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Identification of Genes Regulated by Memantine and MK-801 in Adult Rat Brain by cDNA Microarray Analysis

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In this study, we monitored gene expression profiles using cDNA microarrays after an acute systemic administration of the high affinity *N*-methyl-D-aspartate (NMDA) uncompetitive antagonist MK-801 (1 mg/kg; 4 h), and the clinically used moderate affinity antagonist memantine (25 mg/kg; 4 h) in adult rat brains. From a microarray containing 1090 known genes, 13 genes were regulated by both treatments of which 12 were upregulated and one was downregulated. In addition, 28 and 34 genes were regulated ($\geqslant$1.5- or $\leqslant$0.67-fold change) by either memantine or MK-801, respectively. Genes commonly regulated by both treatments and not previously reported were confirmed by *in situ* hybridization (ISH) and include regenerating liver inhibitory factor-1 (RL/IF-1), GDP-dissociation inhibitor 1 (GDI-1), neural visinin Ca^{2+}-binding protein 2 (NVP-2), neuromedin B receptor, and Na^{+}/K^{+} transporting ATPase 2β. ISH with memantine (5–50 mg/kg) revealed regulation of these genes in other cortical and hippocampal regions. RL/IF-1 induction occurred at 1 h and returned to basal levels by 8 h, consistent with the profile of an immediate early gene. Western blot analysis showed increases ($\sim$30–65%) in GDI-1 protein present in both cytosolic and membrane fractions that were significant in the 84-kDa Rab bound form, suggesting that memantine influences Ras-like GTPase function. Genes regulated by a 5 mg/kg dose of memantine might be important in its therapeutic effects. These findings increase the number of known, differentially altered genes after treatment of uncompetitive NMDA receptor antagonists and suggest broader actions of these agents than previously realized.
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INTRODUCTION

Glutamate receptor blockers with selective action on *N*-methyl-D-aspartate (NMDA) receptors have wide therapeutic potential in a variety of central nervous system (CNS) disorders ranging from the acute neurodegeneration (eg stroke and trauma), chronic neurodegeneration (eg Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) (Danysz *et al*, 1995; Parsons *et al*, 1998; Krystal *et al*, 1999), to symptomatic treatment (eg epilepsy, PD, drug dependence, depression, anxiety, and chronic pain) (Meldrum, 1986; Danysz *et al*, 1995; Krystal *et al*, 1999; Skolnick, 1999).

In these conditions, an excessive release of glutamate is thought to play a major role in triggering neuronal death via NMDA receptors (Choi, 1988; Danysz *et al*, 1995; Parsons *et al*, 1998). NMDA receptor channel blockers represent one group of pharmacologically active agents able to antagonize NMDA receptor activation by acting directly on the phencyclidine recognition site within the channel. Since these agents act in a 'use-dependent' manner, only blocking the channel in the open state (Foster and Wong, 1987; Huettner and Bean, 1988), they have been termed uncompetitive NMDA receptor antagonists.

Dizocilpine maleate (MK-801) is a prototypical NMDA-receptor channel blocker with a high affinity to the ion channel (Wong *et al*, 1986). However, high affinity NMDA receptor channel blockers have been observed to elicit psychotomimetic properties in humans (Krystal *et al*, 1999), and thus have been useful only experimentally *in vitro* and *in vivo*. In contrast, memantine (1-amino-3,5-dimethyladamantane), an uncompetitive NMDA receptor antagonist of moderate affinity to the receptor-associated ion-channel, exhibits fast blocking and unblocking kinetics, high voltage dependency (Kornhuber *et al*, 1991; Parsons *et al*, 1993), and is well tolerated at clinical doses (Parsons *et al*, 1999). Memantine is suggested as a neuroprotective agent for the treatment of several dementias, particularly AD (Görtelmeyer and Erbler, 1992; Muller *et al*, 1995; Parsons *et al*,

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1999). Moreover, memantine and acetylcholinesterase inhibitors are currently the only agents widely used to improve cognition and daily activities in patients with AD (Kilpatrick and Tilbrook, 2002; Winblad *et al*, 2002). Memantine has been approved for clinical use in Europe and is currently undergoing clinical trials in the United States (Kilpatrick and Tilbrook, 2002). Thus, information concerning the molecular pharmacology of memantine is important to understand its actions and to anticipate potential adverse effects.

We sought to characterize the genes with altered expression in adult rat brain after MK-801 and memantine treatment in order to better understand the multiple actions of NMDA/glutamate antagonists. cDNA microarray-based hybridization techniques offer a high throughput platform that allows for rapid and cost-effective screens to detect effects of pharmacological agents (Schena *et al*, 1995; Grunblatt *et al*, 2001; Ueda *et al*, 2002). Here, we utilized a microarray containing 1090 cDNA fragments and *in situ* hybridization (ISH) to determine the gene expression alterations in response to treatment with uncompetitive NMDA receptor antagonists in rat limbic cortex. We were able to identify several genes with altered expression in response to MK-801 and memantine treatment. In addition to already known and identified genes involved in either pharmacological or toxicological effects, the study also uncovered a number of new gene/treatment correlations. Characterization of these genes may lead to better understanding of the molecular pharmacology of uncompetitive NMDA receptor antagonists. More importantly, some of these genes may constitute novel targets for new therapeutic use.

MATERIALS AND METHODS

Male Wistar rats (initial weight 250–280 g, National Laboratory Animal Center, University of Kuopio, Kuopio, Finland) were kept under standardized temperature, humidity, and lighting conditions (12-h light/dark cycles) with free access to water and food. All attempts were made to minimize pain and discomfort of the experimental animals. All animal treatments were approved by the Experimental Animal Committee of the University of Kuopio, and they have been carried out in accordance with the guidelines issued by the Society for Neuroscience.

