



## Expression of D-type cyclins in differentiating cells of the mouse spinal cord

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### Abstract

The D-type cyclins form complexes with the cyclin dependent (CD) kinases CDK4 and CDK6 and promote the G1-S phase transition of the cell cycle by antagonizing the retinoblastoma suppresser protein pRB. In the developing nervous system D-type cyclins show spatially and temporally dynamic patterns of expression. We demonstrated that cyclin D1 was transiently expressed in differentiating spinal cord ventral interneurons while cyclin D3 protein was expressed in differentiating motor neurons and dorsal interneurons. This expression of cyclin D3 in neurons of the mantle zone was extended to all regions of the spinal cord at E15.5. The results suggest that cyclin D1 and D3 have specific functions in differentiating neurons. Similarly, in the developing midbrain-hindbrain region the D-type cyclins were expressed in different subsets of cells. Our results argue in favor of different functions for D-type cyclins during proliferation and differentiation of neural progenitors.

*Key words:* cell cycle, D-type cyclins, midbrain-hindbrain region, neurogenesis, spinal cord.

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### Introduction

The induction of D-type cyclins (cyclins D1, D2 and D3) by mitogens causes a close link between the core cell cycle machinery and the extracellular environment (Matsushime *et al.*, 1991; Sherr, 1996; Sherr and Roberts, 1999). The D-type cyclins associate with the cyclin-dependent kinases CDK4 and CDK6 (Bates *et al.*, 1994a; Meyerson and Harlow, 1994; Tam *et al.*, 1994).

The D-type cyclin-CDK complex phosphorylates the retinoblastoma tumor suppressor protein, pRB and the pRB related proteins p107 and p130 (Bates *et al.*, 1994b; Matsushime *et al.*, 1994; Sherr and Roberts, 1999). This in turn allows the additional phosphorylation of pRB by cyclin E and cyclin A-cdk2 complexes leading to the release of the E2F transcription factors and thereby promoting the G1-S phase transition of the cell cycle (Adams and Kaelin, 1995, Lukas *et al.*, 1995a,b; Adams and Kaelin, 1996; Dyson, 1998).

During mouse embryogenesis the D-type cyclins show highly dynamic expression patterns in different tissues suggesting that unique promotor elements exist in the three genes (Aguzzi *et al.*, 1996). These expression patterns can be overlapping or exclusive in a specific tissue at a determined developmental stage. In the developing nervous

system, for example, only cyclin D2 is expressed in the cerebellar bud between embryonic (E) days 14.5 (E14.5) and E15.5, whereas cyclin D1 and D2, but not D3, are expressed in the subependymal zone of the telencephalon (Aguzzi *et al.*, 1996). During neurulation the expression in the neuroectoderm is regulated along the anterior-posterior and the dorso-ventral axis resulting in distinct patterns for each D-type cyclin and segment specific patterns of cyclin D1 and D2 in the developing hindbrain (Wianny *et al.*, 1998).

As all three D-type cyclins activate CDK4/6, originally they were thought to have redundant functions. This point of view was challenged by the observation that in mice expressing only one single D-type cyclin although this cyclin D is expressed ubiquitously it cannot substitute for the two missing D-type cyclins in all regions of the developing embryo (Ciemerych *et al.*, 2002). For example, mice expressing only cyclin D3 lack normal cerebella while mice expressing only cyclin D2 present neurological abnormalities (Ciemerych *et al.*, 2002). Previous studies have also revealed the expression of cyclin D1 and D2 in post-mitotic cells of the developing nervous system (Tamaru *et al.*, 1993; Ross and Risken, 1994; Wianny *et al.*, 1998; De Falco *et al.*, 2004; Schmetsdorf *et al.*, 2005; Schmetsdorf *et al.*, 2006). These results suggest that the different D-type cyclins have specific functions in proliferating and differentiating cell populations during

neurogenesis. Additionally, expression of D-type cyclins has been implicated in neuronal cell death associated with seizure, ischemia, stress and neurodegenerative diseases (Becker and Bonni, 2005; Hoglinger *et al.*, 2007).

