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Transcriptional Mechanisms Underlying Apoptosis in Cerebellar Granule Neurons

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Abstract

Neuronal apoptosis represents an intrinsic suicide program, by which a neuron orchestrates its own destruction. Although engagement of apoptosis requires transcription and protein synthesis, the complete spectrum of genes involved in distinct temporal domains remained unknown until the advent of genomics. In the last ten years, the genome sequences and the development of high-throughput genomic technologies, such as DNA microarrays, have offered the unprecedented experimental opportunities to explore the transcriptional mechanisms underlying apoptosis from a new systems-level perspective. The present review will go over this genomic approach and illustrate its use to dissecting the multigenic program underlying neuronal apoptosis of cerebellar granule neurons.

Keywords: Apoptosis; DNA microarray; Gene; Mechanisms; Pathway; Transcription; Transcriptome

Introduction

Neuronal apoptosis represents an intrinsic suicide program, by which a neuron orchestrates its own destruction. It is characterized by specific morphological and biochemical events, including fragmentation of nuclear DNA, breakdown of the cellular cytoskeleton, and the bulging out of the plasma membrane (blebbing), which may lead to the detachment of the so-called apoptotic bodies [1,2]. During normal nervous system development, physiologically appropriate neuronal loss contributes to a sculpting process that removes approximately one-half of all neurons born during neurogenesis [3]. Neuronal loss subsequent to this developmental window is physiologically inappropriate for most systems and can contribute to neurological deficits, e.g., neurodegenerative diseases such as Alzheimer's and Parkinson disease [1,4,5]. Elucidating the molecular mechanisms underlying neuronal apoptosis hence may contribute to our understanding of basic developmental biology and to human neuropathology.

Although an extensive number of studies have implicated individual genes or genetic pathways during apoptosis, the complete spectrum of genes involved in distinct temporal domains remained mostly unknown until the advent of genomics. In the last ten years, the genome sequences and the development of high-throughput genomic technologies, such as DNA microarrays, have offered the unprecedented experimental opportunities to explore the transcriptional mechanisms underlying apoptosis in different *in vitro* paradigms [6-16]. The present review will briefly introduce this genomic approach and then illustrate its use to dissecting the multigenic program underlying neuronal apoptosis of cerebellar granule neurons (CGNs), an *in vitro* paradigm that has been extensively utilized to examine the signal transduction mechanisms underlying neuronal apoptosis.

Transcriptional Analysis by DNA Microarray Technology

The most remarkable technology for genome-wide expression analysis is nowadays DNA microarray technology, which permits the quantitative and simultaneous monitoring of the expression levels of thousands of genes under different conditions. DNA-microarray is an orderly arrangement of DNA spots, each containing a unique DNA sequence. DNA spots contain either DNA oligomers or a longer DNA sequence designed to be complementary to a particular mRNA of interest. When a microarray is hybridized to fluorescence-tagged complementary DNAs or RNAs derived from messenger or total RNA, each spot is a target for the mRNA encoded by a gene. Following hybridization, a laser excites the bound cDNAs or cRNAs, and fluorescence intensities from each spot on the slides are collected by a scanner. The intensity of the fluorescence at each array element is proportional to the expression levels of an mRNA. The thing that makes DNA microarrays the most remarkable technology for genome-wide expression analysis is the number of DNA probes that it is possible to place on a microarray. Today, whole-genome microarrays are available for different species and permit the quantitative and simultaneous monitoring of the expression levels of thousands of genes at different time points, under different conditions or tissues. The knowledge of when and under what conditions a gene or a set of genes are expressed often provides strong clues as to their biological role or function. It should be emphasized, however, that expression DNA microarrays measure steady-state mRNA levels, reflecting the equilibrium between mRNA synthesis and degradation. In addition, when the immobilized DNA sequence is complementary to more than one mRNA, such as in the case of alternative splice variants, the fluorescence signal represents a single consensus value for all transcripts. The main challenge in DNA microarray analysis is to extract relevant information from the large amount of data produced. A variety of analytical approaches are available to interpret microarray data and for a more detailed description of microarray technology, the reader is referred to other reviews [17-20].

Transcriptional Changes in Apoptotic Cerebellar Granule Neurons

Cerebellar granule neurons (CGNs) are the most abundant

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neuronal cell type in the mammalian brain and represent, both *in vivo* and *in vitro*, a model of election for the study of neuronal apoptosis [21,22]. Apoptosis of CGNs can be regulated by loss of neurotrophic supply and/or activity-dependent survival signals. The relative contribution of each component varies according to neuronal type and age. During early postnatal development, apoptosis of granule cells is thought to reflect the failure of these neurons to obtain adequate amounts of specific neurotrophic factors [23,24], whereas in the adult, apoptosis of CGNs following mossy fiber axotomy points out to the crucial role of afferent inputs on the survival of these cells.

