Published in final edited form as:

Neuroscience. 2010 March 31; 166(3): 852–863. doi:10.1016/j.neuroscience.2010.01.007.

GENE EXPRESSION PROFILING IN THE DEVELOPING RAT BRAIN EXPOSED TO KETAMINE

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Abstract

Ketamine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, is associated with accelerated neuronal apoptosis in the developing rodent brain. In this study, postnatal day (PND) 7 rats were treated with 20 mg/kg ketamine or saline in six successive doses (s.c.) at 2-h intervals. Brain frontal cortical areas were collected 6 h after the last dose and RNA isolated and hybridized to Illumina Rat Ref-12 Expression BeadChips containing 22,226 probes. Many of the differentially expressed genes were associated with cell death or differentiation and receptor activity. Ingenuity Pathway Analysis software identified perturbations in NMDA-type glutamate, GABA and dopamine receptor signaling. Quantitative polymerase chain reaction (Q-PCR) confirmed that NMDA receptor subunits were significantly up-regulated. Up-regulation of NMDA receptor mRNA signaling was further confirmed by *in situ* hybridization. These observations support our working hypothesis that prolonged ketamine exposure produces up-regulation of NMDA receptors and subsequent over-stimulation of the glutamatergic system by endogenous glutamate, triggering enhanced apoptosis in developing neurons. Published by Elsevier Ltd on behalf of IBRO.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2010.01.007.

Keywords

ketamine; gene expression; microarray; TaqMan; N-methyl-D-aspartate (NMDA) receptor

The general anesthetic ketamine blocks subtypes of glutamate receptors: the N-methyl-D-aspartate (NMDA) receptors. As a dissociative anesthetic, ketamine is commonly used to produce analgesia in children in emergency departments (Kohrs and Durieux, 1998). Clinically, ketamine's role in pediatric anesthesia is well established. However, recent studies have found that ketamine may cause dose-dependent, widespread apoptotic neurodegeneration in immature rat and monkey brains (Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003; Slikker et al., 2007). The window of vulnerability appears restricted to the phase of rapid synaptogenesis, also known as the brain growth spurt period.

Glutamatergic transmission is mediated by receptor families that are classed as ionotropic (iGluRs) or metabotropic (mGluRs). iGluRs are ligand-gated ion channels which can be sub-classified into the following groups based upon their ligand binding properties: NMDA receptors, α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, kainate receptors (KA) and more recently delta receptors. NMDA receptors appear to be heteromeric complexes (Monyer et al., 1992). NMDA-R1 (Grin1) subunits can form homo-oligomeric receptors that are functional, and the presence of this subunit is required to produce detectable NMDA-activated channel currents *in vitro* (Buller et al., 1994). NMDA-R2 (Grin2A–D) subunits produce functional receptors only when co-expressed with NMDA-R1 (Monyer et al., 1992), and co-expression of NMDA-R1 with NMDA-R2 subunits increases their responsiveness to NMDA and yields different functional properties (Buller et al., 1994).

Proposed mechanisms for the developmental neurotoxicity caused by ketamine include a compensatory up-regulation of NMDA receptors and subsequent over-stimulation of the glutamatergic system by endogenous glutamate via this receptor up-regulation (Wang et al., 2005b, 2006; Slikker et al., 2007). This results in dysregulation of calcium signaling, oxidative stress (Wang et al., 2000), and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway (Wang et al., 2006). However, the exact molecular mechanisms underlying ketamine-induced apoptotic neuronal cell death remain elusive.

DNA microarray technology simultaneously detects the perturbation in tens of thousands of genes in one single experiment and is rapidly becoming incorporated into toxicological research. Combined with other bioinformatics tools, it has been widely used to investigate the mechanisms of toxicant action, to search for novel biomarkers of toxicity and to build molecular signatures or models for predictive toxicology (Guo et al., 2006b). To better understand the molecular pathogenesis of ketamine-induced developmental neurotoxicity, the present study was carried out to examine the changes in gene expression profiles in the brains of postnatal day (PND) 7 rats, which have been shown to display a high susceptibility to ketamine-induced apoptotic neuronal cell death.

EXPERIMENTAL PROCEDURES

Animals

Seven-day-old (PND 7) Sprague–Dawley (male and female) rat pups (14–18 g) were used to examine the potential neurotoxic effects of ketamine. All animal procedures were approved by the Institutional Animal Care and Use Committee of the National Center for Toxicological Research (NCTR)/U.S. Food and Drug Administration (FDA) and conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Animal procedures were designed to minimize the number of animals required and their suffering. Ketamine hydrochloride (Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA, USA) was diluted in saline and its identity and purity (>99%) confirmed using HPLC/mass spectrometry.

Drug administration

Rat pups were randomly assigned to either the ketamine-treated or control (saline) groups. Pups were dosed s.c. with either ketamine hydrochloride (20 mg/kg; six injections at 2-hour intervals) (Fort Dodge) or saline using a 30-gauge needle with a volume of 10 μ l/g body weight. Pups were returned to their dam between injections to provide warmth and reduce potential stressors. Control animals received saline at the same time points. In all cases, a 6-h withdrawal period was allowed before animals were sacrificed via decapitation for RNA isolation or *in situ* hybridization or via transaortic perfusion of 0.9% saline and paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.2) for the TUNEL-Assay.

TUNEL (terminal dUTP nick-end labeling)-assay

Condensed or fragmented DNA was assayed using TUNEL staining as previously described (Johnson et al., 1998). Deoxynucleotidyl transferase (TdT), a template-independent polymerase, was used to incorporate biotinylated nucleotides at sites of DNA breaks. The signal was then amplified using avidin-biotin peroxidase, enabling conventional histochemical identification by light microscopy. In brief, brain tissue ($n = 5$ /group; initially perfusion fixed) was equilibrated with sucrose and embedded in optimum cutting temperature (OCT) tissue medium. The frontal cortex (corresponding to Figure 79 as defined by the Atlas of the Developing Rat Brain) (Paxinos et al., 1991) was selected for morphological assessments.

