BMB
reports

Genome wide identification of Staufen2-bound mRNAs in embryonic rat brains

*Marjolaine Maher-Laporte & Luc DesGroseillers**
Département de Biochimie, Université de Montréal, PO Box 6128 Centre Ville, Montréal, Québec, Canada. H3C 3J7

Messenger ribonucleoprotein particles (mRNPs) are used to transport mRNAs along neuronal dendrites to their site of translation. Staufen2 is an mRNA-binding protein expressed in the cell bodies and cellular processes of different brain cells. It is notably involved in the transport of dendritic mRNAs along microtubules. Its knockdown expression was shown to change spine morphology and impair synaptic functions. However, the identity of Staufen2-bound mRNAs in brain cells is still completely unknown. As a mean to identify these mRNAs, we immunoprecipitated Staufen2-containing mRNPs from embryonic rat brains and used a genome wide approach to identify Staufen2-associated mRNAs. The genome wide approach identified 1780 mRNAs in Staufen2-containing mRNPs that code for proteins involved in cellular processes such as post-translational protein modifications, RNA metabolism, intracellular transport and translation. These results represent an additional and important step in the characterization of Staufen2- mediated neuronal functions in rat brains. [BMB reports 2010; 43(5): 344-348]

INTRODUCTION

mRNA transport is critical in many situations to differentially control protein content in domains distant from the cell body (1). In particular, transport and subsequent local translation are important for different aspects of synaptic functions and plasticity (1-3). Several mechanisms of mRNA transport have been described in dendrites ranging from a constitutive transport pathway to an activity-induced transport mechanism of newly transcribed mRNAs (3). It is believed that different mechanisms of mRNA transport are linked to different forms of synaptic activity and/or plasticity.

Upon transcription, nascent mRNAs associate with several factors that form messenger ribonucleoprotein particles (mRNP) (3). Along the way from nuclear export to dendritic anchoring, proteins are added or removed from the mRNP complexes in a dynamic way. These proteins finely control the successive steps that insure proper expression of mRNA at specific times and space. Staufen2 (Stau2), a protein mainly expressed in brain is a well accepted player for mRNA localization (4-9). In brain, Stau2 is expressed in several cell types including neurons and oligodendrocytes. Four Stau2 protein isoforms are generated by differential splicing. Stau2 isoforms bind double-stranded RNAs, are incorporated into mRNPs and display a microtubule-dependent somatodendritic distribution in neurons. In oligodendrocytes, Stau2-containing mRNPs are found in both the cell bodies and myelinating processes. Interestingly, over-expression of Stau2 in dendrites increases the amount of dendritic mRNAs showing the importance of Stau2 for mRNA transport (5). Neurons in which Stau2 has been down-regulated by RNAi show a reduced density of dendritic spines, associated with a change in their morphology (10). These phenotypes result in reduced amplitude of the miniature excitatory postsynaptic currents, a measure of synaptic transmission.

In the somatodendritic compartment, Stau2 associates with the two main types of cytoplasmic mRNA/protein complexes involved in transport: mRNA granules and mRNA transport particles (4, 11). Whereas mRNA particles are devoid of ribosomes, mRNA granules form bigger complexes that are associated with ribosomes. It was suggested that RNA particles might represent the observed transport mRNPs (12). While the distribution and functions of Stau2 in brain cells begin to be elucidated, the identity of transported mRNAs is still unknown, preventing in-depth analyses of the molecular mechanisms involved in specific phenotypes. In this paper, we immunoprecipitated Stau2-containing mRNPs from embryonic rat brains and used a genome wide approach to identify Stau2-associated mRNAs. Several mRNAs coding for proteins mainly involved in protein modification, translation and intracellular trafficking were identified.

