ているか？」「自閉症の発症と予防接種の時期」「水銀含有ワクチンの使用と自閉症頻度の上昇」「自閉症児・者の生物学的サンプル中の水銀濃度」「水銀汚染地域で自閉症が増加しているか？」などの論点で，水銀と自閉症の関連性については，理論的に根拠が乏しいことを浮き彫りしている．

これに対し，2004年にBernardら[8]は，Nelsonらの批判は新たな事実や調査に基づくものではなく，自閉症と水銀の関連を否定する根拠にならないと反論している．

総合的に見て，現時点では自閉症と水銀の関係については一致した見解はみられず，これを積極的に肯定するエビデンスはないと考えられる．

(2) 自閉症と予防接種ワクチンに含まれるチメロサールに関連があるか？

先のNelsonらはこの点に言及し，チメロサールと自閉症の増加の関係は，チメロサールを含有したワクチンが使用されなくなれば明確になる，としている[7]．この疑問に関して，2003年のMadsenらは，両者の因果関係を明確に否定している[9]．現時点では，自閉症とチロメサール含有ワクチンとの間に明確な関連性は見出されていない．

その一方で，チメロサールそのものの安全性には疑問が投げかけられており[10]，自閉症との関連性の有無にかかわらず，チメロサールを含有しない予防接種ワクチンを使用することが望ましい．

(3) 自閉症に水銀キレート療法は有効か？

水銀キレート療法を提唱している論文は前述の3編[1]~[3]であるが，残念ながら有効とする根拠は提示されておらず，症例報告もみられない．したがって，現時点では，自閉症の水銀キレート療法を有効と結論する根拠は見られない．水銀と自閉症の関連性については否定的なことからも，自閉症に対する水銀キレート療法の有効性を支持できる根拠は乏しい．

水銀キレート療法については，
http://www.autism.com/ari/dan/mercurydetox.html から情報を得ることが可能である．

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Case report

Pyruvate dehydrogenase E1α subunit deficiency in a female patient: evidence of antenatal origin of brain damage and possible etiology of infantile spasms

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Abstract

Enlargement of the lateral ventricles and atrophy of the brain were documented ultrasonographically in utero at as early as 28th week of gestation in a female patient with lactic acidosis due to deficiency of the pyruvate dehydrogenase E1α subunit, demonstrating that the changes characteristic of this disease can occur antenatally. The mechanism of infantile spasms in this disease may be linked to mosaicism of the brain cells involving the normal enzyme and the mutant enzyme.

Keywords: Pyruvate dehydrogenase; E1α deficiency; Antenatal brain damage; Infantile spasms

1. Introduction

Pyruvate dehydrogenase complex (PDHC) is an enzyme complex consisting of five enzymes. The first enzyme of the complex, pyruvate dehydrogenase (E1), is a tetramer consisting of two α-subunits and two β-subunits. A deficiency of α-subunit (E1α) is the most common cause of congenital PDHC deficiency with lactic acidemia [1]. The α subunit gene locus is assigned to Xp22.1 [2]; and accordingly, this gene is subjected to random inactivation. E1α deficiency is often associated with various types of brain damage and neurological symptoms [3], and, notably, a significant difference has been shown in symptoms between female and male patients; and infantile spasms have been encountered almost exclusively in female patients. The antenatal origin of such brain damage has been suspected [4–6], but no supportive evidence has yet been found. Mutations in the gene encoding the α-subunit have been documented in patients with E1α deficiency. We herein report a female patient with E1α deficiency who developed infantile spasms, and in whom anomalous development of the brain was antenatally demonstrated.

2. Case report

This female patient was born after 40 weeks of uncomplicated pregnancy to healthy nonconsanguineous parents. The birth weight was 2020 g. A routine ultrasonographic study performed at the 28th week of gestation showed enlargement of the lateral ventricles of the fetus. A similar finding was noted at the 32nd week of gestation (Fig. 1A). Due to early detection of this enlargement, the baby was transferred to a neonatal intensive care unit immediately after birth. Laboratory findings at this time indicated metabolic acidosis and hyperammonemia (151 μmol/l). Auditory-evoked brainstem response was negative. Elevated concentrations of lactic acid (5.8 mmol/l) and pyruvic acid (0.6 mmol/l) in blood were first found at the age of 70 days. The karyotype of the patient was 46, XX. At the age of 6 months, she developed tonic seizures with series formation, which was diagnosed as infantile spasms. An electroencephalographic tracing taken at this time showed modified hypsarrythmia. Because the

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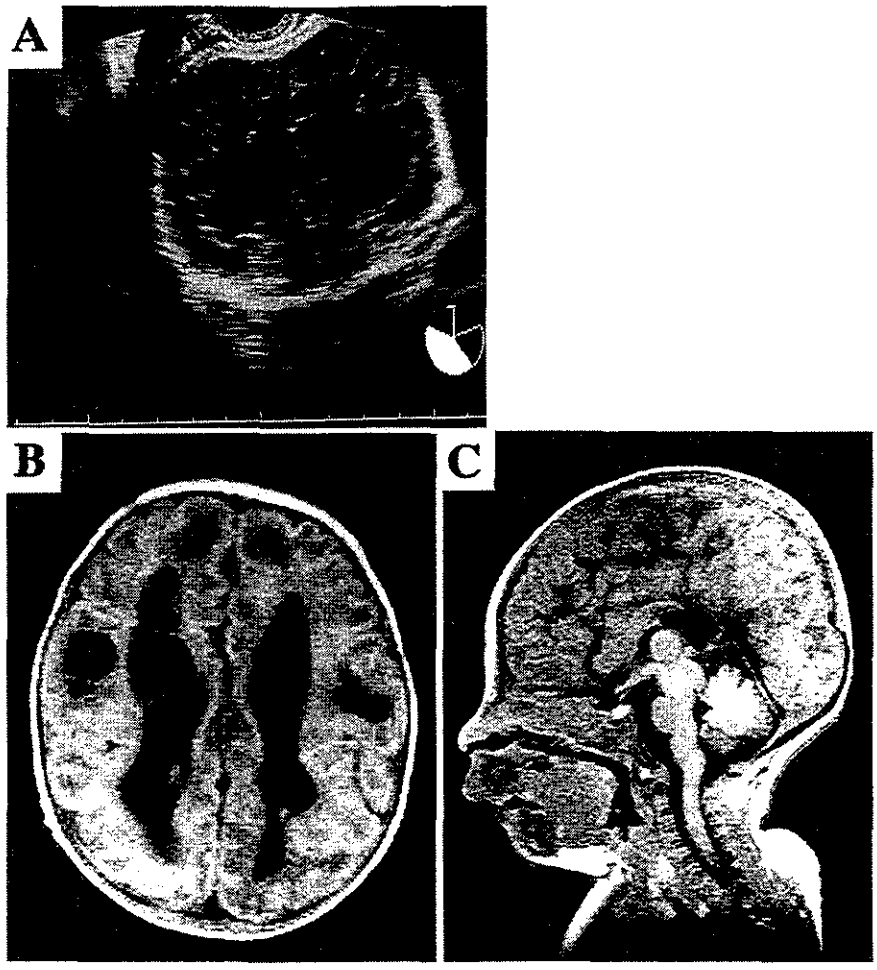

Fig. 1. Enlargement of the lateral ventricles of the fetus was reconfirmed by ultrasonography at the 32nd week of gestation (A). T1-weighted magnetic resonance imaging of the brain recorded at 1 month of age revealed atrophic change of the cerebrum with multiple cystic changes in the brain substance, formation of the intraventricular septum, enlargement of the posterior horns of the lateral ventricles (B), atrophy of the brainstem, and partial agenesis of the corpus callosum (C).

seizure was refractory to vitamin B6, the patient was hospitalized at the age of 6 months for evaluation and management of the seizure. Physical examination on admission revealed a female infant with microcephaly, facial dysmorphism; frontal bossing, laterally upslanting palpebral fissures, depressed nasal bridge, short upturned nose and shark mouth. She had achieved virtually no psychomotor development and had moderate generalized hypotonia with trunkal predominance. The laboratory tests revealed elevated blood concentrations of lactic acid (5.5 mmol/l) and pyruvic acid (0.6 mmol/l), and elevated alanine (1.2 mmol/l) and proline (0.44 mmol/l) levels in serum. Presumptive diagnosis of PDHC E1α deficiency was made at this time. The seizure was ameliorated by the administration of sodium valproate and clonazepam, and the patient was subsequently discharged.

