HHS Public Access

Author manuscript

Exp Biol Med (Maywood). Author manuscript; available in PMC 2015 November 10.

Published in final edited form as:

Exp Biol Med (Maywood). 2015 February ; 240(2): 242–251. doi:10.1177/1535370214551688.

Transcriptome profiling of expression changes during neuronal death by RNA-Seq

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Abstract

The molecular mechanisms underlying neuronal death are poorly understood. One of the most widely used models to study neuronal death are cultured cerebellar granule neurons (CGNs) which undergo apoptosis when switched from a medium containing depolarizing levels of potassium (HK) to a medium with low non-depolarizing levels of potassium (LK). Previously, other labs have used DNA microarray analysis to characterize gene expression changes in LK-treated CGNs. However, microarray analysis is only capable of measuring the status of known transcripts, and expression of low-abundance mRNAs is often not detected by the hybridization-based approach. We have used RNA-Sequencing to conduct a more detailed and comprehensive analysis of gene expression changes in CGNs induced to die by LK treatment. RNA-seq investigates the status of both known transcripts as well as exploring new ones and is substantially more sensitive than the microarray approach. We have found that the expression of 4,334 genes is significantly altered in LK treated CGNs with 2,199 being up-regulated while 2,135 are down-regulated. Genes functioning in cell death and survival regulation, cell growth and proliferation and molecular transport were most affected by LK treatment. Further, a large number of genes involved in nervous system development and function were also deregulated. Analysis of signaling pathways that were affected in LK-induced death included but were not limited to mitochondrial dysfunction and oxidative phosphorylation, consistent with a number of studies showing perturbations of these pathways in neurodegenerative disorders. Thus, our study identifies a large number of new genes that are affected during the process of neuronal death. While a majority of these changes may reflect consequences of the induction of neuronal death, many of the genes that we have identified are likely to be critical and potentially novel mediators of neuronal death, including death associated with neurodegenerative disease.

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SUPPLEMENTAL DATA-Table S1–Table S16.

AUTHOR CONTRIBUTIONS

Experiment was conceived and designed by SRD, DS. Experiments performed by DS. Data was analyzed by MSK, DS. Manuscript was written by DS, MSK, SRD.

Keywords

Cerebellar granule neurons; RNA-Seq; Neuronal death; Gene expression; Transcriptome profiling; Potassium withdrawal

INTRODUCTION

During the development of the vertebrate nervous system about half of all the neurons that are produced are eliminated. This large scale death of neurons serves to ensure proper formation of the nervous system by matching neurons to the target that they innervate and to match innervation to target size[1]. While necessary during nervous system development, abnormal loss of neurons is a characterizing feature of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis[2]. Although the molecular events that lead to neuronal death in each of these neurodegenerative diseases is distinct, the downstream apoptotic process through which neurons die in these disorders are believed to share commonalities to each other as well as to developmentally-regulated neuronal death[3]. Definitely, the mechanisms underlying neuronal death will therefore not only help to understand how neurons die during development, but also the mechanisms regulating neuronal death in various degenerative brain disorders.

Cultures of cerebellar granule neurons (CGNs) have been widely used to study the molecular mechanisms underlying neuronal viability. These neurons are kept healthy and alive by the addition of depolarizing levels of potassium[4–7]. A switch of the medium to low, non-depolarizing levels of potassium induces apoptosis. Survival by elevated potassium, or HK, mimics the survival-promoting effect of neuronal activity during normal neurodevelopment[8]. As in other model systems of neuronal apoptosis[9,10], cell death in this model is believed to require new gene because treatment with transcriptional and translational inhibitors can protect neurons from LK-induced death[11]. Other investigators have used DNA microarray analysis with the CGN model to identify transcriptional alterations that are associated with neuronal death[12,13], however, this method is only capable of measuring the status of known transcripts and does not allow for studying changes in global gene expression. Moreover, expression of mRNAs that are expressed at low levels is often not detected by the hybridization-based microarray analysis. To conduct a more detailed and comprehensive analysis of transcriptome alterations during neuronal death, we have used RNA-Seq analysis, a technique that investigates the status of both known transcripts and allows for exploring new ones[14]. Because of low background signal, RNA-Seq can detect expression and alterations of expression of low abundance transcripts[14–17]. Consistent with these advantages, using RNA-Seq we report that the extent of transcriptional changes during neuronal apoptosis is much larger than previously described using microarray technology[12,13]. We have also looked at the signaling network and pathways that are affected during neuronal death as well as upstream regulators that are likely to be involved. We believe that this study identifies molecules that have thus far not been described as regulators of neuronal death, and may play a key role during developmentally-regulated neuronal death or in the pathogenesis of neurodegenerative disease.

