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Changes in gene expression with increased transglutaminase 2 in a SH-SY5Y cell line

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1. ABSTRACT

Previously we showed that the overexpression of transglutaminase 2 (TGase 2) resulted in activation of NF-κB through polymerization of I-κBα. To explore the pathway of TGase 2-mediated NF-κB activation, a transcriptomic microarray analysis was performed. In a SH-SY5Y cell line transfected with TGase 2, 24 genes were up-regulated at least 1.6-fold and 26 genes were down-regulated, as compared to the wild-type cell line. Detailed analysis resulted in the identification of target genes involved in regulating inflammation, including tribbles homolog 3, peroxiredoxin 4, neuropeptide Y, galanin, and vasoactive intestinal peptide. Our data demonstrate that the increase in TGase 2 in the neuroblastoma causes functional changes in transcriptional regulation, especially in genes associated with inflammation. These changes in gene expression caused by increases in TGase 2 activity may contribute to the pathophysiologic processes of inflammatory diseases.

2. INTRODUCTION

Transglutaminase 2 (TGase 2, E.C. 2.3.2.13) belongs to a family of Ca^{2+}-dependent enzymes that catalyze N^{ε}-(γ-L-glutamyl)-L-lysine isopeptide bond formation between peptide-bound lysine and glutamine residues (1). TGase-mediated crosslinking stabilizes intracellular and extracellular structural proteins for multiple physiological functions such as apoptosis (2), epithelial barriers, and extracellular matrix formation (3). TGase 2 activity is normally low in tissues, but is inappropriately activated in a variety of pathologies including Alzheimer's disease, Huntington's disease, Parkinson's disease, inflammatory myopathies, rheumatoid arthritis, celiac disease, etc. (4). Recently we discovered that TGase 2 plays a role in functional protein activation, such as nuclear factor-κB (NF-κB) activation in an I-κB kinase (IKK)-independent manner (5, 6).Overexpression of TGase 2 causes the continuous activation of NF-κB, which may contribute to chronic inflammation (6). Many stresses,

including oxygen free radicals and pathogens, induce inflammation through IKK-dependent NF-κB activation, which is returned to normal levels by feedback control. However the continuous activation of NF-κB without IKK activation is often observed in chronic inflammation or drug-resistant cancer (7). Chronic inflammation appears to lead to many diseases, such as autoimmune diseases, degenerative diseases, and cancers, which are frequently accompanied by an increase in TGase activity. This suggests that the continuous regulatory failure of TGase activity may result in devastating physiological status (8). We have demonstrated TGase-mediated NF-κB activation in several different cell lines, including human neuroblastoma (SH-SY5Y), human embryonic kidney (EcR293), and mouse microglia (BV-2) (6).

NF-κB activation affects numerous biological phenomena including cell survival, angiogenesis, cell metastasis, drug resistance, and apoptosis (9), but is specifically modulated by the sophisticated dimerization of NF-κB and association with transcription factors. Although we have demonstrated that TGase 2 induces NF-κB activation, there has been little information available concerning the alteration of gene expression by TGase 2 transfection. Herein we have explored the alteration of gene expression in a TGase 2-transfected stable cell line (SH-SY5Y/TG) by transcriptomic analysis. The microarray dataset for TGase 2 is a useful guide for understanding the physiological role of TGase 2 in disease pathogenesis.

3. MATERIALS AND METHODS

3.1. Stable transfection with TGase 2

The previously established human neuroblastoma cell line SH-SY5Y/TG (TGase 2-transfected SH-SY5Y) was used for this study (6). The SH-SY5Y/TG cells were grown in DMEM/F12 (50:50) supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine, penicillin/streptomycin, and hygromycin, as described (6)

3.2. DNA microarrays and gene expression profiling

The Human Array-Ready Oligo Set™ (Version 2.0) containing 70-mer probes for 21,329 genes was obtained from QIAGEN, Inc. Oligo microarrays were produced at the Advanced Technology Center of the National Cancer Institute.

