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NEURO
PHARMACOLOGY

Neuropharmacology 44 (2003) 1089–1099

www.elsevier.com/locate/neuropharm

Regulation of contextual fear conditioning by baseline and inducible septo-hippocampal cyclin-dependent kinase 5

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Received 30 July 2002; received in revised form 24 February 2003; accepted 24 February 2003

Abstract

In this work, we confirm the novel role of cyclin-dependent kinase (Cdk) 5 in associative learning by demonstrating that injection of the Cdk5 inhibitor butyrolactone I into the lateral septum or hippocampus profoundly impaired context-dependent fear conditioning of C57BL/6J mice. However, unlike the inducible up-regulation of Cdk5 and its regulator p35 observed in Balb/c mice, high baseline levels, which were not affected by fear conditioning, were found in C57BL/6J mice. Surprisingly, microinjections of butyrolactone I into the lateral septum or hippocampus significantly decreased baseline Cdk5 activity within the entire septo-hippocampal circuitry, suggesting a functional link between septal and hippocampal Cdk5 activity. Significantly higher levels of the transcription factor Sp4 in the septo-hippocampal system of C57BL/6J mice may account for the high baseline Cdk5/p35 production. On the other hand, the stronger cFos production observed in the lateral septum of fear conditioned Balb/c mice may be responsible, at least in part, for the inducible up-regulation of Cdk5 in this strain. These results suggest that the role of Cdk5 in memory consolidation is strain independent and functionally related to the septo-hippocampal circuitry. However, the molecular regulation of baseline and inducible Cdk5 protein might be different among individual mouse strains and possibly other species. © 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Cyclin-dependent kinase 5; Fear conditioning; Septum; Hippocampus; Sp4; cFos

1. Introduction

Cyclin-dependent kinase (Cdk) 5, a serine/threonine kinase, is a member of the Cdk family of protein kinases. However, unlike other Cdks, Cdk5 is activated by two non-cyclin partners, p35 (Tsai et al., 1994) and p39 (Tang et al., 1995), and seems to function prominently in non-cycling cells. Therefore, Cdk5 activity is mainly restricted to post-mitotic neuronal precursors and mature neurons (Ko et al., 2001; Harada et al., 2001; Dhavan and Tsai, 2001). So far, the physiologic activity of Cdk5 in the brain has been primarily linked to processes involved in brain development (Smith and Tsai, 2002), such as cytoskeletal phosphorylation leading to neuronal

migration (Ohshima et al., 1996; Chae et al., 1997), axon growth (Nikolic et al., 1998), and possibly neurosecretion (Matsubara et al., 1996; Fletcher et al., 1999). Deregulated Cdk5 hyperactivity is thought to be involved in neurodegenerative disorders such as Alzheimer's disease (Patrick et al., 1999) and amyotrophic lateral sclerosis (Patzke and Tsai, 2002). However, the physiological functions of Cdk5 in the adult brain were mainly unknown.

Recent evidence links Cdk5 activity to the regulation of membrane transport (Paglini et al., 2001) and dopamine signaling (Bibb et al., 2001). Moreover, it was demonstrated that phosphorylation of *N*-methyl-D-aspartate receptor subunits by Cdk5 modulates long-term potentiation in hippocampal slices of rats (Li et al., 2001), whereas phosphorylation of P/Q-type voltage-dependent calcium channels by Cdk5 (Tomizawa et al., 2002) may be involved in neurotransmitter release (Tomizawa et al., 2002; Yan et al., 2002). We have recently demonstrated

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that microinjections of the Cdk5 inhibitor butyrolactone I into the septum or hippocampus of Balb/c mice profoundly impaired associative learning as well as its stress-induced facilitation, thereby confirming the growing evidence for a role of Cdk5 in neuronal plasticity (Fischer et al., 2002).

The aim of this work was to further elucidate the novel role of Cdk5 in memory consolidation in the more commonly studied C57BL/6J mice, a strain known to differ from Balb/c mice in several learning paradigms (Francis et al., 1995; Bao et al., 1998; Heyser et al., 1999) including fear conditioning (Chen et al., 1996).

2. Methods

2.1. Animals

Eight-week-old male Balb/c mice (Charles River) and C57BL/6J mice (Centre d'Élevage Janvier, Le Genest-St-Isle, France) were individually housed as described (Radulovic et al., 1999). Experiments were performed in accordance with the European Council Directive (86/609/EEC). The number of mice per experimental group was 3–5 for protein production studies and kinase assays and 8–9 for behavioral experiments.

2.2. Cannulation and injections

Cannulation into the lateral septum (i.s., AP +1 mm, lateral 0.5 mm, depth 3 mm), dorsal hippocampus (i.h., AP –1.5 mm, lateral 1 mm, depth 2 mm) and lateral brain ventricles (i.c.v., AP –0 mm, lateral 1 mm, depth 2 mm) was performed as described (Radulovic et al., 1999). The Cdk5 inhibitor butyrolactone I (50 ng in 0.2% DMSO in artificial cerebrospinal fluid, (aCSF)) or aCSF employed as a vehicle was injected (0.25 µl/side) 15 min before training, memory test or both. In indicated experiments, butyrolactone I was injected immediately after the training. The Cdk5 inhibitor roscovitine (50 ng in 0.2% DMSO/aCSF) was injected i.c.v. 15 min before the training. Vehicle was 0.2% DMSO in aCSF. Only data obtained from mice with correctly inserted cannulae, as verified after methylene blue injection, were included in statistical analysis.

2.3. Context-dependent fear conditioning

Training consisted of a 3-min exposure of the mice to the conditioning box (context) followed by a footshock (2 s, 0.7 mA, constant current) (Milanovic et al., 1998) using a TSE (Bad Homburg, Germany) fear conditioning system. The memory test was performed 24 h later by re-exposing the mice for 3 min to the conditioning context. Freezing, defined as a lack of movement besides heart rate and respiration associated with a crouching posture,

was recorded in 1 s blocks every 10 s by two trained observers (one was unaware of the experimental conditions) during 3 min (a total of 18 sampling intervals). The number of observations obtained as a mean from both observers indicated freezing and was expressed as a percentage of the total number of observations. Control groups of mice were exposed to the context alone (3 min) or immediate footshock (2 s, 0.7 mA, constant current) followed by context (3 min) during the training. The mean activity, activity burst to the shock and percentage of explored area in the context were automatically recorded by an infrared beam system and analyzed by a software developed in collaboration with TSE.

