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Effects on rat thalamic proteome by acute and subchronic MK-801-treatment

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Abstract

Since the symptoms of intoxication with non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonists closely mimic symptoms in patients with schizophrenia, [+]-5-methyl-10,11-dihydro-5H-dibenzo-[*a*,*d*]-cycloheptene-5,10-iminehydrogenmaleate (MK-801)-treated rodents are often used as a model for schizophrenia. In most studies, acute injections of MK-801 to rats have been used, but in some studies, longer periods of treatment have been performed. In our previous work, alterations in mRNA/protein expression were screened in the cerebral cortex of MK-801 treated rats. Different proteins were altered in different treatment courses of MK-801. The main objective of the present study was to evaluate different treatment periods of treatment with MK-801 in rats as a model for schizophrenia. Thalamus proteins from treated (acute, six and 12 days) and control rats were analyzed with two-dimensional gel electrophoresis and mass spectrometry. Our results show that different treatment times of MK-801 to rats give different biochemical results. Therefore, it is important to use the same treatment time in studies that will be compared.

Keywords: MK-801; Schizophrenia; Thalamus, rat; Proteomic; Acute; Subchronic

1. Introduction

Schizophrenia is an organic disorder and the brains of patients with schizophrenia display structural alterations suggestive of brain atrophy or neurodegeneration (Capuano et al., 2002). Imaging studies have revealed reduced size of the thalamus in schizophrenics (Andreasen et al., 1994). Also, a reduction of the synaptic protein rab3a in the thalamus has been reported (Blennow et al., 2000). The thalamus plays a central role in processing and integrating sensory information relevant to emotional and cognitive functions, and several lines of investigation now suggest the possibility of thalamic dysfunction in the pathophysiology of schizophrenia (Oke and Adams, 1987; Jones, 1997; Scheibel, 1997; Andreasen et al., 1998). The neuro- and psychopathology of schizophrenia is still poorly understood, which might be attributed to the paucity of adequate animal models for the disorder (Becker et al., 2003). Current animal models for schizophrenia are not intended to serve as a complete equivalent of the human disorder, instead they are often designed to reflect a specific pathophysiologial mechanism (Marcotte et al., 2001).

Today, multiple lines of evidence have linked abnormalities in glutamatergic function (Goff and Coyle, 2001; Coyle and Tsai, 2004; McCullumsmith et al., 2004), as well as in other monoamine systems (Carlsson et al., 2001) in schizophrenia. *N*-methyl-D-aspartate (NMDA) receptor antagonists such as [+]-5-methyl-10,11-dihydro-5H-dibenzo-[*a*,*d*]-cycloheptene-5,10-iminehydrogenmaleate (MK-801, dizocilpine) and phencyclidine, have psychotomimetic properties, inducing both positive and negative symptoms. In schizophrenics, they aggravate pre-existing

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symptoms of schizophrenia (Javitt, 1987; Javitt and Zukin, 1991; Ellison, 1995; Tamminga et al., 1995; Swerdlow and Geyer, 1998). These findings have led to the use of MK-801-treated rodents as models for schizophrenia (Carlsson and Carlsson, 1990).

In most animal studies of schizophrenia, acute treatment with MK-801 is used (Farber et al., 1996; Carlsson and Svensson, 1990; al-Amin and Schwarzkopf, 1996), but elongated periods of MK-801-treatment have also been performed (Latysheva and Raevskii, 2003). In our previous work, alterations in mRNA and protein levels were screened in the cerebral cortex from rats treated with MK-801 for eight and 18 days. Several of the altered genes/proteins have previously been associated with schizophrenia, supporting the validity of subchronic MK-801-treatment as an animal model of schizophrenia (Paulson et al., 2003). However, different mRNA/protein alterations were found after 8 and 18 days of treatment, suggesting that the changes induced by MK-801-treatment result in normalization of some genes/proteins after 18 days of treatment, by compensatory mechanisms.

In addition to the psychosis induced by single-dose administration, it has also been noted that repeated use of phencyclidine in humans induces a more persistent schizophrenic symptomatology, including psychosis, hallucinations, flattened effect, delusions, formal thought disorder, cognitive dysfunction, and social withdrawal. For a review, see Jentsch and Roth (1999). They concluded that it is long-term administration of NMDA receptor antagonists that produces the effects that are most consistent with schizophrenia (Jentsch and Roth, 1999).

