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Mice lacking p35 display hyperactivity and paradoxical response to psychostimulants

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Abstract

Cyclin-dependent kinase 5/p35 kinase complex plays a critical role in dopaminergic neurotransmission. Dysregulation of dopamine (DA) signaling is associated with neurological and neuropsychiatric disorders. As cyclin-dependent kinase 5 (Cdk5) requires association with p35 for its proper activation, we hypothesized that dysregulation of Cdk5 activity might have an effect on striatal-mediated behavior. We used a mutant mouse, deficient in p35 protein (p35 KO), which displayed reduced Cdk5 activity. Throughout behavioral and biochemical characterization of naïve and psychostimulant-treated mice, we demonstrated that only juvenile p35 KO mice displayed spontaneous hyperactivity, responded with a paradoxical hypolocomotor effect to psychostimulant drugs and exhibited deficit on proper behavioral inhibition. Strong immunolabeling for tyrosine-hydroxylase and high striatal DA synthesis and contents with a low DA turnover, which were reverted by psychostimulants, were also found in mutant mice. Our results demonstrate that p35 deficiency is critically involved in the expression of a hyperactive behavioral phenotype with hyper-functioning of the dopaminergic system, emphasizing the importance of proper Cdk5 kinase activity for normal motor and emotional features. Thus, p35 KO mice may be another useful animal model for understanding cellular and molecular events underlying attention deficit hyperactivity disorder-like disorders.

Keywords: attention deficit hyperactivity disorder, Cdk5/p35, dopamine, hyperactivity, psychostimulants, striatum.

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Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase identified by its close sequence homology to the classic members of the cdk family of regulating cell cycle progression proteins. Cdk5 is a unique member of this family that is active in post-mitotic neurons. Like other Cdks, however, monomeric Cdk5 shows no enzymatic activity and requires association with a regulatory subunit, either p35 or p39, which are brain-specific (Ishiguro *et al.* 1994; Lew *et al.* 1994; Tsai *et al.* 1994; Tang *et al.* 1995). Moreover, *in vivo* evidence has demonstrated that the level of p35 regulating protein is a rate-limiting factor for the up-regulation of Cdk5 activity (Takahashi *et al.* 2005). Several lines of evidence suggest that the Cdk5/p35 complex plays a critical role in normal brain development, regulating neuronal migration and differentiation, axodendritic organization and laminar architecture, trafficking and transport (Nikolic *et al.* 1996; Ohshima *et al.* 1996; Chae *et al.* 1997; Paglini *et al.* 1998; Ratner *et al.* 1998; Kwon and Tsai 1998; Ohshima *et al.* 1999; Kwon *et al.* 1999; Paglini and Caceres 2001; Paglini *et al.* 2001; Smith and Tsai 2002; Dhariwala and Rajadhyaksha 2008). Besides its essential role in brain development, Cdk5 has also been implicated in dopaminergic transmission in the post-natal brain, where it modulates

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Abbreviations used: ADHD, attention deficit hyperactivity disorder; Amph, D-anphetamine sulfate; Cdk5, cyclin-dependent kinase 5; CPu, striatum-caudate putamen; DA, dopamine; DARPP-32, dopamine and cAMP regulated phosphoprotein, molecular mass 32 kDa; DOPAC, 3,4-dihydroxyphenylacetic acid; EPM, elevated plus maze; Mtph, methylphenidate; NAc, nucleus accumbens; NHS, normal horse serum; PBS, phosphate-buffered saline; PKA, cAMP-dependent kinase; TBST, tris-buffered saline-tween 20; TH, tyrosine hydroxylase; WT, wild type.

the efficacy of post-synaptic dopamine/DRD1 signaling through the induction (Bogush *et al.* 2007) and phosphorylation of a key protein, dopamine and cAMP regulated phosphoprotein, molecular mass 32 kDa (DARPP-32) at Thr75, which converts DARPP-32 into an inhibitor of cAMP-dependent kinase A (PKA) (Bibb *et al.* 1999; Fernandez *et al.* 2006). This indicates that Cdk5 is an important molecule in controlling dopamine (DA) neurotransmission in the post-synaptic compartment. Furthermore, the inhibition of Cdk5 activity in the striatum-caudate putamen (CPu) results in increased extracellular DA levels, demonstrating a pre-synaptic function of Cdk5 as a negative regulator of DA release (Chergui *et al.* 2004).

DA is the main neurotransmitter in the nervous system that controls motor performance, emotion, motivation, cognition and several neuroendocrine functions. Dysfunction of the dopaminergic system, mainly in the CPu, has been associated with various neurological and psychiatric pathologies that include Parkinson's and Huntington's diseases, schizophrenia, substance abuse and with the pathogenesis of attention-deficit/hyperactivity disorder (ADHD).

Although Cdk5 activity plays a critical role in dopaminergic transmission, dysregulation of Cdk5 in the CPu and its effects on striatal-mediated behavior, has remained largely unexplored. For this reason, we challenged mutant mice deficient in p35 protein (p35 KO) to different behavioral paradigms and neurochemical analysis, and demonstrated that the juvenile mutant mouse shows locomotor hyperactivity, psychostimulant-induced hypoactivity and deficit in behavioral inhibition, reminiscent of some aspects of ADHD. Strong immunolabeling for tyrosine-hydroxylase (TH) and high levels of striatal DA, which were reverted by psychostimulants, were also found in p35 KO mice. Together, our results demonstrate, for the first time, a critical involvement of the Cdk5/p35 complex in the expression of a hyperactive behavioral phenotype with hyper-functioning of the dopaminergic system. Therefore, we suggest that p35-deficient mouse may be a useful animal model system to provide insights into the basic neural pathways underlying hyperkinetic disorders.

Materials and methods

Animals

Male wild type (WT) and p35$^{-/-}$ (p35 KO) mutant mice were generated by breeding heterozygous mutants, maintained in a 129/SvJ × C57BL/6J background via brother-sister mating (kind gifts of Dr. L.H. Tsai) (Chae *et al.* 1997). Animals were weaned at 21 days of age and genotyped at the end of the experiments, according to Kwon & Tsai (1998). Mice were housed four to five in standard laboratory Plexiglas cages under a 12-h light/12-h dark cycle and a constant temperature (22°C) with free access to food and water. All the experiments were performed during the light cycle, between 10:00 AM to 3:00 PM, at the age of 24 days (P24), weighing 9–11 g at onset of the experiments and in a separated behavioral room. Experiments with adult mice were performed using animals at the age of 2–3 months. During the test, the room was quiet and slightly lit and mice were allowed to acclimatize for 30 min before the onset of each experiment. All animal procedures and care were approved by National Department of Animal Care and Health (SENASA – ARGENTINA) and were in compliance with National Institute of Health general guidelines for the Care and Use of Laboratory Animals. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Open field

The individual animal was tested in an animal activity cage (40 cm in diameter) constructed of opaque plastic walls with two transverse photocells positioned 1 cm above the floor coupled to a computer interface. Horizontal activity was measured by interruptions of infrared photocell beams. Animals, without prior habituation to the activity cages, were placed into them and total locomotor activity was recorded every 10 min, for a total session of 70 min. Observations were monitored by an investigator blinded to genotype condition. Cages were carefully cleaned with a 20% ethanol solution between tests to minimize odor cues.

