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Expression of cyclin E in postmitotic neurons during development and in the adult mouse brain

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ABSTRACT

Cyclin E, a member of the G1 cyclins, is essential for the G1/S transition of the cell cycle in cultured cells, but its roles *in vivo* are not fully defined. The present study characterized the spatiotemporal expression profile of cyclin E in two representative brain regions in the mouse, the cerebral and cerebellar cortices. Western blotting showed that the levels of cyclin E increased towards adulthood. *In situ* hybridization and immunohistochemistry showed the distributions of cyclin E mRNA and protein were comparable in the cerebral cortex and the cerebellum. Immunohistochemistry for the proliferating cell marker, proliferating cell nuclear antigen (PCNA) revealed that cyclin E was expressed by both proliferating and non-proliferating cells in the cerebral cortex at embryonic day 12.5 (E12.5) and in the cerebellum at postnatal day 1 (P1). Subcellular localization in neurons was examined using immunofluorescence and western blotting. Cyclin E expression was nuclear in proliferating neuronal precursor cells but cytoplasmic in postmitotic neurons during embryonic development. Nuclear cyclin E expression in neurons remained faint in newborns, increased during postnatal development and was markedly decreased in adults. In various adult brain regions, cyclin E staining was more intense in the cytoplasm than in the nucleus in most neurons. These data suggest a role for cyclin E in the development and function of the mammalian central nervous system and that its subcellular localization in neurons is important. Our report presents the first detailed analysis of cyclin E expression in postmitotic neurons during development and in the adult mouse brain.

1. Results and discussion

Neurons are generated during development by the proliferation of precursors within proliferating zones. Upon the final cell division the neuron loses its ability to proliferate and migrates to its destination. Cell cycle regulators play crucial roles in controlling neuronal production and in brain histogenesis during development (Dehay and Kennedy, 2007), but they are thought to be unnecessary in terminally differentiated non-cycling mature neurons. However, recent studies have reported that cell cycle regulators are present in healthy adult brains, indicating a possible role for these factors in constantly keeping the cell cycle of mature neurons in check so that neurons will not re-enter an ectopic cell cycle or undergo cell death (Schmetsdorf et al., 2005; Herrup and Yang, 2007).

Although E-type cyclins, which consist of cyclin E1 and cyclin E2, are best known as regulators of the G1/S transition from *in vitro* studies, their importance *in vivo* is not fully understood. Individual knockout (KO) mice for either cyclin E1 or cyclin E2 do not show any obvious phenotypes, but double KO mice for cyclin E1 and cyclin E2 are embryonic lethal due to the lack of endoreplication in trophoblast giant cells, indicating overlapping and dispensable functions of the two factors with respect to cell proliferation (Geng et al., 2003; Parisi et al., 2003).

The present study examined the precise spatiotemporal expression profile of cyclin E in the mouse brain, using western blotting, *in situ* hybridization and immunohistochemistry. The brain regions examined included the cerebral cortex and the cerebellum because neurons in these regions are well organized into laminar structures and the development of these regions has been intensively studied (Chizhikov and Millen, 2003; Dehay and Kennedy, 2007). Our results reveal a profile of cyclin E expression that has not been previously reported, suggesting novel roles for cyclin E in both the developing and mature brain, in addition to its role in cell cycle regulation.

1.1. Expression profile of cyclin E mRNA and protein during cerebral and cerebellar development

The temporal expression of cyclin E was studied by western blotting and was compared with that of cdk2, cdk5 and p35 at

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various developmental stages, from E12.5 to adulthood (Fig. 1). Cyclin E expression was detected at all the stages examined, and postnatal levels were higher in comparison to embryonic levels. These results are comparable with those previously reported (Miyajima et al., 1995). In agreement with results from immunoprecipitation studies, in which expression of cdk2, the binding partner of cyclin E in proliferating cells, was detected in the brain during embryonic development but not in adulthood (Tsai et al., 1993; Miyajima et al., 1995), the present results using western blotting showed that the expression level of cdk2 was highest at E12.5 and then gradually receded during development to be barely detectable in adulthood. Cdk5 and its neuronal activator p35 are both specifically expressed by postmitotic neurons (Tsai et al., 1993). Expression levels of p35 were low during embryonic development and those of cdk5 were relatively unchanged during development and in adulthood, but both factors were expressed at high levels at postnatal stages.

In situ hybridization, using a probe to the 3′ untranslated region of mouse cyclin E, was employed to define the sites of cyclin E mRNA expression in the cerebral cortex and the cerebellum at different developmental stages. In addition, the localization of cyclin E protein was examined by immunohistochemistry, on serial sections adjacent to those used for *in situ* hybridization. Cyclin E localization was further compared with that of cdk5 by parallel immunohistochemistry on serial sections to identify postmitotic neurons, because cdk5 is specifically expressed in postmitotic neurons (Dhariwala and Rajadhyaksha, 2008; Zhang et al., 2008).

