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Identifying the Stress Transcriptome in the Adrenal Medulla Following Acute and Repeated Immobilization

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Abstract

Stress triggers changes in gene expression mediating important adaptive and maladaptive responses. The full repertoire of genes whose expression in the adrenal medulla is altered by stress has not been previously determined. In this study, gene profiling (RAE 230 2.0 Affymetrix) was applied to elucidate global changes in gene expression in adrenal medulla of rats exposed to 2-hour immobilization stress (IMO) once or repeatedly for six consecutive days. The number of transcripts significantly ($p<0.01$) altered with single IMO (651 up, 487 down) was more than with repeated IMO (370 up- 195 down). The annotated transcripts were further analyzed and categorized. The largest numbers of changes were in mRNA levels in the transcription factor and cell signaling categories. Robust changes were also observed in transcripts related to growth factors, apoptosis, neurosecretion/neuropeptides, heat shock proteins, structural proteins, chemokines, cytokines, metabolism/lipid-metabolism, and proteases. Many (>80%) were uniquely induced by single IMO. About half of transcripts changed by repeated IMO were also responsive to single IMO. Pathway analysis was applied to identify direct interactions and common targets among gene products altered by single and repeated IMO. In this paper, we briefly describe the most pronounced changes observed with emphasis on those which may provide new insight into the common and distinct mechanisms whereby the adrenal medulla responses to a first encounter with stress compared to repeated exposure to the same stressor.

Keywords

stress; adrenal medulla; microarray; gene profiling

Introduction

The adrenal medulla plays a key role in the response to stress. Release of epinephrine (Epi) and norepinephrine (NE) from adrenal medulla is among the most rapid responses to stress. Epi and NE are critical in transmitting the perceived threat into action by activating the heart and muscles to prepare for the ‘fight or flight’ response1. This stimulates glycogen breakdown in muscle and liver, gluconeogenesis in the liver, and lipolysis in adipose tissue. It also inhibits insulin release while stimulating glucagons secretion, triggers vasoconstriction and enhances cardiac output [reviewed in2]. Moreover, Epi is key for

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memory of emotionally charged events[3,4]; sympathetic activation and elevated urinary NE and/or Epi concentration are consistently observed in patients with PTSD[5,6].

One of the most important questions in stress research is how are the acute beneficial responses to stress converted to the prolonged detrimental effects. To this end, studies have been directed to comparing the response of the adrenal medulla to acute and chronically repeated stress. Exposure to stress, not only triggers rapid NE and Epi release, but also leads to longer lasting changes in gene expression.

The effect of various stress of different duration on gene expression of catecholamine biosynthetic enzymes in the adrenal medulla has been reviewed[7–10]. Even a few minutes of immobilization stress (IMO) already triggers induction and/or phosphorylation of several transcription factors and elevates transcription of catecholamine biosynthetic enzymes[11–13]. Moreover, exposure to single episode of immobilization stress (IMO) alters the response to subsequent exposure to the same stressor on the next day. For example, the phosphorylation of transcription factor CREB in adrenal medulla is much more pronounced and sustained on the second than on first IMO[12]. However, chronically repeated stress is required to manifest new steady state catecholamine levels[9,14].

The full repertoire of genes whose expression in the adrenal medulla is altered under these conditions has not been previously determined. In the present study, in order to understand long-term consequences of stress, and to obtain a complete picture of changes in gene expression triggered by single and repeated stress, microarray analysis was applied. It enabled us to explore networks of connections among the genes responsive to stress. The findings revealed many previously unidentified targets and potential new biomarkers for adrenomedullary response to stress and help to delineate the mechanisms for the common and distinct responses to acute and repeated exposure to stress.

Methods

Animal procedures

All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Male, murine pathogen-free, Sprague-Dawley rats (280–320 g) were obtained from Taconic Farms (Germantown, NY). The animals were maintained under controlled conditions of a 12-hr light-dark cycle (lights on from 06:00 AM to 18:00 PM) at 23±2 °C with food and water *ad libitum*. IMO was performed as previously described[15–17]. For repeated stress, the animals were immobilized for 2 hr daily for 6 consecutive days. Control groups were not exposed to stress. All animal manipulations were performed between 08:00 AM and 13:00 PM.

Following the last IMO, rats were euthanized by decapitation and both adrenals were dissected from the animals. To isolate the adrenal medulla, a small incision was made to the edge of the cortex and the medulla was gently squeezed out. Subsequently any cortex tissue adhering to the adrenal medulla was carefully removed. It had been estimated by immunocytochemistry that the medulla was more than 95% pure[17]. The right and left adrenal medullae from each individual animal was frozen separately in liquid nitrogen and kept at −80°C.

RNA Isolation

RNA was isolated from two separate immobilization experiments. To minimize sample variability caused by individual differences among animals, each sample was pooled from

left adrenal medulla from 4 individual rats. There were 3-pooled samples (12 animals total) for each group. RNA was extracted using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). The integrity of the RNA was assessed by the A260/A280 ratio which was close to 2.0 and by electrophoresis (Agilent Bioanalyzer 2100).

Microarray

This part of experiment was performed by the NIH Neuroscience Microarray Consortium at UCLA Medical Center (Los Angeles, CA). It is described briefly as follows. Total RNA (≥ 4 μg) from each group was converted to cDNA by using superscript reverse transcriptase and the T7-Oligo (dT) promoter primer kit (Affymetrix, Inc). Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA were purified and served as a template in the subsequent *in vitro* transcription reaction (Affymetrix, Inc.). The *in vitro* transcription reaction was carried by T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix (Affymetrix, Inc.). The biotinylated cRNA targets were cleaned up and fragmented. Each cRNA was hybridized to an individual Affymetrix GeneChip Rat Array Expression 230 2.0 (RAE 230 2.0 array) which was subsequently processed for washing and staining with the antibody stain solution with streptavidin phycoerythrin and the arrays were scanned on the GeneChip Scanner 3000. For detailed protocols see http://www.affymetrx.com.

Microarray Data Analysis

The raw pixel data were uploaded into GeneTraffic (GeneTraffic version 3.2, Iobion Informatics, La Jolla, CA), and all subsequent analyses were performed on a GeneTraffic Server at the Functional Genomics Core Facility of New York Medical College. All the microarray data were analyzed and normalized using a Robust Multi-Chip Analysis (RMA) algorithm with the control data sets as the baseline. According to Iobion Informatics, the median polishing algorithm of RMA helps minimize the effect of noise inherent in microarray data and enhances the discriminating power of the experiment. The quality and the accuracy of each hybridization were checked by Hybridization Annotation and Hybridization Statistics in GeneTraffic program. Statistical analysis was a two-class method comparing the single and repeated IMO probe sets to the probe sets for the controls. The analysis provided the significance level (p-value) for each gene. Differences with a significance of $p < 0.01$ and at least ± 2.0-fold change in gene expression between the respective stress group and absolute control group.

The expression data generated from GeneTraffic were imported into PathwayAssist software (version 3.0, Iobion Informatics) to provide insights into common regulatory mechanisms of the set of genes and the interactions or pathways, or for all relationships among several proteins.

Real-time Quantitative RT-PCR Assay

Changes observed for some genes in microarray analysis were individually confirmed by real-time RT-PCR. The RNA samples used for RT-PCR were the same as for microarray analysis. Mixture for reverse transcription reactions contained 1 μg total RNA, 1 μM random primer (Sigma), 1 mM dNTP mix, 2.5 units of AMV reverse transcriptase, 1 × AMV buffer, 8 units of RNase inhibitor (Roche, Indianapolis, IN) and were incubated at 42°C for 60 min. PCR reactions (25 μl) were set up with 12.5 μl RT2 Real-Time™ SYBR Green PCR Master Mix provided by SuperArray Bioscience Co. (Frecerick, MD), 10.5 μl ddH2O, 1.0 μl gene-specific 10 μM PCR primer pair stock (SuperArray Bioscience Co.), and 1.0 μl template cDNA. The real-time thermal cycler program was recommended by SuperArray manufacture protocol [95°C, 10min; 40 cycles of (95°C, 15 sec; 55°C, 35 sec;

and 72°C, 30 sec)]. The specificity of the amplified target sequences was confirmed with melting curve analysis of the PCR products. For statistics, the values from triplicate pooled samples from individual animals were divided by the mean of the samples from unstressed control group to give relative values. Results were evaluated by Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results and Discussion

Microarray Profiling

Microarray analysis was performed to obtain an unbiased characterization of changes in gene expression in adrenal medulla with single and repeated exposure to IMO stress. Analysis was performed with samples from unstressed controls and rats exposed to single (1× IMO) for 2 hrs or repeated IMO 2 hr daily for 6 consecutive days (6× IMO). The mean values of unstressed controls were taken as baseline. A scatter plot of the changes compared to control group is shown in Fig. 1 Significant changes were observed in expression of a large number of genes. Therefore, we concentrated on the changes which differed significantly ($p<0.01$) and were of up-or down- regulated greater than 2-fold compared to unstressed controls. Using this criteria, we identified 651 genes up-regulated, of which 160 were annotated; and 487 down regulated, of which 64 were annotated. Repeated IMO significantly induced 370 transcripts, of which 78 were annotated and 195 down regulated genes of which 22 were defined.

