

Transcriptomic Profiling of Mice Primary Cortical Neurons in Response to Medium Change

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Abstract

The first *in vitro* system for the culture of primary neurons was developed in 1977. Since then, the culture systems for hippocampal and cortical neurons, as well as neuron cell lines, have improved dramatically. Primary neuronal cultures provide a defined environment where neurons can be studied in isolation from other cell types and are a powerful tool for studying cellular and molecular mechanisms of neuronal development, aging, and death. However, the changes that occur in neurons during culture in relation to cell differentiation and medium composition have not been thoroughly characterized. To study the effect of medium change in primary neurons in culture, we performed a transcriptomic analysis using whole mouse genome microarrays, for neurons between 7 and 10 days *in vitro* after medium change. We show that even though neurons in culture present an expression profile pointing to differentiation with time in culture, medium change induces a transient and partial de-differentiation.

Keywords: Cortical neuron; Neurobasal; Transcriptomic analysis; Medium change

Introduction

Toxic and metabolic insults such as oxygen and glucose deprivation generated by trauma, hypoxia-ischemia, and seizures are extremely harmful for the central nervous system (CNS). Besides this, aging makes the CNS vulnerable to neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Huntington's disease. In these settings, hippocampal and neocortical neurons degeneration is observed. To understand the mechanisms of neuronal dysfunction and degeneration, animal and cell culture model systems are widely used. While animal models can be used to study many aspects of neuronal development and degeneration [1], the molecular, biochemical, and structural determinations are extremely complex or not possible at all, due to the inherent complexity of the nervous system tissue. To better approach these studies *in vitro* systems provide homogenous populations of cells undergoing synchronous and uniform changes.

The first *in vitro* system for the culture of primary neurons was developed by Banker and Cowan [2-4]. It consisted of cultures of hippocampal neurons isolated from 18 day old rat fetuses co-cultured with tissue or hippocampal explants and survived only 2 weeks. Since then, the culture systems for hippocampal and cortical neurons, as well as neuron cell lines, have improved dramatically [5-11] and are widely used in diverse studies.

The development of serum-free media for cell culture [12] represented a major advantage for the culture of neuronal cell lines. Following this, a serum free medium was developed that allowed the culture of hippocampal and cortical neurons [9]. This medium is nowadays widely used for the culture of primary neurons and neuronal cell lines, and is composed of neurobasal medium (NB) supplemented with B-27 [6,8,9]. It was derived from Dulbecco's Modified Eagle's Medium, and was optimized for cell survival and neurite outgrowth of hippocampal neurons with an almost complete absence of glial cells [6,8,9]. The same was also observed in cultures of primary cortical neurons [5]. Primary neuronal cultures provide a defined environment where neurons can be studied in isolation from other cell types that can modify neuronal responses directly or indirectly. The B-27 supplemented NB is useful for studies of neurotoxicology, electrophysiology, gene expression, pharmacology, development, and growth factor effects [9,13]. Thus, primary cultures of cortical neurons are a powerful tool for studying cellular and molecular mechanisms

of neuronal development, aging, and death [14]. However the changes that occur in neurons during culture in relation to cell differentiation and medium composition have not been thoroughly characterized, and this could have a large impact specially in the field of neurotoxicology where many times chemicals are dissolved previously in fresh medium and then added to the cell cultures [15]. Using primary neuron cultures in B-27 supplemented NB medium, and as an approach to determine the effect of time in culture and variations in medium composition due to cell metabolism on primary neurons in culture, we performed a transcriptomic analysis using whole mouse genome microarrays, for primary neurons between 7 and 10 days *in vitro* (DIV) and after medium change.

Material and Methods

Primary cultures of cortical neurons

Swiss mice were used to obtain primary cultures of cortical neurons. Primary cortical neurons were obtained from embryonic day 16-18 Swiss mice. All protocols were approved by the University of Santiago de Compostela Institutional Animal Care and Use Committee. Briefly, cerebral cortices were removed and dissociated by mild trypsinization, followed by mechanical trituration in a DNase solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in Neurobasal medium supplemented with 1% B-27 supplement (Invitrogen), 5 mM L-glutamine, and 1% penicillin/streptomycin. The cell suspension was seeded in 12 well plates pre-coated with poly-D-lysine, and the cell culture was kept in a 95% air and 5% CO₂ atmosphere at 37°C. Culture medium was replaced every 3-4 days. Neurons were employed between 7 and 10 days in culture.