In the present study we analyzed potential functions of D-type cyclins in differentiating neurons of the developing mouse spinal cord and the midbrain-hindbrain region.

## Materials and Methods

Pregnant female mice (*Mus musculus*) with a mixed 129SV/BL background were sacrificed according to institutional guidelines. Embryos were generated by timed-mating, counting the morning after pairing as embryonic day 0.5. Antibody staining experiments on paraffin sections and in situ hybridizations on cryosections were performed as described by Weller *et al.* (2006). Primary antibodies against cyclin D1, D2 and D3 proteins were purchased from Dianova (DLN-12841; DLN-08380; DLN-08391 and DLN-08401). The antibody against Lim1 protein was purchased from Chemicon (AB3200). The antibodies against Islet1, Islet2 and Lim3 proteins were a kind gift from S. Pfaff and T. Jessel (Tsuchida *et al.*, 1994; Shirasaki and Pfaff, 2002). The antibody against neurofilament was purchased from Sigma (N5264). Primary antibodies were visualized with Cy3 or Alexa conjugated secondary antibodies (Jackson Labs) and colorimetrically using the Envision™ +HRP system (DAKO) according to the manufacturer's instructions. Possible cross-reactions of secondary antibodies were excluded by control experiments.

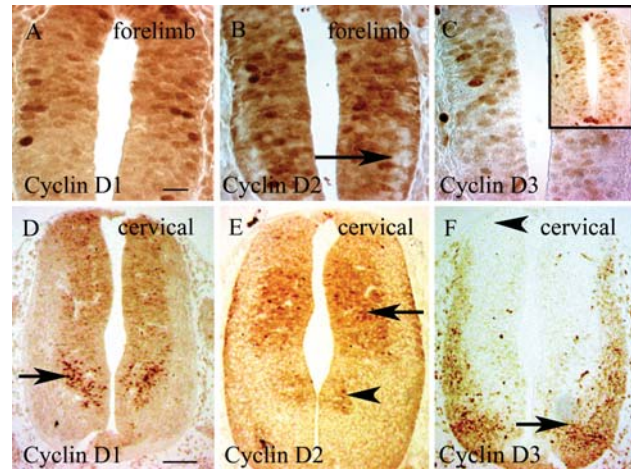
Images were collected using an Axiophot light microscope (Zeiss) in conjunction with a ProgRes 3008 (Jenoptik). Attachment and Image processing was performed with Adobe Photoshop CS software.

## Results

Cyclin D1, D2 and D3 showed different expression patterns in the spinal cord at the E10.5 and E12.5 stages. The results (Figure 1) were summarized in Table 1.

The expression patterns of cyclins D1 and D3 at the E12.5 stage were investigated to ascertain if they were associated with different cell types defined by the expression of patterning genes. To address this question double staining experiments against D-type cyclins and the proteins of patterning genes were performed. Lim1 is expressed in the lateral subdivision of the lateral motor neuron column and in ventral interneurons (LMC<sub>L</sub>; Ensini *et al.*, 1998; Sockanathan and Jessel, 1998; Shirasaki and Pfaff, 2002). It is possible to detect all postmitotic motor neurons with an antibody recognizing Islet1 and Islet 2 (Tsuchida *et al.*, 1994; Pfaff *et al.*, 1996). We found that Lim1 and cyclin D1 were co-expressed at high levels in ventro-lateral spinal cord cells in different regions along the rostro-caudal axis (Figure 2A, B, arrows). The cells

