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Time Courses of pCREB Expression after Dopaminergic Stimulation by Apomorphine in Mouse Brain

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Administration of dopamine agonist, apomorphine (2 mg/kg, s.c.), produces cage climbing behavior in mice that exhibit typical dopaminergic stimulation. The present study investigated the pCREB expression level in several brain regions following apomorphine treatment in order to determine whether the increased the dopaminergic activation produced by apomorphine accompanies the changes in pCREB immunoreactivity. A mouse brain was removed at 0min, 10 min, 30 min, 1 h, 2 h, 7 h, and 24 h after apomorphine treatment. The brain tissue was fixed by an intracardiac perfusion with ice-cold 4% paraformaldehyde in PBS. Immunohistochemical study was conducted using the ABC-DAB method. The data showed that the immunoreactivity of pCREB increased in the striatum, nucleus-accumbens, piriform cortex and the dentate gyrus of the hippocampus of a mouse brain 30 min after the apomorphine treatment. Increased immunoreactivity began to diminish 2 h after the apomorphine treatment in all the brain regions measured. The time course for the pCREB immunoreactivity was similar to the behavioral response induced by the apomorphine treatment. These results suggest that activation of the dopamine receptor is accompanied by an increase in pCREB expression in the mouse brain.

Key words: pCREB, Dopaminergic activation, Climbing behavior, Apomorphine

INTRODUCTION

The dopaminergic system plays a key role in the normal motor function (Albin *et al.*, 1989) and associative learning (Schultz *et al.*, 1997). A dysfunction in dopamine neurotransmission has been linked to CNS disorders, such as schizophrenia, Parkinsons disease, and drug addiction (Hornykiewicz, 1993; Koob and Bloom, 1988). Administration of dopaminergic drugs results in physiological changes as well as behavioral changes. Moreover, dopaminergic drugs rapidly cause changes in gene expression in the striatal neurons (Graybiel *et al.*, 1990). Accumulating evidence suggests that the conversion of extracellular signals into long-term changes in gene expression is mediated by the immediate-early genes (IEGs), such as *c-fos* and the cAMP response element-binding protein (CREB).

CREB is a plasticity-associated transcription factor, which can potentially integrate the cAMP and calcium signals at the gene activation level. Dopaminergic stimulation of the striatal cells also appears to cause changes in CREB phosphorylation (pCREB) and c-fos expression by increasing the intracellular calcium levels, entering through either the voltage sensitive calcium channels or the NMDA receptors (Keefe and Gerfen, 1996; Konradi *et al.*, 1996). Recent studies have shown that dopamine receptor agonists can cause a dramatic activation of a number of IEGs in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the dopaminergic nigrostriatal pathway (Cole *et al.*, 1992; Paul *et al.*, 1992; Robertson *et al.*, 1989). However, the cellular responses in 6-OHDA lesion models to normal stimulation by a dopaminergic agonist are somewhat different, since the 6-OHDA lesion model of Parkinsons disease exhibits the supersensitive state of the dopaminergic systems. The supersensitive state in these Parkinsons models might result in robust CREB phosphorylation and the induction of the gene expression response after dopamine receptor

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stimulation (Robertson *et al.*, 1989; Cole *et al.*, 1994). These amplified cellular responses may result in the accumulation of inaccurate information. Therefore, the dopaminergic behavior-related gene expression caused by dopaminergic stimulation using dopamine agonist in intact animal will provide more accurate information on dopaminergic stimulation in the normal physiological state However, there is a paucity of reports on gene expression after dopaminergic stimulation in an intact animal model.

The apomorphine-induced cage climbing behavior has been used as a convenient method to screen the effects of dopamine agonists or antagonists and to assess the striatal dopamine activity. These behaviors are reduced after the destruction of the striatum and are enhanced by 6-OHDA-induced lesions of dopamine input into the striatum (Protais *et al.*, 1976). In this paper, the time courses of pCREB expression and its regional expression levels in mouse brains after treatment with a dopamine agonist, apomorphine, which produces cage-climbing behavior, is described.

MATERIALS AND METHOD

Animals

ICR male mice (MJ Ltd. Seoul, Korea) weighing 20-26g were used in all the experiments. They were housed at 10-14 mice in a cage with water and food available ad libitum under an artificial 12 h light dark cycle (light on: 7:00 a.m.) with a constant temperature (22 ± 2°C).

