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Effects of Olanzapine on Gene Expression Changes in MK-801-induced Neurotoxicity Using a High-density DNA Microarray

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Abstract

Although the etiology of schizophrenia is known to be linked with the disturbance of glutamatergic and dopaminergic neurotransmission, little is known about the relationship between gene expression and the disease process. To identify genes related to abnormalities in glutamatergic and dopaminergic function, we investigated the effects of olanzapine in the changes of mRNA levels in the animal model of schizophrenia, using a high-density DNA microarray. Olanzapine (3.0 mg/kg, i.p.) significantly reduced hyperlocomotive activities, which was induced by MK-801 (1.0 mg/kg, i.p.). We identified that the expression of 719 genes were significantly altered more than two folds in the prefrontal cortex of the rats treated with MK-801. We selected 15 genes out of them by the changes of the expression pattern in the treatment of Olanzapine and/or MK801 for the further confirmation in RT-PCR. The administration of MK-801 increased the expression of 7 genes (NOS3, Hspb1, Hspa1a, CRH, Serpine1, Igfbp6, Snf1lk) and decreased the expression of 1 gene (Aldh1a2), which was attenuated by olanzapine. One gene (Prss12) was up-regulated after olanzapine treatment although it did not show the significant changes after MK-801 treatment. These results showed that antipsychotic drug, such as olanzapine, may alter the gene expression patterns, which were accompanied by MK-801-induced psychosis. Our results also provide us high-density DNA microarray technology could be potential approaches to find the candidate molecules for the therapeutics and also for the early diagnosis of psychiatric diseases.

Keywords: DNA microarray, Schizophrenia, Olanzapine, MK-801, Gene expression

Although the psychopathology of schizophrenia, a chronic brain disorder, is poorly understood, multiple lines of evidence suggest the disrupted cortical synaptic circuitry as a major deficit. Clinically, schizophrenia is characterized by 1) positive symptoms (such as hallucinations, delusions, and severe thought disorganization), 2) negative symptoms (such as flattened affect, apathy, anhedonia, and social withdrawal), and 3) cognitive symptoms (such as deficits in attention and memory). It was postulated that a number of complex pathophysiological changes underlie this disease. In many possible synaptic events, schizophrenia has linked with abnormalities in glutamatergic function[1-3], as well as in other monoamine systems[4]. In schizophrenia, glutamatergic and dopaminergic neurotransmission are closely related. Thus, it is proposed that schizophrenia is associated with the abnormalities of glutamate and dopamine transmission. Hypofunction of NMDA receptor in the prefrontal cortex generates a dysregulation pattern of dopamine systems, followed by weakness in NMDA-mediated connectivity and synaptic plasticity[5].

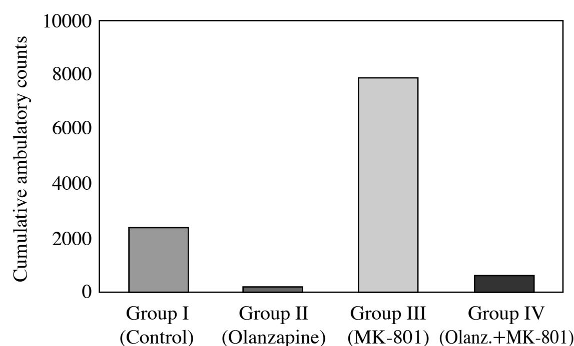
Glutamate is the most common excitatory neurotransmitter in the central nervous system. The glutamate receptor is classified to two groups, ionotropic receptor and metabotropic receptor. In the ionotropic N-methyl-D-aspartate (NMDA) receptor has a major role in schizophrenia. The NMDA receptor regulates ion channels permeable to Ca^{2+}, K^{+}, and Na^{+}, and has also binding sites for, glycine, Zn^{2+} and Mg^{2+} as well as for the synthetic drugs phencyclidine and MK-801 ([+]-5-methyl-10, 11-dihydro-5H-dibenzo-[a, d]-cyclohepten-5, 10-imine hydrogen maleate)[6,7]. These findings led that MK-801 treatment has been used for the animal models of at least some aspects of schizo-

phrenia. In rodents, administration of NMDA receptor antagonists causes hyperactivity, corresponding to positive symptoms in schizophrenia[8]. Although current animal models for schizophrenia are not intended to serve as a complete equivalent of the human disorder, instead they are often designed to represent a specific pathophysiological mechanism[9,10]. In most animal models for schizophrenia studies, acute treatment with MK-801 is used[11-13]. Animals treated with NMDA antagonists, MK-801, exhibit several types of behavior related to a range of symptoms in schizophrenia[14], e.g., hyperlocomotion[15], decreased social behavior[16], impaired performance on cognitive tasks[17], and deficits in sensorimotor gating[18].

