

## Increased expression of cyclin-dependent kinase 5 in induced apoptotic neuron death in rat substantia nigra

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### Abstract

We reported previously that striatal excitotoxic lesion with quinolinic acid of rat pups during the first 2 weeks of postnatal life results in loss of dopaminergic neurons of the substantia nigra (SN) due to induced apoptosis. Here we demonstrate by immunohistochemistry that, following such a lesion, high levels of cyclin-dependent kinase 5 (cdk5) protein are present exclusively in apoptotic cells over and above basal levels of diffuse axonal staining. Furthermore, localization of high levels of cdk5 is associated also with normal developmental programmed cell death in the SN and other regions of the central nervous system, including the cerebral cortex. These findings suggest a novel role for cdk5 during neuron apoptosis and may provide insight into mechanisms of loss of dopaminergic neurons in Parkinson's disease. © 1997 Elsevier Science Ireland Ltd.

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Cyclin-dependent kinase 5 (cdk5) is a threonine/serine kinase [6], originally identified on the basis of sequence similarity to other cyclin-dependent kinases known to play a key regulatory role in cell cycle progression [7,10]. Cdk5 mRNA and protein have widespread basal expression, but levels of expression and kinase activity are highest in brain where they correlate with the extent of neuronal differentiation: cdk5 is highly expressed in post-mitotic neurons in the CNS [18] where it is associated with the cytoskeleton. Despite its high degree of structural similarity to other members of the cyclin-dependent kinase family, cdk5 activity does not appear to depend on association with cyclins but rather, it is activated by binding to a p35 regulatory subunit which is expressed exclusively in neurons of the CNS [8].

The precise role of cdk5 in the CNS is not well understood but recent findings suggest it plays an important part in regulation of phosphorylation of neuronal cytoskeletal proteins. Cdk5 was independently purified as a major brain kinase for the microtubule-associated protein tau [5]

and it phosphorylates major cytoskeletal components of the axon, including the medium and heavy neurofilament forms [7]. Strikingly, hyperphosphorylation of neurofilaments has been demonstrated in diverse neurodegenerative diseases including Parkinson's disease [15] and cdk5 protein is present in Lewy bodies of Parkinson's disease [1]. Cdk5 also phosphorylates the microtubule-associated protein tau *in vitro*, at sites phosphorylated in tau protein isolated from the paired helical filaments of Alzheimer's disease [8] and it has been shown to co-localize with neurofibrillary tangles of Alzheimer's brains [19]. Together, these findings raise the possibility that dysfunction of the phosphorylation cascade involving cdk5 may participate in the pathology of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.

Since the presence of cdk5 is associated with pathological structures in tissues of a diverse set of neurodegenerative diseases, we wished to examine cdk5 expression during neuronal cell death. We have previously shown that natural cell death, with the morphology of apoptosis, takes place postnatally in the substantia nigra (SN) [3,14]. In accord with classic neurotrophic theory, which postulates a dependence of developing neural systems on their targets for trophic support, this death event can be induced in dopami-

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nergic neurons of the SN pars compacta (SNpc) by early target injury with the excitotoxin quinolinic acid (QA) [4,9]. As a first step to determine whether cdk5 plays a role in loss of nigral neurons, and specifically in neuronal apoptosis in our model, we have examined expression of the cdk5 protein in the SN of rats which have undergone striatal target QA lesion. We describe the pattern of cdk5 expression in the developing SN at successive time points after QA lesion and show that apoptotic cell death correlates with increased cdk5 protein expression, temporally and spatially, at a cellular level.