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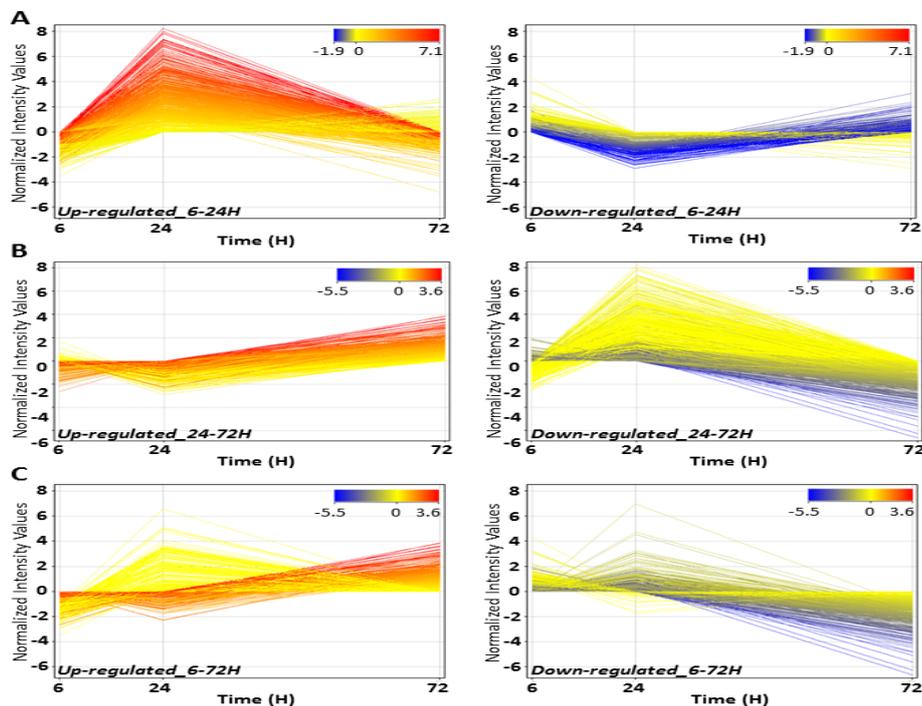


Figure 1: Profile plot of significantly up- and down-regulated genes after medium change. Lines represent average expression profiles for significantly altered transcripts plotted against time. Changes in transcription between different times were identified by anova with $p < 0.05$ using a fold change cut-off ≥ 2 .

RNA extraction, purification, quantification and integrity determination

The Aurum™ Total RNA Mini Kit (BIO-RAD) was used to obtain RNA for microarray assays, following the manufacturer instructions. RNA concentration was determined with a NanoDrop 2000 (THERMO SCIENTIFIC) and integrity was confirmed with a bioanalyzer 2100 (AGILENT) using the kit RNA 6000 nano reagents (AGILENT).

Microarray assay and analysis

To obtain fluorescently labeled RNA for microarray hybridization we used the Low RNA Input Linear Amp Kit (Agilent Technologies) and the Quick-Amp Labeling Kit (Agilent Technologies) and followed the manufacturer instructions. Briefly, Cyanine-3 (Cy3) labeled cRNA was prepared from 1 μg RNA using the Quick-Amp Kit (Agilent Technologies), followed by RNeasy column purification (QIAGEN). Dye incorporation and cRNA yield were checked with a Nanodrop (THERMO SCIENTIFIC) spectrophotometer. Cy3-labelled cRNA (1.5 μg ; specific activity >9.0 pmol Cy3/ μg cRNA) was fragmented at 60°C for 30 minutes in a reaction volume of 25 μl containing 10 X blocking agent (Agilent Technologies), nuclease-free water, and 25 X fragmentation buffer (Agilent Technologies). On completion of the fragmentation reaction, 25 μl of 2 X GE hybridization buffer (HI-RPM0) (Agilent Technologies) was added to the fragmentation mixture which was hybridized to 8X60K SurePrint G3 Agilent Whole Mouse Genome Oligo Microarrays (G4852A) containing 39430 features, for 17 hours at 65°C in a rotating Agilent hybridization oven. After hybridization, microarrays were washed for 1 minute at room temperature with GE Wash Buffer 1 and 1 minute at 37°C GE Wash Buffer 2 (Agilent Technologies). After washing, the microarrays were scanned with a MS200 scanner (ROCHE) using one color scan setting (scan resolution 2 μm). Each slide, containing 8 arrays, was scanned using a single image mode without auto gain and with 100% gain for acquisition. The scanned images were extracted with Feature Extraction Software

