CHAPTER 5

In vivo CREB phosphorylation mediated by dopamine and NMDA receptor activation in mouse hippocampus and caudate nucleus

Brain Research Gene Expression Patterns (2002) 1: 101-106

ABSTRACT

The pattern of CREB phosphorylation was investigated in the caudate nucleus and hippocampus 10 min or 3 h after i.p. injection of dopamine or NMDA receptor agonists alone, or in combination with antagonists. Ten minutes after C57BL/6J mice were injected with either the dopamine D1 receptor agonist SKF-38393 hydrobromide or NMDA, immunoreactivity of phosphorylated CREB (pCREB) was significantly increased in all parts of the caudate nucleus but not in hippocampal regions. However, 3 h after the injection of SKF-38393, pCREB levels in the caudate nucleus did not differ significantly from the pCREB levels in control animals, whereas pCREB levels were still elevated 3 h after NMDA injection. Except for the D1 receptor antagonist SCH-23390, which induced CREB phosphorylation in the caudate nucleus, dopamine and NMDA receptor antagonists had little effect on pCREB levels by themselves. However, the NMDA receptor antagonist CGS-19755 injected i.p. blocked both the NMDA- and SKF-38393-induced rise of pCREB levels in the caudate nucleus. Similarly, the D1 receptor antagonist SCH-23390 inhibited the effects produced by SKF-38393 or NMDA. Interestingly, the D2 receptor antagonist sulpiride also blocked the SKF-38393-triggered rise of pCREB. The results demonstrated that NMDA and dopamine receptors modulate pCREB levels in the caudate nucleus and suggest mutual permissive roles for both receptors.
In earlier experiments, using FOS as a marker, we demonstrated the interdependent relationship of dopamine and NMDA signaling in a brain region-specific manner (Radulovic et al., 2000). The promoter region of c-fos contains a binding site for the cAMP response element-binding protein (CREB). CREB phosphorylation (pCREB) was shown to mediate c-fos expression in response to agents that increase intracellular concentrations of cAMP or Ca2+ (Ginty et al., 1992; Sheng et al., 1991). Here, we investigated the regional distribution of pCREB immunoreactivity after activation of NMDA and dopamine receptors in intact mouse brains to evaluate the possible regional correlation with FOS production. CREB phosphorylation was detected using a specific antibody raised against the serine-133-phosphorylated form of CREB (pCREB) (Ginty et al., 1993).

Unlike FOS production, phosphorylation of CREB was detectable in low levels in most neurons throughout the brain of untreated animals (data not shown). We have previously demonstrated (Radulovic et al., 2000) that FOS production in the hippocampus was highly responsive to the activation of NMDA and dopamine receptors. Surprisingly, i.p. injection of NMDA or the dopamine D1 receptor agonist SKF-38393 did not exhibit any significant effect on the number of pCREB-positive cells in the hippocampus 10 min or 3 h after injection when compared with vehicle-injected animals (Fig. 1).

![Hippocampus](image)

**Figure 1.** Effect of dopamine or NMDA receptor agonists and antagonists on pCREB production in hippocampus. pCREB production in the hippocampus 10 min or 3 h after systemic injection of vehicle or SKF-38393, SCH-23390, sulpiride, NMDA or CGS-19755 as measured by optical density. Bars represent mean ± SEM. Number of mice per group was 6-13.
Previous work has identified two control regions in the c-fos promoter, the cyclic AMP response element (CRE) and the serum response element (SRE), as calcium-responsive promoter/enhancer elements (Sheng et al., 1988). On the basis of our data it might be speculated, that in the hippocampus NMDA and SKF-38393 enhanced FOS production mainly via SRE in view of the observation that neither NMDA nor SKF-38393 induced phosphorylation on serine-133 of CREB which interacts in its phosphorylated form with CRE (Sheng et al., 1991). The D1 receptor antagonist SCH-23390, the NMDA receptor antagonist CGS-19755 and the D2 receptor antagonist sulpiride injected alone did not affect CREB phosphorylation in the hippocampus 10 min after injection (Fig. 1).