2.4. Tone-dependent fear conditioning

Training consisted of a 3-min exposure of the mice to the conditioning box (context) followed by a tone (30 s, 10 kHz, 75 dB SPL) and a footshock (2 s, 0.7 mA, constant current) (Radulovic et al., 1999). The memory test was performed 24 h later by exposing the mice for 3 min to a novel context followed by an additional 3-min exposure to a tone (10 kHz, 75 dB SPL) within the novel context. Freezing was recorded every 10 s by two non-biased observers as described above. A control group of mice was exposed to an unpaired tone and footshock presentation to prove the specificity of tone-dependent freezing. This group was exposed to the context (3 min) followed by shock (2 s) and tone (30 s). The memory test was performed 24 h later by exposing the mice for 3 min to the conditioning context followed by a 3-min exposure to the tone in the novel context.

2.5. Chemicals and antibodies

Butyrolactone I was purchased from Biomol, antibodies for Cdk5 (J-3), p35 (C-19), Sp4 (V-20), Elk-1 (I-20) and pElk-1 (B-4) from Santa Cruz Biotechnology, Sp1 (rabbit polyclonal IgG) and Sp3 (rabbit polyclonal IgG) from Upstate Biotechnologies, cFos from Oncogene Science (rabbit polyclonal) and pErk-1/2 (mouse monoclonal IgG) from Sigma. The dilutions of antibodies used for immunohistochemical and immunoblot analysis were 1:1000 for Cdk5, p35, pElk-1 and Elk-1, 1:2000 for Sp3, 1:4000 for Sp1 and Sp4 and 1:12,000 for cFos. Elk-1 fusion protein (Elk-1 residues 307–428 fused with glutathione S-transferase (GST), 41 kDa) was purchased from Cell Signaling.

2.6. Immunohistochemical analysis

Immunohistochemical analysis was performed and quantified as described (Radulovic et al., 1998a; Kishimoto et al., 2000). Briefly, the total area of the septum of each mouse was outlined at the anatomical coordinates

+0.4 to +0.6 mm anterior to the bregma (Franklin and Paxinos, 1997). For the hippocampus, the coordinates –1.82 to –2.06 mm posterior to the bregma were used. The same threshold was applied for each section and the density of staining was determined automatically with a Macintosh-based imaging system (NIH image). The mean gray values obtained at the level of the corpus callosum, indicating background staining, were subtracted from the total density. Cells positive for cFos were counted as described previously (Pomonis et al., 1997) using the NIH-image analysis system. Nuclei were counted individually and expressed as number of FOS-positive nuclei per 0.1 mm².

2.7. Protein extraction and immunoblot

The septum and hippocampus were collected and lysed in RIPA buffer (Nikolic et al., 1998). The lysates were incubated for 15 min on ice and centrifuged for 15 min, 15,000 × g, 4 °C. The supernatant was collected as cytosolic protein extract. The lysates were subjected to 12.5% SDS-PAGE followed by immunoblotting as described (Radulovic et al., 1998a).

2.8. Immunoprecipitation and kinase assay

For immunoprecipitation, 0.5 µg of total protein was incubated for 1 h at 4 °C with 2 µg anti-cdk5 or pErk antibody, followed by a 30-min incubation on ice with magnetically labeled ProteinG Microbeads. Washing and elution were performed as described in the MAGmol Microbeads user's manual (Milteny Biotec).

For Cdk5 kinase assay, the complexes were not eluted but instead incubated twice with 25 µl reaction buffer, containing kinase buffer (Sharma et al., 1999), 5 µg histone H1 and 5 µCi [γ-p32]-ATP at 30 °C for 20 min, respectively. The flow containing 50 µl phosphorylated substrate solution was analyzed by 12.5% SDS-PAGE followed by autoradiography. For quantification, protein bands corresponding to histone H1 were densitometrically analyzed by using an IBM-based imaging system (WinCam 2.2, Cybertech). To determine Cdk5 kinase activity in vivo, septal and hippocampal lysates were obtained from the mice injected with vehicle or butyrolactone I 15 min before the training and sacrificed 30 min later. The kinase assay was performed as described above, except that total lysates were incubated with histone H1. For pErk-1/2 kinase assay, the dp-Erk-1/2-immunoprecipitated complexes were not eluted from the µMacs column but instead incubated twice with kinase buffer (Sharma et al., 1999), containing 5 µg of Elk-1 fusion protein and 1 µM ATP at 30 °C for 20 min. The eluate containing 50 µl phosphorylated substrate solution was subsequently boiled for 5 min SDS loading buffer containing 0.1% bromophenol blue and analyzed by 12.5% SDS-PAGE followed by immunoblot.

2.9. Statistical analysis

Statistical analysis was performed by unpaired Student's *t*-test or one-way ANOVA followed by Scheffe's test for post-hoc comparison where appropriate. The results are presented as mean ± S.E.

3. Results

3.1. Cdk5 is required for contextual fear conditioning in C57BL/6J mice

To determine whether inhibition of Cdk5 affected associative learning, C57BL/6J mice were injected with the selective Cdk5 inhibitors butyrolactone I (50 ng/mouse) or roscovitine (50 ng/mouse; Bibb et al., 1999) into the lateral brain ventricles (i.c.v.) 15 min before the training. Both inhibitors significantly reduced contextual freezing during the memory test performed 24 h later (Fig. 1A) when compared to the vehicle-injected control group ($F(2,25) = 19.56$, $P < 0.01$) without affecting conditioned freezing to the tone ($F(2,25) = 0.293$, $P = 0.7493$). It was subsequently investigated whether local inhibition of Cdk5 within the lateral septum or dorsal hippocampus also affected fear conditioning. For each i.s. and i.h. injection, four experimental groups were set up in order to control for possible state-dependence of the effects of butyrolactone I on fear conditioning. The injections vehicle/vehicle, butyrolactone I/vehicle, vehicle/butyrolactone I and butyrolactone I/butyrolactone I were performed 15 min before the training and memory test. The mice injected i.s. or i.h. with butyrolactone I/vehicle or butyrolactone I/butyrolactone I froze significantly less (septum, $F(3,26) = 11.65$, $P < 0.01$; hippocampus, $F(3,26) = 10.37$, $P < 0.01$) than vehicle/vehicle and vehicle/butyrolactone I groups (Fig. 1B). The results indicated that the effect of butyrolactone I was not state-dependent and that the inhibitor affected memory consolidation but not memory retrieval.