Thus, nearly 4% of the total genome were found to be significantly ($p<0.01$) altered greater than 2-fold in the rat adrenal medulla by single IMO, and nearly 2% by repeated IMO stress.

Among the annotated transcripts, 35 up-regulated and 4 down-regulated were common and changed with both single and repeated IMO. Thus, most of the changes with single IMO were unique, while nearly half of the defined transcripts up-regulated by repeated IMO were also changed by single IMO.

Based on Gene Ontology, and PubMed publications, combined with knowledge of the physiological function of the adrenal medulla, we organized and categorized all the defined genes changed with single and repeated IMO stress (Fig. 2). In this paper we will briefly describe the most pronounced changes observed. We particularly discuss those changes which may provide new insight into the mechanism whereby the adrenal medulla responds a new experience of stress compared to repeated exposure to the same stressor.

Transcription Factors and Nuclear Protein Related—The greatest numbers of changes were observed with transcription factors. Table 1 shows the changes in transcription factor and nuclear protein related genes. Approximately 20% of the transcripts up-regulated by single IMO were transcription factors. Some were previously identified as transcription factors likely responsible for activation of catecholamine biosynthetic genes with IMO stress, such as *Egr1 and Fra-2*18–20. Others were not previously recognized as IMO responsive genes in the adrenal medulla. The largest changes with single IMO were in *NR4A3* (nuclear receptor subfamily 4, member 3, also know as Nor1, which was increased nearly 100-fold. The elevations of CREM (40-fold) and *gonadotropin inducible ovarian transcription factor 1* (32-fold) were also very pronounced. In some cases, several members of the same transcription factor family were induced, such as: *ATF3 and ATF4; Fra-1 and Fra-2;* nuclear receptor subfamily 4, group A [*NR4A1 (Nurr77 or NGF1-B), NR4A2 (Nurr1),* and *NR4A3 (Nor1)*]. Furthermore, in certain transcription factor families, some members were up-regulated and others were down-regulated, such as *inhibitors of DNA binding 1* and *4* which were up by nearly 4- and 3-fold respectively, while its member 2 was down by about 3-fold.

In addition, changes were observed in some transcription factors containing basic helix-loop-helix motifs, such as *achaete-scute complex homolog-like 1* (*Mash1). Mash1* is a very crucial factor in the development of the sympathoadrenal lineage and noradrenergic differentiation21⁻23. It might be involved in stress mediated hypertrophy of the adrenal gland as described later in the growth factor section. Interestingly, transcripts for *transcription termination factor* and *inhibitor of DNA binding 2* were down regulated, consistent with large increase in transcription with single IMO.

In the nuclear protein-related factors category, we observed induction in some genes involved in nuclear transport, such as *nucleoporin subfamily member karyopherin a*, as well as in *RNA helicase*.

Similar to single IMO, transcription factors were the main category among the changes with repeated IMO, which encompassed 16% of the up regulated gene transcripts. The largest changes were observed in *NR4A3 (Nor1)* (up 54 fold) an din *achaete-scute complex homolog-like 1* (*Mash1),* (up 31 fold). Most of the transcription factors induced with repeated IMO were also elevated by 1× IMO. Only two genes (*Egr2* and *lymphoid enhancer binding factor I*) were uniquely changed with repeated IMO (Table 1).

Cell Signaling Related—The changes in cell signaling related transcripts, including related to kinase/phosphatases, G proteins, calcium signaling, and channels are shown in Table 2. They comprised more than 16% of those up-regulated and about 14% of those down-regulated with single IMO. Among the kinases, the largest changes were observed in *SNF-1 like kinase* (>6-fold) and *MAPK activated protein kinase 2* (4-fold). S*erine/threonine kinase 10* was also elevated (3-fold), while transcript for *polo-like kinase* increased 3.7-fold. This kinase binds calcium and is implicated in long term synaptic plasticity24. In contrast phosphatidylinositol-3-kinase (*PI-3-kinase*) isoforms were down regulated. In addition to changes in kinases, we observed alterations in kinase anchor protein (*PRKA*) family members, with *PRKA protein* 12 up-regulated (4-fold) and *PRKA protein 1* down-regulated (2.7-fold) with single IMO.

The transcripts of several phosphatases were also up-regulated. The largest change was in *dual specificity phosphatase 5* (up 7-fold), which can target Erk1, Erk2. Several isoforms of *protein phosphatase 1*, which de phosphorylate serine/threonine residues were also up-regulated (2 to 5 fold).

The largest change in G-protein related genes was in the transcript for *bradykinin receptor b2* (*BDKRb2*), which is induced more than 6-fold. Stimulation of BDKRb2 activates PKC and triggers release of intracellular calcium25. It also is important in activation of calcium dependent NOS, formation of NO and activation of cGMP pathway26,27. Brakykinin is reported to acts as a secretagogue of medullary catecholamines28. Bradykinin and also induces NO and prostacyclin formation in adrenolmedullary endothelial cells. We have previously shown that bradykinin can elevated TH and DBH gene expression in PC12 cells29. We speculate that bradykinin, via BDKRb2, may be involved in mediating the IMO triggered elevation of *TH* gene expression in the adrenal medulla, which is observed even in rats which underwent hypophysectomy and splanchnic nerve section14. In regard, it is interesting to note that BRKRb2 is higher in adrenal medulla of the stress prone SHR compared to WKY rats30.

Expression of several other genes involved in G-protein coupled signaling were also altered, including: *cAMP inducible exchange factor* (*cAMP-GEF1*) (down >2-fold); *Rab9*, which encodes a small GTP-binding protein and *rabaptin*, which encodes another small protein interacting with Rab5, were reduced by 2–3-fold.

Several calcium-related genes were up-, but not down-, regulated. The greatest changes were observed with *calcitonin/calcitonin-related polypeptide, α* (*CALCA*), which codes for the peptide CGRP and *neurotransmitter-induced early gene protein 4* (*ania-4)* which is implicated in regulation of calcium in neurons31. Two calcium channel related genes were down-regulated and two potassium channel related genes were up-regulated.

Several other signaling related genes were also changed (Table 2). The largest changes were in *homer homolog 1* (up 11-fold) and in *phosphodiesterase 10A* (up at least 9-fold). The transcript for *phosphodiesterase 14B* (*PDE10A, PDE4B*) was also induced. These phosphodiesterases, are involved in both cAMP and cGMP turnover in many physiological and pathophysiological situations, were up-regulated more than 10- and 8-fold.

Many of the changes in signaling related transcripts with single IMO were not observed with repeated IMO. However, among the overlapping changes in response to both single and repeated IMO in kinase/phosphatase genes were *MAPKAPK2* (up >4-fold) and *PRKA anchor protein 1* (down > 4fold). With repeated IMO, several additional kinase- and phosphatase-related transcript were uniquely observed to be up-regulated, such as *calcium/ calmodulin-dependent protein kinase II beta subunit* (3-fold), *p21-activated kinase 3* (5-fold) and *ser/thr-like protein kinase lyk 4* (3.6-fold).

Among G-protein-related only *BDKRb2* was up-regulated by both types of stress. Like with single IMO, 6×IMO also induced mRNA for *ania-4* (6-fold), which has similarity to calcium/calmodulin-dependent kinases and to human doublecortin and it is implicated in regulation of calcium in neurons31. With repeated IMO calcium channel, γ3 as well as ChaK, a dual function transmembrane protein which functions as both a calcium-permeant ion channel and serine/threonine protein kinase; were down regulated about 2–3 fold.

These results implicate many more signally related changes than previously studied. Marked differences in cell signaling pathways in the adrenal medulla with single and repeated exposure to IMO stress and are important to distinguish between molecular processes leading to adaptation to stress or to pathological consequences of chronically repeated stress.

Growth/Apoptosis Related—The changes in growth factor and apoptosis related genes are shown in Table 3. They comprised 11% of the up-regulated and 12% of the down regulated changes with single IMO. The largest change in this category was in *brain derived neurotrophic factor* (*BDNF*), which was elevated 18.4 fold. *EGF receptor* and *inhibin α* genes were up-regulated by about 4.5- and 4-fold. At the same time, the transcript for *FGF receptor activating protein 1* and several differentiation-related genes were down-regulated by more than 2-fold. The changes in growth factor and related genes may be involved in the increased size of adrenal medulla which has been observed following various types of chronic stress32⁻36. However, it is perhaps surprising that even with single IMO, there were already changes in growth factor expression. Fewer growth factor-related genes are changed by the repeated compared to single IMO. However, most of the growth factor related genes changed by 6×IMO were also changed by 1×IMO. *BDNF* showed the largest change also with repeated IMO.