Measurement of apomorphine-induced cage climbing behavior in mice

The cage climbing behavior of the mice was measured by a modification of the method reported by Protais *et al* (1976). Prior to the experiment, apomorphine was dissolved in saline containing 0.1% ascorbic acid. Immediately after a subcutaneous injection of 2 mg/kg of apomorphine, the mice were placed into cylindrical individual cages: 12 cm in diameter and 14 cm in height, with walls comprised of vertical metal bars (2 mm in diameter and 1 cm apart). After a 5-min period of exploratory activity, the climbing behavior was measured at 10, 20, 30, 40, 50 and 60 min intervals for 1 min at each time. The climbing behaviors were scored as an all or none event as follows: four paws on the floor (0 point), forefeet on the bars (1 point), four paws on the bars (2 points).

Measurement of pCREB immunohistochemistry

Two mg/kg of apomorphine was used in this experiment, as the group treated with the 2 mg/kg of apomorphine showed a sub-maximal response in the apomorphine-induced cage climbing behavior experiment using 0.5, 1, 2, and 4 mg/kg. After the apomorphine treatment, the animals were anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.4). The mouse brains were removed at 0min, 10min, 30min, 1h, 2h, 7h, and 24h after the apomorphine treatment. The brain samples were sectioned serially and coronally on a freezing microtome at a 45μm thickness. The immunohistochemical procedure was begun with twice rinsing in 0.1M PBS, which was followed by 2h incubation in order to suppress non-specific absorption in the preincubation solution (0.1M PBS containing 0.2% Triton X-100, 1% bovine serum albumin). To demonstrate the pCREB immunoreactivity, the primary antiserum against pCREB (Cell signaling technology, Beverly, MA, USA) diluted 1:500 in a solution of 0.5% bovine albumin and a preservative sodium azide in 0.1M PBS was used. The sections were incubated in primary antiserum for 16h at room temperature. After a short rinse with PBS, they were reacted using the avidin-biotin peroxidase complex (ABC) method (Vector), and washed twice in 0.1M PBS. The antigens were visualized by 0.02% diaminobenzidine and 0.0045% H_2O_2 at room temperature.

RESULTS AND DISCUSSION

Fig. 1 shows the time course effect of the cage-climbing behavior induced by 2 mg/kg of apomorphine. The climbing behavior increased after 10 min and peaked at 20 min. It then diminished after 30 min. This result is

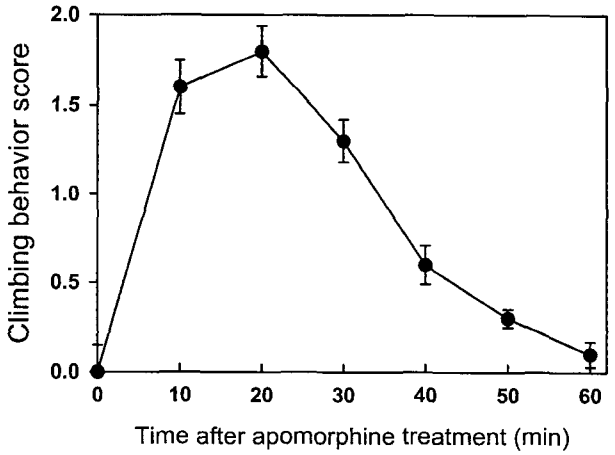

Fig 1. Time-course effects of the climbing behavior induced by apomorphine in mice. After administering 2 mg/kg apomorphine, the climbing behavior was measured at 10, 20, 30, 40, 50 and 60 min intervals for 1 min at each time. The climbing behaviors were scored as all or none events. Animal number of each group was 8-10.