Thiamine hydrochloride at a dose of 10 mg/kg/day was started at the age of 8 months. The patient was readmitted at the age of 11 months, when elevated concentrations of lactic acid (6.2 mmol/l) and pyruvic acid (0.6 mmol/l) in cerebrospinal fluid were found, for the further evaluation. At the age of 13 months, the patient was placed on a high-fat formula. At 16 months of age, after diagnosis had been confirmed by enzyme measurements, administration of sodium dichloroacetate (DCA) was started and maintained at a dose of 50 mg/kg/day for the first 3 months, then tapered to 25 mg/kg/day later (DCA administration had been withheld per parental request until the diagnosis had been confirmed by enzyme analysis). The concentrations of lactic acid and pyruvic acid in blood decreased, with marked fluctuations, shortly after the introduction of DCA and have remained at levels approximately half the pretreatment values. The pretreatment concentrations of lactic acid and pyruvic acid in cerebrospinal fluid were close to those in the blood, and the former levels decreased to approximately two-thirds the pretreatment value 7 days after the initiation of DCA. Nevertheless, no changes in clinical signs have occurred since the introduction of the therapy.

The cranial computerized tomographic (CT) scan on the first day of life showed multiple leukomalacia and enlargement of the lateral ventricles with irregularity of the ventricular walls (figure not shown). Cranial magnetic resonance imaging, first performed at the age of 1 month, revealed, in addition to essentially the same findings as those shown in the previous CT scan, atrophic change of the cerebrum with multiple cystic changes in the brain substance, formations of the intraventricular septum, enlargement of posterior horns of the lateral ventricles (Fig. 1B), atrophy of the brainstem and partial agenesis of corpus callosum (Fig. 1C). The enlargement of the ventricles and atrophic changes of the cerebrum became more marked on the follow-up imagings performed at the ages of 6, 12, and 14 months (figures not shown), irrespective of treatment.

The overall activity (unit, nmol/min/mg protein) of DCA-activated PDHC in cultured skin fibroblasts, measured as described elsewhere [7], was moderately decreased; 0.75 (control range, 2.38 ± 0.60) in the presence of 0.4 mmol/l thiamine pyrophosphate, and 0.53 (control range, 2.31 ± 0.62) in the presence of 1×10^{-4} mmol/l thiamine pyrophosphate. The DCA-activated activity (unit, nmol/h/mg protein) of E1 was 0.69 (control range, 10.7 ± 4.0). Mutational analysis of the E1α gene of the present patient, performed as previously described [7], revealed a deletion of one of the seven base-pair (AGTAAGA) segments of the tandem repeat (nt positions 927–940) in exon 10, creating a termination codon downstream of the deleted segment (nt positions 974–976 in the wild-type sequence).

3. Discussion

It has been postulated that in female patients with E1α deficiency, structural anomalies and degenerative changes in the brain would occur antenatally [4–6]. Observations in our case first demonstrate this postulation, and then confirm that significant retardation of fetal brain development occurs as early as the end of the second trimester in this disease. Additional damage to the brain would be superimposed between that time and delivery because development of the fetal brain becomes more dependent on PDH E1α activity after the mid-organogenesis stage [8]. These observations also suggest that postnatal intervention alone is of limited therapeutic effect. This disease should be included as a differential diagnosis when dilatation of the lateral ventricles is found antenatally.

Scrutiny of clinical records of patients with E1α deficiency [3] revealed that male patients who survived the neonatal period and early infancy generally have a milder phenotype, including mental retardation, ataxia and mild carbohydrate-sensitive lactic acidemia, than female patients, who usually present with seizures, severe developmental delay and structural abnormality of the central nervous system. This observation seems inconsistent with a general rule that in most X-linked dominant diseases, hemizygous male patients usually have a more severe phenotype than heterozygous female patients. A possible explanation for this apparent discrepancy may be as follows. Males who carry a mutant E1α allele that leads to severe deficiency of the enzyme are selected antenatally, and consequently male patients who harbor a mutant E1α allele that results in a less severe enzyme deficiency survive the neonatal period and present as male patients with a mild phenotype. In females, on the other hand, possession of a mutant allele leading to severe enzyme deficiency could still be consistent with a live birth, depending on a pattern of X-inactivation; such individuals would then present a more severe phenotype than male patients who have the mild phenotype.

The pathogenesis of infantile spasms is variable. Neuropathological findings reported in infantile spasms include gross developmental malformations, such as agenesis of the corpus callosum and multifocal dysplastic lesions [9]. The latter is considered to have particular relevance to the mechanism of infantile spasms [10,11]. These cerebral lesions have been observed almost exclusively in female patients with E1α deficiency [3,4], with the exception of one male patient [3]. The vast majority of patients presenting with infantile spasms have been female [12,13]. The presence of such multiple lesions may be linked in part to the pathogenesis of infantile spasms in this disease.

The mosaicism of brain cells may have an implication also in terms of the therapeutic effect of DCA. DCA may not be meaningful in improving pyruvate oxidation in the mutant cells, although it may be effective in reducing the lactic acid released from mutant cells through activation of the enzyme in normal cells. Thus, DCA may have limited therapeutic effect on brain metabolism in female patients.

The seven-base-pair deletion in exon 10 has been reported in multiple patients [14,15]. Clinical presentation among those patients, including the present one, was variable. Though both the nature of mutation and the pattern of the lyonization may affect the phenotype, the former seems to have a more significant influence, as this particular mutation would result in a severe impairment of cellular function in half of the cells of the central nervous system; this is because the mutation is expected to express null activity of the enzyme, given that the wild-type allele and the mutant allele are equally inactivated.

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Noonan Syndrome, Moyamoya-like Vascular Changes, and Antiphospholipid Syndrome

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This reports describes a 12-year-old Japanese female with Noonan syndrome who had antiphospholipid syndrome and moyamoya-like vascular changes. She presented choreic movements in her face and extremities. She manifested phenotypic features of Noonan syndrome with short stature, mental retardation, and a webbed neck. Magnetic resonance angiography revealed occlusion of bilateral internal carotid arteries and moyamoya-like vascular changes around the basal ganglion region. Pimozide completely resolved the patient's choreic movements. Tests for anticardiolipin antibody and lupus anticoagulant were positive. The patient has manifested no symptoms for 2 years with pimozide, aspirin, and growth hormone treatment, without further aggravation of moyamoya-like vascular changes. This article is the first report of Noonan syndrome with antiphospholipid syndrome and moyamoya-like vascular lesions.

Yamashita Y, Kusaga A, Koga Y, Nagamitsu S, Matsuishi T. Noonan syndrome, moyamoya-like vascular changes, and antiphospholipid syndrome. Pediatr Neurol 2004;31: 364-366.

Introduction

Noonan syndrome is characterized by a normal karyotype and clinical features that resemble Turner syndrome. An association between Noonan syndrome and moyamoya has been reported in only two cases [1,2]. In adults, the association between strokes and antiphospholipid antibodies has been established, but such an association with moyamoya disease has not been identified.

This report describes for the first time a first patient with a rare combination of Noonan syndrome, moyamoya-like vascular changes, and antiphospholipid syndrome.

Case Report

In June 2001, a 12-year-old female experienced difficulty in walking because of involuntary movement in the left upper and lower limbs. She was observed at Saga University Hospital. Cranial magnetic resonance imaging was normal. By the end of June, her symptoms improved. She was diagnosed as having antiphospholipid syndrome because of thrombocytopenia and positive anticardiolipin antibody. None of her family members had similar problems. The family did not return to the hospital because the patient was completely well. In February 2002, however, she had chorea again starting from the right hand and extending to the right foot. In April, her chorea extended to the left side and she began to speak less. The family saw a neurosurgeon, and cranial magnetic resonance angiography revealed moyamoya-like vascular changes. The patient was then referred to Kurume University Hospital for further evaluation and treatment.

On admission, her height was 120.8 cm (−5 SD) and her weight was 27 kg (−2 SD). She had multiple purpura lesions in her extremities. She manifested phenotypic features of Noonan syndrome, including a webbed and short neck, low posterior hair lines, low-set and abnormal auricles, and hyperterolism. She manifested no signs of lupus or secondary sexual development. The patient had no history of seizures, and no cardiac lesions were evident. There was no family history of thrombosis.

Neurologically, she was alert, but had dysarthria caused by facial chorea. Her chorea was more prominent in the right side upper and lower limbs with hypotonia. Her deep tendon reflexes were elevated, and the Babinski reflex was positive on the right side. Her full intelligence quotient (Wechsler Intelligence Scale for Children–third version [WISC-III]) was 53; verbal intelligence quotient 60, performance intelligence quotient 55. Her social performance was relatively good, and she was in a mainstream classroom.