RESULTS

Identification of mRNAs in Stau2-containing mRNPs in embryonic brains

In order to identify the mRNA content of Stau2-containing RNPs, extracts of embryonic rat brains were prepared and en-

*Corresponding author. Tel: 514-343-5802; Fax: 514-343-2210;
E-mail: luc.desgroseillers@umontreal.ca

Received 26 January 2010, Accepted 16 March 2010

Keywords: Microarray, mRNA localization, Rat brains, Ribonucleoprotein, Transport particle

dogenous Stau2 was immunoprecipitated using polyclonal anti-Stau2 antibodies and the pre-immune serum as control. To minimize the identification of non-specific mRNAs, two different polyclonal anti-Stau2 antibodies were used in parallel experiments (Fig. 1A). Co-immunoprecipitated mRNAs were purified. Commercially available rat DNA oligonucleotide microarrays from Illumina containing 22,226 probe sets were hybridized with cRNAs derived from Stau2-dependent IPs and controls (Fig. 1B). A total of 12 microarrays were hybridized: four with cRNA probes generated from mRNAs isolated with the anti-Stau2 L1 antibody, two with the anti-Stau2 L2 antibody and six with their respective pre-immune serum. To estimate the validity of these hybridization data, we first generated a PCA plot that translates into a particular space on the plot each measurement and structure of one gene expression data array (13). The PCA plot from the hybridization data of the twelve arrays shows that hybridization data are grouped together according to the antibody used for immunoprecipitation (L1, L2 or PI) (Fig. 1C) indicating reproductive hybridization data. It also indicates that both Stau2-derived hybridizations are different from those of the pre-immune arrays and that anti-Stau2 L1 and L2 antibodies are not perfectly identical. To identify Stau2-associated mRNAs, data were analyzed with the FlexArray 1.2 software (14). We selected mRNAs that were enriched at least 8 fold in both Stau2-dependent IPs (to minimize non-specific hybridization) as compared to controls (P value < 0.002). Using these criteria, 1780 mRNAs were identified (Supplemental Table S1).

RT-PCR validation of identified mRNAs

Several mRNAs identified in the microarray experiments as being enriched in Stau2 mRNPs were validated by RT-PCR experiments. Genes that were enriched less than 6 fold were used as negative controls. Stau2-containing mRNPs were immunoprecipitated from embryonic rat brain extracts using anti-Stau2 antibodies and a pre-immune serum as control. Co-immunoprecipitated mRNA was isolated and RT-PCR amplified using specific primers (Fig. 2). Consistent with the microarray data, several mRNAs were enriched in Stau2-containing complexes as compared to the pre-immune IP. Enrichment was observed with the two different anti-Stau2 antibodies. In contrast, control mRNAs were not enriched in Stau2-dependent immunoprecipitates as compared to those of the pre-immune serum indicating that they were absent from Stau2-containing complexes, as expected from the microarray data. Serial dilutions of the control mRNAs before RT-PCR amplification confirmed that the amplifications were in the linear range (Fig. 2B).

Gene ontology (GO)

Next, we identified pathways in which Stau2-associated mRNAs are involved as a mean to understand the biological functions of Stau2 in brain cells. Probe set lists have been analyzed with the BABELOMICS functional annotation tool (15) to

