Increased Cdk5/p35 activity in the dentate gyrus mediates depressive-like behaviour in rats

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Abstract
Depression is one of the most pervasive and debilitating psychiatric diseases, and the molecular mechanisms underlying the pathophysiology of depression have not been elucidated. Cyclin-dependent kinase 5 (Cdk5) has been implicated in synaptic plasticity underlying learning, memory, and neuropsychiatric disorders. However, whether Cdk5 participates in the development of depressive diseases has not been examined. Using the chronic mild stress (CMS) procedure, we examined the effects of Cdk5/p35 activity in the hippocampus on depressive-like behaviour in rats. We found that CMS increased Cdk5 activity in the hippocampus, accompanied by translocation of neuronal-specific activator p35 from the cytosol to the membrane in the dentate gyrus (DG) subregion. Inhibition of Cdk5 in DG but not in the cornu ammonis 1 (CA1) or CA3 hippocampal subregions inhibited the development of depressive-like symptoms. Overexpression of p35 in DG blocked the antidepressant-like effect of venlafaxine in the CMS model. Moreover, the antidepressants venlafaxine and mirtazapine, but not the antipsychotic aripiprazole, reduced Cdk5 activity through the redistribution of p35 from the membrane to the cytosol in DG. Our results showed that the development of depressive-like behaviour is associated with increased Cdk5 activity in the hippocampus and that the Cdk5/p35 complex plays a key role in the regulation of depressive-like behaviour and antidepressant actions.

Key words: Butyrolactone, Cdk5, chronic mild stress, depression, p35.

Introduction
Depression, with a lifetime prevalence of 16.6%, is a serious psychiatric disorder that leads to a major burden of disease and disability in society (Murray & Lopez, 1997). The chronic mild stress (CMS) procedure has been thoroughly used in preclinical animal studies of the aetiology and pathophysiology of depression (Willner, 1997). Anhedonia induced by CMS has been hypothesized to be a core symptom of depressive disorder and was measured by reduced sucrose preference (Papp et al. 1991; Willner et al. 1987). Animals exposed to CMS also exhibit a variety of behavioural changes, including decreased activity and weight loss (Willner, 1997). Understanding the molecular mechanisms involved in the behavioural response to CMS may help develop therapeutic targets for psychiatric disorders.

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase with high activity in the brain (Ohshima et al. 1996; Tsai et al. 1994). Cdk5 is activated by neuronal-specific proteins p35, p25 (a C-terminal fragment of p35), and p39 through direct binding (Lew et al. 1994; Tsai et al. 1994). Under physiological conditions, Cdk5 is fundamental to the development of synaptogenesis and neurotransmission (Ayala et al. 2007; Fischer et al. 2002, 2005; Tan et al. 2003). However, excessive Cdk5 activation can alter cellular trafficking and transport and contribute to neuronal dysfunction and death (Cruz et al. 2003), ultimately resulting in neurodegenerative diseases (Brion & Couck, 1995; Nakamura et al. 1997, 1998; Patrick et al. 1999; Smith & Tsai, 2002). The amino-terminal myristoylation sequence of mammalian p35 has been proposed to be the key domain anchoring the Cdk5/p35 complex to the cell membrane, and p35...
translocation to the membrane is an important regulator of Cdk5 activity (Sananbenesi et al. 2007; Ubeda et al. 2006). Inhibition of Cdk5 activity in the hippocampus has been recently shown to facilitate the extinction of learned contextual fear, suggesting that Cdk5 could be a potential target for the treatment of emotional disorders (Sananbenesi et al. 2007). Hyperactivity of Cdk5 or failure to regulate Cdk5 activity in the septohippocampal system might contribute to the emergence of excessive anxiety induced by stressful life events (Bignante et al. 2008).

The hippocampus is one of the critical brain regions that contribute directly or indirectly to several of the core symptoms of depression (Warner-Schmidt & Duman, 2006). Magnetic resonance imaging studies have shown a reduction in hippocampal volume in depressed patients (Campbell et al. 2004; Videbech & Ravnkilde, 2004). Hippocampal neurogenesis and neurotrophic factor expression, especially brain-derived neurotrophic factor (BDNF), were decreased by stress and increased by chronic antidepressant administration (Duman & Monteggia, 2006; Schmidt & Duman, 2007). However, whether the Cdk5/p35 complex in the hippocampus is involved in the development or treatment of depression is unknown. In the present study, we investigated the potential role of hippocampal Cdk5/p35 in the regulation of depressive-like behaviours induced by CMS and the effects of antidepressant treatment.

Methods

Subjects

Male Sprague–Dawley rats (240–260 g) were individually housed at a constant temperature (23 ± 2 °C) and maintained on a 12-h light/dark cycle (lights on 08:00 hours) with food and water available ad libitum. All of the animal procedures were performed in accordance with the National Institutes of Health’s ‘Guide for the Care and Use of Laboratory Animals’, and the procedures were approved by the Local Animal Use Committee.