Complete blood count revealed low platelets (90,000). Prothrombin time and activated partial thromboplastin time were mildly prolonged, and lupus anticoagulant 1.76 (normal <1.3), anticardiolipin antibody 100 (normal <10), anti-b2 glycoprotein, and antinuclear antibody were positive. Anti-Sm, anti-RNA, anti-SS-A/Ro, and anti-SS-B/La antibodies were negative. Protein C and S were normal. Her bone age was delayed, and growth hormone secretion was abnormal by both an L-arginine and glucagon loading test. Auditory brainstem response and electroencephalogram were normal. The cranial computed tomography revealed mild

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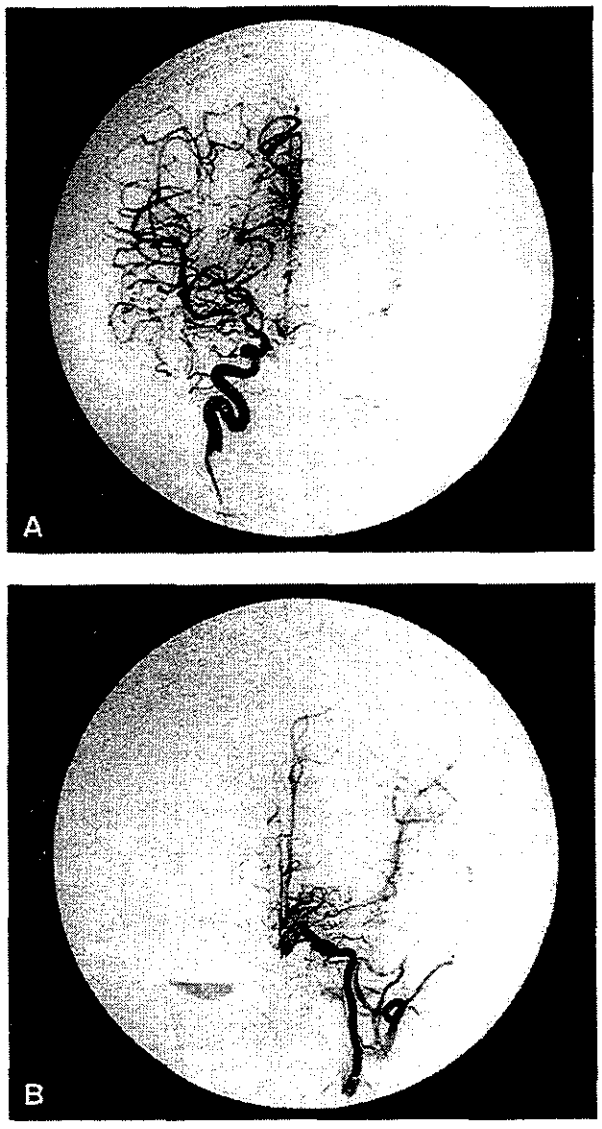

Figure 1. Cerebral angiogram: Right carotid angiogram indicated severe stenosis of internal carotid artery terminal lesion (A), and left carotid angiogram (B) demonstrated severe stenosis in the supraclinoid segment and collateral moyamoya-like vascular changes distal to the stenosis. The left internal carotid artery was narrower than the left side.

atrophy of the parietal lobe in the right hemisphere. The cranial magnetic resonance imaging revealed low-intensity areas in the basal ganglia on the right side. The cranial magnetic resonance angiography documented bilateral stenosis of the internal carotid arteries, which was more prominent on the right side, and moyamoya-like vascular changes in the bilateral basal ganglia and thalami region (Fig 1). Her chromosome count was normal (46 XX).

The patient was treated with a D2 antagonist, pimozide (1.5 mg/day), and her chorea completely resolved by the end of May. She has manifested no symptoms for 2 years with pimozide, aspirin (100 mg/day), and growth hormone treatment, with no further aggravation of moyamoya-like vascular changes.

Discussion

This patient was diagnosed with antiphospholipid syndrome because she had thrombocytopenia, positive anticardiolipin antibody, and lupus anticoagulant. These findings were repeatedly demonstrated at 10-month intervals. Furthermore, the patient manifested characteristic features of Noonan syndrome, including short stature, facial and neck abnormalities, mild mental retardation, and a normal karyotype. On magnetic resonance angiography, she also manifested moyamoya-like vascular changes in bilateral carotid arteries and basal ganglia. She was diagnosed with moyamoya disease because she had underlying Noonan syndrome and antiphospholipid syndrome.

Booth et al. have reported a 7-year-old female with an ischemic event in association with repeated elevation of anticardiolipin antibody [3]. They demonstrated bilateral moyamoya-like vascular changes. The patient was treated with warfarin for 5 months, followed by aspirin. Eleven months after the treatment, a marked improvement in blood flow with decreased stenosis of the left internal carotid was observed. They speculated that the development of moyamoya-like vascular changes might be secondary to initial thrombosis and stenosis of the basal cerebral vasculature with subsequent formation of collateral vessels. Takahashi et al. reported eight children with acute hemiplegia, with three being diagnosed as having infarctions due to moyamoya [4]. Anticardiolipin immunoglobulin G antibody was positive in three of the five with idiopathic infarction, but none with moyamoya disease, suggesting that the etiology of the infarct might be distinct from that in the patients with idiopathic infarction. In contrast, Bonduel et al. reported detecting prothrombotic disorders in 4 of 10 patients with moyamoya disease, suggesting its role in the pathogenesis of moyamoya [5]. Shoning et al. reported eight children who suffered from cerebrovascular ischemia or stroke in which antiphospholipid antibodies were detected. In two patients, stenoses of the basal cerebral arteries were present; a 5-year-old female with moyamoya-like vascular changes manifested improved circulation after treatment with aspirin and intravenous immunoglobulin, whereas a male patient required surgery for encephalo-duro-arterio-synangiosis [6].

There have been only two case reports of Noonan syndrome and moyamoya [1,2]. One of these patients had activated protein C resistance, which was thought to be coincidental. Antiphospholipid antibodies were not measured in these cases. Both patients were treated with aspirin and were responsive to nonsurgical therapy.

Encephalo-duro-arterio-synangiosis surgery was also considered for the patient in the present report; we decided not to pursue this route, however, because pimozide dramatically improved her symptoms and no further recurrence or progression of moyamoya-like vascular change occurred. In patients with moyamoya disease or moyamoya-like vascular changes, the possibility of antiphospholipid syndrome should be considered.

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Effect of methylphenidate on dopamine/DARPP signalling in adult, but not young, mice

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Abstract

Methylphenidate (MPH), a dopamine uptake inhibitor, is the most commonly prescribed drug for the treatment of attention-deficit/hyperactivity disorder (ADHD) in children. We examined the effect of MPH on dopamine- and cAMP-regulated phosphoprotein, M_r 32 kDa (DARPP-32) phosphorylation at Thr34 (PKA-site) and Thr75 (Cdk5-site) using neostriatal slices from young (14–15- and 21–22-day-old) and adult (6–8-week-old) mice. MPH increased DARPP-32 Thr34 phosphorylation and decreased Thr75 phosphorylation in slices from adult mice. The effect of MPH was blocked by a dopamine D1 antagonist, SCH23390. In slices from young mice, MPH did not affect DARPP-32 phosphorylation. As with MPH, cocaine stimulated DARPP-32 Thr34 phosphorylation in slices from adult, but not from young mice. In contrast, a dopamine D1 agonist, SKF81297, regulated DARPP-32 phosphorylation comparably in slices from young and adult mice, as did methamphetamine, a dopamine releaser. The results suggest that dopamine synthesis and the dopamine transporter are functional at dopaminergic terminals in young mice. In contrast, the lack of effect of MPH in young mice is likely attributable to immature development of the machinery that regulates vesicular dopamine release.

Keywords: attention-deficit/hyperactivity disorder, DARPP-32, dopamine, methamphetamine, methylphenidate, neostriatum.

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Attention deficit/hyperactivity disorder (ADHD) is the most common behavioral disorder in children. The behavioral symptoms in children with ADHD are characterized by inattention and/or hyperactivity–impulsivity and are maladaptive and inconsistent with developmental level (American Psychiatric Association 1994). The prevalence of ADHD, based on the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria (American Psychiatric Association 1994), is estimated to be at least 3 to 5% of school-aged children (Solanto 2001). Dysfunction of dopaminergic neurotransmission has been implicated in the pathogenesis of ADHD (Castellanos and Tannock 2002). Neuroimaging studies in ADHD revealed abnormalities in volume or asymmetry of caudate nucleus (Castellanos 2001) and a higher expression of dopamine transporter (DAT) in untreated adult ADHD patients than in controls (Dougherty *et al.* 1999; Krause *et al.* 2000). In studies of candidate genes linked to dopaminergic pathways, two dopaminergic genes, dopamine transporter gene (DAT1) (Cook *et al.* 1995) and dopamine D4 receptor gene (Faraone *et al.* 2001), were found to be associated with susceptibility to ADHD.