In contrast, the number of pCREB-positive cells in the caudate nucleus was strongly increased 10 min (p < 0.001) and 3 h (p < 0.05) after injection of NMDA. SKF-38393 also caused a significant enhancement of CREB phosphorylation 10 min after the injection (p < 0.001), whereas 3 h after injection, no significant difference in pCREB-positive cells between SKF-38393- and vehicle-injected animals was found (Fig. 2,3). Injection of the antagonist SCH-23390 alone significantly enhanced the number of pCREB-positive cells in the caudate nucleus 10 min after injection (p < 0.05). CGS-19755 did not significantly affect CREB phosphorylation (Fig. 2). After sulpiride treatment, the level of pCREB positive cells was not significantly increased (p = 0.08).

**Figure 2.** Effect of dopamine or NMDA receptor agonists and antagonists on pCREB production in the caudate nucleus. pCREB production in the caudate nucleus 10 min or 3 h after systemic injection of vehicle or SKF-38393, SCH-23390, sulpiride, NMDA or CGS-19755 as measured by the number of pCREB-positive nuclei. The number of nuclei was normalized to the average of the vehicle groups. Bars represent mean ± SEM Number of mice per group was 6-13. Statistically significant differences: *, p < 0.05 vs. vehicle.
The NMDA receptor antagonist CGS-19755 prevented the NMDA- and SKF-38393-triggered rise in CREB phosphorylation (Fig. 3). This result suggests that the D1 receptor signaling pathway needs Ca2+ influx through NMDA channels to increase CREB phosphorylation in the caudate nucleus. Activation of D1 receptors in the striatum has been demonstrated to increase phosphorylation of NMDA receptors by a chain of events including cAMP-dependent phosphorylation of the dopamine and cAMP-regulated phosphoprotein (DARPP-32) which, by inhibiting protein phosphatase-1, is believed to inhibit dephosphorylation of NMDA receptors, thus enhancing NMDA receptor-mediated currents (Blank et al., 1997; Snyder et al., 1998). The subsequent increase in Ca2+ influx after D1 receptor activation is inhibited by the NMDA receptor antagonist preventing an SKF-38393-mediated rise of pCREB levels. The D1 receptor antagonist SCH-23390 blocked the increased pCREB levels after SKF-38393 and NMDA injection (Fig. 3). Sulpiride, the D2 receptor antagonist, was also able to prevent the SKF-38393-induced increase of pCREB levels (Fig. 3). This finding was unexpected, since sulpiride and SKF-38393 given alone induced CREB phosphorylation in the caudate nucleus. Similarly, the enhancement of CREB phosphorylation by SCH-23390 and SKF-38393 in the caudate nucleus canceled each other out when both compounds were applied together. Activation of D1 receptors may increase the number of pCREB positive cells via stimulation of adenylyl cyclase in view of the finding that D1 receptors are positively coupled to adenylyl cyclase in striatal neurons (Arnauld et al., 1998). This stimulation of adenylyl cyclase can be blocked by the selective D1 receptor antagonist SCH-23390. On the other hand, blockade of D1 receptors by SCH-23390 alone might shift the balance of D1/D2 receptor activation by endogenous dopamine towards activation of D2 receptors. Activation of D2 receptors has been shown to enhance CREB phosphorylation by activation of protein kinase C and Ca2+/calmodulin-dependent protein kinase requiring DARPP-32 (Yan et al., 1999). In the presence of endogenous dopamine, the blockade of D2 receptors results in an increased activity of adenylyl cyclase (Jaber et al., 1996; Vallone et al., 2000) which might be responsible for the increase of CREB phosphorylation after sulpiride treatment. The observation that blockade of D2 receptors prevented SKF-38393-induced increase of pCREB levels suggests cooperative mechanisms of both receptors to modulate CREB phosphorylation. This view is supported by data of Jung and Schmauss (Jung and Schmauss, 1999) describing that pretreatment of mice with the D2 receptor antagonist eticlopride reduced the magnitude of FOS responses to D1 agonist stimulation in striatum. Thus, maximum FOS or pCREB responses after D1 receptor stimulation may require a steady-state activity of D2 and possibly D3 receptors, which can also be blocked by sulpiride (Levant, 1997).