Unilateral striatal quinolinic acid (QA) lesions were performed at postnatal day (PND) 12 on rat pups of timed pregnant females obtained from Charles River Laboratories (Wilmington, MA). Briefly, pups were anesthetized with Metofane by inhalation and received intrastriatal injection of 480 nmol of QA as previously described [9]. Animals were perfused via the left ventricle with cold 0.9% saline followed by cold 4% paraformaldehyde, 0.1 M phosphate buffer (PB; pH 7.1) for 10 min. Brains were post-fixed at 4°C overnight in 4% paraformaldehyde, 0.1 M PB (pH 7.1) and then placed in cryoprotectant (20% sucrose in 0.1 M PB, pH 7.1) at 4°C overnight prior to rapid freezing. Sections (30 µm) were collected in PBS for the entire SN for immunohistochemistry, and representative striatal sections between Paxinos-Watson planes 8.2 and 10.2 were collected for Nissl staining to confirm striatal location of the QA lesion. The anti-cdk5 primary antibody, rabbit polyclonal antisera C-8 (Santa Cruz), recognizes a C-terminal peptide YFSDFCPP conserved in all mammalian cdk5 proteins. It is monospecific, as verified by Western blot and immunoprecipitation experiments [20]. Nigral sections were incubated overnight with primary antibody, 3 µg/ml in PBS/0.1% BSA at 4°C, followed by biotinylated protein A, in PBS/0.1% BSA for 1 h at room temperature, and then avidin-biotinylated horseradish peroxidase complexes (ABC kit, Vector) at 1:600 dilution for 1 h at room temperature. Sections were then incubated with diaminobenzidine (Aldrich; 50 mg/100 ml Tris, pH 7.6) in the presence of H<sub>2</sub>O<sub>2</sub> and were Nissl counter-stained using thionin. To identify apoptotic cells, the entire SN ipsilateral and contralateral to the QA lesions was scanned at 400× magnification. Profiles were counted as apoptotic only if they contained one or more hyperchromatic chromatin clumps within a cellular profile. Bare chromatin clumps with no surrounding cellular material were not counted. Five to six animals were included for each experimental time point and to ensure that representative sections were included for each animal, two sections of each of Paxinos-Watson planes 4.2, 3.7 and 3.2 were analyzed per animal. For each plane, counts were averaged and results presented here are cumulative counts for the average of each plane within one brain.

Consistent with previous experiments [4,9], apoptotic cells were more numerous 24 h post-lesion, in the SN ipsilateral to the QA lesion (see below). Intense cdk5 immunoreactivity was observed exclusively in apoptotic cells and a

typical example is shown in Fig. 1. This intense staining of apoptotic cells is superimposed on a much lower level of expression in the surrounding neuropil. Tsai and coworkers [18] have previously described increasing levels of cdk5 as neurons differentiate, with maximal levels in post-mitotic neurons, confined to the axons. Consistent with their report, we observed immunoreactivity in fasciculated axons (data not shown). However, the most intense staining in our sections is associated with apoptotic cell bodies, not axons. We observed no staining of sections treated under identical conditions in the absence of primary antibody.

Cdk5-positive apoptotic cells were most numerous ipsilateral to the striatal QA lesion in the SNpc and the SN pars reticulata (SNpr). However, a few such profiles were also observed in the SN contralateral to the lesion, and in normal (non-lesioned) rat SN at PND 12 (data not shown). These cellular profiles in the absence of striatal lesion presumably are due to the natural cell death known to occur in SN [3]. Cdk5-positive apoptotic profiles were not limited to the SN. In QA lesioned brains we noted massive induction of apoptosis in the mammillary body, as previously observed by one of the authors (R.E. Burke, unpublished), and within this structure were numerous apoptotic cells intensely immunostained for cdk5. We also observed small numbers of cdk5-positive apoptotic cells in other midbrain structures and in the cerebral cortex of normal (non-lesioned) rat pups, presumably due to natural cell death. In all regions of the CNS, the appearance of cdk5-positive apoptotic cells we observed was similar to those noted in the SN in Fig. 1.