Methylphenidate (MPH) is the most commonly used agent for the treatment of ADHD in children (Greenhill 2001). MPH and D-amphetamine act as psychomotor stimulatory agents in adults, but as calming agents in children, especially

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Abbreviations used: ADHD, attention-deficit/hyperactivity disorder; cAMP, cyclic AMP; Cdk5, cyclin-dependent kinase 5; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa; DAT, dopamine transporter; METH, methamphetamine; MPH, methylphenidate; PKA, cAMP-dependent protein kinase; PP-1, protein phosphatase-1; TH, tyrosine hydroxylase; TTX, tetrodotoxin, VMAT2, vesicular monoamine transporter 2.

in ADHD patients. It is not understood why the effects of these agents are paradoxical in children (Cirulli and Laviola 2000). MPH binds to DAT and inhibits dopamine uptake into dopaminergic terminals, leading to an increase in extracellular dopamine in the striatum (Nestler *et al.* 2001). In fact, orally administered MPH, at the low dose clinically used for the treatment of ADHD, has been shown to increase extracellular dopamine in healthy adults (Volkow *et al.* 2001). An acute increase in extracellular dopamine, induced by psychostimulants such as methamphetamine and cocaine, results in the activation of locomotor activity in adult rats (Taylor and Jentsch 2001). However, in young pre-weanling rats, psychostimulants fail to stimulate locomotor activity (Laviola *et al.* 1992, 1994) or paradoxically enhance the normal tendency to maintain contact with conspecifics (Campbell and Randall 1977). Both hypodopaminergic (Shaywitz *et al.* 1976; Cardinal *et al.* 2001) and hyperdopaminergic (Giros *et al.* 1996; Viggiano *et al.* 2002) states in animal models are associated with increased locomotor activity. Grace (2001) has hypothesized that a low dopaminergic tone in basal conditions is involved in the pathophysiology of ADHD. MPH at a low dose might exert its therapeutic action by inhibiting DAT and increasing the tonic (basal) level of extracellular dopamine, which in turn would result in the activation of dopamine D2 autoreceptors at dopaminergic terminals. In this condition, the phasic release of dopamine triggered by dopaminergic neuron firing would be reduced and the subsequent activation of dopamine D1 receptor signaling would be attenuated.

DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa) is a cytosolic protein that is selectively enriched in medium spiny neurons in the neostriatum. When DARPP-32 is phosphorylated by cAMP-dependent protein kinase (PKA) on Thr34, it is converted into a potent inhibitor of protein phosphatase-1 (PP-1). DARPP-32 Thr34 phosphorylation leads to an increase in the state of phosphorylation of downstream PP-1 substrates, including various neurotransmitter receptors and voltage-gated ion channels (Greengard *et al.* 1999). In addition to Thr34, DARPP-32 is phosphorylated at Thr75 by cyclin-dependent kinase 5 (Cdk5). DARPP-32 phosphorylated at Thr75 inhibits PKA activity and thereby reduces the efficacy of dopamine signaling (Bibb *et al.* 1999). Mice lacking DARPP-32 exhibit profound deficits in their molecular, electrophysiological and behavioral responses to dopamine, drugs of abuse and anti-psychotic medication, demonstrating the importance of DARPP-32 in most of the actions of dopamine (Fienberg *et al.* 1998). To further characterize the biochemical actions of MPH in young and adult animals, we investigated the effects of MPH on dopamine signaling in neostriatal slices prepared from young and adult mice by analyzing DARPP-32 phosphorylation at Thr34 and Thr75. The results indicate that MPH is able to stimulate D1 receptor signaling in slices from adult mice but not in slices from young mice. This effect was not apparently caused by any immaturity in the dopamine transporter or in the components of D1-dependent signal transduction in neostriatal neurons. Rather, the lack of effect of MPH in young mice was attributed to immature development of the machinery that regulates vesicular dopamine release.

Materials and methods

Preparation and incubation of neostriatal slices

Male C57BL/6 mice at 14–15 days old, 21–22 days old and 6–8 weeks old were killed by decapitation. The brains were rapidly removed and placed in ice-cold, oxygenated Krebs-HCO_3^- buffer (124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 10 mM D-glucose, pH 7.4). Coronal slices (350 μm) were prepared using a vibrating blade microtome, VT1000S (Leica Microsystems, Nussloch, Germany). Striata were dissected from the slices in ice-cold Krebs-HCO_3^- buffer. Each slice was placed in a polypropylene incubation tube with 2 mL of fresh Krebs-HCO_3^- buffer containing adenosine deaminase (10 μg/mL). The slices were pre-incubated at 30°C under constant oxygenation with 95% O_2/5% CO_2 for 60 min. The buffer was replaced with fresh Krebs-HCO_3^- buffer without adenosine deaminase after 30 min of pre-incubation. Slices were treated with drugs as specified in each experiment. Drugs were obtained from the following sources: cocaine from Takeda Chemical Industries (Osaka, Japan); methamphetamine from Dainippon Pharmaceutical Co. (Osaka, Japan); MK801, SKF81297, SCH23390, raclopride, reserpine from Sigma-Research Biochemicals International (St Louis, MO, USA); tetrodotoxin (TTX) from Wako Pure Chemical (Osaka, Japan); CNQX from Tocris Cookson (Bristol, UK). After drug treatment, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at −80°C until assayed.

In some experiments, neostriatal slices were prepared from mice which had been pre-treated with a vesicular monoamine transporter inhibitor, reserpine (5 mg/kg), to deplete vesicular stores of monoamines (Chiueh and Moore 1975; Dobrev *et al.* 1995). Reserpine was dissolved in glacial acetic acid and diluted to 1 mg/mL with water. The final concentration of acetic acid was 1%. The reserpine solution (5 mL/kg) was subcutaneously injected 3 h before killing.

Immunoblotting

Frozen tissue samples were sonicated in boiling 1% sodium dodecyl sulfate (SDS) and boiled for an additional 10 min. Small aliquots of the homogenate were retained for protein determination by the BCA protein assay method (Pierce, Rockford, IL, USA) using bovine serum albumin as standard. Equal amounts of protein (100 μg) were separated by sodium dodecyl sulfate – polyarcylamide gel electrophoresis (SDS–PAGE, 12%), and transferred to nitrocellulose membranes (0.2 μm) (Schleicher and Schuell, Keene, NH, USA) as described (Towbin *et al.* 1979). The membranes were sequentially immunoblotted using a phosphorylation state-specific antibodies raised against a DARPP-32 peptide containing phospho-Thr34, the site phosphorylated by PKA (mAb-23; 1 : 750 dilution) (Snyder *et al.* 1992) and raised against a DARPP-32 peptide containing phospho-Thr75, the site phosphorylated by Cdk5 (1 : 5000 dilution) (Bibb *et al.* 1999). A monoclonal antibody

(C24–5a; 1 : 7500 dilution) generated against DARPP-32 (Hemmings and Greengard 1986), which is not phosphorylation state-specific, was used for re-blotting the membrane in order to determine the total amount of DARPP-32 in samples. None of the experimental manipulations used in the present study altered the total amount of DARPP-32.

Antibody binding was revealed by incubation with a goat anti-mouse horseradish peroxidase-linked IgG (1 : 2000 dilution) (Pierce) or a goat anti-rabbit horseradish peroxidase-linked IgG (1 : 2000–4000 dilution) and the ECL immunoblotting detection system (Amersham, Arlington Heights, IL, USA). Chemiluminescence was detected by autoradiography using Kodak autoradiography film, and phospho-Thr34 and phospho-Thr75 DARPP-32 bands were quantified by densitometry using NIH Image 1.61 software. As the linear range for quantitation of signal density using the ECL detection method is limited to less than 10-fold, we routinely exposed chemiluminescent membranes to film for varying periods of time in order to obtain autoradiograms that provided signals within the linear range for densitometry (Nishi *et al.* 1999). Samples from control and drug-treated slices were analyzed on individual immunoblots. For each experiment, values obtained for treated slices were calculated relative to the value for the control slices. Normalized data from multiple experiments were averaged and statistical analysis was carried out as described in the figure legends.

