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ARTICLE

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Effects of neonatal MK-801 treatment on p70S6K-S6/eIF4B signal pathways and protein translation in the frontal cortex of the developing rat brain

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Abstract

Systemic injections of MK-801, a selective NMDAR antagonist, into neonatal rats induces long-term neurochemical and behavioural changes. It has been suggested that these changes form the neurodevelopmental basis for schizophrenia-like behaviour in rats. In this study, 7-d-old rats were treated with MK-801, and their frontal cortices were examined to investigate the effects on p70S6K-S6 signal pathway and on protein translation, which play crucial roles in the neurodevelopmental process. MK-801, in doses of 0.5 and 1.0 mg/kg, induced a decrease in phosphorylation of p70S6K and its substrates, S6 and eIF4B, in the first 8 h, and no change at 24 and 48 h. These effects were more prominent after two injections of MK-801 than one. Decreased S6 phosphorylation by MK-801 was evident in the prefrontal, cingulate, and insular cortex. In two representative upstream p70S6K-S6 pathways related to ERK1/2 and Akt, changes in ERK1/2-p90RSK phosphorylation were accompanied by changes of p70S6K-S6. Although two MK-801 injections induced a dose-dependent decrease in phosphorylation of Akt and mTOR at 4 and 8 h, a single injection did not produce a significant effect. Protein synthesis rate, measured by [^{3}H]leucine incorporation in frontal cortical tissue, was reduced until 24 h after two MK-801 (1.0 mg/kg) injections. In summary, this study found that neonatal MK-801 treatment induced dysregulation in the p70S6K-S6/eIF4B pathway and protein translation in the frontal cortex of the developing rat brain, which may suggest an important role of protein translation machinery in the MK-801 neurodevelopmental animal model of schizophrenia.

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Key words: Neurodevelopment, *N*-methyl-D-aspartate receptor, protein synthesis, schizophrenia, small ribosomal protein 6.

Introduction

The *N*-methyl-D-aspartate receptor (NMDAR) plays a crucial role in the differentiation, migration, and synaptogenesis of neurons, and thus regulates the developmental process of brain (Contestabile, 2000; Komuro & Rakic, 1993; Sircar, 2000). In rats, peak expression of the NMDAR occurs within the first 2 wk (Ritter *et al*. 2002), which corresponds to the major growth spurt of the brain (Andersen, 2003). During this period, the rodent brain is highly vulnerable to NMDAR antagonism, and the period around postnatal day (PND) 7 shows high sensitivity to NMDAR antagonist-induced apoptotic neurodegeneration (Ikonomidou *et al*. 1999; Lei *et al*. 2008; Lema Tome *et al*. 2006).

Systemic administration of NMDAR antagonists to perinatal rats induces long-term behavioural and neurochemical changes resembling schizophrenia, such as deficits in sensorimotor gating, spatial learning, and working memory (Harris *et al*. 2003; Sircar &

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Rudy, 1998; Stefani & Moghaddam, 2005; Uehara *et al*. 2009; Wiley *et al*. 2003), locomotor activity changes (Harris *et al*. 2003), dysregulation in dopaminergic and glutamatergic neurotransmission (Sircar & Soliman, 2003; Wedzony *et al*. 2005), and decreased parvalbumin-positive neurons (Abekawa *et al*. 2007; Wang *et al*. 2008). The developmental status of PND7 rat brain is assumed to be equivalent to that of the human foetus in the second trimester of development (Clancy *et al*. 2001), which is the period when it has been suggested that the developmental abnormality causing schizophrenia occurs (Bracha *et al*. 1992; Fatemi & Folsom, 2009; Mednick *et al*. 1988). Thus, treatment of rats at around PND7 with MK-801, a selective and non-competitive NMDAR antagonist, has been suggested as a means of inducing a neurodevelopmental model of schizophrenia in rats (du Bois & Huang, 2007). These findings imply that transient disruptions in NMDAR-related signals during this critical period can underpin long-term abnormalities. However, although several studies have suggested possible mechanisms, such as NMDAR antagonist-induced apoptotic neurodegeneration (Dzietko *et al*. 2004; Hansen *et al*. 2004; Ikonomidou *et al*. 1999; Lei *et al*. 2008; Lema Tome *et al*. 2006; Xia *et al*. 2008), it still requires further investigation.

Translational control plays a key role in the long-term modification of neural circuits and behaviour (Costa-Mattioli *et al*. 2009). Synaptogenesis, dendritic arborization, axonal growth and navigation, and differentiation actively occur in the early developing brain, which requires the proper activity of protein translation machinery (Jaworski & Sheng, 2006). In fact, the protein synthesis rate of rat brain in the early postnatal period is significantly higher than in the adult period and declines with age (Fando *et al*. 1980; Sun *et al*. 1995), which suggests a high demand and rapid turnover of protein synthesis in the early neurodevelopmental period.

Protein synthesis is tightly operated by interconnected signal pathways. Initiation is the rate-limiting step in translation, and Akt and mitogen-activated protein kinase (MAPK) pathways are representative of those responsible for initiation of translation (Parsa & Holland, 2004; Ruggero & Sonenberg, 2005). Akt activates mammalian target of rapamycin (mTOR) followed by phosphorylation of eukaryotic translation initiation factor 4E binding protein (4E-BP) and p70 ribosomal S6 kinase (p70S6K) (Proud, 2007; Wang *et al*. 2003). The ERK1/2 pathway is also involved in p70S6K phosphorylation (Bessard *et al*. 2007; Lehman & Gomez-Cambronero, 2002). Activated p70S6K induces phosphorylation of small ribosomal protein

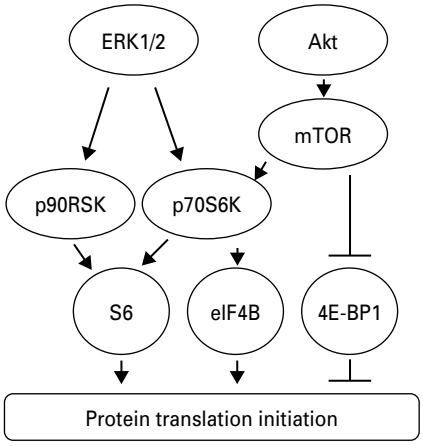

Fig. 1. Schematic diagram illustrating the p70S6K-S6-related protein translation initiation signal pathways. Akt and ERK1/2 pathways can phosphorylate p70S6K, followed by S6 and eIF4B phosphorylation. p90RSK can also phosphorylate S6. In addition, mTOR inactivates the translation repressor 4E-BP, which releases eIF4E to facilitate translation initiation.