A substantial number of apoptosis related transcripts were also changed. In this category, the most robust changes were observed in the induction (22-fold) of *pleckstrin homology-like domain family A, member 1* which can also be induced by growth factors and differentiating agents and it has been implicated in mediating programmed cell death37. Several apoptosis related transcripts were down regulated, such as *caspase 2* and *caspase 12*. With repeated IMO there were less changes in apotosis related genes. From the changes with single IMO only *pleckstrin homology-like domain family A, member 1* was also induced (up 5 fold).

Caspase 9 was down-regulated by about 3 fold following repeated IMO, perhaps consistent with hypertrophy of the adrenal with repeated IMO.

Neurosecretion and Neuropeptide Related—The neurosecretion and neuropeptide related transcripts changed are also shown in Table 3. With single IMO they represent only 3% of the changes. However, several of these neuropeptide-related genes may be important in mediating the response to stress, such as *urocortin 2* (*Ucn2*) (increased 9-fold) *neuromedin* (increased 5-fold), and *urotensin 2* (increased 3-fold). Urocortins are reported to mediate adaptive responses of the cardiovascular system to stressful conditions [reviewed in38]. Ucn2 immunoreactivity was previously observed in TH positive cells in rat adrenal medulla and locus coeruleus39,40. Administration of Ucn2 to PC12 cells increased NE release and phosphorylation of TH41.

More of neurosecretion and neuropeptide related transcripts (8%) were changed with repeated stress than with single IMO. *Neuromedin U* (37 fold) and *urotensin 2* (4 fold) were also increased with repeated IMO. Urotensin 2 is the most potent mammalian vasoconstrictor to date. Plasma urotensin 2 levels are elevated in congestive heart failure patients and may play a role in progression of the disease42. Central neuromedin U is implicated in the stress-induced activation of CRF-containing neurons in the paraventricular nucleus43.

In addition, transcripts of *angiotensin converting enzyme* (*ACE*) and *prepronociceptin* genes were induced, as well as two genes associated with synaptic vesicle function. *Prepronociceptin* was induced by repeated stress by more than 3-fold. Nociceptin, also known as orphanin FQ, is an endogenous ligand for the opioid receptor-like 1 (NOP) receptor and has some structural homology with the endogenous opioid peptide dynorphin A44,45. It is implicating as playing an important role in several physiological functions including pain, anxiety, locomotion, learning, and memory. It is attractive to speculate that the induction of norciceptin may participate in stress triggered nociception.

Stress Related—A number of genes related to stress were induced (Table 3) including *heat shock* 70kD protein *1B* (up over 10-fold) with both single and repeated IMO and h*eat shock* 70kD protein *1A* (up 7 fold) with 1× IMO).

Structural—Some structural related genes (Table 3), such as *desmuslin*, *intercellular adhesion molecule 1*, *myosin heavy chain Myr 8*, also showed more than 2-fold change, and in some cases as much as 5-fold elevated expression. The percentage of changes in structural genes was slightly higher in response to repeated IMO than to single IMO (7.6% vs. 5%)

Immune Related—Although only a few of the changes related to immune function, these changes might be very important. As shown in Table 3, transcripts of *chemokine orphan receptor 1* was induced by 33-fold and *chemokine (C-C) motif ligand 2* was up-regulated by 8-fold with single IMO. One of the cytokine receptor-related genes, *interleukin 4 receptor* was up-regulated by 5-fold. In addition, *chemokine receptor 4* (*CXCR4*) gene expression was more than 8-fold down-regulated. One of the ligands of cytokine receptors, *interleukin 15*, was regulated by more than 2-fold at the same time.

The list of immune response related genes changed by repeated IMO was similar to the genes changed by single IMO. *CXCR4* was severely reduced by 9-fold and *interleukin 15* by more than 4-fold.

Metabolism Related—The results also indicate that 2-hour single IMO could trigger marked changes in metabolism. We found changes of genes that related to enzymes

involved in many metabolic pathways. Most of the up-regulated genes related to metabolism and also to lipid-metabolism in repeated IMO were already up-regulated with single IMO. *Diacylglycerol O-acyltransferase 1*, *methionine adenosyltransferase II, alpha*, *NADH dehydrogenase 1 alpha subcomplex 5*, *poly (ADP-ribose) glycohydrolase* (*PARG*) and *prostaglandin E synthase* (*PTGES*) showed large changes, as they also did with single IMO.

Protease Related—There were some changes in protease related genes, some of which were quite large, such as greater than 5-fold induction of *thrombomodulin*, *metalloprotease with thrombospondin 1*, *plasminogen activator urokinase receptor*, and *serine/cysteine proteinase inhibitor 1*.

Protease related genes, such as *metalloprotease with thrombospondin 1*(14.8-fold) and *thrombomodulin* (2.41-fold) were also increased by repeated IMO. Among the down-regulated genes that related to protease, *calpain 10* was reduced by both types of stress (2.8-, 3.2-fold). *Matrix metalloproteinase 14* (*MMP14*) was up-regulated by more than 4-fold with repeated IMO.

Others—A few of the genes were not easily categorized (Miscellaneous genes in Table 3). The undefined or genes not annotated are given in Table 4.

Validation of Changes of Selected Genes

Selected genes from different categories showing robust changes by microarray analysis were chosen for validation. Real-time RT-PCR was performed to quantify the relative expression levels of mRNA for *bradykinin receptor b2 (BDKRb2)* in both stress groups and *urocortin 2 (Ucn2)*, *phosphodesterase (PDE) 10A*, as well as *chemockine receptor 4 (CXCR4)* in single IMO group (Fig. 3A). To validate changes in repeated stress group, expression levels of *BDKRb2*, *BDNF*, *neuromedin U*, and *Matrix metalloproteinase 14* (*MMP14*) were quantified by real-time RT-PCR as well (Fig 3B).

There was good correspondence between the data obtained from microarray and the results from the real-time RT-PCR analysis. *BDKRb2* and *PDE 10A* gene expression increased about 5-fold and 9-fold in the RNA from the 1× IMO group, which is consistent with the microarray results. *Urocortin 2* showed a greater than 30-fold increase and *CXCR4* was markedly down-regulated by single IMO, which were even greater than the extent of changes from the microarray results. With 6× IMO, *BDKRb2* gene showed about 4-fold increase, *neuromedin U* gene expression increased 5-fold and *BDNF* gene expression induced 9-fold, and *MMP14* about 3-fold, which are similar to the results from the microarray analysis.

Pathway Analysis

The microarray analysis, as detailed above, discovered that hundreds of genes were significantly changed in the adrenal medulla by single as well as by repeated IMO. To further understand how those genes interact with each other and if there are common factors or regulators related to those genes, PathwayAssist software was used to explore networks of connections among the genes responsive to stress. Pathway analysis of direct interactions between the genes which were up- and down-regulated by single (not shown) and repeated IMO (Fig. 4) shows that a number of genes changed by repeated IMO can regulate *Egr1*. Egr1 was previously shown to be induced by stress in the adrenal medulla and to be important for the regulation of TH transcription19،46،47. *ACE*, together with *BDKRb2* and growth hormone receptor together with *SOC3* (*suppressor of cytokine signaling 3*) and *CXCR4*, as well as *BDNF* can regulate *Egr1* gene expression. *BDNF* can inhibit several of the down-regulated genes, such as *caspase 9* and *CXCR4*, the later is activated by the down

regulated *interleukin 15* (*IL15*), and *BDNF*, which interacts with *PARG*. Further downstream interactions of *Egr1* and *Egr2* are connected to *MMP14*, *PTGES* and *inhibin-beta-A* (*INHBA*) and *follistatin* (*FST*), and subsequently to the *CALCA* (*CGRP*) gene product.

Pathway analysis also revealed common targets of the genes changed by both single and repeated IMO stress (Fig. 5). Among the changed genes, *BDNF*, *CALCA* (*CGRP*), and *Egr1* played especially central roles for downstream activation of other target genes. As shown in Figure 5, *BDNF* can trigger activation of *Egr1*, *FOS*, *CREB*, *JUN*, *TH*, and several neuropeptides, such as *vasoactive intestinal peptide* (*VIP*) and *neuropeptide Y* (*NPY*), etc. and it inhibits *CXCR4* gene expression. It is also clear that the activation of *CALCA* could further activate *MAPK1* and *MAPK3* and *TH*. *Egr1*, itself a target of *BDNF*, activated some growth factor and survival related genes, such as *NGF receptor*, *insulin receptor substrate 1* (*IRS1*) and *BCL2*, several MAK kinases and MAP kinase kinases, and transcription factors such as *CREB1*.