Antipsychotic drugs (APDs) can substantially improve the symptoms, but they do not generally cure the disease[19,20]. APDs take time to exhibit their full therapeutic effect, and it has been suggested that molecular adaptations contribute to their mechanism of action[21]. Indeed, APDs induce gene expression and activate the related transcription factors[22-24]. Olanzapine is antipsychotic agent that, like clozapine, exhibits higher $5HT_{2A}$ antagonism than D2 antagonism[25]. Olanzapine, has high affinity to a number of other receptors, including $5HT_{2A}$, $5HT_{2C}$, $5HT_{1A}$, $5HT_6$, $5HT_7$, $5HT_3$, muscarinic, a1-adrenergic, and histamine-H_1 receptors[26]. Additionally, olanzapine facilitates NMDA neurotransmission via affecting on glutamatergic NMDA and AMPA receptors[27]. However, apart from its receptor-binding profile, little is known about the molecular mechanism of olanzapine drug action. Although a number of brain regions have been implicated in the pathophysiology of schizophrenia, the dorsal prefrontal cortex has been singled out as a major site of dysfunction on the basis of considerable clinical, neuroimaging, and postmortem studies and has thus been focused to recent microarray efforts[25,28]. In this study, we detected the change of the expression pattern after olanzapine treatment in the schizophrenia animal model to propose potential candidate molecules in therapeutics.

Movement Behavior Assessment

To confirm whether the psychomimetic rat model employed in this study are built or not properly, we performed a locomotive activity analysis using the Opto-Varimex-3 animal activity meter. We recorded the ambulatory counts and moving distance. Figure 1 shows the cumulative ambulatory counts and the cumulative distance. Olanzapine (3.0 mg/kg, i.p.) treated group showed a little sedative effect compared to control group, and the motor activities were significantly increased only in MK-801 (1.0 mg/kg, i.p.) treated group. MK-801 treated rats showed a hyperlocomotive behavior, a distinctive behavior of schizophrenia animals, indicating that schizophrenia animal model was made successfully in our experimental design. Also, this hyperlocomotive behavior was disappeared in the olanzapine plus MK-801 treated rats, supporting that olanzapine protected effectively from the MK-801-induced toxic effect.



Olanzapine (0 min)	−	+	−	+
MK-801 (after 30 min)	−	−	+	+

Figure 1. The effect of olanzapine based on locomotive activity analysis. In the movement distance, no significant differences were found when vehicles, olanzapine, and olanzapine +MK-801 groups were compared after 90 min. MK-801 group differed significantly from vehicles, olanzapine, and olanzapine+MK-801 groups. Also, in the cumulative ambulatory counts showed the same results with movement distance.

By this observation, we verified the psychomimetic activity of NMDA receptor antagonist, MK-801 and the protective activity of antipsychotic drug, olanzapine.

DNA Microarray Analysis and RT-PCR

We performed the gene expression analysis using microarray of the OpArray Rat genome 27K to search for genes regulated by MK-801, olanzapine and co-administration, MK-801 with olanzapine in the prefrontal cortex. The gene expression data were analyzed using the GenePix Pro 5.1 software (Axon, CA). Quantified files generated through GenePix Pro analysis were imported into GeneSpring GX 7.3.1 software (Silicon Genetics, Redwood City, CA) for data mining. Signal intensity values for all experimental replicates on any given drug-treat were averaged and used for additional analysis. The starting data set represented 28,032 probe sets. Additional filtering was executed to reduce type I errors (i.e., false positives), which result from experimental procedures. Genes assigned