Primary cultures of CGNs have been extensively utilized to examine the signal transduction mechanisms underlying neuronal apoptosis. In this *in vitro* paradigm, CGNs undergo rapid apoptotic cell death within 24 h after removal of serum and lowering of extracellular potassium from 25 to 5 mM [22]. Engagement of apoptosis requires transcription and protein synthesis and the process becomes irreversible during the first 6 hours following induction. Before this "commitment point" CGNs can be rescued by the activation of specific signal transduction pathways or by the treatment with specific neurotrophic factors, such as insulin-like growth factor-1 (IGF1) [22,25] and pituitary adenylyl cyclase-activating polypeptide 38 (PACAP) [26].

Distinct Temporal Gene Expression Profiles Associated with Apoptosis of CGNs

To decipher the transcriptional regulatory elements controlling apoptosis of CGNs, genome-wide expression profiling has been performed in CGNs during the pre-commitment period of apoptosis (Figure 1). By using oligonucleotide microarrays, mRNA expression profiles were monitored in CGNs 3 and 6 h after induction of apoptosis by low [K^+] (5 mM) and serum deprivation (-KS) [7]. Among the 8740 genes interrogated by the microarrays, 421 genes showed significant changes of gene expression [7]. Among these, 69 were down-regulated and 152 up-regulated at 3 h, whereas 93 genes were down-regulated and 204 were up-regulated at 6 h.

To investigate and interpret gene expression data sets, cluster analysis is usually employed in DNA microarray analysis. By grouping together genes that have similar expression profiles, cluster analysis is used for extraction of regulatory motifs, inference of functional annotation, and classification of cell types or tissue samples [8,27]. Most cluster analysis techniques are hierarchical and the resultant classification has an increasing number of nested classes and the result resembles a phylogenetic classification as illustrated in figure 1. An example of hierarchical clustering applied to genes differentially expressed after induction of apoptosis in CGNs is shown in figure 2.

Transcriptional and Post-Transcriptional Regulatory Mechanisms

As previously stated, DNA microarrays measure steady-state levels of mRNAs to observe which of those genes differentially expressed after induction of apoptosis were regulated at the transcriptional or post-transcriptional level, gene expression profiles of CGNs during potassium and serum deprivation were compared in the absence or presence of a transcription inhibitor, Actinomycin-D (ActD) [7]. This analysis demonstrated that most of the gene expression changes observed during apoptosis was under transcriptional control. Treatment with ActD, in fact, reduced the mRNA-expression of most of the genes which were up- or down-regulated during apoptosis. Few of the genes, however, were not affected or actually increased following ActD treatment, indicating the existence of post-transcriptional regulatory mechanisms. A comprehensive picture of transcriptional and post-transcriptional changes associated to CGNs apoptosis is shown in figure 2.

Pathways Analysis of Transcriptional Changes

Apoptosis depend on the activity of an integrated network of genes and their encoded proteins, which almost never work alone but interact with one another in highly structured and incredibly complex ways. In this integrated network it is not important the activity of the single gene and their encoded protein, but the entire components and their interactions. Thus, genes do not act by themselves, but they function in gene networks and molecular pathways and their effects are not independent but often modified by one or several other genes (epistasis) [28].

Identification of differentially expressed genes, as shown in the previous section, represents only the tip of the "iceberg" of a genomic analysis. When genes are analyzed individually, small changes in expression may not pass stringent statistical cut off. Those small changes, however, may show a statistical significance when analyzed, for example, in the context of a pathway. The next paragraphs will illustrate how a more integrated picture of the transcriptional changes during neuronal apoptosis can be obtained by gene ontology and pathway enrichment.