Summary

The stress transcriptome of the adrenal medulla is quite extensive, especially in response to single immobilization stress. The study reveals many previously unidentified targets in the adrenal medulla. For example, extremely large changes were observed among transcription factors in nuclear receptor subfamily members (NR4A1, NR4A2, NR4A3), CREM and Mash1. Among the cell signalling related genes, changes in several kinase and protein phosphatase genes were identified, as well as down regulation of the transcript for the A kinase anchor protein. Considerable differences in cell signalling related genes was observed in response to single and repeated IMO, although bradykinin receptor b2 (BDKRb2) and transcript for CGRP (CALCA) and ania-4 were common to both single and repeated IMO. A large number of growth factor related transcripts were changed, as well as some apoptosis related genes, with several caspases down regulated. While only a few neuropeptides were identified, induction of urocortin 2 with single IMO, and urotensin 2 and neuromedin U with both durations of stress may be very meaningful. Quite a few of the changes were also observed in transcripts related to structural proteins, metabolism and especially lipid metabolism, as well as several chemokine and cytokines or their receptors. In remains to be determined if all of these changes are specifically localized to the adrenal chromaffin cell type. Pathway analyses demonstrate that Egr-1, BDNF and CALCA are likely key players which may regulate tyrosine hydroxylase transcription and thereby Epi/NE biosynthesis. Overall the findings revealed potential new biomarkers for the adrenal medullary response to stress and provide new mechanistic insights into the common and distinct responses to acute and repeated exposure to stress.

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References

1. Cannon W. Organization of physiological homeostasis. Physiological Reviews. 1929; 9:3900–431.
2. Goldstein, DS. Stress, Catecholamines and Cardiovascular Disease. Oxford University Press; Oxford: 1995.
3. McCarty R, Gold PE. Plasma catecholamines: effects of footshock level and hormonal modulators of memory storage. Horm Behav. 1981; 15:168–82. [PubMed: 6265338]

4. Hall JL, Gold PE. Adrenalectomy-induced memory deficits: role of plasma glucose levels. Physiol Behav. 1990; 47:27–33. [PubMed: 2326341]
5. Southwick SM, et al. Role of norepinephrine in the pathophysiology and treatment of posttraumatic stress disorder. Biol Psychiatry. 1999; 46:1192–204. [PubMed: 10560025]
6. Yehuda R, et al. Plasma norepinephrine and 3-methoxy-4-hydroxyphenylglycol concentrations and severity of depression in combat posttraumatic stress disorder and major depressive disorder. Biol Psychiatry. 1998; 44:56–63. [PubMed: 9646884]
7. Kvetnansky R, Sabban EL. Stress and molecular biology of neurotransmitter-related enzymes. Ann N Y Acad Sci. 1998; 851:342–56. [PubMed: 9668625]
8. Sabban EL. Catecholamines in stress: molecular mechanisms of gene expression. Endocrine Regulations. 2007; 41:61–73. [PubMed: 18257649]
9. Sabban EL, Kvetnansky R. Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. Trends Neurosci. 2001; 24:91–8. [PubMed: 11164939]
10. Wong DL, Tank AW. Stress-induced catecholaminergic function: transcriptional and post-transcriptional control. Stress. 2007; 10:121–30. [PubMed: 17514580]
11. Nankova BB, Tank AW, Sabban EL. Transient or sustained transcriptional activation of the genes encoding rat adrenomedullary catecholamine biosynthetic enzymes by different durations of immobilization stress. Neuroscience. 1999; 94:803–8. [PubMed: 10579571]
12. Sabban EL, et al. Stress Triggered Changes in Gene Expression in Adrenal Medulla: Transcriptional Responses to Acute and Chronic Stress. Cell Mol Neurobiol. 2006; 26:845–854. [PubMed: 16691439]
13. Osterhout, C.; Sun, B.; Tank, AW. Regulation of Tyrosine Hydroxylase Gene Transcription Rate by Stress: Use of Transgenic Mice. In: McCarty, R., et al., editors. Stress: Neural, Endocrine and Molecular Studies. Taylor & Francis; Londaon: 2002. p. 107-113.
14. Nankova B, et al. Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress. Proc Natl Acad Sci U S A. 1994; 91:5937–41. [PubMed: 7912437]
15. McMahon A, et al. Regulation of tyrosine hydroxylase and dopamine beta-hydroxylase mRNA levels in rat adrenals by a single and repeated immobilization stress. J Neurochem. 1992; 58:2124–30. [PubMed: 1349344]
16. Kvetnansky R, Mikulaj L. Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. Endocrinology. 1970; 87:738–43. [PubMed: 5453288]
17. Liu X, et al. Increased susceptibility to transcriptional changes with novel stressor in adrenal medulla of rats exposed to prolonged cold stress. Brain Res Mol Brain Res. 2005; 141:19–29. [PubMed: 16169632]
18. Wong DL, et al. Genetic mechanisms for adrenergic control during stress. Ann N Y Acad Sci. 2004; 1018:387–97. [PubMed: 15240394]
19. Papanikolaou NA, Sabban EL. Sp1/Egr1 motif: a new candidate in the regulation of rat tyrosine hydroxylase gene transcription by immobilization stress. J Neurochem. 1999; 73:433–6. [PubMed: 10386997]
20. Nankova BB, et al. Fos-related antigen 2: potential mediator of the transcriptional activation in rat adrenal medulla evoked by repeated immobilization stress. J Neurosci. 2000; 20:5647–53. [PubMed: 10908602]
21. Huber K. The sympathoadrenal cell lineage: specification, diversification, and new perspectives. Dev Biol. 2006; 298:335–43. [PubMed: 16928368]
22. Hirsch MR, et al. Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. Development. 1998; 125:599–608. [PubMed: 9435281]
23. Guillemot F, et al. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell. 1993; 75:463–76. [PubMed: 8221886]
24. Pak DT, Sheng M. Targeted protein degradation and synapse remodeling by an inducible protein kinase. Science. 2003; 302:1368–73. [PubMed: 14576440]
25. Hecquet C, et al. Positive cooperativity between the thrombin and bradykinin B2 receptors enhances arachidonic acid release. Am J Physiol Heart Circ Physiol. 2006; 290:H948–58. [PubMed: 16183725]

26. Abadir PM, et al. Angiotensin II type 2 receptor-bradykinin B2 receptor functional heterodimerization. Hypertension. 2006; 48:316–22. [PubMed: 16754789]
27. Abadir PM, Carey RM, Siragy HM. Angiotensin AT2 receptors directly stimulate renal nitric oxide in bradykinin B2-receptor-null mice. Hypertension. 2003; 42:600–4. [PubMed: 12953015]
28. Murakumo Y, et al. Potentiation by ouabain of bradykinin-induced catecholamine secretion and calcium influx into cultured bovine adrenal chromaffin cells: evidence for involvement of Na+ influx associated with the bradykinin B2-receptor. Life Sci. 1995; 57:PL259–64. [PubMed: 7564901]
29. Gebreyesus K, Kilbourne EJ, Sabban EL. Bradykinin elevates tyrosine hydroxylase and dopamine beta-hydroxylase mRNA levels in PC12 cells. Brain Res. 1993; 608:345–8. [PubMed: 8098650]
30. Qadri F, et al. Kinin B2 receptor localization and expression in the hypothalamo-pituitary-adrenal axis of spontaneously hypertensive rats. Int Immunopharmacol. 2003; 3:285–92. [PubMed: 12639805]
31. Ohmae S, et al. Molecular identification and characterization of a family of kinases with homology to Ca2+/calmodulin-dependent protein kinases I/IV. J Biol Chem. 2006; 281:20427–39. [PubMed: 16684769]
32. Kvetnansky R, et al. Bratislavske Lekarske Listy. 1966; 46:35–41.
33. Scaria KS, Premalatha LS. Cold induced adrenal weight & volume changes in white rats. Indian J Exp Biol. 1967; 5:256–7. [PubMed: 5583862]
34. McCarty R, Horwatt K, Konarska M. Chronic stress and sympathetic-adrenal medullary responsiveness. Soc Sci Med. 1988; 26:333–41. [PubMed: 3279522]
35. Wolman M, et al. Pathological changes in organs of rats chronically exposed to hypoxia. Development of pulmonary lipidosis. Histol Histopathol. 1993; 8:247–55. [PubMed: 8490251]
36. Ulrich-Lai YM, et al. Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. Am J Physiol Endocrinol Metab. 2006
37. Gomes I, et al. A proline- and glutamine-rich protein promotes apoptosis in neuronal cells. J Neurochem. 1999; 73:612–22. [PubMed: 10428057]
38. Nazarloo HP, et al. The roles of corticotropin-releasing factor-related peptides and their receptors in the cardiovascular system. Curr Protein Pept Sci. 2006; 7:229–39. [PubMed: 16787262]
39. Yamauchi N, et al. Distribution of urocortin 2 in various tissues of the rat. J Neuroendocrinol. 2005; 17:656–63. [PubMed: 16159378]
40. Reyes TM, et al. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc Natl Acad Sci U S A. 2001; 98:2843–8. [PubMed: 11226328]
41. Nemoto T, Mano-Otagiri A, Shibasaki T. Urocortin 2 induces tyrosine hydroxylase phosphorylation in PC12 cells. Biochem Biophys Res Commun. 2005; 330:821–31. [PubMed: 15809070]
42. Gruson D, et al. Circulating urotensin II levels in moderate to severe congestive heart failure: its relations with myocardial function and well established neurohormonal markers. Peptides. 2006; 27:1527–31. [PubMed: 16364499]
43. Yokota M, et al. Fos expression in CRF-containing neurons in the rat paraventricular nucleus after central administration of neuromedin U. Stress. 2004; 7:109–12. [PubMed: 15512854]
44. Meunier JC, et al. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature. 1995; 377:532–5. [PubMed: 7566152]
45. Reinscheid RK, et al. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science. 1995; 270:792–4. [PubMed: 7481766]
46. Papanikolaou NA, Sabban EL. Ability of Egr1 to activate tyrosine hydroxylase transcription in PC12 cells. Cross-talk with AP-1 factors. J Biol Chem. 2000; 275:26683–9. [PubMed: 10842166]
47. Nakashima A, Ota A, Sabban EL. Interactions between Egr1 and AP1 factors in regulation of tyrosine hydroxylase transcription. Brain Res Mol Brain Res. 2003; 112:61–9. [PubMed: 12670703]