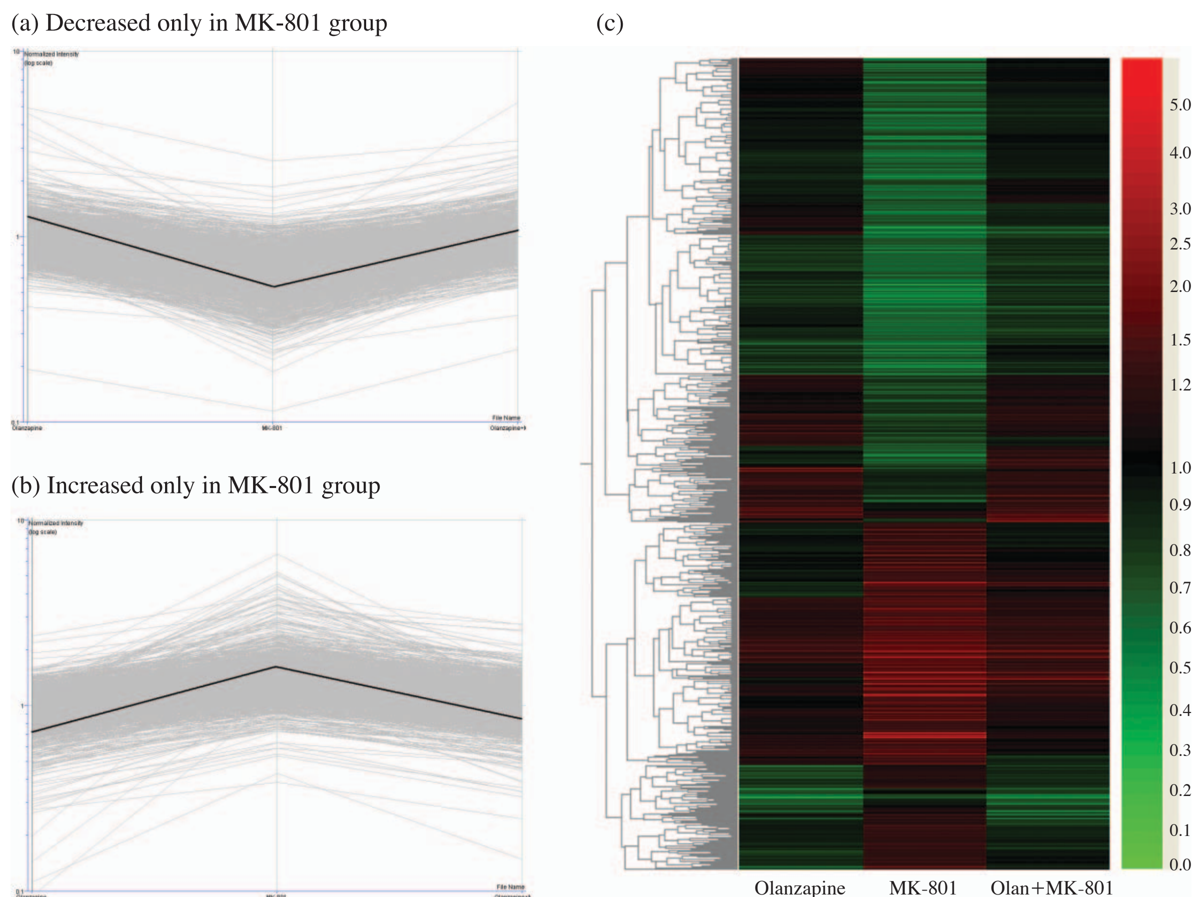


Figure 2. The expression profiles of genes with two clusters, (a) decreased only in MK-801 group (b) increased only in MK-801 group. And (c) shows the hierarchical clustering of these clusters. The expression of all genes in cluster (a) decreases in MK-801 single injection than other groups, it included 2,672 genes. The expression of all genes in cluster (b) increases in MK-801 single injection than other groups, it included 1,825 genes.

a "Flags" call for all drug-treatment were eliminated from the data set and 22,665 probe sets remained.

Acute treatments of 3 kinds to rats resulted in significant ($P<0.05$) upregulation of 196 genes and significant ($P<0.05$) downregulation of 523 genes. MK-801 administration resulted in up-regulated 148 genes, and down-regulated 376 genes. The olanzapine administration caused up-regulated 34 genes, and down-regulated 145 genes. The co-administration, olanzapine plus MK-801, caused up-regulated 61 genes, and down-regulated 31 genes. Acute MK-801 treatment caused the higher impact on the gene regulation than olanzapine and co-administration. And we found that the more genes were in down-regulated expression than in up-regulated expression in MK-801 administration and that the number of genes regulated by MK-801 attenuated with olanzapine.

It was impractical to verify 719 genes changed in microarray assay by RT-PCR and thus we performed gene expression pattern analyses. Probe set lists were filtered using the "Filter on Fold Change" option in GeneSpring. A minimum twofold change in gene expression defined differential expression for this data set. Probe set lists resulting from the comparison of genes expressed on drug-treatment filtered using a twofold cutoff were assigned a biological process with GeneSpring GX 7.3.1 software (Silicon Genetics, Redwood City, CA), annotated by the Gene Ontology Consortium were selected for analysis. Expression pattern clustering was generated using the 22,665 probe sets obtained from data analysis in GeneSpring GX 7.3.1. The following parameters were used: 3 rows, 3 columns, 10,000 iterations. Genes without data in all of the starting conditions were not used for the analysis.

