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BRIEF REPORT

The effects of MK-801 on the phosphorylation of Ser338-c-Raf-MEK-ERK pathway in the rat frontal cortex

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Abstract

MK-801 induces psychotomimetic behavioural changes in animals. ERKs play an important role in the pathogenesis of schizophrenia and in the action of antipsychotics and psychotomimetics. We observed phosphorylation of ERK-signalling-pathway-associated molecules in the rat frontal cortex and their association with rat behaviour after MK-801 administration. After injecting 0.25–1 mg/kg MK-801, ERK phosphorylation decreased compared to vehicle treatment, and rats showed increased locomotion. After 2 mg/kg treatment, ERK phosphorylation increased and rat motility started to decrease. After treating with 4–8 mg/kg, ERK phosphorylation once again decreased and rats showed hypomotility and ataxia. ERK phosphorylation levels were maintained from 15 min to 90 min after 1 or 2 mg/kg treatment. Ser338-c-Raf and MEK phosphorylation showed similar dose-dependent and temporal patterns to those of ERK. Taken together, Ser338-c-Raf-MEK-ERK phosphorylation by MK-801 in the rat frontal cortex showed a specific pattern and may be associated with behavioural changes induced by MK-801.

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Introduction

NMDA receptor hypofunction has been suggested as an aetiology of schizophrenia (Deutsch et al., 2002; Olney and Farber, 1995), and the non-competitive NMDA receptor antagonist MK-801 is known to have psychotomimetic effects that closely resemble the symptoms of schizophrenia (Olney and Farber, 1995). Moreover, behavioural changes induced by MK-801 in rodents, including hyperlocomotion, stereotyped sniffing, and ataxia, are considered pharmacological measures of NMDA hypofunction in living animals (Andine et al., 1999, Deutsch et al., 2002).

The intracellular signal transduction pathways of psychotomimetics have been studied in investigations into the biochemical mechanisms underlying psychotic symptoms. Svenningsson et al. (2003) reported that various psychotomimetics, such as d-amphetamine, lysergic acid diethylamide (LSD) and phencyclidine (PCP), affect the phosphorylation of DARPP-32, GSK-3β, and CREB in the mouse frontal cortex, and our group previously observed that MK-801 influences c-fos expression (Ahn et al., 2002) and the phosphorylation of Akt, GSK-3β, and CREB in the rat frontal cortex (Ahn et al., 2005).

The ERK pathway is known to be an essential component of NMDA receptor-related signal transduction (Bading and Greenberg, 1991; Krapivinsky et al., 2003), and the activity of ERK, downstream of Ras, has been reported to be increased by NMDA activation (Bading and Greenberg, 1991). Moreover, MK-801 reduces the NMDA receptor-mediated hyperphosphorylation of ERK2 in cortical neuronal cultures (Chandler et al., 2001). Schizophrenia has been suggested to be associated with ERK signalling abnormalities in the thalamus and cerebellum (Kyosseva, 2004), and atypical antipsychotics were reported to activate ERK in the mouse cortex and in

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primary cultures of rat cortical neurons (Lu et al., 2004; Valjent et al., 2004). In addition, ERKs are known to be involved in both synaptic plasticity and long-term potentiation (Orban et al., 1999), which are related to the pathophysiology of schizophrenia and the action mechanism of antipsychotics and MK-801 (Carboni et al., 2004).

c-Raf is one of the main effectors to activate the MEK-ERK pathway, and is recruited by GTP-bound Ras (Avruch et al., 1994). The phosphorylation of c-Raf at Ser338 is known to be essential for c-Raf activation (Diaz et al., 1997; Goetz et al., 2003), and the hyperphosphorylation of c-Raf at Ser259 is known to interfere with c-Raf activation (Moelling et al., 2002).

In this study, we observed that the effect of MK-801 on the phosphorylation of the Ser338-c-Raf-MEK-ERK pathway is not unidirectional, and that the phosphorylation level of this pathway in the rat frontal cortex may be related to specific behavioural patterns induced by MK-801.