11.5.1.1 (Agilent Technologies) using default parameters (protocol GE1-QCMT_11.5 and Grid: 028005_D_F20131202).

After extraction, differential gene expression as a consequence of time in culture and response to medium change was determined using GeneSpring software (Agilent Technologies). Gene lists were analyzed for differentially expressed (DE) genes using oneway ANOVA set at $p < 0.05$, with a fold change cutoff = 2. Data mining for significant altered pathways and ontological categories at the biological process level 5, were performed with the DAVID bioinformatics database [16,17].

Results

After 7 days *in vitro* (DIV) the medium was changed and neuron's RNA was extracted after 6, 24, and 72 hours. Microarray analysis of gene expression in primary neurons showed that between the 6 and 24 hours after medium change 2001 genes appeared up-regulated while another 505 genes were down-regulated (Figure 1A). Following these changes in gene expression, analysis of the changes observed between 24 hours and 72 hours post-medium change indicated that 1325 genes were up-regulated between these two time points, while another 2579 were repressed (Figure 1B). Finally when comparing the gene expression profiles between 6 and 72 hours, 899 and 893 genes appeared induced and repressed respectively (Figure 1C).

Analysis of the gene expression profile in relation to time in culture was then performed. A Venn diagram (Figure 2A) for up regulated genes in the three times assayed, showed that only 33 genes increased their expression starting at 6 hours and progressively increasing expression up to 72 hours (Figure 2B). Gene ontology analysis pointed that these genes were mainly involved in biological processes related to the immune response (Figure 2C and Supplementary Table 1). Another 402 genes were induced between the 6 and 72 hours after medium change with no change observed after 24 hours (Figure 2D). The majority of these genes could not be assigned to significant biological