The data were clustered into 9 groups by their expression pattern. We preferred and selected two clusters; 1) increased only in MK-801 group, for exam-

Table 1. Microarray analysis and RT-PCR result of selected 15 genes.

GenBank No.	Genes		Olanzapine	MK-801	Olz+MK-801
<Oxidative Stress-related>					
NM_021838	Nitric oxide synthase 3, endothelial cell	Nos3	Normal	Increase (↑)	Normal
NM_031970	Heat shock 27 kDa protein 1	Hspb1	Normal	Increase (↑)	Normal
NM_031971	Heat shock 70 kD protein 1A	Hspa1a	Normal	Increase (↑)	Normal
NM_031019	Corticotropin releasing hormone	Crh	Normal	Increase (↑)	Normal
NM_053524	NADPH oxidase 4	Nox4	Normal	Decrease (↓)	Decrease (↓)
<Apoptosis-related>					
NM_181086	Tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	Normal	Increase (↑)	Increase (↑)
NM_024388	Nuclear receptor subfamily 4, group A, member 1	Nr4a1	Normal	Increase (↑)	Increase (↑)
NM_053713	Kruppel-like factor 4 (gut)	Klf4	Normal	Increase (↑)	Increase (↑)
NM_012620	Serine (or cysteine) peptidase inhibitor, clade E, member 1	Serpine1	Normal	Increase (↑)	Normal
NM_013104	Insulin-like growth factor binding protein 6	Igfbp6	Normal	Increase (↑)	Normal
<Others>					
NM_053769	Dual specificity phosphatase 1	Dusp1	Normal	Increase (↑)	Increase (↑)
NM_053551	Pyruvate dehydrogenase kinase, isoenzyme 4	Pdk4	Normal	Increase (↑)	Increase (↑)
NM_053504	Peptidase, serine, 12 (neurotrypsin, motopsin)	Prss12	Increase (↑)	Normal	Normal
NM_021693	SNF1-like kinase	Snf1lk	Normal	Increase (↑)	Normal
NM_053896	Aldehyde dehydrogenase family 1, subfamily A2	Aldh1a2	Normal	Decrease (↓)	Normal

ple, normal in olanzapine-upregulation in MK-801-normal in combined treatment , 2) decreased only in MK-801 group, for example, upregulation in olanzapine-normal in MK-801-upregulation (Figure 2).

To identify pathologically-related genes and verify microarray results, we should compare the gene expression levels using real-time quantitative PCR. So, we selected 15 genes under guidelines; belong to above two cluster and significantly changed genes. Additionally, we reviewed their known estimated gene functions, for example, probably being MK-801 induced toxic effect-related. The selected 15 genes are equivalent to approximately 3% of significant regulated 524 genes by MK-801.

The results are shown the Table 1. When we performed expression pattern clustering with RT-PCR results, Serpine1, Igfbp6, Snf1lk, NOS3, Crh, Hspb1, and Hspa1a, have up-regulation by MK-801 and only Aldh1a2 showed down-regulation only in MK-801. Also, Nr4a1, Pdk4, Klf4, and Dusp1, were upregulated in MK-801 and co-administration condition compared with olanzapine treated, and on the contrary, the down-regulating genes, Prss12, and Nox4, were downregulated than in olanzapine treated. Specially, Prss12 was up-regulated by olanzapine, but, attenuated by MK-801. In our study, the comparison between the microarray results and RT-PCR results was showed very high correlation.

Although, 719 gene expression profiles were significantly changed in microarray analysis, we performed RT-PCR to only 15 genes. The genes having similar expression pattern with up-regulated only in MK-801 group or down-regulated only in MK-801 group may be related in psychosis. To verify the relationship between 719 genes and psychosis, there should be further study.

Discussion

It is well known that glutamate exists in high concentration in the brain that are critical in the mediation of psychiatric symptoms such as positive symptoms, negative symptoms, and cognition deficits in schizophrenia, indicating that this amino acid plays important roles in psychosis development[6,29,30]. Increasing evidence suggested that psychosis seems to be caused by abnormal interaction between glutamate and NMDA receptor. However, little is known about gene expression changes related to hypofunction of NMDA receptor in the brain[31]. Microarray analysis is a useful tool for analyzing global gene expression change in brain. Although there have been several studies of gene expression in brain with microarray, most of them have investigated gene expression change by only one compound, MK-801 or other antipsychotics[25,32,33]. In these studies, although several novel gene functions were revealed and confirmed the relationship in synaptic transmission, they used the microarray consisting small numbered genes or

they showed the expression change of the selected gene. We have investigated the global gene expression change using high density microarray OpArray Rat genome 27K (OPRNV3, Operon Biotechnologies, GmbH). This microarray contains whole genome genes to compare the difference between MK-801-induced and olanzapine-induced gene expression profiles.