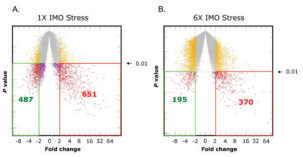

Figure 1.

Statistical plots of changes in gene expression with single and repeated IMO stress. Statistic plots showing the significance of changes in gene expression after single (A) and repeated (B) IMO stress. The X-axis which is the mean log_2 ratio of the current chip intensity over the baseline chip intensity represents the fold change in the gene expression. The Y-axis represents the statistical significance (p value). Each spot in the plots represents one transcript. The number of transcripts that were up-regulated (right side), and down-regulated (left side) by more than 2-fold with significance of $p < 0.01$ are indicated.

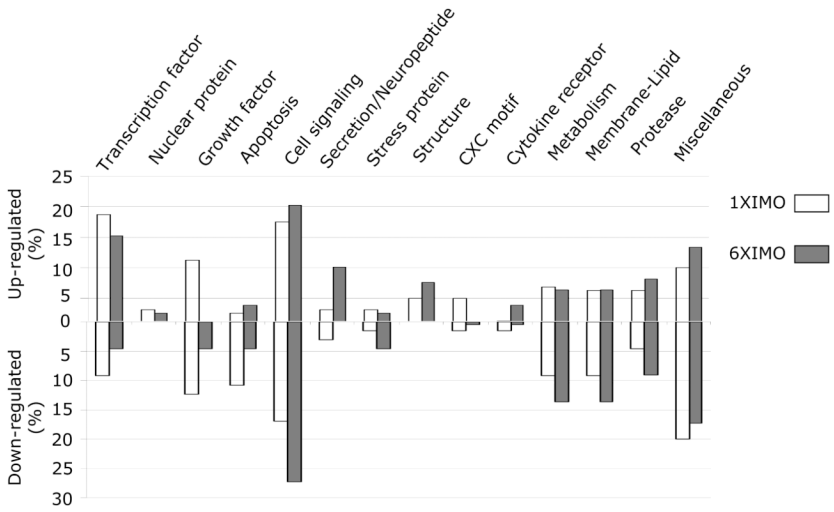

Figure 2.

Percent changes in gene expression with different categories with single and repeated IMO. The percent of the annotated genes up or down regulated with single and repeated IMO are indicated for each category.

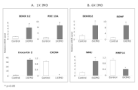

Figure 3.
Validation of selective microarray results from IMO stress experiment by real-time RT-PCR. Quantitative real-time RT-PCR showing the changes in mRNA levels of bradykinin receptor b2 (BDKR b2), phosphodiesterase 10A (PDE 10A), urocortin2, chemokine (C-X-C motif) receptor 4 (CXCR4), BDNF, neuromedin U (NMU) and matrix metalloproteinase 14 (MMP14) with single IMO (A) or repeated IMO (B). The mean for the control is taken as 1.0. * $p < 0.05$ versus unstressed controls.

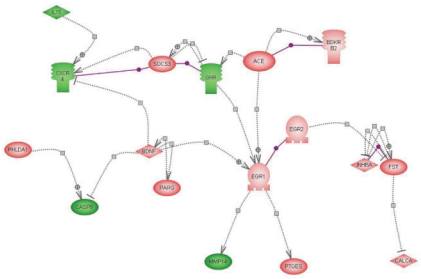

Figure 4.

Direct interaction of changes in gene expression with repeated IMO stress. Pathway analysis showing the direct interactions among genes down- regulated (green, IL15, CXCR4, GHR, CASP9, MMP14)or up-regulated (pink, all others) or by repeated IMO. The symbols are as follows: ligand: ; transcription factor: ; kinase: ; receptor: ; nuclear receptor: ;: binding:: positive regulations

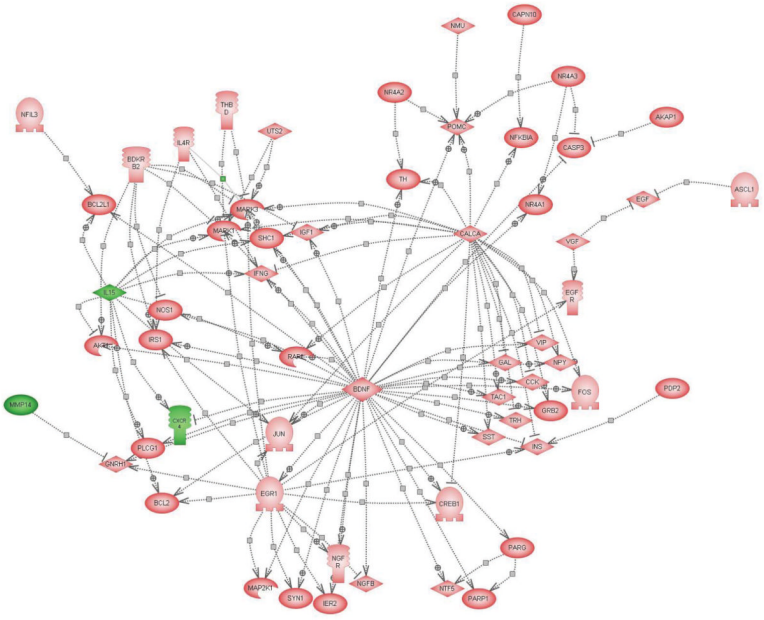

Figure 5.

Putative targets of common changes in gene expression with single and repeated IMO stress. Pathway analysis showing the common targets which are activated or inhibited by the common genes which are decreased (green, IL15, MMP14, CXCR4) or elevated (pink, all others) or by both durations of IMO stress. The symbols are as described in figure above. The abbreviations are as follows: PARP1 [ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)]; BCL2 (B-cell leukemia/lymphoma 2); BCL2L1 (Bcl2-like 1); BDKRB2 (bradykinin receptor B2); BDNF (brain-derived neurotrophic factor); CALCA (calcitonin/calcitonin-related polypeptide, alpha); CREB1 (cAMP responsive element binding protein 1); CASP3 (caspase 3); CD44 (CD44 antigen); CCK (cholecystokinin); EGR1 (early growth response 1); EGF (epidermal growth factor); EGFR (epidermal growth factor) FOS (FOS oncogene); GAL (galanin); GNRH1 (gonadotropin-releasing hormone 1); GRB2 (growth factor receptor bound protein 2); IER2 (immediate early response 2); INS (insulin); IRS1 (insulin receptor substrate 1); IGF1 (insulin-like growth factor 1); IFNG (interferon, gamma); IL15 (interleukin 15); IL4R (interleukin 4 receptor); JUN (Jun oncogene); MMP14 (matrix metalloproteinase 14); MMP9 (matrix metalloproteinase 9); MAPK1 (mitogen activated protein kinase 1); MAPK3 (mitogen activated protein kinase 3); MAP2K1 (mitogen activated protein kinase kinase 1); RAF1 (murine leukemia viral oncogene homolog 1); NGFR (nerve growth factor receptor); NMU (neuromedin U); NPY (neuropeptide Y); NTF5 (neurotrophin 5); NOS1 (nitric oxide synthase 1); NFIL3 (nuclear factor, interleukin 3 regulated); NR4A1 (nuclear receptor subfamily 4, group A, member 1); NR4A2 (nuclear receptor subfamily 4, group A, member 2); NR4A3 (nuclear receptor subfamily 4, group A, member 3); PARG [poly (ADP-ribose) glycohydrolase]; POMC (proopiomelanocortin); PDP2 (pyruvate dehydrogenase phosphatase isoenzyme 2); SST (somatostatin); SHC1 (src homology 2 domain-containing transforming protein C1); SYN1 (synapsin I); TAC1 (tachykinin, precursor 1); THBD (thrombomodulin); AKT1 (thymoma viral proto-oncogene 1); TRH (thyrotropin-releasing hormone); TGFB1 (transforming growth factor, beta 1); TNF (tumor necrosis factor); TH (tyrosine hydroxylase); UTS2 (urotensin 2); VEGF (vascular endothelial growth factor); VIP (vasoactive intestinal peptide); VGF (VGF nerve growth factor inducible).