Materials and methods

Animals and drug treatment

Male Sprague–Dawley rats (150–200 g) were grouped and maintained under a 12-h light/dark cycle with food and water freely available. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

MK-801 (Tocris, Ellisville, MO, USA, dissolved in normal saline) was injected into rats intraperitoneally (i.p.) and control animals received an identical volume of normal saline. To examine the relation between MK-801 dosage and phosphorylation of c-Raf, MEK and ERK phosphorylation, we observed phosphorylation levels 60 min after MK-801 injection because our preliminary results showed that ERK1/2 phosphorylation decreased maximally at 60 min after MK-801 administration. Moreover, it has been reported that MK-801-induced locomotion, stereotyped sniffing, and ataxia are fully and stably developed at 60 min after treatment (Andine et al., 1999). We observed the phosphorylation levels of c-Raf, MEK, and ERK at 15, 30, 60, and 90 min after injection of 1 or 2 mg/kg MK-801 in order to examine the temporal profile of phosphorylation. Three independent experiments were performed at each MK-801 dosage and time-point.

Behavioural experiments

After administration of MK-801, rats were placed in individual standard clear plastic cages ($26 \times 42 \times 18$ cm) bedded with wood chips. We observed and rated the behaviours of rats 60 min after injection of each MK-801 dose for 30 s. We rated locomotion, stereotyped sniffing, and ataxia using the rating scales described by Andine et al. (1999). Locomotion scores were rated from 0 to 5, stereotyped sniffing from 0 to 2, and ataxia from 0 to 3. Higher scores were awarded to more pronounced behavioural patterns. Scores were obtained from three independent experiments.

Western blot analysis

Brains were dissected on ice plates. Frontal cortices were immediately homogenized in 10 vol (v/w) of pre-chilled buffer containing 25 mM Hepes (pH 7.9), 200 mM NaCl, 1.5 mM $MgCl_2$, 0.2% NP-40, 1 mM DTT, 0.5 mM EDTA, 1 mM PMSF, 20 mM β-glycerophosphate, 2 mM NaF, 0.1 mM Na_3VO_4, 2 mg/l leupeptin and protease inhibitor cocktail (Roche, Mannheim, Germany). Homogenates were centrifuged and supernatants were boiled with Laemmli sample buffer. Proteins were then fractionated in 8% SDS–PAGE gel and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Dassel, Germany). Membranes were incubated with ERK1/2, p-ERK1/2 (Thr202/Tyr204), MEK1/2, pMEK1/2 (Ser217/204), c-Raf, p-c-Raf (Ser259) or p-c-Raf-(Ser338) specific antibodies (all from Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:1000–3000 overnight at 4 °C. Membranes were then incubated with anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and signals were detected using an ECL system (Pierce, Rockford, IL, USA). Film exposure was adjusted as indicated by preliminary experiments.

Statistical analysis

At least three independent experiments were performed. The non-parametric Mann–Whitney U test was used to analyse average values, and p values of <0.05 were considered statistically significant.

Results

We examined the effect of MK-801 in the rat frontal cortex on each dose (0.25, 0.5, 1, 2, 4, and 8 mg/kg) 60 min after i.p. injection of MK-801. The phosphorylation of Thr202/Tyr204-ERK showed a different pattern according to the dosage of MK-801. At MK-801 dosages from 0.25 to 1 mg/kg, ERK2 phosphorylation levels markedly reduced compared to vehicle-treated controls. At 2 mg/kg, ERK2 phosphorylation levels increased vs. baseline, and between 4 and 8 mg/kg ERK2 phosphorylation decreased again. We also

