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Molecular Brain Research 117 (2003) 196–205
MOLECULAR BRAIN RESEARCH
www.elsevier.com/locate/molbrainres

Research report

Changes in hippocampal gene expression after neuroprotective activation of group I metabotropic glutamate receptors

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Accepted 8 July 2003

Abstract

Stimulation of group I metabotropic glutamate receptors (mGluRs) has been shown to protect against *N*-methyl-D-aspartate receptor-mediated cell death, but the underlying cellular mechanism is unknown. Using cDNA microarrays we have now compared gene expressions in organotypic hippocampal slice cultures after neuroprotective activation of group I mGluRs with (*S*)-3,5-dihydroxyphenylglycine (DHPG; 10 μM, 2 h) with untreated control cultures. Total RNA was extracted from the cultures immediately after the neuroprotective treatment, reverse transcribed to cDNA with incorporation of $^{[32]}$P-dCTP, and then hybridized to the arrays. Of a total of 1128 genes on the Neuroarray, 33 genes displayed significant changes in expression after DHPG-treatment (six up- and 27 downregulated). These genes have been associated with regulation of synaptic excitation, inflammation, cell adhesion, cell death, and transcription. The small GTPase RAB5B associated with endocytosis emerged as a primary candidate gene for neuroprotection, and its expression was confirmed by Western blot analysis and real time polymerase chain reaction. By providing insight into genes involved in neuroprotection these data may help to identify novel therapeutic targets.
Published by Elsevier B.V.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Excitatory amino acids: excitotoxicity

Keywords: DHPG; cDNA microarray; Hippocampus; mGluR; Neuroprotection; Organotypic slice culture

1. Introduction

Current understanding of nerve cell death following ischemia includes *N*-methyl-D-aspartate receptor (NMDAR) overactivation as a critical mechanism [1]. This knowledge has, however, not yet resulted in effective treatment or prevention strategies. Although blockade of NMDARs is very effective in reducing ischemic cell death in experimental models [2], the clinical use of this approach has been negligible mainly due to psychotogenic side effects [3] of NMDAR antagonists.

We have shown previously that treatment with the selective group I metabotropic glutamate receptor (mGluR) agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) protects neurons from *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity in a concentration-dependent manner [4]. The neuroprotection occurred after the initial prolonged exposure to the agonist and was associated with inhibition of the NMDAR-mediated inward current [4]. In addition to changes in Ca^{2+} homeostasis, production of second messengers IP3 and DAG and activation of protein kinase C (PKC) associated with mGluR activation (e.g., see Ref. [5] for review), recent reports indicate that group I mGluRs can have effects at gene level [6]. To understand whether

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0169-328X/03/$ – see front matter Published by Elsevier B.V.
doi:10.1016/S0169-328X(03)00321-8

changes in gene expression may be involved in regulation of the group I mGluR-induced neuroprotection, we used the cDNA microarray technique. Our goal was to compare gene expression patterns in DHPG-treated organotypic hippocampal slice cultures with untreated control cultures, using a treatment paradigm known to induce neuroprotection.

2. Materials and methods

2.1. cDNA microarray

Details on the development of the NIA-Neuroarray are available at http://www.grc.nia.nih.gov/branches/rrb/dna.htm, and have been reported by Vawter et al. [7]. The array includes 1128 genes, representing transcription factors, synaptic, neuronal, glial, cell-adhesion molecules, kinases, phosphatases, proteases, oncogenes and structural proteins found in the brain. To ensure array reproducibility each array contains two complete sets of spotted cDNA (duplicate genetic element).

2.2. Tissue samples

Tissue to be tested were 2-week-old organotypic hippocampal slice cultures, derived from 7-day-old Wistar rat pups. Briefly, rats were anesthetized with halothane, decapitated, and the brains rapidly removed and placed into an ice-cold stabilization medium [composition: 50% minimal essential medium (MEM) with no bicarbonate or glutamate, 50% calcium- and magnesium-free Hanks' balanced salt solution, 7.5 mM D-glucose, and 20 mM *N*-2-(hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), pH 7.15]. The middorsal part of the two hippocampi were dissected out and cut into 400 μm transverse slices. The slices were separated and excess tissue removed before being placed on 30 mm Millicel-CM 0.4-μm-thick membrane inserts (Millipore, Bedford, MA, USA). The inserts were transferred to six-well culture plates (Corning Costar, Corning, NY, USA) with 1 ml culture medium in each well (composition: 50% MEM, 25% horse serum, 25% Earl's balanced salt solution, D-glucose, HEPES, 5000 units/ml penicillin G, and 5 μl/ml streptomycin sulfate, pH 7.15, without added glutamate). Slices were cultured at 36.5 °C in 100% humidity, in 95% air/5% CO_2 and fed twice a week via 50% medium exchange. After 2 weeks cultures were divided into two groups. One group was treated with the selective group I mGluR agonist DHPG (10 μM for 2 h), which is known to induce neuroprotection against a subsequent exposure to 50 μM NMDA as well as inhibit NMDA-stimulated currents [4]. The control group was exposed to normal culture medium. Immediately after the 2 h treatment the total RNA was extracted from both groups of cultures.

2.3. Total RNA extraction

Total RNA was extracted from approximately 30 mg of hippocampal slice culture tissue, using an RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the protocol provided by the manufacturer. To determine the total RNA yield, a 1:100 dilution was made in RNase-free H_2O, and the A_{260} and A_{260}/A_{280} ratio obtained using a Beckman spectrophotometer (DU-64, Fullerton, CA, USA). The yield of total RNA was between 70 to 90 μg per 100 mg of brain tissue for all brain samples. A sample of the total RNA (1 μg) was analyzed on a BioAnalyzer for 28S and 18S ribosomal RNA visualization. The resulting 28S and 18S ribosomal bands were visualized as well as any degradation of samples. Furthermore, the purity of the total RNA preparation was assessed with the A_{260}/A_{280} ratio which was greater than 1.9. A pool of total RNA (20 μg) was formed for each group using equal amounts of total RNA from each culture.