We quantified the induction of apoptosis in the nigra by striatal QA lesion, and in parallel determined the number of such dying cells expressing high levels of cdk5. Fig. 2

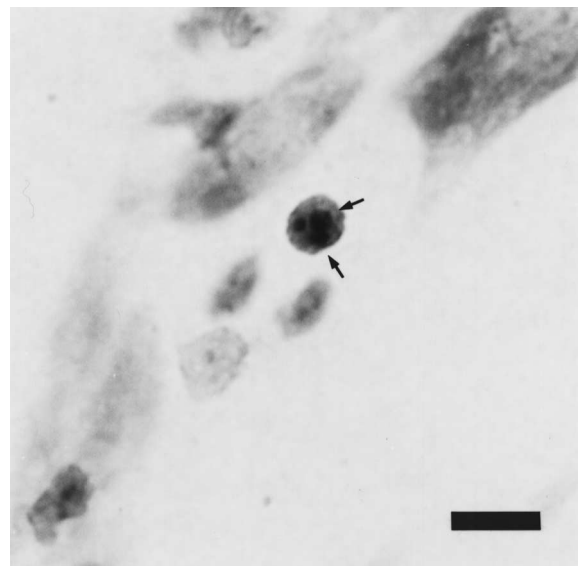


Fig. 1. Typical example of cdk5 immunostaining with Nissl counterstain in SNpc, 24 h post-lesion with QA, reveals that cells undergoing apoptosis in the substantia nigra express high levels of cdk5, stained brown. Apoptotic profiles have multiple discrete, rounded and intensely basophilic chromatin clumps (arrows), not seen in normal neurons. Bar = 10 µm.

demonstrates that the induction of apoptosis (four-fold) in the SNpc ipsilateral to striatal lesion is mirrored by a three-fold induction of cdk5-positive cell profiles at 24 h post-lesion time. Levels of both apoptotic and cdk5-positive cells in the SN are comparable for normal rats and QA-lesioned rats at 4 h post-lesion time, consistent with previous studies [4,9]. Of note, in the presence of induced cell death, only about 20% of apoptotic cells express high levels of cdk5. While studies in tissue sections do not permit precise timing of events, the universal association of cdk5-positive cellular staining with apoptotic chromatin clumps would suggest that cdk5 expression and the formation of clumps are proximate in time. The fact that some of the apoptotic cells detected do not express high levels of cdk5 may reflect induction of cdk5 at a relatively late stage of apoptosis, after chromatin clumps have begun to form. However, we cannot rule out specific differences, perhaps dependent on cell type or differences in penetration of staining reagents, between cell populations that are cdk5-positive and cdk5-negative during programmed cell death in SN.

Tissue lysates, prepared from nigral tissue blocks of lesioned and control animals at 24 hours post-lesion time, were analyzed by Western blot with anti-cdk5 antisera C-8, as described by Zhang and coworkers [20], revealing a single band of 33 kDa, consistent with the reported size of cdk5. The same antisera was used to immunoprecipitate cdk5 protein from the tissue lysates and this was demonstrated to have kinase activity, using histone H1 as a substrate [20]. However, comparison of lysates derived from control and experimental tissues failed to detect a difference in either total cdk5 levels or total cdk5 kinase activity at 24 h post-QA lesion. Two observations may account for this absence of a quantitative effect in these regional biochem-

ical analyses. First, normal rats at this stage have significant levels of active cdk5, consistent with studies in adult brain [7]. Second, the number of apoptotic profiles induced in the nigra by QA lesion is low relative to the total number of cells present (<1%). Therefore, there is an unfavorable signal-to-noise ratio with a small change in activity superimposed on a high baseline level. Thus, this tissue requires analysis at a cellular level, using quantitative anatomical techniques, and at present we are unable to distinguish whether increased immunohistochemical staining reflects induction of cdk5 protein or intracellular redistribution. Nevertheless, these biochemical studies confirm the presence of protein in tissues with the appropriate molecular weight and functional kinase activity.

This study demonstrates for the first time association of cdk5 expression with both induced and natural apoptotic cell death in the CNS. Although here we focus on the substantia nigra, high levels of cdk5 occurred in apoptotic cells in other regions of the CNS, including the cerebral cortex. Zhang et al. [20] have recently described elevated cdk5 expression during mouse development in cells undergoing apoptosis in such diverse tissues as intestinal epithelium, testis and peripheral nerve ganglia, suggesting that cdk5 activation may be a common feature of apoptosis in a variety of settings. Prior to this, a role for cdk5 in cell death had not been explored. However, apoptosis has been associated in many instances with abnormal hyperactivation or over-expression of other members of the cdk family [16].