Drugs

Butyrolactone (Buty) was purchased from Sigma-Aldrich (USA) and dissolved in saline containing 0.2% DMSO. Venlafaxine, mirtazapine, and aripiprazole were purchased from Chengdu Daxi’nan Pharmaceutical Co. Ltd (China). Venlafaxine was dissolved in distilled water. Mirtazapine was dissolved in distilled water containing 0.02% glacial acetic acid. Aripiprazole was suspended in distilled water by adding 2.5% Tween-80.

CMS Protocol and Behavioural Tests

CMS

The CMS protocol was adapted from previous reports with minor modifications (van Riel et al. 2003; Willner et al. 1987). Briefly, rats were subjected to different mild stressors for 21 d. Day 1: cold immobilization for 1 h at 4 °C, tilted cages (45°) for 24 h. Day 2: immobilization for 1 h, crowding for 24 h. Day 3: forced cold swim for 5 min, soiled bedding for 24 h. Day 4: immobilization for 1 h, vibration for 1 h. Day 5: tilted cages (45°) for 24 h, cold immobilization for 1 h at 4 °C. Day 6: forced cold swim for 5 min at 4 °C, crowding for 24 h. Day 7: vibration for 1 h, soiled bedding for 24 h. This schedule was repeated two more times.

Sucrose Preference Test

Rats were trained to adapt to a 1% sucrose solution (w/v) for 48 h at the start of the experiment, in which two bottles of 1% sucrose solution were placed in each cage. After adaptation, rats were deprived of water and food for 24 h, followed by the sucrose preference test, in which rats were housed in individual cages for 12 h and had free access to two bottles containing 1% sucrose or water. The position of the two bottles was interchanged every 6 h during the test. At the end of the 12-h test, sucrose and water consumption (ml) were measured, and sucrose preference (%) was calculated as:

\[
\text{sucrose consumption} \div \text{sucrose consumption + water consumption} \times 100
\]

(Willner et al. 1987).

Open-Field Test

Locomotor activity was measured using the open-field test as previously described (Lin et al. 2005). Briefly, the apparatus consisted of a 75 × 75 × 40 cm square arena divided into 25 equal squares (15 × 15 cm) on the floor of the arena. A single rat was placed in the centre of the cage, and the numbers of crossings (entering the adjacent square line) and rearings (standing on hind legs) were counted for 5 min. All of the behavioural tests and drug administrations were performed during the dark phase.

Intracerebral Cannula Implantation and Intracranial Injections

Rats were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), and guide cannulae (23-gauge, Plastics
One, USA) were implanted bilaterally 1 mm above the cornu ammonis 1 (CA1), CA3, or dentate gyrus (DG). The stereotaxic coordinates were as follows: CA1 [anterior/posterior (AP) -3.8 mm, lateral (L) ±2.0 mm, dorsal/ventral (DV) -2.5 mm], CA3 (AP -3.8 mm, L ±3.8 mm, DV -3.2 mm), DG (AP -3.8 mm, L ±2.0 mm, DV -3.1 mm) (Paxinos et al. 1980; Shirayama et al. 2002). Vehicle or Buty were intracranially microinjected using 10 μl Hamilton syringes (USA) that were connected via polyethylene-50 tubing to 30-gauge injectors (Plastics One). A total volume of 0.5 μl was infused into each side. At the end of the experiments, cannula placements were assessed using Nissl staining. Subjects with misplaced cannulae were excluded from the statistical analysis. Schematic representations of cannula placements in CA1, CA3, and DG are shown in Fig. 1.

Viral-mediated gene transfer

The DNA encoding p35 has been reported previously (Hershberger et al. 1994; Roy & Sapolsky, 2003; Roy et al. 2001, 2002) and was cloned into a pSNAV2.0 vector (Invitrogen, USA) using KpnI/Sall restriction sites, which was provided by GenScript (USA). The pSNAV2.0-p35 plasmid was transfected into BHK-21 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For viral-mediated gene expression, surgery was performed on male Sprague-Dawley rats. Adeno-associated virus (AAV)-p35 (1.4 × 10^9 viral genome particles/ml) or AAV-enhanced green fluorescent protein (EGFP) (3.3 × 10^9 viral genome particles/ml) was injected bilaterally (1.5 μl per side) with guide cannulae over 5 min into DG. Animal behaviours were tested 3 wk after AAV vector injection when transgene expression is known to be maximal (Wallace et al. 2009). The microinjection placements were also evaluated for each rat using Cresyl Violet staining through the infusion site. Animals with incorrect bilateral placements were excluded from the statistical analysis.