MATERIALS AND METHODS

Primary Neuronal Culture, Treatments and viability assay

Cultures enriched in CGNs were prepared from cerebella of 7 day old rat pups and were plated in basal Eagle's minimal medium supplemented with 10% fetal bovine serum, 2 mM L- glutamine, 0.2% gentamycin and 25 mM KCl as described previously[5]. To prevent proliferation of non-neuronal cells, 10μM cytosine arabinoforanoside was added to the culture medium 18–22 h after plating. At 7 days in vitro, CGN cultures were washed and incubated in the serum-free basal Eagle's Minimal medium supplemented with either 25 mM KCl (high potassium, HK) or without KCl (low potassium, LK) for 6 hours, a time, when almost half of the neurons are committed to die and can no longer be rescued. For viability assay, cells were fixed, immunocytochemistry was performed, and viability was quantified by cell morphology using 4′6-diamidino-2-phenylindole hydrochloride (DAPI) staining. Cells with condensed or fragmented nuclei were scored as dead. Unless mentioned otherwise, all viability experiments were performed in duplicate and each experiment was repeated three times. For each experiment, ≥200 cells were counted.

RNA preparation and RT-PCR

Total RNA was isolated from the cultured neurons treated with either HK or LK using Trizol reagent (Life Technologies) according to the manufacturer's guidelines. For reverse transcription, 3 μg of total RNA was used and cDNA was prepared using Superscript cDNA synthesis Kit (Life Technologies). Resulting cDNA was used as a template for PCR to validate the expression data obtained from RNA sequencing analysis. All the Primer pairs were designed using Primer Blast and sequences are mentioned in supplementary table 1.

RNA Sequencing

For sequencing, total RNA was used from either HK or LK treated primary neurons. After an initial quality check on Bioanalyzer (Agilent Technologies), 1μg of total RNA was used and library was constructed using the Trueseq RNA sample preparation kit from Illumina according to the manufacturer's protocol. Triplicate samples of each condition (HK and LK; S1, S2 and S3) were amplified and profile checked for size distribution and peak concentration on Bioanalyzer. Amplified cDNA fragment libraries were sequenced on an Illumina HiSeq2000 at the University of Southwestern Medical Center sequencing facility using default parameters (single end, forward sequencing).

Differential Gene Expression Analysis

Quality assessment of the raw sequencing reads was done using NGS-QC-Toolkit[18]. Sequencing reads with quality score under Phred Score < 20 were discarded. The quality filtered reads were then aligned to rat reference genome RGSC_v3.4 (rn4) using Bowtie2 (v 2.0.6) aligner[19]. Differential gene expression analysis was done using DESeq (v 1.10.1)[20] following the protocols outlined in[21]. Read counts are normalized by taking the median of each gene count across samples and dividing each sample gene count by the relative ratio of library sizes between the calculated median and sample size. Gene counts are scaled to counts per million. Fold change values, raw p-values, and p-values adjusted for multiple

testing using Benjamini-Hochberg are calculated for each gene. Genes were considered significantly differentially expressed if they had a false discovery rate (FDR) of less than 5%.

Significant Gene Ontology (GO) and Pathway Enrichment Analysis

DAVID as well as IPA gene functional annotation and classification tool was used to annotate the list of differentially expressed genes with respective GO terms and GO enrichment analysis was performed for molecular and biological functional categories[22]. A *Rattus norvegicus* specific set of 3797 genes was used as background to calculate the enriched GO functional categories for the differentially expressed genes identified through the RNA-Seq experiment. Functional Gene Ontology groups were selected for significance by using a false discovery rate (FDR) cutoff of 5%.

Significant pathway enrichment analysis was performed using Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, CA, USA). Differentially expressed genes from the RNA expression data are associated with a biological function supported by at least one publication in the Ingenuity Pathways Knowledge Base. Fisher's exact test is then used to calculate the P-value and determine the probability that each biological function is enriched in the dataset due to chance alone. Statistically significant biological pathways were then identified by selection for pathways with Benjamini-Hochberg adjusted p-values < 0.05.

Upstream regulator analysis was done to identify common transcriptional regulators that can explain the observed gene expression data. The analysis was based on using prior knowledge of expected effects between transcriptional regulators and their target genes stored in the Ingenuity Knowledge Base. The analysis examines how many known targets of each transcriptional regulator are present in the RNA expression data and also compares the direction of expression as expected from literature of the relevant transcriptional regulator. Observed gene expression changes can be consistent with the expected direction of expression of which can be in one of two transcriptional regulator activity states: activated or inhibited. Molecules classified as upstream transcriptional regulators include transcription factors, microRNA, kinases and drugs. Functional significance of upstream transcriptional regulators is determined by using a z-score value that measures how well the observed gene expression data matches the literature-derived regulation direction. The absolute value of the z-score determines the likelihood that the regulator predicts the observed expression data was not by chance with positive z-score values indicating regulatory activation and negative z-score values for regulatory inhibition. Fisher's exact test is used in a similar manner as the biological pathway analysis to calculate the p-value and identify statistically significant upstream regulators (p-value<0.05).

