Tyrosine hydroxylase expression and Cdk5 kinase activity in ataxic cerebellum

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Abstract

Ataxia has been associated with abnormalities in neuronal differentiation and migration, which are regulated by Cyclin-dependent kinase 5 (Cdk5). The cerebellum of mice lacking Cdk5 or its activator, p35, resembles those of ataxic *reeler* and *scrambler* mice, suggesting that Cdk5 may contribute to ataxic pathology. As with other ataxic mice, the *pogo/pogo* mouse shows aberrant cerebellar tyrosine hydroxylase (TH) expression. Since Cdk5 phosphorylates and upregulates TH expression, we sought to analyze (i) Cdk5 activity in the *pogo* cerebellum, which exhibits abnormal TH expression, and (ii) TH expression in the cerebellum of *p35*^{−/−} and *p39*^{−/−} mice, which display reduced Cdk5 activity. Interestingly, we found that increased TH expression in the *pogo* cerebellum coincided with reduced Cdk5 activity. However, reduced Cdk5 activity in both *p35*^{−/−} and *p39*^{−/−} cerebellum did not correspond to defects in TH expression. Together, these suggest that abnormal TH expression in the cerebellum might be regulated by mechanisms other than Cdk5 activity.

Keywords

Phosphorylation; Purkinje cells; Cerebellum; Ataxia

Introduction

In order to investigate the etiology of many human diseases of neurological origin, numerous animal models have been generated. Some mutant strains of mice exhibit abnormal cerebellar development as a result of specific cell loss, defective cell migration, and incorrect intercellular communication [1]. The *pogo* mouse is an ataxic mutant that arose spontaneously from an inbred Korean wild mouse [2]. The autosomal recessive mutation, *pogo*, is located on chromosome 8 [3]. Intraspecific backcross between *pogo* and BALB/c mice mapped the *pogo* locus to a region between D8Mit67 and D8Mit240 on chromosome 8. The pathological manifestations of the *pogo* mouse include involuntary movements of

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extremities, difficulty in maintaining posture and side-to-side stability, faulty coordination between movements of the limbs and the trunk, and inability to walk straight [2]. Overt ataxia develops about 2 weeks after birth. The homozygous *pogo* phenotype is similar to tottering mouse (tg/tg), leaner (tgla) and rolling mouse Nagoya (tgrol) but a distinct *pogo* phenotype is frequent falling backwards due to hindlimb stretching [3, 4]. These traits have been associated with defects in the cerebellum that are similar to those observed in the cerebellum of reeler, scrambler, and Cdk5^{-/-} mice [5].

Cdk5 is a proline-directed serine/threonine kinase showing highest activity in postmitotic neurons of the central nervous system, which has abundant amounts of the Cdk5 activating subunits, p35, and p39 [6]. Cdk5 exerts its functions by associating with and phosphorylating a wide range of substrates essential in the regulation of neurite outgrowth, axon guidance, neuronal migration, secretion, dopamine signaling, and cytoskeletal dynamics [6, 7]. The importance of Cdk5 in proper neuronal development is further illustrated in the fact that Cdk5 knock-out mice display severe cortical lamination defects and perinatal death [8]. In the cerebellum, p39 has been shown to colocalize with Cdk5 in a subset of Purkinje cells that form alternating parasagittal band pattern, reflecting a potential role of Cdk5/p39 in the functional compartmentation of tissue [9].

Tyrosine hydroxylase (TH) is a rate-limiting enzyme involved in the biosynthetic pathway of the catecholamines dopamine, norepinephrine, and epinephrine [10]. It has been demonstrated that TH serves as a substrate of Cdk5 [11], and that Cdk5 phosphorylation of TH at serine 31 stabilizes and upregulates the expression and activity of TH [10]. Our previous studies showed that TH expression in the cerebellum was present only transiently during development and is low or absent in normal adult mice [4]. In ataxic mouse mutants, including *tottering/leaner*, *dilute-lethal*, and *pogo*, defects in TH expression in the cerebellar Purkinje cells have been observed [4, 12–16]. For example, in adult *pogo/pogo* mutants, TH is conspicuously expressed in the parasagittal bands of Purkinje cells while adult wild type (wt) mice had barely detectable levels of TH [4]. As Cdk5 regulates TH expression, it is possible that Cdk5 contributes to such abnormality in *pogo* mice. In the current study, we investigated the in vivo relationship between Cdk5 activity and TH expression in the cerebellum using the *pogo*, p35^{-/-}, and p39^{-/-} mice models.