Tissue sample preparation

Rats were decapitated, and brains were extracted based on our previous studies (Li et al. 2010; Lu et al. 2005). Bilateral tissue punches of CA1, CA3, and DG (16-gauge) were obtained and were homogenized with RIPA lysis buffer (Beyotime Biotechnology, China). The cytoplasmic and membrane fractions were separated using the CNM (cytoplasmic, nuclear, membrane) Compartment Protein Extraction kit (K3012010, BioChain Institute, USA). The protein concentrations of all samples were determined using the BCA assay kit (Beyotime Biotechnology).

Western blot assays

Samples were subjected to SDS–PAGE as previously described (Li et al. 2008; Wang et al. 2010). Proteins were transferred to Immobilon-P membranes (Millipore, USA) and were washed with TBST (Tris-buffered saline plus 0.05% Tween-20; pH 7.4) before dipping in blocking buffer overnight at 4°C. Membranes were then incubated with anti-p35 (1:500, Sc-820, Santa Cruz Biotechnology, USA), anti-Cdk5 (1:2000, 05-364, Upstate Biotechnology, USA), anti-SVP (synaptophysin) (1:500, MAB368, Chemicon, USA), or anti-β-actin antibody (1:2000, A5316, Sigma). Subsequently, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or mouse IgG; Santa Cruz Biotechnology and Vector Laboratories, USA, respectively). The blots were incubated with a layer of Super Signal enhanced chemiluminescence substrate mixture (Pierce Biotechnology, USA) and were exposed against X-ray film. Band intensities were quantified using Quantity One software (version 4.0.3) from Bio-Rad Corporation (USA).
Cdk5 activity assays

Cdk5 activity was assayed according to previously published methods (Chen & Chen, 2005; Li et al. 2010). Briefly, tissues from the hippocampus were lysed in RIPA buffer. Cdk5 was immunoprecipitated from the hippocampal homogenates using an anti-Cdk5 antibody (C8 anti-Cdk5, Santa Cruz Biotechnology) added to the antigen-antibody complex, and the reaction was incubated for 2 h at room temperature. Next, 0.5 ml IP buffer (25 mM Tris, 150 mM NaCl; pH 7.2) was added to the immunoprecipitated samples and centrifuged for 2–3 min at 2500 g. The supernatant was discarded. This procedure was repeated several times. To elute the immune complex, 50 ml glycine–HCl buffer; pH 2.5–3.0) was added and incubated for 5 min, after which the tubes were centrifuged for 1–3 min at 2500 g, and the supernatant was collected. This step was repeated one more time, and the two supernatant fractions were combined. The final mixture was incubated with 0.08 mg/ml histone H1 protein (H5505, Sigma) and 0.2 µCi/ml [γ-32P]adenosine triphosphate at 30 ºC for 30 min. Radioactivity was measured with a scintillation counter and corrected with basal activity.

Experimental design

Expt 1: effect of CMS on Cdk5 activity and p35 subcellular distribution in the hippocampus

Rats in CMS groups were subjected to stress daily for 3 wk, whereas animals in control groups were handled daily (n = 10 per group). On day 22, the sucrose preference test was performed, and bodyweight and locomotor activity were assessed. After the behavioural tests, animals were decapitated immediately, and their brains were removed for measurement of Cdk5 activity and membrane and cytosolic p35 levels (n = 5 per group). The correlations between membrane p35 levels and sucrose preference and between membrane p35 levels and Cdk5 activity were performed using Pearson’s correlation analysis (Fig. 2).

Expt 2: effect of Cdk5 inhibition in DG on depressive-like behaviour induced by CMS

Eight groups of rats (n = 7–8 per group) received vehicle (0.2% DMSO) or Buty into DG. Buty and vehicle were microinfused once per week on days 1, 7, 14, and 21 according to previous studies (Luo et al. 2008; Wang et al. 2008). Microinjection of Buty once per week can also prevent brain damage caused by excessive microinfusions. The doses of Buty (25, 50, 100 ng) have been described elsewhere (Sananbenesi et al. 2007). Each intra-hippocampal microinjection was administered between 08:00 and 10:00 hours, 1 h prior to CMS exposure. On day 22, the sucrose preference test was performed, and bodyweight and locomotor activity were assessed. After the behavioural tests, animals were decapitated immediately, and their brains were removed for Cdk5 activity measurement (n = 5 per group) (Fig. 3).

Expt 3: effect of Cdk5 inhibition in CA1 and CA3 on depressive-like behaviour induced by CMS

To examine the subregion-specific effect of Cdk5 in the hippocampus on depressive-like behaviour, we further inhibited Cdk5 activity in CA1 and CA3. Eight groups (n = 9 per group) of rats were microinjected with vehicle or Buty (100 ng) on days 1, 7, 14, and 21. On day 22, the sucrose preference test was performed, and bodyweight and locomotor activity were assessed (Fig. 4).