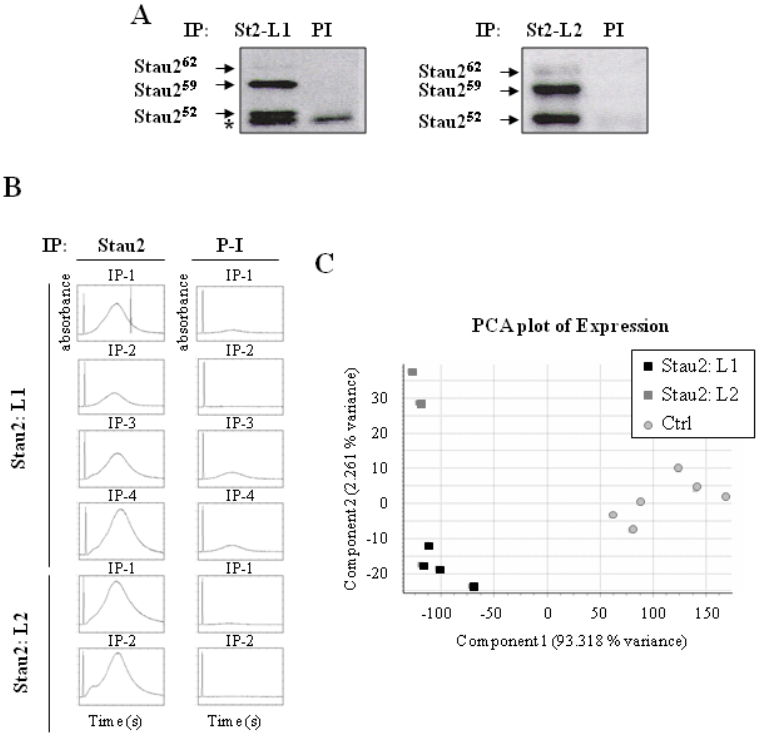

Fig. 1. Immunoprecipitation of Stau2 isoforms. (A) Immunoprecipitation of Stau2 isoforms from embryonic E17-18 rat brain extracts using two different polyclonal anti-Stau2 antibodies, L1 (St2-L1) and L2 (St2-L2). Pre-immune (PI) sera were used as controls. The Stau2^{56} isoform is not visible in these cell extracts. *represents a non-specific IgG band. (B) Analysis of the mRNAs present in Stau2-containing mRNPs. Co-immunoprecipitated mRNAs were purified and used to generate cRNA probes. Probes were analyzed by electrophoresis and visualized on electrophoregrams. (C) Each hybridization profile was plotted on a PCA plot as a mean to determine the reproducibility of the hybridization data.

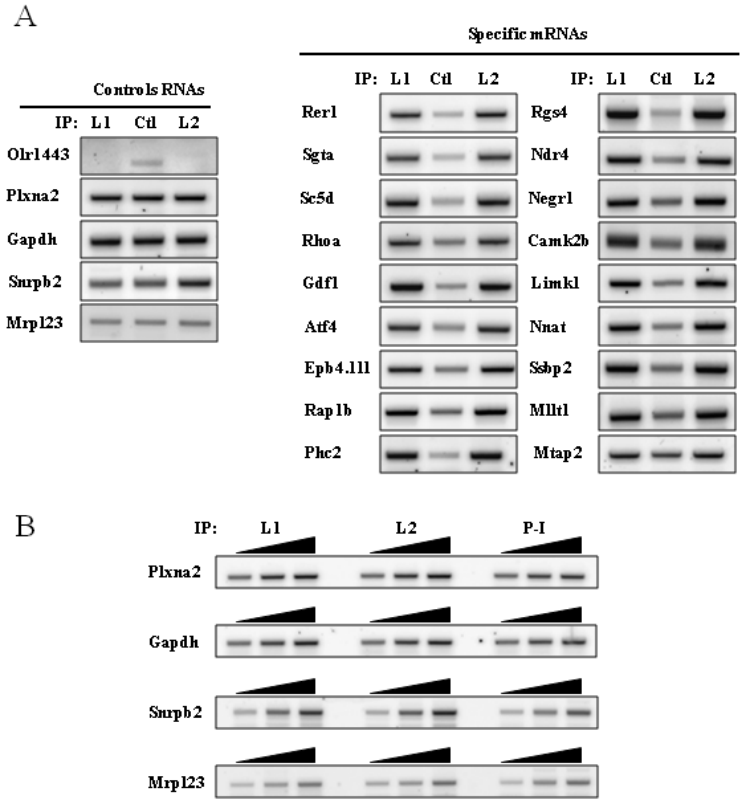

Fig. 2. RT-PCR amplification of selected mRNAs following mRNPs immunoprecipitation. (A) Stau2-containing mRNPs were immunoprecipitated from rat embryonic brains using two different polyclonal rabbit antibodies (L1 and L2) or the pre-immune sera. Co-immunoprecipitated mRNA was purified and RT-PCR amplified with pairs of oligonucleotides (Supplemental Table S4) specific for microarray-identified genes as indicated. Control (left panel) and Stau2-associated (right panel) genes were chosen according to their differential enrichment in Stau2-containing mRNPs according to the microarray data (Supplemental Table S1). (B) Serial dilutions of the control mRNAs after the immunoprecipitation step indicate that the RT-PCR amplification reactions were in the linear range. These results were representative of at least two experiments.

assign cellular functions to mRNAs that were enriched in Stau2-containing RNPs. The result from GO classification revealed that mRNAs associated with Stau2-containing RNPs are involved in cellular processes such as protein modification, RNA metabolism, translation and neurogenesis. Their molecular functions include kinase and phosphatase activities as well as ubiquitin ligase activity (Supplemental Table S2).