2.4. RNA labeling and hybridization

RNA was labelled with $^{[32]}$P-dCTP as previously described [7]. Briefly, total RNA (20 μg from DHPG-treated and control groups) was reverse transcribed to cDNA with reverse transcriptase enzyme in the presence of $^{[32]}$P-dCTP. The $^{[32]}$P-dCTP-cDNA was purified through a spin column by size separation (BioSpin; Bio-Rad, CA, USA) and the heat-denatured probe (~5×10^6 cpm) diluted in 4 ml Microhyb solution (Research Genetics, Carlsbad, CA, USA) and hybridized to the microarray for 16 to 18 h at 50 °C with rotation. Unhybridized probe was removed with two washes with 2× saline sodium citrate at room temperature. The microarray was placed under plastic wrap and exposed to a low-energy phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) for 8 days and scanned in a Phosphorimager 860 (Molecular Dynamics) with 50 μm resolution.

2.5. Statistical analysis

To obtain expression values for each gene on the array, the Phosphorimager scanner data was analyzed using ArrayVision software. Each genetic element was arrayed in duplicate and the binding of each gene was normalized within each array by dividing the total binding of the array and then multiplication by 10^6. The normalized scores were entered into Cyber-T (program available at http://www.igb.uci.edu). Statistical analysis was done using Bayesian approach developed by Baldi and Long [8], and changes were considered significant at $P<0.05$. There were four replicate microarray values for controls and four replicate values for the DHPG pretreatment group.

2.6. Western blot analysis of RAB5B protein levels

Twelve hippocampal slice cultures from each ex-

perimental group (untreated control; 10 μM DHPG treatment; 100 μM DHPG treatment) were weighed and homogenized by sonication (3×20 s) in ice-cold extraction buffer (composition: Tris–HCl 100 mM, EGTA 2.5 mM, EDTA 2.5 mM, sucrose 1.6 mM and mercaptoethanol 0.35%). Samples were adjusted to 50 μg/100 μl, denatured at 95 °C for 4 min, and spun down at 15,000 rpm for 20 s. Equal amounts of adjusted samples were then loaded onto 4–10% polyamide gels, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (75 mA for 2.5 h) and transferred to nitrocellulose membranes. To block nonspecific antibody binding, nitrocellulose membranes were blocked by incubation in 10% dry milk, 1% IgG-free bovine serum albumin (BSA) and 40 μg blocking peptide (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at 4 °C overnight. Visualization of RAB5B protein bands (24 kDa) was done by a 2 h incubation with polyclonal anti-RAB5B antibody (Santa Cruz Biotechnology) diluted 1:100 in TBS-T at room temperature (RT), followed by a 60-min incubation (RT) with HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) diluted 1:1000 in TBS-T. Immunoreactivity was detected by enhanced a chemoluminescence Western blot system (ECL Amersham Pharmacia Biotech), by 1-min exposure to Hyperfilm ECL (Kodak, CA, USA).

2.7. Real time quantitative polymerase chain reaction (PCR)

First strand cDNA from RNeasy-extracted total RNA samples was synthesized in a 50 μl reverse transcriptase reaction according to the protocol supplied by the manufacturer (ABI, Foster City, CA, USA). Briefly, total RNA (2 μg) was equalized with RNase free water to a volume of 19.25 μl, and then combined with reverse transcription reagents (Applied Biosystems) for a total volume of 50 μl. Reagents included 10× Taqman RT buffer (5 μl), 26 mM $MgCl_2$ (11 μl), 10 mM dNTP mix (with dTTP 10 μl total), oligo-dT (2.5 μl), recombinant ribonuclease inhibitor (1 μl), and multiscribe reverse transcriptase (1.3 μl) combined per reaction. The reaction mixture was incubated at 25 °C for 10 min to allow for annealing, at 48 °C for 30 min for the reverse transcription to occur, and then at 95 °C for 5 min to inactivate any enzymatic activity (MJ Research PTC-100). cDNA from this reaction (3 μl) was used in the real time quantitative PCR (Q-PCR).

Real time Q-PCR reactions were carried out in an Applied Biosystems 7000 sequence detection system (ABI) according to the manufacturer's protocol for SYBR® Green PCR using a 25 μl reaction volume. Samples were quantified for RAB5B by comparison to a standard curve generated with known concentrations of PCR product from amplification of rat genomic DNA. The template concentrations for the gDNA standard curve ranged from 10 to 0.001 μg/ml in 10-fold dilution series. A reverse transcription negative (RT−) control was also used to determine the genomic DNA contamination of the starting RNA material. The RT− control samples were also read from the gDNA standard curve. The housekeeping gene, GAPDH, was amplified to determine the integrity and abundance of the starting cDNA. All DHPG, control samples, and RT− samples were run in quadruplicate, and the standards and housekeeping gene samples were run in triplicate. The averages of the corrected concentrations (after subtraction of the RT− contamination) were used in a *t*-test to compare DHPG and control concentrations. The primers used for the RAB5B were:

RAB5B forward 5′-GCAGGGAACAAAGCTGACCTT-3′.

RAB5B reverse 5′-GGAGGATTCTGTAGGCCACCT-TA-3′.

The dissociation curve analysis was examined for each sample and did not contain dimer–primer pairs that would interfere with SYBR green fluorescence. Further the SYBR green master mix (ABI) contains an internal dye used as a reference for the amount of master mix in each sample for normalization of slight variations in pipetting volumes.

3. Results

3.1. Array reproducibility

Comparison of the duplicate genetic elements for each array resulted in a correlation coefficient of $r>0.98$ (Fig. 1A). Images of the array from control and DHPG-treated groups are shown in Fig. 1B.

3.2. Changes in gene expression induced by neuroprotective activation of group I mGluRs

Of the 1128 genes on the Neuroarray, 33 genes were significantly ($P<0.05$) changed as a result of 2-h treatment with the selective group I mGluR agonist DHPG, when compared to untreated controls. From the 33 genes, six were upregulated (fold=1.401 to 1.165), while 27 were downregulated (indicated as negative values; fold=−1.520 to −1.142). Table 1 lists the genes according to the extent of up- or downregulation. Table 2 lists the genes associated with cell death and survival, grouped in five functional categories. The basis for the grouping of the genes and their putative role in neuroprotection are discussed below.