Expt 4: effect of p35 overexpression in DG on depressive-like behaviour induced by CMS

To determine the regulation of p35 overexpression in DG on depressive-like behaviour, four groups of rats were used (n = 8–9 per group) in a 2 (virus: EGFP/p35) × 2 (treatment: EGFP/p35) experimental design: (i) control–EGFP group, (ii) control–p35 group, (iii) CMS–EGFP group, and (iv) CMS–p35 group. One day before the CMS procedure, AAV-EGFP or AAV-p35 were infused into DG. On day 22, the sucrose preference test was performed, and bodyweight and locomotor activity were assessed (Fig. 5).

Expt 5: effect of p35 overexpression in DG on the antidepressant effects of venlafaxine

To assess the effects of p35 overexpression in DG on the antidepressant effects of venlafaxine, four groups of rats (n = 8–9 per group) were subjected to the CMS procedure in a 2 (drug: vehicle/venlafaxine) × 2 (treatment: EGFP/p35) experimental design: (i) vehicle–EGFP group, (ii) vehicle–p35 group, (iii) venlafaxine–EGFP group, and (iv) venlafaxine–p35 group. One day before the CMS procedure, AAV-EGFP or AAV-p35 were infused into DG. Venlafaxine (40 mg/kg i.p.) was administered once daily between 08:00 and 10:00 hours, 30 min prior to stressors of
Fig. 2. Chronic mild stress (CMS) induced depressive-like behaviours as well as increased Cdk5 activity and induced p35 translocation in the hippocampus. The behavioural analysis showed that CMS (a) reduced sucrose preference, (b) reduced bodyweight, and (c) decreased activity compared to the control group (n = 10 per group in the behavioural test). (d) Cdk5 activity increased in the hippocampus after exposure to CMS (n = 5 per group). Cdk5 activity in CMS-treated and control rats was measured by in-vitro phosphorylation on day 22 and is expressed as specific counts (cpm). CMS-treated rats showed (e) greater p35 levels in the membrane and (f) reduction in cytosolic p35 levels in the dentate gyrus (DG) compared to control rats (n = 5 per group). Synaptophysin (SVP) was used as an internal loading control to normalize the membrane fraction of p35, and β-actin was used to normalize the cytosolic fraction of p35. The representative band intensity of the Western blot is shown below the panel. Data are expressed as mean ± S.E.M. * p < 0.05 compared to control group (unpaired t test). (g) CMS induced an increase of Cdk5 activity in DG but not in CA1 or CA3 (n = 5 per group). (h) Cdk5 activity in the hippocampus showed a significant, positive linear correlation with membrane p35 levels (r = 0.688, p < 0.05, n = 10) in DG in control and CMS-treated rats. (i) A significant, negative linear correlation was found between membrane p35 protein levels in DG and sucrose preference in control and CMS-treated rats (r = −0.514, p < 0.05; n = 20).
each day during the CMS procedure for 21 d. On day 22, the sucrose preference test was performed, and bodyweight and locomotor activity were assessed (Fig. 6).

**Expt 6: effects of antidepressants and antipsychotics on Cdk5 activity and p35 translocation in DG**

The aim of this experiment was to explore the possible role of Cdk5 activity change and p35 translocation in DG on the action of antidepressants. Eight groups of rats were treated with vehicle, venlafaxine (40 mg/kg), mirtazapine (20 mg/kg), and aripiprazole (5 mg/kg) intraperitoneally for 21 d in both the control and CMS groups. On day 22, the sucrose preference test was performed. After the behavioural tests, animals were decapitated immediately, and their brains were removed for subsequent Cdk5 activity and p35 level assessment (n = 8 per group for behavioural test; n = 6 per group for kinase activity and Western blot assay, respectively) (Fig. 8).

**Data analysis**

Data are expressed as mean ± S.E.M. The statistical analysis of the behavioural and molecular data in control and CMS-treated rats was performed using unpaired Student’s *t* test. The effects of stress (control/CMS) and treatment (vehicle/Buty) on behaviour, Cdk5 activity, and Cdk5/p35 protein levels and the effects of virus (EGFP/p35) and antidepressant (vehicle/venlafaxine) on behaviour and p35 protein levels were performed using two-way analysis of variance (ANOVA), followed by Tukey’s *post-hoc* test. Values of *p* < 0.05 were considered statistically significant.
as mean induced by CMS (n preference, (p < 0.05), decreased sucrose preference (unpaired t test, (p < 0.05)) were induced by CMS in rats (Fig. 2a–c). Rats that received CMS had significantly elevated Cdk5 activity in the hippocampus (unpaired t test, (p < 0.05)) (Fig. 2d). The protein level of p35 was rate-limiting for Cdk5 activity, especially in the membrane fraction. We then determined whether the elevated level of Cdk5 activity was associated with p35 translocation in the different subregions of the hippocampus. CMS-treated rats had greater membrane p35 levels (p < 0.05) and lower cytosolic p35 levels (p < 0.05) in DG (Fig. 2e, f), whereas Cdk5 levels in DG were unaffected by CMS. We also found that the levels of both p35 and Cdk5 in the membrane and cytosol in CA1 and CA3 sub-regions of the hippocampus were not altered by CMS (data not shown). The results also showed that CMS increased hippocampal Cdk5 activity specifically in DG but not in CA1 or CA3 (p < 0.05, Fig. 2g).

