Cyclin dependent kinase 5 is required for the normal development of oligodendrocytes and myelin formation

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The development of oligodendrocytes, the myelinating cells of the vertebrate CNS, is regulated by a cohort of growth factors and transcription factors. Less is known about the signaling pathways that integrate extracellular signals with intracellular transcriptional regulators to control oligodendrocyte development. Cyclin dependent kinase 5 (Cdk5) and its co-activators play critical roles in the regulation of neuronal differentiation, cortical lamination, neuronal cell migration and axon outgrowth. Here we demonstrate a previously unrecognized function of Cdk5 in regulating oligodendrocyte maturation and myelination. During late embryonic development Cdk5 null animals displayed a reduction in the number of MBP+ cells in the spinal cord, but no difference in the number of OPCs. To determine whether the reduction of oligodendrocytes reflected a cell-intrinsic loss of Cdk5, it was selectively deleted from Olig1+ oligodendrocyte lineage cells. In Olig1Cre/+; Cdk5fl/fl conditional mutants, reduced levels of expression of MBP and PLP mRNA were observed throughout the CNS and ultrastructural analyses demonstrated a significant reduction in the proportion of myelinated axons in the optic nerve and spinal cord. Pharmacological inhibition or RNAi knockdown of Cdk5 in vitro resulted in the reduction in oligodendrocyte maturation, but had no effect on OPC cell proliferation. Conversely, over-expression of Cdk5 promoted oligodendrocyte maturation and enhanced process outgrowth. Consistent with this data, Cdk5−/− oligodendrocytes developed significantly fewer primary processes and branches than control cells. Together, these findings suggest that Cdk5 function as a signaling integrator to regulate oligodendrocyte maturation and myelination.

Introduction

Oligodendrocytes (OLs) the myelin-forming cells of CNS and are derived from oligodendrocyte precursor cells (OPCs) (Raff et al., 1984; Rowitch, 2004). Oligodendrocyte precursors originate in distinct locations and subsequently migrate throughout CNS before differentiating into oligodendrocytes and myelinating target axons (Miller, 1996; Pringle and Richardson, 1993; Sherman and Brophy, 2005). Perturbed myelination during development or failure of remyelination in demyelinating diseases such as multiple sclerosis (MS) leads to axonal damage and irreversible functional loss. Oligodendrocyte development is regulated by a wide range of growth factors (PDGF, neuregulin-1, FGF2), signaling pathways (Shh, LINGO-1, BMP, Wnt and notch signaling) and transcription factors (Olig2, Nkx2.2, Sox10 and MRF) (Barres et al., 1992; Calver et al., 1998; Emery et al., 2009; Fuller et al., 2007; Lu et al., 2000; McKinnon et al., 1990; McKinnon et al., 1993; Mi et al., 2008; Miller et al., 2004; Richardson et al., 1988; Shimizu et al., 2005; Wang et al., 1998). How the roles of these different components are coordinated to regulate the conversion of proliferating OPCs to OLs and subsequent myelination is currently unclear.
Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase with significant homology to cell cycle related Cdns (Hellmich et al., 1992) but has not been implicated in regulating cell cycle progression (Vermeulen et al., 2003). Although Cdk5 is expressed in virtually all tissues, its activity appears to be largely restricted to the nervous system since its critical co-activators, p35 and p39 are highly expressed in the CNS (Lew et al., 1994; Tsai et al., 1994). During neuronal development Cdk5 is important in regulation of cortical lamination, neuronal migration, differentiation and synaptogenesis (Gilmore and Herrup, 2001; Gilmore et al., 1998; Ohshima et al., 1996; Patrick et al., 1998) and loss of Cdk5 leads to deficits of neuronal development and neuronal cell death (Alexander et al., 2004; Cicero and Herrup, 2005). The regulation of Cdk5 activity is complex and may involve the calpain-mediated proteolysis of its co-activators p35 and p39 (Kamei et al., 2007). Alternatively, the non-receptor tyrosine kinases Abl and Fyn, members of the Src-family of tyrosine kinases, phosphorylate Cdk5 at Tyr15, which may modulate its activity (Sasaki et al., 2002; Zukerberg et al., 2000). Not all neuronal cell types are dependent on Cdk5 during development and the spinal cord is less affected than forebrain by the lack of Cdk5 (Yip et al., 2007).