3.3. Western blot analysis of RAB5B protein levels

RAB5B, coding for Ras-associated protein (RAB5B) which is a ubiquitous, small GTPase that facilitates endocytosis [9], was upregulated by DHPG-treatment. RAB5 is essential for the transport of clathrin coated vesicles from the plasma membrane to early endosome, attachment of the vesicles to microtubules, as well as their motility along microtubules [10]. Both NMDA and α-

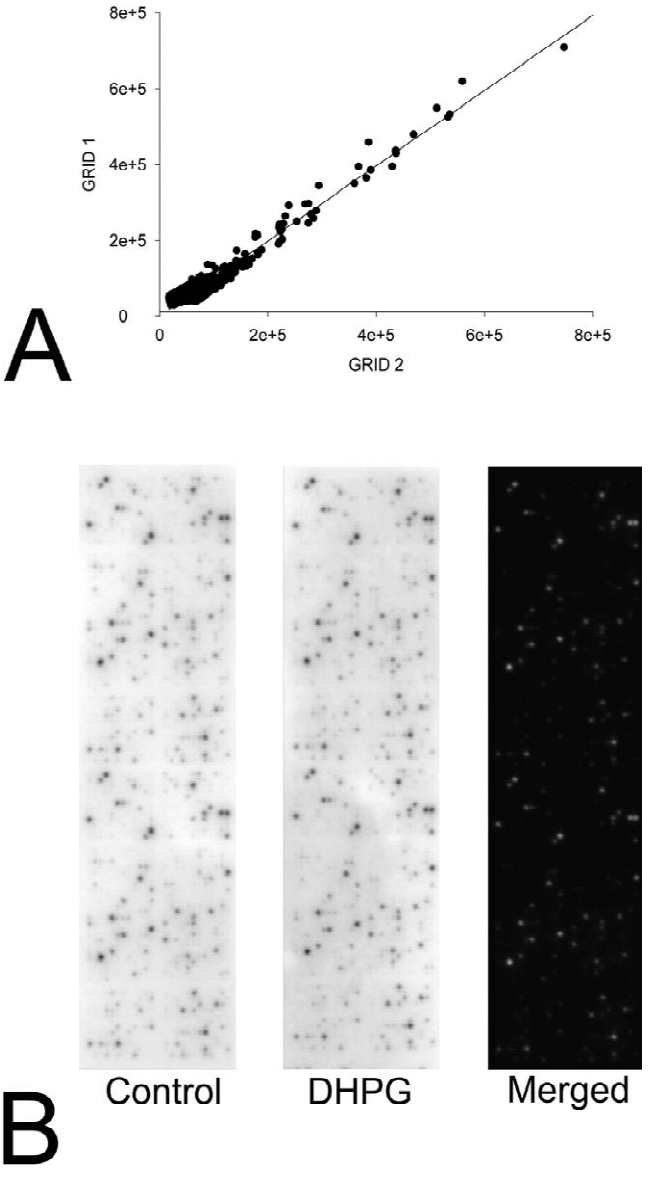

Fig. 1. Correlation of duplicate binding on gene array. (A) To ensure stable binding of the cDNA to the arrays, the duplicate genetic element (grids 1 and 2) of a control array was correlated. This resulted in a correlation coefficient of $r>0.98$. (B) Examples of hybridized arrays from control and DHPG-treated groups. An inverted merged image of controls (green) and DHPG-treated (red) groups illustrates changes in gene expression.

amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are internalized by a clathrin-dependent mechanism [11,12], raising the possibility that DHPG-induced increase in RAB5B could lead to internalization of NMDA and AMPA receptors. Such internalization has been reported to occur in primary hippocampal cultures treated with DHPG [13] and could explain the neuroprotection and reduction of NMDA-mediated currents after a neuroprotective DHPG-treatment [4]. To investigate whether RAB5B was also upregulated on the protein level, we performed Western blotting experiments. Cultures were treated with 10 and 100 μM DHPG for 2 h, the treatment paradigm known to result in concentration-dependent neuroprotection [4]. Western blot analysis performed immediately after the treatment revealed upregulated RAB5B protein levels in a concentration-dependent manner (Fig. 2B).

3.4. Real time quantitative PCR for RAB5B

A standard curve for real time PCR analysis of RAB5B showed a linear response across a 0.001–10 μg/ml range ($r=0.99$). The concentrations for each sample was read from the standard curve and further corrected by the concentration for the RT− samples in each group. After correction, the DHPG pretreatment group showed higher concentration of RAB5B mRNA (0.22 μg/ml±0.04 S.E.M.) compared to the control group (0.05 μg/ml±0.03 S.E.M.). This difference was highly significant ($P=0.012$). DHPG pretreatment increased the RAB5B mRNA by 5.5-fold (Fig. 2A). There was no significant difference for the housekeeping gene GAPDH concentrations between the DHPG pretreatment (27.0 μg/ml±5.8 S.E.M.) and the control group (36.5 μg/ml±2.0 S.E.M.) after correction for the RT− sample ($P=0.17$).

4. Discussion

The present study identified genes associated with the response of brain tissue to the selective group I mGluR agonist DHPG. Our earlier experiments [4] and reports by other researchers [14] have shown that DHPG treatment can reduce ischemic brain tissue damage, making it likely that some of the regulated genes could be involved in neuroprotection. To pursue this possibility of identifying potential candidate genes for neuroprotection, we selected, from the genes listed in Table 1, a group of genes with known functions and divided them into the following five categories: (A) genes associated with regulation of excitatory and inhibitory synaptic transmission, (B) genes associated with regulation of inflammatory processes, (C) genes associated with cell adhesion, (D) genes associated with regulation of cell death and (E) genes associated with regulation of transcription (Table 2).

4.1. Genes associated with regulation of excitatory and inhibitory synaptic transmission

RAB5B, coding for Ras-associated protein (RAB5B) which is a ubiquitous, small GTPase that facilitates endocytosis [9], was upregulated by DHPG-treatment. Activated RAB5 is known to interact with hVPS34-p150 phosphoinositol-3-OH kinase (PI3K) and produce phosphatidylinositol-3-phosphate [PtdIns(3)P], which facilitates tethering of the protein early endosomal autoantigen 1 (EEA1). RAB5 is essential for the transport of

Table 1
Changes in gene expression after neuroprotective activation of group I metabotropic glutamate receptors