Doses and duration of the treatment with uncompetitive NMDA receptor antagonists, MK-801 and memantine, used in this study were based on the previous studies. Memantine intraperitoneally (i.p.) dose 25 mg/kg (4 h) was shown to increase significantly BDNF mRNA levels in the rat brain (Marvanová *et al*, 2001) and produce plasma levels reaching $\sim$6.5 μM concentrations, which are still within the range specific to NMDA receptor affinity (Kornhuber *et al*, 1994). Moreover the neuroprotective dose of memantine in rats has been shown to be 10–30 mg/kg in acute indications (Wenk *et al*, 1994). A 5 mg/kg i.p. dose of memantine in rats produces plasma levels of $\sim$1 μM that can be considered therapeutically relevant (Parsons *et al*, 1999). It has been suggested that neuroprotective doses of MK-801 (0.3–0.8 mg/kg; i.p.) block NMDA receptors in the brain, and these doses produce plasma concentrations within the range of NMDA receptor affinity (Gill *et al*, 1991; Massieu *et al*, 1993).

Rats were injected i.p. with saline (control group of animals) or with 5, 10, 25, and 50 mg/kg of memantine HCl, and killed 1–48 h afterwards as indicated. In all, 1 mg/kg MK-801 (dizocilpine maleate) (RBI, Natick, MA, USA) was applied to rats that were killed 4 h afterwards.

The rats were narcotized with CO_2 before decapitation. Brains were rapidly removed and posterior cingulate and anterior retrosplenial cortices were rapidly and carefully dissected and immediately stored on dry ice to be processed for RNA isolation. Brains for ISH histochemistry were rapidly removed and rinsed in PBS (pH 7.5), placed on dry ice, and stored at -80°C. Coronal brain sections (14 μm) were cut on a Leica CM 3000 cryostat and thaw-mounted onto SuperFrost/Plus (Menzel-Glaeser, Germany) slides. Brain sections from saline (control) and treated rats were comounted onto the same slides and fixed in 4% paraformaldehyde solution, and stored in 95% ethanol at 4°C until use.

cDNA Array

Rat 1.0 Atlas Glass Microarrays for dual color analyses (Clontech Laboratories, Palo Alto, CA, USA) were utilized in this study. The array contains 86 controls and 1090 well-characterized transcripts with assigned function and a broad functional range class. Each gene is represented by 'long oligo', single-stranded cDNA fragments of around 80 bases that lack repetitive elements and have minimal homology with other genes on the array. The full gene list can be found at http://www.uku.fi/aivi/supplementary_information.htm.

RNA Extraction, Probe Synthesis, and Hybridization

Total RNA was isolated from frozen tissue using Trizol reagent (Gibco BRL, MD, USA) according to the manufacturer's recommended protocol. The total RNA was then DNase I treated (Ambion, Inc., Texas, USA). The integrity of RNA was verified with a 1% agarose-formaldehyde minigel and concentrations were obtained by measuring absorbance at 260 nm. Fluorescently labeled probes were synthesized from total RNA (20 μg) by reverse transcription and indirect incorporation of fluorescent dye (Cy3 and Cy5) according to the manufacturer's protocol. Following hybridization (42°C, for 16 h) with fluorescently labeled cDNAs in hybridization chambers, all arrays were washed and then scanned (ScanArray 5000, GSI Lumonics, Inc., Billerica, MA, USA).

Gene Expression Data Analysis

Raw scanned images of Cy3 and Cy5 fluorescence were processed and the signal from each spot was then quantified using QuantArray analysis software (GSI Lumonics, Inc.). The local background was subtracted from the fluorescent values of each spot to obtain an intensity value. For each individual treatment, duplicate microarrays were hybridized with rat brain mRNA samples obtained from two rat individuals. In this hybridization study a 'swapped dyes' design was used. On one microarray, the treatment sample

was labeled with Cy5, and control was labeled with Cy3. The dyes were then switched where the treatment sample was labeled with Cy3 and control was labeled with Cy5. In principle, the ratios of treatment (Cy-5)/control (Cy-3) and treatment (Cy-3)/control (Cy-5) should be approximately equal. In order to account for any differential incorporation of the fluorescent labels and differences in scanning parameters, the signal intensities for Cy3 and Cy5 were globally normalized and the expression ratios between experimental and control samples were determined. Genes were upregulated if they displayed a ratio $\geqslant 1.5$ and downregulated if the ratio was $\leqslant 0.67$ in both replicate dye swap experiments. The cut-off value for gene induction or suppression were chosen based on observed gene expression data, and previously reported microarray profiling studies of brain regions, which showed that the magnitude of gene expression differences is often less than two-fold in brain tissue (Mirnics *et al*, 2000). The analyzed expression data sets of memantine and MK-801 experiments are located on the publicaly available website (http://www.uku.fi/aivi/supplementary_information.htm).

In situ Hybridization

ISH was performed as described previously (Marvanová *et al*, 2001). Briefly, the antisense oligonucleotides of rat RL/IF-1 (5′-GTAGGGCAACTCATCTTCCGTGAATTCTGACT CCG-3′), rat GDI-1 (5′-GGAGATCATGCACACATAGATGT CTGGCTTCCTGT-3′), rat PAK-1 (5′-CTCTGCACACAGC TGCTATCTGGCCTTCATCCATA-3′), rat SHPS-1 (5′-CTG TTTGATTCGGAGGAGGTAGAGGGCAGCCATCA-3′), rat NVP-2 (5′-CACCAGCCCTACTTCTGCATGTCACATTGC AACAG-3′), rat GRP78 (5′-GGCCTGAGAATAGCGAGCAG CAACGTCTCGAGCTT-3′), rat neuromedin B receptor (5′-AACTGCTGTTATCCGAGCTACCGATGCGTGCTACT-3′), rat ARF-3 (5′-GAGCTCATCCTCCGCCAGCATCCTCATCAGC TCTT-3′), rat prostaglandin E2 receptor (5′-CTGCATGC GAATAAGGTTGAGGATCACACTGATG-3′), rat calcium channel β (5′-TTCACTCTGTACCTCAGCCAGGCTGGAGC GAGTGT-3′), rat HSP90β (5′-TCTTCATCAATGCCCAGG CCTAGTTTAATCATGCG3′), rat dopamine D4 receptor (3′-TCGGCAGCCGCCAGGCTCACGATGAAGTAGTTGGT-5′), rat c-fos (5′-GCAGCGGGAGGATGACGCCTCGTAGTCCG CGTTGAAACCCGAGAA-3′), and rat FGF-2 (5′-AGTGTC TAAAGAGAGTCAGCTCTTAGCAGACATTG-3′), rat K^+/Na^+ transporting ATPase 2β (5′-TTTAGCTGGGTCCGGTTGAA CTGGCAGGCACGTT-3′), rat F-spondin (5′-ACACTTCT CCGCCTGCTCCAGATCCTCATTACAGT-3′) mRNAs were 3′ end-labeled to a specific activity of $1–2 \times 10^7$ cpm/pmol using terminal deoxynucleotidyl transferase (MBI Fermentas, Vilnus, Lithuania) and a 30 : 1 molar ratio of α-[^{33}P]-dATP (2000 Ci/mmol, New England Nuclear, Boston, MA) to probe. Hybridization was performed for 21 h at 42°C on paraformaldehyde postfixed sections in the presence of $1–3 \times 10^3$ cpm/μl of labeled probe in buffer containing 50% formamide, 4× standard saline citrate (SSC) (1× SSC: 150 mM NaCl, 15 mM sodium citrate), and 10% dextran sulfate. After incubation, the sections were washed and opposed onto Hyperfilm-βmax films (Amersham Life Sciences, Inc., Buckinghamshire, England) for 5 days to 3 weeks and developed for 5 min in D-19 (Kodak, France). Nonspecific hybridization was determined by adding 100-fold excess of unlabeled oligonucleotide probe to the hybridization buffer. Nonspecific hybridization was not significantly above the background.