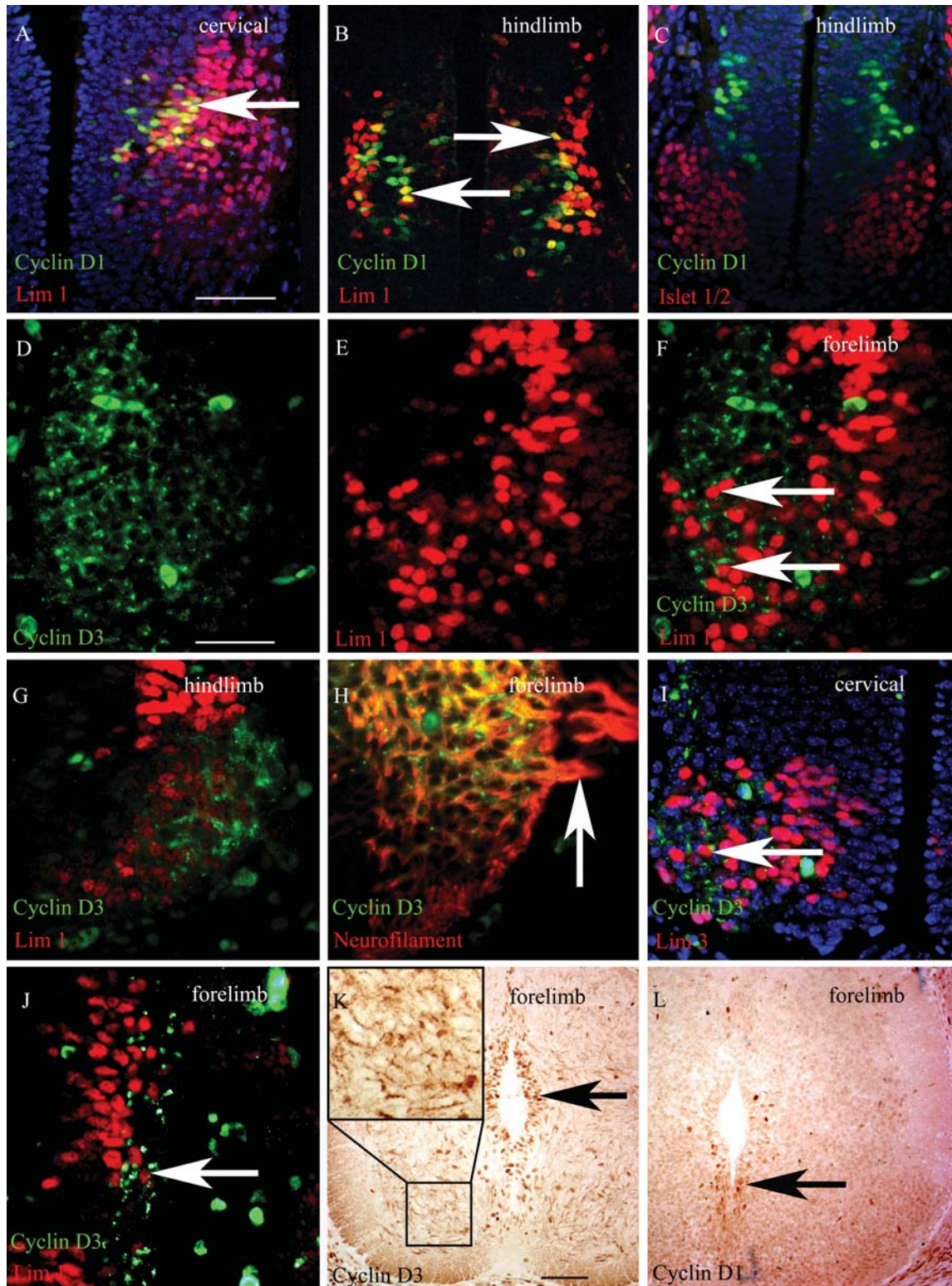
expressing cyclin D1 were located in the spinal cord intermediate and mantle zones. To clarify if these cells were motor neurons or interneurons double stainings with Islet1 and Islet2 were performed. Ventral spinal cord cells expressing cyclin D1 at high levels never expressed Islet1 or Islet2 (Figure 2C), indicating that these cells were not motor neurons but ventral interneurons.