During OPC differentiation Cdk5 activity has been proposed to increase and through the phosphorylation of paxillin (Miyamoto et al., 2007), as well as WAVE2 regulate OPC migration (Miyamoto et al., 2008). In addition, Emx1-Cre mediated Cdk5 conditional mice, showed hypomyelination (He et al., 2011). It is unclear, however whether hypomyelination in these animals reflects Cdk5 loss in oligodendrocyte lineage cells or is secondary to its loss in neurons.

Here we show that Cdk5 expression in the oligodendrocyte lineage is required for the maturation of oligodendrocytes and normal myelination. In Cdk5 knockout animals there is a reduction in the number and distribution of spinal cord MBP+ cells. Conditional deletion of Cdk5 from Olig1+ cells resulted in a reduction of MBP and PLP mRNA expression in the CNS and a reduction in the number of myelinated axons in the optic nerve, while inhibition of Cdk5 delayed OPC differentiation and over-expression promoted OPC differentiation in the absence of changes in cell proliferation. These data suggest that Cdk5 is required for the normal development of mature oligodendrocyte and myelination in the developing CNS and provide insights to speed the development of remyelination strategies in demyelinating diseases such as MS.

Materials and methods

Antibodies

The primary antibodies used in the study include: rabbit polyclonal Cdk5 (Santa Cruz, CA, SC19); mouse monoclonal antibodies of A2B5, O4 and O1 as described previously (Gao et al., 2006; Tsai et al., 2006); rabbit polyclonal antibody anti-NG2 (Millipore, AB5320); rabbit polyclonal Olig2 antibody (Millipore, AB9610); A mouse monoclonal antibody against MBP (Covance, Princeton, NJ) and a polyclonal antibody against EGFP (Invitrogen, A11122).

Animals

Animals were maintained in the Animal Resource Center of Case Western Reserve University School of Medicine using procedures approved by the Institutional Animal Care and Use Committee of the University. Cdk5 heterozygous mice on a C57BL6J genetic background were obtained from Dr. Herrup (Rutgers University) and individual offspring were genotyped by PCR as described previously (Cicero and Herrup, 2005). Studies involving the

Olig1$^{Cre+/+}$: Cdk5$^{fl/fl}$ CKO and age-matched wild type littermate animals were anesthetized using ketamine/xylazine, the brains and the spinal cords were dissected and fixed in 4% paraformaldehyde at 4°C overnight followed in 20% sucrose in PBS overnight. Coronal sections of brains and spinal cords were cryosectioned at 16μm and prepared for in situ hybridization.

**BrdU injection and immunohistochemistry for frozen cryosections**

Animals were injected with 5'-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO, 100μg/g, i.p.) 2 h before sacrifice. Embryos were taken at embryonic day E18, genotyped and fixed in 4% paraformaldehyde at 4°C overnight followed by equilibration in 20% sucrose. Coronal sections of spinal cord were cut at 20μm on a Leica cryostat. For double labeling of BrdU and OPC or OL cell markers, sections were blocked with 5% normal goat serum (NGS) in 0.3% triton in PBS for 1 h, followed by incubation in primary antibody, and subsequently secondary antibody conjugated with Alexa 488 or 596. For BrdU labeling, sections were permeabilized with 2 N HCl for 30 min followed by incubation with anti-BrdU antibody and Alexa conjugated secondary antibodies.