Materials and methods

Animals

Adult (12-months-old) *pogo/pogo* mice used in this study have been described previously [4]. The breeding pairs used to generate p35^{-/-} and p39^{-/-} mice were obtained from Drs. Inez Vincent and Li-Huei Tsai, respectively. All experimental procedures were carried out in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Western blot analysis

Cerebellar tissues were dissected and homogenized in buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecylsulfate, 1% Nonidet P-40,

1 mM phenylmethylsulfonyl fluoride, 1 µg/ml of aprotinin, leupeptin, and pepstatin A. Samples were then sonicated and incubated on ice for 20 min, vortexed and centrifuged at 12,000g for 10 min at 4°C. The supernatants were collected and a Bradford assay was performed to determine the protein concentration. Proteins (100 µg/lane) were separated by SDS-polyacrylamide gels, blotted onto membranes and incubated with the primary antibody of interest [anti-tyrosine hydroxylase from Chemicon Co., Temecula, CA, anti-actin (I-19), anti-Cdk5 (C-8), anti-Cdk5-pTyr15, anti-p35 (C-19) from Santa Cruz Biotechnology Inc, Santa Cruz, CA, anti-phospho tau from Dr. Inez Vincent, and anti-p39 was generated in our laboratory: 9] for 1 h at room temperature. Immunoreactive protein bands were detected using the ECL detection system (New England Biolab, Guelph, Ontario, Canada).

Kinase assay

Histone H1 kinase activity of Cdk5-p35 immunoprecipitates from tissue homogenates was determined as described previously [17]. The reaction was initiated by the addition of 100 µM [γ -³²P] ATP and 100 µM histone H1 peptide. The amount of [γ -³²P] phosphate incorporated into histone was measured using a Beckman LS 5000CE scintillation counter (Beckman Coulter, Fullerton, CA).

Immunohistochemistry

All tissue samples were frozen-sectioned at six-micron and mounted onto saline-coated slides. They were then fixed in cold acetone for 2 min. Using the ImmunoCruz Staining Systems (Santa Cruz Biotechnology Inc, Santa Cruz, CA), serial sections were first blocked with horse serum for 20 min, then incubated with 1:200 TH polyclonal antibody for overnight at 4°C, followed by two washes in PBS each for 2 min. Next, the sections were incubated with biotin-labeled anti-rabbit secondary antibody with avidin–biotin–peroxidase complex for 30 min, followed by two washes in PBS each for 2 min and staining with the HRP substrate containing DAB chromogen and peroxidase substrate for 30 s to 5 min. Sections were immediately dehydrated twice in 95% ethanol each for 10 min, twice in 100% ethanol each for 10 s, and thrice in xylenes each for 10 s. Slides were added with permanent mounting medium, covered with glass coverslip, and observed under a light microscope.

Results

Cdk5 activity is reduced in ataxic *pogo* mouse cerebellum

TH, that is abnormally expressed in the cerebellum of ataxic *pogo* mice, is one of the more recently identified substrates of Cdk5, an enzyme required for normal cerebellar development [4, 7]. It is found that Cdk5 activity can have direct influence on TH stability and expression [10, 11]. To further investigate the relationship between Cdk5 and TH, we sought to examine Cdk5 activity in the *pogo* mouse cerebellum as well as the cerebellum of mice lacking either one of its activator proteins, p35 or p39. As shown in Fig. 1a, Cdk5, p35, and p39 protein levels in *pogo* cerebellum are comparable to those in the wild type. While p35 and p39 knock-out mice lacked their corresponding proteins, their Cdk5 expression is unaffected. In order to examine Cdk5 activity, Cdk5 was immunoprecipitated from cerebellar samples and the immunoprecipitates were tested for the ability to phosphorylate histone H1. Indeed, the *pogo/pogo* cerebellum showed a significantly reduced Cdk5 kinase

activity ($P=0.02$) that was equivalent to that observed in the $p35^{-/-}$ cerebellum (Fig. 1b). While the $p39^{-/-}$ cerebellum also showed reduced level of Cdk5 activity, the lack of p39 did not cause a loss of Cdk5 activity as remarkable as that observed in *pogo/pogo* and $p35^{-/-}$ cerebellum. In order to support our finding that Cdk5 activity is reduced in *pogo* cerebellum, we examined the phosphorylation status of tau, a Cdk5 substrate, in this tissue. As shown in Fig. 2a, tau phosphorylation is reduced in *pogo* compared to wt cerebellum, corroborating with our finding that Cdk5 activity is reduced in the *pogo* cerebellum. However, since the *pogo* and wt cerebella did not show significant differences in Cdk5, p35, and p39 levels, we sought to understand why the *pogo* cerebellum has reduced Cdk5 activity. Potentially, the phosphorylation state of Cdk5 in *pogo* affected its activity. Indeed, we found reduced phosphorylation of Cdk5 at Tyr15 in the *pogo* cerebellum (Fig. 2b), accounting for reduced activity of Cdk5. Together, these results provide a link between the ataxic phenotype and reduced Cdk5 kinase activity.