Results

Effect of MPH on DARPP-32 phosphorylation in neostriatal slices

Neostriatal slices prepared from adult mice (6–8 weeks old) were incubated with MPH to examine the effect on DARPP-32 phosphorylation. Phosphorylation of DARPP-32 at Thr34 was detectable under basal conditions [the stoichiometry of phosphorylation has been estimated to be 0.5–1.0% under basal conditions (Nishi *et al.* 1997)]. Treatment with MPH (100 μM) increased the level of phospho-Thr34 DARPP-32 maximally by ~7-fold within 2 min of incubation (Fig. 1a). The increase in DARPP-32 Thr34 phosphorylation was maximal until 10 min of incubation. Treatment with MPH for 5 min stimulated DARPP-32 Thr34 phosphorylation maximally at a concentration of 100 μM with a half maximal effect at ~10 μM (Fig. 1b). In contrast to the effect on Thr34, treatment with MPH (100 μM) decreased the level of phospho-Thr75 DARPP-32 by ~50% within 2 min of incubation (Fig. 1c). Treatment with MPH for 5 min decreased DARPP-32 Thr75 phosphorylation with a half maximal effect at ~20 μM, which was slightly higher than that for Thr34 phosphorylation (Fig. 1d).

As MPH is known to inhibit dopamine re-uptake into dopaminergic terminals via the dopamine transporter, we examined the role of dopamine in the action of MPH. Pre-treatment of slices with a dopamine D1-type receptor antagonist, SCH233990 (1 μM), did not affect the basal level of phospho-Thr34 DARPP-32, but abolished the stimulatory effect of MPH on Thr34 phosphorylation (Fig. 2a). In the presence of both D1-type and D2-type dopamine receptor antagonist, SCH23390 and raclopride (1 μM), MPH had no effect on Thr34 phosphorylation. Treatment with MPH decreased Thr75 phosphorylation in control conditions, but, in the presence of SCH23390, MPH increased the level of phospho-Thr75 DARPP-32. The MPH-induced increase in Thr75 phosphorylation in the presence of SCH23390 was not seen after pre-treatment with SCH23390 plus raclopride. Raclopride alone had no significant effect on either the basal or the MPH-decreased level of phospho-Thr75 DARPP-32 (data not shown). These results indicate that the effects of MPH on DARPP-32 Thr34 and Thr75 phosphorylation are mediated through an increase in dopamine concentration at extracellular synaptic spaces and requires only the activation of dopamine receptors.

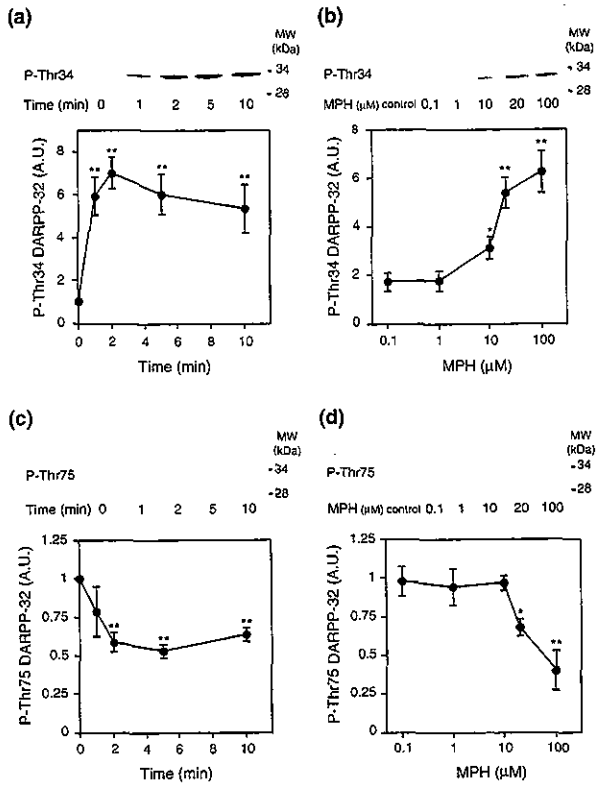

Fig. 1 Methylphenidate (MPH) increases DARPP-32 Thr34 phosphorylation and decreases DARPP-32 Thr75 phosphorylation in neostriatal slices from adult mice. Neostriatal slices were incubated with MPH (100 μM) for the indicated times (a, c) or with the indicated concentrations of MPH for 5 min (b, d). Immunoblots for detection of phospho-Thr34 DARPP-32 (a, b) and phospho-Thr75 DARPP-32 (c, d) are shown in upper panels. The levels of phospho-Thr34 DARPP-32 and phospho-Th75 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for four to eight experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with control; analysis of variance and Newman–Keuls test.

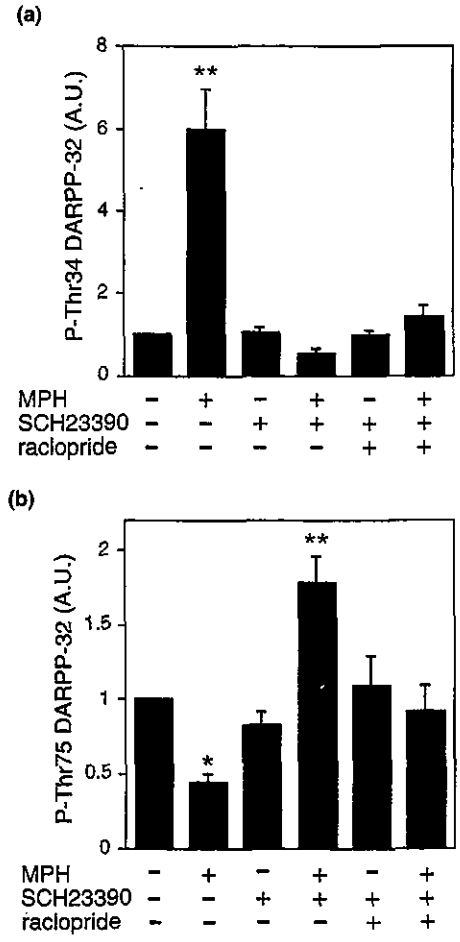

Fig. 2 Effects of methylphenidate (MPH) on DARPP-32 phosphorylation are mediated through activation of dopamine D1-type and D2-type receptors. Neostriatal slices were incubated for a total of 15 min in the absence or presence of a dopamine D1 receptor antagonist, SCH23390 (1 μM), or SCH23390 plus a dopamine D2 receptor antagonist, raclopride (1 μM). SCH23390 or raclopride was added at 0 min, and MPH (100 μM) at 10 min of incubation. The levels of phospho-Thr34 DARPP-32 (a) and phospho-Th75 DARPP-32 (b) were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for five to ten experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with control; analysis of variance and Newman–Keuls test.

Effect of MPH on DARPP-32 phosphorylation in young and adult mice

We next examined the effect of MPH (100 μM) on DARPP-32 phosphorylation in neostriatal slices prepared from young and adult mice (Fig. 3). The amounts of total DARPP-32 in neostriatal slices were similar in young and adult mice, but the basal levels of phospho-Thr34 and phospho-Thr75 DARPP-32 increased from 14–15 days old to 6–8-weeks old by 1.9-fold and 1.5-fold, respectively (data not shown). In slices prepared from 14–15-day-old mice, treatment with MPH (100 μM) had no effect on DARPP-32 phosphorylation either at Thr34 or Thr75 (Figs 3a and d). In 21–22-day-old mice, treatment with MPH (100 μM) slightly increased the level of phospho-Thr34 DARPP-32, but the effect was not statistically significant (Fig. 3b). MPH significantly decreased the level of phospho-Thr75 DARPP-32 at 5 min of incubation (Fig. 3e), but the effect of MPH was less than that in 6–8-week-old adult mice (Fig. 3f).

In contrast to the results with MPH, treatment with a dopamine D1 receptor agonist, SKF81297 (1 μM), increased DARPP-32 Thr34 phosphorylation and decreased DARPP-32 Thr75 phosphorylation similarly in young and adult mice (Table 1). These results suggest that the dopamine D1-type receptor signaling pathway in neostriatal medium spiny neurons is fully functional in young mice, but that the machinery for dopamine release and/or reuptake, or its regulation at presynaptic dopaminergic terminals is immature in young mice.

Effect of cocaine on DARPP-32 phosphorylation in young and adult mice

Cocaine is known to act as a psychostimulant by inhibiting dopamine re-uptake into dopaminergic terminals. The pharmacological action of cocaine would be expected to be similar to that of MPH, and therefore cocaine was examined for its effect on DARPP-32 phosphorylation. Neostriatal slices were treated with 100 μM cocaine, a concentration at which MPH induced maximal effects on DARPP-32 phosphorylation. In adult mice, treatment of neostriatal slices with cocaine transiently increased the level of phospho-Thr34 DARPP-32, with a maximal effect of 5.5-fold within 30 s of incubation (Fig. 4c). The level of DARPP-32 Thr34 phosphorylation then decreased to the basal level by 5 min of incubation. In contrast, in 14–15-day-old mice, treatment with cocaine did not affect the level of phospho-Thr34 DARPP-32 (Fig. 4a). In 21–22-day-old mice, cocaine slightly increased the level of phospho-Thr34 DARPP-32, but the effect was not statistically significant (Fig. 4b). Treatment of neostriatal slices with cocaine did not significantly affect the level of phospho-Thr75 DARPP-32 in either young or adult mice (data not shown).