Coronal cryostat sections (10 μ m) were treated with proteinase K (Roche Diagnostics Corporation, Indianapolis, IN, USA) to dissociate proteins from DNA and the sections were washed in PBS. The sections were covered with H₂O₂ to inactivate endogenous peroxidase, rinsed with PBS and immersed in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 90 min. The reaction mixture was then replaced with TdT (0.3 U/ μ l; Roche) and biotinylated dUTP (0.2 nM/10 U TdT; Roche) in TdT buffer and incubated at 37 °C for 60 min. The reaction was terminated by transferring the sections to buffer (300 mM NaCl, 30 mM sodium citrate) for 15 min at room temperature. The slides were rinsed with PBS, covered with 2% BSA for 10 min at room temperature and rinsed in PBS. The slides were covered with biotin–avidin (1:50 in PBS; Vectastain ABC kit; Vector,

Burlingame, CA, USA) and incubated for 30 min at 37 °C. The reaction product was visualized with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA).

***In situ* hybridization**

An oligonucleotide probe complementary to the mRNA encoding the NMDA receptor NR1 subunit was selected on the basis of cloned cDNA sequences. The sequence of the probe used for *in situ* hybridization was 5'-TTCCTCCTCCTCCTC-ACTGTTACCTT-GAATCGGCCAAAGGGACT (this corresponds to a region that is constant across all NR1 splice variants). It was 3' end-labeled by incubation with [³⁵S]deoxy-ATP (New England Nuclear, Boston, MA, USA) and terminal deoxy-nucleotidyl transferase (Boehringer Mannheim Corp., Indianapolis, IN, USA) to attain specific activities of approximately 5–8 × 10 cpm/μg. The specificity of the probes has been previously described (Monyer et al., 1992).

Coronal sections (10 μm) through the frontal cortex and hippocampus were cut with a cryostat, rinsed in PBS and processed for *in situ* hybridization as described previously (Bartanusz et al., 1993). After an overnight hybridization at 41 °C, slides were washed successively in 4×, 1×, and 0.1× SSC (sodium chloride-sodium citrate solution), quickly dehydrated in ethanol (70%) and air-dried. Autoradiography was performed using Kodak (Rochester, NY, USA) NTB3 emulsion; slides were exposed for 3 weeks at 4 °C. Analyses of *in situ* hybridization autoradiographs were performed on hematoxylin–eosin counterstained sections.

Brain tissue collection and RNA isolation

Rats received six doses of 20 mg/kg ketamine (*n*=10; 5 male and 5 female) or saline (*n*=10; 5 male and 5 female) at 2-hr intervals and were sacrificed by decapitation 6 h following the last injection. Brain tissue (frontal cortex) was collected for RNA isolation. Total RNA from brain tissue was isolated using RNeasy® Lipid Tissue Mini Kits (Qiagen Inc., Valencia, CA, USA). The yield of the extracted RNA was determined spectrophotometrically by measuring the optical density at 260 nm. The purity and quality of the extracted total RNA were evaluated using the RNA 6000 LabChip kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). High quality RNA with RNA integrity numbers (RINs) greater than 8.5 was used for the microarray experiments and TaqMan gene expression assays.

Microarray procedures

To identify signatures in gene expression profiles associated with ketamine treatment, microarray techniques were used to examine the gene expression patterns in the developing PND 7 rat brain. Gene expression profiling was performed using the Illumina Rat Ref-12 Expression BeadChip platform that contains 22,226 probes (Illumina Inc., San Diego, CA, USA).

Sample labeling and quality control of labeled aRNA—For each sample, 200 ng total RNA was labeled using a MessageAmp II-biotin enhanced kit (Applied Biosystems, Foster City, CA, USA). Briefly, double stranded cDNA was synthesized using T7-oligo (dT)

primers and followed by an *in vitro* transcription (IVT) reaction to amplify aRNA while biotin was incorporated into the synthesized aRNA probe. The aRNA probe was then purified and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The size distributions of aRNA were made by running 200 ng of each sample on the Bioanalyzer (Agilent) using the Eukaryotic mRNA Assay with smear analysis.

Sample hybridization—The biotinylated aRNA probe was hybridized to Rat Ref-12 Arrays (Illumina). Labeled aRNA (1.5 μ g) was used for hybridization for each array. The hybridization, washing and scanning were performed according to the manufacturer's instructions with the addition of a 10-min wash in High-temp wash buffer (Illumina) at 55 °C for 10 min in a Scigene Hybex Microarray Incubation System (SciGene Corporation, Sunnyvale, CA, USA) with a water bath insert following overnight hybridization.

Scanning and data outputs—The chips were scanned using a BeadScan 2.3.0.10 (Illumina) at a multiplier setting of “2.” The microarray images were registered and extracted automatically during the scan using the manufacturer's default settings.

Normalization—Raw microarray intensity data were background subtracted and normalized using the cubic spline normalization method per the manufacturer's recommendation.

Data analysis—Gene expression data from the Illumina Rat Ref-12 arrays were input into ArrayTrack™, a software system developed at the NCTR/FDA for the management, analysis, visualization and interpretation of microarray data (<http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/>).

Clustering analysis was conducted within ArrayTrack™. Correlation coefficients were calculated within JMP 7.0 (SAS Institute, Cary, NC, USA). Pathway analysis was conducted using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA) (<http://www.ingenuity.com/>) and gene function analysis of Gene Ontology was conducted using FatiGO+ and GOFFA.

Detection of differentially expressed genes (DEGs)—The lists of significant DEGs were identified using a two-group *t*-test. The criteria used were a *P*-value less than 0.05 and fold changes greater than 1.4. Microarray data generated from 10 treated animals were directly compared to those generated from 10 control animals. The use of additional statistical methods, such as the SAM *t*-test (with a false discovery rate of 0.1), were also explored to identify DEGs (data not shown); however, similar results to the common *t*-test were obtained.

TaqMan gene expression procedures

The expression levels of the following genes were measured by quantitative PCR (Q-PCR) using TaqMan assays (Applied Biosystems). The TaqMan probes included: *Grin1* (*NR1*) (Rn01436038_m1), *Grin2a* (*NR2A*) (Rn01424654_m1), *Grin2b* (*NR2B*) (Rn00680474_m1), *Grin2c* (*NR2C*) (Rn00561364_m1) and *Grin2d* (*NR2D*) (Rn00575638_m1). Two genes,

RNA polymerase II A (*Polr2a*) (Rn01752026_m1) and β -actin (*Actb*) (Rn00667869_m1), were used for endogenous controls.