Reduced Cdk5 activity does not cause abnormal TH expression

Since *pogo*, $p35^{-/-}$ and $p39^{-/-}$ mice showed reduced Cdk5 kinase activity, we asked whether such defect affects the cerebellar expression of TH. Consistent with previous findings [4], immunohistochemical analysis revealed that TH expression in the *pogo/pogo* cerebellum formed clear parasagittal bands of Purkinje cells, which were most apparent in the caudal regions (Fig. 3a, arrow). Conversely, no such TH pattern was observed in the cerebellum of both $p35^{-/-}$ and $p39^{-/-}$ mice, which appeared indistinguishable from the wt mouse cerebellum. The dramatic difference in cerebellar TH immunoreactivity between *pogo* and wt, $p35^{-/-}$ and $p39^{-/-}$ mice was more evident at higher resolution (Fig. 3b) of the cerebellar caudal areas (indicated by arrows in Fig. 3a). Results of Western blot analysis of whole cerebellar homogenates (Fig. 3c) coincided with the TH immunohistochemical data, where only the *pogo/pogo* cerebellum had increased TH protein expression. These results indicate that reduced Cdk5 activity cannot account for abnormal TH expression in the cerebellum.

Discussion

Cdk5 and TH have progressively been implicated as essential requirements for normal developmental processes in the brain and an increasing but limited number of studies have demonstrated a link between Cdk5 and TH. For example, one study identified TH as a substrate of Cdk5 that is specifically phosphorylated at Serine 31 [11] and another demonstrated that TH phosphorylation by Cdk5 enhances TH stability and expression [10]. Furthermore, it was found that the level of Cdk5 and TH protein expression in the ventral tegmental area of the mouse brain was elevated following administration of cocaine for 10 days [18].

Many ataxic mice such as *pogo*, *staggerer*, *reeler*, *weaver*, and *meander tail* share similar features in aberrant structuring of neurons in the cerebellum [3, 4, 19–22]. Since Cdk5 regulates neuronal migration [7], it may be involved in the formation of cerebellar defects in ataxic mice. In addition, since TH expression is increased in ataxic mice, and TH phosphorylation at Ser31 by Cdk5 increases TH stability and expression, it seems reasonable

to hypothesize that Cdk5 activity would be increased in ataxic *pogo* cerebellum. Interestingly, however, we observed a remarkable decrease in Cdk5 kinase activity, which corresponds to reduced phosphorylation of tau, in *pogo/pogo* cerebellum. Reduced Cdk5 activity in ataxic *pogo* was comparable to that of *p35*^{-/-}, and the latter resembles chimeric *Cdk5*^{-/-} ↔ *Cdk5*^{+/+} mice, which do not exhibit noticeable ataxia [5]. Therefore, although deficient Cdk5 activity in *pogo* may contribute to abnormal cerebellar lamination associated with the ataxic phenotype, it appears that Cdk5 activity is not involved in the upregulation of TH expression in the ataxic *pogo* cerebellum. Consistent with previous observations, Cdk5 activity was only slightly affected in *p39*^{-/-} mutants. Although *p39* has been firmly established to be a biological activator of Cdk5, recent evidence indicates that the presence of *p35* can compensate for the lack of *p39* while *p39* can only minimally rescue the absence of *p35*. This is highlighted by the observation that whereas *p35*^{-/-} mice exhibit some brain defects and adult mortality, *p39*^{-/-} mice lack significant phenotypic abnormalities [23]. While reduced Cdk5 activity in *p35*^{-/-} can be attributed to the absence of *p35*, we found that reduced Cdk5 activity in *pogo* is due to reduced phosphorylation of Cdk5 at Tyr15. This indicates that reduced activity of cAbl and subsequent decrease in phosphorylation of Cdk5 Tyr15 (a specific target of cAbl) account for reduced Cdk5 activity in the *pogo* cerebellum. Indeed, it has been demonstrated that phosphorylation of Cdk5 by c-Abl at Tyr15 enhances its activity [24]. In separate studies, we also found that phosphorylation of Cdk5 at Ser159 by a brain-specific protein complex affects its activity [25].

pogo, *p35*^{-/-} and *p39*^{-/-} cerebella all have reduced Cdk5 activity, but in contrast to *pogo*, which showed increased TH level, *p35*^{-/-} and *p39*^{-/-} have reduced TH levels. Our latter finding is compatible with the observation of Moy and Tsai [10] in *Cdk5*^{-/-} embryonic striatum and whole brain lysates. However, it is intriguing that reduced Cdk5 activity in the *pogo/pogo* mutant correlated with ectopic expression of TH, which forms parasagittal bands of Purkinje cells and shows an overall elevation of TH protein expression in the cerebellum, contradicting the previous report that Cdk5 upregulates the expression of TH [10]. The discrepancy may be explained by activity of other factors that modulate TH expression in *pogo* mice. While Cdk5 may phosphorylate and influence the stability of TH, other TH expression promoting factors may cause the upregulation of TH level and abnormal TH expression in the *pogo* cerebellum. For instance, striatal-derived trophic substances have been shown to induce TH expression [26], but more studies are clearly required to understand the mechanism behind increased cerebellar TH expression in the ataxic *pogo* mouse model.