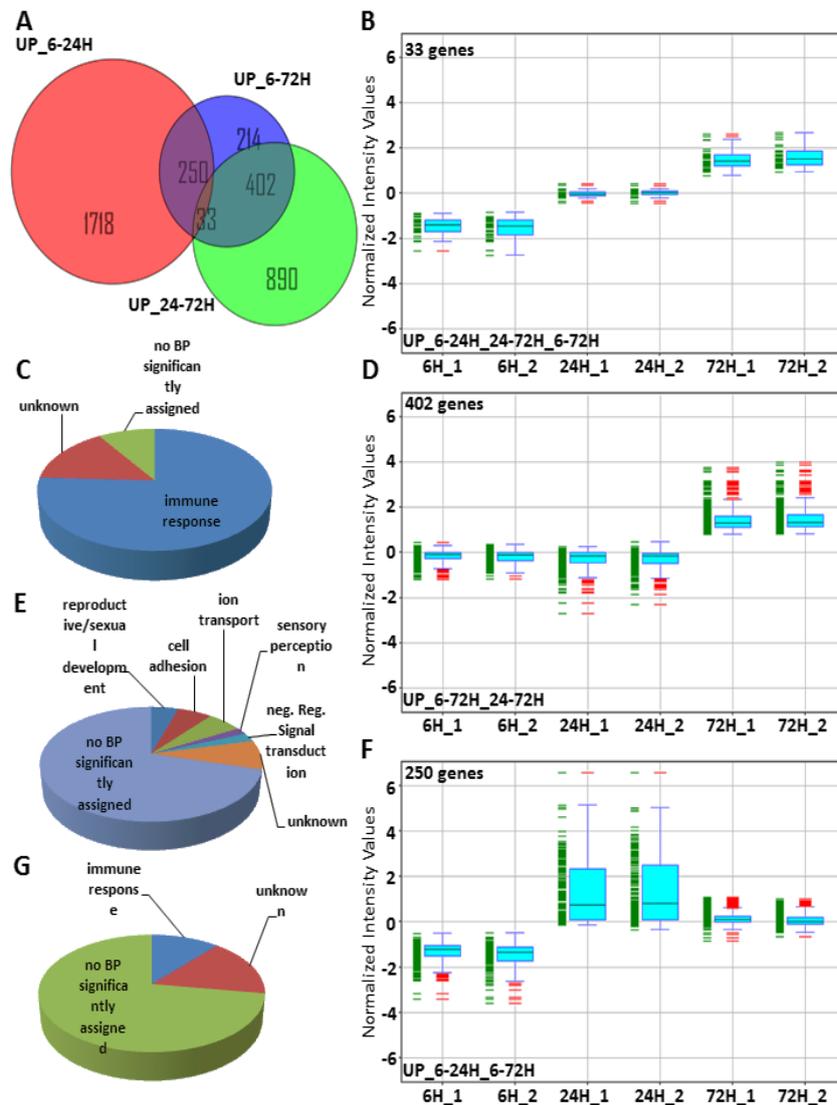


Figure 2: (A) Venn diagram for genes up-regulated between 6-24 hours, 24-72 hours, and 6-72 hours. (B) Box whisker plot of transcripts which progressively increased between 6 and 72 hours. (C) Biological processes significantly up-regulated between 6 and 72 hours identified using the DAVID database (Supplementary Table 1 for detailed information). (D) Box whisker plot of genes with increased expression between 24 and 72 hours after medium change. (E) Biological processes significantly up-regulated between 24 and 72 hours identified using the DAVID database (See supplementary Table 1 for detailed information). (F) Box whisker plot of genes with increased expression between 6 and 24 that remain increased up to 72 hours after medium change. (G) Biological processes significantly up-regulated between 24 and 6 hours that remain increased after 72 hours identified using the DAVID database (Supplementary Table 1 for detailed information).

processes after gene ontology analysis. The remaining genes are involved in development, ion transport, adhesion, sensory perception, and regulation of signal transduction (Figure 2E). Finally, similarly to the previous case, the 250 genes with up-regulated expression between 24 and 72 hours, remained in this state after 72 hours (Figure 2F) and could not be assigned to any biological process. Those assigned are involved in the immune response (Figure 2G). Venn diagram was also performed for repressed genes (Figure 3A). This showed that the expression of only 8 genes progressively decreased between 6,24 and 72 hours (Figure 3B). No significant biological process involving these genes was identified after ontology analysis. Genes repressed during the first 24 hours after medium change, which remained in this state until 72 hours, also produced no significant biological process after ontology analysis (Figure 3C). The repression observed after 24 hours of medium change involved much more genes (Figure 3D), which are involved in biosynthesis/metabolism, development, transcription regulation,

regulation of cell proliferation, response to unfolded protein and neuron differentiation (Figure 3E).

To analyze how medium change transiently affected gene expression at 24 hours that reversed with time in culture at 72 hours, a clustering analysis was performed. After 72 hours in culture, most of the DE genes in the first 24 hours after medium change reverted their expression profile (Figure 4). Expression analysis showed that 1596 genes were induced in the first 24 hours after medium change but returned to their initial state at 72 hours (Figure 5A). Another 345 genes appeared repressed after 24 hours of medium change, increasing their expression between 24 and 72 hours (Figure 5B). Ontology analysis showed that transiently induced genes are involved in positive regulation of cell proliferation, immune response, biosynthesis/metabolism, and positive regulation of transcription. Transiently repressed genes are involved in the RHO/RAS signal transduction, and ion transport. Among these, several are important mediators/regulators of neuron function and development (Table 1).