It is important to note that pre-training injections of butyrolactone I into the lateral septum did not affect locomotor activity (mean ± S.E.M.: butyrolactone I, 4.479 ± 0.423 , vehicle, 3.993 ± 0.236 ; $t(1,17) = 0.972$, $P = 0.3449$) and exploration (mean % of explored area ± S.E.M.: butyrolactone I, 57.236 ± 3.869 , vehicle, 58.876 ± 2.881 ; $t(1,17) = -0.333$, $P = 0.7429$) in response to the context or the activity burst in response to the shock (mean ± S.E.M.: butyrolactone I, 43.122 ± 3.089 , vehicle, 38.621 ± 3.904 ; $t(1,17) = 0.913$, $P = 0.3739$). Similarly, i.h. application of the inhibitor did not alter any of these behavioral parameters (mean activity: butyrolactone I, 4.665 ± 0.363 , vehicle, 4.378 ± 0.335 ; $t(1,17) = -0.565$, $P = 0.5805$; percentage of explored area, butyrolactone I, 53.014 ± 3.528 , vehicle,

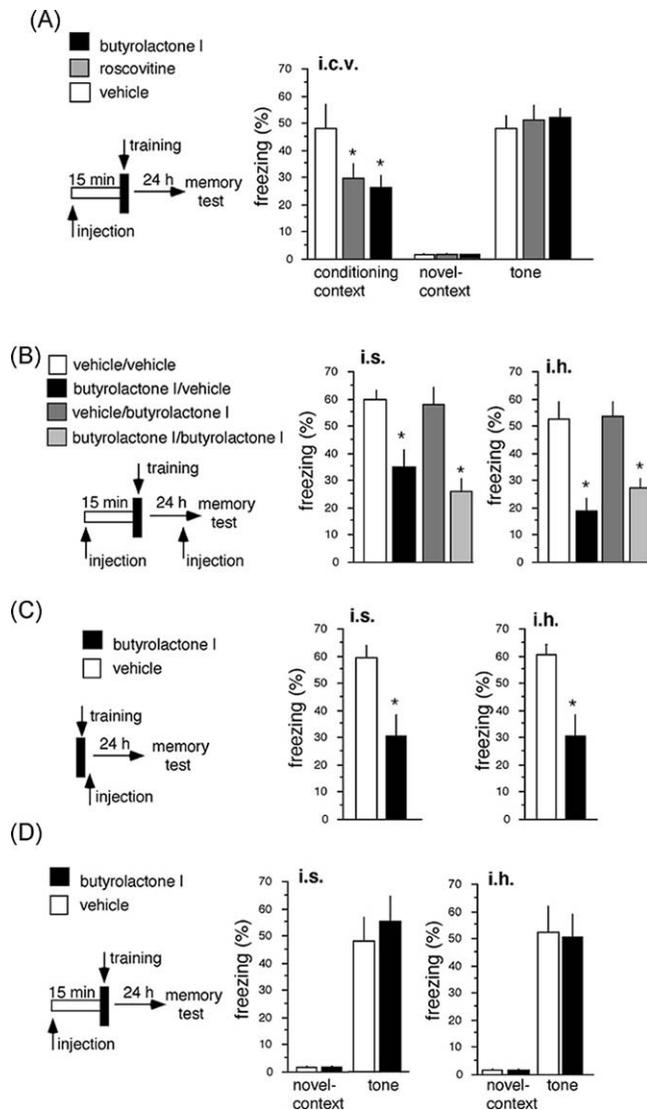


Fig. 1. Butyrolactone I prevents context-dependent fear conditioning. (A) Butyrolactone I (50 ng/mouse) or roscovitine (50 ng/mouse) was injected into the lateral brain ventricles (i.c.v.) 15 min before training. Freezing to the conditioning context, a novel context and tone was measured 24 h later. Both inhibitors significantly impaired freezing to the conditioning context when compared to the vehicle-injected control ($*P < 0.01$). Tone-dependent fear conditioning was not affected by either inhibitor. (B) Effect of butyrolactone I injected into the lateral septum (i.s.) or dorsal hippocampus (i.h.) 15 min before context-dependent fear conditioning or/and memory test on freezing behavior during the test. Statistically significant differences: $*P < 0.01$ vs. vehicle/vehicle and vehicle/butyrolactone I groups. (C) Effect of butyrolactone I injected i.s. or i.h. immediately after context-dependent fear conditioning on freezing behavior during the memory test. Statistically significant impairment was demonstrated after both i.s. and i.h. injections of butyrolactone I ($*P < 0.01$ vs. vehicle). (D) Effect of butyrolactone I injected i.s. or i.h. 15 min before tone-dependent fear conditioning on freezing behavior during the memory test. No significant differences were observed between groups. The number of mice was 8–9 for each group.

54.746 ± 3.150 ; $t(1,17) = -0.367$, $P = 0.7184$; activity burst to shock, butyrolactone I, 41.241 ± 3.799 , vehicle, 34.482 ± 3.637 ; $t(1,17) = -1.284$, $P = 0.2186$).

The conclusion that butyrolactone I selectively affected memory consolidation was supported by the findings demonstrating a significant impairment of fear conditioning by immediate post-training i.s. ($t(1,17) = 8.790$, $P < 0.01$) and i.h. ($t(1,15) = 7.476$, $P < 0.01$) injections of butyrolactone I when compared to vehicle-injected mice (Fig. 1C).

Tone-dependent fear conditioning was not affected by i.s. or i.h. injections of butyrolactone I (septum, $t(1,17) = 0.141$, $P = 0.7116$; hippocampus, $t(1,15) = 0.083$, $P = 0.777$), as determined by similar freezing of butyrolactone I- and vehicle-injected mice during the memory test to the tone presented in a novel context (Fig. 1D). On the basis of these results, it was concluded that septo-hippocampal Cdk5 selectively affected contextual fear conditioning of C57BL/6J mice.

It should be mentioned that under the conditions employed in the experiments, the freezing to tone reflected associative learning and not a non-specific freezing response. This was demonstrated by the significant impairment of freezing to the tone ($t(1,14) = 219.54$, $P < 0.001$) after an unpaired presentation of tone and shock, when compared to a paired presentation during the training (freezing (%) \pm S.E.M.: unpaired, 1.58 ± 1.52 ; paired 49.30 ± 2.66). In contrast, both groups exhibited similar freezing to the conditioning context (freezing (%) \pm S.E.M.: unpaired, 60.6 ± 3.07 ; paired 54.80 ± 3.98 ; $t(1,14) = 1.122$, $P = 0.308$).