A gene or its encoded protein has not only a name/symbol or an expression value, but several ontologies or functional annotations [8,27]. Common functional annotations are those listed in the Gene Ontology (GO) database (www.geneontology.org), a controlled vocabulary of terms that describes the roles of genes and proteins in all organisms [29]. GO is comprised of three independent ontologies: 1) biological process describes biological goals accomplished by one or more ordered assemblies of molecular functions; 2) cellular component describes locations, at the levels of subcellular structures

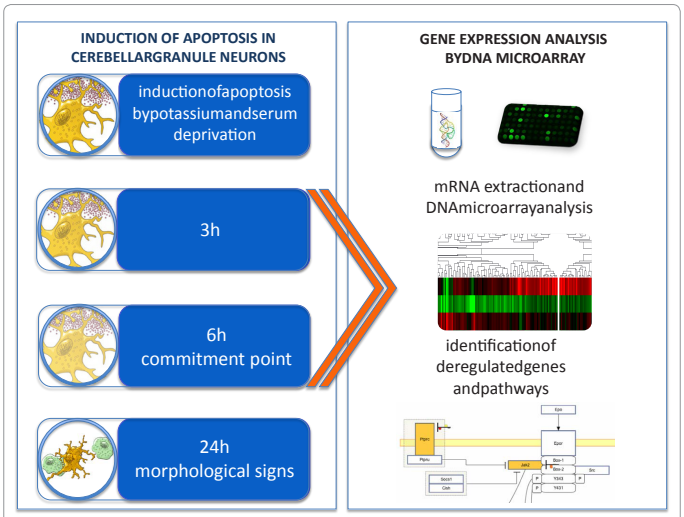

Figure 1: The genomic approach used for uncovering transcriptional mechanisms underlying apoptosis of CGNs. Primary cultures of CGNs undergo rapid apoptotic cell death within 24 h after removal of serum and lowering of extracellular potassium [22]. Engagement of apoptosis requires transcription and protein synthesis and the process becomes irreversible during the first 6 hours following induction. Expression profiling by microarray analysis of CGNs during the pre-commitment period of apoptosis (3 and 6 h) was used to decipher the transcriptional changes underlying apoptosis [7]. The genomic approach starts with RNA extraction, moves through DNA microarray analysis, and ends with the identification of deregulated genes and pathways.

and macromolecular complexes; 3) molecular function describes activities, such as catalytic or binding activities, at the molecular level. Biological process, molecular function and cellular component are all attributes of genes, gene products or gene-product groups and each of these may be assigned independently. The relationships between a gene product (or a gene-product group) to biological process, molecular function and cellular component are one-to-many, reflecting the biological reality that a particular protein may function in several processes, contain domains that carry out diverse molecular functions, and participate in multiple alternative interactions with other proteins, organelles or locations in the cell. Through the use of GO terms, a number of software tools (http://www.geneontology.org/GO.tools.shtml) are able to perform gene ontology enrichment analysis of high-throughput experimental results, such as gene expression microarray data, and discover statistically significantly enriched GO terms among a given gene list.

In addition to the GO terms described above, many other annotations are nowadays linked to a specific gene/protein. Examples of these are the associated disease of phenotype (OMIM Links), publications (Medline links), chromosomal location, interacting drug, functional domain, and functional pathway. This last, in particular, represents a set of consecutive signals or metabolic transformations that have been confirmed as a whole by experimental data. Thousand of pathways are nowadays available (for a list of biological pathway related resources see: http://www.pathguide.org/) and different informatics tools have been developed that enable to analyze gene expression changes in the context of pathways. Some of these public and private resources are described below.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) [30] is a free resource that contains a comprehensive collection of databases for genes, pathways and ligands for several organisms, together with web-accessible tools for the retrieval of pathways and the annotation of gene lists. The Gene Map Annotator and Pathway Profiler (GenMAPP) [31] are freely available programs for viewing and analyzing gene expression data in the context of biological pathways. Examples of private resources include MetaCore (http://www.genego.com/), Ingenuity Pathways Analysis (http://www.ingenuity.com/), Pathway Assist (http://www.ariadnegenomics.com/) and Genespring (www.agilent.com).

Most of the genes differentially expressed in CGNs after induction of apoptosis can be assigned to functional categories, subcellular compartments and pathways based on their translated products. Some of them have been previously related to apoptosis in CGNs or other cellular systems, whereas others provide a significant number of unique and novel entry points. In many cases, genes with common biological functions or in the same metabolic pathway showed coordinated expression [7]. For space limitation, only two examples of pathways differentially affected following induction of apoptosis of CGNs will be discussed in the following paragraphs and in figures 3 and 4.