6 (S6) and eukaryotic translation initiation factor 4B (eIF4B) (Raught *et al*. 2004; Ruvinsky & Meyuhas, 2006). Phosphorylation of S6 can be regulated by not only p70S6K, traditionally known to be the main upstream kinase, but also by p90RSK, which mediates the Ras-ERK1/2 signal pathway (Pende *et al*. 2004; Roux *et al*. 2007). Among the phosphorylation sites of S6, p90RSK exclusively phosphorylates at Ser235/236 (Pende *et al*. 2004; Roux *et al*. 2007). Therefore, p70S6K and S6, regulated by Akt and ERK pathways, can act as one of the critical points regulating initiation of protein translation (Fig. 1).

Activity of NMDARs affects protein translation, and related signal pathways including ERK1/2, Akt, and mTOR (Chandler *et al*. 2001; Gong & Tang, 2006; Gong *et al*. 2006; Sutton & Chandler, 2002) and protein synthesis plays an important role in synaptic plasticity regulated by the glutamatergic system (Kelleher *et al*. 2004; Nicoll & Malenka, 1999). Moreover, we have previously reported that MK-801 treatment affects Akt and ERK1/2 pathways (Ahn *et al*. 2005, 2006; Seo *et al*. 2007) and mTOR/p70S6K-related pathways in the frontal cortex of adult rat brain (Yoon *et al*. 2008).

Taken together, the evidence suggests that MK-801 treatment of rats in the early postnatal period may induce changes in protein translation and related signal pathways in the brain and thus disrupt the normal neurodevelopmental process. In this study, p70S6K-S6-related signal pathway, regulated by ERK1/2 and Akt, and protein synthesis rate were investigated after MK-801 treatment at PND7 in the frontal cortex of the developing rat brain.

Methods

Animals

Animals were treated in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Formal approval to conduct this experiment was obtained from the Animal Subjects Review Board of Seoul National University Hospital. PND7 female Sprague–Dawley rats (weighing 12–16 g) were housed with a maternal rat under a 12-h light/dark cycle (lights on 08:00 hours) with food and water available *ad libitum*. We chose female rats because they have previously shown more prominent long-term behavioural changes than male rats in response to neonatal MK-801 treatment (Harris *et al*. 2003). Drug injections were performed outside the cage under a heating lamp, and the rat pups were returned to the cage immediately after the drug injections.

Drug treatment

We administered either one or two (8 h apart) subcutaneous (s.c.) injections of MK-801 (Tocris, USA; dissolved in normal saline) or normal saline to rats at PND7, a period showing high vulnerability to the treatment of MK-801 (Ikonomidou *et al*. 1999). The rat pups were randomly assigned to either the MK-801 or the normal saline groups.

MK-801 doses of 0.1, 0.5, and 1.0 mg/kg, were used. The rat pups were then sacrificed by decapitation and the frontal cortices were dissected at 1, 4, 8, 24, and 48 h after their last drug injection in order to analyse molecular changes. The effects of MK-801 treatment in developing rat brain are dose- and time-dependent. For example, regarding the effects on apoptotic neurodegeneration, the threshold dose of MK-801 for inducing apoptotic damage was 0.25 mg/kg, and damage was evident from 4 to 24 h after the MK-801 treatment, but at 48 h no apoptotic signs remained (Ikonomidou *et al*. 1999).

Sample preparations and Western blot analysis

Whole extracts of frontal cortex were used for immunoblot analysis. Frontal cortices were immediately homogenized in a glass–Teflon homogenizer in 10% v/w ice-cold RIPA(+) buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton, 1% sodium deoxycholate, and 0.1% SDS] containing 1 mM DTT, protease inhibitor cocktail (Sigma-Aldrich), and 1 mM PMSF (Sigma-Aldrich). Subsequent steps for immunoblot analysis was performed as described previously (Seo *et al*. 2007). Antibodies against actin (Sigma-Aldrich), p70S6K, S6, eIF4B, ERK1/2, p90RSK, Akt, GSK-3β, mTOR, 4E-BP (Santa Cruz Biotechnology, USA), p-p70S6K (Thr389), p-S6 (Ser240/244 or Ser235/236), p-eIF4B (Ser442), p-ERK1/2 (Thr202/Tyr204), p-p90RSK (Thr359/Ser363), p-Akt (Ser473), p-mTOR (Ser2448 or Ser2481), p-4E-BP (Thr37/46) (Cell Signaling Technology, USA) were used as primary antibodies at dilutions of 1:1000 to 3000. They were incubated overnight at 4 °C, followed by a second incubation with anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The signal was detected with the ECL system (Pierce, USA). Results were quantified by densitometry and band intensity was corrected for background by subtraction using the TINA program (Raytest, Germany).

Immunohistochemistry

For immunohistochemistry, different animals from those in the immunoblotting experiments were used. These rat pups were treated in the same way except for the method of analysis. The rat pup brains were extracted at 8 h after two injections of MK-801 (1.0 mg/kg). Brains were post-fixed with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M PBS (pH 7.4) for 2 h, briefly equilibrated in glycerol (20% in PBS), and sectioned at 20 mm on a freezing microtome (Leitz, Germany). Tissue sections were rinsed three times in PBS and incubated in 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. After extensive washing in 0.1 M PBS (three times for 10 min each), sections were preincubated in 10% normal goat serum (Jackson ImmunoResearch Laboratories, USA) for 1 h. The sections were incubated with antibody against p-S6 (Ser240/244) at a 1:1500 dilution at 4 °C overnight. After rinsing in 0.1 M PBS, a refined avidin–biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules was employed for amplification using a LSAB+kit/HRP (Dako Corporation, USA). The sections were incubated in DAB substrate and subsequently mounted with DPX mountant (Fluka, Switzerland). Images of the regions of interest (area 220 μm $\times$ 300 μm) were digitally collected and counted using a computerized image analyser (Leica Application Suite V3, Germany). For each animal, the mean count of p-S6 positive neurons per examined brain region, including the prefrontal, cingulate, insular, and orbital cortex (Paxinos & Watson, 1998: Fr2, Cg3, AI, and LO and VLO, fig. 8), was determined.

Double-label immunofluorescence

The sections were blocked for 1 h with 3% BSA in TBST, and incubated with primary antibodies [1:100 anti-p-S6 (Ser240/244) and 1:100 anti-neuronal nuclear protein (Neu-N; Chemicon, USA)] at 4 °C overnight. After washing in TBST, the sections were incubated with secondary antibodies (1:100 Alexa Fluor 488 and 555; Molecular Probes, USA) for 1 h at room temperature. The sections were then fluorescence-labelled, nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), mounted with DPX mountant, and analysed on a Meta confocal microscope (Model LSM 510; Carl Zeiss MicroImaging Inc., Germany) equipped with four lasers (Diode 405, Argon 488, HeNe 543, and HeNe633). Each channel was separately scanned using a multitrack PMT configuration to avoid cross-talk between fluorescent labels. Images were acquired and three-dimensionally reconstructed using Zeiss LSM software.