First strand cDNA synthesis—cDNA was prepared using a High-Capacity cDNA Archive Kit (Applied Biosystems). Briefly, total RNA (2 μ g) was reverse-transcribed in a final volume of 20 μ l with random primers at 25 °C for 10 min followed by 37 °C for 120 min according to the manufacturer's instructions.

TaqMan assays—Each assay was run in triplicate for each RNA sample. Total cDNA (25 ng) in a 25 μ l final volume was used for each assay. Assays were run with Universal Master Mix (2x) without AmpErase UNG on an Applied Biosystems 7000 Real-Time PCR System using universal cycling conditions (10 min at 95 °C; then 15 s at 95 °C and 1 min at 60 °C for 40 cycles).

Data normalization and analysis—Two endogenous control genes, *Actb* and *Polr2a*, were used for normalization. Each replicate cycle threshold (C_T) was normalized to the average C_T of the two endogenous controls on a per sample basis. The comparative C_T method was used to calculate relative quantification of gene expression (Livak and Schmittgen, 2001). The following formula was used to calculate the relative amount of the transcripts in the ketamine-treated samples (treated) and the saline-treated samples (control), and both were normalized to the endogenous controls. $C_T = C_T(\text{treated}) - C_T(\text{control})$.

C_T is the difference in C_T between the target gene and endogenous controls by subtracting the average C_T of controls. The fold-change for the ketamine-treated sample relative to the control sample = 2^{-C_T} .

Sensitivity detection and identification of differentially expressed genes (DEGs)—TaqMan assay's quantification was decided by the C_T value. The C_T value for each reaction was determined by setting the same threshold value across all reactions. A gene was considered not detectable when $C_T > 32$. A list of DEGs was identified using a two-tailed *t*-test calculated based on C_T values. Changes were considered statistically significant if a *P*-value was less than 0.05.

RESULTS

Ketamine-induced neurodegeneration

The repeated ketamine exposure produced elevated neuronal cell death as indicated by an increased number of TUNEL-positive cells in the developing (PND 7) rat brain (Fig. 1). In controls (Fig. 1A), only a few TUNEL-positive cells were observed, however, numerous darkly stained TUNEL-positive cells exhibiting typical nuclear condensation and fragmentation, indicative of enhanced apoptotic cell death, were observed (Fig. 1B) in ketamine-treated rat pups.

DNA microarray data quality

In this study, gene expression profiles for the developing brains of rats treated with or without ketamine were determined using whole genome-wide gene expression microarray

analyses. Both the control group and ketamine-treated group contained 10 biological replicates, therefore, a total of 20 microarrays were analyzed. To assess the overall quality and reproducibility of the DNA microarray data, Pearson's correlation coefficients for pair-wise $\log_2$ intensity correlations were calculated. It is worth mentioning that the Pearson's correlation matrix of 20 arrays was calculated based on all data points (22,226 probes), with no filtering applied for evaluation of microarray quality. The median rank correlation was 0.965 across all 20 arrays with the range of 0.953–0.980, indicating the data from the microarrays were very reproducible.

Hierarchical Cluster Analysis (HCA) (Fig. 2) and Principal Component Analysis (PCA) (Fig. 3) were employed to describe the gene expression profiles for controls and ketamine-treated samples. Two apparent clusters were revealed in both HCA and PCA with the samples separating well into control and treatment groups. These results indicate that clear treatment effects are detectable in ketamine-treated samples. It should be noted that the data from a total of 22,226 probes (the entire dataset), without a specific cut off, were utilized for the clustering analysis.

Statistical and functional analysis of DEGs

Fold-change criteria combined with a minimum P -value cut off derived from an appropriate t -test is considered a straightforward method for identifying DEGs (Guo et al., 2006a). Because there was no clear separation between male and female rats in either the ketamine-treated or control groups by either HCA (Fig. 2) or PCA (data not shown), sex differences were not taken into consideration when identifying the DEGs. In addition, to further differentiate the effects of sex and ketamine on the sources of variation in gene expression, two-way ANOVA was performed. It was determined that sex effects were nearly the same as random error, and the effects of ketamine were remarkably higher than those of sex, random error or the combination of both (data not shown). These results indicated that male and female animals display similar gene expression profiles after ketamine administration. Therefore, sex differences were excluded when analyzing DEGs.

To obtain a complete picture of the DEGs, a criterion of $P < 0.05$ with various fold-changes were used to generate a comprehensive list of DEGs. The numbers of DEGs at various fold changes with a $P < 0.05$ are shown in Table 1. The overall changes were relatively small, with only 60 DEGs observed using a fold-change greater than 2.0, and 10 DEGs with a fold-change greater than 3.0. The 819 DEGs associated with a fold-change greater than 1.4 and $P < 0.05$ were arbitrarily selected to do further bioinformatics analyses, and a complete list of these DEGs is presented in Supplementary Table 1.

Functional enrichment of Gene Ontology terms using FatiGO+ and GOFFA

FatiGO+ is a web-based bioinformatics tool for the functional profiling of genome-scale experiments specifically oriented for the interpretation of microarray experiments (Al-Shahrour et al., 2007). We applied the 819 DEGs in response to ketamine treatment to perform the functional enrichment of Gene Ontology terms by using FatiGO+. FatiGO+ produced 31 significant terms associated with Biological Process, 11 significant terms related to Molecular Function, and nine significant terms relevant to Cellular Component.

Details are shown in Table 2. Of note, a total of 35 genes were found associated with the Gene Ontology term “cell death” and 46 genes were associated with the GO term “receptor activity.” Additional GO analyses were conducted using GOFFA (Sun et al., 2006) which produced 32 genes associated with the process of apoptosis (Table 3).

Pathway analysis of the DEGs

Ingenuity Pathway Analysis (IPA) software was used to determine the most relevant biological pathways from the significant DEGs. Out of the 819 DEGs, 516 were identified by IPA software and were used to map the canonical pathways contained in the database. Up to 18 pathways were found to be significantly altered with the $-\log(P\text{-value})$ greater than 1.4 (equivalent to $P < 0.05$). These significant canonical pathways and linked genes are presented in Table 4. It should be mentioned that four receptor signaling pathways (glutamate, GABA, dopamine and aryl hydrocarbon) were positively identified. Alterations in the glutamate receptor signaling pathway is in line with our hypothesis that ketamine triggers compensatory up-regulation of the NMDA receptor, which is an ionotropic receptor for glutamate, leading to over-excitation of the signaling pathway mediated by endogenous glutamate. Also of interest is that circadian rhythm signaling, which is related to behavioral patterns, and the complement system, which is associated with the clearance of apoptotic neurons, were significantly changed, reminiscent of the behavioral changes and accelerated apoptosis observed after ketamine administration. It is worth noting that all five genes involved in the complement system that were identified in this study were up-regulated.

