validation. Previously, we reported qRT-PCR validation for genes in ionotropic glutamate pathways(16). These experiments were not repeated here. Genes coding for various receptors, pathway components, and channel subunits and isoforms were measured by qRT-PCR to validate the microarray data. We chose genes that conferred to the coordinate regulation in each pathway or receptor class described in the paper to validate the overall coordinate regulation described. Primers were selected in pairs using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and tested by qRT-PCR prior to the validation study (sequences listed in Supplemental Table S11). Primers were screened and those that produced high CT values (>35) were discarded.

qRT-PCR was performed on an ABI Prism 7000 machine (Applied Biosystems, Foster City, CA) using SYBR Green I as a fluorescent reporter (iTaq mastermix, Bio-Rad, Hercules, CA), taking triplicate measures to determine the threshold cycle (C_T), and employing the $\Delta\Delta C_T$ method to detect differential gene expression (19). Mitochondrial ribosomal protein S18C (MRPS18C) was used as a housekeeping gene for 60 minutes after onset of hypertension. Cytochrome P450, family 2, subfamily d, polypeptide 22 (CYP2D6) was used as a housekeeping gene for 90 and 105 minutes after onset of hypertension because MRPS18C was showing differential expression at this time point. These genes were chosen because they exhibited high gene expression but no differential expression in the microarray measurements. 150ng of RNA from each sample was a reverse transcribed (Superscript II, Invitrogen) using random primers to generate template for the qRT-PCR reaction. About 1.5ng of qRT-PCR template was used per PCR reaction and each measurement was made in triplicate. The fluorescence threshold for each gene was calculated by finding the threshold that was in the linear range of the PCR cycles, but minimized the variance of the respective cycle thresholds. For each sample, the cycle thresholds for the gene of interest were subtracted from the cycle thresholds for the housekeeping gene and the median of the differences was taken (to eliminate outliers) yielding a ΔC_T. Any genes that produced large standard deviations (>0.3) of triplicate C_Ts for more than half of the samples were discarded (results were considered not reproducible). A one factor, two sample ANOVA was performed on the eight normalized ΔC_T values (4 biological replicates, two conditions) and a p-value was

obtained. The possibility that one animal pair was anomalous was eliminated by repeating this calculation four times removing one pair each time. If any of these p-values were less than 0.05, then the gene was classified as differentially expressed at that time point. If the direction of differential expression matched that of the microarray, the microarray data was classified as validated for that gene and time point. Of the 42 measurements taken, the qRT-PCR validated 22 of the microarray predictions, disagreed with 14 of the microarray measurements, and was inconclusive about 6 of the predictions. Supplemental Table S12 details the qRT-PCR results. qRT-PCR raw data, cycle thresholds, and R code used to make these calculations are provided in the supplement at http://www.dbi.tju.edu/hypertension.

Pathway model development and coloring. A list of receptors expressed in the NTS that have been implicated in the regulation of blood pressure was curated from the pharmacological literature (Table 3). The signaling pathways of these receptors were curated and placed in an *in silico* prototypical neuron model using a public resource named CellDesigner version 3.1 (17). This *in silico* model represents the known molecular components of 27 receptor pathways implicated in baroreflex response. A set of 25 of the 27 pathways were obtained from the Panther Pathways Database (21), http://www.patherdb.org. The remaining two pathways (AT1R and GABA) were constructed from curated literature and subsequently added to the model. The *in silico* model is available as an XML file in the systems biology markup language (SBML) format. Gene identifiers were mapped to pathway components through the Panther Pathways Database and manual curation. The clone identifiers from the microarray data were mapped to gene identifiers using UniGene. The gene identifiers were mapped to pather

component identifiers by uploading all gene identifiers to Panther database and downloading and parsing the resulting HTML. The *in silico* model Pather Pathways components were colored according to the gene expression data at each time point.

Detailed Pathway Discussion

cAMP/PKA pathway

Adenylate cyclase (AC) and PKA are both up regulated in response to the one hour pulse hypertension and are subsequently down regulated after the pulse was completed (Table 2). AC is modulated by the alpha subunit of G-proteins (Gs activates while Gi inactivates AC) (27). AC converts ATP into cyclic AMP (cAMP), which in turn activates PKA. PKA is known to activate L-type voltage dependent calcium channels (VDCC) (4, 14). Localization of L-type VDCC's on post-synaptic dendrites suggests little role in synaptic transmission and a larger role in coupling synaptic excitation to activation of transcription factors thought to contribute to neuronal plasticity. L-type calcium channels have been shown to regulate the transcription of a number of immediate early genes (c-fos, jun-B, zif268, and fos-B) cultured cortical neurons (22). Ca^{2+} influx through L-type channels is an effective activator of Cyclic-AMP Response Element Binding (CREB) protein transcription factor (1). Further, when PKA is translocated to the nucleus it activates the CREB transcription factor with a rich array of regulated genes (25). If we infer that up and down gene regulation confer to activation and inhibition, AC and PKA activation during acute hypertension suggest a pathway

for multiple activations of CREB and other calcium-dependent transcription factors and subsequent transcriptional events leading to modulation of neuronal plasticity. AC and PKA inactivation after the one hour hypertensive pulse was completed suggest that the transcriptional program initiated by acute hypertension is being turned off.