Behavioral pharmacological experiments

Mice were placed in the open field for an initial period of 10 min and then were treated with an i.p. injection of different doses of D-amphetamine sulfate (Amph) (0.2, 0.5 and 1.0 mg/kg) (Parafarm® Buenos Aires, Argentina) or Methylphenidate (Mtph) (0.5, 1.0 and 2.0 mg/kg) (Ritalina; Novartis, Argentina) dissolved in saline solution (NaCl 0.9%) and were placed again in the open field. Control mice were injected with saline solution. After injection, locomotor activity was recorded every 10 min for a period of 60 min.

Elevated plus maze

Elevated plus maze (EPM) apparatus consists of a plus shape of black Plexiglas with two opposite open arms (30 × 5 cm) and two opposite closed arms (30 × 5 × 16 cm), placed at a height of 40 cm above the floor. The testing room was quiet and dimly lit. Each mouse was placed in the central square of the maze to begin the test. All entries to both arms and the time spent in open arms were recorded for 5 min. An entry was considered when the two front paws of the animal were inside an arm. Observations were monitored by an investigator blinded to genotype condition and the maze was carefully cleaned after each test with a 20% ethanol solution. All these experiments were performed with naïve animals.

Tyrosine hydroxylase immunohistochemistry

WT and p35 KO mice were anesthetized by i.p. injection with an overdose of 30% chloral hydrate (0.1 mL/100 g) and perfused transcardially with a blood-washing solution consisting of 0.8% sucrose, 0.8% sodium chloride and 0.4% glucose followed by 4% paraformaldehyde in 0.2 M borate buffer pH 7.5 (Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany). The brains were left in the skull overnight at 4°C, and then removed and placed in 30% sucrose until they sank. The brains were cut coronally at 40 μm on a freezing microtome (Reicher-Jung Hn40, Leica Microsystems, Wetzlar, Germany) and the sections were collected

serially and processed for immunohistochemistry. Sections were washed three times in 0.01 M phosphate-buffered saline (PBS) to remove residual fixative. Prior to immunostaining, sections were placed in a mixture of 3% H_2O_2 and 10% methanol for 1 h, later rinsed in 0.01 M PBS and then incubated for 1 h at 25°C in blocking serum [5% normal horse serum (NHS) in 0.01 M PBS]. The sections were then transferred directly into anti-TH primary antibody diluted 1 : 1000 in 1% NHS, 0.05% Triton X 100, and gently agitated at 4°C for 48 h. Following incubation, sections were washed three times in PBS and placed in 1 : 200 goat anti-mouse IgG biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) in 1% NHS PBS for 2 h. The sections were then washed three times in PBS and incubated in avidin-biotin complex (Vectastain ABC Kit, Vector Laboratories) for 2 h. The sections were washed thrice in PBS and then immersed in 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 to visualize the reaction product. The reaction was stopped with PBS and the sections were mounted on subbed slides, dried on a slide warmer, dehydrated and coverslipped with DPX (Fluka, Biochemika, Sigma Aldrich, Chemie GmbH, Steinheim, Germany). Sections were examined with an Olympus CX40 (Tokyo, Japan) microscope.

Western blot analysis

Naïve mice were killed by cervical dislocation and the nucleus accumbens (NAc) and CPu from WT and p35 KO mice were quickly dissected over ice and homogenized in cold lysis buffer ristocetin-induced platelet agglutination (RIPA) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% c, 1 μg/mL Aprotinin, 1 μg/mL Leupeptin, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL Pepstatin, and 0.2 mM Sodium Orthovanadate). After centrifugation (15 000 *g* at 4°C), supernatants were assayed for protein concentration (DC Protein Assay Kit, Bio-Rad Laboratory, Hercules, CA, USA) and equal amounts of protein (20 μg) were separated into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes in a Tris-glycine buffer, 20% methanol. Membranes were then blocked in 5% skim milk in Tris-buffered saline-Tween 20 (TBST) (20 mM Tris, pH 7.5, 150 mM NaCl plus 0.05% Tween-20) and incubated overnight at 4°C with their primary antibodies diluted in TBST. Monoclonal antibody anti-Tyrosine Hydroxylase (clone LNC1, 1 : 1000, Chemicon International, Billerica, MA, USA) and α-Tubulin (clone DM1A, 1 : 2000, Sigma-Aldrich, Saint Louis, MO, USA) were used. Membranes were then washed three times in TBST and incubated with a secondary horseradish peroxidase-conjugated antibody (1 : 2000, anti-mouse IgG-HRP, Jackson ImmunoResearch Laboratory, West Grove, PA, USA) for 1 h at 37°C. After five washes with TBST, the blots were developed using enhanced chemiluminescence detection (ECL, Amersham Life Science, Buckinghamshire, UK). Chemiluminescence was detected by autoradiography using Agfa medical X-Ray Film (Buenos Aires, Argentina). Densitometry analyses of western blot bands were quantified on scanned films by Scion Image for Windows (Scion Corporation Frederick, MD, USA) analysis software. Values of proteins of interest were normalized to α-tubulin and the ratios were then used to perform statistical analysis.

The same procedure was conducted in saline- or Amph- (0.5 mg/kg) or Mtph- (0.5 mg/kg) administered WT and p35 KO mice 30 min after injection.

CPu tissue content of DA, metabolites and L-DOPA

Naïve mice were killed by cervical dislocation and the CPu from WT and p35 KO mice were quickly dissected over ice and were homogenized and deproteinized in cold 0.2 M perchloric acid (1/40 w/v). After centrifugation (10 min at 15 000 *g*), supernatants were filtered through a 0.22 μm polyvinylidene difluoride membrane (Millipore, Sao Paulo, SP, Brazil). Levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and L-DOPA accumulation were analyzed using HPLC with electrochemical detection, only for the measurement of DA we performed a previous extraction in aluminium oxide standardized for chromatographic adsorption as described by Revol *et al.* (Revol *et al.* 1994). DA, DOPAC and L-DOPA were separated on a reverse-phase column (Zorbax Eclipse XDB, C-8, 4.6 × 150 mm, 3.5 μm, Agilent Technologies, Inc. Santa Clara, CA, USA) with a mobile phase consisting of 0.03 M monochloroacetic/citric acid, 0.1 mM EDTA, 5 mM potassium chloride, and 3% acetonitrile (pH 3.2), at a flow rate of 0.8 mL/min and detected by a 3-mm glass carbon electrode (HP 1049) +0.75 V. The volume of injection was 80 μL and the peak heights were measured by HP 1100 ChemStation (Agilent Technologies, Inc.).