We found the significant changes of 520 genes in microarray analysis by MK-801 alone-treated group. And these genes were classified to 9 clusters by the expression pattern clustering. Because we focused preferentially the toxic effect, the hypofunction of NMDA receptor by MK-801, we selected 15 genes under two criteria; a) significantly changed in selected 520 genes, b) included in two clusters, increased or decreased only in MK-801 alone-treated group compared with other groups.

Among the selected 15 genes, 7 genes (NOS3, Hspb1, Hspa1a, CRH, Serpine1, Igfbp6, and Snf1lk) were up-regulated, and 1 gene (Aldh1a2) was down-regulated by an administration of MK-801. And olanzapine treatment attenuated MK-801-induced gene expression changes in these changes. In contrast, one gene (Prss12) was up-regulated in olanzapine-treated group and attenuated by MK-801. And we could classify 15 genes into three group, oxidative stress-related, including NOS3, Hspb1, Hspa1a, CRH and NOX4, apoptosis-related, including Tnfrsf12a, Nr4a1, Klf4, Serpine1 and Igfbp6, and Prss12, Snf1lk, Aldh1a2, Dusp1 and Pdk4 in various function-related gene group (Table 1).

Hspb1 (heat shock protein 27) and Hspa1a (heat shock protein 70) were well known their function[34], especially the protection to neuronal loss from brain damages, for example ischemic stress[35]. Also these genes were overexpressed in the prefrontal cortex of subjects with schizophrenia[36]. Nitric oxide synthases produce nitric oxide and have 3 isozymes, NOS1 (neuronal NOS), NOS2 (inducible NOS) and NOS3 (endothelial NOS). The over activation of NMDA receptor by high concentrated glutamate resulted in oxidative stress in neuronal cells, it mediated by NOS1. In our results, however, NOS1 did not change (in our microarray), and NOS3 was increased in only MK-801 alone-treated group. This result is corresponded with recent study that NOS3 gene may be involved in the pathogenesis of tardive dyskinesia in patients with schizophrenia[37]. Moreover, intrathecal administration of uncompetitive NMDA receptor antagonist ketamine reduced NOS1 expression in monoarthritic rats but increased the expression of NOS2 and NOS3[38]. These results suggest that blockade of NMDA receptors produces complex regulatory changes in the expression of NOS isoforms.

CRH, corticotrophin releasing hormone, play critical roles in regulating behavioral and neuroendocrine responses to stress. Previous evidence that CRH could active as excitatory transmitter and neuroprotectant in ischemic condition provides CRH might play a important roles in the cascade of events leading to cellular damages[39].

All of the apoptosis-related genes, Tnfrsf12a, Nr4a1, Klf4, Serpine1 and Igfbp6 are up-regulated in MK-801 alone-treated rat. Overexpression of Serpine1 and Igfbp6 were attenuated by olanzapine. Serpine1, serine (or cysteine) peptidase inhibitor, clade E, member 1, is called Plasminogen activator inhibitor-1 (PAI-1), a principal inhibitor of plasminogen activators. Aldosterone-mediated contribution to glomerulosclerosis is partly due to up-regulation of PAI-1 expression, and spironolactone can ameliorate glomerulosclerosis via the inhibition of PAI-1 expression in streptozotocin-diabetic rats. Also, PAI-1 is a glycoprotein of the serpin superfamily and one of the major targets is regulated by TGF-β[40]. Direct relationship of Serpine 1 with schizophrenia was not elucidated yet. Igfbp6, insulin-like growth factor binding protein 6, is up-regulated by estrogen and TGF-β. Igfbp6 is a negative effector of oligodendrocyte survival and differentiation. Oligodendrocyte cells involve myelination of neuronal cell. Also Insulin-like growth factor 1 (IGF-1) is well known for its survival and growth-promoting effects on oligodendrocytes *in vitro*[41]. Klf4, Kruppel-like factor 4 (gut), is broadly involved in differentiation and development, growth-related signal transduction, cell proliferation, apoptosis, and angiogenesis, and induced in response to heat stress. But, it was not known the relationship with schizophrenia till now. Klf4 exhibits antiapoptotic activity following gamma-radiation-induced DNA damage and KLF4 work by activating expression of the cell cycle arrest gene, p21(WAF1/CIP1), and by inhibiting the ability of p53 to transactivate expression of the proapoptotic gene, BAX[42]. However, in our microarray analysis, p21 is up-regulated, while p53 and BAX is not changed.