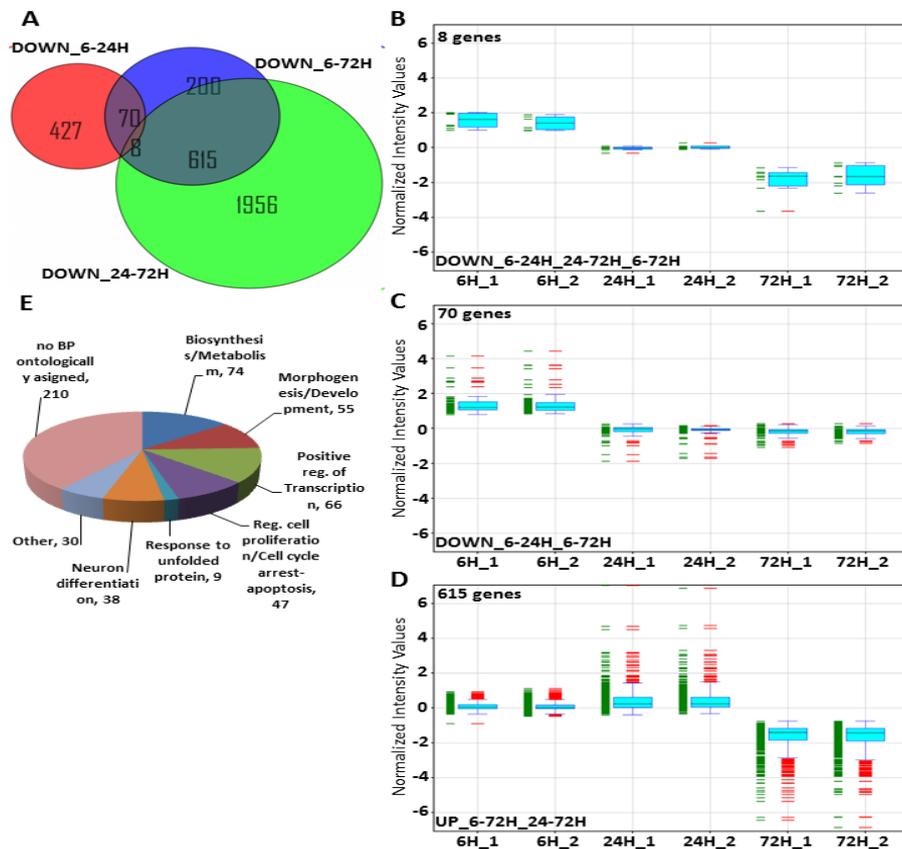


Figure 3: (A) Venn diagram for genes down-regulated between 6-24 hours, 24-72 hours, and 6-72 hours. (B) Box whisker plot of transcripts which progressively decreased expression between 6 and 72 hours. (C) Box whisker plot of genes with decreased expression between 6 and 24 hours that remain repressed up to 72 hours after medium change. (D) Box whisker plot of genes with decreased expression between 24 and 72 hours after medium change. (E) Biological processes significantly down-regulated between 24 and 72 hours identified using the DAVID bioinformatic database (Supplementary Table 2 for detailed information).

Discussion

The results presented in this work show that medium renewal induced an important change in gene expression in primary neurons, which are incubated for several days in culture to achieve cell differentiation. In the first 24 hours after medium change, the repression and induction of hundreds of genes is observed, many of which recover to the initial state after 72 hours. This implies that gene expression alteration is a direct consequence of medium change and not of the cell differentiation that is in progress. Among the 33 genes that progressively increased their expression since medium change and up to 72 hours, are several involved in the immune response (CD38, PF4, FCGR2B, TGM2, C1QA, C1QB, C1QC). Of note is the progressive increase in the expression of C1QA, C1QB, C1QC, which code for the complement protein C1Q protein, formed by three subunits (C1Q α , C1Q β , and C1Q γ) [18]. This protein, which is involved in synapse elimination and is a critical mediator of synaptic refinement and plasticity, has been shown to increase in aged mouse brains [19]. The increase of this protein reported in this work, evidence that primary neurons in culture, in the conditions described in this work, gradually differentiate with incubation time resembling normal neurons ageing. This protein is also considered neuroprotective since it increases neuronal survival and neurite outgrowth in the presence of β -amyloid induced neurotoxicity [20], and also triggers a gene expression program that enhances neurite outgrowth limiting the neuronal stress *in vitro* [21].

Several ionic channels, involved in specific neuronal functions,

showed an increased expression after 10 DIVS when compared to the expression observed after 8 and 7 DIVS. This is probably a consequence of neurons differentiation and is not affected by medium change, since no effect was observed after 6 and 24 hours of medium renewal.