Table 1

Transcription Factors and Nuclear Protein Related Genes

Probe Set ID	UniGene ID	UniGene Name	1×IMO Fold C (Up)	Probe Set ID	UniGene ID	UniGene Name	6×IMO Fold C (Up)
1369765_at	Rn.32936	**achaete-scute complex homolog-like 1**	12.65	1369765_at	Rn.32936	**achaete-scute complex homolog-like 1**	30.99
1369268_at	Rn.9664	activating transcription factor 3 (ATF3)	13.45	1369738_s_at	Rn.10251	**cAMP responsive element modulator**	13.07
1367624_at	Rn.2423	activating transcription factor 4 (ATF4)	2.29	1368321_at	Rn.9096	**early growth response 1 (Egr1)**	8.09
1369737_at	Rn.10251	**cAMP responsive element modulator**	**39.82**	1387306_a_at	Rn.89235	early growth response 2 (Egr2)	2.72
1369738_s_at			22.5	1368775_at	NA	**gonadotropin inducible ovarian TF 1**	17.5
1368813_at	Rn.6975	CCAAT/enhancer binding, δ	3.57	1387028_a_at	Rn.2113	**inhibitor of DNA binding 1**	3.23
1387343_at			2.9	1375120_at	Rn.22987	**inhibitor of DNA binding 4**	2.08
1368321_at	Rn.9096	**early growth response 1 (Egr1)**	8.23	1370138_at	Rn.21926	lymphoid enhancer binding factor 1	6.86
1368489_at	Rn.11306	fos-like antigen 1 (Fra-1)	3.77	1368488_at	Rn.54147	**nuclear factor, interleukin 3, regulated**	10.63
1387530_a_at	Rn.10962	fos-like antigen 2 (Fra-2)	3.61	1387410_at	Rn.88129	**NR4A2**	6.73
1368775_at	NA	**gonadotropin inducible ovarian TF 1**	**32.06**	1369067_at	Rn.62694	**NR4A3**	**54.05**
1368726_a_at	NA	gonadotropin inducible ovarian TF 2	10.51	1373632_at	Rn.8139	**TAF9 RNA polymerase**	3.03
1387270_at	Rn.12188	hematopoietically expressed homeobox	3.58	1387169_at	Rn.24106	**transducin-like enhancer of split 3**	3.76
1368546_at	Rn.9802	HIV type I enhancer binding protein 2	2.64				
1388587_at	Rn.23638	immediate early response 3	2.91				
1387028_a_at	Rn.2113	**inhibitor of DNA binding 1**	3.52				
1394022_at	Rn.22987	**inhibitor of DNA binding 4**	2.82				
1387788_at	Rn.15806	Jun-B oncogene	7.83				
1387260_at	Rn.7719	Kruppel-like factor 4 (gut)	10.13				
1370968_at	Rn.2411	nuclear factor kappa B p105 subunit	2.73				
1368488_at	Rn.54147	**nuclear factor, interleukin 3, regulated**	15.43				
1386935_at	Rn.10000	NR4A1	8.85				
1369007_at	Rn.88129	**NR4A2**	6.17				
1369067_at	Rn.62694	**NR4A3**	**97.62**				
1387200_at	Rn.45339	oligodendrocyte transcription factor 1	2.12				
1387684_at	Rn.96181	peroxisome proliferator activated R δ	5.15				
1370224_at	Rn.10247	signal transducer and activator of T 3	2.38				
1373632_at	Rn.8139	**TAF9 RNA polymerase II**	2.05				

Probe Set ID	UniGene ID	UniGene Name	1×IMO Fold C (Up)	Probe Set ID	UniGene ID	UniGene Name	6×IMO Fold C (Up)
1387169_at	Rn.24106	**transducin-like enhancer of split 3**	2.97				
1372211_at	Rn.3818	v-maf	1.97				
1387870_at	Rn.82737	zinc finger protein 36	3.07				
			(**Down**)				(**Down**)
1368870_at	Rn.3272	inhibitor of DNA binding 2	2.62	1368376_at	Rn.10712	NR0B2	5.48
1368073_at	Rn.6396	interferon regulatory factor 1	2.58				
1373471_at	Rn.43927	ring finger protein 166	2.18				
1387732_at	Rn.64629	transcription termination factor	2.13				
1387624_at	Rn.10845	upstream transcription factor 1	3.17				
1399006_at			2.58				
	Nuclear protein related		**(Up)**		**Nuclear protein related**		**(Up)**
1382756_at	Rn.6272	karyopherin alpha	2.65	1370004_at	Rn.11098	H2A histone family, member Y	3.27
1368747_at	Rn.11324	nucleoporin 98	3.69	1373032_at	Rn.9453	musculoskeletal	4.34
1388198_at	Rn.11099	nucleoporin p58	2.55				
1367761_at	Rn.2947	nudE nuclear distribution gene E	2.19				
1387912_at	Rn.3436	RNA helicase	4.83				

Bold – Genes changed by both types of stress; Fold C – Fold change

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Table 2

Cell Signaling Related Genes

Probe Set ID	UniGene ID	UniGene Name	1×IMO Fold C (Up)	Probe Set ID	UniGene ID	UniGene Name	6×IMO Fold C (Up)
		Kinase/Phosphatase related genes				**Kinase/Phosphatase related genes**	
1368869_at	Rn.122094	A kinase (PRKA) anchor protein 12	4.07	1367942_at	Rn.3494	acid phosphatase 5	2.02
1368868_at			3.07	1398251_a_at	Rn.9743	cal/cal-dependent pk II beta	3.23
1388686_at	Rn.12942	Down syndrome critical region homolog 1	4.44	1372299_at	Rn.92509	cyclin-dependent k inhibitor 1C	4.01
1368124_at	Rn.10877	dual specificity phosphatase 5	7.01	1371446_at	Rn.6276	**MAPK-activated protein kinase 2**	4.48
1371446_at	Rn.6276	**MAPK-activated protein kinase 2**	4.28	1368902_at	Rn.10128	p21-activated kinase 3	5.24
1368106_at	Rn.12100	polo-like kinase 2	3.72	1382307_at	Rn.58447	protein phosphatase 1(12A)	2.3
1384262_at	Rn.30046	**protein phosphatase 1(3B)**	5.09	1384262_at	Rn.30046	**protein phosphatase 1(3B)**	5.05
1386971_at	Rn.37758	protein phosphatase 1(10)	3.86	1380045_at	Rn.30021	**PDP isoenzyme 2**	4.19
1398807_at	Rn.4143	protein phosphatase 1B, β	2.73	1370509_at			4.05
1371136_at			2.21	1371943_at	Rn.4052	Ser/Thr-like protein kinase lyk4	3.56
1380045_at	Rn.30021	**PDP isoenzyme 2**	4.12				
1370509_at			3.82				
1367936_at	Rn.4190	serine/threonine kinase 10	3.08				
1367802_at	Rn.4636	serum/glucocorticoid regulated kinase	2.03				
1368597_at	Rn.42905	SNF1-like kinase	8				
1368596_at			6.72				
1368254_a_at	Rn.18522	sphingosine kinase 1	2.74				
			(**Down**)				**(Down)**
1369069_at	Rn.91372	**A kinase (PRKA) anchor protein 1**	2.66	1369069_at	Rn.91372	**A kinase (PRKA) anchor protein 1**	4.24
1370923_at	Rn.36170	expressed in non-metastatic cells 6	2.52				
1370100_at	Rn.22497	PI-3-kinase, class 2	2.08				
1369655_at	Rn.30010	PI-3-kinase, class 3	2.61				
	G-protein related genes		**(Up)**			**G-protein related genes (6×IMO)**	**(Up)**
1370649_at	Rn.9845	**bradykinin receptor b2**	6.59	1370649_at	Rn.9845	**bradykinin receptor b2**	14.24
1370650_s_at			3.89	1370650_s_at			4.97
1387596_at	Rn.10543	thrombin receptor-like 1	2.36	1369624_at	Rn.91303	prolactin releasing hormone	7.21