Among the other genes, the expression changes of Snf1lk and Aldh1a2 were attenuated by olanzapine. Snf1lk, SNF1-like kinase and salt-inducible kinase-1, was only up-regulated in MK-801 alone-treated rat. Snf1lk represses cAMP response element-binding protein (CREB) activity both in the nucleus and in the cytoplasm. CREB, a transcription factor involved in numerous physiological processes, regulates gene expression in a phosphorylation dependent manner[43]. In the yeast *Saccharomyces cerevisiae*, Snf1 protein kinase is also activated by stresses, notably glucose

limitation[44]. Aldh1a2, aldehyde dehydrogenase family 1, subfamily A2, is only down-regulated in MK-801 alone-treated rat. Aldh1a2 is called retinal dehydrogenases (RalDHs), it involve the oxidation of retinal to retinoic acid and the regulation of retinoic acid production. Retinoic acid plays important roles in cellular differentiation and proliferation in various tissues including the liver[45].

On the contrary, Prss12, peptidase-serine, 12 (neurotrypsin, motopsin), was only up-regulated in olanzapine alone-treated rat and attenuated by MK-801. The extracellular proteolytic action of neurotrypsin involves structural reorganizations associated with learning and memory operations and the loss of Prss12 causes mental retardation[46].

Kamphuis, W. *et al.*[47], analyzed the gene expression changes after ischemic preconditioning in the rat. They found that the group of altered genes contained

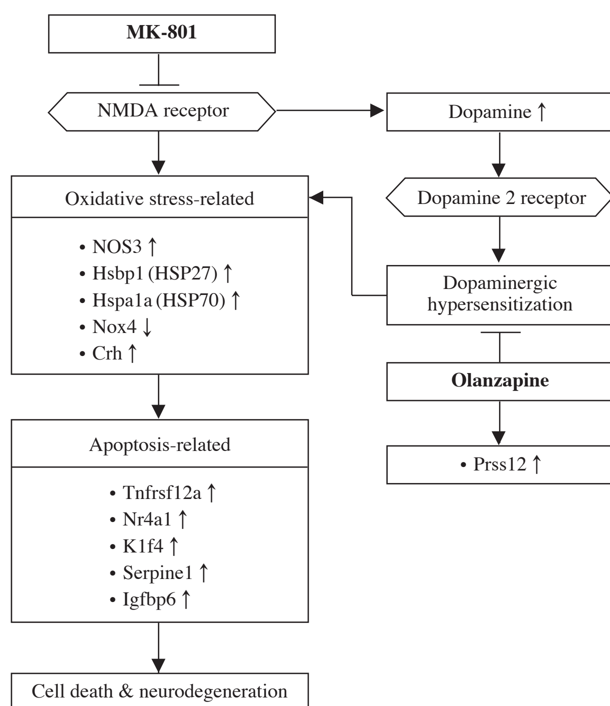


Figure 3. Proposed changes in gene expression in rat treated with an NMDA receptor antagonist, MK-801 and Dopamine 2-receptor antagonist, olanzapine. The blockade of NMDA receptor by MK-801 induced hypofunction of NMDA receptor, which cascades to change in the expression profile of oxidative-related and apoptosis-related genes. Also, D2 receptor antagonism by olanzapine blocks dopaminergic hypersensitivity by increased dopamine level and protects MK-801 -induced psychosis. This proposed schimatic diagram was supported by attenuating with olanzapine of gene expression changes by MK-801, although we need more study for the precise relationship between the gene expression changes and the blockade of NMDA receptor by MK-801.

a significant overrepresentation of genes involved in aminoacyl-tRNA synthetase activity, amino acid transport, regulation of transcription, and cell death. When our results compared their results, we could find that Nr4a1, Snf1lk, Hsbp1, Klf4 are overlapped with both results. It may support that ischemic preconditioning and NMDA receptor antagonist, MK-801 can attenuate general neuronal cell death through similar gene regulation pathway. Based on our results, we could propose the relationship of the surveyed genes, respectively (Figure 3).