Cdk5 activity in the hippocampus showed a positive linear correlation with membrane p35 levels in DG (r = 0.688, (p < 0.05) (Fig. 2h). These data indicated that increased Cdk5 activity is attributable to elevated levels of membrane p35 in DG, which is consistent with previous findings in which membrane p35 was shown to be an important regulator of Cdk5 activity (Sananbenesi et al. 2007).

To determine the potential links between p35 protein levels and depressive-like symptoms, we explored the correlations between membrane p35 levels in DG and sucrose preference in control and CMS rats. The results showed that sucrose preference negatively correlated with membrane p35 expression in DG (r = −0.514, (p < 0.05) (Fig. 2i). Thus, rats that consumed less sucrose solution were those with higher membrane p35 in DG. These data suggested that increased Cdk5 activity correlates with depressive-like behaviours via p35 translocation from the cytosol to the membrane in DG.

### Results

**CMS increased Cdk5 activity in the hippocampus and induced p35 translocation in DG**

Decreased sucrose preference (unpaired t test, (p < 0.05)), bodyweight (p < 0.05), activity (p < 0.05) were induced by CMS in rats (Fig. 2a–c). Rats that received CMS had significantly elevated Cdk5 activity in the hippocampus (unpaired t test, (p < 0.05)) (Fig. 2d). The protein level of p35 was rate-limiting for Cdk5 activity, especially in the membrane fraction. We then determined whether the elevated level of Cdk5 activity was associated with p35 translocation in the different subregions of the hippocampus. CMS-treated rats had greater membrane p35 levels (p < 0.05) and lower cytosolic p35 levels (p < 0.05) in DG (Fig. 2e, f).

**Inhibition of Cdk5 in DG prevented depressive-like behaviour induced by CMS**

To further investigate whether the Cdk5/p35 complex is functionally related to elevated depressive-like symptoms, we tested the effects of Buty on depressive-like behaviour. The experimental design is shown in Fig. 3a. The data showed that Buty (50 and 100 ng) inhibited Cdk5 activity in DG (Fig. 3b) and increased sucrose preference, bodyweight gain, and locomotor activity (Fig. 3c–e) in CMS-treated rats. Two-way ANOVA with two between-subjects factors, including stress (control and CMS) and Buty dose (0, 25, 50, 100 ng), revealed main effects of stress (F$_{1,33}$ = 56.84, (p < 0.001) and Buty dose (F$_{3,32}$ = 8.57, (p < 0.001) and a stress × Buty dose interaction (F$_{3,32}$ = 4.09, (p < 0.05) for Cdk5 activity.

DG infusions of Buty reversed the decrease in sucrose preference, bodyweight gain, and locomotor activity induced by CMS (Fig. 3c–e). Two-way ANOVA with two between-subjects factors, including stress (control and CMS) and Buty dose (0, 25, 50, 100 ng), revealed main effects of stress (F$_{1,49}$ = 261.15, (p < 0.001); F$_{3,48}$ = 466.87, (p < 0.001); F$_{3,48}$ = 20.19, (p < 0.001), Buty dose (F$_{3,49}$ = 5.72, (p < 0.01); F$_{3,49}$ = 3.28, (p < 0.05), Fig. 3b).
Inhibition of Cdk5 activity in CA1 or CA3 did not alter depressive-like behaviour induced by CMS

To determine whether Cdk5 inhibition in other sub-regions of the hippocampus can reverse depressive-like behaviour induced by CMS, we infused the Cdk5 inhibitor Buty into CA1 and CA3 sub-regions. Two-way ANOVA with two between-subjects factors, including stress (control and CMS) and Buty dose (0 and 100 ng), indicated that the infusion of 100 ng Buty into either CA1 or CA3 had no effect on sucrose preference (CA1 group: $F_{1,32}=1.04$, $p>0.05$; CA3 group: $F_{1,32}=3.03$, $p>0.05$), bodyweight (CA1 group: $F_{1,32}=0.28$, $p>0.05$; CA3 group: $F_{1,32}=0.21$, $p>0.05$), and locomotor activity (CA1 group: $F_{1,32}=2.06$, $p>0.05$; CA3 group: $F_{1,32}=3.10$, $p>0.05$) (Fig. 4a–c), suggesting that the Cdk5
inhibition-induced reversal of depressive-like symptoms is specific to DG.