Total RNAs were isolated from SH-SY5Y/TG cultured cells using the Trizol method. Total RNAs from wild-type SH-SY5Y cells were used as the reference for all microarray experiments. To obtain gene expression data from TGase2-over-expressing cells, 20 μg of total RNA were used to produce fluorescence (Cy-5 or Cy-3)-labeled cDNAs. At least two hybridizations were carried out for each culture, using a dye-swap strategy to eliminate dye-labeling bias as previously described (10,11). Data transformation and normalization of gene expression data were performed as previously described (10, 11).

3.3. Pathway analysis

PathwayAssist™ (version 2.5, Ariadne Genomics) was used to explore the functional relationships among the genes with altered expression in TGase 2-over-expressing cells. This method explores gene-interaction networks represented in the ResNet™ database. ResNet™ is a comprehensive database of molecular networks compiled by a proprietary natural-language-processing algorithm and applied to the entire PubMed database. The database contains more than 500,000 functional links for about 50,000 proteins, extracted from 4.5 million PubMed abstracts and full-text articles.

3.4. Western blotting

Protein extracted from each group of cells (20 μg/well) was applied to 4-20%-gradient SDS gels using Tricine buffers and then transferred to PVDF membranes. For western blotting using polyclonal anti-PRDX4 IV (anti-rabbit anti-peroxiredoxin IV; Lab Frontier, Seoul, Korea), polyclonal anti-TRIB3 (anti-rabbit anti-tribbles homolog 3 drosophila; Imgenex, San Diego, CA, USA), monoclonal anti-HSPA8 (anti-mouse anti-heat shock 70 kDa protein 8; Abcam, Cambridge, UK), monoclonal anti-NDUFB6 (anti-mouse anti- NADH dehydrogenase ubiquinone 1 beta subcomplex; Mitoscience, Eugene, OR, USA), and polyclonal anti-LDH (anti-lactate dehydrogenase; goat, Research Diagnostics, Inc., Flanders, NJ, USA), the concentration of the primary antibody was 5 μg/ml and that of the secondary antibody was 0.1 μg/ml. The western blots were developed by enhanced chemiluminescence (Pierce, Rockford, IL, USA). The data was analyzed by densitometric scanning using the NIH image program.

4. RESULTS AND DISCUSSION

4.1. Experimental setup and statistical analysis

We characterized gene expression profiles of TGase 2-transfected SH-SY5Y (SH-SY5Y/TG) by using DNA microarrays containing 21,767 oligonucleotide probes representing 21,329 unique genes. To estimate and minimize the variation from cell cultures, we prepared total RNAs from two independent cell cultures and used them to prepare cDNAs labeled with fluorescent dyes. A reference fluorescent cDNA probe was prepared from pooled total RNA from the parental cell line (SH-SY5Y). To minimize labeling biases, total RNAs from each cell culture were labeled with the reciprocal fluorochrome in duplicate experiments. We excluded all gene that had any missing data points from quadruplicate experiments and then applied the F-test ($P<0.01$) to exclude gene whose expression patterns did not change significantly.

Figure 1 shows that in TGase 2-transfected SH-SY5Y cells 24 genes were induced by 1.6- fold or more, whereas 26 genes were repressed by at least 1.6- fold. The induction of a random set of selected genes was confirmed by western blotting (Figure 2).

4.2. Genes that are up-regulated and associated with regulating inflammation

Peripheral corticotropin-releasing hormone (CRH) is thought to have proinflammatory effects. Using the experimental autoimmune encephalomyelitis (EAE) model, Benou *et al.* showed that CRH-deficient mice are resistant to EAE, with a decrease in the clinical score as