Translation assays in vitro

The protein synthesis rate was measured using a cell-free system validated for the brain (Burda *et al*. 1994; Cosgrove & Rapoport, 1986; Fando & Wasterlain, 1980). Tissues from frontal cortices were homogenized in 2.5 vol. of the homogenization buffer [0.32 M sucrose, 50 mM Hepes-KOH (pH 7.54), 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM DTT], using five passes of a glass–Teflon homogenizer with a motor-driven pestle. Then the homogenates were centrifuged at 11220 ***g*** for 10 min at 4 °C. The supernatant (post-mitochondrial supernatant; PMS) was used for *in-vitro* protein synthesis assay. Protein concentrations were determined by Bradford assay. Reactions were performed three times for each sample using a 150 μg PMS for each reaction. The reaction was performed at 37 °C for 45 min. The complete reaction mixture in a final volume of 0.1 ml contained 150 μg PMS, 0.32 M sucrose, 50 mM Hepes buffer (pH 7.54), 200 mM potassium acetate, 5 mM magnesium acetate, 2.5 mM dithiotheitol, 1 mM ATP, 1 mM GTP, 500 μM creatine phosphate, 50 μg/ml creatine phosphokinase, and 50 μCi/ml [^{3}H]leucine. The reaction was stopped by adding 1 ml distilled ice-cold water, after which 0.5 ml of 1 M KOH containing 2 mg/ml unlabelled leucine was added, and the mixture was incubated at 37 °C for an additional 10 min to release labelled amino-acid bound to tRNA and chilled. The proteins were precipitated by adding 1 ml of 25% TCA (containing 2 mg/ml unlabelled leucine) and storing at 4 °C overnight. The precipitated proteins were collected on Whatman GF/C glass-fibre filters presoaked in 10% TCA by vacuum filtration, then washed with 10% TCA, and dried. The filters were counted for radioactivity after agitation for 60 min with liquid scintillation cocktail (PerkinElmer, USA).

Statistical analysis

Immunoblot results are expressed as relative optical densities (ODs). The OD of phosphorylation level was normalized by each OD of corresponding total protein. The relative ODs of immunoreactivity (%) are reported as the mean ± standard error. Each group consisted of 4–6 animals for immunoreactivity analysis. The mean of the relative OD of each group was compared with that of the vehicle control using a one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. To compare the mean values of vehicle control and MK-801-treated groups from immunohistochemistry and protein translation assay, an independent *t* test was performed. p values <0.05 were deemed statistically significant. All tests were performed using SPSS 12.0 for Windows (SPSS Inc., USA).

Results

Effects of MK-801 treatment on p70S6K, S6, and eIF4B in the frontal cortex of PND7 rats

Changes in phosphorylation of p70S6K at Thr389, which reflects the activity of p70S6K (Dufner & Thomas, 1999; Pearson *et al*. 1995), were examined after MK-801 treatment at PND7. At 1 h after single treatment of MK-801 ($F=5.10$, d.f. $=3$, $p=0.01$), 1.0 mg/kg MK-801 significantly reduced immunoreactivity of p-p70S6K ($p=0.04$), at 4 h ($F=2.53$, d.f. $=3$, $p<0.01$), 0.5 and 1.0 mg/kg both induced a significant decrease ($p<0.01$ for both), at 8 h ($F=10.24$, d.f. $=3$, $p<0.01$), 0.5 and 1.0 mg/kg also induced a significant decrease ($p=0.01$, $p<0.01$, respectively). Phosphorylation levels of S6, a major substrate of p70S6K, at both Ser240/244 and Ser235/236 were analysed. Phosphorylation of S6 at Ser240/244 is regulated by p70S6K (Raught *et al*. 2004; Ruvinsky & Meyuhas, 2006), and p90RSK phosphorylates S6 at Ser235/236 (Pende *et al*. 2004; Roux *et al*. 2007). Immunoreactivitiy of p-S6 (Ser240/244 and Ser235/236) showed the similar pattern of changes as did of p-p70S6K. In addition, phosphorylation of eIF4B (Ser442), another substrate of p70S6K, changed in a similar way. At 24 and 48 h, no significant changes were found in the immunoreactivity of p-p70S6K, p-S6, or p-eIF4B following the single MK-801 treatment (Fig. 2).

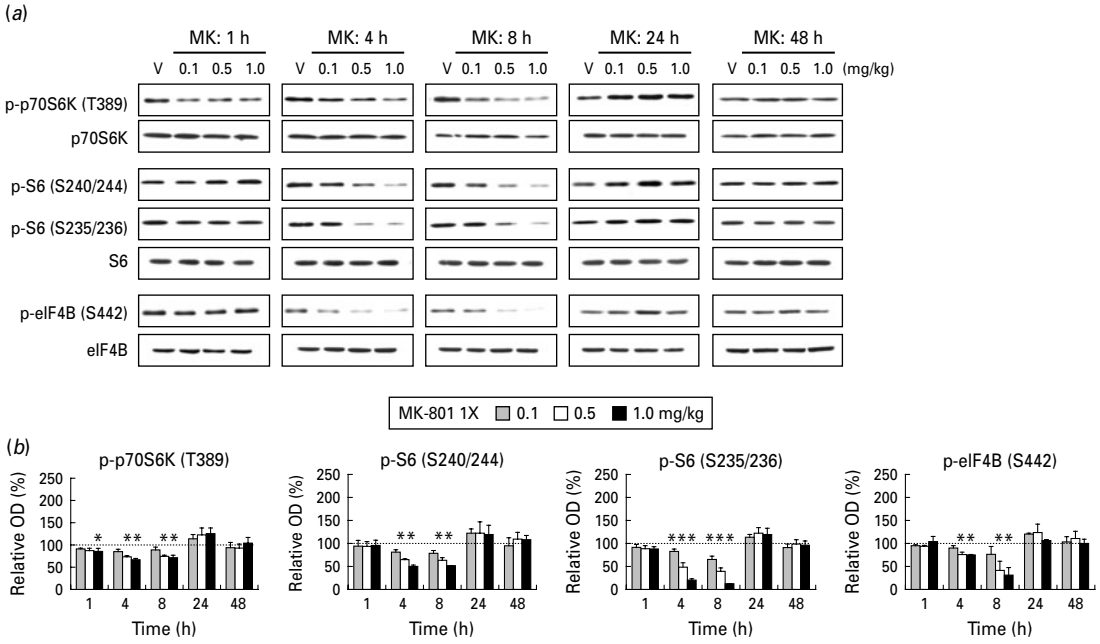

Fig. 2. Time- and dose-dependent changes in the phosphorylation level of p70S6K and its substrates, S6 and eIF4B, in the rat frontal cortex after a single injection of MK-801 at PND7. A single injection of MK-801 at PND7 induced a dose-dependent decrease in immunoreactivity of p-p70S6K (Thr389), p-S6 (Ser240/244 and Ser235/236), and p-eIF4B (Ser442) up to 8 h after injection. (*a*) Representative immunoblots of the rat frontal cortex at 1, 4, 8, 24, and 48 h after a single MK-801 injection for the indicated doses; V indicates vehicle-treated controls. (*b*) Quantification of the immunoblot data using a densitometric analysis of band intensity. Data are expressed as the relative optical density (OD) and given as average values and standard errors ($n = 4$–6 for each treatment group). The relative ODs are percentages of the OD of each vehicle control. The asterisks (*) indicate statistically significant differences in each immunoreactivity value compared to the vehicle control ($p < 0.05$).