Immunoblotting

Tissues from posterior cingulate and anterior retrosplenial cortices were rapidly dissected and well homogenized in 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, Protease inhibitors and 0.1 mM GDP). The homogenate was spun for 10 min at 600 *g* at 4°C to remove nuclear fractions and intact cells. The supernatant was transferred to SW 40 swinging bucket rotor tubes and centrifuged at 100 000 *g* for 30 min at 4°C. After centrifugation, the supernatant containing cytosolic fraction was removed to a separate tube and the obtained pellet containing membrane protein was resuspended in 250 μl of lysis buffer. All protein fractions were stored in −80°C until use. After measuring protein concentrations (Dc Protein Assay, BioRad, Richmond, CA, USA), samples consisting of equal amounts of protein (20 μg) were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, Jeene, NH) and then blocked with 3% bovine serum albumin (BSA) in TBS containing 0.1% Tween 20 (TBS-T). Immunoblotting was carried out with the rabbit GDI-1 (GDP-dissociation inhibitor 1) (Amersham Life Sciences, Inc.) and anti-actin (c-11, goat polyclonal IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies using 1 : 1000 dilutions at 4°C overnight. Filters were rinsed in water twice and in PBS containing 0.05% Tween twice. Bound immunoglobulins were detected with anti-rabbit (Amersham) or anti-goat (Santa Cruz Biotechnology) horseradish peroxidase-conjugated secondary antibodies at 1 : 1000 dilution for 1 h at room temperature, rinsed, and followed by chemiluminescence detection using luminol (Sigma-Aldrich, Helsinki, Finland) as the substrate. Signals were exposed to films for 1–10 min.

Data Analysis

Optical densities from immunoblot and ISH films were quantified by a video-based image analysis system (MCID M4, Imaging Research, Inc., St Catharines, Ontario, Canada). The anatomical brain regions were determined according to Paxinos and Watson (1986). For ISH autoradiograms, ^{14}C-microscales were exposed simultaneously to convert optical densities to nCi/g statistical analysis was performed with ANOVA followed by Dunnett's *post hoc* test.

RESULTS

Expression Array Data Analysis

To examine the molecular mechanisms contributing to the effect of MK-801 and memantine, mRNA expression was profiled using Clontech Rat 1.0 glass arrays containing 1090 genes of known function. Expression levels and profiles of genes were obtained after saline and MK-801 (1 mg/kg; 4 h),

or saline and memantine (25 mg/kg; 4 h) systemic administrations in rat posterior cingulate and anterior retrosplenial cortices. Duplicate microarrays were hybridized and the ratios (treated *vs* control) were calculated for each individual array. Analysis of both arrays from each treatment revealed that 28 genes were differentially altered after memantine administration (Tables 1 and 3; Figures 1 and 2) while 34 genes were differentially altered after MK-801 administration (Tables 2 and 3; Figures 1 and 2). A total of 13 genes were regulated by both treatments of which 12 were upregulated and one was downregulated (Table 3).

ISH Analysis of Candidate Genes

ISH was used to independently verify the results from previous microarray analyses as well as to determine brain expression distribution, dose response, and time course. ISH from five to six rat brains treated with MK-801 (i.p.; 1 mg/kg; 4 h) or memantine (i.p.; 5–25 mg/kg; 4 h) was performed for several up- or downregulated candidate genes. Candidate genes selected for ISH analysis were based on groups of genes regulated by both treatments, by only memantine, or by only MK-801.

ISH Analysis of Candidate Genes after Memantine Treatment

From the microarray analysis, 28 candidate genes were identified of which five were previously confirmed by the literature, and 10 were confirmed by ISH with one false positive (Tables 1 and 3). Moreover, we confirmed the regulation of memantine selectively altered genes: PAK-1, SHPS-1, calcium channel β, and F-spondin. Additionally, one false negative was observed (c-fos). Quantification of these results can be found as supplemental material (http://www.uku.fi/aivi/supplementary_information.htm).

Regenerating liver inhibitory factor-1 (RL/IF-1), GDI-1, SHPS-1, PAK-1, and neural visinin Ca^{2+}-binding protein 2 (NVP-2) were further subjected to study memantine-induced dose response and time course. mRNA levels of RL/IF-1 gene was significantly increased in all investigated brain regions ($32 \pm 3.4\%$) (Figures 1a and 2a). Gene expression levels of GDI-1 were significantly increased in retrosplenial cortex with an increase of $25 \pm 4.4\%$ and in all studied hippocampal regions with an increase of $29 \pm 4.4\%$ (Figures 1b and 2a). NVP-2 mRNA level was decreased in retrosplenial and temporal cortices ($86 \pm 1.0\%$), and $22 \pm 5.2\%$ increased in DG (Figures 1c and 2a). Moreover, the gene SHPS-1 was significantly increased $14 \pm 1.0\%$ in retrosplenial and temporal cortices, and $26 \pm 6.5\%$ in DG (Table 4). Furthermore, gene expression of PAK-1 gene was induced with $28 \pm 4.4\%$ increase in studied cortical regions, and $31 \pm 2.4\%$ in hippocampal DG, CA1 and CA3 regions (Table 4).