The same procedure was conducted in saline- or Amph- (0.5 mg/kg) or Mtph- (0.5 mg/kg) administered WT and p35 KO mice 30 min after injection. L-DOPA accumulation was measured 30 min after administration of 100 mg/kg NSD 1015 (3-hydroxybenzylhydrazine dihydro-chloride; Sigma-Aldrich).

Statistical analysis

Data were analyzed using the Statistica 5.1 program (Statsoft, Inc., Tulsa, OK, USA). Statistical analyses of biochemical and behavioral data were performed using one- or two-way ANOVA. Significant main effects indicated by the two-way ANOVA were further analyzed through Fisher's LSD *post hoc* test (Fisher's least significance difference test with a Type I error set at 0.05; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Results

Juvenile p35-deficient mouse exhibits hyperactivity and paradoxical response to psychostimulants

Spontaneous locomotor activity of juvenile (P24) and adult (P80–90) mice was tested in an open field. Juvenile p35 KO mice exhibited two and a half-fold spontaneous hyperlocomotion compared with the WT mice (WT: 380.50 ± 32.4 vs. p35 KO: 926.38 ± 83.47, $n = 6$ each genotype). In contrast, adult p35 KO mice did not manifest locomotor hyperactivity (WT: 252 ± 28.77 vs. p35 KO: 300.25 ± 34.03, $n = 5$ each genotype). Statistical analyses strongly suggest that the hyperactive phenotype is exclusive for juvenile mice (Fig. 1a).

The augmented horizontal locomotor activity displayed by juvenile p35 KO mice was significantly different over time except during the first 10 min. WT mice showed a progressive diminution of locomotor activity, while p35 KO mice hyperlocomotion remained raised during the rest of the test (Fig. 1b).

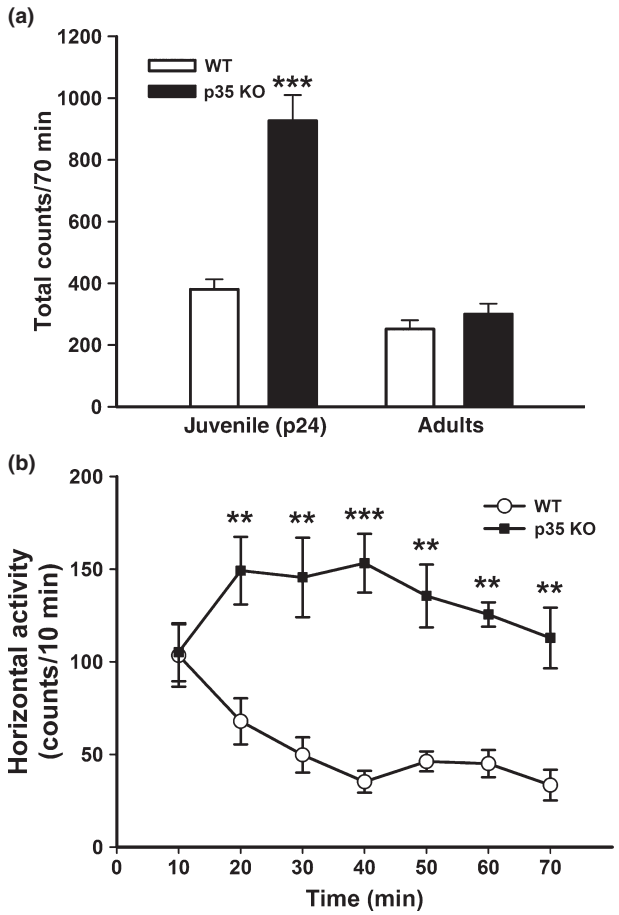

Fig. 1 Juvenile p35 KO mice displayed more than two-fold horizontal spontaneous hyperactivity compared with wild type (WT) mice. (a) cumulative counts of locomotor activity of juvenile and adult mutant mice in 70 min tests in the open field; two way ANOVA revealed a main effect of age and genotype and interaction between both factors $F(1,18) = 21.344$, $p = 0.0021$. Significant differences were observed only in juvenile mice (Fisher's LSD test). (b) locomotor activity during 70 min, to explore genotype differences, one-way ANOVA were performed for each time point: all Fs(1,10) > 13.59 and all ps < 0.004; $n = 6$ each genotype. Error bars represent standard error of the mean (SEM). $^{**}p < 0.01$, $^{***}p < 0.001$.

Finding that p35 KO mice displayed hyperactivity and considering that drugs that regulate dopaminergic transmission, such as Amph or Mtph, are effective in ameliorating behavioral symptoms in hyperactive rodents, like the spontaneously hypertensive rat (Sagvolden *et al.* 1992), 6-Hydroxydopamine–lesioned neonatal rat and mouse (Shaywitz *et al.* 1976; Avale *et al.* 2004), DA transporter knockout mouse (Gainetdinov *et al.* 1999), among others (Sagvolden *et al.* 2005), we were encouraged to test these drugs in the hyperactive p35 KO mice. In normal rodents, these psychostimulants are potent inducers of locomotor hyperactivity. When we tested them in the two genotype conditions, a drastic paradoxical hypolocomotor effect was observed in p35 KO mice, whereas WT mice showed the classical hyperlocomotor response to all Amph and Mtph challenge doses (Fig. 2a and b). Thus, the same doses of Amph (0.2, 0.5 and 1.0 mg/kg) or Mtph (0.5, 1.0 and 2.0 mg/kg) elicited a significant decrease of locomotor activity in p35 KO mice, and the scores were similar to those in WT animals after saline-injection (Fig. 2a and b). Notably, saline-injected-p35 KO mice displayed identical hyperlocomotion activity scores to those observed in their control WT mice stimulated with all the challenge doses of Mtph or Amph, except the highest one (1.0 mg/kg) (Fig. 2a and b). The hypolocomotor responses of p35 KO mice to the action of both psychostimulants were similar at the different doses used and remained sustained throughout the test. An example of chosen doses of Amph and Mtph is shown in Fig. 2d and e.