The main objective of the present study was to evaluate different treatment courses for MK-801-treatment in rats, biochemically, as a model for schizophrenia. Rats were treated with MK-801 as three different treatment courses: acute, 6, and 12 days. Thalamus proteins from treated and control rats were analyzed with two-dimensional gel electrophoresis (2-DGE), and altered proteins were identified using mass spectrometry (MS).

2. Materials and methods

2.1. Subjects

Sprague–Dawley rats (B&K Universal, Sollentuna, Sweden) (n=24, male, 220–260 g) were injected subcutaneously with physiological saline 5 ml/kg (0.9% w/v NaCl (aq.)) (controls) or MK-801 (Research Biochemicals, MA, USA) 0.7 mg/kg, (treated) and were divided into four groups with eight rats in each: control, acute, 6 days and 12 days of treatment. All rats received injections throughout 12 days (Table 1). The control group got saline for 12 days. The acute group got saline for 11 days and MK-801 on the last day. The 6 days group got saline for 6 days and MK-801 for 6 days. The 12 days group got MK-801 for 12 days. Measurements of locomotor activity (results not shown) were performed on day 12, at 90 min after the final injection. On day 13, approximately 24 h after the final injection, locomotor activity was again measured, and decapitation was performed. The brains were immediately removed and put in an ice-chilled petri dish, then dissected according to the method described by Carlsson and Lindqvist (Carlsson and Lindqvist, 1973). The dissected brain material (thalamus) was stored at −80 °C pending biochemical analysis. The Ethics Committee of Göteborg University approved the study.

2.2. Two-dimensional electrophoresis

The proteomic procedure was performed as described previously with some modifications (Paulson et al., 2004). Briefly, thalamus, approx. 25–50 mg wet weight, was homogenized, delipidized in chloroform/methanol/water (4/8/3 v/v). The pellet was dissolved in 50 μl sample buffer (20 mM Tris–HCl, 35 mM sodium dodecyl sulphate (SDS)). The samples were ultrasonicated for 15 min and finally boiled (95–100 °C) for 2–3 min. Protein sample, 100 μg (10 μl), was dissolved in 65 μl rehydration buffer (9 M urea, 4% immobilised pH gradient buffer, bromphenolblue) and 65 μl isobuffer (9 M urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanosulfonate hydrate (CHAPS), 35 mM Tris, 65 mM dithiothreitol, 15 mM iodoacetic acid, bromphenolblue). The first dimension was carried out using immobilised pH gradient strips, pH 5–8, 7 cm (BioRad, Hercules, CA, USA), in a Protean IEF Cell (BioRad). Active rehydration at 50 V was performed for 12 h, followed by focusing for 20,000 Vh. The second dimension was carried out using the Nu-PAGE gel system on 10% Bis–Tris gels (1 well) from Novex (San Diego, CA, USA) combined with 3-[N-morpholino] propane sulfonic acid (MOPS) running buffer (50 mM MOPS, 50 mM tris, 3.5 mM SDS, 0.8 mM EDTA) at a constant voltage (200 V, 50 min). The gels were stained with SYPRO Ruby Protein Stain (Molecular Probes, Eugene, OR, USA). Image acquisition and analysis were performed on a Fluor-S MultiImager (BioRad). The protein spots were detected, quantified and matched using the PD-Quest 2D-gel analysis software, version 7.1. The gels were normalized according to the total protein density of detected spots in each gel. Only proteins with significantly altered levels (Mann–Whitney P<0.05) in the MK-801 treated rats as compared with controls were taken into account. Altered proteins were excised for identification by mass spectrometry. The protein digestion method was performed as previously described (Davidsson et al., 2002). Briefly, the gel pieces were digested with porcine trypsin (Promega, Madison, WI, USA) and the peptides were extracted with formic acid and acetonitrile. The digested protein sample was dried and then dissolved in 10 μl 0.1% TFA v/v.

Table 1
Administration of MK-801 to rats used in this study

Group	Day												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Control	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Diss
Acute	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline	MK-801	Diss
6 Day	Saline	Saline	Saline	Saline	Saline	Saline	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	Diss
12 Day	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	MK-801	Diss

Sprague–Dawley rats (n=32, male, 220–260 g) received subcutaneous injections (5 ml/kg) of vehicle (saline, 0.9%w/v), (controls) or MK-801 (0.7 mg/kg) acute or once a day for 6 and 12 days. Day 13 and approximately 24 h after final injection, decapitation was performed.