**Figure 1** - Consecutive sections through the mouse spinal cord (SC) are shown. Protein expression of D-type cyclins at the E10.5 embryonic stage at the forelimb-level (A-C) and the E12.5 embryonic stage at the cervical level (D-F). (A) Cyclin D1 is expressed in all or most cells but at higher levels in the dorsal SC. (B) Although cyclin D2 shows equal expression levels in the majority of the SC, the protein is missing in some mantle zone cells (arrow). (C) Cyclin D3 is expressed at higher levels in the dorsal spinal cord and at the highest levels in the floor plate and in a small cell population of the roof plate. (D) Expression of cyclin D1 in the ventricular and the intermediate zone. Highest protein levels are found ventrally (arrow). (E) Expression of cyclin D2 dorsally in cells of the ventricular and the intermediate zones (arrow) and at weak levels in a ventral cell population (arrowhead). (F) Expression of cyclin D3 in the mantle zone (arrow) is missing in the dorsal region (arrowhead). Dorsal on the top; Ventral at the bottom. Scale bars: In A, 50  $\mu$ m for A-C; In D, 50  $\mu$ m for D-F and the inset of C.

**Table 1** - 1. Expression of D-type cyclins in the developing mouse spinal cord. The number of plus symbols indicates the staining intensity (+++ > ++ > +) for each D-type cyclin in the spinal cord at embryonic stages E10.5 and E12.5.

Embryonic stages	Cyclin		
	D1	D2	D3
<b>E10.5</b>			
Dorsal spinal cord	+++	+++	++
Ventral spinal cord	++	+++	+
Roof plate	+++	+++	+++
Floor plate	++	+++	+++
<b>E12.5</b>			
Dorsal ventricular zone and intermediate zone	++	+++	+
Ventral ventricular zone and intermediate zone	+++	++	+
Dorsal mantle zone	+	+	++
Ventral mantle zone	+	+	+++



**Figure 2** - Sections through the spinal cord (SC) E12.5 embryonic stage showing fluorescent staining (A-J) and through the spinal cord E15.5 embryonic stage showing colorimetric staining (K, L). (A-C) Cyclin D1 (green) co-localizes with Lim1 (red; A, B, arrows), but not with Islet 1/2 (red; C). (D-G) Cyclin D3 (green) is expressed ventrally in Lim1 (red) positive cells at the forelimb (D-F) and the hind limb level (G). In (F) the overlay of (D) and (E) is shown. (H) Cells expressing cyclin D3 show neurofilament (red) positive axon extensions typical of motor neurons (arrow). (I) Cyclin D3 (green) positive ventro-lateral motor neurons expressing Lim3 (red, arrow). (J) Dorsal Lim1 (red) positive interneurons expressing cyclin D3 (green, arrow). (K) Cyclin D3 is expressed in cells around the lumen (arrow) and in a punctuated pattern in the whole SC. The inlay shows a ventro-lateral part of the spinal cord at a higher magnification. (L) Cyclin D1 is expressed in cells ventrally of the lumen (arrow). Dorsal on the top; Ventral at the bottom. Scale bars: In A, 50  $\mu$ m for A-C; In D, 25  $\mu$ m for D-J; In K, 50  $\mu$ m for K, L.