A dose response to memantine (5–50 mg/kg) was performed with the RL/IF-1, GDI-1, SHPS-1, and PAK-1 genes (Table 4). The lowest dose of memantine used, 5 mg/kg, which can be considered clinically relevant, was able to increase significantly mRNA levels of GDI-1 and PAK-1 in several regions of rat hippocampus. The second lowest dose of memantine (i.p.; 10 mg/kg; 4 h) was able to increase

Table 1 Profiling of Genes Altered by Memantine Administration (i.p.; 25 mg/kg; 4 h) in Rat Limbic Cortex

Representative gene name	GenBank#	Swissprot#	Mem#1	Mem#2	Conf.
Transcription activators/repressors					
RL/IF-1[a]	X63594	Q63746	1.86	1.72	+
Growth factors/receptors					
TrkB[a]	M55291	Q63604	1.58	1.64	L
Chaperones/heat shock proteins					
HSP70[a]	Z27118	Q63718	1.80	2.00	L
HSP90β	S45392	P34058	0.65	0.64	+
GRP78[a]	M14050	P06761	1.72	1.97	–
Vesicle- and synaptic-related genes					
GDI-1[a]	U07952	P50398	1.73	1.64	+
NVP-2[a]	D13125	P35332	0.63	0.52	+
Syntaxin 1A	M95734	P32851	1.55	1.53	
Neurotransmitter/hormone receptor					
NMDAR2B[a]	M91562	Q00960	1.57	1.61	L
Retinoid xα receptors	L06482	Q05343	1.56	1.61	
G-protein-coupled receptors					
Neuromedin B receptor[a]	U37058	P24053	1.76	1.59	+
Neuropeptides					
NPY[a]	M20373	P07808	1.52	1.64	L
CRF[a]	M54987	P01143	1.62	1.53	L
Osmotic balance regulators					
SCNB2	U37026	P54900	1.55	1.51	
CACNA1	M59786	P22002	1.62	1.50	
Na^+/K^+ transporting ATPase 2β[a]	J04629	P13638	1.69	1.52	+
Ca^{2+} transporting ATPase-isoform 2[a]	J03754	P11506	1.67	1.52	
Calcium channel β	X61394	P54283	1.56	1.69	+
Complex lipid/xenobiotic metabolizing enzymes					
CYP4A8	M37828	P24464	1.69	1.64	
CYP2C11	P08683	Q64554	1.57	1.76	
GST7-7	X02904	P04906	1.55	1.54	
Intracellular kinases					
PAK-1	U23443	P35465	1.91	1.91	+
Intracellular phosphatases					
Protein phosphatase 2A[a]	M23591	P11082	1.54	1.59	

Table Continued…

Table 1 *(Continued)*

Representative gene name	GenBank#	Swissprot#	Mem#1	Mem#2	Conf.
Glucose metabolism and regulation					
Pyruvate dehydrogenase kinase	L22294	Q63065	1.54	1.54	
Miscellaneous group					
SHPS-1	D85183	BAA12734	1.55	1.51	+
F-spondin	M88469	P35446	1.88	1.66	+
CPR	M12516	P00388	0.64	0.62	
Antigen peptide transporter 1	X57523	P36370	0.66	0.65	

Expression ratios shown represent the ratio obtained with each of microarray duplicate and indicate normalized, non-$\log_2$-transformed ratios of memantine- over saline-treated animals.
[a]Gene also regulated by MK-801. Symbols for confirmation (Conf.): +, confirmed by ISH; −, false positive; L, confirmation by literature. PAK-1, α serine/threonine kinase; HSP70, heat shock protein 70; GRP-78, 78 kDa glucose regulated protein precursor; RL/IF-1, regenerating liver inhibitory factor 1; GDI-1, GDP-dissociation inhibitor 1; CYP2C11, cytochrome P450 IIC11; CYP4A8, cytochrome P450 IVA8; trkB, tyrosine kinase receptor; NMDAR2B, *N*-methyl-D-aspartate receptor 2B subunit; NPY, neuropeptide Y; CRF, corticotropin-releasing factor; CACNA1, voltage-dependent P/Q-type calcium channel α-1A subunit; GST7-7, glutathione *S*-transferase P; SHPS-1, receptor-like protein with SH2-binding site; SCNB2, sodium channel β2 subunit; HSP90β, heat shock protein 90β, CPR, NADPH-cytochrome P450 reductase; NVP-2, neural visinin Ca^{2+}-binding protein 2.

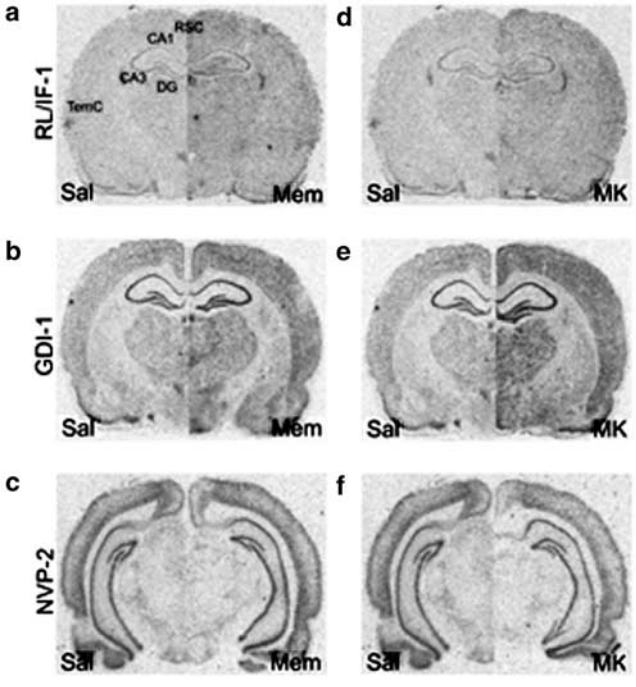

Figure 1 Acute systemic administration of memantine and MK-801 regulated gene expression of candidate genes in rat brain. Representative autoradiograms from ISH showing differential gene expression of RL/IF-1 (a, d), GDI-1 (b, e), and NVP-2 (c, f) after saline (Sal), memantine (Mem), and MK-801 (MK) treatments. Retrosplenial cortex (RSC), temporal cortex (TemC), dentate gyrus (DG), hippocampal regions (CA1 and CA3).