Two MK-801 injections induced more prominent changes in the immunoreactivity of p-p70S6K, p-S6, and p-eIF4B. The changes in the immunoreactivity of p-p70S6K were not significant at 1 h after two MK-801 injections. At 4 h ($F = 36.20$, d.f. = 3, $p < 0.01$), 0.1, 0.5, and 1.0 mg/kg MK-801 significantly reduced immunoreactivity of p-p70S6K ($p < 0.01$ for all), and at 8 h h ($F = 21.16$, d.f. = 3, $p < 0.01$), 0.5 and 1.0 mg/kg both induced a significant decrease ($p < 0.01$ for both). At 24 h ($F = 9.29$, d.f. = 3, $p < 0.01$), increased immunoreactivity was found with statistical significance in the groups receiving 0.5 and 1.0 mg/kg MK-801 ($p = 0.03$, $p < 0.01$, respectively). Immunoreactivity of p-S6 (both Ser240/244 and Ser235/236) showed a similar pattern of change to p-p70S6K. Immunoreactivity of p-eIF4B changed similarly. At 48 h, no statistically significant changes were observed in the immunoreactivity of p-p70S6K, p-S6, and p-eIF4B (Fig. 3). No changes were found in the total levels p70S6K, S6, and eIF4B (Figs 2 and 3).

Immunohistochemistry was performed after two injections of MK-801 (1.0 mg/kg). Based on cell morphology, p-S6 (Ser240/244) immunoreactivity was localized in the cytoplasmic part of the neurons, and the density of p-S6 (Ser240/244) positive neurons was decreased in the prefrontal cortex at 8 h after two MK-801 injections compared to that of vehicle control groups (Fig. 4*a*). The p-S6 (Ser240/244) positive cells per cortical region examined were counted. The p-S6 (Ser240/244) positive cells were significantly reduced in MK-801-treated samples compared to those of vehicle-treated controls in the prefrontal ($t = 5.33$, d.f. = 6, $p < 0.01$), cingulate ($t = 4.08$, d.f. = 6, $p < 0.01$), and insular ($t = 5.16$, d.f. = 6, $p < 0.01$) cortices (Paxinos & Watson, 1998) (Fig. 4*b*). Next, we performed immunofluorescence analysis to investigate whether the cells stained with p-S6 (Ser240/244) antibody were of neuronal origin, as observed on cell morphology. Immunoreactivity of p-S6 was co-localized with Neu-N, a specific neuronal protein (Mullen *et al*. 1992), which was decreased in the prefrontal cortex at 8 h after two MK-801 injections (Fig. 4*c*). In addition, immunoreactivity of p-S6 was not co-localized with immunoreactivity of DAPI (4′,6-diamidino-2-phenylindole), a fluorescence stain labelling cell nuclei through binding to DNA (Kubista

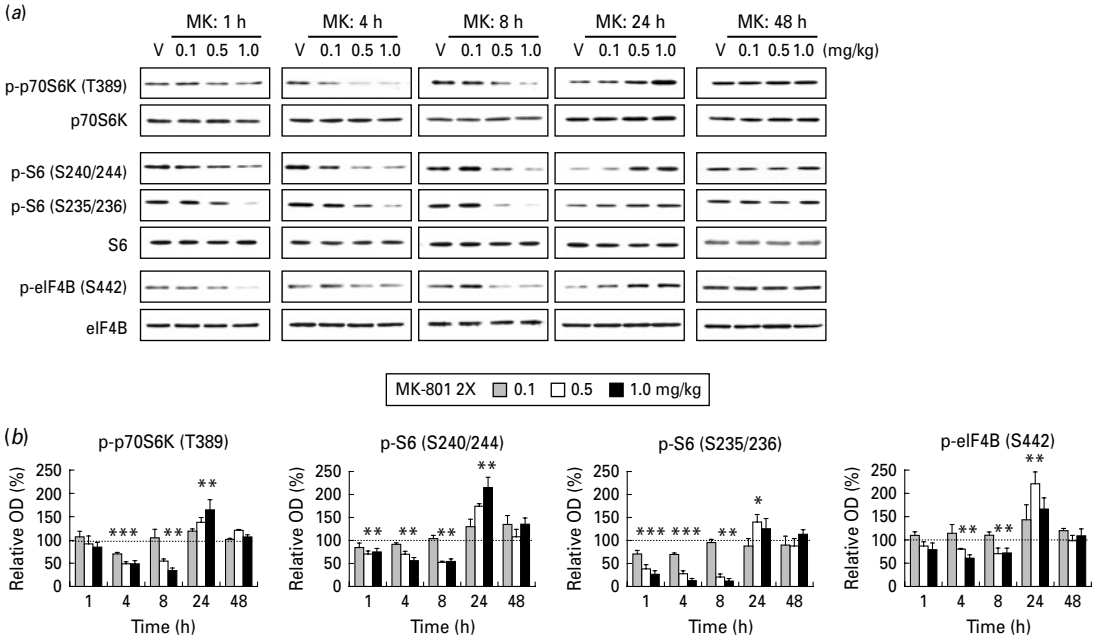

Fig. 3. Time- and dose-dependent changes in the phosphorylation level of p70S6K and its substrates, S6 and eIF4B, in the rat frontal cortex after two MK-801 injections at PND7. Two MK-801 injections at PND7 induced a decrease in immunoreactivity of p-p70S6K (Thr389), p-S6 (Ser240/244 and Ser235/236), and p-eIF4B (Ser442) in the first 8 h, an increase at 24 h, and no change at 48 h. (*a*) Representative immunoblots of the rat frontal cortex at 1, 4, 8, 24, and 48 h after two MK-801 injections at the indicated doses. (*b*) Quantification of the immunoblot data using a densitometric analysis of band intensity. Data are expressed as described in the legend to Fig. 2 ($n=4$–6 for each treatment group).

et al. 1987) (Fig. 4*d*). MK-801 treatment reduced the immunoreactivity of p-S6 in the cytoplasm of the neurons in the rat cortical regions, including frontal, cingulate, and insular cortices.