RESULTS

RNA-Seq analysis and global gene expression profiles in neurons primed to die

Switching cultured CGNs from HK medium to LK medium induces neuronal apoptosis. Although cell death is not observed until 12 h, the neurons are committed to death between 4 – 6 hours of LK-treatment[23–25]. We have therefore conducted our RNA-Seq analysis using

RNA from HK and LK treated cultures prepared at 6 h after treatment. Three RNA samples from three separate CGN cultures were used. We confirmed by RT-PCR analysis that expression of c-jun, a widely recognized marker of neuronal death, was robustly induced in the three RNA samples before RNA-Seq was conducted (data not shown). The three sets of HK and LK samples were sequenced on one lane of the Illumina HiSeq 2000 platform (Illumina, San Diego, CA). Raw reads were mapped to the reference rat genome (rn4). Around 70% of total reads were successfully mapped to the reference genome. Detailed mapping statistics are listed in Table 1. First, clustering analysis of the expression data shows the samples are grouped according to their HK or LK classification. Correlation matrix of the six samples from the three sets of CGN cultures are given in supplementary data (Fig. S1). Using criteria of FDR adjusted p-value of less than 0.05, a total number of 4334 genes were identified to be differentially expressed in the two conditions (Table 1.2). Out of these differentially expressed genes 2,199 genes were up-regulated and 2,135 genes were down-regulated under LK condition. The 10 most up- and down-regulated genes in LK are listed in Table 2. A complete list of genes that are significantly regulated is provided in supplementary data (Table S2–4). To validate our sequencing data, we performed RT-PCR analysis using RNA from HK and LK-treated CGN cultures for 4 genes which, based on our RNA-Seq results, were up-regulated in LK (*Trim 17, Slc6a7, Btbd17, Stmn4*), and 4 more that were found to be down-regulated (*Npas4, Igfbp3, Tmem 196, Car7)* (Fig.1). These genes were chosen because they are potentially relevant to neuronal death but had not been previously focused as neuronal death-causing molecule. For example, *Slc6a7* (Solute Carrier Family 6 (Neurotransmitter Transporter), Member 7), is a member of GABA neurotransmitter gene family and functions as a Proline transporter. *Stmn4* (Stathmin like 4) exhibits microtubule-destabilizing activity. The IGFBP family (insulinlike growth factor binding proteins) represents a family of conserved proteins that share the ability to bind IGF-I and IGF-II and are known to either inhibit or potentiate the action of IGF[26]. *Npas4* is an activity dependent neuronal transcription factor with a key role in social and cognitive functions relevant to developmental disorders. Although the extent of alteration was not as much as indicated by RNA-Seq, we confirmed using RT-PCR that the expressions of these genes were either up- or down-regulated in accordance with the RNA-Seq data. We further validated our RNA-Seq data by examining whether our list of differentially-expressed genes included those already known in the literature to be deregulated during neuronal death. For example, *c-Jun, Igfbp1, Egr-1, Caspase-3, Caspase-6* and *p73*, which are known to be up-regulated[27–31], showed a significantly increased expression in our RNA seq data (Table S3). On the other hand expression of *Igfbp5*, *c-Fos* and *Npas4*, previously reported to be down-regulated[32,33], was in the list significantly down-regulated genes (Table S4). These results provide confidence that the RNA-Seq analysis was successful in identifying genes that are differentially-expressed in CGNs during LK-induced death.

Functional annotation of the global gene expression profile and pathway analysis

To investigate the physiological processes targeted by the differentially expressed genes in response to LK-treatment, we carried out a GO classification analysis of the differentially expressed genes using IPA. Fig.2 lists the functional categories with significant p-value < 0.01. Based on the GO analysis, a particularly large number of differentially expressed genes are found to be associated with “Cell Death and Survival” (154 genes up-regulated

and 125 genes down-regulated). The second highest group of significant functional categories is related to ion transport and molecular secretion. When looked at the list of up- and down-regulated genes in LK (Table 3), highly significant annotations were found to be “Molecular Transport”, and “Cellular Growth and Proliferation”, where 147 and 132 genes, respectively, were found to be up-regulated in LK. On the other hand functional annotations enriched in down-regulated genes category were “Nucleic Acid Metabolism”, “Small Molecule Biochemistry”, and “Energy Production”. Categories such as “Cell Death and Survival” were more or less equally represented in both up-regulated and down-regulated genes (Table 3). When we looked at the physiological system development and functions, most significant annotations were in “Behavior” and “Nervous System Development and Function” in the up-regulated and down-regulated gene categories, respectively (Table 3). A complete list of all the Gene Ontology term enrichments is provided as supplementary data (Table S5–S7).

In order to investigate pathways involved in neuronal death after potassium deprivation, the differentially expressed gene data set was processed through IPA. Table 4 lists the top five significantly dysregulated pathways under LK condition. The two most significantly dysregulated canonical pathways in the down-regulated genes category include mitochondrial dysfunction and oxidative phosphorylation. Contrary to this, “Protein Kinase A Signaling” was the most perturbed pathway in the up-regulated genes category (Table 4). One interesting finding was that the “Ubiquitination Pathway” also appeared to be significantly dysregulated where 47 out of 232 molecules were down-regulated after LK-treatment (Table 4). We also performed network analysis using IPA to identify significantly enriched networks based on the RNA-Seq data. The 2 most significant networks are shown in Fig.3 for genes that are up-regulated (A and B) and down-regulated (C and D) in LK. The up-regulated genes category was largely associated with “Nervous System Development and Function” and “Energy Production”. On the other hand, networks like “Cell Death and Survival”, “Tumor Morphology”, “Neurological Diseases”, “Molecular Transport” and “Cell Signaling” was associated with the category of genes down-regulated after LK treatment. Detailed lists of all the pathways and networks are given in supplementary data (Table S8–S13).