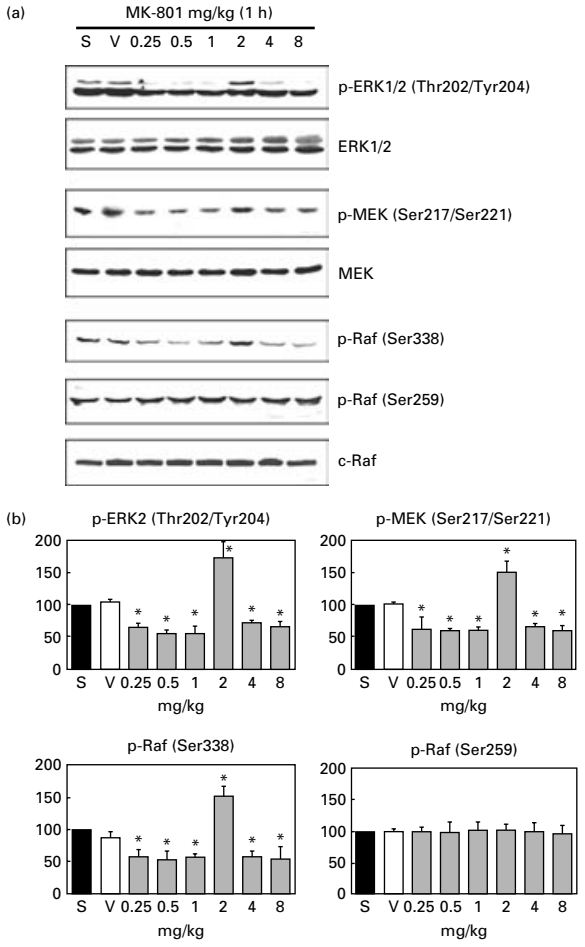

Figure 1. MK-801 dose-dependent patterns of c-Raf, MEK and ERK phosphorylation in the rat frontal cortex. (a) Immunoblots of rat frontal cortex 60 min after treatment with MK-801 at the indicated doses. (b) Quantification of immunoblot data by densitometry. Three independent experiments were performed. Data are expressed as relative optical densities (ODs) and represent the average values and standard deviations of three independent experiments. The relative ODs are quoted as percentages vs. the OD of the sham-treated group. S and V indicate the sham- and vehicle-treated controls respectively. The asterisks (*) indicate significant difference of OD of each dose from the OD of vehicle ($p < 0.05$, Mann–Whitney U test).

observed an ERK1 signal (44 kDa, upper bands in Figure 1a) above ERK2, and phospho-ERK1 above phospho-ERK2. These phospho-ERK1 signals were much weaker than ERK2 and could not be quantified, but they showed a similar pattern to those of phospho-ERK2 (Figure 1).

The phosphorylation of Ser217/Ser221-MEK, immediately upstream of ERK, and of Ser338-c-Raf showed a similar dose-dependent pattern to that of ERK. At doses of 0.25–1 mg/kg MK-801, the phosphorylation levels of Ser217/Ser221-MEK and Ser338-c-Raf decreased. Phosphorylation increased when MK-801 was administered at 2 mg/kg and decreased again when MK-801 was administered at 4–8 mg/kg. However, MK-801 administration did not affect Ser259-c-Raf phosphorylation or the total amounts of ERK, MEK, or c-Raf (Figure 1).

Rat behaviour also changed with MK-801 dosage. At baseline, after i.p. injection of an equivalent volume of normal saline, locomotion, stereotyped sniffing, and ataxia scores were 0.33 ± 0.58, 1.33 ± 0.58 and 0.00 ± 0.00 (mean $\pm$ S.D.) respectively. At MK-801 doses of 0.25–1 mg/kg, rats showed increased locomotion and stereotyped sniffing (locomotion, stereotypy, and ataxia at 1 mg/kg: 1.67 ± 0.58, 2.00 ± 0.00 and 1.33 ± 0.58 respectively). However, after treatment with 2 mg/kg MK801, locomotion decreased, although it was still higher than that of vehicle-treated rats (locomotion, stereotypy, and ataxia; 0.33 ± 0.58, 1.67 ± 0.58 and 3.00 ± 0.00 respectively). Moreover, from 4 to 8 mg/kg MK801, rats showed hypomotility and ataxia (locomotion, stereotypy and ataxia: 0.00 ± 0.00, 0.00 ± 0.00 and 3.00 ± 0.00 respectively). These behavioural changes seem to be related to MK801-induced phosphorylation level changes in Thr202/Tyr204-ERK2, Ser217/Ser221-MEK and Ser338-c-Raf in the rat frontal cortex. At lower MK-801 dosages (0.25–1 mg/kg), rat motility increased whereas ERK, MEK, and Ser338-c-Raf phosphorylation decreased, and when rats showed a transition from hypermotility to hypomotility at 2 mg/kg MK-801, ERK, MEK, and Ser338-c-Raf phosphorylation increased significantly vs. baseline. Moreover, when the rats showed hypomotility and ataxia these phosphorylation levels decreased again.

After administration of 1 mg/kg MK-801, ERK2 phosphorylation was observed to be reduced from 15 min post-injection and this was maintained until 90 min. Ser338-c-Raf and MEK phosphorylation levels were also attenuated from 15 to 90 min after MK-801 injection (Figure 2).

After injecting 2 mg/kg MK-801, ERK2 phosphorylation was increased from 15 min to 90 min post-injection; Ser338-c-Raf and MEK showed similar patterns to those of ERK (Figure 3).