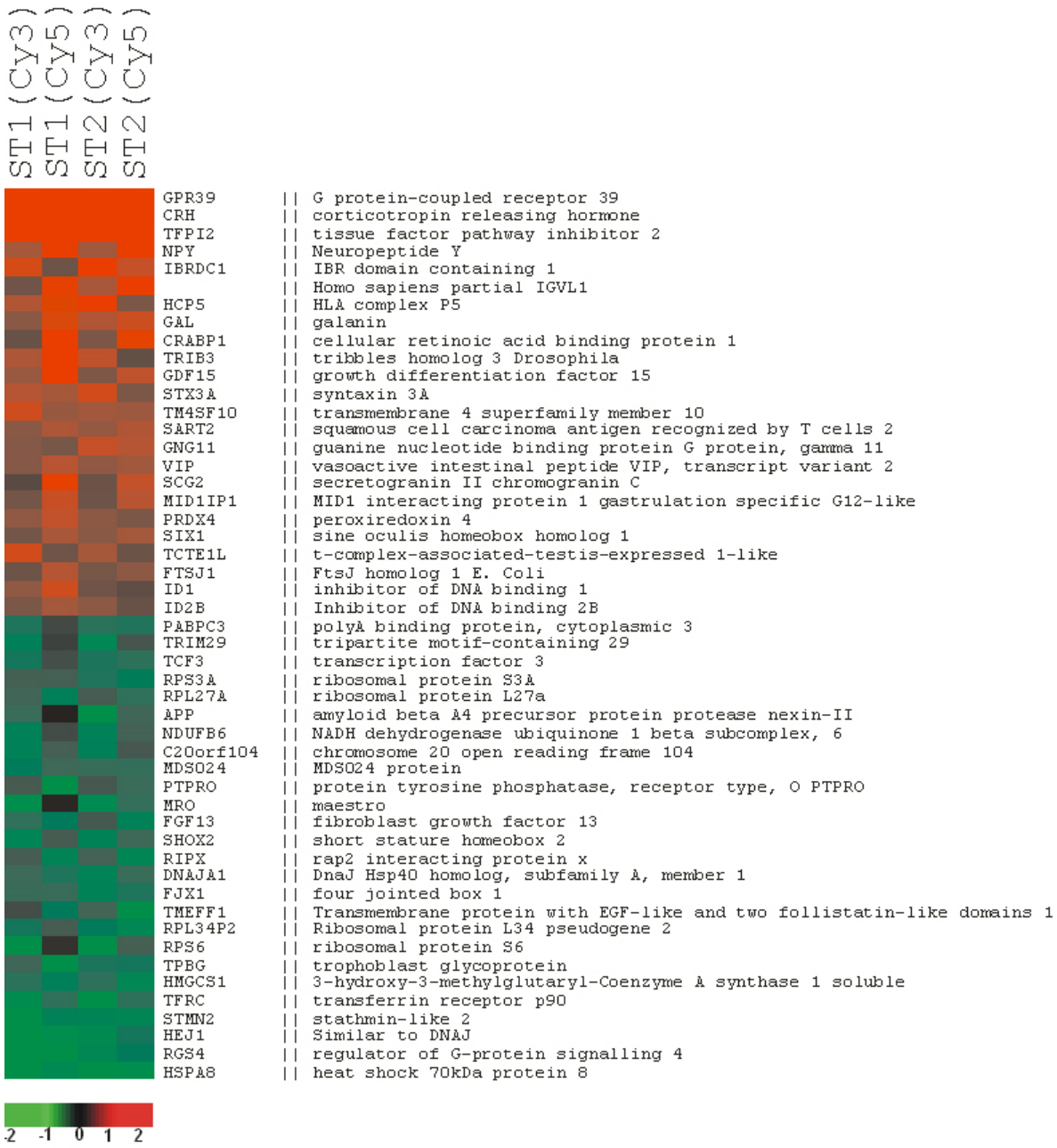

Figure 1. Altered gene expression in TGase 2-over-expressing SH-SY5Y cells. Fifty genes with expression ratios that were at least 1.6-fold different relative to the reference expression in the average of four microarray experiments were selected for analysis. The data are presented in a matrix format in which the rows represent individual genes and the columns represent repeated experiments. Each cell in the matrix represents the expression level of a gene in an individual experiment. The red and green colors reflect high and low expression levels, respectively, as indicated by the scale bar (log2 transformed scale).

well as in cellular infiltration of the central nervous system (12). Coincidently, increased TGase expression has been reported in EAE (13).