On the other hand, clustering analysis showed that the expression of several genes was significantly altered 24 hours after medium change (8 DIVS) but reverted this profile 48 hours later (10 DIVS). Transiently down-regulated genes were identified to be involved in ion transport, transcription regulation, cell adhesion, and differentiation. Among ion channels affected were the potassium voltage-gated channels KCNC1 (Kv3.1), KCNG2 (Kv6.2), and KCNA4 (Kv1.4). The Kv3.1 channel subtype, that is widely distributed in the central nervous system playing a pivotal role in high-frequency repetitive firing of mammalian neurons [22,23], has been shown to be involved in neuronal differentiation [24], while the Kv1.4 channel subtype has been shown to be a component of the rapidly activating and rapidly inactivating voltage-gated A-type K⁺ current, IA, which acts regulating intrinsic excitability of cortical neurons [25]. Another channel subtype transiently down-regulated was SCN8A, which encodes sodium channel Na_v1.6, one of the major voltage-gated sodium channels responsible for the generation and propagation of action potentials in mammalian neurons. It is present in the dendrites and soma of most neurons in the central and peripheral nervous systems [26,27]. In glutamatergic neurons this channel is strongly associated to the axon initial segment [28-31]. Besides the previously described channels, chloride channels and acid sensing channels also appeared down-regulated. These changes point to de-

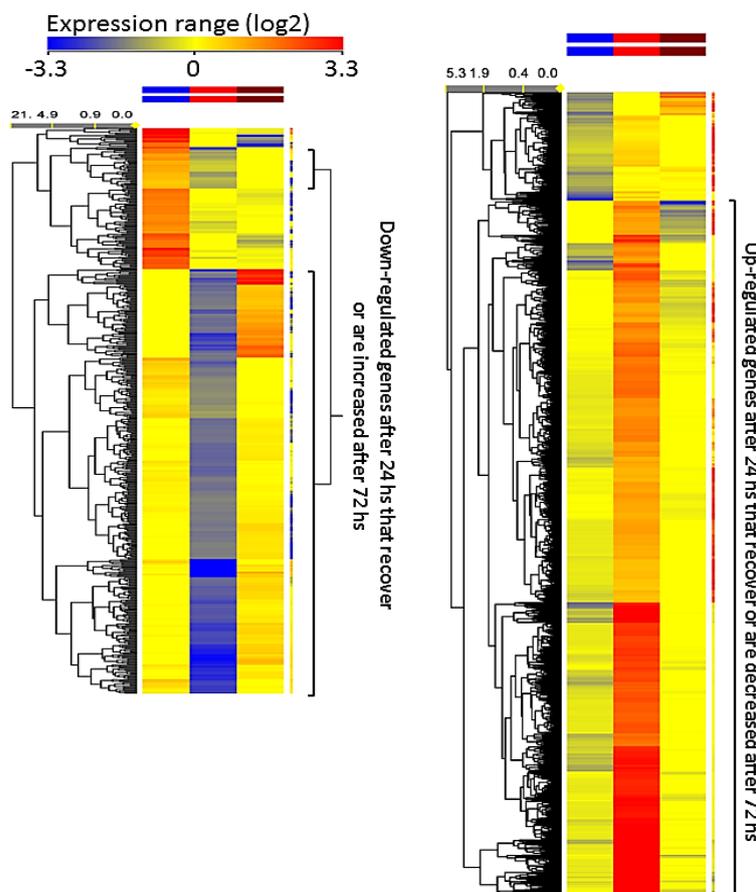


Figure 4: Heat map obtained after hierarchical clustering of genes up- or down-regulated between 6 and 24 hours after medium change. Clusters of genes that are increased or decreased after 24 hours that revert the respective state after 72 hours are identified.