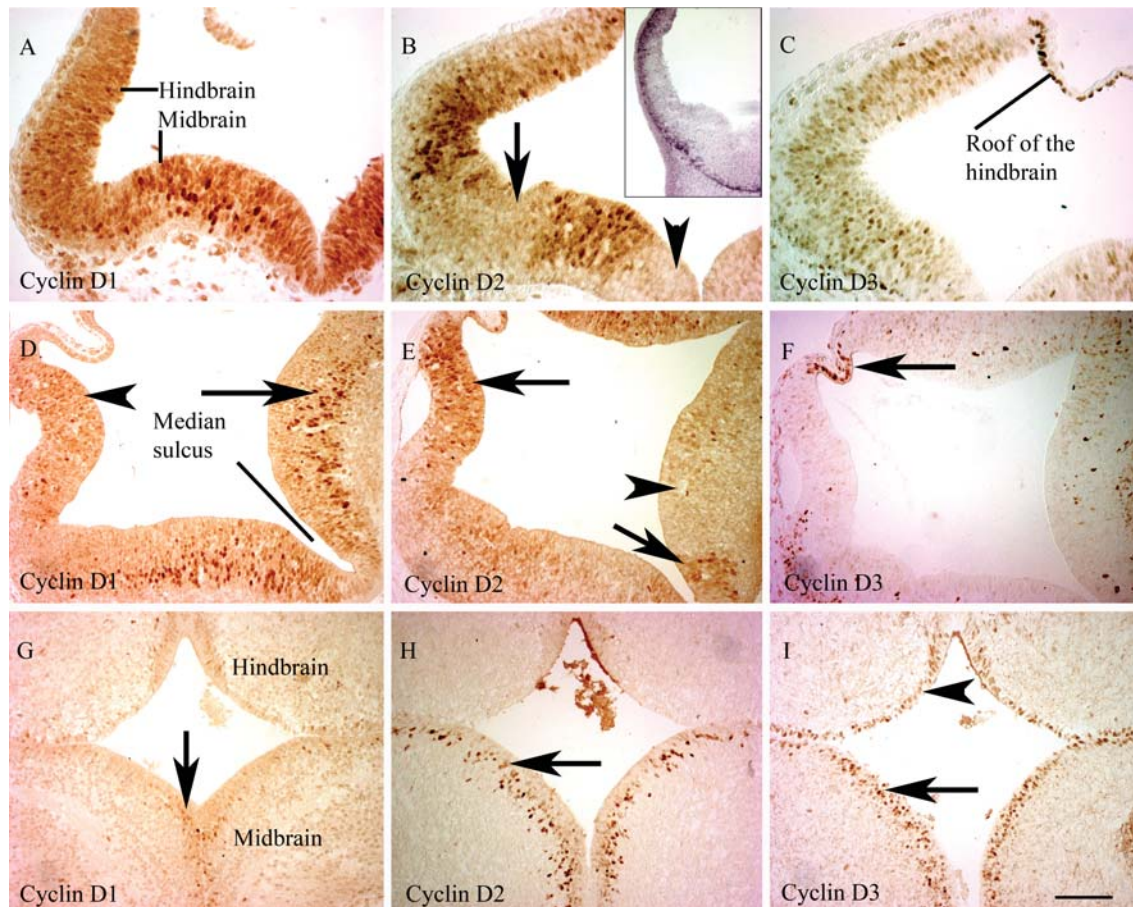
The cyclin D3 protein showed a punctuated expression pattern in marginal zone cells (Figure 2D). These cyclin D3 spots were localized exclusively in the cytoplasm (Figure 2I). The expression of cyclin D3 overlapped in the ventral spinal cord with Lim1 at the forelimb and hind limb levels (Figure 2D-G, arrows) and Islet1/2 (not shown). Additionally, cyclin D3 co-localized with Lim3 (Figure 2I, arrow), a marker of the medial motor neuron column (MMC; Sockanathan and Jessel, 1998). Double staining demonstrated that the motor neurons expressing cyclin D3 at the E12.5 stage, extended axons (Figure 2H, arrow). Additionally, cyclin D3 was expressed in Lim1 positive dorsal interneurons (Figure 1J, arrow).

These results suggest that cyclin D3 is expressed in most, or all, differentiated neurons located in the mantle zone. To address the question of whether this expression of cyclin D3 is extended to other differentiated cells at later

stages, the expression was analyzed at the E15.5 stage. At the E15.5 stage, the punctuated pattern of cyclin D3 protein is present in all or most intermediate and mantle zone cells (Figure 2K), while whole cell bodies were stained in the ventricular zone (Figure 2K, arrow). At the same developmental stage, cyclin D1 was expressed in cells of the ventral ventricular zone (Figure 2L, arrow) while cyclin D2 was absent in the whole spinal cord (not shown).

To prove whether the D-type cyclins were also expressed in distinct subsets of cells in the brain, the expression of D-type cyclins in the midbrain-hindbrain region (Figure 3) were analyzed. The results (Figure 3) were summarized in Table 2.

In contrast to cyclin D1 and D2, the expression level of cyclin D3 was high in the hindbrain at the E15.5 stage (Figure 3I, arrowhead). Moreover, in the midbrain cyclin D3 was expressed in cells closer to the ventricle than those expressing cyclin D2 (Figure 3H, I, arrows).