Neuropeptide Y (NPY), a major brain neurotransmitter, is expressed in neurons of the hypothalamic arcuate nucleus that project mainly to the paraventricular nucleus, an important site of NPY release. NPY synthesis in the arcuate nucleus is thought to be regulated by several factors, notably insulin, which may exert an inhibitory action. The effects of NPY injected into the paraventricular nucleus and other sites include hyperphagia, reduced energy expenditure and weight gain, insulin secretion, and stimulation of corticotropin and

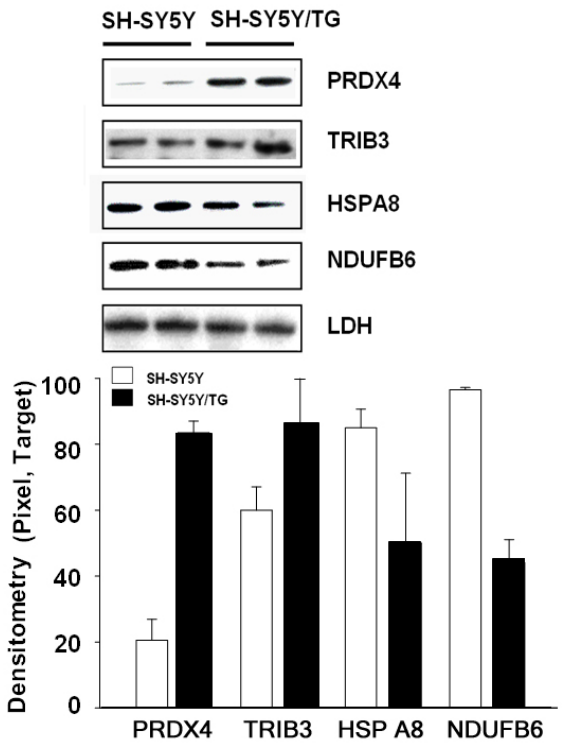

Figure 2. Western blot analysis shows that the levels of gene expression are consistent with the data analyzed by microarray. Peroxiredoxin IV (PRDX4) and Tribbles homolog 3 (TRIB3) increase the gene expression while gene expressions of heat shock 70kDa protein 8 (HSPA8) and NADH dehydrogenase (ubiquinone) 1β subcomplex 6 (NDUFB6) are decreased in TGase 2 transfected SH-SY5Y cells. Densitometry reveals a 3-fold increase in PRDX4 containing thioredoxin peroxidase activity. LDH was used for the control.

corticosterone release (14). Therefore uncontrolled NPY expression may contribute to the development of diabetes. Although the detailed mechanism is unclear, the levels of NPY in the plasma of patients with inflammatory myositis are significantly higher than those in the controls (15). Interestingly, we previously reported that an increase in TGase 2 expression is closely associated with pathological lesions of inclusion body myositis (IBM) (16).

Interferon (IFN)-γ is one of the most important microglia stimulators *in vivo*, participating in inflammation and Th1 activation/differentiation. IFN-γ-mediated signaling involves the activation of the Jak/STAT1 pathway. The neuropeptide vasoactive intestinal peptide (VIP) is a potent microglia-deactivating factor that inhibits the production of proinflammatory mediators *in vitro* and *in vivo* (17). That report showed that VIP inhibits Jak1-2 and STAT1 phosphorylation and the binding of activated STAT1 to the IFN-γ-activated site motif in IFN regulatory factor-1 and the CD40 promoter and to the IFN-stimulated response element motif of the IP-10 promoter. Through its effect on the IFN-γ-induced Jak/STAT1 pathway, VIP is able to control the gene expression of IP-10, CD40, and iNOS, three microglia-derived mediators that play essential roles in several pathologies, i.e. inflammation and autoimmune disorders (17). Therefore VIP appears to be a negative modulator of inflammation induced by TGase 2. In contrast, neuropeptides including NPY and VIP also have been postulated to be involved in the mechanisms of some dermatoses, including psoriasis, eczema and atopic dermatitis, and various inflammatory skin disorders (18-20).

Galanin (GAL) is present in enteric nerves lining the gastrointestinal tract, where it is normally involved in regulating intestinal motility by binding to the galanin-1 receptor (Gal1R) subtype expressed in smooth muscle cells. In contrast, although epithelial cells lining the colon do not normally express Gal1R, this protein is up-regulated by the inflammation-associated transcription factor NF-κB. It has been shown that the murine colitis induced by dextran sulfate sodium is associated with increased Gal1R expression as well as with increased colonic fluid secretion. GAL and Gal1R up-regulation in colonic epithelial cells results in increased intestinal Cl^- secretion (21).