Fatty acid metabolism

The coordinated increase of seven enzymes that are key regulators of beta-oxidation of fatty acids, were found in apoptotic CGNs: Acadl (acyl-CoA dehydrogenase, long chain), Acsl1 (acyl-CoA synthetase long-chain family member 1), Cpt1a (carnitine palmitoyltransferase 1a), Cpt1b (carnitine palmitoyltransferase 1b), Cpt2 (carnitine palmitoyltransferase 2), Decr1 (2,4-dienoyl CoA reductase 1), and Eci1 (enoyl-CoA delta isomerase 1) (Figure 3). Induction of this pathway may reflect an increased demand in energy production during the energy-requiring apoptotic program, also in view of the rapid and progressive impairment of oxidative phosphorylation during the same time period [32].

Classical apoptotic pathway

Several genes conventionally designated as apoptosis regulators were found differentially expressed in CGNs and are represented in figure 4. Two of these genes encode Tnfrsf21 (tumor necrosis factor receptor superfamily, member 21; also known as DR6) [33] and Igf1r (insulin-like growth factor 1 receptor) [34], two receptors implicated in neuronal apoptosis. An inhibitor of the NF-κB pathway, Nfkbia (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), was up-regulated following induction of CGNs, supporting the inhibitory role of NF-κB in CGN apoptosis [35-41]. Reduced expression of the anti-apoptotic Bcl2l1 (Bcl2-like 1, also known as Bcl-xl) was observed following induction of CGN apoptosis. This gene encodes a Bcl-2 family protein that prevents apoptosis [42-45]. In apoptotic CGNs, another Bcl-2 family member, Hrk (Harakiri, also known as neuronal death protein DP5), was found up-regulated. Activation of Hrk is known to occur in a c-jun dependent manner during apoptosis of CGNs [46-48]. Consistent with these and other studies [49,50] is the observed up-regulation of c-jun (jun proto-oncogene) following induction of apoptosis in CGNs. Differential expression of another transcriptional regulator, Myc (myelocytomatosis oncogene) [51] was found in apoptotic CGNs. Increased expression of Casp3 (caspase 3), a cysteine-aspartic acid protease that is considered an important regulator and a marker of apoptotic processes [38,52] was evident both at 3 h and 6 h after induction of apoptosis. Finally, an increased expression of Irf1 (interferon regulatory factor 1) was also evident after induction of CGN apoptosis. Although the role of Irf1 in CGNs is unknown, the over-expression of this tumor suppressor gene has been linked to apoptosis in other cell types [53,54].

Conclusion

While demonstrating the utility of a genomic approach as a means

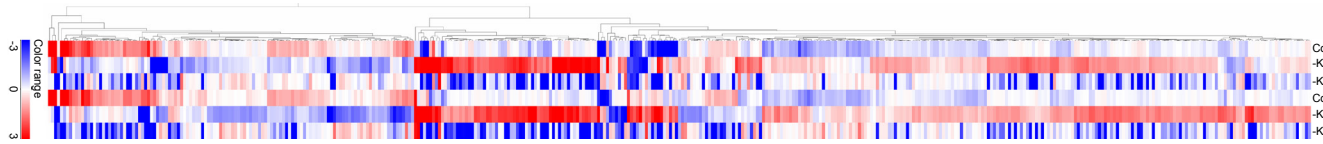

Figure 2: Genes differentially expressed after induction of apoptosis in CGNs. To induce apoptosis, cultures were switched in serum-free medium containing 5 mM KCl in the absence (-KS) or presence of 10 μM Actinomycin D (-KS + ActD). Control cells (control) were maintained in complete medium. 421 genes showed significant changes of gene expression following induction of apoptosis at 3 h and/or 6 h [7]. A hierarchical clustering algorithm (similarity measure: euclidean; linkage rule: complete) was used to order these genes in a dendrogram, in which the pattern and length of the branches reflects the relatedness of the expression levels in different experimental conditions. Data are presented in a matrix format: each row represents a single gene and each column an experimental condition. The averaged normalized intensity from replicates is represented by the color of the corresponding cell in the matrix. Blue, white and red cells, respectively, represent transcript levels below, equal or above the median abundance across all conditions. Color intensity reflects the magnitude of the deviation from the median (see scale at the bottom).