Gene symbol	Gene name	Fold change	*P* value	Class
CNTN6	Contactin 6	1.401	0.004	Neural adhesion molecule
RAB18	Ras-associated protein RAB18	1.317	0.037	Ras-related small GTPase
HTR2B	5-Hydroxytryptamine (serotonin) receptor 2B	1.262	0.007	Serotonin receptor 2B
E2F1	E2F transcription factor 1	1.216	0.008	Transcription factor
RAB5B	Ras-associated protein RAB5B	1.214	0.014	Ras-related small GTPase
RPMS12	Ribosomal protein, mitochondrial, S12	1.165	0.033	Ribosomal protein S12
IL2RG	Interleukin 2 receptor, gamma	−1.530	0.033	Lymphokine IL2-receptor subunit
BMP1	Bone morphogenetic protein 1	−1.497	0.017	Metalloprotease, member of TGFbeta superfamily
PRKCBP2	Protein kinase C binding protein 2	−1.493	0.024	Oligodendrocyte lineage transcription factor 2
BMP7	Bone morphogenetic protein 7	−1.452	0.031	Metalloprotease, member of TGFbeta superfamily
SMARCA5	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5	−1.404	0.003	Regulator of chromatin
HOX11	Homeo box 11	−1.401	0.043	Homeo box
HOXD3	Homeo box D3	−1.392	0.028	Homeo box
GNA15	Guanine nucleotide binding protein, alpha 15	−1.388	0.023	G-protein (Gq-class)
COL6A3	Collagen, type VI, alpha 3	−1.388	0.037	Collagen
PIGK	Phosphatidylinositol glycan, class K	−1.387	0.037	Glycosylphosphatidylinositol (GPI) anchor protein
RELN	Reelin	−1.373	0.022	Extracellular adhesion protein
PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	−1.368	0.390	Phosphatidylinositol 3-kinase subunit
DTNA	Dystrobrevin, alpha	−1.362	0.029	Sarcolemma protein
UBCH10	Ubiquitin-conjugating enzyme E2-C	−1.359	0.033	Regulator of mitosis
SRF	Serum response factor	−1.356	0.023	DNA essential for transcription of growth factors
IL2RB	Interleukin 2 receptor, beta	−1.342	0.024	Lymphokine IL2-receptor subunit
CUL3	Cullin 3	−1.330	0.041	Regulator of protein degradation
RAP2A	RAP2A, member of RAS oncogene family	−1.323	0.029	Ras-associated protein
GABARAP	GABA(A) receptor-associated protein	−1.318	0.015	Protein linking GABA(A)-receptors to cytoskeleton
KCNJ13	Potassium inwardly-rectifying channel, subfamily J, member 13	−1.315	0.015	Inward-rectifying potassium channel
GRIK2	Kainate receptor GluR6	−1.294	0.023	Kainate receptor GluR6
SEMA3C	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	−1.258	0.038	Secreted regulator of growth cone
RAB2	RAB2, member RAS oncogene family	−1.241	0.030	Ras-associated protein
HPK1	Hematopoietic progenitor kinase	−1.227	0.032	Mitogen-activated protein kinase kinase kinase kinase
PI7	Protease inhibitor 7	−1.207	0.043	Glial-derived neurite promoting factor
ITGA3	Integrin, alpha 3 subunit	−1.174	0.045	Integrin receptor subunit
ITGAX	Integrin, alpha X, alpha polypeptide	−1.142	0.034	Integrin receptor subunit

clathrin coated vesicles from the plasma membrane to early endosome, attachment of the vesicles to microtubules, as well as their motility along microtubules [10]. Both NMDA and AMPA receptors are internalized by a clathrin-dependent mechanism [11,12], raising the possibility that DHPG-induced increase in RAB5B could lead to internalization of NMDA and AMPA receptors. Such internalization has been reported to occur in primary hippocampal cultures treated with DHPG [13] and could explain the neuroprotection and reduction of NMDA-mediated currents after a neuroprotective DHPG-treatment [4]. Alternatively, downregulation of NMDA receptors or their subunits by DHPG could provide explanation of the NMDA current reduction; this explanation however, is not supported by unchanged expression of NMDAR subunits (NR1, NR2A and NR2C) on the array. Our preliminary experiments with antisense oligonucleotides against RAB5B showed a reduced DHPG neuroprotective effect in antisense- but not scrambled-oligonucleotide-treated cultures [15]. Western blot analysis showed that DHPG in a concentration-dependent manner increased the expression of RAB5B protein in the hippocampus. RAB5B mRNA was also measured by a quantitative real time PCR and shown to be increased by 5.5-fold following DHPG pretreatment. Taken together, these data suggest that upregulation of RAB5B mRNA and protein, and internalization of NMDARs, may represent a fast and efficient mechanism to regulate such NMDA-dependent processes as cell death.

GRIK2 (kainate receptor, GluR6) which codes for the kainate receptor subunit GluR6, was downregulated by DHPG-treatment. Genes for other kainate receptor subunits (KA1, KA2) on the array were not affected. How could GRIK2 downregulation facilitate neuroprotection? Application of low concentrations of kainate have been shown to enhance synaptic transmission in perforant path synapses,

Table 2
Grouping of genes into classes with possible putative neuroprotective effects

Gene symbol	Gene name	Fold change	*P* value	Putative function in neuroprotection [Refs.]
(A) Genes associated with regulation of excitatory and inhibitory synaptic excitation				
RAB5B	RAB5B, member RAS oncogene family	1.214	0.014	Internalization of NMDA-receptors [9–13]
GRIK2	Kainate receptor GluR6	−1.294	0.023	Inhibition of excitatory synaptic transmission [16,17]
GABARAP	GABA(A) receptor-associated protein	−1.318	0.015	Unclustering of GABA(A)-receptors, increased inhibition [20]
(B) Genes associated with inflammatory processes				
IL2RG	Interleukin 2 receptor, gamma	−1.530	0.033	Reduction of inflammation, reduction of NMDA-mediated excitotoxicity [21,22,25,26]
IL2RB	Interleukin 2 receptor, beta	−1.342	0.024	Reduction of inflammation, reduction of NMDA-mediated excitotoxicity [21,22,25,26]
(C) Cell adhesion and anchor genes				
ITGA3	Integrin, alpha 3 subunit	−1.174	0.045	Inhibition of NMDA-receptor maturation, reduced activation of MAPK cascades [30–32]
ITGAX	Integrin, alpha X, alpha polypeptide	−1.142	0.034	Inhibition of NMDA-receptor maturation, reduced activation of MAPK cascades [30–32]
CNTN6	Contactin 6	1.401	0.004	Facilitation of long term depression [33]
(D) Genes associated with cell death				
SEMA3C	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	−1.258	0.038	Reduced glutamate-stimulated NMDA-receptor activation [34]
DTNA	Dystrobrevin, alpha	−1.362	0.029	Reduced NOS signaling [35,36]
HPK1	Hematopoietic progenitor kinase	−1.227	0.032	Inhibition of JNK/SAPK pathways [44,45]
BMP1	Bone morphogenetic protein 1	−1.497	0.017	Decreased formation of synapses, reduced activation of MAPK cascades [32,48,49]
BMP7	Bone morphogenetic protein 7	−1.452	0.031	Decreased formation of synapses, reduced activation of MAPK cascades [32,48,49]
(E) Genes associated with transcription				
E2F1	E2F transcription factor 1	1.216	0.008	Repression of E2F-responsive genes [50–52]

but this effect was absent in GluR6-deficient mice [16]. Long-term potentiation (LTP) was also reduced and short-term facilitation impaired in mossy fibers lacking GluR6 [17]. It is therefore possible that the DHPG-induced reduction of GRIK2 expression could reduce the excitability of CA3 and dentate granule cells by reducing LTP and preventing facilitation of synaptic transmission contributing to neuroprotection.