Quantitative (Q)-PCR validation of the microarray results

Q-PCR assays were used to verify the results of the gene expression changes measured using microarrays, with particular focus on mechanistically important genes, such as those involved in the glutamate receptor signaling pathway. As shown in Table 5, based on triplicate measurements for each RNA sample, the genes for *Grin1*, *Grin2a* and *Grin2c* were significantly up-regulated in ketamine-treated animals compared to controls. It should be noted that the Q-PCR data correlated well with the microarray results (Table 5). The observed pattern of change was nearly identical using the two different techniques, although the Q-PCR method appeared more sensitive than the microarray.

Altered NMDA receptor expression (*in situ* hybridization)

Because ketamine is an NMDA receptor antagonist, it seemed plausible that the localization of the most severe brain damage (apoptotic neurons) in the frontal cortical area might correspond to alterations in NMDA receptor expression. In both controls and ketamine-treated (20 mg/kg×6 injections) rats, NMDA receptor NR1 subunit mRNA was prominent. The autoradiographic density (labeling) for NR1 subunit mRNA was up-regulated in the frontal cortex of rats treated with ketamine compared with control (Fig. 4).

DISCUSSION

The vulnerability of the immature rat brain to ketamine-induced neurotoxicity has been previously examined by our laboratory (Zou et al., 2009). Rat pups (PND-7) were exposed to 5, 10, or 20 mg/kg ketamine (dose response) in single or multiple injections (three or six

injections) once every 2 h (time course profile). PND 7 is a period characterized by intense synaptic remodeling and a period of myelin formation. At PND 7 most afferent pathways are already present in their target areas. However, their final distribution and synaptic targets are still immature. Thus, PND 7 is thought to be the most sensitive stage to disruption by ketamine and was selected for this study of associated changes in gene expression. Enhanced apoptotic cell death was not detected in the frontal cortex from animals exposed to 5 or 10 mg/kg of ketamine in single or multiple injections compared with their controls. However, a remarkable increase in apoptotic neurodegeneration was observed in layers II and III of the frontal cortex and several major brain regions including striatum, hippocampus, thalamus and amygdala after 20 mg/kg×6 injections of ketamine. Multiple ketamine injections (20 mg/kg given six times, once every 2 h) produce the most severe neuronal damage (~10-fold increase) in the frontal cortex versus a threefold increase (or less) in striatum, hippocampus, thalamus, and amygdala as revealed by immunocytochemical and morphological approaches. No significant effects were observed in animals given either one or three injections of 20 mg/kg ketamine. These data suggest that the frontal cortex is the brain region most vulnerable to ketamine-induced neurotoxicity during development.

Doses of ketamine administered to children are in the range of 5–10 mg/kg i.m. for induction of anesthesia. However, there appears to be a significant difference in sensitivity to ketamine-induced anesthesia between humans and animals such as rodents and nonhuman primates. In the present study, ketamine doses of 20 mg/kg were given to PND-7 rat pups in order to attain a light anesthetic state which is characterized by pup immobility and failure to nurse after a brief period of excitation (paddling/swimming motions) following each ketamine injection. The results (enhanced apoptotic cell death) are consistent with those from previously published reports in which plasma levels in 7-day-old rat pups were noted to be higher than those in human subjects anesthetized with ketamine (Scallet et al., 2004).

In this study, brain tissues from frontal cortical levels, where the most severe neuronal damage occurred after exposure to ketamine, were selected for RNA isolation and microarray analysis. Consistent with the TUNEL assay and previous *in vivo* data, microarray data also indicated apoptosis is involved in ketamine neurotoxicity. A total of 32 genes were found to be involved in apoptosis. Of these 32 genes, 15 were up-regulated and 17 were down-regulated in exposed animals. It has been reported that apoptosis-related genes comprise a group that has two distinct modes of operation: pro-apoptosis or anti-apoptosis. In response to various inducers such as stressful stimuli or sustained elevation of intracellular calcium, the ultimate fate of neuronal cells is determined by the roles of those apoptosis-related genes in regulating the life/death cell balance. The mechanism(s) underlying ketamine-induced neuronal cell death have not been fully elucidated. However, microarray data from this study indicate that the majority of up-regulated genes were pro-apoptotic (*Agt*, *Clu*, *Gjb6*, *Hrk*, *Igfbp3*, *Inpp5d*, *Jun*, *Mal*, *Rassf5* and *Txnip*). Upregulation of the antiapoptotic genes *Alb*, *Apoe*, and *Cryab* may inhibit apoptosis (Mao et al., 2004). *Bag3* (Bcl2-associated athanogene 3) was found to be down-regulated and it has been reported that silencing the expression of *Bag3* leads to reduced protein levels of Bcl-XL, Mcl-1 and Bcl-2 in colon cancer cells and increased apoptosis (Jacobs and Marnett, 2009). One critical gene, *Cebpb* [CCAAT/enhancer binding protein (C/EBP)], acts as a major regulator of metabolic homeostasis and is involved in many cellular processes, such as

differentiation, growth, immune responses, neoplastic growth, development of the reproductive system, and pro- and anti-growth pathways (Gade et al., 2008).

In genes that were down-regulated (17 genes) approximately half are pro-apoptotic and the other half are antiapoptotic genes. The over-expression of *Acvr1c* suppresses apoptotic effects and *Amigo2* acts as an antiapoptotic factor (Hashimoto et al., 1998; Ono et al., 2003). *Bnip3* encodes cellular proteins that interact with Bcl-2. In cortical cells cyanide induces a rapid up-regulation of *Bnip3* expression, followed by caspase-dependent cell death (Prabhakaran et al., 2007). Down-regulation of *Cd24*, *Cdc2a*, and disruption of the *Rasa1* gene in the early development of mice induces apoptosis of neuronal cells (Smith et al., 2006; Lapinski et al., 2007). In neocortical and hippocampal tissues, apoptotic effects can be demonstrated following *Ahr* activation (Kajta et al., 2009).