**Overexpression of p35 in DG did not influence depressive-like behaviour**

Given our findings that CMS significantly increased Cdk5 activity in DG, we sought to determine whether Cdk5 up-regulation alters depressive-like behaviour. Four groups of rats were used in a 2 (stress: control and CMS) × 2 (virus: EGFP and p35) factorial design to test the effects of p35 overexpression in DG on depressive-like behaviour (Fig. 5a). The statistical analysis revealed significant effects of CMS on sucrose preference (F(1,29) = 30.88, p < 0.001), bodyweight gain (F(1,29) = 76.09, p < 0.001), and locomotor activity (F(1,29) = 30.88, p < 0.001). DG overexpression of p35 did not alter sucrose preference (F(1,29) = 0.042, p > 0.05), bodyweight (F(1,29) = 0.52, p > 0.05), or locomotor activity (F(1,29) = 0.20, p > 0.05) compared to the AAV-EGFP group in both control and CMS-treated rats (Fig. 5b–d). The statistical analysis showed that overexpression of p35 in DG increased Cdk5 activity (F(1,29) = 5.56, p < 0.05) and membrane (F(1,11) = 135.31, p < 0.001) and cytosol (F(1,11) = 153.18, p < 0.001) p35 protein levels in the control group compared to AAV-EGFP injection group (Fig. 5e–g).

**Overexpression of p35 in DG reversed the antidepressant-like effects of venlafaxine in CMS model**

The experimental design is shown in Fig. 6a. The antidepressant venlafaxine significantly increased sucrose preference in the AAV-EGFP group compared to vehicle-treated AAV-EGFP group (p < 0.05), but this effect of venlafaxine was abolished by AAV-p35 treatment (p < 0.05). Two-way ANOVA with two between-subjects factors, including venlafaxine treatment (0 and 40 mg/kg) and virus (EGFP and p35), revealed a significant effect of virus (F(1,29) = 6.69, p < 0.05; F(1,29) = 12.24, p < 0.01; F(1,29) = 8.21, p < 0.01)
and venlafaxine ($F_{1,20} = 6.29, p < 0.05$; $F_{1,29} = 8.88, p < 0.01$; $F_{1,20} = 10.05 p < 0.05$) and a virus x venlafaxine interaction ($F_{1,20} = 5.65, p < 0.05$; $F_{1,29} = 12.05, p < 0.01$; $F_{1,20} = 9.24, p < 0.01$) on sucrose preference, bodyweight gain, and locomotor activity, respectively (Fig. 6b–d). These findings indicate that the behavioural responses to the antidepressant venlafaxine can be prevented by p35 overexpression. Furthermore, we determined the effect of CA1 and CA3 p35 overexpression on depressive-like behaviours. The results revealed that overexpression of p35 in CA1 and CA3 did not alter sucrose preference, bodyweight and locomotor activity (Fig. 7). Thus, the specific role of DG Cdk5 in the involvement of depressive-like behaviour has been further confirmed.

**Antidepressants but not antipsychotics altered p35 translocation from the membrane to the cytosol in DG**

The behavioural tests showed that sucrose preference, bodyweight and locomotor activity were increased by treatment with venlafaxine and mirtazapine, but not aripiprazole, in the CMS-treated group (Fig. 8a–c). Two-way ANOVA with two between-subjects factors, including drug treatment (vehicle, venlafaxine, mirtazapine, aripiprazole) and stress (control and CMS), was used to analyse the effects of drug treatment on behavioural measures, Cdk5 activity and p35 protein levels. The analysis revealed significant effects of drug treatment ($F_{1,36} = 8.59, p < 0.01$; $F_{3,56} = 6.35, p < 0.01$; $F_{3,56} = 10.57, p < 0.001$) and stress ($F_{1,56} = 62.42, p < 0.001$; $F_{1,56} = 322.28, p < 0.001$; $F_{1,56} = 62.18, p < 0.001$) and a drug treatment x stress interaction ($F_{3,56} = 5.14, p < 0.01$; $F_{3,56} = 6.37, p < 0.01$; $F_{3,56} = 3.64, p < 0.05$) on sucrose preference, bodyweight gain and locomotor activity, respectively. Cdk5 kinase activity assay revealed that antidepressants venlafaxine and mirtazapine reduced Cdk5 activity induced by CMS ($p < 0.05$), while the antipsychotic aripiprazole had no such effect (Fig. 8d). The data analysis from Western blot assay showed that venlafaxine and mirtazapine, but not aripiprazole, increased cytosol p35 and decreased membrane p35 levels in DG in the CMS-treated group (Fig. 8e, f). Two-way ANOVA revealed significant effects of drug treatment ($F_{1,40} = 36.61, p < 0.001$) and stress ($F_{1,40} = 178.35, p < 0.001$) and a venlafaxine treatment x stress interaction ($F_{3,40} = 33.81, p < 0.001$) on membrane p35 levels. The analysis of cytosolic p35 levels revealed significant effects of drug treatment ($F_{3,40} = 36.54, p < 0.001$) and stress ($F_{1,40} = 245.76, p < 0.001$) and a drug treatment x stress interaction ($F_{3,40} = 35.73, p < 0.001$) on cytosolic p35 levels. These results showed that venlafaxine- and mirtazapine-treated rats had decreased Cdk5 activity through p35 translocation from the membrane to the cytosol in DG, which is consistent with the reduced depressive-like behaviour in CMS-treated rats. In contrast, the antipsychotic aripiprazole did not affect Cdk5 activity and p35 translocation in DG.