Effect of methamphetamine on DARPP-32 phosphorylation in young and adult mice

Another type of psychostimulant, methamphetamine, increases the availability of dopamine through the use of a mechanism that requires DAT but is distinct from that of MPH and cocaine (Nestler *et al.* 2001). In adult mice, treatment of neostriatal slices with methamphetamine (100 μM) increased the level of phospho-Thr34 DARPP-32 maximally by 9.5-fold at 2 min of incubation (Fig. 5c). The level of phospho-Thr75 DARPP-32 decreased by over 50% after 1 min of incubation (Fig. 5f). Effects of methamphetamine on DARPP-32 phosphorylation both at Thr34 and

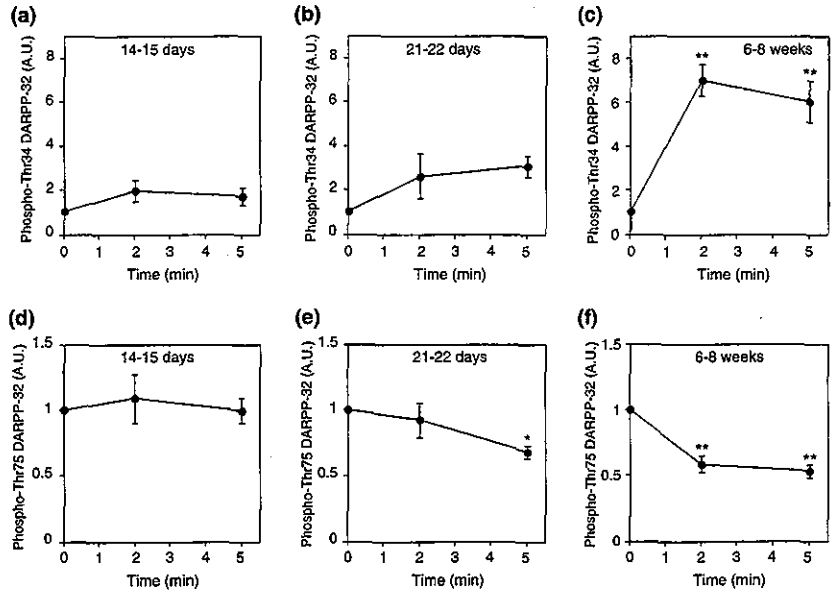

Fig. 3 Effects of methylphenidate (MPH) on DARPP-32 phosphorylation in neostriatal slices from young and adult mice. Neostriatal slices were prepared from 14–15-day-old (a, d), 21–22 day-old (b, e) and 6–8-week-old (c, f) mice. Slices were incubated with MPH (100 μM) for 2 or 5 min. The levels of phospho-Thr34 DARPP-32 (a–c) and phospho-Thr75 DARPP-32 (d–f) were quantified by densitometry, and the data were normalized to values obtained for untreated slices. The data for 6–8 week-old mice (c, f) are reproduced from Fig. 1 for comparison. *Data represent means* ± SEM for five to six experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with control; analysis of variance and Newman–Keuls test.

Table 1 Effects of a dopamine D1 receptor agonist, SKF81297, on DARPP-32 phosphorylation in neostriatal slices from young and adult mice

Age of mice	phospho-Thr34 DARPP-32 (fold increase)	phospho-Thr75 DARPP-32 (fold increase)
14–15 days	8.21 ± 0.63**	0.704 ± 0.105**
21–22 days	8.49 ± 1.20**	0.596 ± 0.112**
6–8 weeks	8.22 ± 1.55**	0.496 ± 0.059**

Neostriatal slices, prepared from 14–15-day-old, 21–22-day-old and 6–8-week-old mice, were incubated with SKF81297 (1 μM) for 5 min. The amounts of phospho-Thr34 and phospho-Thr75 DARPP-32 were quantified by densitometry. *Data represent means* ± SEM for six experiments. $^{**}p < 0.01$ compared with untreated slices from the same age (A.U. = 1).

Thr75 were maximal at a concentration of 100 μM with a half maximal effect at ~10 μM. Notably, the effects of methamphetamine on DARPP-32 phosphorylation at Thr34 and Thr75 were completely blocked by the dopamine D1 antagonist, SCH23390 (1 μM) (insets in Figs 5c and f).

In 14–15- and 21–22 day-old mice, treatment with methamphetamine (100 μM) significantly increased DARPP-32 Thr34 phosphorylation (Figs 5a and b) and decreased DARPP-32 Thr75 phosphorylation (Figs 5d and e), although the magnitude of the effects in young mice was slightly less than that in adult mice.

Effects of MPH and methamphetamine on DARPP-32 phosphorylation in reserpine-pre-treated mice

Neostriatal slices were prepared from adult mice, which had been pre-treated with a vesicular monoamine transporter

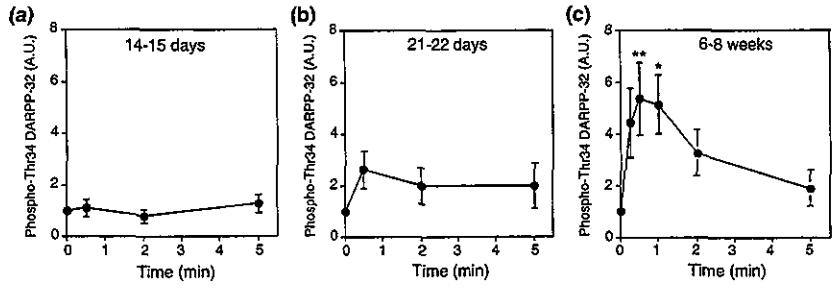

Fig. 4 Effects of cocaine on DARPP-32 phosphorylation in neostriatal slices from young and adult mice. Neostriatal slices were prepared from 14-15-day-old (a), 21–22-day-old (b) and 6–8-week-old (c) mice. Slices were incubated with cocaine (100 μM) for the indicated times. The levels of phospho-Thr34 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for five to 12 experiments. $^*p < 0.05$, $^{**}p < 0.01$ compared with control; analysis of variance and Newman–Keuls test.

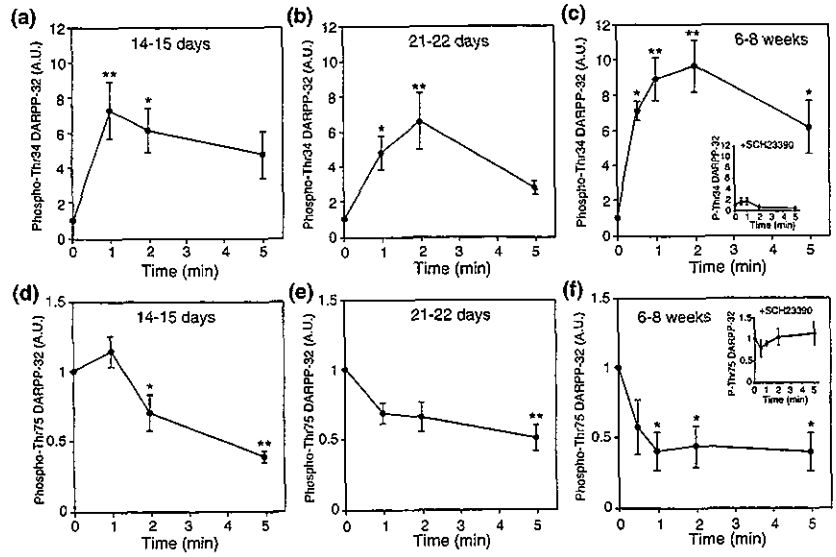

Fig. 5 Effects of methamphetamine on DARPP-32 phosphorylation in neostriatal slices from young and adult mice. Neostriatal slices were prepared from 14–15-day-old (a, d), 21–22-day-old (b, e) and 6–8-week-old (c, f) mice. Slices were incubated with methamphetamine (100 μM) for the indicated times. (insets for c and f) Neostriatal slices from adult mice were pre-treated with a dopamine D1 receptor antagonist, SCH23390 (1 μM), for 10 min, followed by the addition of methamphetamine (100 μM) for the indicated times. The levels of phospho-Thr34 DARPP-32 (a–c) and phospho-Thr75 DARPP-32 (d–f) were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for seven to nine experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control; analysis of variance and Newman–Keuls test.

inhibitor, reserpine (5 mg/kg), for 3 h to deplete the vesicular stores of dopamine. In neostriatal slices from reserpine-pre-treated mice, MPH (100 μM) failed to stimulate the phosphorylation of DARPP-32 at Thr34 (Fig. 6a). The effect of cocaine (100 μM) on DARPP-32 Thr34 phosphorylation was also abolished in reserpine-pre-treated mice (data not shown). In contrast, methamphetamine (100 μM) increased DARPP-32 Thr34 phosphorylation similarly in reserpine-treated and untreated mice (Figs 5c and 6b).