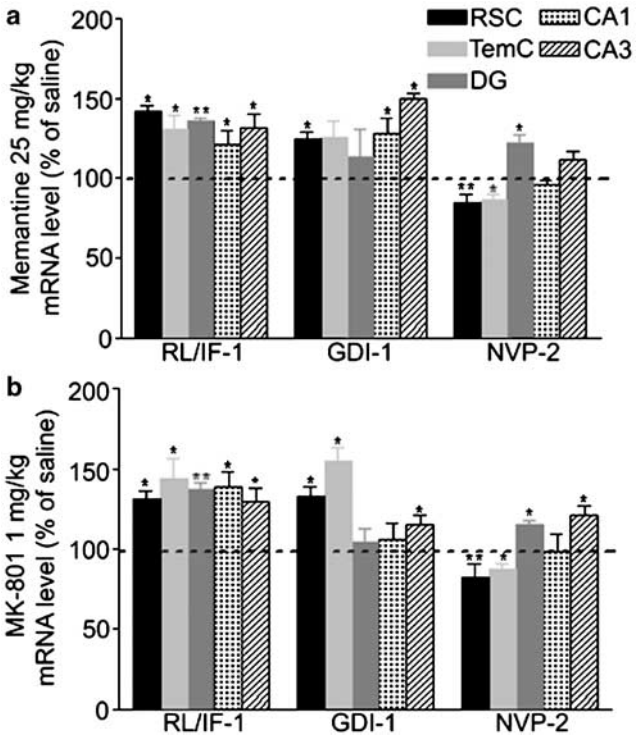

Figure 2 The effect of acute memantine (25 mg/kg) and MK-801 (1 mg/kg) systemic administration in rat brain. The rats were i.p. injected with 25 mg/kg of memantine or 1 mg/kg of MK-801 and killed 4 h later. In all, 14 μm coronal brain sections were hybridized by radioactive ISH with oligonucleotide probes. Exposure time for autoradiograms was 1–3 weeks. Histograms of the region-expression levels are shown for memantine (a) and for MK-801 (b) acute administration. Data are mean ± SEM obtained from five to six brains in each group. Significant differences between saline and treated rats are indicated: $*p<0.05$; $**p<0.01$, ANOVA followed by Dunnett's *post hoc* test. Retrosplenial cortex (RSC), temporal cortex (TemC), dentate gyrus (DG), hippocampal regions (CA1 and CA3).

significantly PAK-1 mRNA level in all studied cortical and hippocampal regions (Table 4). With increased dose of memantine, mRNA levels of RL/IF-1, GDI-1, and PAK-1 were also induced in other brain regions, and the effect was more pronounced. SHPS-1 mRNA levels were increased only after high doses of memantine (i.p.; 25–50 mg/kg; 4 h) (Table 4).

Peak induction of RL/IF-1 was observed in the cingulate and parietal cortices, and striatum (CPu) at 1 and 4 h after administration of memantine (i.p.; 25 mg/kg; 4 h) (Figure 3). Furthermore, peak induction of SHPS-1 mRNA was observed in retrosplenial cortex at 4 and 8 h after memantine (i.p.; 25 mg/kg; 4 h) administration (data not shown). Other brain regions may also be regulated by acute MK-801 and memantine administration but were not quantitated.

ISH Analysis of Candidate Genes of MK-801 Treatment

From the microarray analysis, 34 candidate genes were identified of which nine were previously confirmed by the literature, and seven were confirmed by ISH with two false positives (Tables 2 and 3). Moreover, we confirmed the regulation of MK-801 selectively altered genes: FGF-2,

Table 2 Profiling of Genes Altered by MK-801 Administration (i.p.; 1 mg/kg; 4 h) in Rat Limbic Cortex

Representative gene name	GenBank#	Swissprot#	MK#1	MK#2	Conf.
Immediate early genes/oncogenes					
c-fos	X06769	P12841	1.79	1.71	+; L
ear-3	U10995	Q62681	2.37	2.05	
Transcription activators/repressors					
STAT-3	X91810	P52631	0.63	0.66	
RL/IF-1[a]	X63594	Q63746	2.00	1.72	+
Growth factors/receptors					
FGF-2	M22427	P13109	1.70	1.55	+; L
TrkB[a]	M55291	Q63604	1.83	1.82	L
P75NTR	X05137	P07174	2.45	2.20	
Activin type receptor	S48190	P38444	2.00	2.01	
Chaperones/heat shock proteins					
HSP70[a]	Z27118	Q63718	2.00	2.43	L
GRP78[a]	M14050	P06761	2.06	1.85	–
Vesicle- and synaptic-related genes					
GDI-1[a]	U07952	P50398	1.90	1.67	+
ARF-3[b]	L12382	P16587	1.96	1.72	–
NVP-2[a]	D13125	P35332	0.64	0.38	+
Synaptotagmin 3	D28512	P40748	1.95	1.65	
Syntaxin-binding protein 1	U06069	Q64320	2.06	2.27	
Amphiphysin	Y13381	O08838	1.60	1.69	
Neurotransmitter/hormone receptor					
Dopamine 4 receptor	M84009	P30729	1.60	1.83	+
NMDAR2B[a]	M91562	Q00960	1.66	1.89	L
Metabotropic receptor 2	M92075	P31421	2.16	2.47	
G-protein-coupled receptors					
Neuromedin B receptor[a]	U37058	P24053	2.32	2.10	+
Vasopressin V2 receptor	Z11932	Q00788	1.51	1.61	
Thromboxane A2 receptor	D21158	P34978	1.70	2.09	
Prostaglandin E2 receptor	U94708	Q62928	0.45	0.22	+
Somatostatin 2 receptor	M93273	P30936	0.60	0.50	L
Neuropeptides					
NPY[a]	M20373	P07808	1.60	1.91	L
CRF[a]	M54987	P01143	1.58	1.86	L
Osmotic balance regulators					
CCHB3	M88751	P54287	1.69	1.80	
Na^+/K^+ transporting ATPase 2β[a]	J04629	P13638	2.04	1.93	+
Ca^{2+} transporting ATPase-isoform 2[a]	J03754	P11506	1.91	1.65	
Intracellular phosphatases					
Inositol polyphosphate 4-phosphatase IIα	U96920	AAB72151	0.63	0.56	
Protein phosphatase 2A[a]	M23591	P11082	1.52	1.65	
LAR	L11586	CAA5895	1.75	1.63	
Glucose metabolism and regulation					
Fructose-biphosphate aldolase A	M12919	P09117	1.74	2.02	L
Miscelaneous group					
Neuronal pentraxin receptor	AF005099	O35764	2.08	2.60	