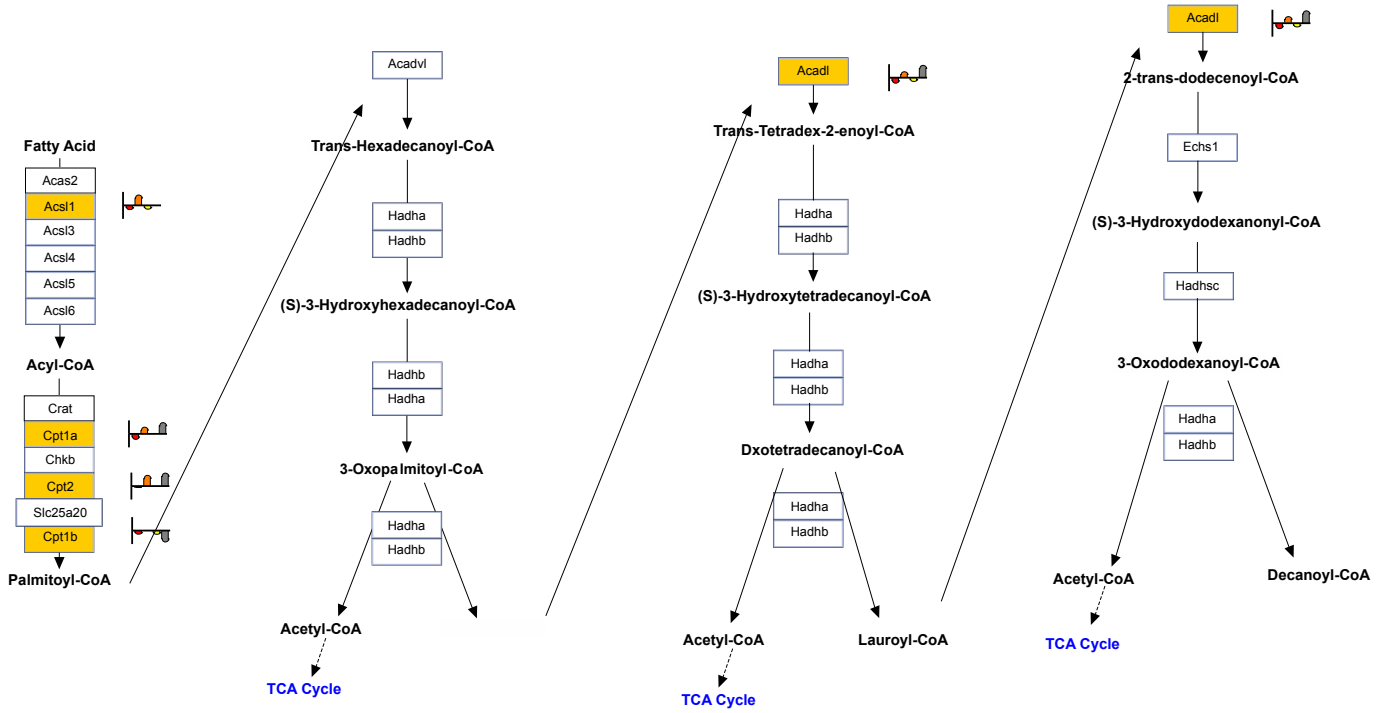

Figure 3: Beta-oxidation pathway. The coordinated increase of seven enzymes that are key regulators of beta-oxidation of fatty acids, were found in apoptotic CGCs: Acadl (acyl-CoA dehydrogenase, long chain), Acsl1 (acyl-CoA synthetase long-chain family member 1), Cpt1a (carnitine palmitoyltransferase 1a), Cpt1b (carnitine palmitoyltransferase 1b), Cpt2 (carnitine palmitoyltransferase 2), Decr1 (2,4-dienoyl CoA reductase 1), and Eci1 (enoyl-CoA delta isomerase 1). Differentially expressed genes are highlighted in yellow and their expression in four experimental conditions are indicated with bar graphs (Control 3 h, first bar in red; -KS 3 h, second bar in orange; Control 6 h, third bar in yellow; -KS 6 h, fourth bar in grey).