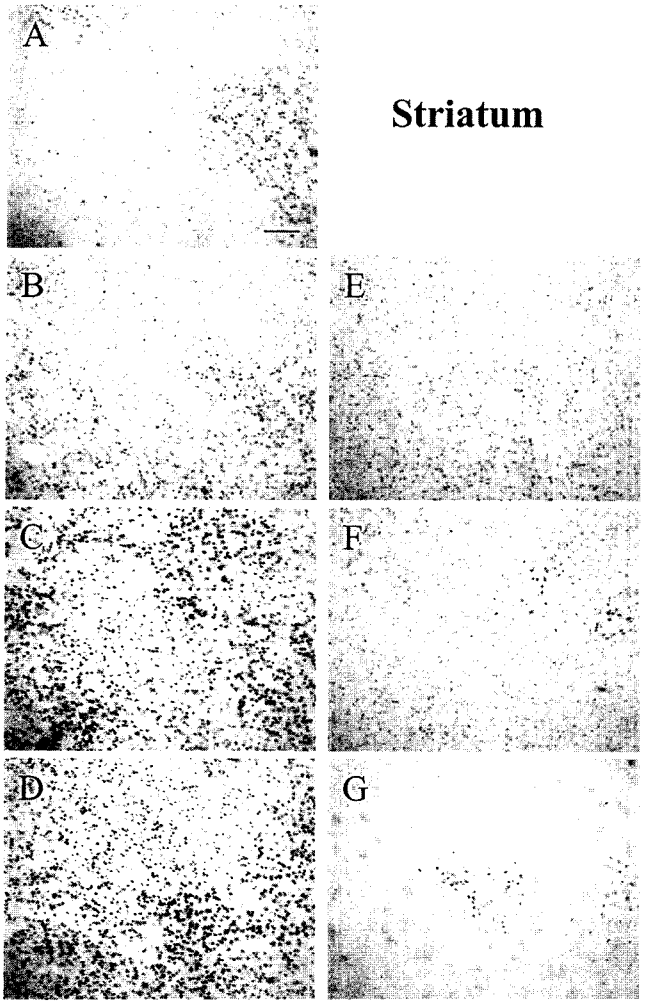

Fig. 2. pCREB immunoreactivity following apomorphine treatment in the striatum of mouse brain. A, 0min; B, 10min; C, 30min; D, 1h; E, 2h; F, 7h; G, 24h after apomorphine treatment. Scale bar, 200ìm.

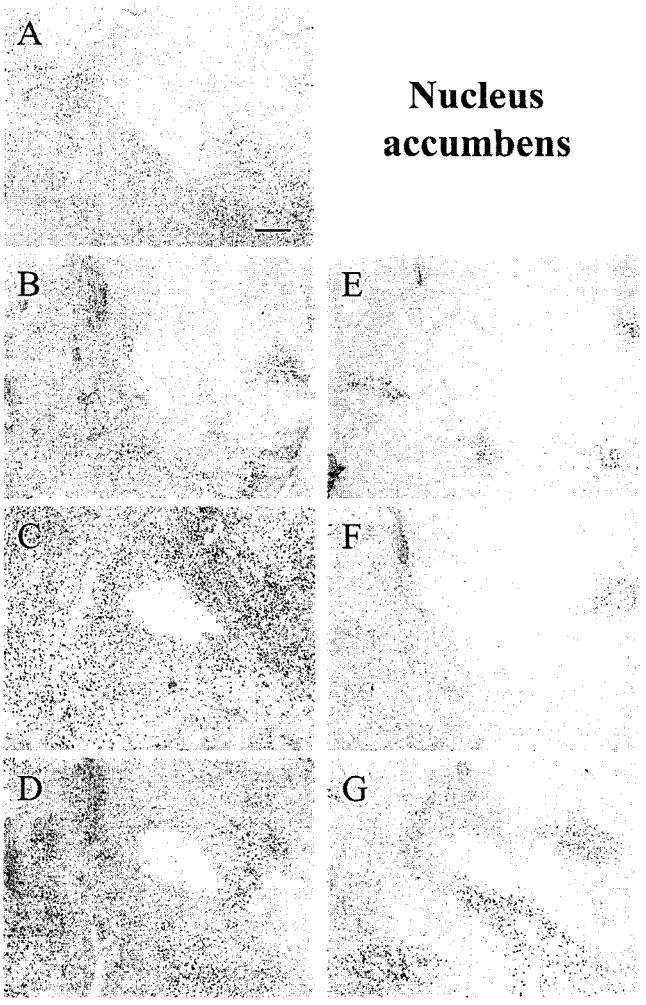

Fig. 3. pCREB immunoreactivity following apomorphine treatment in the nucleus accumbens of mouse brain. A, 0min; B, 10min; C, 30min; D, 1h; E, 2h; F, 7h; G, 24h after apomorphine treatment. Scale bar, 200ìm.

identical with the results reported elsewhere (Protais *et al.*, 1976; Costentin *et al.*, 1976). Therefore, the climbing data in this study shows that the dopamine receptors are stimulated by apomorphine, a mixed dopamine receptor agonist.