DISCUSSION

Stau2 is an RNA-binding protein mainly expressed in different cells of the brain (4, 6) and its importance for mRNA transport in dendrites (5, 8) and synaptic functions (10) has been established. RNA transport is a complex process that relies on a highly heterogeneous population of mRNA particles and granules (3). The differential presence/absence of RNA-binding proteins and other cofactors in each mRNP determines the roles of each mRNP complex and the fate of associated mRNAs. These proteins regulate gene expression at the post-transcriptional level in a mechanism widely used by mammalian cells to control protein synthesis with acute precision (16, 17). Identification of Stau2-bound mRNAs in embryonic brain cells is a further step for our understanding of Stau2 functions in agreement with the post-transcriptional regulon theory (18). This theory stipulates that RNA-binding proteins such as Stau2 mediate the coordinate post-transcriptional expression of bound mRNAs according to cell signalling. The major molecular functions associated with mRNAs identified in Stau2-containing mRNPs in brains are related to protein modifications such as phosphorylation, dephosphorylation and ubiquitination. This is very interesting since these processes are well characterized in neurons and oligodendrocytes and most of the signalling events are regulated at least in part by protein modifications (19, 20). A significant reduction in the local synthesis of kinases and/or phosphatases could easily explain the reduction of dendritic spines and the increase of elongated filopodia-like structures that were observed following Stau2 down-regulation in neurons (10). Other major Stau2-associated GO groups include intracellular transport, translation, RNA metabolism, neurogenesis and synaptic functions. This classification suggests that Stau2 mRNPs may carry mRNAs whose translation generates proteins involved in the regulation of their own transport, translation and degradation allowing an auto-stimulatory process after an initial signalling event.

According to our microarray data that identified β-*actin* mRNA as a target of Stau2, it was previously shown that the down-regulation of Stau2 by RNA interference in mature neurons caused a significant reduction in β-*actin* mRNA expression level and fewer dendritic β-*actin* mRNPs (10). To explain these results, it was proposed that Stau2 binds to β-*actin*

mRNA and controls its fate. Our microarray results indicate that, indeed, Stau2 binds to β-*actin* mRNA. However, its role in the post-transcriptional regulation of β-*actin* and/or other mRNAs in brain cells is not yet clear. Stau2 may influence mRNA transport and/or localization in cellular processes since previous work has implicated Stau2 in dendritic mRNA transport (5, 8, 9). Alternatively, based on known functions of its paralog Stau1, Stau2 may also control the stability or the translation of specific mRNAs. Indeed, Stau1 elicits mRNA decay of a sub-population of transcripts when bound to their 3′UTR (21) and enhances translation of other mRNAs when bound to their 5′UTR (22). This latter function of Stau1 is consistent with a role of *Drosophila* Staufen in *oskar* mRNA translation once localized to the posterior pole (23, 24). It will be interesting to determine the role of Stau2 in the post-transcriptional regulation of bound mRNAs.

Using the Affimetrix platform for microarray analyses, we recently identified mRNAs that are associated with over-expressed Stau2-HA_3 in the human epithelial HEK293 cell line (25). Despite the fact that the present study identified mRNAs bound to endogenous Stau2 using the Illumina technology, it is relevant to compare these mRNA populations as a mean to get some clues on Stau2 mRNA-binding specificity in different tissues. Comparison of gene symbols and/or orthologs identified 218 common Stau2-associated mRNAs in the two studies (Table S3). Considering that 983 of the 1780 Illumina probe sets have a gene symbol that is shared by Affymetrix probe sets, we estimate that only 22% of the mRNAs associated with endogenous Stau2 in brains are also present in Stau2-HA_3-containing mRNPs in human. This relatively low percentage of common mRNAs suggests that either we have characterized a specialized sub-population of mRNPs expressed in brains or that the sub-population of Stau2-bound mRNAs differs in different tissues probably due to the differential availability of mRNAs and cofactors that constitute the mRNPs. It makes sense that mRNAs required for local translation in specialized sub-cellular domains such as distal dendrites of neurons or myelinating processes of oligodendrocytes are different from those required in epithelial cells.