In summary, we established a psychotomimetic animal model of schizophrenia using a neurotoxic agent, an NMDA receptor antagonist, MK-801 and confirmed the anti-psychosis effect of dopamine 2-receptor antagonist, olanzapine by movement behavior assessment. In microarray analysis, we performed the comparative analysis of gene expression profiles using the prefrontal cortex of these rats and OpArray Rat genome 27K containing whole rat genome. We selected 15 genes with focusing the effect of MK-801 for RT-PCR study. When we compared our RT-PCR results of 15 genes with established data, we know that our results correspond with the established gene characteristics, and have sufficient reliability. These results suggest a close relationship in gene expression level between glutamatergic neurotransmission and dopaminergic neurotransmission in schizophrenia. Future studies should reveal relevance to schizophrenia in other genes, for example Nr4a1, Prss12, Snf1lk, Aldh1a2, Dusp1 and Pdk4.

Methods

Animals and Drugs

Eight male Sprague Dawley rats with an average weight of 250 g were obtained from OrientBio, Sungnam, Korea and used for the experiment. These animals were housed in a temperature and humidity-controlled environment with a 12-h light/dark cycle and had access to food and water ad libitum. The animals were allowed for 1 week for habituation to the colony before the drug administration.

General reagents were purchased from Sigma (St. Louis, MO), whereas molecular biology reagents were obtained from Ambion, New England Biolabs (Beverly, MA), and Promega (Madison, WI). The following drugs were used in these studies: (+)-MK-801 maleate (Tocris Cookson Ltd., Bristol, UK) and olanzapine (Sigma-Aldrich, St. Louis, MO, USA). Olanzapine (3.0 mg/kg, ip) was dissolved in 0.1N acetic acid, pH 5-6 and MK-801 (1.0 mg/kg, ip) was dissolved in 0.9% saline. This dose of MK-801 was based

on previous reports and was expected to cause hyper-locomotion[14,48].

Drug Treatment

Rats were randomly assigned to one of four groups: (a) vehicle 1 (0.1 N acetic acid solution) and vehicle 2 (0.9% saline solution), (b) olanzapine (3 mg/kg) and vehicle 2, (c) vehicle 1 and MK-801 (1 mg/kg), (d) olanzapine (3 mg/kg) and MK-801 (1 mg/kg).

The dosage for olanzapine was chosen based on values obtained from the literature that correspond to clinically relevant human-equivalent dosage[49]. Although Kusumi, I. *et al.*[49] injected olanzapine at 1 or 2 mg/kg, ip, into rats for 3 weeks, we should selected dosage of olanzapine (3 mg/kg) for solid efficacy.

Rats were treated with olanzapine 30 min prior to MK-801 treatment and locomotion activity was measured for 2 hrs. Animals were killed after the behavior assessment was completed. The prefrontal cortex was dissected, stored in Ambicon's RNAlater solution for 1 hr and then frozen at $-70°C$ in deep freezer for future assays.

Measurement of Locomotion Activities

Motor activities in an open field was determined by using the Opto-Varimex-3 animal activity meter (Columbus Instruments Inc., Columbus, OH, USA)[16-18]. The Opto-Varimex activity sensors utilize high-intensity, modulated infrared light beams to detect animal motion.

Animals were housed in transparent cages ($17 \times 17 \times 8$ inches) through which 30 infrared beams pass in the horizontal plane, 15 on each axis. This device differentiates non-ambulatory movements (scratching, gnawing) from ambulation on the basis of consecutive interruption of the infrared monitoring beams. An additional row of infrared beams in the horizontal plane (15 on each axis) about 10 cm above the floor was used to count the vertical movements. During the activity measurements, animals had no access to food or chow. All studies were performed under strictly standardized conditions in the dark room for 120 minutes[50]. The counting numbers of the total movements, ambulatory movements were separately recorded to reflect the motor activities of rats.

Preparation of Fluorescent DNA Probe and Hybridization

Total RNA was extracted from the drugs treated rat prefrontal cortex using the TRI REAGENT (MRC, OH) according to the manufacturer's instructions. Each total RNA sample (30 μg) was labeled with Cyanine (CY3) or Cyanine (Cy5) conjugated dCTP (Amersharm, Piscataway, NJ) by a reverse transcription reaction using reverse transcriptase, SuperScrip ll (Invitrogen, Carlsbad, California). The labeled cDNA mixture was then concentrated using ethanol precipitation method. The concentrated Cy3 and Cy5 labeled cDNAs were resuspended in 30 μL of hybridization solution (GenoCheck, Korea). After two labeled cDNAs were mixed, placed on OpArray Rat genome 27K (OPRNV3, Operon Biotechnologies, GmbH) and covered by a MAUI FL chamber (Biomicro systems, Inc. UT). The slides were hybridized for 12 hr at 62°C MAUI system (Biomicro systems, Inc. UT). The hybridized slides were washed in $2 \times$ SSC, 0.1% SDS for 2 min, $1 \times$ SSC for 3 min, and then $0.2 \times$ SSC for 2 min at room temperature. The slides were centrifuged at 3,000 rpm for 20 sec to dry.