Proliferating progenitor cells are organized in a pseudostratified epithelium during development of the cerebral cortex at E9-E11. At E9.5, cyclin E mRNA was detected in the whole depth of the neuroepithelium (Fig. 2A). Intense immunoreactivity (ir) for cyclin E was detected in the neuroepithelium, excluding the deepest layer where dividing cells are present (Fig. 2B). No cdk5-ir was detected at this stage. Around E12, neuronal precursor cells, which are destined to be cortical neurons, accomplish their terminal mitosis to exit the cell cycle, leave the proliferating zone and migrate outward to the pial surface to form the preplate (pp), which is the earliest layer to form in the laminar organization. Cell proliferation, meanwhile, is limited to the inner neuroepithelial layer, the ventricular zone (vz). At E14.5, the pp is divided into two regions, the marginal zone (mz) and the cortical plate (cp) by the arrival of later-generated cortical neurons. At E12.5 and E14.5, cyclin E mRNA was detected throughout the depth of the neuroepithelium, but the signal in the middle part was slightly weaker in comparison to the luminal and superficial layers (Fig. 2C and F). Cyclin E–ir was detected in cells in the vz and the pp at E12.5 (Fig. 2D). Cdk5-positive postmitotic neurons were confined to the pp (Fig. 2E). At E14.5, cyclin E-ir was detected in cells within the vz and the subventricular zone (svz) but was reduced in the cp and the mz, although there were some cells that showed intense cyclin E-ir in the mz (Fig. 2G). At P0, cyclin E mRNA was detected at levels above background in the molecular layer (ml) and the cp, but the intermediate zone and the vz showed very little hybridization (Fig. 2I). At this stage, cyclin E-ir was detected in a number of cells in the cp and in occasional cells that were localized beneath the pia in the most superficial aspect of the ml, but few cells in the vz exhibited cyclin E-ir (Fig. 2J). Cdk5-ir was confined to cells within the cp at E14.5 and P0 (Fig. 2H and K). Cyclin E mRNA was detected in cells within layers 2–6 in the adult cerebral cortex (Fig. 2L and L′), whereas expression in layer 1 was closer to background (Fig. 2L). Both cyclin E-ir and cdk5-ir were detected in cells within layers 2–6 (Fig. 2M and N). Higher magnification views showed that the two proteins were expressed in the nuclei, cytoplasm and apical processes of pyramidal neurons and that cyclin E but not cdk5 was expressed in glial cells (Fig. 2 M′, N′).

In the cerebellar primordium, which is formed around E14, neuronal progenitor cells are located in two distinct progenitor zones, the vz and the more dorsally located rhombic lip (rl). Both cyclin E mRNA and protein for cyclin E were detected throughout the E14.5 cerebellar primordium and the signals were highest in the vz (Fig. 2O and P). Newly differentiating neurons, including Purkinje cells, leave the vz and migrate within the differentiating zone (dz), and cells exiting the rl migrate over the primordium to form the external granular layer (egl), where progenitor cells destined to be granule cells are proliferating. At P0, the Purkinje cell layer (pcl), which is formed underneath the egl, showed strong cyclin E mRNA expression, whereas the expression in other regions was detectable at levels closer to background (Fig. 2Q). In the adult cerebellum, cyclin E mRNA levels were high in the pcl, but low levels were also detected in the molecular cell layer (mcl) and in the internal granular layer (igl) (Fig. 2T). At P0 and in adulthood, intense immunoreactivities for cyclin E and cdk5 were detected in cells within the pcl (Fig. 2R, S, U, V). Intense cyclin E-ir was also detected in the processes of Bergmann glia (indicated by arrowheads in Fig. 2U), which were negative for cdk5 (Fig. 2V).

These results demonstrate that cyclin E is expressed by terminally differentiated neurons in the two brain regions. Cyclin E is found in mature neurons of the primate cerebral cortex (Lukaszewicz et al., 2005) and in the rat hippocampus (Burke et al., 2006), which supports the current findings.

The tightly overlapping distributions of cyclin E mRNA and protein indicate that cyclin E mRNA is translated into cyclin E protein in the same regions. This validates the specificity of the cyclin E probe and of the cyclin E antibody used for *in situ* hybridization and immunohistochemistry, respectively.

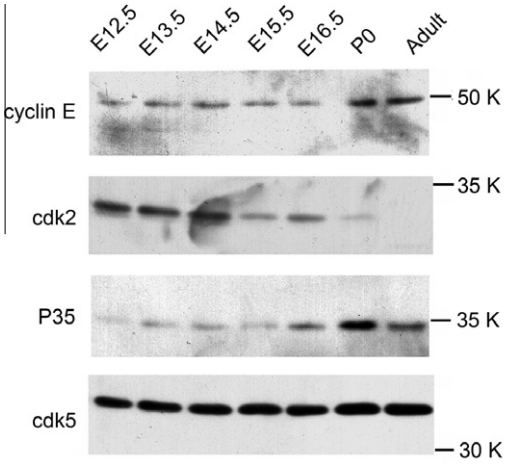

Fig. 1. Temporal expression profile of cyclin E during mouse cerebral development. Extracts prepared from mouse embryonic, postnatal and adult cortices are subjected to Western blot analysis with specific antibodies. The antibodies include cyclin E and p35, and their binding partners, cdk2 and cdk5. Cyclin E is expressed in the brain at all stages from embryonic day 12.5 (E12.5) through to adulthood (Adult), and the levels at postnatal day 0 (P0) and in the Adult were observed to be the highest.

1.2. Cyclin E is expressed in both proliferating and non-proliferating cells during development of the cerebral and cerebellar cortices

The distribution of cyclin E and of some cell cycle regulators, including cdk2, cyclin A and p27, was examined using double-label immunofluorescence with proliferating cell nuclear antigen (PCNA), a marker for proliferating cells. Distribution was examined in two brain regions, the cerebral cortex at E12.5 and the cerebellum at P0-P1, where both actively proliferating and non-proliferating cells are simultaneously present.