MATERIALS AND METHODS

Immunoprecipitation

Immunoprecipitation of Stau2-containing mRNPs was performed on cell extracts prepared from whole brains of E17-E18 Sprague Dawley rat embryos. Cells were dissociated with a manual putter and lysed in the ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% Triton X-100, 15 mM EGTA, 1 mM DTT and complete EDTA-free protease inhibitor cocktail (Roche)] containing 2 U/ml rRNAsin (Invitrogen) as described (25). All further manipulations of the brain lysates were performed at 4°C. Cell lysates were centrifuged at 9,300 g for 10 min to remove nuclei and cell debris. After centrifugation, supernatants were incubated with rabbit polyclonal anti-Stau2 antibodies or pre-immune serum (4) for 2 hours at 4°C, then with a 50% protein A-sepharose slurry for 2 hours at 4°C as described before (26), except that Escherichia coli tRNA (Sigma-Aldrich) was added to a final concentration of 200 mg/ml. Immune complexes were washed five times with the lysis buffer and eluted from the resin by heating at 95°C for 5 minutes in elution buffer (100 mM Tris-HCl pH 7.4, 200 mM DTT and 4% SDS). RNAs were isolated by Trizol (Invitrogen) extraction according to manufacturer instructions.

Microarray hybridization and RT-PCR validation

RNA pellets were solubilized in water and residual contaminants were removed using an RNA nanoprep column (Stratagene). Biotinylated cRNA probes were synthesized by the TotalPrep RNA labelling kit (Ambion). Illumina rat genome arrays (RatRef-12_V1 Expression BeadChip comprising 22,226 probe sets) were used for hybridization according to Illumina guidelines. Hybridized chips were scanned using an Illumina iScan System. Results were recorded using the BeadStudio software platform. To identify mRNAs that specifically copurify with Stau2, signal intensities obtained for specific IPs were compared with the baseline signal intensities of control IPs using the FlexArray 1.2 software (14). Each probe set presenting a fold enrichment over control of more than 8 (t-test P value $<$ 0.002) was scored as a potential Stau2-associated mRNA. Complete microarray analysis results can be found in the Supplemental Data. Microarray data have been deposited in the GEO database and are available through the series accession number GSE16437. For validation, RT-PCR was performed as described before (25, 27, 28). Primer pairs are listed in Supplemental Table S4.

Acknowledgements

We thank Louise Cournoyer for her help with tissue cultures; the Genome Quebec Innovation Centre, in particular André Ponton, for microarray screening and analysis. This work was supported by operating and group grants from the Canadian Institutes of Health Research (CIHR) to LDG. MML was supported by a studentship from the Natural Sciences and Engineering Research Council of Canada (NSERC).

REREFENCES

1. Bassell, G. J., Oleynikov, Y. and Singer, R. H. (1999) The travels of mRNAs through all cells large and small. *Faseb. J.* **13**, 447-454.
2. Sanchez-Carbente, M. and DesGroseillers, L. (2008) Understanding the importance of mRNA transport in memory. *Prog. Brain Res.* **169**, 41-58.
3. Sossin, W. S. and DesGroseillers, L. (2006) Intracellular trafficking of RNA in neurons. *Traffic* **7**, 1581-1589.
4. Duchaine, T. F., Hemraj, I., Furic, L., Deitinghoff, A., Kiebler, M. A. and DesGroseillers, L. (2002) Staufen2 isoforms localize to the somatodendritic domain of neurons and interact with different organelles. *J. Cell Sci.* **115**,