Identification of upstream regulators involved in gene regulation

To identify the different molecules that mediate the observed transcriptional changes, we performed an upstream regulator analysis with IPA. Factors that can be identified as potential upstream regulators include kinases, nuclear receptors, other transcription factors and some micro RNAs. A list of top 8 upstream molecules in up-and down-regulated category are listed in Table 5. Most notable upstream regulator in the up-regulated gene category was found to be protein kinase A (PKA). Other important molecules include Ephrin receptor B1 (EPHB1) and Upstream Transcription factor 1 (USF1). Under down-regulated genes, the most important upstream molecule was predicted to be Myc and adrenergic receptor beta. Upstream regulator analysis also revealed micro RNA 221 (miR-221) as a regulatory molecule targeting cyclin dependent kinase inhibitor1 (Cdkn1). A complete list of all the upstream regulators is provided in the supplementary data (Table S14–S16).

DISCUSSION

We have used RNA-Seq to obtain a global overview of changes in gene expression that occur during neuronal apoptosis. The paradigm we used, LK-treated CGNs, is one of the most commonly used experimental systems to study neuronal death and significant insight into the mechanism of neuronal death, including death associated with neurodegenerative diseases, has come from this model. We find that a total of 4,334 genes are differentially expressed after LK-treatment, with 2,199 genes being up-regulated and 2,135 genes being down-regulated. These numbers of genes showing up or downregulation during neuronal apoptosis is much more than what others have found earlier[12,13]. For example, Desagher et al., 2005, using microarray could find only 368 genes to be differentially expressed. Out of these, 278 genes were significantly upregulate and 90 genes were significantly down-regulated in apoptotic neurons. Similarly, Chiang et al., 2001, again using a cDNA array analysis could pick only 790 genes showing down regulation. Similarly, using other paradigms of neuronal death such as NGF-deprived sympathetic neurons[34] a relatively smaller number of genes were identified as differentially-expressed. For example, Kristiansen et al. (2011) described using NGF-deprived sympathetic neurons and the same FDR value as we did, that 813 genes were down-regulated by NGF-deprivation compared with 415 that were up-regulated. However, again these results were obtained using microarray technology.

Although both microarray analyses and RNA-Seq can pull up false hits, based on our analysis we are confident that our screen has identified genes that are actually differentially regulated. Before our RNA samples were used for RNA-Seq, we made sure that expression of c-jun was up-regulated in all three sets of RNA from LK-treated samples. Once RNA-Seq data was obtained, we confirmed that genes previously shown to be regulated by LK-treatment were in the list of genes identified in our RNA-Seq experiment. Moreover, we validated by RT-PCR analyses that other genes not previously documented to display altered expression in paradigms of neuronal death but identified in the RNA-Seq analyses we conducted, were in fact differentially expressed. Not surprisingly, alterations were most abundantly observed among genes that function in the regulation of cell death and survival. Genes functioning in the regulation of molecular transport or cell proliferation also showed extensive deregulation. Our analysis also reveals that expression of genes connected to signaling pathways mediating mitochondrial dysfunction and oxidative phosphorylation are also affected. This is consistent with numerous reports showing that key roles for mitochondrial dysfunction and oxidative stress in experimental models neuronal death and in neurodegenerative disorders[35–40].

One very interesting finding from our RNA-seq analysis was dysregulation of the protein ubiquitination pathway following LK-treatment of CGNs. Involvement of ubiquitin/proteasome system (UPS) impairment has been known to be of much significance in neurodegenerative disorders[41]. Even in non-neuronal systems, a large amount of data has been published showing a link between the UPS and cell death (see[42] for review). A large number of molecules involved in apoptosis are known to be regulated by the ubiquitin–proteasome[43–46]. Analysis of the present data also revealed that a number of cell cycle related genes were deregulated following potassium withdrawal. This was not surprising

considering the fact that deregulation of cell cycle control leads to an abortive reentry of mature differentiated post mitotic neurons and results in apoptosis rather than neuronal proliferation[47–51].

Previously, studies related to neuronal death involved in most of the neurodegenerative disorders has largely focused on the roles of protein-coding genes; however, further studies have shown that disease pathogenesis can also be modulated through regulatory mechanisms mediated by small non-coding RNAs (reviewed in[52]). Our study further supports the idea that micro RNAs can also be a therapeutic targets in neuronal death as we found micro RNA as an upstream regulatory molecule affecting changes in the expression of genes downstream. Specifically miR-221 was found to be a regulatory molecule targeting cyclin dependent kinase inhibitor1 (Cdkn1). Selective Cdk inhibitors have earlier been shown to limit neuroinflammation and progressive neurodegeneration after brain injury[53]. In addition to miR-221, miR-183 was also found a regulatory molecule targeting EGR1 (Early Growth Response 1) and IRS1 (Insulin Receptor Substrate 1). EGR1 belongs to the EGR family of proteins and functions as a transcriptional regulator, playing an important role in mitogenesis and differentiation. On the other hand, IRS1 is associated with the AKT signaling pathway.