In a study by Lee et al. [27], it was found that the abnormally high levels of TH in *pogo* cerebellum are not phosphorylated at Ser40, leading to the conclusion that *pogo* TH are not activated and not involved in catecholamine synthesis. However, it should be noted that TH can be phosphorylated by various protein kinases, including PKA, PKG, PKC, CaMPKII, PKC, MAPKs [28] and Cdk5 [10], at specific serine sites, and phosphorylation at some of these sites, including Ser19 and Ser31, has been associated with increased TH activity [28]. Therefore, whether overexpressed TH in the *pogo* cerebellum is truly inactive and uninvolved in catecholamine synthesis remains to be elucidated.

Although a clear link between Cdk5 and ataxia has not yet been demonstrated, it cannot be ruled out that reduced Cdk5 activity may contribute to some cerebellar pathologies in the ataxic *pogo* mouse. To our knowledge, Cdk5 activity has not been examined in reeler, scrambler, or other ataxic mice. Thus, the current study presents, for the first time, the analysis of Cdk5 activity in an ataxic mouse model (*pogo*). It is interesting, however, that tau, a Cdk5 substrate, is hyperphosphorylated in reeler brain [29]. Since reelin downregulates Cdk5 activity under normal conditions, Cdk5 may remain active in reeler mouse neurons, resulting in failure of postmitotic neurons to adhere to one another in the presumptive marginal zone, and thus, the disruption of cortical layers in reeler brains [30]. The scrambler phenotype, which overlaps with reeler, results from mutations in mDab1 (mouse disabled 1), a putative adapter protein for tyrosine kinases that binds directly to known reelin receptors [31]. However, how Cdk5 activity could be linked to the scrambler phenotype remains to be investigated. In this study, we show that, while Cdk5 stabilizes and enhances TH expression [10], abnormalities in ataxic *pogo* cerebellar TH expression cannot be directly and exclusively linked to Cdk5 activity.

Acknowledgments

We thank Seung-Hyuk Chung for his excellent technical assistance. This project was supported by an operating grant from Canadian Institutes of Health Research to K-Y. Lee, an Alberta Heritage Foundation for Medical Research Senior Scholar.

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Fig. 1.

Cdk5 activity is reduced in the cerebellum of *pogo/pogo*, *p35^{-/-}*, and *p39^{-/-}* mice. **(a)** Western blot analysis of Cdk5, p35, and p39 in wild type, *pogo/pogo*, *p35^{-/-}*, and *p39^{-/-}* cerebellar homogenates. Actin was used as a loading control. Immunoreactive protein bands were detected using the ECL detection system (New England Biolab, Guelph, Ontario, Canada). **(b)** Cdk5 was immuno-precipitated from 250 μ g of cerebellar homogenates using the C-8 Cdk5 antibody (Santa Cruz Biotech.). Kinase activity of immuno-precipitates was determined by incubating with histone H1 (9–18) peptide and [γ -³²P] ATP/Mg²⁺ at 30°C for 1 h. Values represent the mean $\pm$ SEM ($n = 9$)

Fig. 2.

The pogo cerebellum shows reduced phosphorylation of tau and Cdk5-pTyr15. Western blot analysis of wild-type (+/+) and pogo/pogo cerebellar homogenates using (a) phospho-tau (PHF-1) and (b) Cdk5-pTyr15 antibodies. For loading controls, the blots were reprobbed with an actin antibody and a phosphorylation-independent Cdk5 antibody (C-8) for (a) and (b), respectively

Fig. 3.

TH is conspicuously present in *pogo/pogo* but not in *wt*, *p35*^{-/-} and *p39*^{-/-} adult cerebellum. (a) Selective transverse cerebellar sections were presented here with immunostaining for TH. "LC" indicates internal positive controls: TH-expressing locus coeruleus (LC) cells. Scale bar = 1 mm. (b) Enlarged microscopic images of areas indicated by arrows in (a) show the difference in TH expression between *pogo/pogo* and *wt*, *p35*^{-/-} and *p39*^{-/-}; *n* = 3. TH expression in *pogo/pogo*, *p35*^{-/-}, and *p39*^{-/-} cerebellum showed the same pattern in juvenile mice. (c) Western blot analysis of TH in homogenates of *pogo/pogo*, *p35*^{-/-} and *p39*^{-/-} mouse cerebellum