Growth/differentiation factor-15 (GDF-15) is a divergent TGF-β family member. GDF-15 induction after organ injury is a hallmark of many tissues. This suggests that GDF-15 is an early mediator of the injury response in kidney and lung and may regulate inflammation (22).

Tribbles homolog 3 (TRIB3) is a representative negative-feedback molecule and a p65-interacting negative regulator of NF-κB-dependent transcription (23). TRIB3 also is a NF-κB-inducible protein. Over-expression of TRIB3 inhibits NF-κB-dependent transcription induced by tumor necrosis factor (TNF) or downstream signaling proteins (23). TRIB3 is critically involved in a novel negative feedback control pathway of NF-κB-induced gene expression, as well as in disrupting Akt activation (24). TGase 2 activates the PI3K/Akt pathway in the human chronic myelogenous leukemia K562 cell line, which results in NF-κB activation (25). Our data show that an increase in TGase 2 expression induces cellular retinoic acid-binding protein 1 (CRABP1) as in Figure 1, which inhibits PI3K/Akt signaling through a retinoic acid receptor-dependent mechanism. (Figure 1) (26). Therefore CRABP1 and TRIB3 induced by TGase 2 appear to be feedback molecules that block PI3K/Akt signaling.

Thrombin activates NF-κB through the MAPK pathway via protease-activated receptor-1 (PAR-1) activation, which results in the increase of cyclooxygenase-2 (COX-2). Tissue factor pathway inhibitor 2 (TFPI2) is induced by a COX-2-dependent pathway. The up-regulation of TFPI2 expression by thrombin could, in turn, down-regulate thrombin production and contribute to the limiting of blood coagulation (27). Therefore, TGase 2-mediated NF-κB activation appears to induce TFPI2 as a feedback mechanism.

Reduction-oxidation (redox) plays a critical role in NF-κB activation. Diverse stimuli appear to utilize reactive oxygen species (e.g. hydrogen peroxide) as

common effectors for activating NF-κB. Antioxidants govern intracellular redox status and many such molecules can reduce H_2O_2. Peroxiredoxin 4 (PRDX4) is a secretable protein that may exert its protective function against oxidative damage by scavenging reactive oxygen species in the extracellular space (28). Therefore, PRDX4 functions as an immediate regulator of H_2O_2-mediated activation of NF-κB (29). PRDX4 induction by TGase might be coordinated with the activation of negative feedback molecules for NF-κB, such as TRIB3 and TFPI-2. TGase-mediated NF-κB induces nitric oxide (NO) synthesis in microglia (6). There has been a report that NO is a physiological substrate for mammalian peroxidases that catalyze NO at sites of inflammation (30). Thus, toxicity from increased NO might be reversed by increased PRDX4.

4.3. Genes that are down-regulated

The tomoregulin (transmembrane protein with EGF-like and two follistatin-like domains 1, TMEFF1) ectodomain, which contains two follistatin modules and a single epidermal growth factor (EGF)-like domain, sheds during development (31). Interestingly, the proinflammatory cytokines, IL-1β and TNF-α, induce tomoregulin-ectodomain shedding in human glioma cells. It appears that this shedding process is induced via activation of the NF-κB signaling pathway. This suggests that inflammation-induced injury increases with tomoregulin shedding, which may contribute to tissue growth and repair in the central nervous system (32).

NADH dehydrogenase (ubiquinone) 1β subcomplex 6 (NDUFB6) is one of the key genes associated with the hereditary autosomal dominant inclusion body myopathy of Paget disease and frontotemporal dementia (IBMPFD) (33). Energy metabolism is down-regulated because ultrastructural mitochondrial changes and ragged-red fibers are common in patients with IBM (34). We have reported that increased TGase 2 expression is closely associated with inflammatory myopathies including IBM (16). Using the guinea pig myositis model, Gendek *et al.* confirmed that TGase 2 is a potential diagnostic marker for idiopathic inflammatory myopathies (35). Therefore, increased TGase 2 may down-regulate NDUFB6 expression, which results in loss of energy metabolism in IBM.