2.3. Mass spectrometry and data base searches

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analyses were performed in reflectron mode with a MALDI-TOF mass spectrometer (Reflex III, Bruker-Franzen Analytik, Germany; or a MALDI-LR mass spectrometer Micromass, Manchester, UK). The mass spectra, acquired with the Bruker instrument, were initially mass-calibrated by external calibration, using a known peptide mixture, and were later recalibrated internally by using trypsin porcine auto-digestion peptides as mass-calibrants. Peptide samples analyzed in the Micromass instrument were deposited directly on the probe (0.5 μl), mixed with an equal volume of matrix solution (12 mg/ml α-cyano-4-OH-cinnamic acid in acetonitrile/water 1:1), and allowed to dry under ambient conditions. An external lock mass of adrenocorticotropic hormone, $[M+H]^+$ 2465.199 was used for spectral calibration; post-acquisition calibration was accomplished using an internal lock mass of an autolytic peptide from porcine trypsin of mass $[M+H]^+$ 2211.105.

The protein database search tools "ProFound" (http://129.85.19.192/profound_bin/WebProFound.exe) and MASCOT (http://www.matrixscience.com) were used to compare the monoisotopic m/z values of the tryptic fragments to proteins in the NCBI database. A mass deviation of 75 ppm was used, one missed cleavage, and *Rattus norvegicus* were specified.

Samples for which unambiguous protein identity (>95% confidences) was not determined with MALDI-TOF-MS were further investigated throughout acquisition of fragment ion data in an electrospray quadrupole time-of-flight instrument (Q-Tof, Micromass, Manchester, UK) (Table 1). Zip-Tip$_{C18}$ (Millipore) enriched samples in acetonitrile: 0.1% formic acid were sprayed from gold-coated glass capillaries using a nanoflow electrospray source. Argon was used as the collision gas. MS/MS spectra were post-processed using MassLynx (Micromass) and used without further interpretation for database searches using MASCOT against all entries in the NCBI nr database. Only those protein identities that were obtained with >95% confidences by MALDI-MS and/or ESI-MS/MS were considered.

3. Results

The main objective of the present study was to evaluate different treatment periods of MK-801 in rats biochemically. Rats were treated with MK-801 in three different treatment courses: acute, 6, and 12 days. Proteins displaying altered levels in MK-801-treated rats were quantified and identified from the 2-D gels using PD-Quest software and MS. For analysis of quantitative differences, about 230 protein spots were taken into account.

Measured on day 12 (after 11 days of vehicle treatment), acute administration of MK-801 induced, as expected, a profound increase in forward locomotion as compared to vehicle-injected controls. The locomotor response was approximately twice as high in animals treated with MK-801 for 6 or 12 days (results not shown) as compared to acute.

Regarding effects on the proteome, in the "acute group" the levels of 16 proteins were altered (seven increased), in the "6 day group" 22 (14 increased), and 30 in the "12 day group" (14 increased), compared with controls. Six altered protein levels were unique to the acute group. Eight altered protein levels were unique to the "6 day group", and 15 altered protein levels were unique to the "12 day group". One protein level was altered in both the acute group and the "6 day group". Two protein levels were altered in both the acute group and in the 12 days group. However, these two protein levels had trends in the same direction in the 6 day group as in the 12 day group. Six protein levels were altered in the 6 day group and in the 12 days group but not in the acute group. Seven protein levels were altered in all three groups.

Twelve of the altered proteins were identified by MALDI-TOF MS and/or ESI-q-TOF MS (Table 2, Fig. 1). Four of the identified proteins have previously been associated with schizophrenia in the literature: heat shock proteins 60 and 72, dihydropyrimidinase related protein-2 (DRP-2), and neuron-specific enolase (NSE). None of these protein levels were altered in the acute group. Three of these proteins were altered in the 6 day group, and two were altered in the 12 day group (Table 2).