To summarize, our study identifies a large number of genes previously unconnected to the regulation of neuronal death. While a majority of these changes may reflect consequences of the induction of neuronal death, many of the genes that we have identified are likely to be critical mediators of neuronal death. It is tempting to speculate that these genes may contribute to the pathogenesis of neurodegenerative diseases and could represent molecules that can be targeted in the development of therapies for these fatal brain disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by a grant from the NIH (R01 NS040408) to SRD. We thank Dr. Michael Zhang and Dr. Yunfei Wang from the University of Texas at Dallas for their suggestions and help in the discussion of our data.

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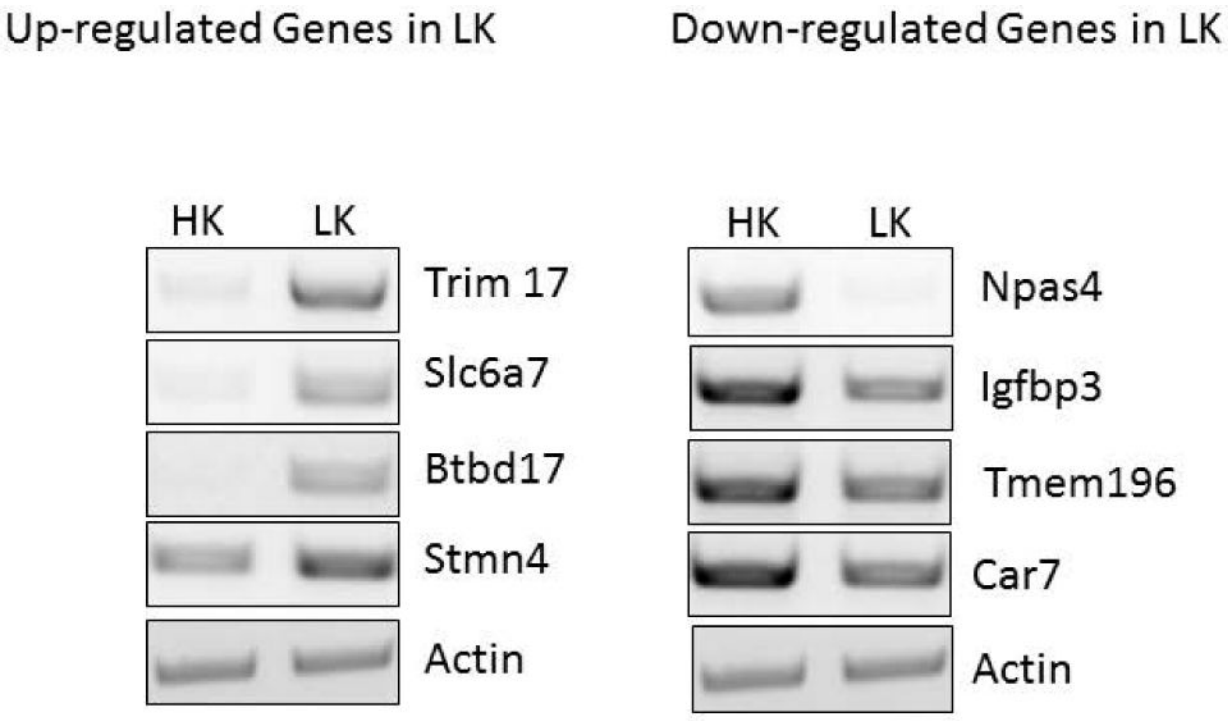

Figure 1.

RT-PCR validation of up-regulated and down-regulated genes after LK treatment. Primary cultures of CGN were treated with HK or LK for 6h on day 7 in vitro and RNA was used for RT-PCR. Actin was used as a normalization control. Primer pairs used to amplify cDNA are given in supplementary data (Table S1).

