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Changes in gene expression after phencyclidine administration in developing rats: a potential animal model for schizophrenia

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ABSTRACT

Repeated administration of phencyclidine (PCP), an *N*-methyl-D-aspartate (NMDA) receptor antagonist, during development, may result in neuronal damage that leads to behavioral deficits in adulthood. The present study examined the potential neurotoxic effects of PCP exposure (10 mg/kg) in rats on postnatal days (PNDs) 7, 9 and 11 and the possible underlying mechanism(s) for neurotoxicity. Brain tissue was harvested for RNA extraction and morphological assessments. RNA was collected from the frontal cortex for DNA microarray analysis and quantitative RT-PCR. Gene expression profiling was determined using Illumina Rat Ref-12 Expression BeadChips containing 22,226 probes. Based on criteria of a fold-change greater than 1.4 and a *P*-value less than 0.05, 19 genes including NMDAR1 (*N*-methyl-D-aspartate receptor) and four pro-apoptotic genes were up-regulated, and 25 genes including four anti-apoptotic genes were down-regulated, in the PCP-treated group. In addition, the schizophrenia-relevant genes, Bdnf (Brain-derived neurotrophic factor) and Bhlhb2 (basic helix-loop-helix domain containing, class B, 2), were significantly different between the PCP and the control groups. Quantitative RT-PCR confirmed the microarray results. Elevated neuronal cell death was further confirmed using Fluoro-Jade C staining. These findings support the hypothesis that neurodegeneration caused by PCP occurs, at least in part, through the up-regulation of NMDA receptors, which makes neurons possessing these receptors more vulnerable to endogenous glutamate. The changes in schizophrenia-relevant genes after repeated PCP exposure during development may provide important information concerning the validation of an animal model for this disorder.

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1. Introduction

Schizophrenia is a chronic, severe, and disabling brain disorder that affects about 1% of the world's population (Bromet and Fennig, 1999). It is characterized by abnormalities in the perception or expression of reality. Schizophrenia affects multiple cognitive-behavioral domains, comprising positive symptoms (e.g., delusion, hallucination, and paranoia), negative symptoms (e.g., loss of motivation, affective blunting and social withdrawal), and cognitive symptoms. Despite intensive studies, its molecular etiology remains enigmatic. Because many drugs that ameliorate psychotic symptoms in patients with schizophrenia are dopamine receptor blockers, much attention has been devoted to the dopamine hyperactivity hypothesis. However, it is difficult to explain the disease solely in terms of an abnormally overactive dopaminergic transmitter system (Olney and Farber, 1995). Several studies on families, twins, and adoptions suggest the importance of genetic factors in etiology; while clinical studies, advanced imaging techniques such as magnetic resonance imaging (MRI), and novel neuroanatomical markers have provided evidence that schizophrenia is a neurodevelopmental disorder (Sawa and Snyder, 2002). Some investigators have suggested that NMDA receptor hypofunction may underlie certain features of schizophrenia (Olney and Farber, 1995). In fact, early in 1980, Kim et al. (1980) first proposed that decreased glutamatergic activity may be involved in the etiology of schizophrenia. The accretion of evidence in support of the hypothesis that hypofunction of NMDA receptors contributes to the

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symptoms of schizophrenia, has provided the first compelling alternative to the dopamine hypothesis, even a more important role in the endophenotype of schizophrenia than dopamine (Coyle, 2006). In addition to the abnormalities in GABAergic neurotransmission in schizophrenia (Reynolds and Harte, 2007), the involvement of the phosphatidylinositide 3-kinase (PI3K) pathway, has also been suggested to contribute to schizophrenia (Brazil and Hemmings, 2001; Kalkman, 2006).

To better understand the mechanisms of schizophrenia, animal models have been developed, although none of the current animal models can serve as a complete animal equivalent to the human disorder. For example, an insurmountable obstacle of animal models is the inability to exhibit certain schizophrenia deficits, such as verbal behavior, making it impossible to produce a comprehensive animal model of schizophrenia. The *N*-methyl-D-aspartate (NMDA) receptor antagonists, including phencyclidine (PCP) and ketamine, seem to be capable of inducing both positive and negative symptoms of schizophrenia, including cognitive dysfunction in normal patients (Javitt and Zukin, 1991; Snyder, 1980; Tamminga, 1998); these drugs also profoundly exacerbate both positive and negative symptoms in schizophrenia patients (Lahti et al., 1995; Malhotra et al., 1997). Because of the more comprehensive psychopathology induced by PCP, many researchers have studied the effects of PCP in humans and animals to gain insights into the mechanisms of schizophrenia. Two separate groups (Jentsch et al., 1997; Johnson et al., 1998) reported that repeated PCP administration in adult rats produced behavioral, cellular and biochemical deficits sensitive to antipsychotics. Wang et al. (2001) demonstrated that perinatal PCP administration produces behavioral deficits in puberty. However, the behavioral deficits and underlying neuronal plasticity observed in adult animals may not be the same as the changes in the developing brain initiated by a perinatal insult. It has been demonstrated that some cases of schizophrenia may be the result of an insult during the prenatal or perinatal period (Benes et al., 1991; Murray et al., 1992; Pilowsky et al., 1993), though the functional consequences of the insult are not apparent until after puberty, when the affected neural networks reach maturity (Weinberger, 1987). Since it is a popular schizophrenia animal model, PCP-treated animals have been used to test some specific mechanistic hypotheses. It is evident that no single molecular event could be completely explanatory of the pathophysiology of schizophrenia. Therefore, a complete study of gene expression changes in this model would be helpful. In the present study, an animal model was developed using perinatal rats and repeated PCP administration to explore the brain gene expression profile and provide further evidence to validate an animal model of schizophrenia.