It has been reported that the 6-OHDA-lesioned striatum displays an increased dopamine responsiveness compared with the unlesioned striatum, and this can be further modulated by D1 stimulation (Juncos *et al.*, 1989). Moreover, many studies have suggested that dopamine receptor agonists can cause a dramatic activation of a number of IEGs. However, these results have mostly studied dopamine receptor activation in the supersensitive state in rats with 6-OHDA lesions of the dopaminergic nigrostriatal pathway. Therefore, the time course of pCREB expression was investigated using immunohistochemical analysis in mice normally activated by apomorphine.

The results showed that the pCREB immunoreactivity was markedly increased in the striatum 10min to 30min after the apomorphine treatment. These results suggest that the time-course of the increase in the climbing behavior by apomorphine is similar to the time-course of the increase in the pCREB expression level. Robertson *et al.* (1989) reported that dopaminergic drugs rapidly cause changes in *c-fos* expression in the striatal neurons. It has been reported that CREB is essential for the expression of the *fos* family genes in the intact but not in the dopamine-denervated striatal neurons (Andersson *et al.*, 2001). Therefore, it is proposed that the pCREB gene expression may be responsible for the alterations in the intact striatal physiology. Therefore, our data suggests that the pCREB expression in the striatum may be closely related to the apomorphine-induced cage climbing

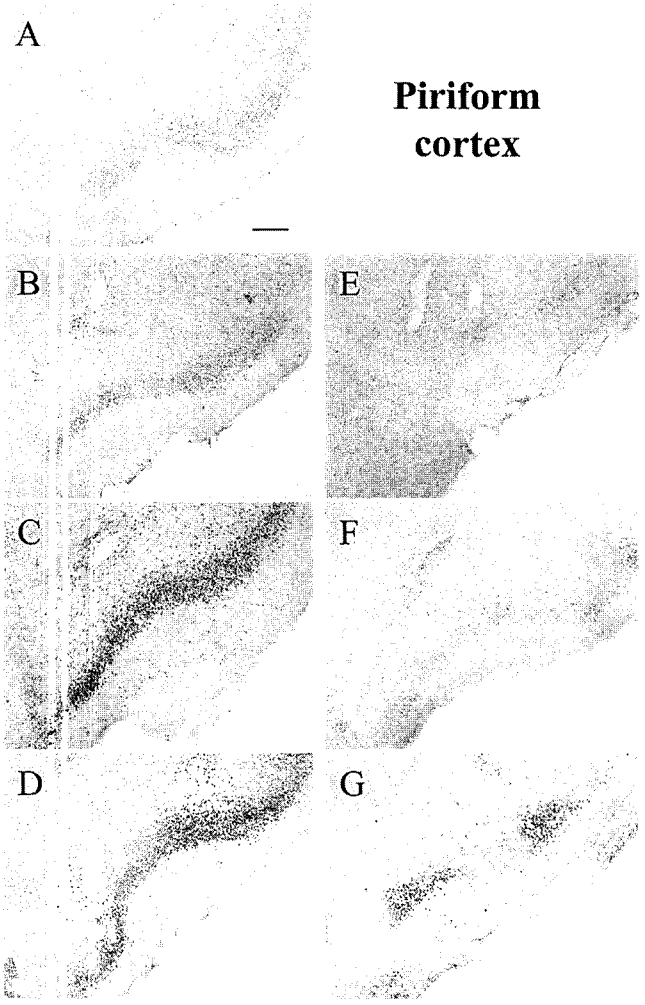

Fig. 4. pCREB immunoreactivity following apomorphine treatment in the piriform cortex of mouse brain. A, 0min; B, 10min; C, 30min; D, 1h; E, 2h; F, 7h; G, 24h after apomorphine treatment. Scale bar, 200μm.