Effects of MK-801 treatment on ERK1/2 and p90RSK in the frontal cortex of PND7 rats

Single treatment of MK-801 also induced dose- and time-dependent changes in the immunoreactivity of p-ERK1/2 (Thr202/Tyr204) and p-p90RSK (Thr359/Ser363), a downstream kinase of ERK1/2, in the frontal cortex. Immunoreactivity of p-ERK1/2 was significantly decreased at 1 h ($F=18.87$, d.f.$=3$, $p<0.01$) with 0.5 and 1.0 mg/kg MK-801 ($p<0.01$ for both), and at 4 h ($F=25.42$, d.f.$=3$, $p<0.01$) with 0.5 and 1.0 mg/kg MK-801 ($p<0.01$ for both). At 8 h, no significant differences were found and, at 24 h ($F=3.68$, d.f.$=3$, $p=0.03$), an increased immunoreactivity of p-ERK1/2 was observed with 0.5 mg/kg MK-801 ($p=0.02$). Immunoreactivity of p-p90RSK showed a pattern of changes similar to those observed in p-ERK1/2. At 48 h, no significant changes were found in the immunoreactivity of either p-ERK1/2 or p-p90RSK (Fig. 5).

Two MK-801 injections induced more prominent changes in the immunoreactivity of both p-ERK1/2 and p-p90RSK. The immunoreactivity of p-ERK1/2 significantly decreased at 1 h ($F=56.03$, d.f.$=3$, $p<0.01$) with 0.1, 0.5, and 1.0 mg/kg MK-801 ($p<0.01$ for all), and at 4 h ($F=4.30$, d.f.$=3$, $p=0.02$) with 1.0 mg/kg MK-801 ($p=0.01$). Immunoreactivity recovered at 8 h and significantly increased at 24 h ($F=20.76$, d.f.$=3$, $p<0.01$) with 0.5 and 1.0 mg/kg MK-801 ($p<0.01$ for both). At 48 h ($F=5.76$, d.f.$=3$, $p=0.01$), immunoreactivity of p-ERK1/2 decreased with 1.0 mg/kg MK-801 ($p=0.01$). Immunoreactivity of p-p90RSK showed a similar pattern of changes to those observed in p-ERK1/2, but there were no significant changes at 24 and 48 h. Total levels of ERK1/2 and p90RSK did not change after either one or two MK-801 injections (Fig. 5).

Effects of MK-801 treatment on Akt, mTOR, and 4E-BP in the frontal cortex of PND7 rats

Immunoreactivity p-Akt (Ser473) did not show any significant change after a single MK-801 treatment. mTOR can be activated by phosphorylation at Ser2448 (Chiang & Abraham, 2005; Mothe-Satney *et al*. 2004).

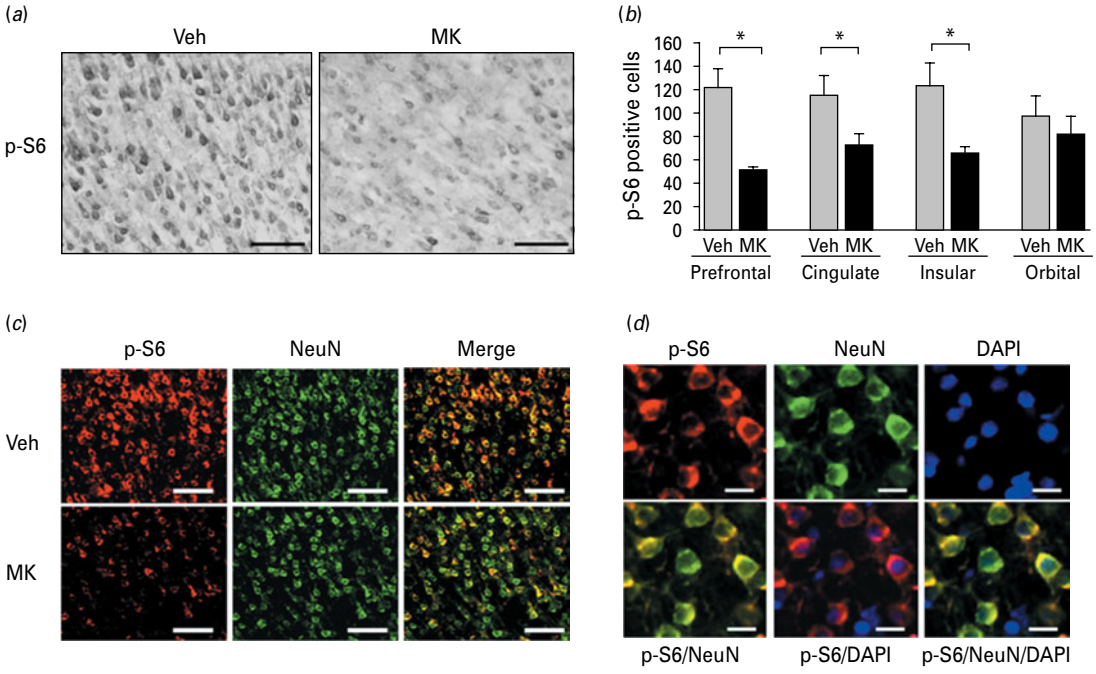

Fig. 4. Decreased immunostaining of p-S6 co-localized with immunofluorescence of Neu-N in the rat frontal cortex after two MK-801 injections (1.0 mg/kg). (*a*) Representative microscopic images taken from sections stained for p-S6 (Ser420/424) in rat prefrontal cortex (Fr) (Paxinos & Watson, 1998). All captured images were obtained 8 h after two MK-801 injections (1.0 mg/kg) or vehicle (normal saline). (*b*) The number of positive cells for p-S6 were counted and were found to be decreased in prefrontal (Fr), cingulate (Cg), and angular insular (AI), but not in orbital (LO and VLO) cortical regions (Paxinos & Watson, 1998) after MK-801 treatment. Bars represent the average cell counts in each section and standard errors. The asterisks (*) indicate statistically significant differences in each value compared to vehicle control ($p<0.05$). (*c*, *d*) Decreased immunofluorescence of p-S6 (red) was co-localized with that of Neu-N (green), but not with that of DAPI (blue), in rat prefrontal cortex (Fr) (Paxinos & Watson, 1998). Magnification bar: (*a*, *c*) 50 μm, (*d*) 10 μm. V and MK indicate vehicle-treated control and MK-801, respectively.