**RNA in situ hybridization**

Digoxigenin-labeled riboprobes against Mbp, Pgp96, Pdgfrα were used to perform RNA in situ hybridization as described previously (Lu et al., 2002). For analysis of proliferation, the conjunction of Pdgfrα in situ hybridization and immunostaining for BrdU were performed as described previously (Xin et al., 2005). Controls and Olig1$^{Cre+}$/Cdk5$^{fl/fl}$ CKO at P7 and P14 were injected (i.p.) with 100 mg/kg BrdU for 4 h before sacrifice of the animals. Sections were hybridized with the Pdgfrα riboprobe (Lu et al., 2002). After fixation with 4% parafomaldehyde for 15 min, sections were treated with 2 N HCl in PBS for 30 min at 37 °C and rinsed in PBS. Sections were blocked with 0.1% NP-40 and 5% normal goat serum in PBS for 1 h at room temperature. After addition of rat polyclonal anti-BrdU antibody (1:100 dilution; Sigma, St. Louis, MO) at 4 °C overnight, the sections were treated using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA); HRP was detected with diamobenzidine (Sigma, St. Louis, MO) according to the manufacturer’s instructions.

**Tissue preparation for EM analysis**

Both spinal cord and optic nerve of Olig1$^{Cre+}$/; Cdk5$^{fl/fl}$ CKO and control mice were dissected at P14 and were fixed in glutaraldehyde (2%) and paraformaldehyde (4%) followed by 1% OsO4, block stained with saturated uranyl acetate, dehydrated through graded alcohols and embedded in Epon 812. Sections were cut in a plane parallel to the surface of the slice. To assess the extent of
myelination and compact myelination, ultrathin sections were examined on a Jeol 100CX electron microscope at 80 kV. To compare the relative thickness of myelin sheaths in the Cdk5 KO and WT animals the g ratios were calculated from at least 35–50 axons in the anterior funiculus of the spinal cord. The proportion of myelinated axons was quantified from the same region of the spinal cord and optic nerve in control and transgenic animals and a minimum of 35–50 axons were assayed.

Preparation and characterization of spinal cord cell cultures

Mixed brain or spinal cord cell cultures were prepared either from E18 Cdk5 KO or postnatal day 2 (P2) Sprague-Dawley rat pups (SD, Harland Animal Resource, USA) as previously described (Kerstetter et al., 2009) and plated at a density of 2 x 10^4 cells/coverslip in DMEM supplemented with 1% FBS-c (HyClone), 10 ng/ml PDGF (PDGF-AA, Sigma) and N2 supplement (Invitrogen). Cultures were maintained at 37°C/5% CO2 and the growth medium was changed every second day. Highly enriched populations A2B5+ or O4+ OPCs were prepared from spinal cord of P1 SD rat pups by immunopanning as previously described (Robinson and Miller, 1996).

Spinal cord explant cultures were prepared from E18 Cdk5−/− and WT mouse embryos. Cervical and thoracic segments of the spinal cord were chopped into 1 mm pieces and transferred to PLL-coated coverslips. BD MatrigelTM Basement Membrane Matrix, Growth Factor Reduced (Cat# 354230, BD Bioscience) was applied with 1% FBS-c (HyClone), 10 ng/ml PDGF (PDGF-AA, Sigma) and N2 supplement (Invitrogen). In some experiments PDGF (50 ng/ml) was added.

Labeling with monoclonal antibodies A2B5 (1:3), O4 (1:4) and O1 (1:4) was performed as live cells as previously described (Nishiyama et al., 1999; Zhang and Miller, 1996). Cells were incubated in primary antibody for 30 min followed by fluorescence-conjugated Alexa mouse IgM 488 or 596 secondary antibodies. Cells were then fixed in 4% paraformaldehyde for 20 min. For double labeling, second primary antibodies – rabbit Cdk5 (1:200), mouse MBP (1:500) or rabbit EGFP (1:500) were applied to cells and incubated overnight at 4°C followed by incubation with fluorescence-conjugated secondary Alexa 488 or 596-conjugated antibody.