Expression ratios shown represent the ratio obtained with each of the microarray duplicate and indicate normalized, non-$\log_2$-transformed ratios of MK-801- over saline-treated animals.
[a]Gene also regulated by memantine. Symbols for confirmation (Conf.): +, confirmation by ISH; –, false positive; L, confirmation by literature. p75NTR, low affinity neurotrophin receptor; HSP70, heat shock protein 70; GRP-78, 78 kDa glucose regulated protein precursor; RL/IF-1, regenerating liver inhibitory factor 1; ARF3, ADP-ribosylation factor 3; trkB, tyrosine kinase receptor; GDI-1, GDP-dissociation inhibitor 1; NMDAR2B, *N*-methyl-D-aspartate receptor 2B subunit; NPY, neuropeptide Y; CCHB3, voltage-gated dihydropyridine-sensitive L-type calcium channel β3 subunit; CRF, corticotropin-releasing factor; D4 receptor, dopamine D4 receptor; LAR, leukocyte common antigen-related tyrosine phosphatase; FGF-2, fibroblast growth factor 2; STAT-3, signal transducer and activator of transcription 3; NVP-2, neural visinin Ca^{2+}-binding protein 2.
[b]ARF3 gene was found to be upregulated on cDNA microarray, however, ISH revealed significant downregulation, with the peak reduction at 1–4 h after MK-801 treatment.

prostaglandin EP2 receptor, and dopamine D4 receptor. Additionally, one false negative was observed (HSP90β). Quantification of these results can be found as supplemental material (http://www.uku.fi/aivi/supplementary_information.htm).

Expression of RL/IF-1 was increased in retrosplenial and temporal cortices ($38 \pm 6.5\%$) as well as in hippocampal regions ($35 \pm 3.1\%$) (Figures 1d and 2b). mRNA expression of GDI-1 was also increased in retrosplenial cortex ($33 \pm 5.9\%$) and CA3 hippocampal region ($15 \pm 5.3\%$) (Figures 1e and 2b). NVP-2 mRNA level was decreased in retrosplenial and temporal cortices ($85 \pm 2.5\%$). Moreover, an acute administration of MK-801 increased mRNA levels of the NVP-2 gene in hippocampal DG ($15 \pm 5.9\%$) (Figures 1f and 2b).

Table 3 The Number of Genes Similarly and Differentially Regulated by Memantine and MK-801 in Rat Limbic Cortex by Microarray and ISH Analysis

Altered gene groups	Mem gene no.	MK gene no.	Mem+MK gene no.
Upregulated			
All revealed genes	24	29	12
Previously reported[a]	5	8	5
Not previously reported	19 (8)	21 (5)	7 (4)
Downregulated			
All revealed genes	4	5	1
Previously reported[a]	0	1	0
Not previously reported	4 (2)	4 (2)	1 (1)

Numbers of genes shown represent the results observed from two microarray experiments of each indicated treatment. The number in the parentheses represents the number of candidate genes confirmed by ISH.
[a]Gene previously reported in the literature to be regulated by NMDA antagonists. Memantine 25 mg/kg (Mem) and MK-801 1 mg/kg (MK).

Table 4 Memantine (M) (i.p.; 5–50 mg/kg; 4 h) Dose Response of Gene Expression in Rat Brain Regions

Gene	Region	M 5 mg/kg	M 10 mg/kg	M 25 mg/kg	M 50 mg/kg
RL/IF-1	RSC	117±8.7	133±8.1**	142±3.2**	170±9.5**
	TemC	96±9.5	110±4.7	131±2.5*	161±10*
	DG	105±5.0	106±7.5	136±2.5**	151±8.5*
	CA1	105±7.8	115±8.1	121±8.3*	134±5.3**
	CA3	105±7.8	115±6.5	131±8.6*	160±11*
GDI-1	RSC	115±8.1	123±7.7*	125±4.4*	131±5.3**
	TemC	101±11	94±8.6	125±10*	133±12*
	DG	122±10	117±11	114±17	118±22
	CA1	119±8.0*	115±3.3*	128±9.5*	141±13*
	CA3	121±3.1**	128±5.8**	150±3.8*	162±8.5**
PAK-1	RSC	110±6.5	116±3.2*	130±7.5*	133±8.2*
	TemC	110±3.2	117±3.7*	125±8.8*	131±4.2**
	DG	120±8.2*	130±8.9**	134±1.8**	189±11*
	CA1	115±6.5	121±6.3*	132±8.5*	125±3.3**
	CA3	116±8.3	119±3.2*	126±6.8*	143±3.3**
SHPS-1	RSC	101±5.6	110±8.1	115±3.2*	125±3.8*
	TemC	104±4.2	105±3.2	113±0.1*	121±4.5*
	DG	108±14	112±8.4	126±6.5*	147±7.6*
	CA1	106±7.6	107±6.7	104±8.1	111±6.5
	CA3	107±9.0	107±7.5	110±4.9	109±5.2

All values (±SEM) are expressed as percentage of the saline-treated treatment period matched control sections ($n = 5$–6, ANOVA followed by Dunnett's *post hoc* test). *$p < 0.05$; **$p < 0.01$.