GABARAP coding for GABA-A receptor-associated protein, which is a member of membrane trafficking and/or fusion protein family [18], was downregulated by DHPG-treatment. It binds to the γ-2 subunit of the GABA-A receptor, and is ubiquitously expressed in the brain [19]. It has been suggested that GABARAP may be involved in intraneuronal receptor sorting and targeting that precedes and/or initiates receptor clustering at the synapse [20]. Clustering of GABA-A receptors can dramatically modulate kinetic properties of these receptors and significantly reduce affinity for GABA ([20] and Refs. therein). DHPG-induced downregulation of GABARAP may contribute to neuroprotection by unclustering of GABA-A receptors with resulting increase in receptor affinity for GABA and possibly decreased excitability of neurons.

4.2. Genes associated with inflammatory processes

IL2RB and IL2RG coding for Interleukin 2 receptor β and γ subunits (also known as CD122 and CD132, respectively) were downregulated by DHPG-treatment. It is important that upregulation of these genes has been associated with brain injury (for reviews see Refs. [21] and [22]). The IL2γ-receptor subunit is shared by 4 interleukin receptor complexes: IL4, IL7, IL9, and IL15 [23], whereas the IL2β-receptor subunit is shared with IL15. The chemokine IL2 has been found to potentiate NMDAR-mediated currents in mesolimbic neurons [24] and stimulate neurotoxic nitric oxide (NO) production [25]. Moreover, IL9 has been found to potentiate excitotoxic injury in newborn pups [26]. The potentiation of excitotoxicity via IL2 receptors may be a significant contributor to NMDA-induced cell death, which, if suppressed, may reduce NMDA-mediated toxicity.

4.3. Cell adhesion and anchor genes

ITGA3 and ITGAX coding for the α3 and an α subunit of the integrin receptor proteins, were downregulated by

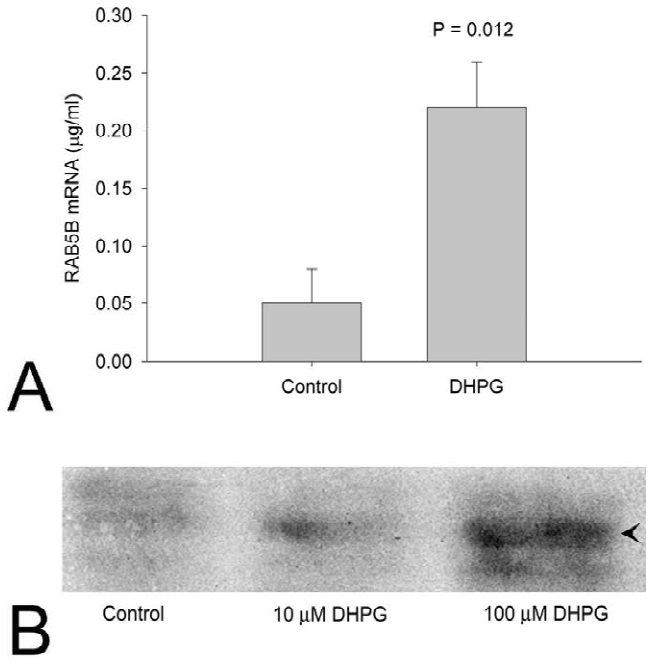

Fig. 2. Real time PCR and Western blot analysis of RAB5B levels. (A) Quantitative real time PCR analysis of untreated cultures (control) and cultures treated for 2 h with 10 μM DHPG. Note significant ($P=0.012$, *t*-test) increase in RAB5B mRNA levels (5.5 fold) from 0.05 ± 0.03 to 0.22 ± 0.04 μg/ml. (B) Western blot analysis of RAB5B protein levels after 2 h DHPG (10 μM and 100 μM) treatment. Note a concentration-dependent increase in the RAB5B band intensity (arrowhead) in cultures treated with DHPG in comparison compared to untreated (control) cultures.

DHPG and are involved in cell–cell and cell–matrix interactions, respectively [27]. Integrins are abundantly expressed in hippocampal neurons and glial cells [28]. Most integrins possess an Arg–Gly–Asp (RGD) binding site, recognized by a number of extracellular matrix and cell surface proteins [29]. Blockade of this site with synthetic peptides inhibits integrin binding, prevents maturation of NMDARs, and reverses hippocampal LTP [30]. In addition, the proteins also activate intracellular signaling pathways such as focal adhesion kinase (FAK), Src, and the MAPK-cascade [31]. Inhibition of MAPK is known to protect neurons from NMDA toxicity [32]. These data support the idea that inhibition of integrin signaling by DHPG may contribute to neuroprotection.

CNTN6 coding for Contactin 6 (also known as NB3), was upregulated by DHPG and is a glycosylphosphatidylinositol (GPI)-anchored neural cell adhesion molecule belonging to the immunoglobulin superfamily. Although most cell adhesion molecules are thought to be involved in LTP (see Ref. [33] and references therein), contactin-deficient mutant mice display normal LTP, but no long-term depression (LTD; [33]). Assuming that Contactin 6 has the same function in rat as it does in mouse, upregulation of this gene could facilitate induction of LTD, which could lead to reduction of synaptic excitation and likely contribute to neuroprotection.

4.4. Genes associated with cell death

SEMA3C coding for Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, semaphoring 3C (also known as semaphorin E and collapsin) was downregulated by DHPG-treatment. Interaction of semaphorin with their receptors induces cell death [34] and it is likely, that downregulation of SEMA3C by DHPG may increase neuronal survival.