Transfection of OPCs in spinal cord cell cultures

Cells were collected from dissociated spinal cord cultures and centrifuged at 100 rpm at room temperature. The cell pellet was re-suspended to a density of 5 x 10^6 cells/100 μl in O2 Nucleofection solution (Amaza rat oligodendrocytes Nucleofector kit, Amaza). 100 μl of Nucleofection solution containing 5 x 10^5 cells was added to RNAi for Cdk5 (~4 μg/ml) or Cdk5-EGFP plasmids (2 μg/ml) as well as control pEGFP-C1 plasmid or a scrambled siRNA plasmid followed by electroporation using the Amaza nucleofection apparatus with the program O-17 (Amaza) according to the manufacturer’s instructions. Cells were plated on PLL-coated coverslip at 2 x 10^4/coverslip and grown for 2–4 days in growth media as described above.

Quantification and Statistics

Cell counts: Blinded cell counts of cultured cells and labeled cells in age and location matched frozen sections were performed on at least three coverslips or sections per condition from three separate experiments using a Leica Fluorescence microscope with a 20 x or 40 x objective lens and a reticule (10 x 10 grid). For each experiment, seven fields were randomly selected on each coverslip or section. The total number of immunopositive cells (A2B5+, O4+, O1+ or MBP+) and the total number of DAPI stained cells were calculated and compared between treated cultures and non-treated controls. For quantification of MBP immunostaining in the tissue sections, the average of total numbers of MBP+ cells for per spinal cord section was calculated and compared between Cdk5−/− and wild type animals at E18. To quantify the proportion of myelinated axons in the spinal cord, the anterior funiculus region in ventral spinal cord was selected and the at least 200 axons assayed in three animals of each genotype. To quantify the effects of loss of Cdk5 on the cytoarchitecture of oligodendrocytes the number of primary processes and the number of branches were assayed in the mixed brain cell cultures of WT and Cdk5−/− cells (E18+ 5DIV or 9DIV). For foot print analyses, the average footprint area of at least 50 individual O4+ cell or MBP+ cell was measured by ImageJ from WT and Cdk5−/− cultures treated with PDGF. Paired 2-way t-tests were used for statistical analysis between treated and non-treated groups and the significance level determined as P < 0.05.

Results

Reduction of MBP+ cells during development in Cdk5 knockout animals

To determine whether the lack of Cdk5 influenced the development of oligodendrocytes, sections through the spinal cord of wild type and Cdk5−/− littermates were labeled with the antibodies for OPCs (Olig2) and mature OLs (MBP) and the relative number of cells assayed. The number of MBP+ cells was reduced in the spinal cord of Cdk5−/− animals compared to WT littermates at E18 (Fig. 1G, J). For example, the mean number of MBP+ cells/section in the wild type spinal cord was approximately 50 ± 1.8 and this number was reduced to 18 ± 1.6 in Cdk5−/− animals (Fig. 1M). The distribution of MBP+ oligodendrocytes was also more restricted in Cdk5−/− animals compared to their wild type littermates. In Cdk5 mutant animals MBP+ cells were primarily localized to ventral midline regions of the spinal cord, while in littermate controls MBP+ cells were distributed more widely throughout the dorso-ventral axis with a higher concentration in ventral regions. The reduction in the number of MBP+ cells in Cdk5−/− mice was not a reflection of a reduction in progenitor cells since the number of Olig2+ OPCs was not significantly different between WT and Cdk5−/− animals. Previous studies suggested that Cdk5 regulated OPC migration (Miyamoto et al., 2008) in vitro, however no significant difference in the distribution of Olig2+ OPCs was detected between Cdk5−/− and WT animals. These data suggest that in the absence of Cdk5 the generation of mature oligodendrocytes is compromised while the generation of OPCs is relatively less affected. The transition from OPCs to oligodendrocytes has been linked to the proliferative status of OPCs and to assess changes in cell division animals were pulsed with BrdU during E16–E18 and the average numbers of dividing OPCs in region matched sections of spinal cord compared between WT and Cdk5−/− animals. No significant differences in OPC proliferation were apparent between WT (61 BrdU+ cells ± 1.4) and Cdk5−/− (56 BrdU+ cells ± 1.7) littermates (Fig. 1B and E; H and K) suggesting the Cdk5 is not required for OPC proliferation and dispersal but is required for the timely development of mature oligodendrocytes.