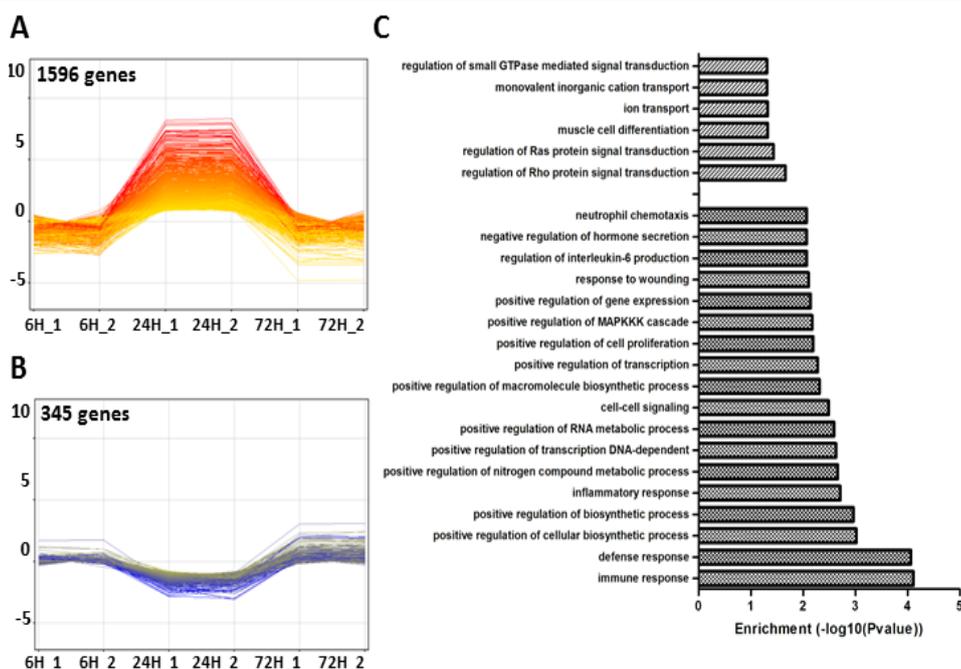


Figure 5: (A) Gene expression profile of induced genes after 24 hours of medium change that recover the expression the expression level observed after 6 hours. (B) Gene expression profile of repressed genes after 24 hours of medium change that recover the expression the expression level observed after 6 hours. (C) Biological processes significantly up- and down-regulated for identified genes, identified using the DAVID database (Supplementary Table 2 for detailed information).

GenBank accession	Gene symbol ¹	Gene Name ²	Function ³
Ion Transport			
NM_001112739	KCNC1	Potassium Voltage-Gated Channel, Shaw-Related Subfamily, Member 1	Mediates the voltage-dependent potassium ion permeability of excitable membranes
NM_001190373	KCNQ2	Potassium Voltage-Gated Channel, Subfamily G, Member 2	Potassium channel subunit. Modulates channel activity by shifting the threshold and the half-maximal activation to more negative values
NM_009899	CLCA1	Chloride Channel Accessory 1	May be involved in mediating calcium-activated chloride conductance
NM_030601	CLCA2	Chloride Channel Accessory 2	Plays a role in modulating chloride current across the plasma membrane in a calcium-dependent manner, and cell adhesion
NM_007384	ASIC2	Acid-Sensing (Proton-Gated) Ion Channel 2	Cation channel with high affinity for sodium, which is gated by extracellular protons and inhibited by the diuretic amiloride.
NM_026214	KCTD4	Potassium Channel Tetramerization Domain Containing 4	Paralog of this gene is KCTD1
NM_021275	KCNA4	Potassium Voltage-Gated Channel, Member 4	Mediates the voltage-dependent potassium ion permeability of excitable membranes
NM_011773	SLC30A3	Solute Carrier Family 30 (Zinc Transporter), Member 3	Involved in accumulation of zinc in synaptic vesicles
NM_001077499	SCN8A	Sodium Channel, Voltage Gated, Type VIII, Alpha Subunit	Mediates the voltage-dependent sodium ion permeability of excitable membranes
AK085398	SLC16A2	Solute Carrier Family 16, Member 2 (Thyroid Hormone Transporter)	Very active and specific thyroid hormone transporter
NM_001012434	KCTD14	Potassium Channel Tetramerization Domain Containing 14	Paralog of this gene is KCTD1
RHO/RAS Protein Signal Transduction			
NM_013710	FGD2	FYVE, RhoGEF And PH Domain Containing 2	Activates CDC42, a member of the Ras-like family of Rho- and Rac proteins, by exchanging bound GDP for free GTP
NM_177566	ARHGEF15	Rho Guanine Nucleotide Exchange Factor (GEF) 15	Specific GEF for RhoA activation. Negatively regulates excitatory synapse development by suppressing the synapse-promoting activity of EPHB2
NM_012026	ARHGEF28	Rho Guanine Nucleotide Exchange Factor (GEF) 28	Functions in axonal branching, synapse formation and dendritic morphogenesis
NM_153804	PLEKHG3	Pleckstrin Homology Domain Containing, Family G	An important paralog of this gene is ARHGEF6
NM_018775	TBC1D8	TBC1 Domain Family, Member 8	May act as a GTPase-activating protein for Rab family proteins
NM_139206	ARAP3	ArfGAP With RhoGAP Domain, Ankyrin Repeat And PH Domain 3	Phosphatidylinositol 3,4,5-trisphosphate-dependent GTPase-activating protein that modulates actin cytoskeleton remodeling by regulating ARF and RHO family members
Cell Differentiation/Morphogenesis			
NM_001126047	SEMA4C	Sema Domain, Immunoglobulin Domain (Ig), Transmembrane Domain And Short Cytoplasmic Domain, (Semaphorin) 4C	Cell surface receptor for PLXNB2 that plays an important role in cell-cell signaling. Required for normal brain development, axon guidance and cell migration
NM_007601	CAPN3	Calpain 3, (P94)	Calcium-regulated non-lysosomal thiol-protease
NM_009523	WNT4	Wingless-Type MMTV Integration Site Family, Member 4	Ligand for members of the frizzled family of seven transmembrane receptors
NM_023047	DPYSL5	Dihydropyrimidinase-Like 5	May have a function in neuronal differentiation and/or axon growth
NM_011912	VAX2	Ventral Anterior Homeobox 2	Transcription factor that may function in dorsoventral specification of the forebrain
NM_019626	CBLN1	Cerebellin 1 Precursor	Required for synapse integrity and synaptic plasticity
Negative Regulation of Transcription			
NM_008718	NPAS1	Neuronal PAS Domain Protein 1	May play a role in late central nervous system development by modulating EPO expression in response to cellular oxygen level
NM_001168502	ZFP57	ZFP57 Zinc Finger Protein	Acts by controlling DNA methylation during the earliest multicellular stages of development at multiple imprinting control regions
NM_021455	MLXIPL	MLX Interacting Protein-Like	Transcriptional repressor. Binds to the canonical and non-canonical E box sequences 5'-CACGTG-3'
NM_007804	CUX2	Cut-Like Homeobox 2	May be a transcription factor involved in neural specification.
NM_001204276	HDAC7	Histone Deacetylase 7	Responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4)
¹ Official gene symbol ² Official gene name ³ Source: GeneBank			