Mice lacking p35 display less anxiety-like behaviors

The fact that p35 deficiency in the juvenile mouse seems to be critical to displaying spontaneous hyperlocomotion, as well as its sensitivity to the major pharmacotherapies, Mtph and Amph, which were able to revert the hyperactivity, led us to study the role of p35 in anxiety-like behaviors. For this purpose, we challenged WT and p35 KO mice on the EPM, an approach/avoidance conflict paradigm that is resolved on the basis of a proper risk assessment of environmental cues (Avale *et al.* 2004; Mormede *et al.* 2002). As expected, normal juvenile WT mice showed high avoidance for the open unprotected arms of the EPM, with few entries (Fig. 3a) and less time spent in them (Fig. 3b). In contrast, p35 KO mice entered the open arms more frequently and spent significantly more time in them compared with WT mice (Fig. 3a and b). The total number of entries to all arms was not different between WT and p35 KO mice (Fig. 3c), indicating that the motivation to explore the arms of the EPM was similar in both genotypes.

What happens with dopamine neurotransmission components in p35-lacking mice?

It has been demonstrated that Cdk5/p35 complex is a crucial regulator of post-synaptic function mediated by DA signaling in the striatum (Bibb *et al.* 1999, 2001; Nishi *et al.* 2002; Takahashi *et al.* 2005). Nevertheless, its role in pre-synaptic DA signaling has been little explored. It is already known that Cdk5 activity is an important regulator of TH (Kansy *et al.* 2004; Moy and Tsai 2004), the rate-limiting enzyme in DA biosynthesis. Thus, we investigated whether DA synthesis is affected in p35 KO hyperactivity mouse. Initially, we evaluated TH immunoreactivity and expression levels in the dorsal CPu and nucleus accumbens (NAc) of both WT and p35 juvenile mutant mice. A strong immunoreactivity of TH was found in the CPu and NAc of brain sections from p35 KO mice compared with their control WT animals (Fig. 4a). In addition, western blot analysis on CPu and NAc extracts prepared from p35 KO mice and control

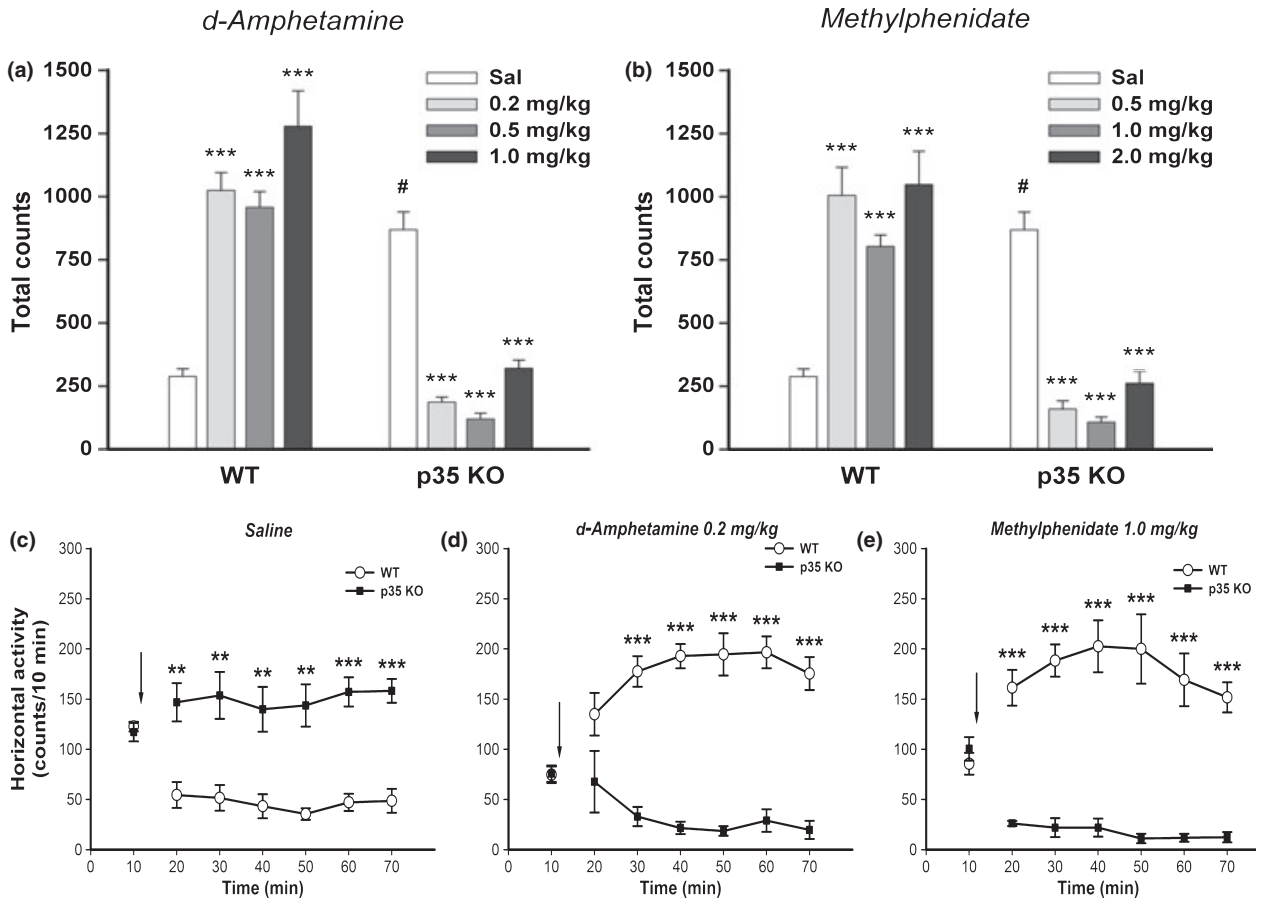

Fig. 2 Paradoxical hypolocomotor response to psychostimulants in p35 KO mice. (a) amphetamine (0.0, 0.2, 0.5 and 1.0 mg/kg, i.p.) induced hyperlocomotion in control wild type (WT) mice ($n = 25$). Conversely, the same doses of amphetamine provoked a hypolocomotor effect when given to p35 KO mice ($n = 27$) [amphetamine × genotype interaction, $F(3,44) = 49.84$ $p = 0.0022$, two-way ANOVA followed by the Fisher's LSD test]. (b) a similar effect was observed with the administration of methylphenidate (0.0, 0.5, 1.0 and 2.0 mg/kg, i.p.) [methylphenidate × genotype interaction, $F(3,40) = 57.41$, $p < 0.00001$, WT: $n = 22$; p35 KO: $n = 25$, two-way ANOVA followed by the Fisher's LSD test]. Bars represent the mean of cumulative counts of locomotor activity in 60 min; error bars represent SEM. Horizontal locomotor activity during 60 min of WT and p35 KO mice after injection (indicated with arrows) of (c) saline [saline × genotype interaction, $F(6,60) = 5.57$, $p = 0.00012$, $n = 10$], (d) amphetamine 0.2 mg/kg [amphetamine × genotype interaction, $F(6,66) = 15.15$, $p < 0.0001$, $n = 11$] and (e) methylphenidate 1.0 mg/kg [methylphenidate × genotype interaction, $F(6,54) = 13.56$, $p < 0.0001$, $n = 13$]; tested in open field. Note that the hypolocomotor effect in p35 KO mice of both psychostimulants was sustained throughout the time that the test was run. For all the treatments, one-way ANOVA were performed for each time point, for saline: all F's(1,9) > 14.89 and all p's < 0.003; for Amph: all F's(1,10) > 56.87 and all p's < 0.0001, and for methylphenidate: all F's(1,12) > 35.61 and all p's < 0.0001.