Deletion of Cdk5 in Olig1+ cells results in reduced expression of Mbp and Plp1

The Cdk5 null animals have multiple defects and die before birth limiting their usefulness in the analyses of the later stages
of oligodendrocyte maturation and myelination. To determine whether the reduction in MBP+ cells in Cdk5−/− animals reflected the loss of Cdk5 function in cells of the oligodendrocyte lineage, Cdk5 was specifically deleted in Olig1+ cells using Cre-lox technology (Fig. 2A). In contrast to Cdk5−/− animals, the Olig1Cre+/−; Cdk5fl/fl (Cdk5 cKO) animals are viable and fertile. Comparison of the mRNA expression of the myelin components PLP and MBP by in situ hybridization, however, showed a substantial reduction in the expression of these myelin genes in Cdk5 cKO animals compared to littermate controls in the corpus callosum and overlying cortex at postnatal day 7 (Fig. 2B−E). This phenotype was not restricted to early postnatal development, rather comparable analyses at P14 showed the phenotype to be relatively more severe than at P7 (Fig. 2F−I). In the P7 spinal cord quantification of plp mRNA positive cells revealed a significant reduction in Cdk5 cKO animals (29 ± 2.8 in gray matter and 138 ± 6.3 in white matter) compared to WT littermates animals (49 ± 3.0 in gray matter and 207 ± 7.6 in white matter, P < 0.001; Fig. 2J−L). As in Cdk5−/− animals, quantification of the number of PDGFA+ OPCs and their relative level of proliferation revealed no significant differences in either the total number of OPCs or their incorporation of BrdU in Cdk5 cKOs compared to littermate controls (Fig. 2M−Q).

Deletion of Cdk5 in Olig1+ cells results in a reduction of myelinated axons in Olig1Cre+/−; Cdk5fl/fl cKO

The reduction in the number of mature oligodendrocytes persisted during development, was widespread, and resulted in a reduction in the level of myelination. In the spinal cord of Olig1Cre+/−; Cdk5 cKO animals in situ hybridization at P14 showed reduced expression of MBP mRNA compared to littermate controls (Fig. 3A, B). This reduction was relatively uniform in all regions of the spinal cord with clear reductions in both dorsal and ventral white matter suggesting the majority of oligodendrocytes were affected by the loss of Cdk5. Consistent with the reduction in myelin gene expression, ultrastructural analysis revealed a significant reduction of myelinated axons and compact myelin in P14 spinal cord of Olig1Cre+/−; Cdk5 cKO compared to littermate controls (Fig. 3C−F). The relative proportion of myelinated axons was reduced from 44.9% ± 2.4 in control animals to 30.5% ± 2.9 in the Olig1Cre+/−; Cdk5 cKO animals (Fig. 3I). Analyses of the g ratios between WT and Olig1Cre+/−; Cdk5 cKO animals revealed a reproducible reduction in the thickness of myelin sheaths in Olig1Cre+/−; Cdk5 cKO animals (Fig. 3E, F). To determine whether the reduction in myelination was present in other white matter regions, the proportion of myelinated axons was compared in the optic nerves of wild type and Olig1Cre+/−; Cdk5 cKO animals. Consistent with the findings in the spinal cord in Olig1Cre+/−; Cdk5 cKO animals, an apparent reduction in the proportion of myelinated axons (5.7% ± 0.6) was seen in the optic nerve compared to littermate WT (18.3% ± 1.1) controls (Fig. 3G−J, K) and as in the spinal cord there was an increase in the g ratio of Olig1Cre+/−; Cdk5 cKO optic nerve myelinated axons (Fig. 3L). Taken together these data suggest Cdk5 is important for the generation of the correct cohort of oligodendrocytes and subsequent myelin formation throughout the CNS.