PI3K pathway

PI3K is up regulated initially after the one hour hypertensive pulse and after 45 minutes it is subsequently down regulated (Table 2). A different pattern of down regulation during elevated blood pressure and up regulation in the time points following the one hour hypertensive pulse is followed by many of the downstream PI3K pathway members such as phosphostase and tensin homolog (PTEN), protein kinase B (PKB or AKT), cell division cycle 42 homolog (CDC42), c-Jun amino-terminal kinase (JNK) and Jun oncogene (Supplemental Table 2; Figure 2). The major action of PI3K is phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP3) while PTEN reverses this process(20). PIP3 activates 3-phosphoinositide dependent protein kinase (PDPK) which activates protein kinase B (PKB) (20). PKB is the major effector of the PI3K pathway and has many roles including activation of nitric oxide synthases (NOS) to produce nitric oxide (NO) (20). NO has been shown to be essential in the bradycardia component of the baroreflex mediated through glutamate receptors (7). Our data indicates an up regulation of genes that encode proteins involved in the production of nitric oxide (PI3K, AKT/PKB, nNOS; Supplemental Table 2; Figure 2) in the time points after the

one hour hypertensive pulse. This suggests a physiologically functional role for NO in return to normotensive state following acute hypertension stimuli. Such a regulation is in agreement with studies in SHR rats that show reduced mRNA and protein expression of inducible nitric oxide synthase in the rostral ventrolateral medulla (RVLM) (5). PI3K may be involved in changing synaptic strength by mediating the trafficking of AMPA receptors(3) and calcium channels(31) to the membrane. Finally, there may be gene expression changes involved in PI3K mediated activation of c-jun, which would initiate a cascade of transcriptional responses (30).

PKC pathway

$G_{\alpha q}$ proteins activate PKC through phospholipase C (PLC) (11). Our data shows down regulation of $G_{\alpha q}$ mRNA during hypertension and up regulation after the one hour hypertensive pulse was completed (Table 2). PKC subunits overall are down regulated at all of the time points studied. PKC subunits beta and eta, was down regulated at all time points measured throughout the experiment although they were significant after one hour of hypertension and 15 and 30 minutes after the one hour hypertensive pulse (see Table 2). PKC subunits epsilon and zeta was significantly down regulated after one hour of hypertension and 45 minutes after the one hour pulse respectively. PKC subunits delta and gamma had mixed regulation. Components upstream and downstream of PKC are not coordinately regulated except one endpoint of the PKC signal transduction pathway: transcription factor Fyn. Fyn is down regulated during acute hypertension and subsequently up regulated when blood pressure after the one hour hypertensive

pulse (Table 2). Fyn activates c-Fos, which is an immediate early gene mediates the transcription of many genes. The Fyn familiy of kinases is also a potent effector of the PI3K pathway (13). PKC also suppresses inwardly rectifying potassium channels (28), sodium channels (12) N-type calcium channels(8), and L-type calcium channels (2, 14). PKC may also activate certain L-type calcium channels(14). The effects of PKC on channels are not visible using gene expression data alone, and follow up studies with PKC inhibitors and activators will be necessary.

MAPK pathway

Several genes belonging to the Ras family, a key signaling component upstream of MAPK, were up regulated at 30 minutes after the one hour hypertensive pulse but not regulated during the acute hypertension stimulus (Table 2). The MAPK pathway activates a number of transcription factors that initiate changes in cell composition such as channel and receptor density and increased concentration of intracellular signaling pathway proteins. For example, activation of angiotensin receptor 1 causes the MAPK signaling pathway to initiate transcription of fos and jun, which in turn initiates the transcription of catecholamine synthesis and transport enzymes (30). As summarized in Table 1 and illustrated in Figure 2, the MAPK pathway is more associated to receptors involved in a bradycardic response rather than a tachycardic response. The up regulation of Ras suggests a role in recruiting bradycardic receptors during return to normotensive blood pressure levels. Components downstream of Ras were not coordinately regulated. While our analysis does not indicate coordinated regulation of

canonical MAPK pathways in the limited time frame studied, it is possible that a specific instance of the pathway consisting of particular component subtypes may be coordinately regulated, and combining multiple instances into a canonical pathway is convoluting the present analysis.

SNARE pathway

Although the SNARE pathway is not a typical signaling pathway, it is particularly important in neurons because it plays an important role in neurotransmitter release (i.e. presynaptic exocytosis) (24) and provides a swift means to regulate receptor and channel density (i.e. postsynaptic endocytosis) (6, 10, 18). Vesicle associated membrane proteins (VAMP) were down regulated after the one hour hypertensive pulse was completed. Syntaxins (except syntaxin 2) showed the opposite regulation pattern. VAMPs are bound to the plasma membrane and are essential in the process of vesicle fusion to the plasma membrane. Syntaxins are attached to the vesicle and bind to VAMPs for exocytosis in a calcium dependent manner. Calcium influx from VDCC's cause neurotransmitter release by physical and functional linkage between N- and P/Q-type calcium channels and syntaxins (24, 26). Finally, VAMP is necessary in vesicle mediated endocytosis in order to mediate postsynaptic receptor and channel endocytosis and keep a constant pool of presynaptic vesicles available (without having to resort to clathrin coated pits) (9, 23). Regulation of VAMPs and syntaxins could be an effective means of enabling increased presynaptic neurotransmitter release and postsynaptic modification of the receptor and channel populations during acute hypertension.

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