3.2. Butyrolactone I prevents memory consolidation by decreasing baseline Cdk5 activity within the septo-hippocampal circuitry

It was demonstrated that Cdk5 protein levels transiently increase in response to fear conditioning in Balb/c mice (Fischer et al., 2002). To determine whether transient activation of Cdk5 plays a role in the acquisition of conditioned fear of C57BL/6J mice, the production of Cdk5 protein was measured in the septo-hippocampal system at different time points after context-dependent fear conditioning. Control groups consisted of naive mice, mice exposed to the context without footshock, and mice exposed to an immediate footshock followed by context. These training conditions did not affect the mean activity ($F(2,27) = 1.875$, $P = 0.56$), exploratory behavior ($F(2,27) = 1.12$, $P = 0.38$) or activity burst to the shock ($t(1,18) = 0.96$, $P = 0.28$) during the training. However, during the context-dependent memory test performed 24 h later, the mice exposed to context followed by shock exhibited significantly lower activity ($F(2,27) = 14.3$, $P < 0.01$) and stronger freezing behavior ($F(2,27) = 21.88$, $P < 0.001$) than the mice of the context and immediate shock groups (Fig. 2A). Thus,

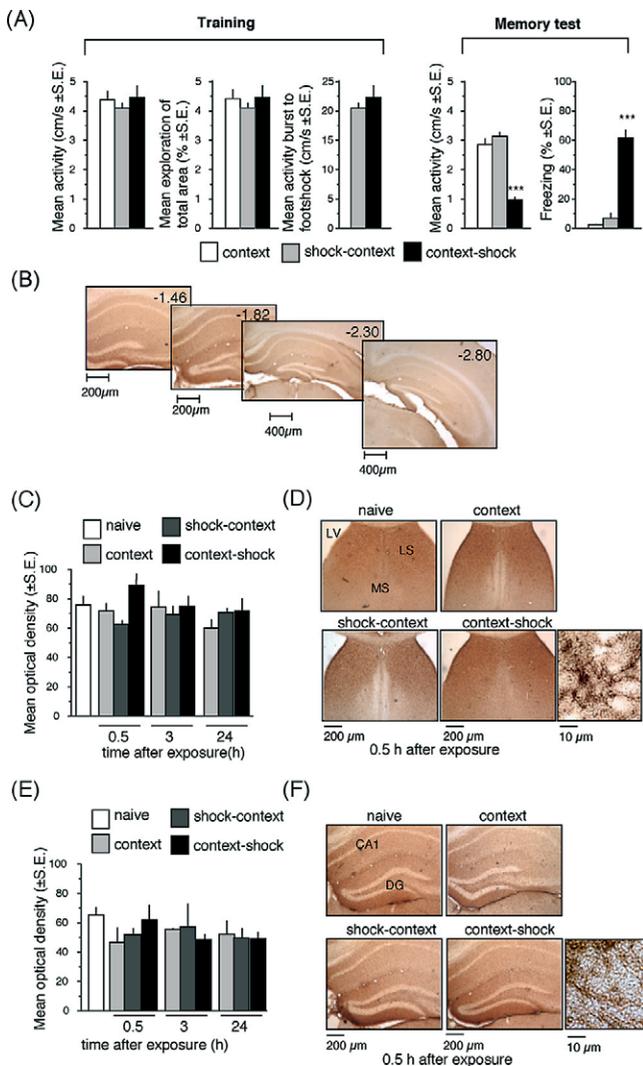


Fig. 2. Septo-hippocampal Cdk5 protein production after fear conditioning. (A) Mean activity, exploratory behavior, activity burst to footshock and freezing behavior during exposure of the mice to context, shock–context or context–shock and the subsequent memory test performed 24 h later. Statistically significant differences: *** $P < 0.001$ vs. context and immediate shock groups (B) Hippocampal Cdk5 production in mice exposed to context-dependent fear conditioning and sacrificed 0.5 h later. (C) Mean density of Cdk5 protein levels in the septum of the mice (four/group) exposed to fear conditioning or control paradigms at different time points after treatment. No statistically significant differences were observed. (D) Representative photomicrographs of the experiment described in (C) for the time point 0.5 h after treatment. Right: high magnification photomicrograph of Cdk5 protein production of the context–shock group. (E) Cdk5 protein levels in the hippocampus of the mice (four/group) exposed to fear conditioning or control paradigms at different time points after treatment. No statistically significant differences were observed. (F) Representative photomicrographs of the experiment described in (E). Right: high magnification photomicrograph of the DG area of the context–shock group. CA1, hippocampal subfield; DG, dentate gyrus; LSI, lateral intermediate septum; LV, lateral ventricle; MS, medial septum.

the latter training conditions did not result in associative learning (Atkins et al., 1998; Milanovic et al., 1998) and were therefore employed to delineate the impact of associative learning on Cdk5 production from the effects of non-associative learning and unconditioned stress responses to footshock. In view of the ability of dorso-hippocampal injection of butyrolactone I to impair context-dependent fear conditioning as well as the observation that high levels of Cdk5 were found in the dorsal but not ventral hippocampus (Fig. 2B), further immunohistochemical evaluations were performed in the dorsohippocampal area. Surprisingly, no difference of septal ($F(1,3) = 0.970$, $P = 0.498$) and hippocampal ($F(1,3) = 0.739$, $P = 0.730$) Cdk5 protein production was observed among the groups (Fig. 2C–F).

A possibility remained that the Cdk5 activity increased in response to associative learning, independent of changes in the protein level. However, Cdk5 immunoprecipitated from septo-hippocampal lysates did not display significant differences in histone H1 phosphorylation ($F(6,10) = 1.144$, $P = 0.4157$) in response to fear conditioning or control paradigms (Fig. 3A,B). The specific kinase activity in the samples was demonstrated by the ability of butyrolactone I to inhibit histone H1 phosphorylation of Cdk5 immunoprecipitates (Fig. 3C), without affecting Elk-1 phosphorylation by pErk-1/2 immunoprecipitates (Fig. 3D). Thus, it was shown that butyrolactone did not affect the activity of the Erk-1/2 mitogen-activated protein kinases.