Probe Set ID	UniGene ID	UniGene Name	1×IMO Fold C (Up)	Probe Set ID	UniGene ID	UniGene Name	6×IMO Fold C (Up)
1387908_at	Rn.54720	DEXRAS1 (Dexras1)	4.33				
1384979_at	Rn.92385	G protein-coupled receptor 50	2.53				
1368029_at	Rn.4368	guanine nucleotide binding protein	2.07				
			(Down)				**(Down)**
1371635_at	Rn.3566	transmembrane domain protein regulated	2.27	1370449_at	Rn.87082G	protein-coupled receptor 105	2.42
	Calcium related genes		**(Up)**		**Calcium related genes (6×IMO)**		**(Up)**
1387276_at	Rn.80575	**ania-4**	4.78	1387276_at	Rn.80575	**ania-4**	6.08
1370050_at	Rn.7208	ATPase	2.56	1369117_at	Rn.90085	**cal/cal-related polypeptide, a**	2.87
1369116_a_at	Rn.90085	**cal/cal-related polypeptide, a**	15.17	1370000_at	Rn.41602	nucleobindin	2
1369117_at			5.61				
1370775_a_at			3.39				
1369886_a_at	Rn.23560	calcium binding protein 1	2.1				
	Channel related genes (1×IMO)		**(Up)**		**Channel related genes (6×IMO)**		**(Up)**
1387477_at	Rn.64577	potassium channel K12	3.01				
1368751_at	Rn.10878	potassium voltage-gated S3	3.44				
			(Down)				**(Down)**
1368343_at	Rn.10970	potassium voltage-gated channel, H2	2.18	1370757_at	Rn.81221	calcium channel, 3	2.3
1369674_at	Rn.10257	purinergic receptor P2X, 5	3.51	1369059_at	Rn.86991	ChaK	3.29
	Other Signaling related genes		**(Up)**		**Other Signaling related genes**		**(Up)**
1368605_at	Rn.30041	adaptor protein	2.38				
1369468_at	Rn.48736	frizzled homolog 4 (Drosophila)	2.97				
1370454_at	Rn.37500	homer homolog 1 (Drosophila)	11.33				
1370997_at			8.78				
1370669_a_at	Rn.44869	phosphodiesterase 10A	10.63				
1368438_at			8.96				
1369044_a_at	Rn.37733	phosphodiesterase 4B	2				
			(Down)				**(Down)**

Probe Set ID	UniGene ID	UniGene Name	1×IMO Fold C (Up)	Probe Set ID	UniGene ID	UniGene Name	6×IMO Fold C (Up)
1368660_at	Rn.42899	cAMP-GEFI	2.23	1398299_at	Rn.105776	Rho GEF 11	2.14
1387499_a_at	Rn.51153	phosducin-like	2.61	1393955_at	Rn.79380	WD-containing protein	2.19
1370196_at	Rn.14548	protein inhibitor of activated STAT 3	2.94	1371027_at	Rn.21799	Cas-Br-M ectropic RTS b	3.02
1387186_at	Rn.35289	RAB9, member RAS oncogene family	2.48				
1369024_at	Rn.3228	rabaptin	3.21				

Bold – Genes changed by both types of stress; Fold C – Fold change

Table 3

Other Major Changes in Gene Expression with Single and Repeated IMO Stress

Probe Set ID	UniGene ID	UniGene Name	(1×IMO) Fold C	Probe Set ID	UniGene ID	UniGene Name	(6×IMO) Fold C
Growth factor related genes			**Up**	**Growth factor related genes**			**Up**
1386994_at	Rn.27923	B-cell translocation gene 2	5.86	1368677_at	Rn.11266	**brain derived neurotrophic factor**	14.42
1386995_at			4.71	1387244_at	Rn.87514	**cell growth regulator**	5.02
1370823_at	Rn.25267	BMP and AMB inhibitor	2.73	1387951_at	Rn.18841	**decay-accelarating factor**	3.1
1368677_at	Rn.11266	**brain derived neurotrophic factor**	18.36	1369012_at	Rn.9874	inhibin beta-A	3.37
1387244_at	Rn.87514	**cell growth regulator**	7.08				
1387951_at	Rn.18841	**decay-accelarating factor**	4.72				
1370830_at	Rn.37227	epidermal growth factor receptor	4.51				
1387663_at	Rn.10454	glia maturation factor, β	7.67				
1386908_at	Rn.1484	glutaredoxin 1 (thioltransferase)	2.72				
1367705_at			2.17				
1387124_at	Rn.8831	inhibin alpha	3.75				
1387922_at	Rn.4346	late gestation lung protein 1	2.07				
1370174_at	Rn.2232	MDP response gene 116	3.59				
1367874_at	Rn.4169	ras homolog gene family, member Q	4.23				
1386967_at			2.76				
1369867_at	Rn.96242	sialyltransferase 8 A	2.48				
1396101_at	Rn.10647	stanniocalcin 1	4.49				
1387623_at			2.04				
1387280_a_at	Rn.32261	tumor-associated protein 1	4.07				
			Down				**Down**
1370327_at	Rn.24747	COMM domain containing 5	2.55	1368924_at	Rn.2178	growth hormone receptor	5.19
1370204_at	Rn.94200	FGF receptor activating protein 1	2.13				
1368618_at	Rn.30028	growth factor receptor bound protein 14	3.03				
1398785_at	Rn.6775	multiple endocrine neoplasia 1	2.05				
1392743_at	Rn.77753	myc induced nuclear antigen	2.64				
1398867_at	Rn.19573	neuronal differentiation-related gene	2.59				
1370336_at	Rn.15599	pregnancy-induced growth inhibitor	2.36				

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1387427_at	Rn.51136	RAD50 homolog (S. cerevisiae)	2.17				
1387153_at	Rn.34221	reversion induced LIM gene	2.58				
	Apoptosis related genes		**Up**		**Apoptosis related genes**		**Up**
1367752_at	Rn.40101	breast cancer anti-estrogen resistance 1	2.16	1395237_at	Rn.107482	annexin V-binding protein ABP-7	4.09
1368860_at	Rn.40778	**pleckstrin homology-like domain, A1**	21.8	1378247_at	Rn.20681	ELL associated factor 2	3.68
1368025_at	Rn.9775	DNA-damage-inducible transcript 4	5.41	1368860_at	Rn.40778	**pleckstrin homology-like domain, A1**	5
1370319_at	Rn.2923	peptidylprolyl isomerase F	2.2				
			Down				**Down**
1367842_at	Rn.19953	amyloid beta precursor protein-binding, B1	2.41	1368652_at	Rn.32199	caspase 9	2.87
1387055_at	Rn.4279	APP-binding protein 1	2.55				
1387605_at	Rn.81078	caspase 12	2.22				
1367890_at	Rn.1438	caspase 2	2.3				
1370044_at	Rn.22800	Fas apoptotic inhibitory molecule	3.1				
1390434_at	Rn.18545	TNFRSF1A-associated via death domain	4.17				
1371131_a_at	Rn.2758	upregulated by 1,25-dihydroxyvitamin D-3	2.34				
	Secretion/Neuropeptide related genes		**Up**		**Secretion/Neuropeptide related genes**		**Up**
1369717_at	Rn.47720	**neuromedin U**	5	1370028_at	Rn.10149	angiotensin 1 converting enzyme 1	2.42
1370408_at	Rn.8865	putative small membrane protein NID67	2.37	1369116_a_at			2.59
1387569_at	Rn.74043	synaptic vesicle glycoprotein 2c	2.49	1370507_at	Rn.11279	disks large-associated protein 4	4.87
1369619_at	Rn.153037	urocortin 2	8.73	1369717_at	Rn.47720	**neuromedin** U	37.48
1368805_at	Rn.48886	**urotensin 2**	3.17	1368369_at	Rn.87935	prepronociceptin	3.52
				1368805_at	Rn.48886	**urotensin 2**	3.83
				1390257_at	Rn.28719	vesicle-associated membrane protein	2.14
			Down				**Down**
1372950_at	Rn.90025	blocked early in transport	2.55				
1369414_at	Rn.64627	syntaxin binding protein 3	2.83				
	Stress related genes		**Up**		**Stress related genes**		**Up**