WT littermates showed an approximately two-fold increase of TH levels in the CPu and NAc of p35 KO mice compared with WT subjects (Fig. 4b).

Given the high expression levels of TH, mainly in the CPu of p35 KO mice, we next turned to biochemical measurements of DA contents, synthesis and turnover in animals of both genotypes. To explore DA synthesis, we determined the accumulation of L-DOPA in mice pretreated with the DOPA-decarboxylase inhibitor NSD-1015, which blocks the conversion of L-DOPA to DA (Carlsson *et al.* 1977). HPLC-coupled electrochemical detection consistently revealed that CPu from juvenile p35 KO mice accumulated more L-DOPA than CPu from WT mice (Fig. 4c). Moreover, total CPu tissue DA content was found to be significantly increased in p35 KO mutant mice compared with WT animals (Fig. 4d). In contrast, analysis of CPu TH expression levels, DA contents and turnover (DOPAC/DA ratio) in adult p35 KO mice were normal and indistinguishable from those of WT animals (Fig. 5).

When Amph or Mtph were administered to animals of both genotypes, 30 min before killing, the total CPu tissue content of DA was significantly decreased in p35 KO mice, to the same extent as WT mice receiving either saline or psychostimulant drugs (Fig. 6a). Interestingly, CPu DOPAC

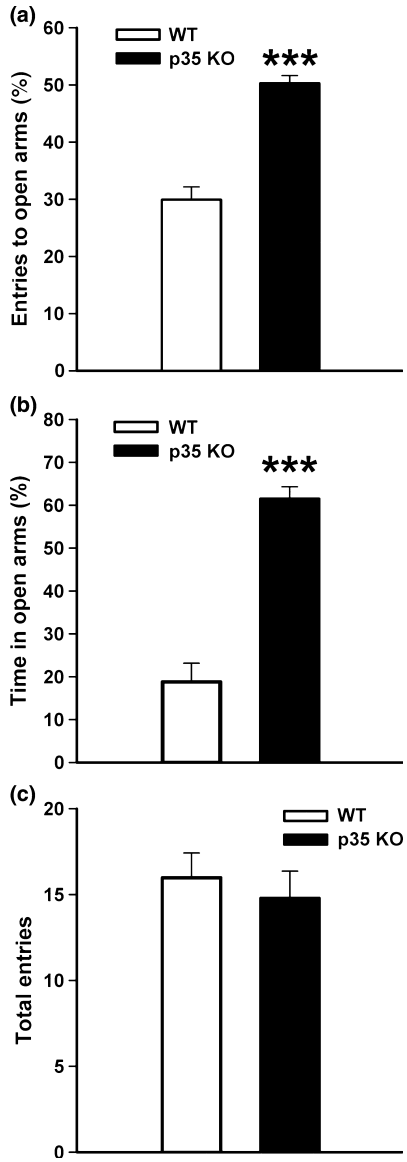

Fig. 3 P35 KO mice displayed less anxiety-like behavior. Mice of both genotypes were challenged in the elevated plus maze. (a) percentage of entries into open arms [$F(1,10) = 45.79$, $p < 0.0001$]; (b) percentage of time inside open arms [$F(1,10) = 53.67$, $p < 0.0001$] and (c) total entries into all arms showed no difference among the two groups [$F(1,10) = 0.316$, $p = 0.58$]. Wild type (WT): $n = 7$; p35 KO: $n = 5$. All statistical analyses were performed through one-way ANOVA. Bars represent the mean ± SEM. ***$p < 0.001$.

content from saline-injected p35 KO mice was lower than from WT animals in the same condition (Fig. 6b), resulting in an approximately 44% decrease in DOPAC/DA ratio in p35 KO mice (Fig. 6c). Furthermore, DOPAC levels and DOPAC/DA ratio in p35 KO mice after psychostimulant treatment were similar to those in WT animals after saline injection (Fig. 6b and c).