In combination with our previous findings (Radulovic et al., 2000), the present results show a clear brain region-specific interaction of NMDA and the dopamine signaling system on the level of immediate early genes. Several studies have reported an interaction of both transmission systems in learning and memory (Adriani et al., 1998), especially in the encoding of spatial information. Since CREB has been associated with various forms of memory formation (Frank and Greenberg, 1994), it may represent a molecular target involved in the modulation of learning and memory processes by glutamatergic and dopaminergic neurotransmission.
Figure 3. Representative photomicrographs of the caudate nucleus of agonist and antagonist-injected animals. D1, D2 and NMDA receptor antagonists block the SKF-38393- or NMDA-induced production of pCREB in caudate nucleus. Animals received either agonist alone or antagonist 30 min before agonist injection. Scale bar = 100 µm.
METHODS AND PROTOCOLS

All animal procedures are in strict accordance with the guidelines of the animal experimentation committee of the State Government of Lower Saxony.

Subjects
Eight-week-old, male, inbred C57BL/6J mice ± 25 g were obtained from Elevage Janvier (Le Genest St Isle, France). Animals were housed individually in macrolon cages placed in an environmentally controlled room (22 ± 1°C, 55 ± 10% humidity) maintained on a 12 h light/dark cycle (light on between 7 am and 7 pm). Water and standard pelleted diet were available ad libitum. Mice underwent treatment at least one week after adaptation to the housing conditions.

Treatment
Treatment was performed essentially as described (Radulovic et al., 2000). All drugs were injected i.p. SKF-38393 (7.5 mg/kg) was injected as D1 receptor agonist and NMDA (30 mg/kg) was used as NMDA receptor agonist. Antagonist studies were performed with D1 receptor antagonist SCH-23390 (1 mg/kg), D2 receptor antagonist sulphiride (50 mg/kg) and NMDA receptor antagonist CGS-19755 (10 mg/kg). When both agonist and antagonist were injected i.p., the antagonist was injected 30 min before i.p. injection of the agonist. All substances were dissolved in phosphate-buffered saline (PBS) to their final concentration. When necessary, the pH of the injected solution was adjusted to 7.4 with NaOH or HCl. Each mouse received 100 µl of drug solution. Control animals were injected with 100 µl of the appropriate vehicle. Experiments were always performed at the same time of the day.

Immunohistochemistry
At 10 min or 3 h after the final treatment animals were deeply anaesthetized with 0.02 ml/g b/w. avertin (tribromethanol 1 g; amylalcohol 0.81 g; millipore 71.49 g; pH 7.4). Then, they were transcardially perfused with ice cold 0.1 M phosphate buffer (PB; pH 7.4) followed by 4% paraformaldehyde solution (in 0.1 M PB, pH 7.4). Brains were postfixed and subsequently dehydrated in 10%, 20% and 30% sucrose in 0.01 M phosphate buffered saline. Immunohistochemical staining of free-floating coronal sections (50 µm) was performed as described previously (Radulovic et al., 2000) and summarized here. After elimination of endogenous peroxidase activity and a preincubation step, sections were incubated for 48 h with rabbit anti-pCREB antibody (1:3000 dilution; New England Biolabs). Subsequently, sections were incubated with biotinylated goat anti-rabbit antibody (1:200; Vector ABC kit) and with the ABC complex (Vector ABC kit) before being reacted with diaminobenzidine (DAB). Sections were mounted on gelatin-coated slides, air-dried, dehydrated, coverslipped with Eukitt, and examined using light microscopy.

Quantification and Analysis
The number of pCREB positive cells was counted in mouse hippocampus and caudate nucleus with a Macintosh-based image analysis system (NIH Image), as was described previously (Radulovic et al., 1998). Counting was performed at a magnification X10. The anteroposterior (AP) coordinates relative to bregma of the areas (Franklin and Paxinos, 1997) included for detailed analysis were: AP +0.98 caudate nucleus and AP –1.34 hippocampus. In the caudate nucleus individual nuclei were counted and expressed as number of pCREB positive nuclei per 0.1 mm². The density of stained cells in the hippocampus made it impossible to measure individual cells. Therefore, computerized microdensity was used to determine the intensity of staining. The data reflect optical density of the pyramidal cell layer, determined after subtraction of the mean gray value found for the stratum oriens. Statistical analysis of immunohistochemical data was performed using ANOVA and the Bonferroni-Dunn test for posthoc comparisons.
ACKNOWLEDGMENTS

We would like to thank Dr. Jelena Radulovic for helpful discussions and Birgit Grafelmann and Eleanor Chen who worked as students in our laboratory.

REFERENCES