Discussion

We examined the effect of MK-801 on the c-Raf-MEK-ERK pathway in the rat frontal cortex in vivo. The phosphorylation levels of Ser338-c-Raf, MEK, and ERK were reduced after administration of 0.25–1 or

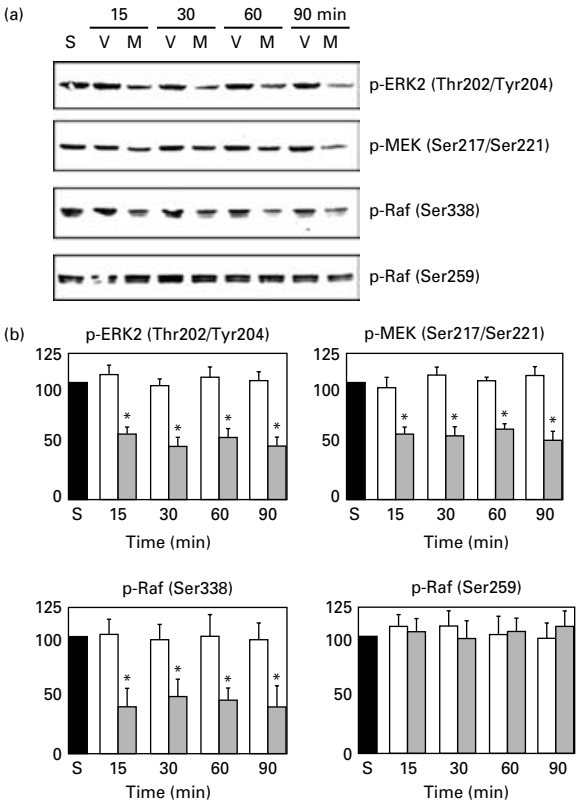

Figure 2. Temporal patterns of c-Raf, MEK and ERK phosphorylation in the rat frontal cortex after administration of 1 mg/kg MK-801 i.p. (a) Immunoblots of rat frontal cortex after treatment with MK-801 (1 mg/kg) for the indicated periods. (b) Quantification of immunoblot data by densitometric analysis of band intensities (performed as described in Figure 1 legend). ■, Sham (S); □, vehicle (V); ▨, MK-801.

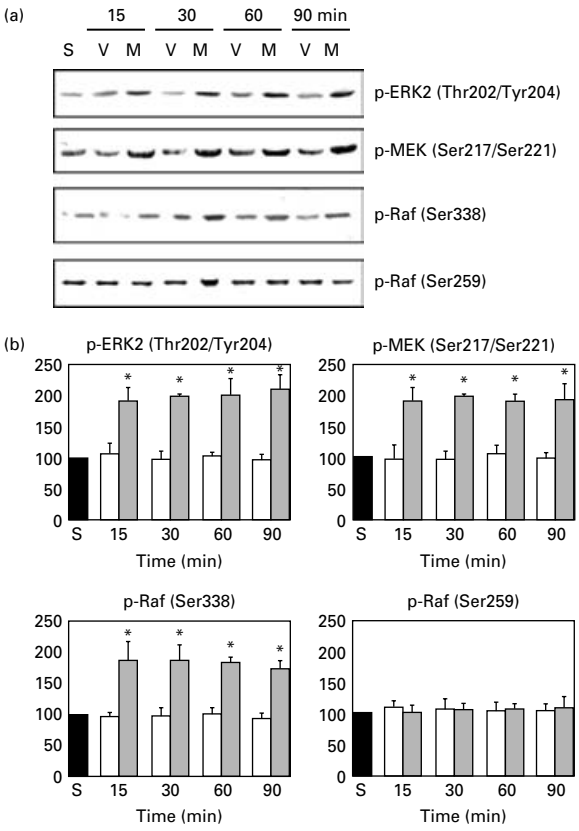

Figure 3. Temporal patterns of c-Raf, MEK and ERK phosphorylation in the rat frontal cortex after administration of 2 mg/kg MK-801 i.p. (a) Immunoblots of the rat frontal cortex at the indicated times after treatment with 2 mg/kg MK-801. (b) Quantification of immunoblot data by densitometry (performed as described in Figure 1 legend). ■, Sham (S); □, vehicle (V); ▨, MK-801.

4–8 mg/kg MK-801, but increased after administration of 2 mg/kg MK-801. Moreover, these phosphorylation level changes were maintained from 15 to 90 min after administration of 1 or 2 mg/kg MK-801. These results suggest that the Ser338-c-Raf-MEK-ERK pathway in the rat frontal cortex may be one of the signalling pathways involved in the action mechanism of MK-801.