**Figure 3** - Consecutive sections through the midbrain-hindbrain region at embryonic stages E10.5 (A-C), E12.5 (D-F) and E15.5 (G-I) showing colorimetric staining of D-type cyclins. (A, D, G) Cyclin D1 protein is detectable in cells of all cycle phases at E10.5 (A), at stage E12.5 mainly in S-phase cells of the midbrain (B, arrow), at weak levels in cells of the hindbrain (D, arrowhead) and in few cells close to the median sulcus at stage E15.5 (G). (B) Cyclin D2 protein is not expressed in a region of the dorsal midbrain (arrow) and around the median sulcus whereas the RNA is present at equal levels in S-phase cells of all regions (inlay). (E, H) At stage E12.5 cyclin D2 is expressed in the hindbrain and around the median sulcus (E, arrows), it is missing in the dorsal midbrain (E, arrowhead) and is up-regulated in the midbrain at E15.5 (H, arrow). (C, F, I) The expression of cyclin D3 at stage E10.5 (C) is reduced to the roof of the hindbrain at stage E12.5 (F) and present again in the whole midbrain-hindbrain region at stage E15.5 (I). Dorsal on the top; Ventral at the bottom. Scale bar: In I, 100  $\mu$ m for A-I.

**Table 2** - Expression of D-type cyclins in the developing mouse mid and hindbrain region. The number of plus symbols indicates the staining intensity (+++ > ++ > +) for each D-type cyclin in the spinal cord at embryonic stages E10.5, E12.5 and E15.5. A bar (-) denotes negligible staining.

Embryonic stages	Cyclin		
	D1	D2*	D3
E10.5			
Midbrain	+++	++ (do) +++ (ve)	++
Hindbrain	+++	+++	++
Roof of the hindbrain	+++	+++	+++
Median sulcus	+++	-	++
E12.5			
Midbrain	+++	+	+
Hindbrain	++	++	+
Roof of the hindbrain	+	++	+++
Median sulcus	+++	++	+
E15.5			
Midbrain	+	+++	++
Hindbrain	-	-	++

\* do = dorsal, ve = ventral.

## Discussion

At the E10.5 stage, all three D-type cyclins were expressed in most of the spinal cord cells but cyclin D1 and D3 showed higher expression levels in the dorsal half of the spinal cord. Wianny *et al.* (1998) found that the dorso-ventral gradient of the cyclin D1 transcript also occurs in the spinal cord of 7-9 somite-stage embryos. However, in our study we found that at the E10.5 stage cyclin D2 was not missing from the floor plate and also that cyclin D3 was not expressed only ventrally, as was reported for the transcripts of the genes in 7-9 somite stage embryos by Wianny *et al.* (1998). This may have been due to altered expression patterns of these genes during the time course of spinal cord development and differences in protein and RNA expression patterns. We found that at the E10.5 stage cyclin D3 was expressed at higher levels in the floor and the roof plate than in the other spinal cord cells and that few cells of the mantle zone failed to express cyclin D2. The cyclin D3 expression pattern changed completely at the E12.5 stage. At this stage cyclin D3 was expressed in a punctuated pattern in mantle zone cells. It was possible to attribute this expression to ventral motor neurons and dorsal interneurons by the co-expression of Lim1 and Lim3 in these regions. Zhao *et al.*, (1995) have pointed out that spinal cord neurons are generated between the E10 and E14 stages, which suggests that the expression of cyclin D3 in cells of the whole spinal cord at the E15.5 stage has extended to more differentiated neurons. Similarly, Sumrejkanchanakij *et al.* (2003) reported that cyclin D1 became predominantly cytoplasmatic in cortical cells as they underwent terminal differentiation.

Cyclin D3 is expressed in the absence of CDK4/6 during the differentiation of myoblasts and subsequently in myotubes (Kiess *et al.*, 1995) and is also expressed in compartments of columnar gastrointestinal and stratified squamous epithelia where differentiation takes place (Bartkova *et al.*, 1998). Our results suggest an unknown function of cyclin D3 in differentiating neurons. Sicinska *et al.* (2003) reported that cyclin D3 knock-out mice are viable and fertile but display lymphoid abnormalities, which could mean that the remaining cyclins can compensate for the loss of function of cyclin D3 in motor neurons. On the other hand, the spastic leg-clasping reflex in cyclin D1 deficient mice argues in favour of an essential role of cyclin D1 during spinal cord development (Fantl *et al.*, 1995; Sicinski *et al.*, 1995).