There also is a loss of NDUFB6 activity in Huntington's disease (HD) (36). Energy metabolism dysfunction is a very well-known characteristic of HD pathogenesis (37). Coincidently, TGase 2 expression is upregulated in the HD brain, which, in part, contributes to the formation of insoluble nuclear inclusions (38). Recently, we reported that the loss of mitochondrial function may be related to the loss of mitochondrial aconitase activity due to TGase 2-mediated crosslinking (39). Although we showed an increase in crosslinkage in the mitochondrial fraction from HD patients, the pathological process is difficult to explain since it requires that TGase 2 enter the mitochondrial matrix without a signal peptide. In the case of TGase 2-mediated down-regulation of NDUFB6 expression, TGase 2 activation may simply reduce energy metabolism at the transcriptional level. Western blotting of proteins from TGase 2-transfected cells with anti-NDUFB6 clearly shows a decrease in NDUFB6 (Figure 2). Therefore the reduced energy metabolism in neurodegenerative diseases associated with increased TGase 2 expression, such as Huntington's (22), Alzheimer's (40), and Parkinson's diseases (41), is likely due to the increase in TGase 2.

4.4. Gene network analysis

The global network of interconnections among genes that show significant differences in TGase 2-over-expressing cells was analyzed with the PathwayAssist Program (Figure 3). The connections among the up-regulated genes, GAL, NPY, and VIP, are highlighted in Figure 3. Although neuropeptides, including NPY and VIP, are effective medicines for various diseases including septic shock and rheumatoid arthritis, they also are involved in the mechanisms of some dermatoses – psoriasis, prurigo, vitiligo, eczema and atopic dermatitis, and various inflammatory skin disorders (18-20). Therefore, increased expression of NPY and VIP may play key roles in both inflammation and anti-inflammation, depending on the type of stress and the type of signal pathway. Further analysis of inter-signaling pathways may reveal the potential roles of these three factors in TGase 2-transfected cells.

Interestingly, inter-connected genes in the network, such as brain-derived neurotrophic factor (BDNF) (42), nerve growth factor beta polypeptide (NGFβ) (42, 43) and leukemia inhibitory factor (LIF) (44), are also related to NF-κB signaling. Pro-inflammatory cytokines, such as interleukin-1β and TNF-α, synergistically stimulate microglial transcription of NGF (43) and LIF (44). NF-κB inhibition reduces IL-6 and LIF transcription stimulated by IL-1α and TNF-α. This suggests that NF-κB mediates signaling between IL-1α and TNF-α receptors and the expression of LIF and IL-6 in endometrial epithelial cells (44). Moreover, an increase in NGF levels is found throughout the brains of Alzheimer's disease patients that present neuroinflammatory pathogenesis. NGF and BDNF themselves do not induce the nuclear translocation of NF-κB. However, co-stimulation of TNF-α and neurotrophic factors such as NGF and BDNF greatly increase the nuclear translocation of NF-κB (42). This suggests that TGase 2-mediated NF-κB activity may be enhanced by neurotrophic factors. Exploring the interrelationships of the pathways for TGase 2 and neurotrophic factors will be of great interest for understanding neurodevelopment and neuroinflammation.

5. SUMMARY

Our dataset clearly shows that the expression of many genes is associated with the regulation of inflammation. This is an intriguing result since TGase 2 expression appears to be increased in inflammatory diseases and TGase 2 is able to activate proinflammatory transcriptional factors such as NF-κB. As expected, the induction of inflammatory molecules often triggers negative feedback molecules such as the inhibitory subunit