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1387282_at	Rn.102906	crystallin, alpha C (Hsp22)	6.21	1368247_at	NA	**heat shock 70kD protein 1B**	11.87
1388721_at			6	1388850_at	Rn.3277	heat shock protein 1, alpha	2.42
1368852_at	Rn.64562	DnaJ-like protein	1.96				
1370912_at	Rn.1950	heat shock 70kD protein 1A	7.01				
1368247_at	NA	**heat shock 70kD protein 1B**	13.76				
1378002_at	Rn.58449	osmotic stress protein 94 kDa	3.9				
			Down				**Down**
1367741_at	Rn.4028	ubiquitin-like domain member 1	2.06	1382809_at	Rn.28931	cold inducible RNA binding protein	3.89
	Structural protein related genes		**Up**		**Structural protein related genes**		**Up**
1372658_at	Rn.46362	desmuslin	2.83	1370053_at	Rn.90059	discs	2.75
1370017_at	Rn.10968	emerin	4.89	1387031_at	Rn.32904	endoplasmic retuclum protein 29	2.35
1387202_at	Rn.12	intercellular adhesion molecule 1	4.11	1387843_at	Rn.2743	follistatin	7.51
1388932_at	Rn.62616	laminin, alpha 5	2.55	1388244_s_at	Rn.999	laminin receptor 1	2.47
1371682_at	Rn.3135	microtubule-associated protein 1 LC3 α	3.01	1371682_at	Rn.3135	microtubule-associated protein 1 LC3 α	3.68
1370478_at	Rn.48756	**myosin heavy chain Myr 8**	5.25	1370478_at	Rn.48756	**myosin heavy chain Myr 8**	12.57
1370697_a_at	Rn.107975	nexilin	2.19				
1370875_at	Rn.773	villin 2	3.83				
	CXC-motif related genes		**Up**		**CXC-motif related genes**		**Up**
1367973_at	Rn.4772	chemokine (C-C motif) ligand 2	7.14				
1367940_at	Rn.12959	chemokine orphan receptor 1	33.36				
			Down				**Down**
1370097_a_at	Rn.44431	**chemokine (C-X-C motif) receptor 4**	8.5	1370097_a_at	Rn.44431	**chemokine (C-X-C motif) receptor 4**	9.1
1373661_a_at			6.3	1373661_a_at			6.18
1389244_x_at			5.9	1389244_x_at			5.13
	Cytokine related genes		**Up**		**Cytokine related genes**		**Up**
1388233_at	Rn.14523	cytokine inducible SH2-containing protein	6.8	1387180_at	Rn.10758	interleukin 1 receptor, type II	3.83
1368134_a_at	Rn.10471	**interleukin 4 receptor**	5.12	1368134_a_at	Rn.10471	**interleukin 4 receptor**	4.51

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				1369584_at	Rn.81237	suppressor of cytokine signaling 3	2.36
			Down				**Down**
1368375_a_at	Rn.2490	**interleukin 15**	2.47	1368375_a_at	Rn.2490	**interleukin 15**	4.63
	Metabolism related genes		**Up**		**Metabolism related genes**		**Up**
1367982_at	Rn.97126	aminolevulinic acid synthase 1	7.44	1370375_at	Rn.10202	**glutaminase 2 (liver, mitochondrial)**	5.42
1370964_at	Rn.5078	arginosuccinate synthetase	6.23	1371350_at	Rn.41420	**methionine adenosyltransferase II, α**	2.91
1383248_at	Rn.7038	flavin containing monooxygenase 5	3.13	1386951_at	Rn.100240	**NADH dehydrogenase 1 α 5**	5.51
1370375_at	Rn.10202	**glutaminase 2 (liver, mitochondrial)**	5.78	1370354_at	Rn.13634	**poly (ADP-ribose) glycohydrolase**	4.94
1386914_at	Rn.3862	guanosine monophosphate reductase	7.07	1371762_at	Rn.108214	retinol binding protein 4	4.65
1374903_at	Rn.8807	I-branching β-1,6-ASATF	2.32				
1371350_at	Rn.41420	**methionine adenosyltransferase II, α**	2.41				
1386951_at	Rn.100240	**NADH dehydrogenase 1 α 5**	6.13				
1370191_at	Rn.6290	ornithine decarboxylase antizyme inhibitor	2.12				
1369785_at	Rn.18690	PP amidotransferase	2.92				
1370354_at	Rn.13634	**poly (ADP-ribose) glycohydrolase**	6.05				
1386981_at	Rn.6085	solute carrier family member 1	2.07				
			Down				**Down**
1373838_at	Rn.44467	α 1,3-fucosyltransferase Fuc-T	2.39	1388044_at	Rn.44844	6-PF-2-kinase/fructose-2,6-BP 2	2.2
1367991_at	Rn.22161	glucosidase 1	2.29	1387973_at	Rn.10170	cytochrome P450 4F4	4.31
1386983_at	Rn.11080	hydroxymethylbilane synthase	2.48	1379885_at	Rn.6404	flavin containing monooxygenase 4	2.78
1387187_a_at	Rn.37420	N-acetyltransferase 1	2.62				
1368756_at	Rn.9674	thioesterase domain containing 1	3.76				
1389689_at	Rn.43153	valyl-tRNA synthetase 2-like	2.55				
	Lipid-Metabolism related genes		**Up**		**Lipid-Metabolism related genes**		**Up**
1367915_at	Rn.252	**diacylglycerol O-acyltransferase 1**	11.93	1390549_at	Rn.101807	adiponectin receptor 2	2.18
1387630_at	Rn.4243	fatty acid elongase 1	4.83	1387796_at	Rn.11318	arachidonate 12-lipoxygenase	2.01
1394401_at	Rn.46942	fatty acid elongase 2	6.32	1367979_s_at	Rn.107152	cytochrome P450, subfamily 51	5.68
1388108_at			4.74	1367915_at	Rn.252	**diacylglycerol O-acyltransferase 1**	6.08

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1387233_at	Rn.7040	hydroxysteroid (17-beta) dehydrogenase 7	4.7	1368878_at	Rn.10780	**isopentenyl-diphosphate delta isomerase**	7.8
1368878_at	Rn.10780	**isopentenyl-diphosphate delta isomerase**	5.04	1368020_at	Rn.10288	mevalonate (diphospho) decarboxylase	2.89
1368570_at	Rn.54479	lecithin-retinol acyltransferase	17.89	1368015_at	Rn.7730	**prostaglandin E synthase**	10.5
1368683_at	Rn.87449	oxidised LDL receptor 1	15.44	1368014_at			3.05
1368014_at	Rn.7730	**prostaglandin E synthase**	4.97				
1368527_at	Rn.44369	prostaglandin-endoperoxide synthase 2	12.27				
1367855_at	Rn.88169	scavenger receptor class B, member 1	4.57				
1386956_at			4.54				
			Down				**Down**
1367638_at	Rn.13468	malonyl-CoA decarboxylase	2.09	1388211_s_at	Rn.37524	cytosolic acyl-CoA thioesterase 1	2.78
1369070_at	Rn.29982	peroxisomal biogenesis factor 12	2.04				
1387064_at	Rn.4065	peroxisomal membrane protein 3	3.23				
1396866_s_at			3.00				
	Protease related genes		**Up**		**Protease related genes**		**Up**
1368223_at	Rn.7897	**metalloprotease with thrombospondin 1,1**	8.1	1387135_at	Rn.98788	metalloproteinase domain 15	2.35
1392894_at	Rn.64635	fibrinogen-like 2	2.45	1368223_at	Rn.7897	**metalloprotease with thrombospondin 1,1**	4.77
1367800_at	Rn.107102	plasminogen activator	4.1	1368595_at	Rn.3117	matrix metalloproteinase 24	2.1
1387269_s_at	Rn.82711	plasminogen activator, urokinase receptor	6.92	1368901_at	Rn.88295	**thrombomodulin**	2.41
1392264_s_at	Rn.29367	serine/cysteine proteinase inhibitor, 1	10.36				
1368519_at			4.37				
1387812_at	Rn.950	Subtilisin - like endoprotease	3.3				
1367712_at	Rn.25754	tissue inhibitor of metalloproteinase 1	2.08				
			Down				**Down**
1368904_at	Rn.8875	**calpain 10**	2.79	1368904_at	Rn.8875	**calpain 10**	3.24
1375951_at	Rn.88295	**thrombomodulin**	7.57				
1368900_at			5.95				
1368901_at			5.19				
1367966_at	Rn.10902	dipeptidylpeptidase 3	2.14	1367860_a_at	Rn.10371	matrix metalloproteinase 14	4.26

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	Miscellaneous genes		**Up**		**Miscellaneous genes**		**Up**
1368111_at	Rn.11149	**BTB domain containing 2**	3.84	1368111_at	Rn.11149	**BTB domain containing 2**	2.91
1370951_at	Rn.12038	ER transmembrane protein Dri 42	2.25				
1370950_at			2.42				
1370177_at	Rn.10677	poliovirus receptor	11.92				
			Down				**Down**
1373034_at	Rn.103351	tryptophan rich basic protein	2.42				

Bold – Genes changed by both types of stress; Fold C – Fold change