Microarray Data Analysis

Hybridized slides were scanned with the Axon Instruments GenePix 4000B scanner and the scanned images were analyzed with the software program GenePix Pro 5.1 (Axon, CA) and GeneSpring GX 7.3.1 (Sillicongenetics, CA). Spots that were judged as substandard by visual examination of each slide were flagged and excluded from further analysis. Spots that had dust artifacts or spatial defects were manually flagged and excluded. To filter out the unreliable data, spots with signal-to-noise (signal-background-background SD) below 10 were not included in the data. Data were normalized by Global, lowess, print-tip and scaled normalization for data reliability. Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes. Data were clustered groups of genes that behave similarly across a drug treated experiments using GeneSpring GX 7.3.1 (Sillicongenetics, CA). We used an algorithm, based on the Pearson correlation, to separate the gene of similar patterns.

Real-time Quantitative PCR

Real-time quantitative PCR was performed in triplicate in 384-well plates. A 384-well high-throughput analysis was performed by using the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems) and white colored 384-well plates (ABgene, Hamburg, Germany) for intensification of the fluorescent signals by a factor of three. The system operates using a thermal cycler and a laser that is directed via fiber optics to each of 384 sample wells. The fluorescence emission from each sample is collected by a charge-coupled device-camera and the quantitative data were analyzed using the Sequence Detection System software (SDS version 2.0, PE Applied Biosystems).

Table 2. Primer sequence.

Gene	Forward primer sequence	Reverse primer sequence	Size
Aldh1a2	AGC AAG CGC TCC ATA CTG TT	GGA CTC AAA GCC CAT GTC AG	77 bp
Crh	GCA GAG CAG TTA GCT CAG CA	AAG CGC AAC ATT TCA TTT CC	75 bp
Dusp1	CAT TCC CGA TGA CAT ACA CG	AAA CCA ACA CTG GCT TCG TC	195 bp
Hspa1a	GTC TCA AGG GCA AGA TCA GC	AGA GTC CAG CCA GGA GAT GA	80 bp
Hspb1	CCC AAA GCA GTC ACA CAA TC	TCC AGA CTG TTC CGA CTC TG	93 bp
Igfpb6	ATA AGG CCC AGT CCT GTT CA	CTG CTT GCG GTA GAA ACC TC	153 bp
Klf4	CGT CAG AGG AAG AGG AAG CA	GCA GTT GGA GAA CTT TGG CT	118 bp
Nos3	ACT GCG TCG CTT CAT TAG GT	GAG TTC TTA AAT CGG CAG CCT	89 bp
Nox4	CCT CCA TCA AGC CAA GAT TC	TAT CGA TGC AAA CGG AGT GA	165 bp
Nr4a1	CTC AAG CTG GAG GAC TTG GT	CGT GGG TGA CAA GAA GAA CA	126 bp
Pdk4	CCT GTG ATG GAC AAT TCA CG	CCC ATA GCC TGA CAT GGA AT	123 bp
Prss12	ATG CTC TGT GCT GGA AAC CT	TAA GTG GTC CTC CGC TGT CT	73 bp
Serpine1	GCT CCT GGT CAA CCA CCT TA	TGG AGA TGT AAC GGA TGC AG	150 bp
Snf1lk	TGA AGA TGG TTT CGG ACT CA	GAG GTC CTC AGT GCA AGG AA	134 bp
Tnfrsf12	TGG TTT CTG GTT TCC TGG TC	CAG CCT TCT CCA CCA GTC TC	85 bp

Reaction mixtures contained 10 pmol/μL of each primer and 2X SYBR Green PCR Master Mix (PE Applied Biosystems), which includes the HotStarTaq DNA-Polymerase in an optimized buffer, the dNTP mix (with dUTP additive), the SYBRs Green I fluorescent dye, and ROX dye as a passive reference. Each of the 384-well real-time quantitative PCR plates included serial dilutions (1, 1/2 and 1/4) of cDNA, which were used to generate Relative standard curves for genes. All primers (Table 2) were amplified using the same conditions. Thermal cycling conditions 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 60°C for 30 s, 72°C for 30 s.

In order to exclude the presence of unspecific products, a melting curve analysis of products was performed routinely after finishing amplification by a high-resolution data collection during an incremental temperature increase from 60°C to 95°C with a ramp rate of 0.21°C/sec.

We then converted real-time PCR cycle numbers to gene amounts (ng) on the basis of the equation. The real-time PCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System (PE Applied Biosystems).

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