We identify apoptotic cells in this study by morphologic criteria at the light microscope level and in this model we have previously confirmed that these cells are indeed undergoing apoptosis, as assessed by electron microscopy and 3' end-labeling [9]. We believe that the apoptotic cells we detect in SN are derived from neurons since we have demonstrated in previous studies that induced cell death occurred in a defined population of dopaminergic neurons in the SNpc [9].

It has recently been hypothesized that programmed cell death may play a role in the pathogenesis of neurodegenerative disease [17], and while much remains to be clarified, evidence in support of this hypothesis has been reported for Alzheimer's disease [2] and Parkinson's disease [11]. However, it has been unclear how programmed cell death might be linked with known cytoskeletal pathology associated with those disorders. Our results suggest that induction of cdk5 during programmed cell death may provide such a mechanism, given its important role in the regulation of phosphorylation of neuronal cytoskeletal proteins, including neurofilament proteins and the microtubule-associated protein tau. Changes in cdk5 activity, either by expression of dominant negative mutants in cultured neurons [12] or by targeted disruption of cdk5 in mice [13] have major consequences for cytoskeletal structure, neuronal behavior and brain development. However, how regulation of cytoskeletal organization may play a role in the events of programmed cell death is unknown. It will therefore be of

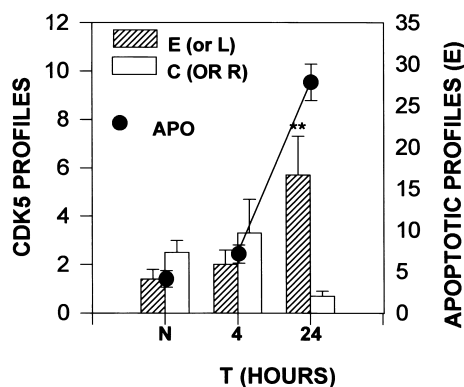


Fig. 2. Quantitative analysis of the induction of cdk5-positive cells and apoptotic profiles in the SNpc, following unilateral QA striatal lesion on PND 12. Lesioned brains were examined at 4 and 24 h post-lesion and the lesioned (experimental, E) side was compared with both the unlesioned (control, C) side of the same animal, and to each side (L, left and R, right) of the SN of unlesioned (normal, N) animals. Striatal QA lesions lead to an induction of cdk5-positive apoptotic profiles in parallel with total apoptotic profiles in SNpc at 24 hours post-lesion time (\*\* $P < 0.0001$ , ANOVA). Cdk5-positive cells are represented by histograms. The number of apoptotic cells (APO) on the experimental side is shown. The number of apoptotic profiles on the unlesioned side was not significantly different among the groups, and is not shown. Similar results were observed in SNpr.

particular interest to examine the pattern of programmed cell death in brains of mice lacking cdk5 expression. In relation to our specific finding of induced expression of cdk5 in neurons of the SNpc in a model of augmented programmed cell death, it is of interest that cdk5 has recently been identified as a component of Lewy bodies, a pathologic hallmark of Parkinson's disease [1]. This report provides a link between expression of cdk5 and neuronal cell death in the substantia nigra, so the role of cdk5 in nigral cell loss in Parkinson's disease warrants further attention.