Table 1: Significantly down-regulated genes after 24 hs of medium change (8 DIVs).

differentiation of neurons. This notion is reinforced by the depression of the nucleotide exchange factor (GEFs) that activate the decrease in the expression of the small GTPases RHO/RAS. These GTPases are involved in neuronal development, through the regulation of actin and microtubule cytoskeleton [32]. We observed down-regulation of the GEFs ARHGEF15 (Ephexin5), which encodes a nucleotide exchange factor that negatively regulates synapse development limiting the number of excitatory synapses that neurons receive and, therefore, controlling synaptogenesis [33], ARHGEF28 (RGNEF), recently associated with the adult onset of amyotrophic lateral sclerosis [34], FGD2, an activator of Cdc42 (Rho GTPases family member), and of ARAP3, which inhibits RhoA activity leading to neurite outgrowth in response to nerve growth factor [35]. Other genes involved in neuron differentiation and morphogenesis (SEMA4C, CAPN3, WNT4, DPYSL5, VAX2, CBLN1) appeared also down-regulated again pointing to de-differentiation. These results imply that care should be taken in studies involving alterations in the above described genes and/or proteins since medium change could obscure or lead to misinterpretations of results.

Induced genes after 24 hours of medium change showed the expected response to renewal of nutrients and growth factors also pointing to partial loss of differentiation in cultured neurons. Among the genes induced are those involved in positive regulation of cell proliferation, biosynthesis/metabolism, and positive regulation of transcription all expected after medium renewal in cells in culture [36].

Taken together, the results indicate that in the time frame studied (7-10 days) a medium change at day 7 in culture induces a partial and transient de-differentiation that is reversed with time in culture as seen at day 10. In the same interval, several markers characteristic of differentiated neurons progressively increase from day 7 to day 10 with no interference observed after medium change.

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