A possibility remained that in C57BL/6J mice microinjection of butyrolactone I could impair fear conditioning by affecting baseline Cdk5 activity. To determine this possibility, the in vivo Cdk5 kinase activity after butyrolactone I injection was analyzed. Butyrolactone I injected into the hippocampus 15 min before training significantly reduced hippocampal Cdk5 activity 0.5 h after fear conditioning ($t(1,6) = 57.652$, $P < 0.05$).

Unexpectedly, i.h. injection (Fig. 4A,B) also significantly reduced the Cdk5 activity in the lateral septum ($t(1,6) = 284.939$, $P < 0.01$). Similar results were obtained when butyrolactone I was injected into the lateral septum (Fig. 4A,B), as demonstrated by a significant reduction of Cdk5 activity in both septum ($t(1,6) = 320.725$, $P < 0.01$) and hippocampus ($t(1,6) = 189.876$, $P < 0.01$). These results suggested that the effect of butyrolactone I on context-dependent fear conditioning in C57BL/6J mice (Fig. 1) could be mediated by inhibition of baseline Cdk5 activity within the septo-hippocampal circuitry. To exclude the possibility that butyrolactone I injected i.h. inhibited septal Cdk5 activity and vice versa due to diffusion, separate naive mice were i.s. ($n = 3$) or i.h. ($n = 3$) injected with methylene blue and decapitated 45 min later. This injection schedule was selected to mimic the injections of butyrolactone I (15 min before training) resulting in decreased Cdk5 activity (30 min after training). After i.h. or i.s.

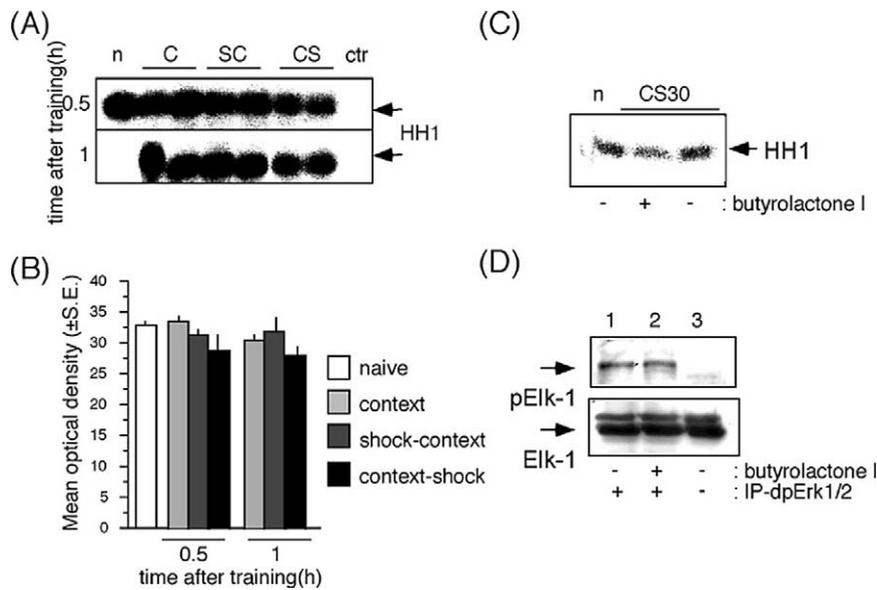


Fig. 3. Cdk5 activity within the septo-hippocampal circuitry is not affected by fear conditioning. (A) Representative results of histone H1 phosphorylation by Cdk5 immunoprecipitated 0.5 or 1 h after fear conditioning or control paradigms. Septo-hippocampal lysates were obtained from naive mice (n, three/group) or after exposure to context (C), shock–context (SC) and context–shock (CS), respectively. A control (ctr) sample was treated identically but incubated without lysate. (B) The results were repeated in two independent experiments and the signals were analyzed densitometrically. No significant differences were observed. (C) Representative results of histone H1 phosphorylation by Cdk5 immunoprecipitated hippocampal lysates of naive mice or 0.5 h after fear conditioning (three mice/group) in the presence or absence of butyrolactone I (10 μ M). The experiment was repeated three times. Butyrolactone I decreased histone H1 phosphorylation by 50%. (D) Representative results of Elk-1 phosphorylation by dpErk1/2 immunoprecipitated from mice 0.5 h after fear conditioning (three mice/group) without (lane 1) or in the presence of butyrolactone I (10 μ M, lane 2). As a control-experiment, lysates were incubated without antibody against dpErk1/2 (lane 3). No difference on Elk-1 phosphorylation was observed in the presence of butyrolactone I.

injection, methylene blue staining was only found in the injected area, but not within the entire septo-hippocampal system (Fig. 4C,D). Consistently, injections of methylene blue performed for each mouse after the behavioral experiments revealed that spread of methylene blue outside these areas (observed in a few mice with slightly displaced cannula and therefore excluded from behavioral analysis) correlated closely with reduced effects of butyrolactone I. On this basis, it was assumed that methylene blue may be used as a good estimate to verify the local amount of the inhibitor.

3.3. Baseline protein levels of Cdk5 and p35 are higher in C57BL/6J than in Balb/c mice

To investigate whether the production of Cdk5 and p35 differed between strains, baseline and fear conditioning-induced levels of these proteins were analyzed in C57BL/6J and Balb/c mice. Significantly higher septo-hippocampal protein levels of Cdk5 and p35 were observed in naive C57BL/6J mice (Fig. 5A–C) when compared to levels in naive Balb/c mice (Cdk5, $t(1,6) = -10.710$, $P < 0.001$; p35, $t(1,6) = -15.756$, $P < 0.001$).

Moreover, in comparison to naive animals, fear conditioning resulted in a significant increase of Cdk5 and p35 in the septo-hippocampal system of Balb/c mice

(Cdk5, $t(1,6) = 12.095$, $P < 0.001$; p35, $t(1,6) = 14.352$, $P < 0.001$). In contrast, the high baseline Cdk5 and p35 protein production of C57BL/6J mice (Fig. 5D,E) was not affected by fear conditioning (Cdk5, $t(1,6) = 0.190$, $P = 0.8559$; p35, $t(1,6) = -0.197$, $P = 0.85$). These findings demonstrated that Cdk5 and p35 proteins were inducible after fear conditioning of Balb/c but not C57BL/6J mice.