**Discussion**

In the present study, we found that CMS induced an increase of Cdk5 kinase activity in DG, but not in CA1 or CA3, accompanied by p35 translocation from the cytosol to the membrane in DG. Inhibition of Cdk5 in DG, but not CA1 or CA3 reversed the depressive-like behaviour induced by CMS. These findings revealed
that DG Cdk5 plays a critical role in the regulation of depressive-like behaviour while CA1 or CA3 Cdk5 have no such effects. To further confirm the specific role of DG Cdk5 in the involvement of depression, we determined the effects of overexpression of p35 in DG, CA1 and CA3 on depressive-like behaviours. The results showed that overexpression of p35 in DG, CA1 and CA3 did not alter depressive-like behaviours while DG p35 overexpression blocked the anti-depressant effects of venlafaxine on depressive-like behaviour. Finally, the antidepressants venlafaxine and mirtazapine, but not the antipsychotic aripiprazole, altered the redistribution of membrane p35 to the cytosol in DG. These data reveal a unique and previously unrecognized function of the Cdk5/p35 complex in the regulation of the depressive-like phenotype and behavioural responses to antidepressant treatment.

**Cdk5 activity and p35 translocation in the hippocampus**

The accumulation of Cdk5 has been demonstrated in several diseases, including Alzheimer’s disease (Liu et al. 1995; Takahashi et al. 2000), Parkinson’s disease (Brion & Couck, 1995), amyotrophic lateral sclerosis...
(Bajaj et al. 1999; Nguyen et al. 2001), and cocaine addiction (Li et al. 2010). These data indicate a positive association between Cdk5 activity and neurodegenerative and stress-related psychiatric disorders. Full activation of Cdk5 requires Cdk5-specific activators, such as p35 and p39, which are expressed only in post-mitotic neurons (Lew et al. 1994; Tsai et al. 1994). p35 translocation to the membrane from the cytosol has also been shown to be critical for Cdk5 activity (Sananbenesi et al. 2007; Ubeda et al. 2006). We found that increased membrane and decreased cytosolic p35 in DG were observed simultaneously in stressed rats. These data suggest that chronic stress caused the translocation of p35 from the cytosol to the membrane in DG, with subsequently increased Cdk5 activity in the hippocampus. Additionally, we found that Cdk5 activity in the hippocampus had a positive linear correlation with membrane p35 levels in DG. As previously reported, p35 is a neural-specific regulatory subunit of Cdk5 and is responsible for the activation of Cdk5 in the brain (Tsai et al. 1994).

The present findings that increased Cdk5 activity induced by CMS parallels the translocation of p35 from the cytosolic fraction to the membrane in DG, are consistent with a previous study showing that the brain N-terminal p35 myristoylation signal may anchor Cdk5 on the cell membrane to activate Cdk5 (Tsai et al. 1994). Therefore, we can speculate that CMS-induced Cdk5 activity might mainly act on p35 translocation from the cytosol to the membrane in DG, causing an aberrant distribution of p35 in the membrane and cytosol within the hippocampus and ultimately participate in the development of depressive-like behaviour.

Specific role of Cdk5 in DG in the regulation of depressive-like behaviour

The present study showed that increased membrane p35 correlated with increased depressive-like behaviour, suggesting that inhibition of membrane-associated Cdk5 activity might alleviate depressive-like symptoms. As expected, the administration of Buty, which acts through competitive inhibition of adenosine triphosphate binding to prevent Cdk5 activation (Kitagawa et al. 1993, 1994), into DG suppressed depressive-like behaviour induced by chronic stress. In contrast, Buty microinjections into CA1 or CA3 did not prevent the depressive symptoms, indicating that Cdk5 activation is specific to DG. The specific role of p35 in DG in the regulation of depressive-like behaviour can be explained by the differential vulnerability of hippocampal subregions. The CA1 subregion is highly susceptible to ischaemia and glutamate-mediated excitotoxicity (Gee et al. 2006; Wang et al. 2005). CA3 also has high levels of endogenous glucocorticoids and is susceptible to chronic stress (Conrad, 2008), whereas DG is one of the critical subregions responsible for adult neurogenesis, which is a target for the development of novel antidepressant medications (Duman, 2004; McEwen, 1999).