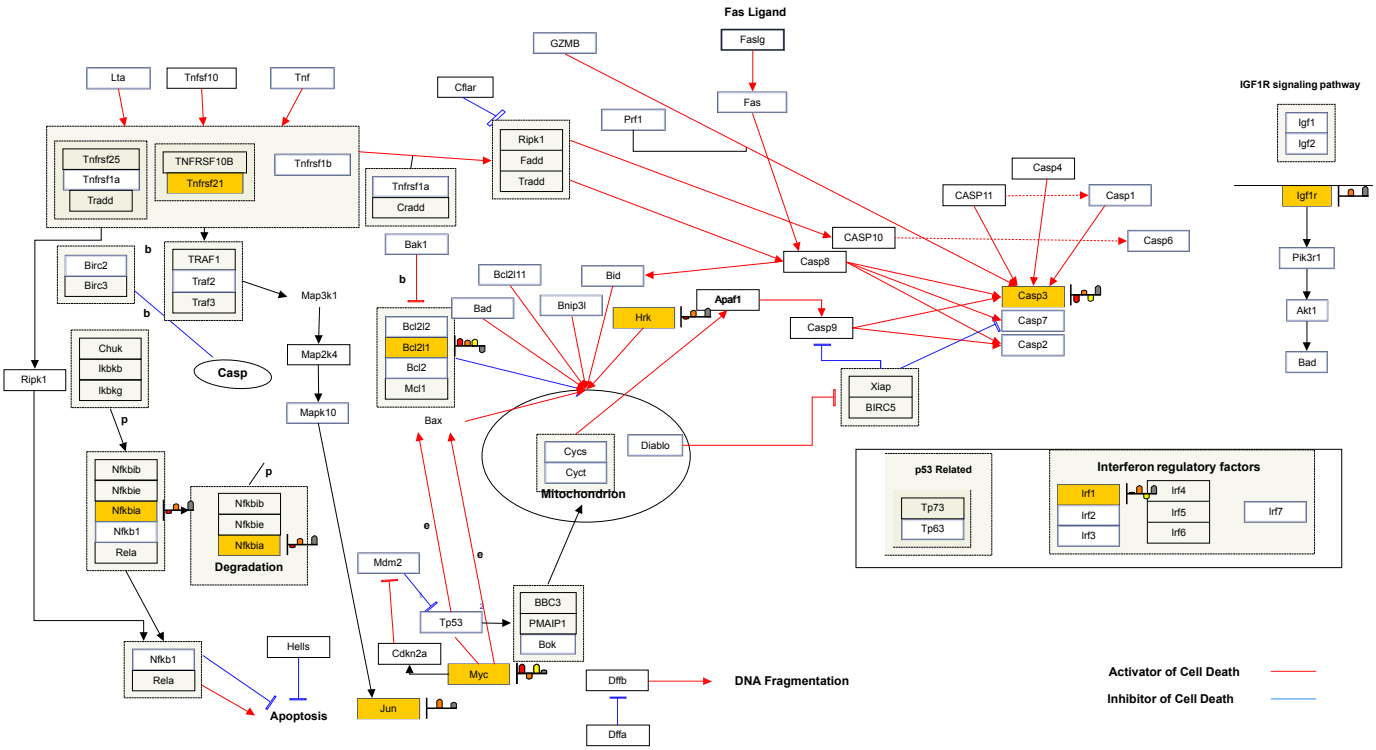

Figure 4: Classical apoptotic pathway. Several genes conventionally designated as apoptosis regulators are represented in this pathway. Nine of these genes were differentially expressed in CGNs: Casp3 (caspase 3), c-jun (jun proto-oncogene), Bcl2l1 (Bcl2-like 1), Hrk (Harakiri), Igf1r (insulin-like growth factor 1 receptor), Irf1 (interferon regulatory factor 1), Myc (myelocytomatosis oncogene), Nfkbia (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), Tnfrsf21 (tumor necrosis factor receptor superfamily, member 21).

of dissecting the multigenic program underlying neuronal apoptosis, the studies illustrated represent just a glimpse of this complex phenomenon. It should be emphasized that the microarray provides estimates of changes in mRNA levels that cannot be correlated with the amount and function of the gene products. Translation and post-translational modifications of many gene products and protein turnover have dramatic effects on function, and these cannot be inferred from expression analysis alone.

Most of the changes observed in CGNs seem to be pro-apoptotic, while others could be adaptive and represent an attempt for survival. Each of them, having a larger or smaller specific weight may contribute to the development of life or death. The exact role and functional relationships of the genes implicated are presumably those we cannot yet recognize. Gene expression profiles unlock virtually unexplored frontiers and we will learn as we explore them. Systematic characterization of expression patterns associated with apoptosis in different pathophysiological conditions and in distinct temporal domains will provide a framework for interpreting the biological significance of the expression patterns observed in CGNs. Such a challenging task has just begun by characterizing gene expression profiles in cortical neurons exposed to amyloid b-protein (b–AP) [9], whose toxicity is considered the leading mechanism proposed for neuronal death in Alzheimer's disease. Among the genes differentially expressed following bAP treatment, 70 were in common with those differentially expressed during apoptosis of CGNs [7]. Although preliminary, these data suggest the existence of both common and diverse mechanisms responsible of neuronal cell death. Knowledge of the mechanisms and pathways that determine apoptosis and are aberrant in pathological conditions will pave the way for new pharmacological approaches.

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