3.4. Differential expression of transcription factors may account for the strain-specific expression of Cdk5 and p35

Different levels of transcription factors might underlie the described strain-specific protein production of Cdk5 and p35. To investigate this possibility, the protein production of the transcription factors Sp4, Sp1, Sp3 and cFOS was analyzed immunohistochemically in the septo-hippocampal system of Balb/c and C57BL/6J mice. Statistical comparisons were performed by two-way ANOVA employing Strain (Balb/c and C57BL/6J) and Experimental Group (naive and fear conditioning) as factors.

The production of Sp4 depended significantly on Strain ($F(1,8) = 87.107$, $P < 0.001$) and Experimental Group ($F(1,8) = 5.136$, $P < 0.05$). Post-hoc analyses demonstrated significantly higher baseline levels of Sp4

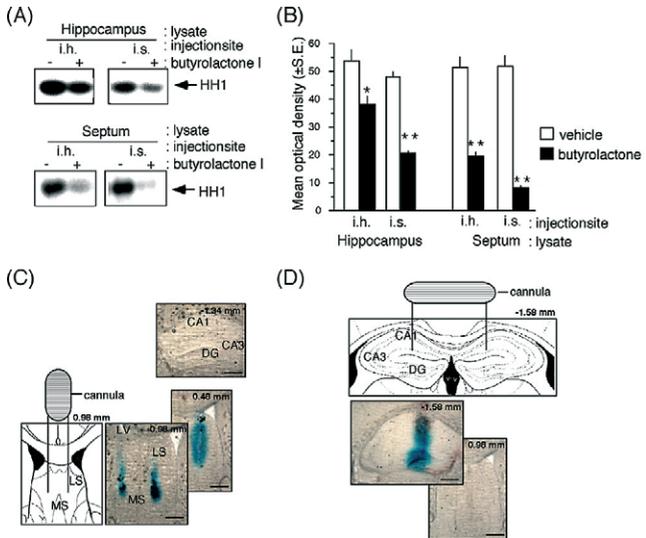


Fig. 4. Butyrolactone I decreases Cdk5 activity within the septo-hippocampal circuit during memory consolidation. (A) The mice (four/group) were injected i.s. or i.h. with butyrolactone I or vehicle 15 min prior to context-dependent fear conditioning and decapitated 0.5 h after the training. Hippocampal and septal lysates were prepared from each group and tested for Cdk5 activity. Both i.s. and i.h. injection of butyrolactone I dramatically decreased histone H1 phosphorylation within the hippocampus (upper panel). Similar results are obtained for the septum (lower panel). (B) The results described in (A) were repeated in two independent experiments and the signals were analyzed densitometrically. Statistically significant differences: $**P < 0.05$ vs. corresponding vehicle injection, $***P < 0.01$ vs. corresponding vehicle injection. (C) Representative photomicrograph of septal and hippocampal region. The mice (three/group) were injected i.s. with methylene blue diluted in vehicle and decapitated 45 min later. Placement of the cannula is indicated. Methylene blue staining was restricted to the lateral septum, as no diffusion to the hippocampus was observed. Scale bar, 400 μ m. Numbers indicate anatomical position from bregma. (D) Representative photomicrograph of septal and hippocampal region. The mice (three/group) were injected i.h. with methylene blue diluted in vehicle and decapitated 45 min later. Placement of the cannula is indicated. Methylene blue staining was restricted to the hippocampus, as no diffusion to the septum was observed. Scale bar, 400 μ m. Numbers indicate anatomical position from bregma. CA1, CA3, hippocampal subfields; DG, dentate gyrus; LS, lateral septum; LV, lateral ventricle; MS, medial septum.

($P < 0.001$) in all of the investigated areas of C57BL/6J when compared to Balb/c mice (Fig. 6A–C). After fear conditioning, Sp4 levels in the lateral septum of C57BL/6J mice decreased significantly when compared to their baseline levels ($P < 0.05$).

The levels of Sp3 (Fig. 6D) did not depend significantly on Strain ($F(1,8) = 0.128$, $P = 0.7218$) or Experimental Group ($F(1,8) = 1.298$, $P = 0.2602$) in the investigated brain regions. Similarly, the levels of Sp1 (Fig. 6E) did not depend significantly on the tested factors (Strain, $F(1,8) = 0.08$, $P = 0.9305$; Experimental Group, $F(1,8) = 2.403$, $P = 1.277$). The only significant difference obtained by post-hoc analysis indicated decreased Sp1 production in the dentate gyrus of C57BL/6J mice when compared to their naive controls ($P < 0.05$).