A previous study showed that induced overexpression of CREB in DG, but not CA1 or CA3, was associated with antidepressant-like effects (Chen et al. 2001a). Similarly, BDNF, downstream of the cAMP pathway, increased in DG in depressed subjects treated with antidepressant medications at the time of death, compared to subjects not treated with antidepressants (Chen et al. 2001b). Therefore, we suggest the possibility that the specific effect of Cdk5 activation in DG on depressive behaviour is attributable to the impairment of hippocampal DG neurogenesis induced by CMS. We subsequently hypothesize that p35 overexpression may induce depressive symptoms in normal rats. The present results showed that p35 overexpression could not induce depressive-like behaviour in normal rats or exacerbate the depressive-like phenotype in stressed rats. However, p35 overexpression in DG can block the antidepressant-like efficacy of chronic venlafaxine treatment. This finding indicates that although reduced Cdk5 activity is required for an antidepressant response, increased Cdk5 activation does not induce a depressive-like phenotype in this animal model. This could be partially attributable to the fact that p35 overexpression did not impair neuronal function in granule cells of DG, or another mechanism may involve the interaction between Cdk5 activation and p35 levels in depressive-like behaviour.

Additionally, we found that overexpression of p35 in DG reversed the antidepressant-like effect of venlafaxine but did not influence depressive-like behaviour. The following reasons might explain this contradiction. It has been evidenced that p35 translocation from the cytosol to the membrane is an important regulator of Cdk5 activity (Sananbenesi et al. 2007; Ubeda et al. 2006). Although the current findings reveal that overexpression of p35 induced an increase level of both membrane and cytosol p35 protein, the increased extent of membrane p35 level presumably could not up-regulate Cdk5 activity and subsequently could not change the depressive-like behaviour. In the present study, we also found that the antidepressant venlafaxine decreased membrane p35 and increased cytosol p35 levels in DG in the CMS-treated group. The decreased membrane p35 level of venlafaxine
treatment might be blunted by overexpression of AAV-p35; therefore, overexpression of p35 in DG reversed the antidepressant-like effect of venlafaxine, but did not alter depressive-like behaviour.

Mechanisms underlying the role of Cdk5 activation in antidepressant treatment

Numerous proteins and genes have been identified as substrates of Cdk5, one of which is DARPP-32 (dopamine and cAMP-regulated phosphoprotein of Mr 32 kDa). Activation of Cdk5 consequently phosphorylates DARPP-32 at the threonine-75 (Thr\(^{75}\)) position (Greengard, 2001; Liu et al. 2001). The key role of DARPP-32 in the biochemical and behavioural effects of antidepressants has also been demonstrated. Acute and chronic treatment with fluoxetine decreased DARPP-32 phosphorylation at Thr\(^{75}\), and a similar finding was observed with serotonin-incubated hippocampal slices (Svenningsson et al. 2002). Moreover, BDNF, which regulates neuronal survival and synaptic plasticity, has been hypothesized to be involved in the mechanisms underlying the regulation of DARPP-32. Chronic treatment with several different antidepressants, including fluoxetine, increased BDNF levels in the frontal cortex and hippocampus in experimental animals (Nibuya et al. 1995). Altogether, the present results suggest that inactivation of the Cdk5/DARPP-32 signalling pathway might be a potential mechanism underlying the behavioural response to chronic venlafaxine treatment.

Notably, a previous study found that Cdk5, which is one target gene of ΔFosB, was increased by chronic electroconvulsive seizure (ECS) treatment in the hippocampus (Chen et al. 2000). This finding appears to be inconsistent with our present results, in which chronic venlafaxine and mirtazapine treatment decreased DG membrane p35 levels and ultimately reduced Cdk5 activity in CMS-treated rats. We assume that one possibility for this discrepancy is that ECS and chemical antidepressants produced the therapeutic effect probably through different downstream pathways of Cdk5. Venlafaxine- and mirtazapine-induced Cdk5 down-regulation might lead to a reduction in hyperphosphorylation of the cytoskeleton by Cdk5 hyperactivity. Consequently, the down-regulation of hyperphosphorylation of the cytoskeleton reduces neuronal death (Gong et al. 2003; Lee et al. 2000). Up-regulation of Cdk5 by ECS might promote the growth and sprouting of hippocampal neurons (Duman et al. 1997; Li et al. 2002). Based on these studies, we assume that the extent to which Cdk5 was activated might trigger different processes that induce neuronal survival or death. However, this hypothesis requires further investigation.

Concluding remarks

In summary, we demonstrated that the regulation of Cdk5 activity via translocation of cytosolic p35 to the membrane is associated with depressive-like behaviours evoked by CMS. Moreover, Cdk5 was a crucial mediator implicated in the regulation of the behavioural effects of antidepressants. Cdk5 has been associated essentially with neuronal differentiation and neurodegenerative processes, and the critical regulation of the Cdk5/p35 complex could be a potential target for the development of novel therapies for the treatment of depression.

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Statement of Interest

None.

References


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