Study of GDI-1 Protein Induction

Acute administration of memantine (i.p.; 10 and 25 mg/kg; 8 h) significantly increased protein levels of Rab/GDI-1 complex (~84 kDa) in the cytosolic fraction (29±5.3%; 64±6.8%), and also in the membrane fraction of retrosplenial cortex (33±4.2%; 52±1.8%) (Figure 4). Cytosolic and membrane levels of free GDI-1 (~56 kDa) were unchanged after memantine administration.

DISCUSSION

Agents acting as noncompetitive antagonists of NMDA/glutamate receptor induce the expression of several genes in limbic cortical regions, such as the cingulate, retrosplenial, and entorhinal cortices (Sharp *et al*, 1991; Gass *et al*, 1993; Tomitaka *et al*, 1996; Marvanová *et al*, 2001). In this study, we have applied expression profiling of MK-801 (1 mg/kg) and memantine (5–50 mg/kg) in the posterior cingulate and anterior retrosplenial cortices to look more carefully at induced genes, and to delineate molecular pharmacologic effects of NMDA/glutamate receptor antagonists in the brain.

We screened the expression of 1090 known rat genes representing about 2% of entire rat genome by using cDNA microarrays. We identified 13 genes regulated by both NMDA receptor antagonists of which 12 genes were upregulated and one was downregulated. In addition, 28 genes were regulated by memantine and 34 were regulated by MK-801 administration. Confidence in the microarray results are supported by (1) identification of genes previously known to be altered by NMDA receptor antagonist treatment, (2) verification of novel regulated genes by ISH, and (3) verification by immunoblotting.

Antagonist of NMDA receptor subtype of glutamate receptor, protect against brain damage in neurological disorders (Danysz *et al*, 1995; Parsons *et al*, 1998; Krystal *et al*, 1999). However, in the previous studies it was observed that administration of NMDA receptor antagonists with high affinity or at high doses, can produce psychotomimetic effect in humans and neurotoxicity in specific neuronal subpopulations in the CNS of rodents, in particular neurons of the limbic cortex. Injured neurons in layers III and IV of the posterior cingulate and anterior retrosplenial cortex are characterized by abnormal cytoplasmic vacuoles (Olney *et al*, 1989; Sharp *et al*, 1991). At a 1 mg/kg dose of MK-801, this vacuole formation is reversible and not detected after 24 h (Olney *et al*, 1989). Therefore, it is likely that not only genes related to beneficial effects of MK-801 and memantine treatments, but also genes related to neurotoxic or stress-response effects of cortical cells are revealed in this study. In this study, we observed increases in mRNA levels of c-fos and HSP70 in the retrosplenial and cingulate cortex of adult rat treated with 25 mg/kg memantine and 1 mg/kg MK-801. Moreover, we observed increased transcriptional production of FGF-2 after MK-801 treatment.

Genes coding for a diverse range of proteins whose function is related to secretion, synaptic vesicle trafficking, and regulation of membrane trafficking were shown to be upregulated by different NMDA receptor antagonist treatments. GDI-1 (RabGDI, GDI-α) transcript level was

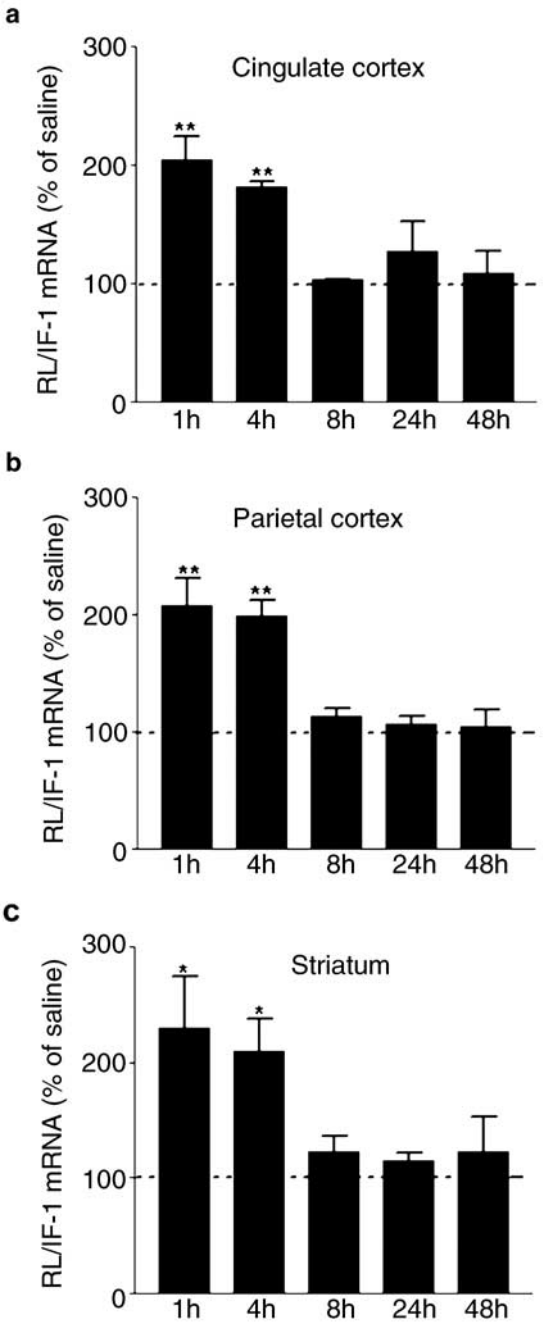

Figure 3 The time course of memantine effect on RL/IF-1 mRNA expression. The rats were i.p. injected either with saline and killed after 4 h or with memantine (25 mg/kg) and killed 1, 4, 8, 24, or 48 h later. Optical densities were quantified by MCID/M4-analysis system from cingulate cortex (a), parietal cortex (b), and striatum (c) for RL/IF-1 gene. Values are expressed as percentage of saline (mean ± SEM from three rats in each treatment group). Significant differences between saline and memantine treated rats are indicated: $*p<0.05$; $**p<0.01$, ANOVA, Dunnett's *post hoc* test.