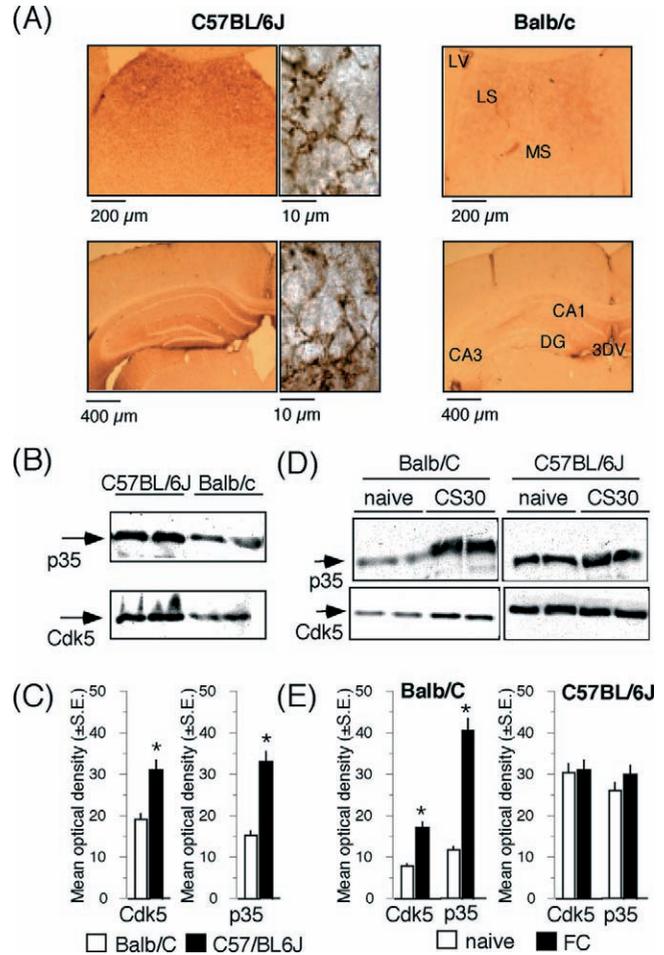


Fig. 5. Baseline protein levels of Cdk5 and p35 in C57BL/6J mice are similar to levels in Balb/c mice induced by fear conditioning. (A) Cdk5 protein levels in the septum and hippocampus of naive C57BL/6J (left panel, small picture shows high magnification of Cdk5 positive neurons within the LS and DG, respectively) are higher than in naive Balb/c mice (right panel). (B) Immunoblot analyses of Cdk5 and p35 protein in naive C57BL/6J and Balb/c demonstrated lower baseline protein levels for Balb/c mice. (C) The results shown under (B) were repeated in two independent experiments and analyzed densitometrically. Higher baseline protein levels of Cdk5 and p35 in C57BL/6J mice were confirmed ($*P < 0.001$ vs. Balb/c mice). (D) Septo-hippocampal lysates of both strains were obtained from naive mice and mice decapitated 0.5 h after context-dependent fear conditioning. The production of Cdk5 and p35 analyzed by immunoblot shows that protein levels are inducible in Balb/c mice, whereas protein levels are not increasing above baseline levels in C57BL/6J. (E) The results shown under (D) were repeated in two independent experiments and analyzed densitometrically. Whereas Cdk5 and p35 were found to increase significantly in response to fear conditioning in Balb/c mice ($*P < 0.001$ vs. naive), no difference was observed for C57BL/6J mice. CA1, hippocampal subfield; D3V, dorsal third ventricle; LSI, lateral intermediate septum; LV, lateral ventricle; MS, medial septum.

The production of cFOS protein in the septo-hippocampal system (Fig. 6F) was low to undetectable in naive animals, however, a significant increase was observed in both strains after fear conditioning ($F(1,8) = 25.47$, $P < 0.001$). This increase was significantly stronger in the lateral septum of Balb/c mice when com-