It should be mentioned that the microarray data analysis did not detect changes in the expression of several regulatory apoptotic genes, such as the caspase-3 and Bcl-2 gene families (Slikker et al., 2007; Wang et al., 2005b, 2006). These observations may be related to the timing of tissue sampling for the microarray analyses. Analyses at different times after ketamine exposure may show very different effects.

The present study also identified disruptions in glutamate receptor signaling, synaptic long-term potentiation, PTEN (phosphatase and tensin homolog deleted on chromosome 10) signaling, pyrimidine metabolism, circadian rhythm signaling, etc., using IPA software. In addition, this study observed unique alterations in dopamine receptor signaling and the complement system, and also found remarkable changes in 32 genes related to apoptosis. More specific and sharper differences did exist in that the Troponin T1 (*Tnnt1*) gene was significantly induced in the adult mouse brain (Lowe et al., 2007), but this was not observed in our system. Conversely, while a significant up-regulation of the *Grin1* (*NR1*) and *Grin2* (*NR2*) genes was observed in this study, the previous report did not show similar results (Lowe et al., 2007). These similarities and differences most likely reflect the unique response patterns of adult and developing brains toward ketamine administration. Together, these data indicate that potential anesthetic-induced neurotoxicity may depend on the dose of anesthetic, the duration of exposure, the route of administration, the receptor subtype activated, and the stage of development or maturity at the time of exposure.

The gene expression of the NMDA receptor subunit gene, *Grin1* (*NR1*), was significantly up-regulated in ketamine-treated rat pups as detected in microarray experiments and subsequently confirmed with TaqMan analyses. The NMDA receptor NR1 subunit is widely distributed throughout the brain and is the fundamental subunit necessary for NMDA channel function. In the present study, by using *in situ* hybridization techniques to detect the relative densities of NMDA receptor NR1 subunits, a potential parallel relationship between enhanced apoptosis and NMDA receptor expression levels was examined. Our *in situ* data provided direct evidence that repeated ketamine exposure results in a substantial increase in autoradiographic density (labeling) of NR1 subunit mRNA in the frontal cortex and hippocampus. These data indicate that ketamine-induced pathological change is closely associated with a remarkable up-regulation of NMDA NR1 sub-unit mRNA.

It is possible that increased expression of *Grin1* (*NR1*) was accompanied by an altered expression of other glutamate receptor subunits. The present microarray analyses revealed an increase in *Grin2a* (*NR2A*; 1.5 fold) and *Grin2c* (*NR2C*; 1.7 fold), but no significant effects were observed in *Grin2b* (*NR2B*) or *Grin2d* (*NR2D*). It should be noted that NMDA-R2 subunits produce functional receptors only when co-expressed with NMDA-R1 (Monyer et al., 1992) and heteromeric complexes increase the responsiveness to NMDA and yield different functional properties (Buller et al., 1994). Our findings are consistent with those of previous *in situ* hybridization and immunoblotting data that demonstrated a compensatory up-regulation of NMDA-R1 and NMDA-R2 receptors following prolonged exposure to NMDA receptor antagonists (Wang et al., 2005a; Slikker et al., 2007; Zou et al., 2009).

NMDA receptor density has been shown to increase in cultured cortical neurons after exposure to the NMDA receptor antagonists D-AP5, CGS-19755, and MK-801, but not after exposure to the AMPA/kainate receptor antagonist CNQX (Williams et al., 1992). In the present study, no significant changes were detected in the gene expression patterns of AMPA or kainate receptors after repeated ketamine exposure. These findings support our previous pharmacological data showing that application of the non-NMDA receptor antagonist, CNQX, or nifedipine (an antagonist of the L-type voltage sensitive calcium channel) did not produce a significant protective effect against ketamine-induced neuronal apoptosis (Wang et al., 2005b). We hypothesize that continuous blockade of NMDA receptors by ketamine causes a compensatory up-regulation of NMDA receptors and this up-regulation makes neurons bearing these receptors more vulnerable, after ketamine withdrawal, to the excitotoxic effects of endogenous glutamate, because this up-regulation of NMDA receptors allows for the accumulation of toxic levels of intracellular calcium even under normal physiological conditions. This hypothesis is also supported by data from previous *in vitro* studies where it was shown that co-administration of antisense oligonucleotides that specifically target NMDA receptor NR1 and NR2A subunit mRNAs were able to block the neuronal damage induced by ketamine or phencyclidine (PCP) (Wang et al., 2005a,b, 2006). To determine if ketamine directly increases calcium levels and to further elucidate the mechanisms of ketamine-induced neurotoxicity, calcium influx experiments are currently being conducted in our laboratory to measure intracellular calcium after ketamine treatment.

In the present study, NF- κ B genes were significantly up-regulated (1.6-fold). These results are consistent with our previous data (Wang et al., 2006) that demonstrated ketamine produces a remarkable increase in the translocation of NF- κ B into the nucleus. The protection against ketamine-induced cortical neuronal cell death by a peptide inhibitor of NF- κ B translocation, SN-50, suggests that there is a causal relationship between these effects. There is evidence in the literature suggesting that the transcriptional regulation of target genes by NF- κ B is tissue-specific and possibly gene-specific within a given cell type (Shou et al., 2000). Whether ketamine-induced NF- κ B gene over-expression is specifically responsible for the apoptotic pathways observed in this study is still unknown. Resolution of this question will require additional experiments.

Perturbation of the complement system is an interesting new finding of the present study. It is worth noting that five relevant genes (*C1QB*, *C1QC*, *C1QA*, *C2* and *C4BPB*) were up-regulated, strongly suggesting an enhanced activation of the complement system. Three

pathways, that is, the classic pathway, the lectin pathway and alternative pathway, have been proposed to activate the complement system (Francis et al., 2003). All the up-regulated genes identified here are solely involved in the classic pathway. Activation of the complement system, especially via the classic pathway, is well-recognized as an important means to selectively remove apoptotic cells (Francis et al., 2003). Recent evidence also highlights the significance of complement activation in “tagging unwanted synapses for removal” during developmental synaptic refinement, a notion supported by the observation that elevated *CIq* expression is directly associated with disrupted synapse loss in a glaucoma mouse model (Stevens et al., 2007). We propose that ketamine triggers enhanced apoptosis in the developing brain, which then leads to subsequent activation of the complement system to help remove apoptotic brain cells. Dysregulated complement activation, however, could be detrimental to the physiological process of brain development and normal maturation. Investigations using chemical inhibitors and genetic manipulations are needed to confirm the role of the complement system in ketamine-induced developmental neurotoxicity.