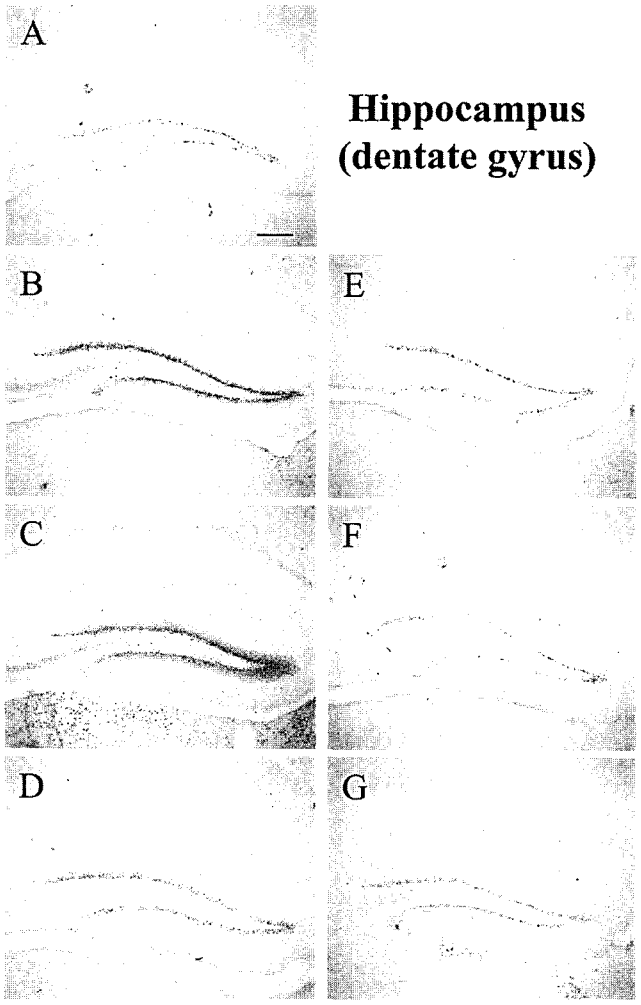

Fig. 5. pCREB immunoreactivity following apomorphine treatment in the dentate gyrus of hippocampus of mouse brain. A, 0min; B, 10min; C, 30min; D, 1h; E, 2h; F, 7h; G, 24h after apomorphine treatment. Scale bar, 200μm.

behavior through the activation of the dopamine receptors in mice.

Pioneering experiments first identified two dopamine receptor subtypes, a D1 receptor, which is coupled to G_s/G_{olf} and increases cAMP production, and a D2 receptor, which is coupled to G_i/G_o and has a negative influence on cAMP (Kebabian and Calne, 1979). Increased cAMP levels can activate protein kinase A, which phosphorylates the transcription factor CREB. D1 stimulation of the striatal cells also appears to cause changes in CREB phosphorylation and *c-fos* expression by increasing the intracellular calcium level, entering through either the voltage-sensitive calcium channels or the NMDA receptors (Konradi and Heckers, 1995; Keefe and Gerfen, 1996). It is believed that apomorphine treatment increases the cAMP levels by stimulating the dopamine receptors, and the protein kinase A, which is activated by cAMP, phosphorylates the CREB. In these experiments, the pCREB immunoreactivity was also increased in the nucleus accumbens, piriform cortex, and the dentate gyrus of the hippocampus 10min, 30min, and 1 h after apomorphine treatment. These results show that the increase in pCREB expression occurs in the striatum as well as the other brain regions by apomorphine treatment, unlike the 6-OHDA-lesioned model. Therefore, the fact that pCREB expression is increased in the diverse brain regions may imply that the projections of the dopaminergic neurons from the ventral mecephalon are segregated into the mesostriatal, mesolimbic, and mesocortical systems (Deutch *et al.*, 1988; Fallon and Moore, 1978). These results suggest that the increase in pCREB expression in the other brain regions may contribute to the development of the apomorphine-induced climbing behaviors. Another explanation is that

dopamine receptor stimulation may result in the activation of other receptors, such as the NMDA receptor, which increases the intracellular calcium level. This hypothesis is supported by the reports that NMDA receptor antagonists prevent apomorphine-induced cage climbing behavior (Kim *et al.*, 1996; Jang and Lee, 2001) and the activation of *c-fos* in response to a direct-acting dopamine agonists (Paul *et al.*, 1992).

Overall, this study suggests that pCREB expression increases in the mouse brain region treated with apomorphine, and this increase may be closely related to the climbing behavior associated with apomorphine by activating the dopamine receptors in mice.

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