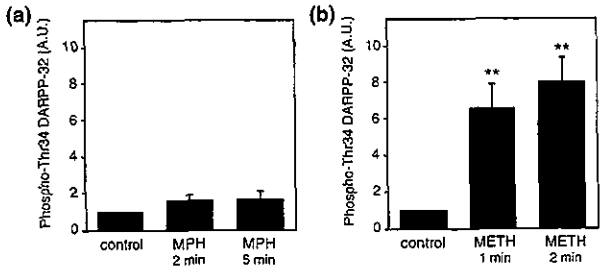

Fig. 6 Effects of methylphenidate (MPH) and methamphetamine (METH) on DARPP-32 Thr34 phosphorylation in neostriatal slices prepared from reserpine-pretreated mice. Neostriatal slices were prepared from adult mice pretreated with a vesicular monoamine transporter (VMAT) inhibitor, reserpine (5 mg/kg, subcutaneous injection), for 3 h. Neostriatal slices were incubated with MPH (100 μM) (a) or methamphetamine (100 μM) (b) for the indicated times. The levels of phospho-Thr34 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for five to 12 experiments. $^{**}p < 0.01$ compared with control; analysis of variance and Newman–Keuls test.

Effects of MPH and methamphetamine on DARPP-32 phosphorylation in the presence of TTX or NMDA and AMPA receptor antagonists

To further characterize the mechanisms by which MPH and methamphetamine enhance the action of dopamine on DARPP-32 Thr34 phosphorylation, the effects of MPH and methamphetamine were examined in the presence of a sodium channel blocker, TTX, or ionotropic NMDA- and AMPA-type glutamate receptor antagonists, using neostriatal slices prepared from adult mice. Pre-treatment of slices with TTX (1 μM) or an NMDA receptor antagonist, MK801 (100 μM), plus an AMPA receptor antagonist, CNQX (20 μM), did not significantly affect the basal level of phospho-Thr34 DARPP-32. Pre-treatment with TTX attenuated the stimulatory effect of MPH (100 μM) on DARPP-32 Thr34 phosphorylation (Fig. 7a). The effect of MPH was also attenuated by pre-treatment with MK801 plus CNQX. Pre-treatment with either MK801 alone or CNQX alone did not significantly attenuate the stimulatory effect of MPH on DARPP-32 Thr34 phosphorylation (data not shown). In contrast to MPH, the stimulatory effect of methamphetamine

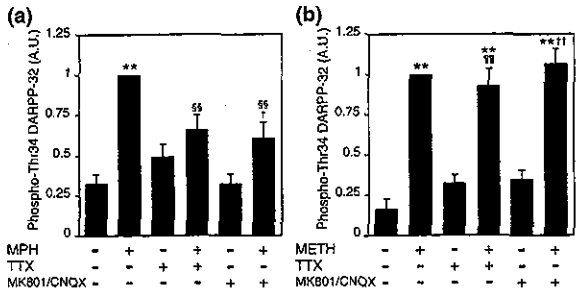

Fig. 7 Effects of methylphenidate (MPH) and methamphetamine (METH) on DARPP-32 Thr34 phosphorylation in the presence of TTX or NMDA and AMPA receptor antagonists. Neostriatal slices from adult mice were incubated for a total of 15 min in the absence or presence of TTX (1 μM) or an NMDA receptor antagonist, MK801 (100 μM), plus an AMPA receptor antagonist, CNQX (20 μM). TTX or MK801/CNQX was added at 0 min, and MPH (100 μM) (a) or methamphetamine (100 μM) (b) at 10 min of incubation. The levels of phospho-Thr34 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained with slices treated with MPH or methamphetamine alone. Data represent means ± SEM for seven to 20 experiments. $^{**}p < 0.01$ compared with control, §§$p < 0.01$ compared with MPH alone, ¶¶$p < 0.01$ compared with TTX alone, †$p < 0.05$, ††$p < 0.01$ compared with MK801 plus CNQX; analysis of variance and Newman–Keuls test.

(100 μM) on DARPP-32 Thr34 phosphorylation was not attenuated by pre-treatment with TTX or MK801 plus CNQX (Fig. 7b). The data suggest that the effect of MPH, but not that of methamphetamine, on DARPP-32 phosphorylation depends on the basal release of dopamine from vesicular stores that is regulated by TTX-sensitive and NMDA and AMPA receptor-dependent processes.

Discussion

We have demonstrated in adult mice that MPH stimulates dopamine/D1 receptor/DARPP-32 signaling in neostriatal neurons, probably by inhibiting dopamine reuptake into presynaptic dopaminergic terminals. The effect of MPH was mimicked by cocaine. In contrast to adult mice, MPH and cocaine were not able to stimulate dopamine/D1 receptor/DARPP-32 signaling in neostriatal neurons from young mice. The lack of the effect of MPH and cocaine in young mice is likely due to the immature development of the machinery that regulates dopamine release from vesicular stores at dopaminergic terminals.

MPH increased DARPP-32 Thr34 phosphorylation and decreased DARPP-32 Thr75 phosphorylation in neostriatal slices from adult mice. The effects of MPH on DARPP-32 phosphorylation both at Thr34 and Thr75 were abolished by the dopamine D1-type antagonist, SCH23390. The results are consistent with previous reports showing that activation of dopamine D1-type receptors leads to the phosphorylation of DARPP-32 at Thr34 by PKA (Nishi *et al.* 1997) and the dephosphorylation of DARPP-32 at Thr75 by protein phosphatase-2A (Nishi *et al.* 2000). As PKA is tonically inhibited by phospho-Thr75 DARPP-32 in basal conditions (Bibb *et al.* 1999), 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). Thus, MPH increases the concentration of extracellular dopamine by inhibiting DAT and, thereby, potentiates the action of spontaneously released dopamine acting at D1 receptors in medium spiny neurons (Fig. 8).

In adults, MPH binds to DAT and increases extracellular dopamine in the striatum by inhibiting dopamine uptake with a potency similar to that of cocaine (Pan *et al.* 1994; Kuczenski and Segal 1997). Moreover, Volkow *et al.* (2001) have found that oral administration of MPH at doses used for the treatment of ADHD increases extracellular dopamine in the striatum of adult humans, using positron emission tomography. MPH binds to the norepinephrine transporter with five times lower affinity than it does to DAT, but still might increase extracellular norepinephrine (Pan *et al.* 1994). MPH has weak potency both in binding to the serotonin transporter and in inhibiting serotonin uptake (Pan *et al.* 1994; Kuczenski and Segal 1997). Recent studies have shown that serotonin can increase the phosphorylation of DARPP-32 at Thr34 in neostriatal slices (Svenningsson *et al.* 2002). However, as all the effects of MPH on DARPP-32 phosphorylation were blocked by D1 and D2 antagonists, it appears that the major target of MPH in the striatum is DAT.

In slices from young mice at 14–15 and 21–22 days old, MPH failed to stimulate DARPP-32 Thr34 phosphorylation and Thr75 dephosphorylation. In contrast, the dopamine D1 agonist, SKF81297, increased DARPP-32 Thr34 phosphorylation and decreased Thr75 phosphorylation in slices from young mice as effectively as in adult mice, indicating that dopamine D1 receptor/DARPP-32 signaling in medium spiny neurons is already functional in 14-day-old mice. The expression of dopamine D1-type receptors in the striatum is very low but detectable at birth, and reaches adult levels at 2–3 weeks (Rao *et al.* 1991; Schambra *et al.* 1994). The expressions of $G\alpha_{olf}$ (Sakagami *et al.* 1995), Gγ7 (Morishita *et al.* 1999), adenylyl cyclase type V (Matsuoka *et al.* 1997), PKA (Massa *et al.* 1991) and DARPP-32 (Hemmings *et al.* 1992) in the striatum at 2 weeks of age are already high and comparable with those in adults. Thus, the developmental expression patterns of dopamine D1 receptor signaling components support the functional maturation of D1 receptor/DARPP-32 signaling in medium spiny neurons at 2 weeks of age. Therefore, the lack of the effects of MPH in young mice cannot be due to the immaturity of postsynaptic dopamine signaling.