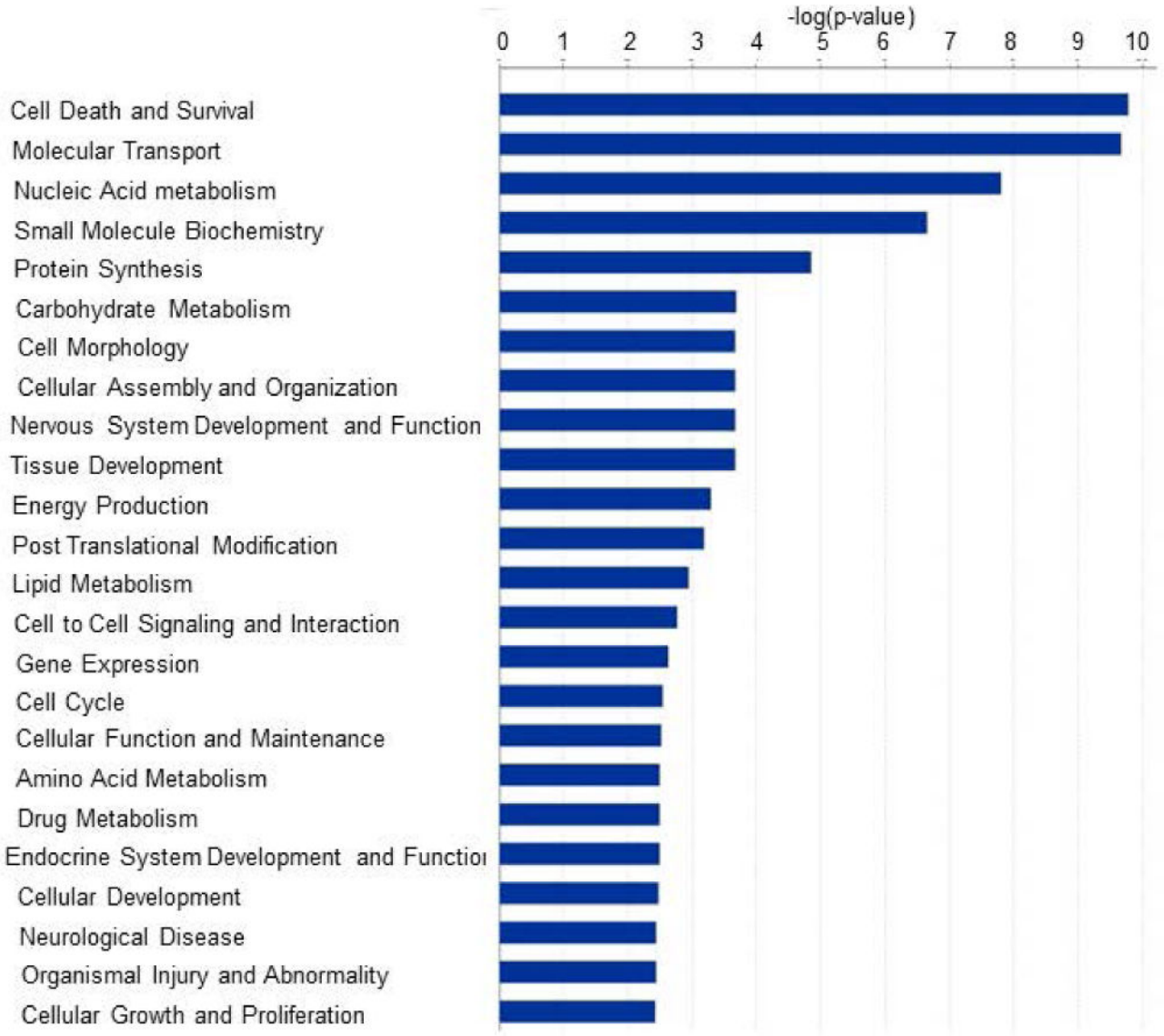

Figure 2.

Graphical representation of biological functions derived from GO enrichment of differentially expressed genes in CGNs treated with HK and LK. DAVID and IPA gene functional annotation and classification tool was used to annotate the list of differentially expressed genes with respective GO terms. Bars represent –log (p-value) in each functional category. Functional GO enrichments were selected for significance by using a false discovery rate FDR cutoff of 5%.