However, phosphorylation at Ser2481 can also activate mTOR regardless of the status of phosphorylation at Ser2448 (Copp *et al*. 2009; Peterson *et al*. 2000; Sekulic *et al*. 2000). Therefore, we examined the phosphorylation of mTOR at both Ser2448 and Ser2481. The immunoreacitivity of p-mTOR (both Ser2448 and Ser2481) did not change significantly after a single MK-801 treatment. The immunoreactivity of p-4E-BP (Thr37/46), one of the substrates of mTOR, also showed no significant change (Fig. 6).

After two MK-801 injections, immunoreactivity of p-Akt decreased at 4 h ($F=5.20$, d.f. $=3$, $p=0.01$) with 0.5 and 1.0 mg/kg ($p=0.04$, $p=0.02$, respectively) and at 8 h ($F=8.96$, d.f. $=3$, $p<0.01$) with 0.5 and 1.0 mg/kg MK-801 ($p<0.01$, $p=0.03$, respectively). In addition, immunoreactivity of p-mTOR (Ser2481), reflecting kinase activity of mTOR (Copp *et al*. 2009; Peterson *et al*. 2000; Sekulic *et al*. 2000), significantly decreased at 4 h ($F=35.07$, d.f. $=3$, $p<0.01$) with 0.5 and 1.0 mg/kg (for both $p<0.01$) and at 8 h ($F=7.44$, d.f. $=3$, $p<0.01$) with 1.0 mg/kg MK-801 ($p=0.02$). Immunoreactivity of p-4E-BP showed no significant changes. The total protein levels of Akt, mTOR, and 4E-BP did not change after either one or two MK-801 injections (Fig. 6).

Changes in protein synthesis rate with MK-801 treatment

Based on the findings concerning the molecules related to the initiation of protein translation, it was expected that the protein synthesis rate in the frontal cortex could be affected by MK-801 treatment. To determine the protein translation rate in the frontal cortex after MK-801 treatment, an *in-vitro* translation assay, validated for the analysis of protein translation rate in brain tissue samples, was used to measure the incorporation of [^{3}H]leucine in precipitated proteins, using PMS of frontal cortical tissue (Burda *et al*. 1994; Cosgrove & Rapoport, 1986; Fando & Wasterlain, 1980; Fando *et al*. 1980). First, we confirmed the previous report that the protein synthesis rate in the

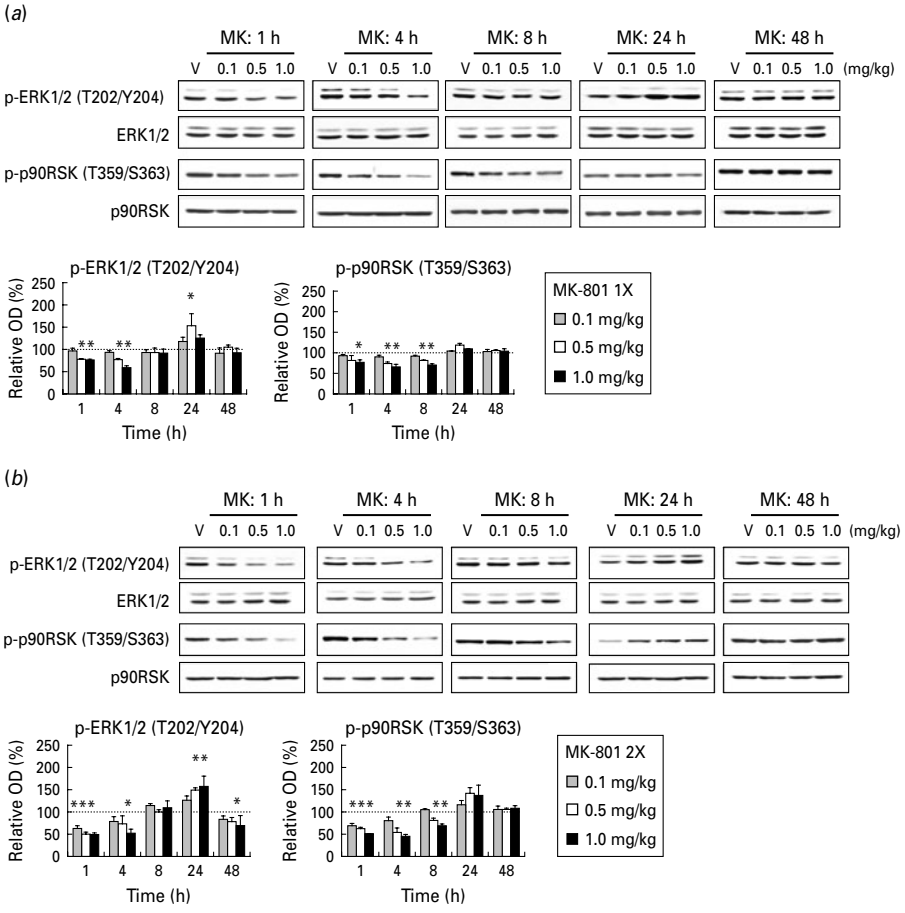

Fig. 5. Time- and dose-dependent changes in p-ERK1/2 and p-p90RSK after a single or two MK-801 injections. Treatment of MK-801 at PND7 induced a decrease in the immunoreactivity of p-ERK1/2 (Thr202/Tyr204) and p-p90RSK (Thr359/Ser363) in the first 8 h, an increase at 24 h, and no change at 48 h. These changes were more prominent after two MK-801 injections than after a single treatment. Representative immunoblots of the rat frontal cortex at 1, 4, 8, 24, and 48 h after (*a*) a single or (*b*) two MK-801 injections for the indicated doses. Data are expressed as described in the legend to Fig. 2 ($n = 4$–6 for each treatment group). V and MK indicate vehicle-treated control and MK-801, respectively.

frontal cortex of the Sprague–Dawley rat brain in the early postnatal period is higher than that in the adult period (Sun *et al*. 1995). In our experimental condition, the protein translation rate of the frontal cortex of PND7 rats was 2.8-fold higher than that of PND45 rats ($t = 8.72$, d.f. $= 6$, $p < 0.01$). Next, we examined the protein translation rate at 8 and 24 h after two injections of 1.0 mg/kg MK-801, when the phosphorylation of p70S6K, S6, and eIF4B decreased and then recovered or increased, respectively. At 8 h after two injections of MK-801 (1.0 mg/kg), incorporation of [^{3}H]leucine was 70.6% of that of the vehicle control ($t = 3.68$, d.f. $= 6$, $p < 0.01$), and at 24 h, it was 73.1% of that of the vehicle control group ($t = 3.13$, d.f. $= 6$, $p < 0.01$). Taken together, these findings indicate that the protein synthesis rate was significantly reduced in the frontal cortex at 8 and 24 h after two injections of 1.0 mg/kg MK-801 (Fig. 7).