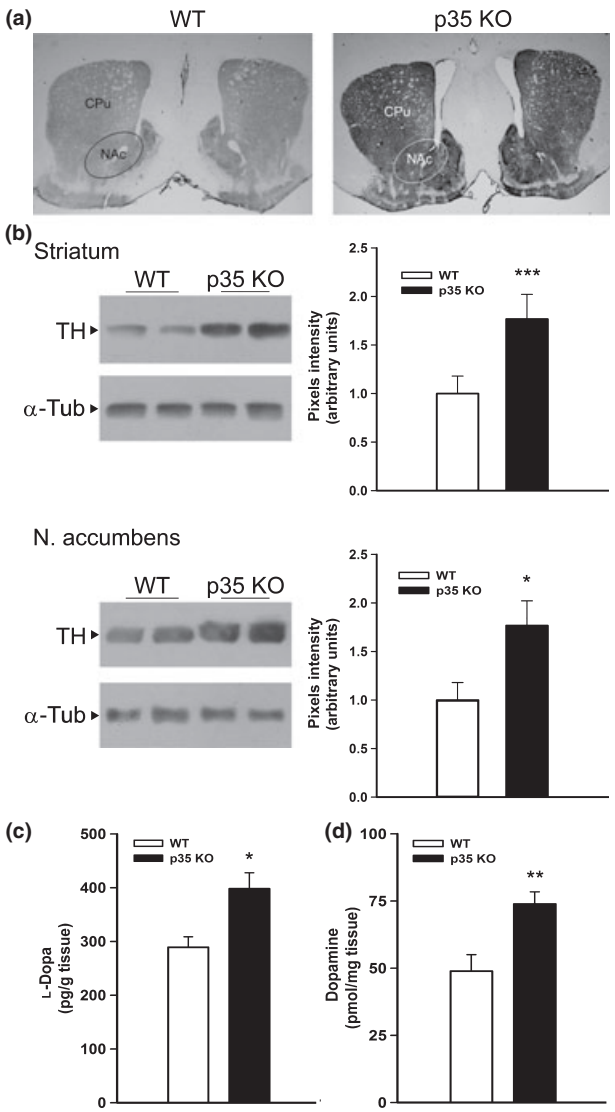

Fig. 4 Tyrosine hydroxylase (TH) protein levels and dopamine synthesis and contents are highly elevated in p35 KO mice. (a) TH immunohistochemistry in coronal brain slices of juvenile naïve wild type (WT) and p35 KO mice at the level of the dorsal striatum-caudate putamen (CPu) and nucleus accumbens (NAc). Note the strong TH immunolabeling in CPu and NAc of p35 KO mice. (b) Representative western immunoblots showing the expression levels of TH in total homogenates of CPu and NAc of naïve WT and p35 KO mice. CPu: $F(1,20) = 20.519$, $p = 0.0002$; $n = 22$. NAc: $F(1,12) = 6.797$, $p = 0.0229$; $n = 14$. (c) Accumulation of L-DOPA in the CPu of naïve WT and p35 KO mice pretreated with the DOPA-decarboxylase inhibitor NSD-1015 [$F(1,17) = 6.679$, $p = 0.019$; $n = 19$]. (d) CPu dopamine contents in naïve WT and p35 KO mice [$F(1,11) = 10.139$, $p = 0.0087$; $n = 13$]. All statistical analyses were performed through one-way ANOVA. (b) Each lane corresponds to an individual animal and bar graphs represent the mean value of the densitometric quantification of TH normalized with α-tubulin (α-Tub) ± SEM; (c) and (d) Bar graphs represent the mean value ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

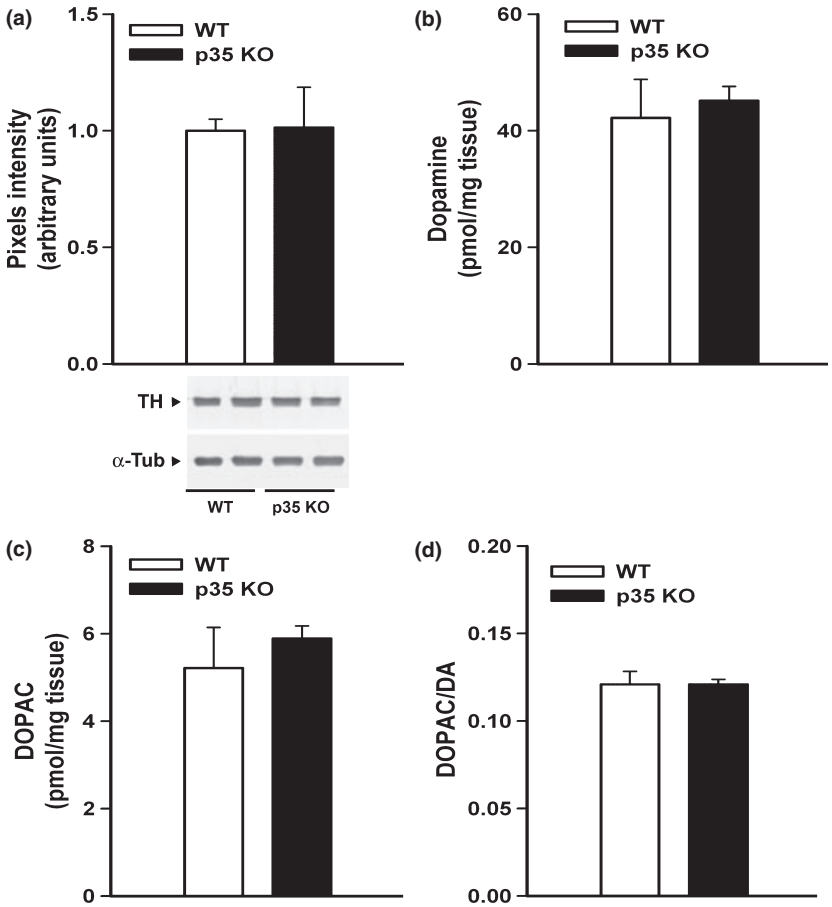

Fig. 5 Tyrosine hydroxylase (TH) protein levels, dopamine (DA) content and turnover are normalized in adult p35 KO mice. (a) Representative western immunoblots showing the expression levels of TH in total homogenates of striatum-caudate putamen (CPu) of adults naïve wild type (WT) and p35 KO mice. Note that TH expression levels are similar in both genotypes ($n = 25$). (b) CPu DA contents in adults naïve WT and p35 KO mice. (c) CPu 3,4-dihydroxyphenylacetic acid (DOPAC) contents in adults naïve WT and p35 KO mice. (d) DOPAC/DA ratios in adults naïve WT and p35 KO mice. DA and DOPAC levels and DA turnover show no differences among both genotypes ($n = 16$). All statistical analyses were performed throughout One-way ANOVA. Bars represent the mean value ± SEM.

These results led us to evaluate if CPu TH expression levels in p35 KO mice are diminished under stimulated conditions. To further address this possibility, total homogenates of CPu from WT and p35 KO animals receiving either saline or psychostimulants (Amph or Mtph), 30 min before killing, were tested by western blot. These analyses revealed a significant decrease in TH expression levels only in p35 KO mutant mice treated either with Amph or Mtph, while TH levels in WT animals remained invariable under saline or psychostimulant conditions (Fig. 6d).

Discussion

Dysregulation of DA signaling is associated with neurological and neuropsychiatric disorders, such as Parkinson's disease, schizophrenia and more recently, ADHD. Previous works have indicated that Cdk5 regulates DA neurotransmission in some neuropsychiatric diseases, especially in drug addiction (Meyer *et al.* 2008; Benavides *et al.* 2007; Mlewski *et al.* 2008; Busceti *et al.* 2008). In this study we demonstrated, for the first time, a critical involvement of Cdk5/p35 complex in the expression of a hyperactive behavioral phenotype with hyper-functioning of the dopaminergic system. By using mutant mice lacking p35, which display reduced Cdk5 activity, we observed that juvenile mice display spontaneous hyperactivity and respond with a paradoxical hypolocomotor effect to psychostimulant drugs, such as Amph or Mtph; also p35 KO mice seems to exhibit deficit of proper behavioral inhibition and/or reduced anxiety-related behavior. DA has been proved to be implicated in this mouse syndrome, as high expression levels of TH are found in relevant brain areas, and DA contents are dramatically augmented in CPu of p35 KO mice, while Amph or Mtph treatments revert DA concentration and TH expression similar to those in WT mice after saline injection. In spite of the high DA concentration, these mutant mice produce less DOPAC than their WT littermates, resulting in a low turnover of DA in basal condition.