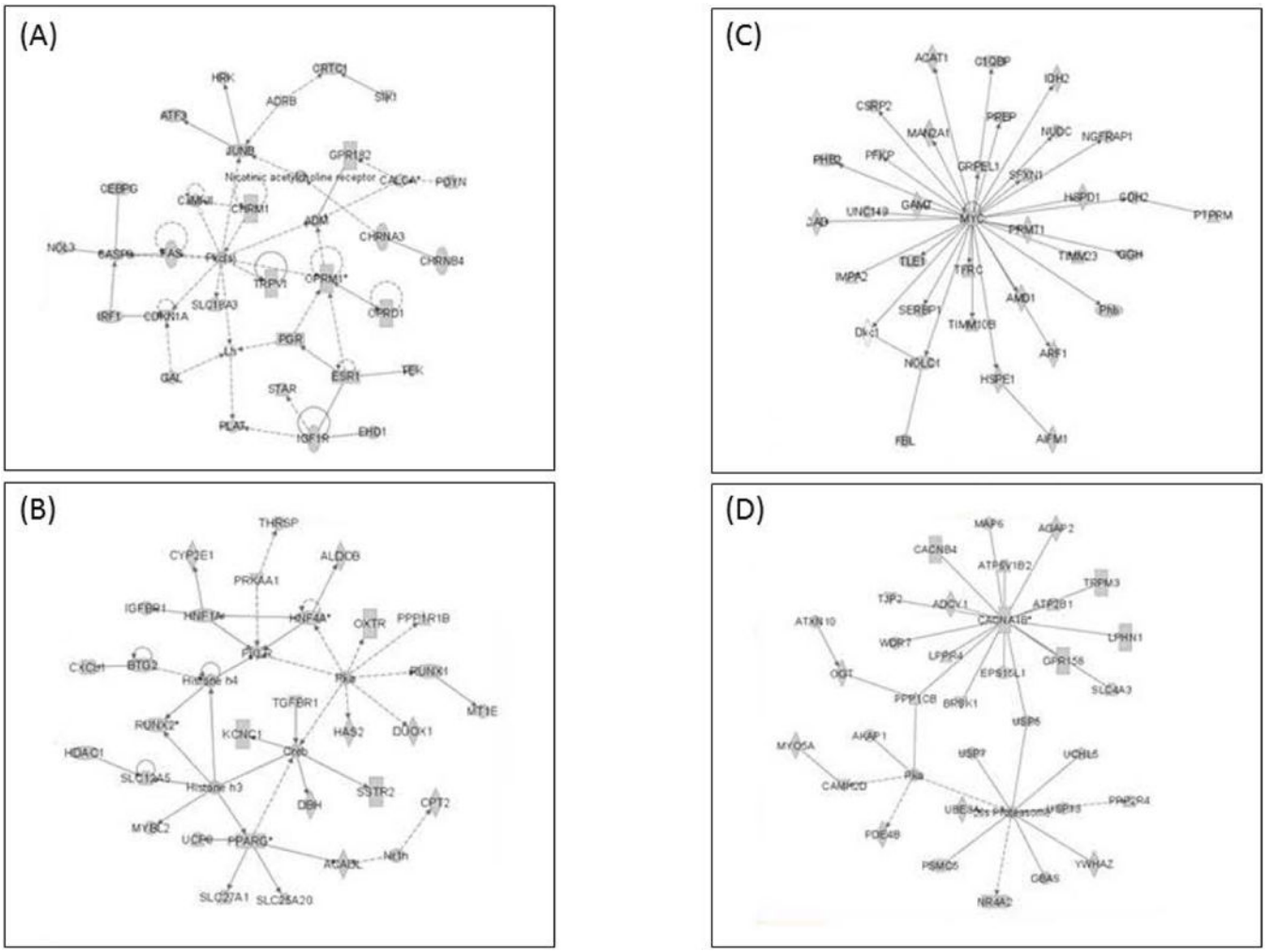

Figure 3.

Top 2 significant networks developed from the differentially expressed up-regulated (A and B) and down-regulated (C and D) genes in primary CGN cultures after LK treatment. (A) Network associated with “Organismal Injury and Abnormalities”, “Nervous System Development and Function” and “Inflammatory Response” (B) Network associated with “Energy Production”, “Lipid Metabolism” and “Small Molecule Biochemistry” (C) Network associated with “Cell Death and Survival”, “Tumor Morphology” and “Energy Production”. (D) Network associated with “Neurological Disease”, “Molecular Transport” and “Cell Signaling”.

Table 1

RNA from three separate sets of CGN cultures treated with HK or LK were used for RNA-Seq. Raw reads were mapped to the reference rat genome (rn4). Table 1.1 shows detailed mapping statistics of the sequencing reads obtained from the three sets of RNA samples in either condition. Differential expression analysis was performed using DEseq. Table 1.2 shows the numbers of genes differentially expressed after LK treatment of CGNs, calculated after applying FDR cutoff of adjusted p-value < 0.05

1.1

High Potasium (+25mM KCI, HK)

	Mapped			**Uniquely mapped**			**% of Total mapped**		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Exon-exon	4757117	4495316	3736405	4313864	4079542	3385667	16.91	16.87	16.01
Exon-intron	226170	199771	201238	215838	190137	192172	0.8	0.75	0.86
Total exon	23908062	22694951	19824076	22084046	20997447	18393002	84.96	85.19	84.97
Total intron	4231747	3944936	3507883	3460041	3206800	2897428	15.04	14.81	15.03
Total gene	28139809	26639887	23331959	25544087	24204247	21290430	100	100	100

Low Potasium (+5mM KCI, LK)

	Mapped			Uniquely mapped			% of Total mapped		
	1	2	3	1	2	3	1	2	3
Exon-exon	4328289	4105310	3853533	3924946	3738989	3493850	16.27	16.11	15.29
Exon-intron	210037	199432	203481	199715	189776	193824	0.79	0.78	0.81
Total exon	22578563	21682543	21465130	20960096	20195084	20021671	84.89	85.08	85.2
Total intron	4018031	3802487	3729792	3184725	3000245	2983964	15.11	14.92	14.8
Total gene	26596594	25485030	25194922	24144821	23195329	23005635	100	100	100

1.2

Differential Expression	**Gene Count**
Up-regulated	2199
Down-regulated	2135
Total	4334

Table 2

RNA from three separate sets of CGN cultures treated with HK or LK were used for RNA-Seq. List of top 10 (A) up-regulated genes and (B) down-regulated genes after LK treatment. Fold change values, raw p-values and p-values adjusted for multiple testing were calculated using Benjamini-Hochberg test. Genes were considered significantly differentially expressed if they had a false discovery rate of less than 5%.