The present study found that seven genes associated with the circadian rhythm signaling pathway were significantly changed after ketamine administration, indicating that ketamine might lead to significant disruption of the neural regulation of circadian rhythms. Specifically, four clock genes including *Per1*, *BHLHB3*, *NR1D1* and *BHLHB2* were up-regulated. These genes function as key negative regulators (i.e., act to prevent transcription) of the transcriptional/translational feedback loop that forms the basis of the circadian clock cycles (Dispersyn et al., 2008). The biological consequences of ketamine-induced up-regulation of these clock genes might be prolonged circadian cycles. The gene *ADCYAP1*, which encodes the pituitary adenylyl cyclase-activating peptide that selectively maintains the light response of the circadian system (Colwell et al., 2004), was down-regulated 1.4-fold by ketamine. Up-regulation of *Grin1* (*NR1*) and *Grin2c* (*NR2C*), which produces light-like phase shifts in the circadian clock (Mintz et al., 1999) might offset this response. Investigations are currently in progress to determine if ketamine induces behavioral alterations in developing rats, with a special focus on circadian cycles.

CONCLUSION

A total of 462 down-regulated genes and 357 up-regulated genes were identified using a criteria of >1.4-fold change and a $P < 0.05$. Among them, 32 genes are associated with apoptotic pathways. Perturbations of NMDA-type glutamate and other receptor signaling were identified by IPA software. Q-PCR confirmed that NMDA receptor genes including *Grin1* (*NR1*), *Grin2a* (*NR2A*) and *Grin2c* (*NR2C*) were significantly up-regulated. The ketamine-induced up-regulation of NMDA receptor *Grin1* (*NR1*) mRNA signaling was further confirmed by *in situ* hybridization. These observations suggest that repeated ketamine exposure causes compensatory up-regulation of NMDA receptors and subsequent over-stimulation of the glutamatergic system triggering enhanced apoptosis in developing neurons.

When taken together, the morphological, *in situ* and microarray data imply that the frontal cortex is the brain region most vulnerable to ketamine-induced neurotoxicity during

development, and that neuronal survival in the early phases of apoptotic cascades depends mostly on the balance between pro- and anti-apoptotic factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by an Interagency Agreement (IAG 224-07-007) between NCTR/FDA and the National Institute for Environmental Health Sciences (NIEHS)/ National Toxicology Program (NTP), the Center for Drug Evaluation and Research (CDER)/FDA, and the National Institute of Child Health and Human Development (NICHD).

Abbreviations

DEGs	differentially expressed genes
GO	gene ontology
iGluRs	ionotropic glutamate receptors
HCA	hierarchical cluster analysis
IPA	ingenuity pathway analysis
KA	kainate receptors
mGluRs	metabotropic glutamate receptors
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
PCA	principal component analysis
PND	postnatal day
PTEN	phosphatase and tensing homolog deleted on chromosome 10
Q-PCR	quantitative polymerase chain reaction
RINs	RNA integrity numbers
TdT	deoxynucleotidyl transferase
TUNEL	terminal dUTP nick-end labelling

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TUNEL-Assay (*PND-7 rat pups*)

Fig. 1. Ketamine-induced neurodegeneration in PND 7 rats assessed by TUNEL labeling. Representative photographs indicate that TUNEL-positive cells are more numerous in layers II and III of the frontal cortex in the ketamine treated rat brain (B). Only a few TUNEL-positive cells were observed in the control (saline treated) rat brain (A). Scale bar=60 µm.

Fig. 2.

Hierarchical Cluster Analysis (HCA) of expression profiles for control and ketamine-treated groups. The log₂ intensity of the entire gene set was scaled by Z-score transformation, and then these values were hierarchically clustered using 1-r distance metric and average linkage. Each column represents the results from an individual animal. Each row represents the log₂ intensity values of the 20 samples for one particular gene. Samples are labeled according to the convention of Treatment_Animal ID_Gender. Ket, ketamine treatment; CTR, vehicle control; F1-20, animal ID; M, male; F, female.

Fig. 3. Principal component analysis (PCA) of gene expression profiles for ketamine-treated groups and the concurrent controls. The intensity of entire gene set was used; no specific cut off was applied for the analysis. The autoscaled method was used for the PCA. The circles and triangles indicate controls and ketamine treatments, respectively.

***In Situ* Hybridization (Frontal Cortical Levels) (NMDA Receptor NR1 Subunit mRNA)**

Fig. 4. NMDA receptor NR1 subunit mRNA abundance in PND 7 rats. *In situ* hybridization was performed on rat brain sections (coronal) using a ^{35}S -labeled oligonucleotide probe specific for the NMDA receptor NR1 subunit. Panel (I) is a film autoradiography that provides a general view of NMDA receptor NR1 subunit mRNA expression in the frontal cortical areas from both control and ketamine-treated rats. Panel (II) illustrates that the autoradiographic density (labeling) for NR1 subunit mRNA was higher in ketamine-treated (20 mg/kg $\times$ 6 injections) rat brain frontal cortex (B) compared to control (A). Scale bar=90 μm .

Table 1

Number of differentially expressed genes (DEGs) in the brain of PND 7 rats after ketamine treatment using various fold-change criteria and a $P < 0.05$

Fold-change	Total no. DEGs	No. up-regulated DEGs	No. down-regulate DEGs
>1.3	1485	641	844
>1.4	819	357	462
>1.5	468	197	271
>1.6	264	116	148
>1.7	166	76	90
>1.8	115	59	56
>1.9	78	40	38
>2.0	60	32	28
>2.1	43	25	18
>2.2	24	12	12
>2.5	17	9	8
>3.0	10	5	5

Table 2

Functional enrichment of the 819 differentially expressed genes having Gene Ontology terms using FatiGO+