Methamphetamine, another type of psychostimulant, has been shown to act as a dopamine releaser instead of a dopamine uptake inhibitor (Nestler *et al.* 2001).

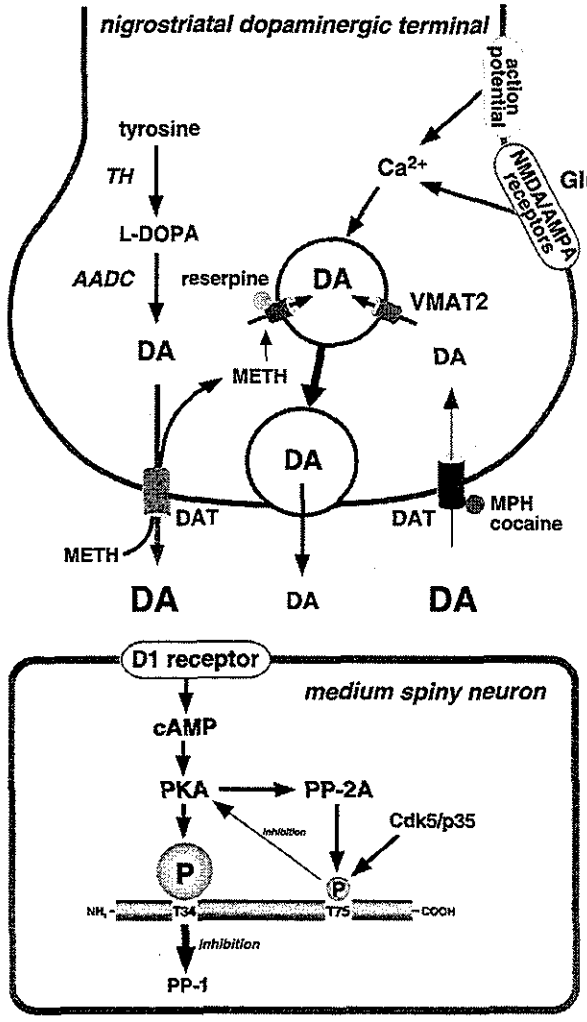

Fig. 8 Model illustrating the action of methylphenidate (MPH), cocaine and methamphetamine (METH) at nigrostriatal dopaminergic terminals of adult mice. MPH and cocaine potentiate the action of dopamine released from vesicular stores by inhibition of the dopamine transporter (DAT), resulting in increased activation of dopamine D1 receptor/DARPP-32/PP-1 signaling in medium spiny neurons. Methamphetamine, taken up by dopaminergic terminals via DAT, inhibits accumulation of dopamine into vesicles, and thereby increases dopamine content inside terminals, leading to the reverse transport of dopamine via DAT. In this way, methamphetamine stimulates the release of non-vesicular dopamine and activates dopamine D1 receptor/DARPP-32/PP-1 signaling.

Methamphetamine is taken up by DAT into dopaminergic terminals, and inhibits the uptake of dopamine into vesicles (Fig. 8). Inhibition of dopamine uptake results in an increased dopamine concentration inside dopaminergic terminals, leading to the reverse transport of dopamine via DAT from dopaminergic terminals to the extracellular space. Methamphetamine increased DARPP-32 Thr34 phosphorylation and decreased Thr75 phosphorylation by activating dopamine D1-type receptors in neostriatal slices from adult mice. In slices from young mice, the changes in DARPP-32 phosphorylation at Thr34 and Thr75 in response to methamphetamine were comparable with those in slices from adult mice. Moreover, the effects of methamphetamine, but not of MPH, were observed after depletion of vesicular stores of dopamine by reserpine. Together, these results suggest that methamphetamine uptake via DAT, inhibition of vesicular uptake of dopamine via VMAT2 (Hansson *et al.* 1998; Schutz *et al.* 1998), synthesis of dopamine by tyrosine hydroxylase (Coyle and Axelrod 1972; Baker *et al.* 1982), and dopamine release by reverse transport via DAT are functional at dopaminergic terminals as early as 14 days. Although there are reports showing that methamphetamine-induced hyperthermia is independent from methamphetamine-induced neurotoxicity, which requires DAT (Cappon *et al.* 1997; Itzhak *et al.* 2000), it is unlikely that DAT-unrelated mechanisms are relevant to the regulation of DARPP-32 Thr34 phosphorylation in neostriatal slices examined in the present study.

It has been reported that, in the rat striatum, DAT density is low at birth and gradually increases through 2 weeks, followed by a rapid increase until puberty (2–3 months old) (Coulter *et al.* 1997; Moll *et al.* 2000). In the mouse striatum, mRNA levels for DAT in 14-and 21-day-old mice, detected using *in situ* hybridization, were 67 ± 6 and 82 ± 10% of that in adult mice, respectively (Svenningsson, unpublished observations), demonstrating that rat and mouse show similar developmental patterns of DAT expression in the striatum. Our data with methamphetamine suggest that DAT is functional even at the lower level of expression in young mice, and that the lack of effect of MPH on DARPP-32 phosphorylation is not likely due to the functional immaturity of DAT. This interpretation is supported by the fact that DAT activity is up-regulated at a young age (Gordon *et al.* 1995). However, we cannot completely rule out the possibility that some aspect of DAT function is involved in the lack of response to MPH, as an association of the DAT gene with ADHD (Cook *et al.* 1995) and an elevated striatal DAT density in adult ADHD patients (Dougherty *et al.* 1999) have been reported.

Electrically evoked or KCl-evoked dopamine release from rat striatal slices remains stable from early post-natal days to 1 year of age (De Vries *et al.* 1992; Gordon *et al.* 1995). Moreover, the expression of synapsins (Melloni and DeGennaro 1994) and of proteins required for the fusion of vesicles to the presynaptic membrane such as the SNARE complex (Hepp and Langley 2001) are already high at birth, suggesting that dopamine can be released even in young mice if dopaminergic terminals are properly stimulated. Our evidence indicates that, in the slice preparation used in this study, dopamine is spontaneously released from vesicular stores in a TTX-sensitive and NMDA and AMPA receptor-dependent manner (Fig. 8). Glutamatergic inputs to the

striatum have been shown to stimulate the release of dopamine (Desce *et al.* 1992; Ohta *et al.* 1994). We have recently reported that activation of ionotropic NMDA and AMPA glutamate receptors is required for the release of dopamine by neurotensin in neostriatal slices (Matsuyama *et al.* 2002). Glutamatergic innervation at the striatum is delayed relative to dopaminergic innervation. Glutamatergic synapse formation at dendritic spines of medium spiny neurons is achieved at post-natal day 21 or later (Sharpe and Tepper 1998), and electrophysiological properties of medium spiny neurons become adult-like at ~5th post-natal week, following the functional maturation of glutamatergic excitatory inputs (Tepper *et al.* 1998). In young mice, inhibition of DAT by MPH might not induce an increase in extracellular dopamine as a result of the low glutamatergic driving force for dopamine release. In turn, lack of regulation of dopamine release would explain why the effects of MPH on DARPP-32 phosphorylation are lacking in young mice.

MPH acts as a stimulatory agent in adults, but as a calming agent in children, especially in ADHD patients (Volkow *et al.* 1995; Greenhill 2001). The paradoxical effects of MPH in children are not clearly understood. Studies using mice lacking DARPP-32 indicated that the phosphoprotein plays an important role in the regulation of locomotor activity by psychostimulants (Fienberg *et al.* 1998). Lack of activation of dopamine/D1 receptor/DARPP-32 signaling by MPH in young mice might explain why MPH does not stimulate psychomotor function in young patients. However, at the present time, we cannot obviously explain why MPH works as a calming agent instead of a stimulatory agent. For the treatment of ADHD, a low dose of MPH is orally administered (Greenhill 2001). Pharmacokinetics of orally administered MPH *in vivo* are largely different from those in slice experiments (Wargin *et al.* 1983; Volkow *et al.* 2002), and therefore key elements that explain the calming effect of MPH in ADHD patients might not have been detected in this study. Alternatively, a neurotransmitter other than dopamine with a calming effect could be involved in the therapeutic effect of MPH. We speculate that, in adults, MPH predominantly activates dopamine signaling and overwhelms any effects of other neurotransmitters, resulting in the increase in psychomotor function. In children, MPH cannot activate dopamine signaling, but possibly activates neurotransmitter signaling other than dopamine, resulting in the decrease in psychomotor activity. Elucidation of the neurotransmitter system predominantly activated by MPH in children would help in the design of new therapeutic agents for ADHD.

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