Cdk5 is expressed in the different stages of OPCs and oligodendrocytes and selectively regulates oligodendrocyte maturation

The reduction in mature oligodendrocytes and myelination may reflect a role for Cdk5 in multiple aspects of oligodendrocyte development. Characterization of the expression of Cdk5 in cells of the oligodendrocyte lineage in vitro revealed its expression throughout oligodendrocyte development in the majority of cells. In cultures of pan-purified rat A2B5+ cells virtually all cells expressed Cdk5 in the perinuclear cytoplasm and in the nucleus (Fig. 4A−C) and this expression was maintained as the cells developed into O4+ cells (Fig. 4D−F). Mature oligodendrocytes...
Fig. 2. The expression of *Mbp* and *Plp1* mRNA is reduced during postnatal development in *Olig1*<sup>Cre+</sup>; *Cdk5*<sup>fl/fl</sup> cKO animals compared to *Olig1*<sup>Cre+</sup>; *Cdk5*<sup>fl/+</sup> littermate controls. (A) An outline of the genetic approach of conditional deletion of *Cdk5* in olig1+ cells used in the current study. Exons II-V encoding vital *Cdk5* catalytic-domain components were flanked with loxp elements and excised by Olig1-Cre. (B–E) At P7, *in situ* hybridization reveals a reduction of *Mbp* and *Plp1* mRNA in the corpus callosum and overlying cortex of *Olig1*<sup>Cre/+</sup>; *Cdk5*<sup>fl/fl</sup> cKO (C & E) compared to their WT littermates (B, D). F–I the reduced expression mRNA for *Mbp* and *Plp1* is more pronounced at P14 in the corpus callosum of *Olig1*<sup>Cre+</sup>; *Cdk5*<sup>fl/fl</sup> cKO (G, I) compared to their WT littermates (F, H). (J–L) at P7 spinal cord, the total number of *plp1*+ cells in both gray matter (GM) and white matter (WM) is significantly reduced in *Olig1*<sup>Cre+</sup>; *Cdk5*<sup>fl/fl</sup> cKO revealed by *in situ* hybridization. No significant difference is seen in the number of *pdgfra*+ OPCs following a 2 h pulse of BrdU by double labeling of BrdU+ (brown)/*pdgfra*+ (blue) cells (arrows) in the cortex (Ctx) and in corpus callosum (CC) between WT (M, O) and *Olig1*<sup>Cre+</sup>; *Cdk5*<sup>fl/fl</sup> cKO (N, P). Quantitative data is shown in bar graphs Q. The data represent the mean ± SD taken from at least 8 sections from 3 independent animals of each genotype. Scale bar=25 μm.
Fig. 3. Conditional deletion of Cdk5 in Olig1+ cells results in myelination defects. Reduced myelination in the spinal cord and the optic nerve of Olig1\textsuperscript{Cre}\textsuperscript{+}; Cdk5\textsuperscript{fl/fl} cKO. (A–B) In situ hybridization of mRNA expression of Mbp shows reduced level in the spinal cord of Olig1\textsuperscript{Cre}\textsuperscript{+}; Cdk5\textsuperscript{fl/fl} cKO at P14 compared to their WT littermates. (C–J) At P14 Olig1\textsuperscript{Cre}\textsuperscript{+}; Cdk5\textsuperscript{fl/fl} cKO contained significantly reduced number of myelinated axons compared to their WT littermates in both spinal cord (C–F) and the optic nerve (G–J). The relative thickness of the myelin was also reduced in the Olig1\textsuperscript{Cre}\textsuperscript{+}; Cdk5\textsuperscript{fl/fl} cKO animals and g ratio for spinal cord and optical nerve axons shows a significant reduction in Olig1\textsuperscript{Cre}\textsuperscript{+}; Cdk5\textsuperscript{fl/fl} cKO (J, L). Quantitative analysis of the proportion of myelinated axons in the spinal cord and optic nerve at P14 reveals a significant reduction in the absence of Cdk5 in oligodendrocyte lineage cells (I, K). $P < 0.001$. Scale bar = 25 μm.
identified through expression MBP+ also expressed Cdk5 predominantly in their cell bodies (Fig. 4G–I). The expression of Cdk5 was not restricted to cells of the oligodendrocyte lineage during development but as previously described (Yip et al., 2007) was also expressed in neurons and NG2+ cells of adult rat spinal cord (Fig. 4J–L).