Various studies have revealed that, apart from their function in the cell cycle, D-type cyclins may be involved in the regulation of the physiological functions of specific neuronal subpopulations (De Falco *et al.*, 2004; Becker and Bonni, 2005; Schmetsdorf *et al.*, 2006). The loss of cyclin D2 in the mouse cerebellum affected the differentiation of stellate and granule interneurons (Huard *et al.*, 1999). We found that cyclin D1 is localized in Lim1-positive interneurons but totally absent in Islet1- and Islet2-positive motor neurons. This could mean that cyclin D1 plays a specific role during differentiation of spinal cord interneurons, although expression is, presumably, transient since we found no up-regulation of cyclin D1 at the E15.5 stage. Alternatively cyclin D1 might play a role during apoptotic cell death of interneurons. Many studies have revealed the up-regulation of cyclin D and other cell cycle regulating factors before apoptosis of neuronal cells (Timisit *et al.*, 1999; Fischer *et al.*, 2001; Ino and Chiba, 2001; Katchanov *et al.*, 2001; Liu and Green, 2001; Sumrejkanchanakij *et al.*, 2003; Becker and Bonni, 2004; Becker and Bonni, 2005). Programmed cell death is an integral process in the development of the nervous system (Oppenheim, 1991; Meier *et al.*, 2000) and, therefore, an apoptotic function of D-type cyclins in spinal cord and midbrain-hindbrain cells cannot be excluded.

We also found that, similar to the situation in the spinal cord, D-type cyclins were expressed in partially overlapping and different compartments along the dorso-ventral axis of the developing midbrain-hindbrain region. At E10.5 cyclin D2 RNA was expressed at high levels in cells leaving the cell cycle (Figure 3B, inset) but this expression was not correlated with the up-regulation of the cyclin D2 protein in the same region. This argues in favor of post-transcriptional control of cyclin D2 protein expression in differentiating neurons. Alternatively, high levels of cyclin D2 RNA could be localized in S-phase cells but, however, this would imply that in these neural progenitors cyclin D2 has a function only in the daughter cells during the next cell cycle. The observation that at E15.5 cyclin D2 protein was found at high levels in midbrain cells, obviously leaving the

cell cycle (Figure 3H, arrow), supports the idea that cyclin D2 is up-regulated in differentiating neurons. However, we found no up-regulation in the hindbrain. In contrast, at the E15.5 stage, cyclin D3 was up-regulated in both midbrain and hindbrain ventricular zone cells. (Figure 3I, arrow, arrowhead)

The question of whether the up-regulation of the distinct D-type cyclins leads to differences in the length of the G1 phase in neural precursor cells has not been addressed as yet. It is known that the total length of the cell cycle of neural precursors changes between the E11 and E16 stages in the telencephalon due mainly to the lengthening of the duration of the G1 phase (Takahashi *et al.*, 1995). Additionally, it has been shown that lengthening of the G1 phase drives neural precursor cells into differentiation (Iacopetti *et al.*, 1999; Calegari and Huttner, 2003; Calegari *et al.*, 2005).

Different functions of the D-type cyclins in the determination of the length of the G1 phase could contribute mainly to the dynamic expression patterns observed in proliferating cells of the nervous system. These roles of D-type cyclins in proliferating cells could be combined with additional functions during the differentiation of specific cell types in the nervous system. Future experiments should elucidate the specific functions of the D-type cyclins during mammalian neurogenesis in proliferating neural precursor cells and differentiating neurons.

In summary, cyclin D1 and cyclin D3 were found to be expressed in differentiating cells at the E12.5 stage. High levels of cyclin D1 protein were expressed in spinal cord ventral interneurons, whereas cyclin D3 protein was expressed in differentiated dorsal interneurons and ventrally in motor neurons. The expression of cyclin D3 in mantle zone neurons was extended to all regions of the spinal cord at the E15.5 stage. The results suggest that cyclins D1 and D3 have specific functions in differentiating neurons and, similarly, that in the developing midbrain-hindbrain region the D-type cyclins are expressed in different subsets of cells.

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