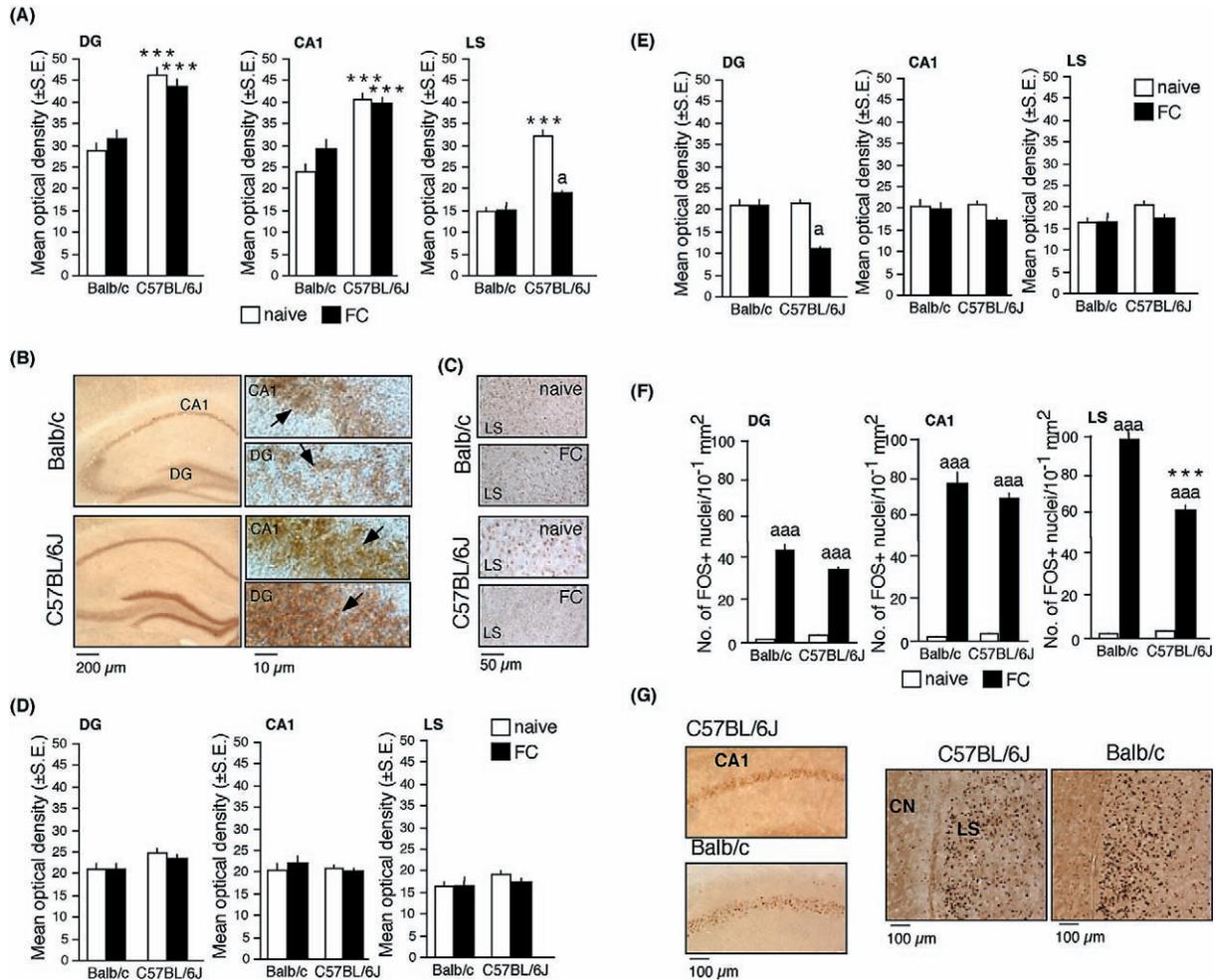


Fig. 6. Comparison of Sp1, Sp3, Sp4 and cFos protein production under baseline conditions and 0.5 h after fear conditioning of C57BL/6J and Balb/c mice. (A) Densitometric quantification of the immunostaining (five mice/group) for Sp4 showing significantly higher baseline protein levels in C57BL/6J mice within the hippocampus regions and the lateral septum. The level of Sp4 in the lateral septum was significantly lower in fear conditioned C57BL/6J mice than in their naive controls. (B) Representative photomicrographs of the hippocampus region quantified under (A), arrows indicate Sp4 positive cells. (C) Representative high magnification photomicrographs of the lateral septum region quantified under (A). (D) Densitometric quantification of the immunostaining (five mice/group) for Sp3 revealed no significant differences between strains or in response to fear conditioning. (E) Densitometric quantification of the immunostaining (five mice/group) for Sp1 showing no significant differences in baseline protein levels between strains. Fear conditioning significantly decreased Sp1 protein levels in the DG of C57BL/6J mice. (F) Quantification of the immunostaining for cFOS (five mice/group). In both strains, the mean number of cFOS positive nuclei increases significantly in response to fear conditioning. In the lateral septum, this response was significantly stronger for Balb/c mice. (G) Representative photomicrographs of the results described in (F) Statistically significant differences: $***P < 0.001$ vs. corresponding group of Balb/c mice, $^aP < 0.05$ vs. naive group of the same strain, $^{aaa}P < 0.001$ vs. naive group of the same strain. CA1, hippocampal subfield, DG, dentate gyrus; LS, lateral septum; CN, caudate nucleus; FC, fear conditioning.

pared to C57BL/6J mice, as revealed by a significant Strain difference ($F(1,8) = 22.35$, $P < 0.001$).

4. Discussion

By demonstrating that microinjection of butyrolactone I significantly decreased fear conditioning and Cdk5 activity in the septo-hippocampal circuitry, we showed that Cdk5 activity was required for context-dependent fear conditioning of C57BL/6J mice. Although a number of studies employing electrolytic or neurotoxic lesions

reported a selective involvement of the ventral but not dorsal hippocampus in context-dependent fear conditioning (Maren et al., 1997; Richmond et al., 1999), acute pharmacologic manipulations (Young et al., 1994; Barrientos et al., 2002) as well as reversible lesions (Sacchetti et al., 1999) demonstrated that the dorsal hippocampus contributed significantly to the acquisition of context-dependent fear. In line with these observations, single injection of butyrolactone I into the dorsal hippocampus, the predominant source of hippocampal Cdk5, impaired fear conditioning significantly.

This finding is in agreement with our previous studies

in Balb/c mice, demonstrating that associative learning and its facilitation by stress requires Cdk5 activity (Fischer et al., 2002). However, in contrast to Balb/c mice, fear conditioning had no effect on Cdk5/p35 protein levels and Cdk5 activity in C57BL/6J mice. The finding that butyrolactone I injected into the septum not only inhibited septal but also hippocampal Cdk5 activity and vice versa indicated that Cdk5 activity within septo-hippocampal circuitry was functionally linked. In this respect, it is interesting that lesions of the fornix, the massive fiber bundle connecting the hippocampus with the septum, that disrupt associative learning in rats (Taubenfeld et al., 1999) also prevent phosphorylation and activation of cAMP response element binding protein (Creb) and the induction of several transcription factors in the hippocampus (Taubenfeld et al., 2001). Moreover, it was previously demonstrated that septo-hippocampal Cdk5 is mainly localized in cholinergic neurons (Fischer et al., 2002), suggesting that Cdk5 activity within the septo-hippocampal circuitry may be modulated by cholinergic neurotransmission. Taken together, these findings suggest multiple functional links between septal and hippocampal Cdk5 activity and its phosphorylation substrates involved in memory consolidation.

The observation that the baseline levels of Cdk5 and p35 were significantly higher in C57BL/6J in comparison to Balb/c mice indicated that up-regulation was not necessary for the acquisition of conditioned fear in C57BL/6J mice. Thus, it may be speculated that increased baseline levels of Cdk5 and possibly other proteins involved in memory consolidation may in part account for the better learning ability of C57BL/6J mice in the context-dependent fear conditioning paradigm (Chen et al., 1996). This view is supported by recent studies suggesting that the quantity of mRNA transcribed to proteins in the brain may directly reflect differences in the quality of cognitive abilities (Enard et al., 2002).

The high baseline protein production of Cdk5 and p35 in C57BL/6J mice most likely resulted from specific transcriptional activity. Recently, it was demonstrated that the GC-rich element binding factors Sp1, Sp3 and Sp4 are essential for the expression of p35 in neurons (Ross et al., 2002). However, little is known about the distribution of members of the SP transcription factor family in the central nervous system. The 5'-region of the *Cdk5* mouse promoter is also GC-rich, and furthermore, contains an AP-1 binding site, two AP-2 binding sites and a cAMP-responsive element (Ishizuka et al., 1995). The presence of an AP-1 binding site is in agreement with studies demonstrating induced *Cdk5* levels after $\Delta FosB$ overexpression in transgenic mice (Bibb et al., 2001). The significantly higher septo-hippocampal baseline levels of Sp4 demonstrated in C57BL/6J mice may indicate that Sp4 is one of the transcription factors

responsible for the strong baseline Cdk5 and p35 levels. On the other hand, immediate early genes like *cFOS* (Radulovic et al., 1998b) that respond to a broad range of environmental stimuli may mediate the observed up-regulation of Cdk5 and p35 in response to fear conditioning and stress in Balb/c mice. Indeed, the up-regulation of the cFos protein in response to fear conditioning within the septum of Balb/c mice was significantly stronger compared to C57BL/6J mice. This result suggested that *cFOS* as well as other inducible immediate early genes may account for the observed inducible Cdk5 and p35 levels in Balb/c mice.

In conclusion, in addition to Balb/c mice (Fischer et al., 2002), in vivo Cdk5 activity within the septo-hippocampal circuitry was found to be crucial for memory consolidation of context-dependent fear conditioning of C57BL/6J mice, thereby indicating that the role of Cdk5 in memory consolidation is conserved among different mouse strains and possibly other species. It was shown that septal Cdk5 activity could influence the Cdk5 activity within the hippocampus and vice versa, suggesting a direct link between septal and hippocampal Cdk5 activity. Furthermore, the results indicated that different transcriptional mechanisms might account for the regulation of Cdk5 activity in Balb/c and C57BL/6J mice. The characterization of the roles of Sp4 and cFOS as possible up-stream activators of Cdk5 will be of great importance for the further understanding of the physiological roles of Cdk5 as well as its dysregulation in neurodegenerative disorders.

Acknowledgements

This research was supported by the Max Planck Society. We would like to thank A. Burgdorf for assistance with the preparation of the manuscript.

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