Increased proteins not previously associated with schizophrenia in the literature include: pyruvate dehydrogenase E1 (PDC-E1), titin, tropomodulin 2, and α-internexin. Decreased proteins not previously associated with schizo-

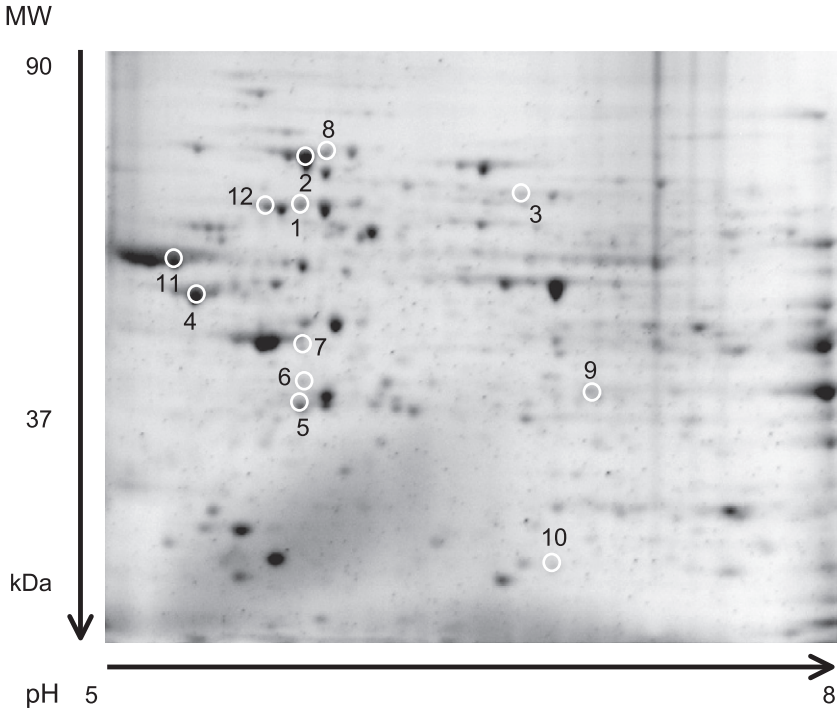

Fig. 1. Proteins from rat thalamus were separated on pH 5–8 immobilised pH gradient strips, followed by Nu-PAGE gels and dyed with SYPRO-Ruby staining. The proteins were then detected and compared using the PD-Quest software, and analyzed by MALDI-TOF MS and/or ESI-qTOF-MS/MS. Selected spots (1–4) represent rat proteins with significantly different levels, and these have earlier been connected to schizophrenia.

phrenia in the literature include: grp 75, 3-hydroxyisobutyratedehydrogenase, neuronal protein 22, and H+-transporter ATPase β-subunit (Table 2).

4. Discussion

We have previously shown that different treatment courses of MK-801 give different altered gene and protein patterns (Paulson et al., 2003, 2004). In our earlier studies many of the altered proteins in the 8 day group were not changed after 18 days of treatment. In order to evaluate different treatment courses for MK-801 biochemically, rats were treated with MK-801 in three different treatment courses: acute, 6, and 12 days.

In most studies found in the literature, acute treatment with MK-801 was used (Farber et al., 1996; Carlsson and Svensson, 1990; al-Amin and Schwarzkopf, 1996), but in some studies longer period of treatment have been performed (e.g. Latysheva and Raevskii, 2003). In the present study, most proteins were altered after 6 and 12 days of treatment compared with the acute group. Most proteins earlier associated with schizophrenia were in the 6 day group as compared with the acute group. Four of the identified thalamic proteins, heat schock protein 60 (Mazeh et al., 1998; Schwarz et al., 1999), heat shock protein 72 (Schwarz et al., 1999), DRP-2 (Johnston-Wilson et al., 2000), and neuron-specific enolase NSE (Vermuyten et al., 1990), have previously been associated with schizophrenia in the literature (Table 2). In this study, DRP-2 was decreased in schizophrenic patients and NSE was increased, mimicked by our findings in rats (Table 2). None of these proteins was altered in the acute group as compared with controls. In contrast, the levels of heat shock protein 60, heat shock protein 72 and NSE were altered in the 6 day group, whereas the levels of heat shock protein 60 and DRP-2 were altered in the 12 day group (Table 2).

The level of heat shock protein 60 was increased after 6 and 12 days of MK-801-treatment. The level of heat shock protein 72 was increased after 6 days of treatment. However, increased expression of heat shock proteins is induced by exposure of cells and tissues to extreme conditions that cause stress, so this is not unique for schizophrenia (Kopecek et al., 2001).