While the Olig1 conditional deletion studies strongly implicate a requirement for Cdk5 in the development of oligodendrocytes they provide limited insights into which specific biological processes are influenced. The preferential effect of Cdk5 loss on MBP+ cells in the mutant animals suggests that Cdk5 is predominantly involved in later stages of oligodendrocyte maturation. Consistent with this notion treatment of P1 pan-purified A2B5+ spinal cord OPCs with the Cdk inhibitor roscovitine (10 μM) (Gray et al., 1999; Jorda et al., 2012) had a small but not significant effect on the numbers of NG2+ cells (control 15.9±2.1; experimental 10.8±2.1) and Olig2+ (control 18.6±1.5; experimental 12.4±1) cells that developed (Fig. 5A–I, T). By contrast, the number of MBP+ cells that developed was significantly reduced in roscovitine treated cultures compared to controls (control 20.1±1.8: experimental 8.1±1.7, p < 0.05) (Fig. 5M–R, T). Not only were the numbers of MBP+ cells reduced but the morphological complexity of the residual MBP+ cells was also reduced following roscovitine treatment such that the number of MBP+ cells with long and complex branched processes or sheets of membrane was decreased compared to controls (Fig. 5M–R). The reduction in the number of mature oligodendrocytes in roscovitine treated cultures is not a consequence of enhanced proliferation of OPC blocking their differentiation. Pulsing OPC cultures with BrdU for 7 h prior to analyses revealed and no significant changes in the proportion of NG2+ (control 21.2±2.1: experimental 20±2.3) or Olig2+ (control 19.2±1.9: experimental 17.1±2.2) OPCs that incorporated BrdU compared to controls (Fig. 5S, n=3) suggesting that Cdk5 is not critical for OPC proliferation but, rather is important for the transition of newly formed oligodendrocytes to mature oligodendrocytes.

Knockdown of endogenous Cdk5 delays oligodendrocyte maturation while over-expression of Cdk5 promotes MBP+ cell appearance

The roscovitine studies suggest that kinase activity is important for the maturation of oligodendrocytes. To define the specific role of Cdk5 in oligodendrogenesis, Cdk5 was targeted by RNAi in spinal cord mixed cell cultures via electroporation using Amaxa Nucleitransfecto and the development of oligodendrocytes were assessed. In cultures transfected with Cdk5 RNAi endogenous Cdk5 was markedly down-regulated after 2 days (Fig. 6Ab-b″) while in control cultures the majority of cells expressed Cdk5 (Fig. 6Aa-a″), indicating that the Cdk5 RNAi effectively suppresses endogenous Cdk5 expression. Analysis of the oligodendrocyte development in Cdk5 RNAi treated cultures demonstrated that the complexity of the processes of O4+ cells lacking Cdk5 was reduced, resulting in
Fig. 5. Blocking Cdk5 with roscovitine inhibits oligodendrocyte maturation but does not affect OPC proliferation. (A–F) The number of NG2+ cells present in A2B5 pan purified cell cultures was only slightly reduced following inhibition of Cdk5 activity for 4 days by treatment with roscovitine and their proliferation as measured by incorporation of BrdU was not significantly different. (G–L) The number of Olig2+ cells present in A2B5 pan purified cell cultures was slightly reduced following inhibition of Cdk5 activity for 4 days by treatment with roscovitine and their proliferation as measured by incorporation of BrdU was not significantly different. (M–R) the number of MBP+ cells that developed in A2B5 pan purified cell cultures was significantly reduced following inhibition of Cdk5 activity for 4 days by treatment with roscovitine. The morphology of MBP+ cells in roscovitine treated cultures was notably less complex than in vehicle treated control cultures. The cells contained fewer processes and lacked characteristic membrane sheets of mature MBP+ cells. Quantitation is shown in histogram (S, T) and represents the mean ± SD taken from at least 3 independent coverslips from 3 different experiments. Bar=25 μm.
A cell body with relatively few short processes (Fig. 6Bb-b″). A similar reduction in the complexity of MBP+ cell processes was seen in MBP+ cells following Cdk5 RNAi treatment (Fig. 6Bc-c″). Oligodendrocytes in the cultures that escaped electroporation retained Cdk5 expression and developed normal process arborizations (Fig. 6Be-e″). These findings are consistent with the effects of pharmacological inhibition of Cdk5 and suggest that it plays a critical role in regulating oligodendrocytes development and maturation. Quantification of the proportion of Cdk5+/MBP+ cells demonstrated a significant decrease in cells subjected to Cdk5 knockdown compared to controls (Fig. 6D). While not all cells in the cultures had reduced expression of Cdk5 none of the cells that lacked Cdk5 had detectable expression of MBP, while cells that retained Cdk5 exhibited normal MBP expression, suggesting reduced or delayed maturation of oligodendrocytes in the absence of Cdk5.