Discussion

Treatment of MK-801 on PND7 rats induced time- and dose-dependent changes in the phosphorylation level of p70S6K-S6/eIF4B and reductions in protein translation in the frontal cortex of developing rat brain. Phosphorylation of p70S6K and its substrates, S6 and eIF4B, decreased dose-dependently from 1 to 8 h after MK-801 treatment. Decreased phosphorylation of S6 was evident in broad cortical regions, including the prefrontal, cingulate, and insular cortices. Among the representative upstream signal pathways of p70S6K-S6-, Akt- and ERK1/2-related pathways,

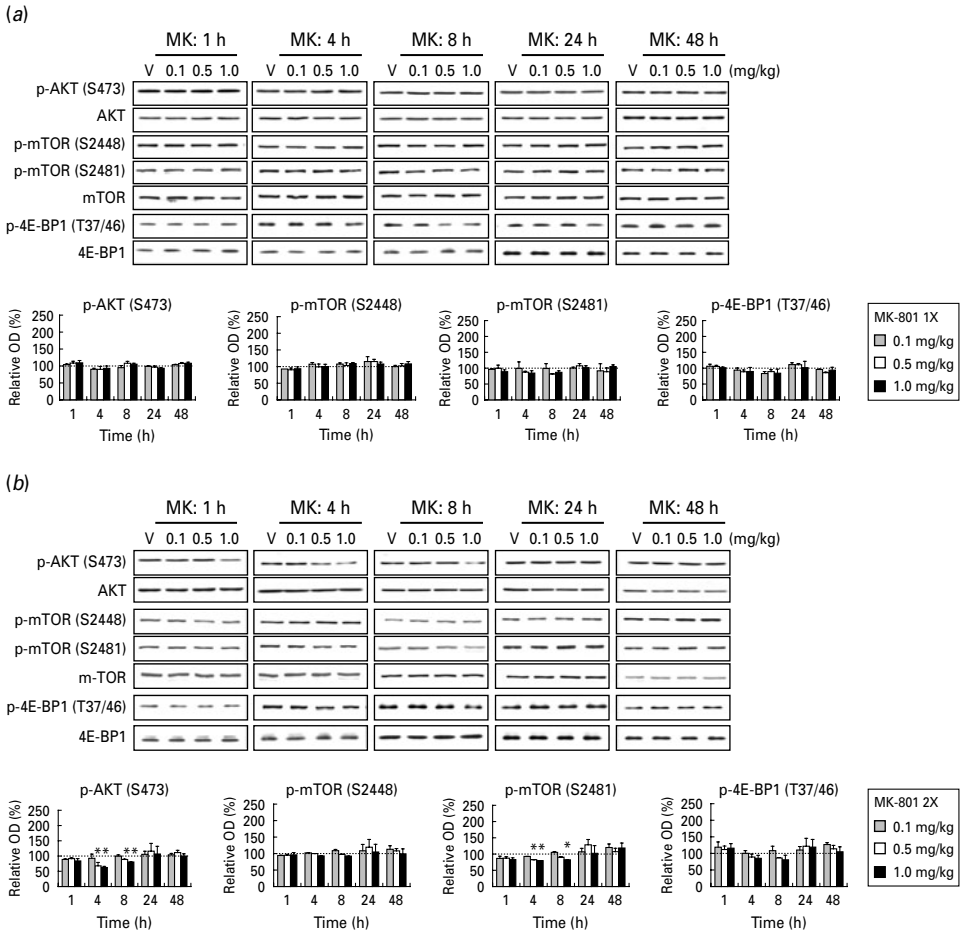

Fig. 6. Effects of MK-801 treatment on p-Akt, p-mTOR, and p-4E-BP1. After two MK-801 injections immunoreactivity of p-Akt (Ser473) was decreased at 4 and 8 h with 0.5 and 1.0 mg/kg, and that of p-mTOR (Ser2481) was decreased at 4 h with 0.5 and 1.0 mg/kg and at 8 h with 1.0 mg/kg MK-801. Representative immunoblots of the rat frontal cortex at 1, 4, 8, 24, and 48 h after (*a*) a single or (*b*) two MK-801 injections for the indicated doses. Data are expressed as described in the legend to Fig. 2 ($n = 4$–6 for each treatment group). V and MK indicate vehicle-treated control and MK-801, respectively.

phosphorylation of ERK1/2 and p90RSK changed in a similar dose- and time-dependent manner observed in p70S6K and S6. The protein synthesis rate was reduced at 8 and 24 h after two MK-801 (1.0 mg/kg) injections. Taken together, these findings indicate that neonatal MK-801 treatment induced dysregulation in the p70S6K-S6 pathway and protein translation in the frontal cortex of the developing rat brain.

p70S6K and its major substrate, S6, play important roles in protein translation (Dufner & Thomas, 1999; Ruvinsky & Meyuhas, 2006). Activated p70S6K induces phosphorylation of S6 and eIF4B, which promotes the initiation of protein translation (Raught *et al*. 2004). S6 is a component of the 40S ribosomal subunit (Nygard & Nilsson, 1990), and phosphorylation of S6 enhances protein synthesis via recruitment of the 7-methylguanosine cap complex or translation of the 5′ tract of oligopyrimidine mRNA, which encodes for translation initiation factors and ribosomal protein subunits (Meyuhas, 2008; Ruvinsky & Meyuhas, 2006; Roux *et al*. 2007). eIF4B functions as a co-factor of an RNA helicase, eIF4A, and enhances the translation rate of mRNA with 5′ unstructured regions (Rogers *et al*. 2001). This suggests that the activity of p70S6K and S6 correlates with protein translation activity.

In our study, the protein synthesis rate was analysed using a cell-free translation system to demonstrate that protein translation was affected along with the dysregulated p70S6K-S6 signal pathways in the developing rat brain in response to MK-801 treatment. The *in-vitro* cell-free translation assay system used in our study has been utilized and validated for brain,

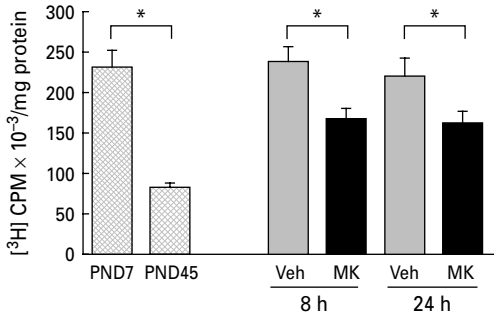

Fig. 7. Decreased protein synthesis rate after two MK-801 injections (1.0 mg/kg). An *in-vitro* translation assay was used to measure the incorporation of [^{3}H]leucine in precipitated proteins, using post-mitochondrial supernatant of frontal cortical tissue. The protein translation rate of the frontal cortex of PND7 rats was significantly higher than that of PND45 rats, and the protein synthesis rate was significantly reduced in the frontal cortex at 8 and 24 h after two injections of 1.0 mg/kg MK-801 ($n=4$ for each treatment group). V and MK indicate vehicle-treated control and MK-801, respectively. The asterisks (*) indicate statistically significant differences ($p<0.05$).

to accurately reflect the changes that occur *in vivo* (Burda *et al*. 1994; Cosgrove & Rapoport, 1986; Fando & Wasterlain, 1980). Initial rapid changes in the activity of protein translation are associated with the related regulatory mechanism, such as states of phosphorylation of translation factors and specific RNA binding proteins. However, over the longer term, the control of protein synthesis is related to alterations in the cellular capacity for protein synthesis, such as changes in the levels of translation factors and ribosomes (Proud, 2007). Therefore, decreased phosphorylation of p70S6K-S6/eIF4B until after 8 h could result in a longer-term reduction in the protein synthesis rate until 24 h after MK-801 treatment.