The profound spontaneous hyperactivity exhibited by juvenile p35 KO mice exceeds more than two-fold the activity of the WT control littermates (Fig. 1). Furthermore, these mice showed a notable increase of TH expression levels and accumulated more L-DOPA following DOPA decarboxylase inhibition, with a concomitant increase of DA contents in the CPu (Fig. 4). In agreement with our results, pharmacological inhibition of Cdk5 increases evoked dopamine release (Chergui *et al.* 2004). These observations may be consistent with a loss of an inhibitory

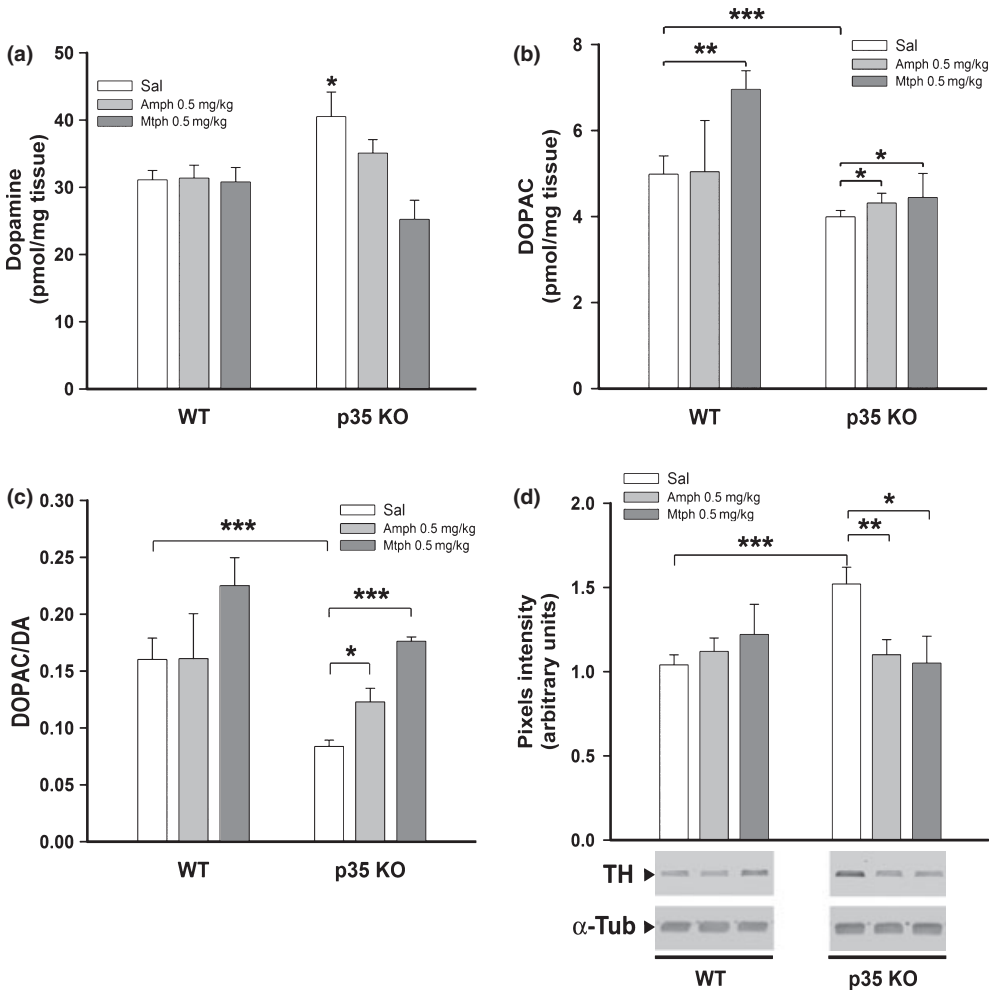

Fig. 6 Psychostimulant drugs revert dopamine (DA) contents, turnover and tyrosine hydroxylase (TH) expression levels in p35 KO mutant mice. (a) striatum-caudate putamen (CPu) DA contents in wild type (WT) and p35 KO mice treated with saline (Sal), amphetamine (Amph) or methylphenidate (Mtph) [treatments × genotype interaction, $F(2,22) = 3.681$, $p = 0.042$]. Note the significant decrease of DA contents in Amph- or Mtph-treated p35 KO mice. (b) CPu 3,4-dihydroxyphenylacetic acid (DOPAC) contents in WT and p35 KO mice treated with Sal, Amph or Mtph, [treatments × genotype interaction, $F(2,22) = 6.985$, $p = 0.015$]. Note the significant increase of DOPAC levels in both psychostimulant treatments in p35 KO mice. (c) DOPAC/DA ratios [treatments × genotype interaction, $F(2,22) = 8.548$, $p = 0.002$]. Note that the treatment of p35 KO mice with psychostimulants revert the DOPAC/DA ratio. (d) Representative western immunoblots showing the expression levels of TH in total homogenates of CPu of WT and p35 KO mice treated with Sal, Amph or Mtph, [treatments × genotype interaction, $F(2,50) = 3.852$, $p = 0.0109$; $n = 56$]. Note the significant decrease of TH levels in both psychostimulant treatments in p35 KO mice. Each lane corresponds to an individual animal and bar graphs represent the mean value of the densitometric quantification of TH normalized with α-tubulin (α-Tub) ± SEM. All statistical analyses were performed throughout two-way ANOVA followed by Fisher's LSD test. Bars represent the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

D2-like autoreceptor, considering that the rate at which TH synthesizes DA is thought to be modulated, in part, through feedback inhibition via stimulation of pre-synaptic D2-like autoreceptors (Sulzer *et al.* 2005). Moreover, it has been demonstrated that Cdk5 is an important regulator of TH activity through stabilization of TH protein levels (Moy and Tsai 2004). Therefore, the lack of p35 in juvenile mice, which display reduced Cdk5 activity, appears to have important consequences in DA neurotransmission. Despite the high DA synthesis in p35 KO mice, they display a low turnover of DA (DOPAC/DA ratio) in basal condition. This reduced metabolism of DA in p35 KO mice can manifest in a number of different scenarios including increased DA synthesis but altered striatal DA release, decreased DA uptake because of abnormal functioning of the DA and/or vesicular monoamine transporters or decreased monoamine oxidase activity, among others. Unfortunately, because of technical difficulties in implanting a microdialysis guide cannula in pre-weaning mice, striatal extracellular dopamine measures remain to be tested in future studies.

The fact that Cdk5 inhibition increases DA release in the CPu (Chergui *et al.* 2004), and changes in striatal levels of Cdk5 were also associated with the onset of levodopa-induced dyskinesias (Aubert *et al.* 2005), demonstrates a pre-synaptic action of Cdk5; but this protein kinase also regulates dopaminergic transmission at post-synaptic level. Thus, Cdk5 inhibitors stimulate dopaminergic neurotransmission both by increasing extracellular levels of DA, which can activate D1 receptor-mediated PKA signaling, and by relieving the post-synaptic Thr75-DARPP-32-mediated inhibition of PKA (Bibb *et al.* 1999). Therefore, PKA is tonically inhibited by phospho-Thr75 DARPP-32 in basal conditions, and Thr75 dephosphorylation results in the dis-inhibition of PKA and the amplification of D1 receptor/PKA/phospho-Thr34 DARPP-32/PP-1 signaling (Nishi *et al.* 2000). In agreement with this, p35 KO mice exhibit a 75% reduction in the basal level of phospho-Thr75 DARPP-32 (Bibb *et al.* 1999), and the high levels of striatal DA (this study), acting at D1 receptors, may lead p35 KO mice to a continuous state of PKA dis-inhibition, resulting in a hyperactive phenotype. In line with this argument and considering that during the normal course of brain development, p35 signal gradually declines toward adulthood (Delalle *et al.* 1997), we also demonstrated that the spontaneous hyperactivity is silenced in adult p35 mutant mice and that DA turnover and TH expression levels are normal and identical from those of WT animals (Fig. 5).