DTNA coding for α-dystrobrevin [also known as dystrobrevin or dystrotrophin-related protein 3 (DRP3) and D18S892E] was downregulated by DHPG. DTNA is found in the brain [35] and is a component of the dystrophin-containing glycoprotein complex (DGC) associated with nitric oxide synthase (NOS) signaling. In the absence of DTNA, NOS signaling, which is associated with increased cell death, is impaired [36]. A review of the literature reveals that NOS have probably a dual role in cell survival. Exposure to exogenous NO could be toxic, it could also be toxic when its induction is stimulated, e.g., by high glucose concentration; in that situation, L-NAME, a NOS inhibitor that reduced NO production, increased survival [37] but had no effect on the background level of cell death. Similar survival promoting effects of NOS inhibitors on TNF-alpha/lipopolysacharide-induced differentiated PC12 cell death were reported by Heneka et al. [38]. In primary cultures of rat cortical neurons NOS inhibitors were found to protect neurons against L-glutamate (500 μM)-induced toxicity [39]. Studies on NMDA induced toxicity were less conclusive and there are reports on NOS inhibitor-mediated neuroprotection [40] as well as lack of it [41]. A competitive NOS inhibitor rescued a significant proportion of cortical neurons subjected to anoxia [43]. However, NO producing compounds were found to promote survival of naïve PC12 cells and in medium lacking serum or neuronally differentiated PC12 cells in medium lacking neuronal growth factor (NGF) [42]. Together these data lend support to the idea that DTNA downregulation may contribute to DHPG-mediated neuroprotection.

HPK1 coding for hematopoietic progenitor kinase (also known as mitogen-activated protein kinase kinase kinase kinase; MAP4K) was downregulated by DHPG-treatment. HPK1 activates the JNK/SAPK kinase pathway [44] which leads to cell death [45]. It is not unlikely that downregulation of the HPK1 expression could be neuroprotective.

BMP1 and BMP7 coding for bone morphogenetic proteins 1 and 7 were downregulated by DHPG. BMPs belong to the transforming growth factor-β (TGF-β) superfamily and are involved in cellular growth, differentiation, and repair in many tissues [46]. BMP-receptors are abundantly expressed in the hippocampus [47]. Their signaling pathways include stimulatory modification of

Smad proteins and activation of RAS, RAF, and, subsequently, MEK1/2 and ERK1/2 [48]. Activation of MEK1/2 and ERK1/2 are associated with NMDAR stimulation [49] and inhibition is neuroprotective [32]. Decreased BMP expression seen in our experiments may inhibit NMDA-induced ERK1/2 activation and contribute to neuroprotection. Heteromeric BMP proteins have higher activity than homomeric proteins, thus, inhibition of BMP1 and BMP7 expression may have a greater impact than would inhibition of a single BMP subtype.

4.5. Genes associated with transcription

E2F1 coding for E2F transcription factor 1, which was upregulated by DHPG, binds and inactivates retinoblastoma gene product associated with apoptosis and reduces infarct size following cerebral ischemia [50,51]. E2F is a gene silencer in neurons and repression of E2F-responsive genes may be required for neuronal survival because neuronal death due to DNA damage or growth factor withdrawal was associated with reversal of repression (derepression) of E2F-responsive genes [52]. Results of our study are consistent with the model whereby repression and derepression of the E2F-dependent genes (B- and C-myb) is associated with neuronal survival and death [52].

4.6. Methodological considerations

We used cDNA microarray analysis to examine genes associated with DHPG-mediated neuroprotection in organotypic hippocampal cultures. There were 33 genes that were up- or downregulated following a 2-h DHPG treatment of the organotypic hippocampal cultures. The change in the gene expression profile does not pretend to be comprehensive, nor complete, as genes that might have been affected by group I mGluR activation were not necessarily on the array, despite its content of more than 1,100 brain-related genes. Moreover, changes in gene expression reported here represent one particular time point excluding genes that are potentially regulated later. The concentration of DHPG used in this study is consistent with the drug concentration used in other neuroprotection studies [4] and is optimally neuroprotective.

As described in the Materials and Methods section we chose the Bayesian 'rationalized' *t*-test approach for statistical analysis which allows supplementing the weak empirical estimates of single-gene variances across a small number of replicates with more robust estimates of variance obtained by pooling genes with similar expression levels [53]. Results of simulations and comparison of data analyzed with a two-fold expression cut-off and the Bayesian method suggest that large fold changes in expression were often associated with *P*-values not indicative of statistical change in Bayesian analysis, and conversely, subtle fold changes were often highly significant as judged by the Bayesian analysis [8]. Therefore, small but highly significant changes seen in our study may be a reflection of the statistical analysis method we used. Analysis of one candidate gene (which showed a modest increase on microarray) using two independent methods of validation, showed that it was highly upregulated both at the mRNA level and protein level, and lends further support to this idea.

It should also be noted that several caveats apply for interpretation of the present results. First, post-transcriptional changes (e.g., immediate changes in phosphorylation and compartmentalization of proteins) are not measured by microarray. Second, the steady-state level of mRNA is assumed to be measured by microarray, since the present experimental conditions do not determine whether the rates of gene transcription or gene stability are altered during neuroprotection. Nonetheless, by screening a large number of brain-relevant genes, we can determine parallel neuroprotective mechanisms unlikely to be found by classical one gene at a time approach.

5. Concluding remarks

RAB5B, which is highly expressed in the brain [54] emerged as a main candidate gene that could contribute to neuroprotection possibly through regulation of membrane trafficking [55]. It is possible that DHPG-induced increase in RAB5B protein could lead to internalization of NMDA and AMPA receptors. Such internalization has been reported to occur in primary hippocampal cultures treated with DHPG [13] and might explain the neuroprotection and reduction of NMDA-mediated currents after a neuroprotective DHPG-treatment observed previously [4]. The role of RAB5B in DHPG-mediated neuroprotection is supported by quantitative real time PCR and Western blot data showing RAB5B mRNA and protein upregulation after pre-treatment with DHPG, as well as our preliminary findings from antisense experiments showing lack of DHPG-mediated neuroprotection in anti-RAB5B-treated cultures [15]. Other genes with no apparent connection to each other seem to act in concert to achieve neuroprotection. These included genes associated with regulation of neuronal excitability, NOS signaling, and reduction in MAPK activation. We conclude that neuroprotection by group I mGluR activation is not restricted to a single pathway but may involve multiple mechanisms acting in concert.

Acknowledgements

The study was supported by VISN 22 MIRECC (A.B.), the Penzner Foundation (M.P.V.), the Fuhrmann Foundation, the Codan Foundation, the FP5-EU grant (QLK3-CT-2001-00407) and the Faculty of Health Sciences, Universi-

ty of Southern Denmark, Odense (M.B.). Dr. Kevin G. Becker, DHHS, NIH, National Institute of Aging, Baltimore, MD, USA, is gratefully acknowledged for providing the Neuroarrays. The authors acknowledge Liwei Fang for tissue culture preparation and Western blot analysis as well as technical help by Ryan Vu and Kevin Overman.