Some of the altered identified proteins have also been associated with other disorders in the brain. For example, NSE is altered in Creutzfeldt–Jacob disease, meningeal hemorrhage, thrombosis, and in Guillain–Barré syndrome (Vermuyten et al., 1990). Heat shock protein 72 and grp 75 are down-regulated in Downs syndrome (Yoo et al., 2001). DRP-2 is altered in Downs syndrome, bipolar disorder, Alzheimer's disease and depression (Johnston-Wilson et al., 2000; Weitzdoerfer et al., 2001; Castegna et al., 2002).

Increased proteins not previously associated with schizophrenia in the literature include: PDC-E1 (Matuda et al., 1991), titin (Horowits et al., 1986), tropomodulin 2

Table 2
Identified altered proteins in thalamus from MK-801-treated rats

Spot no. in Fig. 1	Accession no. NCBI	Protein identity	MS	MS/MS	Sequence coverage %	No. of identified peptides	Probability score (ProFound)	Probability score (Mascot)	Theoretical pI/mass (kDa)	Intensity (mean±S.D.)			
										Control	Acute injection	6 Days	12 Days
(A)													
1	1334284	Heat shock protein 60	–	×	–	1	–	38	5.3/58.08	4119±957	NS	6560±375	5436±955
2	347019	Heat shock protein 72	×	–	46	24	2.40	–	5.4/71.14	352±874	NS	4330±2810	NS
3	1351260	DRP-2	×	–	20	11	4.41	–	6.0/62.66	2013±804	NS	NS	852±774
4	2465396	NSE	×	–	39	10	–	97	5.03/47.51	1270±805	NS	2145±1312	NS
(B)													
5	1352624	PDC-E1	×	–	35	8	–	65	5.94/39.34	6384±5015	10,561±7194	13,479±3930	NS
6	27700611	titin	×	–	2	13	–	60	5.24/1310.68	500±387	1524±587	NS	897±408
7	23396879	tropomodulin 2	×	–	23	8	–	95	5.34/39.47	1370±904	2255±1123	NS	NS
8	1000439	grp 75	×	–	39	21	–	210	5.87/73.98	1448±922	442±711	350±295	226±207
9	27710396	3-hydroxyiso-butyratedehydro-genase	×	–	22	6	–	76	8.73/35.68	1659±309	1256±83	NS	1112±65
10	18252579	neuronal protein 22	×	–	38	7	–	116	6.84/22.66	1893±580	NS	NS	1253±300
11	1374715	H+-transp. ATP-synthase β-subunit	×	–	49	17	2.28	–	4.9/51.18	11,335±2179	7883±1297	NS	NS
12	55622	α-internexin	×	–	34	16	2.27	–	5.2/56.27	905±424	2437±1414	2608±1345	2807±1073

Proteins from thalamus were separated using two-dimensional electrophoresis and identified by MS. The protein spots were quantified from Sypro Ruby stained gels using PD-Quest software. Only the proteins significantly ((Mann–Whitney) $P<0.05$) altered were reported. Protein identities obtained with >95% confidence levels by MALDI-TOF MS and ESI-q-TOF MS were considered. The proteins in group A have previously been associated with schizophrenia in the literature, whereas the proteins in group B have not. NS=not significant.

(Pittenger et al., 1994), and α-internexin (Fliegner et al., 1990). Decreased proteins not previously associated with schizophrenia in the literature include: grp 75 (Massa et al., 1995), 3-hydroxyisobutyratedehydrogenase (Rougraff et al., 1989), neuronal protein 22 (Fan et al., 2001), and H+-transporter ATPase β-subunit (Hubbard and McHugh, 1996) (Table 2). All these proteins are involved in either the cytoskeleton or in mitochondrial function, further supporting this animal model for schizophrenia. Changes in mitochondrial function (oxidative metabolism) have been implicated in schizophrenia pathology (Marchbanks et al., 1995; Whatley et al., 1996, 1998; Prince et al., 1999; Maurer et al., 2001). Some of the proteins whose levels were altered by MK-801 in this study were not identified in our earlier studies. This could have different explanations; different gel and buffer systems were used in the different papers. Also, proteins that were identified in earlier studies could not be identified in the previous studies and vice versa. Also, in the first study, rats received injections of vehicle or MK-801 once a day, five times a week to mimic the fluctuating nature of schizophrenic disease. All the injections started the same days. In the present study, rats received injections all days with no interruptions and all the injections were finished the same day.

Our results shows that different treatment times of MK-801 to rats give different biochemical results. Therefore, it is important to use the same treatment time in studies that will be compared.

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