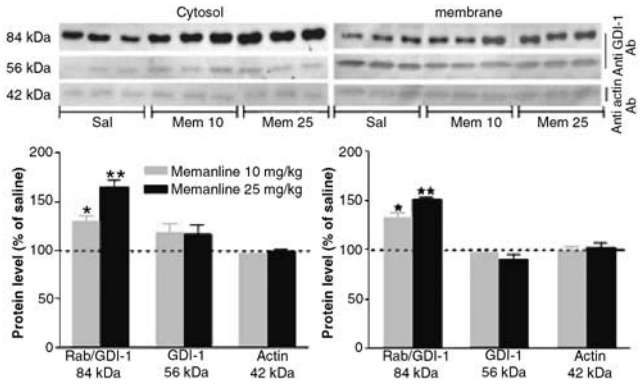

Figure 4 Western blot analysis of GDI protein expression in retrosplenial cortex after memantine treatment. The rats were injected with memantine (i.p.; 10 or 25 mg/kg) for an 8 h time period, and protein from the dissected retrosplenial cortex was obtained from cytosolic and membrane fractions. Proteins were run on SDS/polyacrylamide gels and transferred to nitrocellulose filters. The filters were probed either with anti-GDI-1 or anti-actin antibodies. Values are expressed as percentage of saline. Data shown are mean ± SEM from three rats. $*p<0.05$; $**p<0.01$, significantly different compared to saline-treated rats (ANOVA, Dunnett's *post hoc* test).

increased by both NMDA receptor antagonists. The lowest dose of memantine increased GDI-1 mRNA levels in the hippocampal subfields, CA1 and CA3, and also in the cortex after higher doses. Moreover, we observed significantly increased levels of Rab/GDI-1 protein complex in membrane and cytosolic fractions of rat retrosplenial cortices. However, since GDI-1 interacts with most Rab proteins of which approximately 40 have been identified, we were unable to determine the specific Rab protein(s) involved in this process. The production of GDI-1 protein is critical for vesicular membrane transport in both the endocytic and also exocytic pathways via regulation of Ras-like GTPases of the Rab family proteins (Pfeffer *et al*, 1995; Martinez and Goud, 1998; Luan *et al*, 1999; Seabra *et al*, 2002). Moreover, GDI-1 has an important role *in vivo* to suppress hyperexcitability of the CA1 pyramidal neurons, which changes the firing properties of the neurons (Ishizaki *et al*, 2000).

Consistent with previous reports demonstrating that MK-801 and memantine stimulate hypothalamic–puituitary–adrenal activity by activating corticotropin-releasing factor or cortocoliberin (CRF) in the hypothalamus (Lee *et al*, 1994; Zhou *et al*, 1998), we found the increased transcript levels of CRF after both treatments. Neuropeptide Y (NPY) and neuromedin B receptor were also upregulated by both treatments, whereas somatostatin receptor 2 was downregulated after MK-801. Taken together, these results suggest effects on multiple neuropeptide systems.

Memantine and MK-801 elicited also induction of the immediate early gene RL/IF-1 in several brain regions. The RL/IF-1 gene is the rat homolog of human MAD-3, and possesses I-κB activity of broad specificity and thus it is able to inhibit the binding of p50–p60 NF-κB, c-Rel-p50, and RelB-p50, but not homodimeric NF-κB to the κB site (Tewari *et al*, 1992). NF-κB is a ubiquitous transcription factor that regulates transcription of multiple genes involved in immune and inflammatory responses (Baeuerle and Henkel, 1994). Previous studies have reported that inappropriate regulation of NF-κB/Rel-mediated transcription is associated with pathological conditions including acute inflammatory responses, toxic/septic shock, acute phase reactions, atherosclerosis, radiation damage, and also with several neuropathologies (Grilli *et al*, 1993; Baldwin, 1996; O'Neill and Kaltschmidt, 1997). In post-mortem tissue from patients with neurodegenerative disease or cultured

neurons exposed to neurotoxic stimuli, increased NF-κB activity in cells has been reported (Kaltschmidt *et al*, 1995; Terai *et al*, 1996; Hunot *et al*, 1997). Furthermore, several studies have demonstrated that stimulation of both NMDA and non-NMDA glutamate receptors strongly stimulate NF-κB *in vitro* (Guerrini *et al*, 1995; Kaltschmidt *et al*, 1995). Additionally, another gene involved in immune and inflammatory responses, prostaglandin EP2 receptor (Narumiya *et al*, 1999), was downregulated.

This study revealed several new regulated transcripts in the brain after acute memantine or MK-801 treatment. While a 25 mg/kg dose of memantine causes more general expression changes in RL/IF-1 and GDI-1 transcripts, as shown by representative autoradiograms, regional changes were observed only at lower doses of memantine. These findings suggest caution in over extrapolation of gene expression changes, especially in cases like RL/IF-1 and GDI-1, where the ISH expression pattern is diffuse and regional differences may be subtle and revealed only by lower doses. Moreover, these changes were observed and reported only after acute administration. In a clinical setting of AD treatment, memantine is used chronically in relatively low doses. The 25 mg/kg dose results in plasma levels reaching $\sim$6.5 μM concentration that are still specific to NMDA receptor antagonism, although it is approximately seven times higher than the typical therapeutic values ($\sim$1 μM) (Kornhuber *et al*, 1994). The current study was designed to identify early effects of memantine treatment and potential new drug targets for NMDA receptor antagonists. Thus, future studies in a rat model should use chronic administration of lower memantine doses (1–5 mg/kg) to mimic the clinical regimen, and in this way reveal gene candidates responsible for its beneficial therapeutical effect. Moreover, as the rat genome project advances toward completion, whole genome expression array analysis could eventually be possible to perform.

In summary, we have used microarrays to study differentially expressed genes in response to acute treatments of high and modest affinity NMDA receptor antagonists. This study identified new genes not previously implicated in the molecular pharmacologic actions of high affinity and medium affinity NMDA receptor channel blockers, MK-801 and memantine, in the brain. Some caution should be taken in extrapolation of our results since only a portion of the microarray results were confirmed by independent methods. Nonetheless, genes regulated by a clinically relevant dose of memantine might be important for its therapeutic effects, and could potentially constitute new targets for investigation. Further and more detailed characterization of these gene relationships to neural function may lead to a better understanding of neurodegenerative processes and ultimately their treatment.

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