References

[1] D.W. Choi, S.M. Rothman, The role of glutamate neurotoxicity in hypoxic–ischemic neuronal death, Annu. Rev. Neurosci. 13 (1990) 171–182.
[2] J.J. Vornov, R.C. Tasker, J.T. Coyle, Delayed protection by MK-801 and tetrodotoxin in a rat organotypic hippocampal culture model of ischemia, Stroke 25 (1994) 457–464.
[3] J.H. Krystal, A. Anand, B. Moghaddam, Effects of NMDA receptor antagonists: implications for the pathophysiology of schizophrenia, Arch. Gen. Psychiatry 59 (2002) 663–664.
[4] M. Blaabjerg, Neuroprotection against NMDA excitotoxicity by group I metabotropic glutamate receptors is associated with reduction of NMDA stimulated currents, Exp. Neurol., in press.
[5] B.S. Meldrum, Glutamate as a neurotransmitter in the brain: review of physiology and pathology, J. Nutr. 130 (2000) 1007S–1015S.
[6] L. Mao, J.Q. Wang, Group I metabotropic glutamate receptor-mediated calcium signalling and immediate early gene expression in cultured rat striatal neurons, Eur. J. Neurosci. 17 (2003) 741–750.
[7] M.P. Vawter, T. Barrett, C. Cheadle, B.P. Sokolov, W.H. Wood III, D.M. Donovan, M. Webster, W.J. Freed, K.G. Becker, Application of cDNA microarrays to examine gene expression differences in schizophrenia, Brain Res. Bull. 55 (2001) 641–650.
[8] P. Baldi, A.D. Long, A Bayesian framework for the analysis of microarray expression data: regularized *t*-test and statistical inferences of gene changes, Bioinformatics 17 (2001) 509–519.
[9] M.J. de Hoop, L.A. Huber, H. Stenmark, E. Williamson, M. Zerial, R.G. Parton, C.G. Dotti, The involvement of the small GTP-binding protein Rab5a in neuronal endocytosis, Neuron 13 (1994) 11–22.
[10] M. Zerial, H. McBride, Rab proteins as membrane organizers, Nat. Rev. Mol. Cell Biol. 2 (2001) 107–117.
[11] H.Y. Man, J.W. Lin, W.H. Ju, G. Ahmadian, L. Liu, L.E. Becker, M. Sheng, Y.T. Wang, Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization, Neuron 25 (2000) 649–662.
[12] K.W. Roche, S. Standley, J. McCallum, L.C. Dune, M.D. Ehlers, R.J. Wenthold, Molecular determinants of NMDA receptor internalization, Nat. Neurosci. 4 (2001) 794–802.
[13] E.M. Snyder, B.D. Philpot, K.M. Huber, X. Dong, J.R. Fallon, M.F. Bear, Internalization of ionotropic glutamate receptors in response to mGluR activation, Nat. Neurosci. 4 (2001) 1079–1085.
[14] U.H. Schroder, T. Opitz, T. Jager, C.F. Sabelhaus, J. Breder, K.G. Reymann, Protective effect of group I metabotropic glutamate receptor activation against hypoxic/hypoglycemic injury in rat hippocampal slices: timing and involvement of protein kinase C, Neuropharmacology 38 (1999) 209–216.
[15] A. Arnett, L. Fang, R. Vu, C. Reist, A. Baskys, Inhibition of DHPG-induced neuroprotection with antisense oligonucleotides against RAB5B, Soc. Neurosci. Abstr. (2003).
[16] A. Contractor, G.T. Swanson, A. Sailer, S. O'Gorman, S.F. Heinemann, Identification of the kainate receptor subunits underlying modulation of excitatory synaptic transmission in the CA3 region of the hippocampus, J. Neurosci. 20 (2000) 8269–8278.
[17] A. Contractor, G. Swanson, S.F. Heinemann, Kainate receptors are involved in short- and long-term plasticity at mossy fiber synapses in the hippocampus, Neuron 29 (2001) 209–216.
[18] V.N. Bavro, M. Sola, A. Bracher, M. Kneussel, H. Betz, W. Weissenhorn, Crystal structure of the GABA(A)-receptor-associated protein, GABARAP, EMBO Rep. 3 (2002) 183–189.
[19] J.E. Coyle, S. Qamar, K.R. Rajashankar, D.B. Nikolov, Structure of GABARAP in two conformations: implications for GABA(A) receptor localization and tubulin binding, Neuron 33 (2002) 63–74.
[20] M.B. Kennedy, Sticking together, Proc. Natl. Acad. Sci. USA 97 (2000) 11135–11136.
[21] G.Z. Feuerstein, X. Wang, F.C. Barone, Inflammatory gene expression in cerebral ischemia and trauma. Potential new therapeutic targets, Ann. N. Y. Acad. Sci. 825 (1997) 179–193.
[22] B. Zhao, J.P. Schwartz, Involvement of cytokines in normal CNS development and neurological diseases: recent progress and perspectives, J. Neurosci. Res. 52 (1998) 7–16.
[23] J.M. Petitto, Z. Huang, M.K. Raizada, C.M. Rinker, D.B. McCarthy, Molecular cloning of the cDNA coding sequence of IL-2 receptor-gamma (gammac) from human and murine forebrain: expression in the hippocampus in situ and by brain cells in vitro, Brain Res. Mol. Brain Res. 53 (1998) 152–162.
[24] J.H. Ye, L. Tao, S.S. Zalcman, Interleukin-2 modulates *N*-methyl-D-aspartate receptors of native mesolimbic neurons, Brain Res. 894 (2001) 241–248.
[25] L. Brunetti, Nitric oxide: a gas as a modulator of neuroendocrine secretions, Clin. Ter. 144 (1994) 147–153.
[26] M.A. Dommergues, J. Patkai, J.C. Renauld, P. Evrard, P. Gressens, Proinflammatory cytokines and interleukin-9 exacerbate excitotoxic lesions of the newborn murine neopallium, Ann. Neurol. 47 (2000) 54–63.
[27] A.E. Aplin, A. Howe, S.K. Alahari, R.L. Juliano, Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins, Pharmacol. Rev. 50 (1998) 197–263.
[28] J.K. Pinkstaff, J. Detterich, G. Lynch, C. Gall, Integrin subunit gene expression is regionally differentiated in adult brain, J. Neurosci. 19 (1999) 1541–1556.
[29] E. Ruoslahti, RGD and other recognition sequences for integrins, Annu. Rev. Cell Dev. Biol. 12 (1996) 697–715.
[30] P. Chavis, G. Westbrook, Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse, Nature 411 (2001) 317–321.
[31] D.D. Schlaepfer, T. Hunter, Integrin signalling and tyrosine phosphorylation: just the FAKs?, Trends Cell Biol. 8 (1998) 151–157.
[32] S.D. Skaper, L. Facci, P.J. Strijbos, Neuronal protein kinase signaling cascades and excitotoxic cell death, Ann. N. Y. Acad. Sci. 939 (2001) 11–22.
[33] K.K. Murai, D. Misner, B. Ranscht, Contactin supports synaptic plasticity associated with hippocampal long-term depression but not potentiation, Curr. Biol. 12 (2002) 181–190.
[34] C. Fankhauser, R.M. Friedlander, V. Gagliardini, Prevention of nuclear localization of activated caspases correlates with inhibition of apoptosis, Apoptosis 5 (2000) 117–132.
[35] D.J. Blake, R. Hawkes, M.A. Benson, P.W. Beesley, Different dystrophin-like complexes are expressed in neurons and glia, J. Cell Biol. 147 (1999) 645–658.
[36] R.M. Grady, R.W. Grange, K.S. Lau, M.M. Maimone, M.C. Nichol, J.T. Stull, J.R. Sanes, Role for alpha-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies, Nat. Cell Biol. 1 (1999) 215–220.
[37] K. Koshimura, J. Tanaka, Y. Murakami, Y. Kato, Involvement of nitric oxide in glucose toxicity on differentiated PC12 cells: prevention of glucose toxicity by tetrahydrobiopterin, a cofactor for nitric oxide synthase, Neurosci. Res. 43 (2002) 31–38.
[38] M.T. Heneka, P.A. Loschmann, M. Gleichmann, M. Weller, J.B. Schulz, U. Wullner, T. Klockgether, Induction of nitric oxide synthase and nitric oxide-mediated apoptosis in neuronal PC12 cells after stimulation with tumor necrosis factor-alpha/lipopolysaccharide, J. Neurochem. 71 (1998) 88–94.
[39] X. Vige, A. Carreau, B. Scatton, J.P. Nowicki, Antagonism by