	Level	GO accession	P
Biological process			
Regulation of biological process	3	(GO:0050789)	1.52E-07
Death	3	(GO:0016265)	1.79E-05
Cellular developmental process	3	(GO:0048869)	1.75E-04
Response to stress	3	(GO:0006950)	6.71E-04
Regulation of biological quality	3	(GO:0065008)	1.09E-03
Response to biotic stimulus	3	(GO:0009607)	9.59E-03
Response to endogenous stimulus	3	(GO:0009719)	9.59E-03
Multicellular organismal development	3	(GO:0007275)	2.90E-02
Anatomical structure development	3	(GO:0048856)	2.90E-02
Circulation	3	(GO:0008015)	4.86E-02
Regulation of cellular process	4	(GO:0050794)	3.36E-05
Negative regulation of biological process	4	(GO:0048519)	2.19E-04
Cell differentiation	4	(GO:0030154)	5.24E-04
Alcohol metabolic process	4	(GO:0006066)	9.81E-04
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4	(GO:0006139)	2.10E-03
Signal transduction	4	(GO:0007165)	5.18E-03
Regulation of developmental process	4	(GO:0050793)	1.18E-02
Oxygen and reactive oxygen species metabolic process	4	(GO:0006800)	2.00E-02
Positive regulation of biological process	4	(GO:0048518)	2.90E-02
Lipid metabolic process	4	(GO:0006629)	3.90E-02
Cellular component disassembly	4	(GO:0022411)	4.55E-02
Regulation of metabolic process	4	(GO:0019222)	4.71E-02
Cell surface receptor linked signal transduction	5	(GO:0007166)	9.10E-10
Negative regulation of cellular process	5	(GO:0048523)	7.84E-04
Cell development	5	(GO:0048468)	1.14E-02
G-protein coupled receptor protein signaling pathway	6	(GO:0007186)	3.01E-13
Cell death	6	(GO:0008219)	2.38E-04
Steroid metabolic process	6	(GO:0008202)	6.70E-03
Regulation of cell-cell adhesion	6	(GO:0022407)	1.43E-02
Sterol metabolic process	7	(GO:0016125)	2.84E-02
Sterol biosynthetic process	8	(GO:0016126)	4.73E-02
Molecular function			
Receptor activity	3	(GO:0004872)	8.88E-07
Protein binding	3	(GO:0005515)	2.95E-06
Ion binding	3	(GO:0043167)	1.82E-04
Structural constituent of ribosome	3	(GO:0003735)	1.72E-03
Enzyme inhibitor activity	3	(GO:0004857)	4.62E-02
Transmembrane receptor activity	4	(GO:0004888)	1.12E-11

	Level	GO accession	P
Metal ion binding	4	(GO:0046872)	8.22E-04
Cation binding	4	(GO:0043169)	3.98E-03
G-protein coupled receptor activity	5	(GO:0004930)	7.78E-16
Rhodopsin-like receptor activity	6	(GO:0001584)	1.78E-14
Olfactory receptor activity	7	(GO:0004984)	2.25E-17
Cellular component			
Membrane-bound organelle	3	(GO:0043227)	2.17E-04
Neuron projection	5	(GO:0043005)	3.91E-02
Dendrite	6	(GO:0030425)	1.34E-03
Intracellular organelle	6	(GO:0043229)	1.34E-03
Ribonucleoprotein complex	6	(GO:0030529)	3.09E-03
Cytoplasm	6	(GO:0005737)	3.75E-03
Intracellular membrane-bound organelle	7	(GO:0043231)	1.12E-05
Dendritic spine	7	(GO:0043197)	2.63E-02
Ribosome	8	(GO:0005840)	2.73E-04

Table 3

Apoptosis related genes identified by GOFFA

	Gene symbols	Gene names
1	<i>Acvr1c</i>	Activin A receptor, type IC
2	<i>Ahr</i>	Aryl hydrocarbon receptor
3	<i>Alms1</i>	Alstrom syndrome 1
4	<i>Amigo2</i>	Adhesion molecule with Ig like domain 2
5	<i>Atp7a</i>	ATPase, Cu + + transporting, alpha polypeptide
6	<i>Bnip3</i>	BCL2/adenovirus E1B 19 kDa-interacting protein 3
7	<i>Bub1b</i>	Budding uninhibited by benzimidazoles 1 homolog, beta
8	<i>Cd24</i>	CD24 antigen
9	<i>Cdc2a</i>	Cell division cycle 2 homolog A (S. pombe)
10	<i>Inhba</i>	Inhibin beta-A
11	<i>Myc</i>	Myelocytomatosis oncogene
12	<i>Ntf3</i>	Neurotrophin 3
13	<i>Pak7_predicted</i>	p21 (CDKN1A)-activated kinase 7 (predicted)
14	<i>Pdia2_predicted</i>	Protein disulfide isomerase associated 2 (predicted)
15	<i>Rasa1</i>	RAS p21 protein activator 1
16	<i>Tnfrsf11b</i>	Tumor necrosis factor receptor superfamily, member 11b
17	<i>Unc5c</i>	unc-5 homolog C (C. elegans)
18	<i>Agtr</i>	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
19	<i>Alb</i>	Albumin
20	<i>Apoe</i>	Apolipoprotein E
21	<i>Bag3</i>	Bcl2-associated athanogene 3
22	<i>Cebpb</i>	CCAAT/enhancer binding protein (C/EBP), beta
23	<i>Clu</i>	Clusterin
24	<i>Cryab</i>	Crystallin, alpha B
25	<i>Gjb6</i>	Gap junction membrane channel protein beta 6
26	<i>Hrk</i>	Harakiri, BCL2 interacting protein (contains only BH3 domain)
27	<i>Igfbp3</i>	Insulin-like growth factor binding protein 3
28	<i>Inpp5d</i>	Inositol polyphosphate-5-phosphatase D
29	<i>Jun</i>	Jun oncogene
30	<i>Mal</i>	Myelin and lymphocyte protein, T-cell differentiation protein
31	<i>Rassf5</i>	Ras association (RalGDS/AF-6) domain family 5
32	<i>Txnip</i>	Thioredoxin interacting protein

Genes 1–17 were down-regulated and genes 18–32 were up-regulated.