2. Material and methods

2.1. Animal treatment

Sprague–Dawley rat pups (both male and female) were used and randomly divided into either a PCP-treated or control (saline) group. 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. PCP was acquired from the National Institute on Drug Abuse (Rockville, MD, USA) and was dissolved in 0.9% NaCl (saline vehicle).

Similar to previous studies (Wang et al., 2001, 2003), rat pups received 10 mg/kg PCP, s.c. ($n = 10$, 5 animals for the histochemical study and 5 animals for DNA microarray analyses) or saline, s.c. ($n = 10$, 5 animals for the histochemical study and 5 animals for DNA microarray analyses) on postnatal days (PNDs) 7, 9 and 11. The animals were returned to their dams between injections. A previous study in the rat (James and Schnoll, 1976) has shown that 24 h after PCP treatment the concentrations of PCP in the blood and brain are nearly undetectable. Therefore, on PND 12, 24 h after the final PCP administration, pups for microarray analyses were sacrificed and their brains were collected for RNA isolation. Meanwhile, pups for the histochemical study received a transaortic perfusion of 0.9% saline and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2).

2.2. RNA extraction from brains

Tissues from the frontal cortex were collected for RNA extraction. Total RNA was isolated with RNeasy® Lipid Tissue Mini Kits (Qiagen Inc., Valencia, CA). The extracted RNA was assessed spectrophotometrically by measuring the optical density at 260 nm. RNA purity and quality were evaluated using RNA 6000 LabChip Kits and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). High quality RNA with RNA integrity numbers (RINs) greater than 8.5 was used for the microarray experiments and TaqMan gene expression assays.

2.3. Microarray analysis

To assess gene expression profiles in PCP-treated rat brains, microarray techniques were employed to examine the gene expression patterns in the PND 12 rat brains. Gene expression profiling was performed using the Illumina Rat Ref-12 Expression BeadChip platform containing 22,226 probes (Illumina Inc., San Diego, CA). Gene expression data from the Illumina Rat Ref-12 arrays were analyzed using 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/). Microarray data from five treated animals were compared to those from four control animals (one array from the control group was not used in the data analysis because of poor array quality). The differentially expressed genes (DEGs) were selected using criteria of a *P*-value less than 0.05 and a fold-change greater than 1.4 (up or down). This straightforward gene selection method, which combines a *P*-value cutoff (e.g. 0.05) and a fold-change ranking, has been extensively demonstrated to result in a higher level of concordance among lists of DEGs when the same set of samples were reanalyzed with different microarray platforms or in different laboratories with the same microarray platform (Guo et al., 2006; Shi et al., 2006). The use of a $P < 0.05$ criterion controls the chance of false discovery (*i.e.* calling a gene as differentially expressed when it is in fact not), whereas ranking the remaining genes that meet the $P < 0.05$ criterion by the fold-change criterion allows for genes with a larger magnitude of differential expression to be reproducibly identified as truly differentially expressed, thus balancing the sensitivity, specificity, and reproducibility of DEGs from a microarray gene expression study (Shi et al., 2008). It should be noted that the choice of a cutoff for fold-change, like for any other statistical measures, is arbitrary. The choice can be influenced by many factors such as the degree of the inherent differences between the groups of study samples and the number of DEGs that the researchers are willing to consider. For studies with larger between-group differences, such as in comparing different tissue types, a more stringent fold-change cutoff may be applied, whereas for studies with smaller between-group differences, such as comparing the same brain tissue type before and after chemical treatment, a less stringent fold-change cutoff may be needed to make sure that some genes with biological significance and a statistically significant *P*-value are included. In this study, the combination of $P < 0.05$ and fold-change greater than 1.4 resulted in a reasonable number of genes selected as differentially expressed on which biological interpretation was focused. It is important to point out that some genes that do not meet these selection criteria may indeed be differentially expressed, but with different levels of confidence.

2.4. TaqMan gene expression assays

The expression levels of the following genes were measured by quantitative RT-PCR (Q-PCR) using TaqMan assays (Applied Biosystems, Foster City, CA). The TaqMan probes included Bdnf (Brain-derived neurotrophic factor) (Rn01484924_m1), Bhlhb2 (basic helix-loop-helix domain containing, class B, 2) (Rn00584155_m1), and Grin1 (NR1) (Rn01436038_m1). Two genes, Polr2a (RNA polymerase II A) (Rn01752026_m1) and Actb (β-actin) (Rn00667869_m1), were used for endogenous controls.

Each assay was run in triplicate for each RNA sample. Total cDNA (20 ng) in a 20 μl final volume was used for each assay. Assays were run with Universal Master Mix (2x) without AmpErase UNG on a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA) using universal cycling conditions (10 min at 95 °C; then 15 s at 95 °C and 1 min at 60 °C for 40 cycles).

2.4.1. First strand cDNA synthesis

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

2.4.2. 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 quantitation of gene expression (Livak and Schmittgen, 2001). The following formula was used to calculate the relative amount of the transcripts in the PCP-treated samples (treated) and the saline-treated samples (control), and both were normalized to the endogenous controls. $\Delta\Delta C_T = \Delta C_T(\text{treated}) - \Delta C_T(\text{control})$. ΔC_T is the difference in C_T between the target gene and endogenous controls by