NG-nitro-L-arginine of L-glutamate-induced neurotoxicity in cultured neonatal rat cortical neurons. Prolonged application enhances neuroprotective efficacy, Neuroscience 55 (1993) 893–901.
[40] V.L. Dawson, T.M. Dawson, E.D. London, D.S. Bredt, S.H. Snyder, Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures, Proc. Natl. Acad. Sci. USA 88 (1991) 6368–6371.
[41] S.J. Hewett, J.A. Corbett, M.L. McDaniel, D.W. Choi, Inhibition of nitric oxide formation does not protect murine cortical cell cultures from *N*-methyl-D-aspartate neurotoxicity, Brain Res. 625 (1993) 337–341.
[42] S.E. Farinelli, D.S. Park, L.A. Greene, Nitric oxide delays the death of trophic factor-deprived PC12 cells and sympathetic neurons by a cGMP-mediated mechanism, J. Neurosci. 16 (1996) 2325–2334.
[43] K. Maiese, I. Boniece, D. DeMeo, J.A. Wagner, Peptide growth factors protect against ischemia in culture by preventing nitric oxide toxicity, J. Neurosci. 13 (1993) 3034–3040.
[44] M.C. Hu, W.R. Qiu, X. Wang, C.F. Meyer, T.H. Tan, Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade, Genes Dev. 10 (1996) 2251–2264.
[45] Y. Luo, H. Umegaki, X. Wang, R. Abe, G.S. Roth, Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway, J. Biol. Chem. 273 (1998) 3756–3764.
[46] D.I. Israel, J. Nove, K.M. Kerns, R.J. Kaufman, V. Rosen, K.A. Cox, J.M. Wozney, Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo, Growth Factors 13 (1996) 291–300.
[47] D.A. Charytoniuk, E. Traiffort, E. Pinard, O. Issertial, J. Seylaz, M. Ruat, Distribution of bone morphogenetic protein and bone morphogenetic protein receptor transcripts in the rodent nervous system and up-regulation of bone morphogenetic protein receptor type II in hippocampal dentate gyrus in a rat model of global cerebral ischemia, Neuroscience 100 (2000) 33–43.
[48] M. Melnick, H. Chen, Z.Y. Min, T. Jaskoll, The functional genomic response of developing embryonic submandibular glands to NF-kappaB inhibition, BMC Dev. Biol. 1 (2001) 15.
[49] J.D. English, J.D. Sweatt, A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation, J. Biol. Chem. 272 (1997) 19103–19106.
[50] S.T. Hou, D. Callaghan, M.C. Fournier, I. Hill, L. Kang, B. Massie, P. Morley, C. Murray, I. Rasquinha, R. Slack, J.P. MacManus, The transcription factor E2F1 modulates apoptosis of neurons, J. Neurochem. 75 (2000) 91–100.
[51] T.F. Gendron, G.A. Mealing, J. Paris, A. Lou, A. Edwards, S.T. Hou, J.P. MacManus, A.M. Hakim, P. Morley, Attenuation of neurotoxicity in cortical cultures and hippocampal slices from E2F1 knockout mice, J. Neurochem. 78 (2001) 316–324.
[52] D.X. Liu, L.A. Greene, Regulation of neuronal survival and death by E2F-dependent gene repression and derepression, Neuron 32 (2001) 425–438.
[53] G.W. Hatfield, S.P. Hung, P. Baldi, Differential analysis of DNA microarray gene expression data, Mol. Microbiol. 47 (2003) 871–877.
[54] C. Bucci, A. Lutcke, O. Steele-Mortimer, V.M. Olkkonen, P. Dupree, M. Chiariello, C.B. Bruni, K. Simons, M. Zerial, Co-operative regulation of endocytosis by three Rab5 isoforms, FEBS Lett. 366 (1995) 65–71.
[55] J. Callaghan, S. Nixon, C. Bucci, B.H. Toh, H. Stenmark, Direct interaction of EEA1 with Rab5b, Eur. J. Biochem. 265 (1999) 361–366.