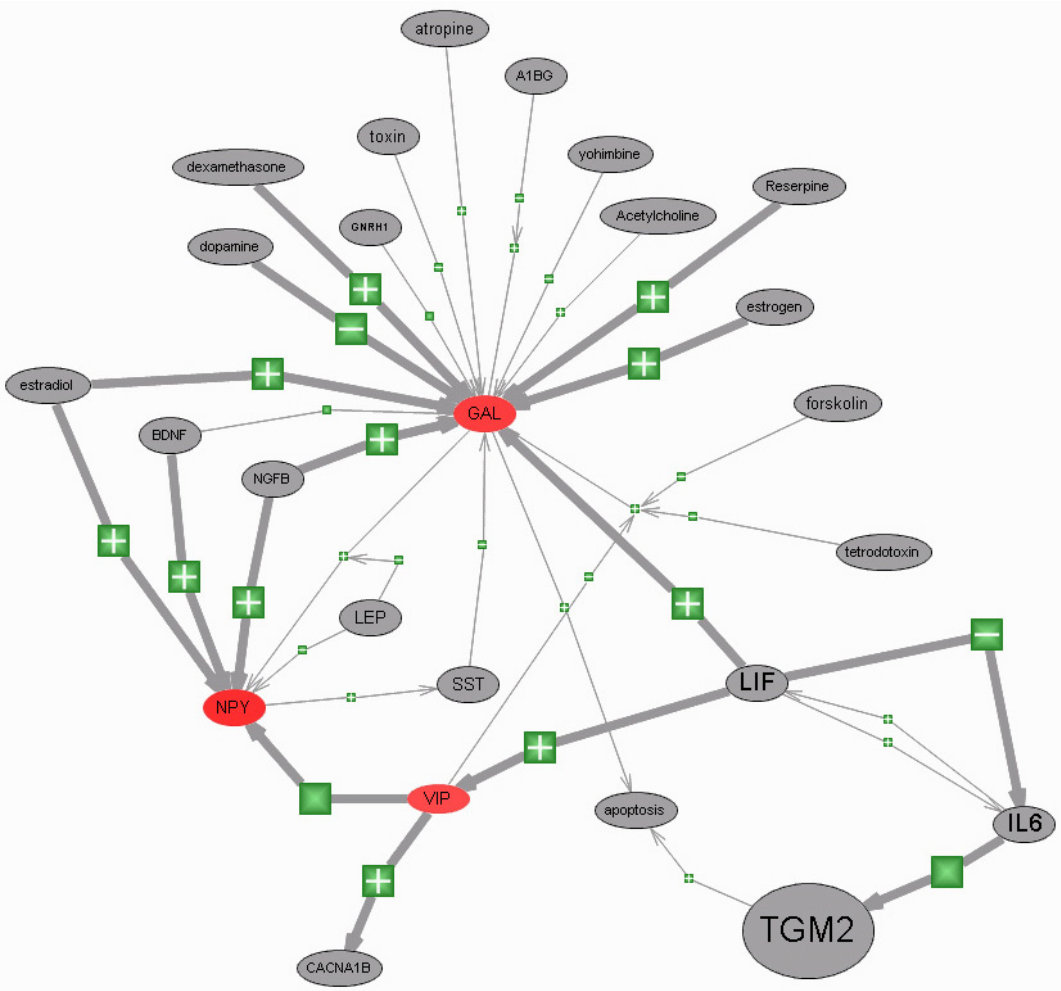

Figure 3. Global network of interconnections among genes with significantly different expression in TGase 2-over-expressing cells. The genes in Figure 1 were used in the analysis. Up-regulated genes in TGase 2-over-expressing cells are indicated in red. Genes in gray color are associated with the regulated genes. Each line and arrow represents functional and/or physical interactions and the direction of regulation as reported in the literature. Green squares refer to the type of regulation: '+', positive; '−', negative; 'blank', unknown. GAL, galanin (neuropeptide hormone); BDNF, brain-derived neurotrophic factor; NGFB, nerve growth factor, beta polypeptide; LEP, lipopolysaccharide binding protein; SST, somatostatin; NPY, neuropeptide Y; VIP, vasoactive intestinal peptide; IL6, interleukin 6; LIF, leukemia inhibitory factor; TGM2, transglutaminase 2.

α of NF-κB (I-κBα) to re-establish homeostasis after the cause of the stress is eliminated. Here we have categorized genes associated with the regulation of inflammation. An increase in TGase 2 expression induces proinflammatory molecules such as peripheral CRH, as well as inflammatory negative-feedback molecules such as TRIB3. TGase 2 plays a key role in modulating responses to inflammatory stress. This suggests that homeostatic control will be disrupted if TGase 2 regulation fails. Deregulation of TGase 2 expression in diseases will be measured in the near future to elucidate the detailed mechanisms.

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Abbreviations: I-κBα, inhibitory subunit α of NF-κB; IKK, I-κB kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; TGase, transglutaminase; TNF-α, tumor necrosis factor-alpha

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