Psychostimulant drugs enhance activity in normal rodents, but exert a calming effect in various hyperactive animals, such as like ADHD models (Russell *et al.* 2005; Sagvolden *et al.* 2005; van der Kooij and Glennon 2007). Amph and Mtph enhanced spontaneous locomotor activity in WT mice, but, in contrast, activity in juvenile p35 KO animals was substantially attenuated (Fig. 2). The observed hyperlocomotor response to low doses of psychostimulants in WT animals, contrary to some previous reports (Xu *et al.* 2000; Berridge *et al.* 2006; Tilley and Gu 2008), is likely caused by the lack of a period of habituation to the locomotor chamber before receiving the injections. However, it is important to point out that the activity scores of p35 KO naïve or saline-injected mice are identical to those observed in both psychostimulant-injected WT animals at all doses tested, except for the highest Amph dose.

Notably, the hypolocomotor response of juvenile p35 KO mice to the action of Amph or Mtph is correlated with the significant decrease of striatal DA contents and TH expression and with the increase of DA turnover (DOPAC/DA), all of them to similar values to those observed in WT animals after saline injection (Fig. 6). In agreement with these findings, it has been demonstrated a significant decrease in the density of TH-immunoreactive fibers, immediately after Mtph administration, in regions implicated in the etiology of ADHD, particularly in the striatum (Gray *et al.* 2007). Therefore, treatment with psychostimulants reverts the behavioral phenotype as well as the neurochemical patterns in p35 KO mice. It is possible that the calming effect of psychostimulants in p35 KO mice may be the result of autoreceptor stimulation that decreases DA synthesis and release (Solanto 1998). This speculation is supported by the pre-synaptic/antagonist and post-synaptic/agonist hypotheses outlined by Seeman and Madras (1998), who proposed that tonic levels would suppress phasic DA release, so that psychostimulants would function as antagonists, thus correcting a DA excess rather than a deficit. Our findings are in line with this hypothesis, because the excessive DA synthesis and high TH expression levels exhibited by p35 KO mice were reversed by the treatment with Amph or Mtph. This premise is further supported by the selective inhibition of TH phosphorylation at Ser40 and reduction of its activity, following by the decrease of DA synthesis after activation of DA D2 autoreceptors, in striatum (Lindgren *et al.* 2001). Post-translational modification, such as phosphorylation, plays an important role in regulating TH function; and alterations in TH levels and enzymatic activity lead to significant changes in catecholamine levels (Dunkley *et al.* 2004). Moreover, and also supporting our findings, the phosphorylation of TH at Ser31 by Cdk5/p35 plays a critical role in the protein stability and in the absence of a proper Cdk5 activity, the protein is unstable and thus more susceptible to degradation (Moy and Tsai 2004). This would also explain the rapid fall in TH levels in psychostimulants-injected mutant p35 KO mice.

The deficiency of p35 protein in the mouse also seems to be critical in anxiety-like behaviors. The EPM, an animal model test of anxiety (Pellow *et al.* 1985; Cruz *et al.* 1994), is an approach/avoidance conflict paradigm in which the mice assess the potential risk or reward associated with safe or unsafe novel areas (Avale *et al.* 2004; Mormede *et al.* 2002). Normal p35 WT rodents avoid entering the open unprotected arms, and therefore the number of entries and the time spent in them are reduced. In contrast, the behavior exhibited in the EPM by juvenile p35 KO animals showed a clear reduction in the conflict approach/avoidance; moving indiscriminately into either protected or unprotected arms and the time spent in both is similar, indicating less basal anxiety-like behavior and/or a possible abnormal evaluation of the potential environment risks. Moreover, the motivation to explore the arms is similar in both genotypes because the total number of entries to all arms did not differ between genotypes (Fig. 3). These EPM behavioral patterns are thought to reflect impairment in behavioral inhibition, where juvenile p35 KO mice might be unable to inhibit inappropriate responses. Impaired behavioral inhibition using EPM was observed previously in mice lesioned perinatally with 6-hydroxydopamine (Avale *et al.* 2004). In addition, similar behaviors were reported in some animal models of ADHD (Mill 2007), one of the most well-characterized of which is

the spontaneously hypertensive rat that displayed the ADHD-like characteristic of decreased anxiety-like behavior in the EPM (Howells *et al.* 2009). Furthermore, prior findings showed that adult p35 KO mice attenuated the usual increase in anxiety-like behavior produced by the inescapable stressful stimulus (Bignante *et al.* 2008). However, as p35 KO animals display cortical neuronal migration deficits and anatomical impairments (Chae *et al.* 1997), and considering that the prefrontal cortex is particularly important in inhibitory control over behavior (Sowell *et al.* 2003; Aron and Poldrack 2005), we cannot rule out that these abnormalities could be related to this emotional feature. More specific tests should be conducted in the future to explore in detail this aspect.

The fact that juvenile p35 KO mice are hyperactive, show an impairment in behavioral inhibition and exhibit paradoxical response to psychostimulants, which are capable of reverting the behavioral and neurochemical phenotype, surprisingly resemble those described in animal models of ADHD (Sagvolden *et al.* 2005; Russell *et al.* 2005; Russell 2007). Despite the similarities between mutant mice and humans with ADHD, it is unlikely that their phenotypes are identical. Nonetheless, our results emphasize the importance of the proper function of Cdk5/p35 kinase activity in DA system for normal motor activity and emotional features, and suggest that common mechanisms may underlie some of their behaviors and responses to psychostimulants. Moreover, the availability of a mouse model to test the participation of targeted gene mutation, related to dopaminergic pathways, that mimics the key hallmarks of this human disease, including hyperactivity and paradoxical response to psychostimulants, is of high value. In view of the high prevalence of ADHD in school-age children and that most of these patients are chronically medicated with psychostimulants (Berman *et al.* 2009), we consider that the mutant mouse deficient in p35 protein may represent a useful animal model, alongside others (Sagvolden *et al.* 2005), to understand the cellular and molecular neurobiology mechanisms underlying this complex and heterogeneous disorder, and further investigations into this mouse model could provide new insights about the mechanisms of action of drugs used to treat ADHD.

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