Table 4

Significant canonical pathways and associated genes identified by Ingenuity Pathways Analysis software

Pathways	Log (P)	Gene symbols and changes in the expression level
Circadian rhythm signaling	3.78E+00	NR1D1 (↑2.8) BHLHB2 (↑1.6) BHLHB3 (↑1.5) GRIN2C (↑1.5) PER1 (↑1.4) GRIN1 (↑1.4), ADCYAP1 (↓1.4)
Biosynthesis of steroids	2.84E+00	CYP46A1 (↑1.4), DUI (↓1.9), HMGCR (↓1.7), EBF (↓1.5), SQLE (↓1.5), FDFT1 (↓1.5)
Pyrimidine metabolism	2.70E+00	POLR3F (↑1.7) POLR2F (↑1.5) CANT1 (↑1.4) NME4 (↓2.0), TYMS (↓1.9), DHODH (↑1.8), DPYSL3 (↓1.8), POLG (↓1.7), RRM2 (↑1.6), POLE2 (↓1.5), POLD (↓1.5), POLA1 (↓1.4), DCTD (↓1.4), POLR3H (↓1.4), NR (↓1.4)
GABA receptor signaling	2.42E+00	GABRD (↑1.7) DNMI (↑1.5) NSF (↑1.5) SLC32A1 (↑1.4), GABRB2 (↓1.6), GABRG2 (↓1.5), UBQLN1 (↓1.5)
IGF-1 signaling	2.34E+00	IGFBP1 (↑2.2) JUN (↑1.9) IGFBP3 (↑1.6) CASP9 (↑1.5) MRAS (↑1.4), RASA1 (↓1.9), PTK2 (↓1.5), RRAS2 (↓1.4), FOXO1 (↓1.4), PRKAG2 (↓1.4)
Acute phase response signaling	2.19E+00	AGT (↓2.9) ALB (↓2.1) JUN (↑1.9) ITIH3 (↑1.7) TF (↑1.7) NFKB1A (↑1.6) C2 (↑1.5) CEBPPB (↑1.5) HMOX2 (↑1.5) C4BPB (↑1.4) MRAS (↑1.4), TNFRSF11B (↓1.7), JAK2 (↓1.5), SOC35 (↓1.5), RRAS2 (↓1.4)
VDR/RXR activation	2.17E+00	IGFBP1 (↑2.2) PRKCG (↑1.6) IGFBP3 (↑1.6) CEBPPB (↑1.5) SERP1NB (↑1.5) IL12A (↑1.4) RXRQ (↓1.6), YY1 (↓1.4), FOXO1 (↓1.4)
LXR/RXR activation	2.00E+00	APOE (↑1.6) SCD (↑1.5) SREBF1 (↑1.5) ABCG1 (↑1.5) ABCA1 (↑1.5), HMGCR (↓1.7), TNFRSF11B (↓1.7), RXRQ (↓1.6)
Complement system	1.85E+00	CIQB (↑2.1) CIQC (↑1.9) CIQA (↑1.6) C2 (↑1.5) C4BPB (↑1.4)
LPS/IL-1 mediated inhibition of RXR function	1.69E+00	JUN (↑1.9) GSTO2 (↑1.8) SULT1A1 (↑1.8) HMGCS2 (↑1.7) SMOX (↑1.7) APOE (↑1.6), MGST2 (↑1.6) ABCG1 (↑1.5) SOD3 (↑1.5) ABCA1 (↑1.5) ACSBG1 (↑1.5) SREBF1 (↑1.5), HS3ST5 (↓1.8), TNFRSF11B (↓1.7), ALDH18A1 (↓1.6)
Inositol phosphate metabolism	1.54E+00	ITPKA (↑2.0) INPP5D (↑1.5) PLCD4 (↑1.5) PMPCA (↑1.5), INPP5F (↓1.6), PRPF4B (↓1.6), PAK7 (↓1.6), PIP4K2A (↓1.5), CDC2 (↓1.5), ISYNA1 (↓1.4), PLCL1 (↓1.4)
PDGF signaling	1.52E+00	JUN (↑1.9) INPP5D (↑1.5) MRAS (↑1.4), MYC (↓1.9), RASA1 (↓1.9), JAK2 (↓1.5), RRAS2 (↓1.4)
Glutamate receptor signaling	1.47E+00	SLC17A7 (↑1.8) SLC1A2 (↑1.7) GRIN2C (↑1.5) GRIN1 (↑1.4) GRIK5 (↑1.4), GRM3 (↓1.6)
Aryl hydrocarbon receptor signaling	1.43E+00	JUN (↑1.9) GSTO2 (↑1.8) MGST2 (↑1.6), CCND1 (↓2.0), MYC (↓1.9), AHR (↓1.8), CCNA2 (↓1.8), RXRQ (↓1.6), ALDH18A1 (↓1.6), RB1 (↓1.5), POLA1 (↓1.4)
14-3-3-mediated signaling	1.43E+00	JUN (↑1.9) TUBA4A (↑1.9) GFAPI (↑1.6) PRKCG (↑1.6) PLCD4 (↑1.5) MRAS (↑1.4), PRPF4B (↓1.6), PAK7 (↓1.6), CDC2 (↓1.5), RRAS2 (↓1.4), FOXO1 (↓1.4), PLCL1 (↓1.4)
PTEN signaling	1.42E+00	CNKSR3 (↑1.6) INPP5D (↑1.5) CASP9 (↑1.5), MRAS (↑1.4), CCND1 (↓2.0), PTK2 (↓1.5), RRAS2 (↓1.4), FOXO1 (↓1.4)
Synaptic long term potentiation	1.39E+00	PRKCG (↑1.6) GRIN1 (↑1.4) GRIN2C (↑1.5), PPP1R14B (↓1.7), ADCY8 (↓1.6), GRM3 (↓1.6), RRAS2 (↓1.4), MRAS (↑1.4), PRKAG2 (↓1.4)
Dopamine receptor signaling	1.35E+00	COMT (↑2.1) PPP1R1B (↑1.9) SMOX (↑1.7) ADCY8 (↑1.6) ADCY9 (↑1.5), PPP1R14B (↓1.7), PRKAG2 (↓1.4)

The symbols ↑ and ↓ indicate up- and down-regulated genes respectively. Numbers following the ↑ or ↓ symbols represent the fold-change for the gene expression level. The up-regulated genes are in bold.

Table 5

Selective validation of the microarray results by Q-PCR

Gene symbols	Fold-change (Q-PCR)	Fold-change (microarray)
<i>Grin1 (NR1)</i>	1.8 *	1.5 *
<i>Grin2a (NR2A)</i>	1.5 *	1.2
<i>Grin2b (NR2B)</i>	1.0	0.9
<i>Grin2c (NR2C)</i>	1.7 *	1.5 *
<i>Grin2d (NR2D)</i>	1.2	1.1

* $P < 0.05$, as compared to the control.