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- [1] Brion, J.-P. and Couck, A.-M., Cortical and brainstem-type Lewy bodies are immunoreactive for the cyclin-dependent kinase 5, *Am. J. Pathol.*, 147 (1995) 1465–1476.
- [2] Cotman, C.W. and Anderson, A.J., A potential role for apoptosis in neurodegeneration and Alzheimer's disease, *Mol. Neurobiol.*, 10 (1995) 19–45.
- [3] Janec, E. and Burke, R.E., Naturally occurring cell death during postnatal development of the substantia nigra of the rat, *Mol. Cell. Neurosci.*, 4 (1993) 30–35.
- [4] Kelly, W.J. and Burke, R.E., Apoptotic neuron death in rat substantia nigra induced by striatal excitotoxic injury is developmentally dependent, *Neurosci. Lett.*, 220 (1996) 85–88.
- [5] Kobayashi, S., Ishiguro, K., Omori, A., Takamatsu, M., Arioka, M., Imahori, K. and Uchida, T., A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule, *FEBS Lett.*, 335 (1993) 171–175.
- [6] Lew, J. and Wang, J.H., Neuronal cdc2-like kinase, *Trends Biol. Sci.*, 20 (1995) 33–37.
- [7] Lew, J., Winkfein, R.J., Paudel, H.K. and Wang, J.H., Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34<sup>cdc2</sup>, *J. Biol. Chem.*, 267 (1992) 25922–25926.
- [8] Lew, J., Zhong, Q., Huang, Q.-Q., Paudel, H.K., Matsuura, I., Matsushita, M., Zhu, X. and Wang, J.H., Structure, function, and regulation of neuronal cdc2-like protein kinase, *Neurobiol. Aging*, 16 (1995) 263–270.
- [9] Macaya, A., Munell, F., Gubits, R.M. and Burke, R.E., Apoptosis in substantia nigra following developmental striatal excitotoxic injury, *Proc. Natl. Acad. Sci. USA*, 91 (1994) 8117–8121.
- [10] Meyerson, M., Enders, G.H., Wu, C.-L., Su, L.-K., Gorke, C., Nelson, C., Harlow, E. and Tsai, L.-H., A family of human cdc2-related protein kinases, *EMBO J.*, 11 (1992) 2909–2917.
- [11] Mochizuki, H., Goto, K., Mori, H. and Mizuno, Y., Histochemical detection of apoptosis in Parkinson's Disease, *J. Neurol. Sci.*, 137 (1996) 120–123.
- [12] Nikolic, M., Dudek, H., Kwon, Y.T., Ramos, Y.F.M. and Tsai, L.-H., The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation, *Genes Dev.*, 10 (1996) 816–825.
- [13] Ohshima, T., Ward, J.M., Huh, C.-G., Longenecker, G., Veeranna, Pant, H.C., Brady, R.O., Martin, L.J. and Kulkarni, A.B., Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death, *Proc. Natl. Acad. Sci. USA*, 93 (1996) 11173–11178.
- [14] Oo, T.F. and Burke, R.E., The time course of developmental cell death in phenotypically defined dopaminergic neurons of the substantia nigra, *Dev. Brain Res.*, 98 (1997) 191–196.
- [15] Schmidt, M.L., Murray, J., Lee, V.M.-Y., Hill, W.D., Wertkin, A. and Trojanowski, J.Q., Epitope map of neurofilament protein domains in cortical and peripheral nervous system Lewy bodies., *Am. J. Pathol.*, 139 (1991) 53–65.
- [16] Shi, L., Nishioka, W.K., Th'ng, J., Bradbury, E.M., Litchfield, D.W. and Greenberg, A.H., Premature p34<sup>cdc2</sup> activation required for apoptosis, *Science*, 263 (1994) 1143–1145.
- [17] Thompson, C.B., Apoptosis in the pathogenesis and treatment of disease, *Science*, 267 (1995) 1456–1462.
- [18] Tsai, L.-H., Takahashi, T., Caviness, V.S. Jr. and Harlow, E., Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system, *Development*, 119 (1993) 1029–1040.
- [19] Yamaguchi, H., Ishiguro, K., Takashima, A., Lemere, C.A. and Imahori, K., Preferential labeling of Alzheimer neurofibrillary tangles with antisera for tau-protein kinase (Tpk) I, glycogen-synthase kinase-3-beta and cyclin-dependent kinase-5, a component of Tpk-II, *Acta Neuropathol.*, 92 (1996) 232–241.
- [20] Zhang, Q., Ahuja, H.S., Zakeri, Z.F. and Wolgemuth, D.J., Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling, *Dev. Biol.*, 183 (1997) 222–233.