In the brain of early postnatal period rats, synaptogenesis, differentiation, proliferation, and migration actively occur (Andersen, 2003), all of which requires a high rate of protein synthesis. In fact, the local cerebral protein synthesis rate in the rat during normal postnatal development is highest in the early postnatal period (Fando *et al*. 1980; Sun *et al*. 1995). We also found that the protein synthesis rate in the fontal cortex of PND7 rats was significantly higher than that in PND45 rats, and MK-801 treatment reduced the general protein synthesis rate in the frontal cortex of PND7 rats.

The frontal cortex has been suggested as a key region related to the behavioural abnormalities of animals in response to NMDAR antagonists (Jackson *et al*. 2004; Jentsch & Roth, 1999; Takahata & Moghaddam, 2003). Moreover, the brain regions most affected by ketamine-induced acute psychotic states in humans are in the prefrontal cortex (Breier *et al*. 1997). MK-801 treatment in the early postnatal period also induces long-term impairments in cognitive set-shifting abilities and working memory, which reflects dysfunctions in frontal cortical regions (Stefani & Moghaddam, 2005). A reduction in the protein synthesis rate in early development in response to MK-801 treatment could contribute to these long-term behavioural changes, which are related to frontal cortical dysfunction.

The ERK1/2 and Akt pathways are both strongly linked to translational control, but the ERK1/2 signal pathway is more highly sensitive to levels of postsynaptic activity than is the Akt pathway (Sutton *et al*. 2007). After MK-801 treatment at PND7, the ERK1/2 signal pathway was affected more prominently than the Akt signal pathway was. The PI3K-Akt and ERK1/2 pathways have been shown to cooperatively regulate p70S6K activity, which correlates with the phosphorylation at Thr389 in linker domain of p70S6K (Dufner & Thomas, 1999; Pearson *et al*. 1995). After a MK-801 treatment, phosphorylation changes in p70S6K at Thr389 were accompanied by changes in ERK1/2 and p90RSK. Phosphorylation of S6 can be regulated by not only p70S6K but also by p90RSK, which mediates the Ras-ERK1/2 signal pathway (Pende *et al*. 2004; Roux *et al*. 2007). Among the phosphorylation sites of S6, such as Ser235, Ser236, Ser240, Ser244, and Ser247, p90RSK regulates phosphorylation of S6 at Ser235/236 (Pende *et al*. 2004; Roux *et al*. 2007). The phosphorylation of S6 at both Ser240/244 and Ser235/236 was affected by the MK-801 treatment. These findings could suggest that the role of the ERK1/2 signal pathway in the regulation of p70S6K-S6/eIF4B in developing rat brains is affected by MK-801 treatment.

The effects of perinatal treatment with MK-801 and PCP on ERK1/2 and Akt pathways of developing rodent brains have been reported. A single injection of PCP inhibits PI3K-Akt signal pathway and activates GSK-3β in the brain of PND7 rats and in cortical neuronal cells, which are related to neuronal apoptosis (Lei *et al*. 2008). Treatment with 0.5 mg/kg MK-801 at PND7 was found to reduce ERK1/2 kinase activity in rat cingulate and retrosplenial cortices at around 4 and 8 h after one, 4 h after two, and 12 h after three consecutive injections (8 h apart) of MK-801 (Hansen *et al*. 2004). In addition, a single injection of 0.5 mg/kg MK-801 decreased phosphorylation of ERK1/2 and Akt in cingulate and retrosplenial cortices at 30 and 60 min

after MK-801 treatment (Dzietko *et al*. 2004). These findings are in accord with our findings despite several differences, which may result from different experimental conditions, such as the brain regions examined, time-points, doses of NMDAR antagonists, and possible differences between MK-801 and PCP. The findings of Dzietko *et al*. (2004) focus on the changes in ERK1/2 and Akt signal pathways related to apoptotic neurodegeneration, whereas our findings highlight the role of the ERK1/2 and Akt signal pathways in the translational control machinery involved in the dysregulation of protein synthesis induced by MK-801 treatment.

In-vivo systemic treatment of NMDAR antagonists can induce complex changes in neurotransmission and intracellular signal pathways in the brain. For example, systemic treatment of MK-801 was suggested to induce excitotoxicity of cortical regions in the brain as a result of disinhibition of GABA neurotransmission, and dysregulations of various neurotransmissions including glutamatergic system have been reported (Greene, 2001; Lorrain *et al*. 2003; Moghaddam *et al*. 1997; Olney & Farber, 1995). PCP treatment of PND7 rats was reported to increase membrane levels of NR1 and NR2B, NMDAR subunits, in the rat frontal cortex (Anastasio & Johnson, 2008). The response to NMDAR antagonists varies depending on the developmental stage of the rats (Ikonomidou *et al*. 1999). Moreover, alterations in signal pathways in the brain can vary depending on the treatment duration and dose of NMDAR antagonists. Therefore, the initial reductions followed by recovery or increase in phosphorylation of p70S6K-S6/eIF4B after MK-801 treatment in the brain of PND7 rats may result from these complicated effects of systemic treatment of NMDAR antagonists. These effects require further clarification.

In conclusion, we found that early postnatal treatment with MK-801, a selective NMDAR antagonist, induced a reduction in the protein synthesis rate, along with dysregulations in the p70S6K-S6/eIF4B signal pathways in the frontal cortex of the developing rat brain. Dysregulations in protein translation and related signal pathways during this critical period of brain growth, induced by perinatal treatment of NMDAR antagonists, disrupt the proper construction of neural circuits and cause long-term behavioural modifications, which may relate to neurochemical and behavioural changes resembling schizophrenia. These findings could contribute to an understanding of the developmental underpinnings of protein translation regulation in the MK-801 neurodevelopmental rat model of schizophrenia.

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Statement of Interest

None.

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