3285-3295.
5. Tang, S. J., Meulemans, D., Vazquez, L., Colaco, N. and Schuman, E. (2001) A role for a rat homolog of staufen in the transport of RNA to neuronal dendrites. *Neuron* **32**, 463-475.
6. Thomas, M. G., Martinez Tosar, L. J., Loschi, M., Pasquini, J. M., Correale, J., Kindler, S. and Boccaccio, G. L. (2005) Staufen recruitment into stress granules does not affect early mRNA transport in oligodendrocytes. *Mol. Biol. Cell* **16**, 405-420.
7. Monshausen, M., Gehring, N. H. and Kosik, K. S. (2004) The mammalian RNA-binding protein Staufen2 links nuclear and cytoplasmic RNA processing pathways in neurons. *Neuromolecular Med.* **6**, 127-144.
8. Jeong, J. H., Nam, Y. J., Kim, S. Y., Kim, E. G., Jeong, J. and Kim, H. K. (2007) The transport of Staufen2- containing ribonucleoprotein complexes involves kinesin motor protein and is modulated by mitogen-activated protein kinase pathway. *J. Neurochem.* **102**, 2073-2084.
9. Kim, K. C. and Kim, H. K. (2006) Role of Staufen in dendritic mRNA transport and its modulation. *Neurosci. Lett.* **397**, 48-52.
10. Goetze, B., Tuebing, F., Xie, Y., Dorostkar, M. M., Thomas, S., Pehl, U., Boehm, S., Macchi, P. and Kiebler, M. A. (2006) The brain-specific double-stranded RNA-binding protein Staufen2 is required for dendritic spine morphogenesis. *J. Cell Biol.* **172**, 221-231.
11. Mallardo, M., Deitinghoff, A., Muller, J., Goetze, B., Macchi, P., Peters, C. and Kiebler, M. A. (2003) Isolation and characterization of Staufen-containing ribonucleoprotein particles from rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2100-2105.
12. Kiebler, M. A. and Bassell, G. J. (2006) Neuronal RNA granules: movers and makers. *Neuron* **51**, 685-690.
13. Khatua, S., Peterson, K. M., Brown, K. M., Lawlor, C., Santi, M. R., LaFleur, B., Dressman, D., Stephan, D. A. and MacDonald, T. J. (2003) Overexpression of the EGFR/FKBP12/HIF-2alpha pathway identified in childhood astrocytomas by angiogenesis gene profiling. *Cancer Res.* **63**, 1865-1870.
14. Blazejczyk, M., Miron, M. and Nadon, R. (2007) FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, http://genomequebec.mcgill.ca/FlexArray
15. Al-Shahrour, F., Minguez, P., Vaquerizas, J. M., Conde, L. and Dopazo, J. (2005) BABELOMICS: a suite of web tools for functional annotation and analysis of groups of genes in high-throughput experiments. *Nucleic Acids Res.* **33**, W460-464.
16. Hieronymus, H. and Silver, P. A. (2004) A systems view of mRNP biology. *Genes Dev.* **18**, 2845-2860.
17. Moore, M. J. (2005) From birth to death: the complex lives of eukaryotic mRNAs. *Science* **309**, 1514-1518.
18. Keene, J. D. (2007) RNA regulons: coordination of post-transcriptional events. *Nat. Rev. Genet.* **8**, 533-543.
19. Walsh, C. T., Garneau-Tsodikova, S. and Gatto, G. J., Jr. (2005) Protein posttranslational modifications: the chemistry of proteome diversifications. *Angew. Chem. Int. Ed. Engl.* **44**, 7342-7372.
20. Yang, X. J. (2005) Multisite protein modification and intramolecular signaling. *Oncogene* **24**, 1653-1662.
21. Kim, Y. K., Furic, L., Desgroseillers, L. and Maquat, L. E. (2005) Mammalian Staufen1 recruits Upf1 to specific mRNA 3'UTRs so as to elicit mRNA decay. *Cell* **120**, 195-208.
22. Dugre-Brisson, S., Elvira, G., Boulay, K., Chatel-Chaix, L., Mouland, A. J. and DesGroseillers, L. (2005) Interaction of Staufen1 with the 5' end of mRNA facilitates translation of these RNAs. *Nucleic Acids Res.* **33**, 4797-4812.
23. Kim-Ha, J., Kerr, K. and Macdonald, P. M. (1995) Translational regulation of oskar mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell* **81**, 403-412.
24. Micklem, D. R., Adams, J., Grunert, S. and St Johnston, D. (2000) Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. *EMBO J.* **19**, 1366-1377.
25. Furic, L., Maher-Laporte, M. and DesGroseillers, L. (2008) A genome-wide approach identifies distinct but overlapping subsets of cellular mRNAs associated with Staufen1- and Staufen2-containing ribonucleoprotein complexes. *RNA* **14**, 324-335.
26. Duchaine, T., Wang, H. J., Luo, M., Steinberg, S. V., Nabi, I. R. and DesGroseillers, L. (2000) A novel murine Staufen isoform modulates the RNA content of Staufen complexes. *Mol. Cell Biol.* **20**, 5592-5601.
27. Ryu, N. K., Yang, M. H., Jung, M. S., Jeon, J. O., Kim, K. W. and Park, J. H. (2007) Gene expression profiling of rewarding effect in methamphetamine treated Bax-deficient mouse. *J. Biochem. Mol. Biol.* **40**, 475-485.
28. Yang, M. H., Yoo, K. H., Yook, Y. J., Park, E. Y., Jeon, J. O., Choi, S. H., Park, S. Y., Woo, Y. M., Lee, M. J. and Park, J. H. (2007) The gene expression profiling in murine cortical cells undergoing programmed cell death (PCD) induced by serum deprivation. *J. Biochem. Mol. Biol.* **40**, 277-285.