A) Up-regulated Genes in LK			B) Down-regulated Genes in LK		
Gene	**Fold Change**	**FDR**	**Gene**	**Fold Change**	**FDR**
Klrd1	82.48	1.94E-07	Npas4	50.83	5.02E-04
Slcl7a1	79.85	2.33E-07	Pthlh	27.06	3.47E-74
Ccin	74.27	3.21E-06	Txlnb	19.86	3.87E-07
Calhm1	71.54	1.92E-07	Bag2	15.25	1.37E-90
Sohlh1	50.58	3.44E-04	Car7	14.61	7.46E-14
Stra6	47.56	1.14E-07	Cacng6	13.82	7.95E-29
Slcl7a3	46.26	4.76E-54	Cytip	13.25	5.32E-29
Dusp9	41.64	6.65E-08	Igfbp3	13.20	7.25E-35
Kcnj15	38.81	7.71E-04	Irx3	12.43	4.21E-16
Gpr113	36.52	5.43E-38	RGD1304931	11.61	1.04E-69

Exp Biol Med (Maywood). Author manuscript; available in PMC 2015 November 10.

Table 3

List of the top 5 functional annotations derived from GO enrichment analysis of differentially expressed up-regulated and down-regulated genes in primary CGN cultures after LK treatment. GO enrichments were classified into two categories i) Molecular and Cellular Function, ii) Physiological System development and function. Table also shows the number of molecules associated with each category.

Up-regulated in LK		
Molecular and Cellular Functions	**p- value**	**# molecules**
Molecular Transport	7.52E-09 – 4.87E-02	147
Cellular growth and Proliferation	2.04E-07 – 3.86E-02	132
Cell death and Survival	4.28E-07 – 4.64E-02	154
Gene Expression	4.25E-05 – 3.49E-02	83
Amino Acid Metabolism	1.24E-04 – 4.20E-02	29
Physiological system Development and Function		
Behavior	171E-05 – 4.20E-02	45
Endocrine System Development and Function	1.24E-04 – 2.62E-02	22
Cardiovascular System Development and Function	1.32E-03 – 3.86E-02	34
Organismal Development	1.32E-03 – 4.41E-02	45
Tissue Morphology	1.32E-03 – 3.86E-02	13
Down-regulated in LK		
Molecular and Cellular Functions		
Nucleic Acid Metabolism	2.97E-09 – 1.27E-02	55
Small Molecule Biochemistry	2.97E-09 – 4.44E-02	91
Energy Production	2.96E-07 – 1.27E-02	20
Cell Death and Survival	1.40E-05 – 4.76E-02	125
Cellular Function and Maintenance	5.01E-05 – 3.80E-02	95
Physiological system Development and Function		
Nervous System Development and Function	2.56E-03 – 4.25E-02	87
Tissue Development	2.56E-03 – 4.25E-02	67
Endocrine System Development and Function	5.25E-03 – 5.25E-03	3
Embryonic Development	6.25E-03 – 3.54E-02	23
Organ Morphology	1.20E-02 – 1.20E-02	3

Table 4

List of the top 5 canonical pathways derived from IPA analysis of differentially expressed up- and down-regulated genes in primary CGN cultures after LK treatment. Fisher's exact test was used to calculate the P-value and determine the probability that each biological function is enriched in the dataset due to chance alone. Statistically significant biological pathways were then identified by selection for pathways with Benjamini-Hochberg adjusted p-values < 0.05.

Top Canonical Pathways	p- value	Ratio
Mitochondrial Dysfunction	1.30E-11	74/156
Oxidative Phosphorylation	474E-11	53/100
TCA Cycle II (Eukaryotic)	2.59E-05	14/21
Protein Kinase A Signaling	3.86E-05	113/354
Dopamine-DARP32 Feedback in cAMP signalling	5.27E-05	57/156
Pathways Formed under Up-regulated Genes inLK		
Protein Kinase A Signaling	5.40E-06	70/354
Tweak signaling	5.85E-06	14/35
Role of PKR in interferon induction and Antiviral Response	1.81E-05	15/43
Axonal guidance signaling	2.25E-05	74/406
Hepatic cholestasis	7.80E-05	31/133
Pathways formed under Down-regulated Genes in LK		
Mitochondrial dysfunction	5.18E-25	68/156
Oxidative Phosphorylation	6.11E-25	53/100
TCA Cycle II (Eukaryotic)	2.77E-09	14/21
Protein Ubiquitination Pathway	3.60E-05	47/232
Glutaryl-CoA Degradation	5.02E-05	7/11.

Table 5

List of the top 8 upstream regulator molecules derived from IPA analysis of differentially expressed up- and down-regulated genes in primary CGN cultures after LK treatment. Functional significance of upstream transcriptional regulators is determined by using a Z-score value. Fisher's exact test was used to calculate the p-value and identify statistically significant upstream regulators.

Upstream regulators	p-value
Up-regulated in LK	
Pka	9.54E-07
EPHB1	1.31E-04
USF1	7.51E-04
SP3	7.51E-04
HNF1A	7.51E-04
P38 MAPK	9.68E-04
PRKAA2	1.46E-03
AGTR2	2.05E-03
Down-regulated in LK	
Myc	1.28E-04
ADRB	1.10E-02
KAT5	3.26E-02
Hsp90	3.33E-02
NRF1	3.33E-02
PPP1CC	3.33E-02
Setd3	3.33E-02
FSHR	4.91E-02