ERK phosphorylation was reduced with 1 mg/kg MK-801, but increased by 2 mg/kg MK-801. In addition, we reported that c-fos induction and Akt-GSK-3β phosphorylation were increased by 1 mg/kg MK-801, and then started to decrease with 2 mg/kg MK-801. The ERK phosphorylation level decreased again with 4–8 mg/kg MK-801, and c-fos expression and phosphorylation of Akt and GSK-3β were also attenuated (Ahn et al., 2002, 2005). These results suggest that intracellular signalling pathways respond differently to different MK-801 dosages, and that ERK phosphorylation differences after 0.25–1 mg/kg, 2 mg/kg, or 4–8 mg/kg administration of MK-801 reflect different states of neuronal activation in the rat frontal cortex.

Interestingly, rat behaviour and ERK phosphorylation levels in the rat frontal cortex depended on MK-801 dosage. At a dose of 1 mg/kg MK-801, rats showed increased locomotion. However, at 2 mg/kg MK-801, rat motility started to reduce, and at >2 mg/kg rats showed hypomotility and ataxia. Andine et al. (1999) reported that rats showed maximal behavioural activation in terms of locomotion and stereotyped sniffing at 1 mg/kg MK-801, whereas, at 3 mg/kg, they showed extensive ataxia without locomotion. It has been reported that a blood–brain barrier penetrating MEK inhibitor (SL327) blocking the ERK pathway increased locomotion time and distance travelled in a large open field in a manner similar to

that induced by amphetamine (Einat et al., 2003), which is known to induce schizophrenia-like psychosis. Therefore, it may be presumed that specific patterns of ERK signalling pathways in the rat frontal cortex may have some association with the state of brain activation, which could result in specific patterns of behaviour – including the psychotomimetic effect of MK-801.

The changes in ERK phosphorylation by MK-801 could be explained in several ways. First, ERK signalling may be positively or negatively regulated by PI3-kinase (PI3K). The activation of ERK by NMDA receptor stimulation has been reported to completely or partially depend on PI3K activity (Chandler et al., 2001; Opazo et al., 2003), although, it has also been suggested that PI3K activation inhibits ERK activation (Zimmermann and Moelling, 1999). The relationship between PI3K and ERK is known to be dependent on cell type, type of stimulus, and strength of signal (Duckworth and Cantley, 1997; Wennstrom and Downward, 1999; Zhuang et al., 2004). Previously, our group observed that MK-801 increases the phosphorylation of Ser9-GSK-3β, Ser133-CREB, and Ser473-Akt in the rat frontal cortex, thus implicating PI3K pathway activation (Ahn et al., 2005). According to our results, administration of MK-801 from 0.25 to 1 mg/kg caused ERK phosphorylation to decrease, but Akt-GSK-3β phosphorylation to increase; however, at 2 mg/kg MK-801, ERK and Akt-GSK-3β phosphorylation increased, and at 4–8 mg/kg both decreased. According to these results, the interaction in the phosphorylation between ERK and Akt-GSK-3β may not show apparent cross-talk. Furthermore, it has been reported that Akt can phosphorylate c-Raf on Ser259, and that this results in ERK activity inhibition (Moelling et al., 2002), but the phosphorylation on Ser259 was unaffected by MK-801 treatment, as previously mentioned. Under our experimental conditions, changes in ERK phosphorylation did not seem to be attributable to an interaction between the PI3K and ERK pathways.

Second, reduced Ser338-c-Raf-MEK-ERK phosphorylation may have been due to the inactivation of NMDA receptor activity by MK-801. However, this hypothesis is incompatible with the finding that MK-801 at a dose of 2 mg/kg increased ERK phosphorylation, but MK-801 at a higher dosage reduced ERK phosphorylation. Chandler et al. (2001) reported that the dose–response curve for the stimulation of phospho-ERK2 by NMDA is biphasic in cortical neuronal cultures, and that the possibility that MK-801 influences ERK activity via a mechanism independent of the NMDA receptor cannot be excluded (Olney and Farber, 1995). Taken together with our findings, these observations suggest that a simple linear relation between NMDA receptor activity and ERK phosphorylation is unlikely.

Finally, changes in ERK phosphorylation induced by MK-801 could be explained by an alteration in the deactivation of kinase, i.e. a changed phosphatase activity. The contribution of phosphatase activation cannot be ruled out as a possible reason for the change in altered ERK phosphorylation by MK-801, because we did not measure phosphatase activities. Further studies on the regulation of phosphatase activity by MK-801 may shed new light on this possibility.

In summary, the present study shows that the effect of MK-801 on the phosphorylation of the Ser338-c-Raf-MEK-ERK pathway in the rat frontal cortex is not unidirectional, and that the phosphorylation of this pathway in the rat frontal cortex may play an important role in specific patterns of behaviour, including the psychotomimetic effect, induced by MK-801.

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Statement of Interest

None.

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