To determine whether increasing the expression of Cdk5 enhanced the maturation of oligodendrocytes, cultures were transfected with Cdk5-EGFP (Fig. 6C) or a randomly scrambled siRNA (not shown) and labeled with O1+ or anti-MBP+ antibodies. Overexpression of Cdk5 resulted in O1+ (Cb-b″) and MBP+ oligodendrocytes (Cd-d″). More than 6% of the MBP+ cells had an increased complexity of their processes in cultures driven to overexpress Cdk5-EGFP compared to control. Quantitative data were shown in graphics. Scale bar = 25 μm.

The extent of oligodendrocyte process outgrowth and branching is significantly reduced in the absence of Cdk5.

To assess in more detail the effects of Cdk5 on the elaboration of a mature morphological oligodendrocyte phenotype, the morphology of WT and Cdk5−/− cells was compared in vitro. While there was no significant difference in the proportion of O4+ or O1+...
oligodendrocytes that developed in the CDK5−/− cultures (Figs. 7A(a–d)) the number of MBP+ cells was significantly reduced (Fig. 7A(e–f)). With maturation, oligodendrocytes acquire an increasingly complex morphology and while a slight reduction in the number of processes and the extent of branching was apparent in O4+ cell lacking Cdk5 the effect was significantly more pronounced in mature MBP+ cells (Fig. 7A(e–f)). For examples while control MBP+ cells had a mean of 5.1 ± 0.3 primary process and 19.8 ± 1.5 branches, Cdk5−/− cells had a mean of 3.4 ± 0.23 primary process and 9.4 ± 0.75 branches.

PDGF does not rescue reduced oligodendrocyte maturation in Cdk5−/− E18 spinal cord explant culture

One possible mechanism that might account for the perturbation of oligodendrocyte differentiation in the absence of Cdk5 is a limited response to PDGF, a major OPC mitogen and survival factor for newly generated oligodendrocytes (Raff et al., 1988; Richardson et al., 1988). To determine if the lack of mature oligodendrocytes in Cdk5−/− animals could be reversed by exposure to PDGF, spinal cord explants from WT and Cdk5−/− animals were grown in the
presence of exogenous PDGF (10 ng/ml) for 7 days. Addition of PDGF failed to rescue the perturbed development or morphology of oligodendrocytes in Cdk5−/− cultures. The number of O4+ and MBP+ cells was decreased in Cdk5−/− derived culture compared to WT (Fig. 7Bb and d) and the MBP+ cells that did develop had few short processes (Fig. 7Bd) compared to the morphology of WT MBP+ cells that possessed long highly branched processes (Fig. 7Bc). The quantitative analysis of average footprint for individual MBP+ cells of WT or Cdk5−/− with PDGF treatment showed a substantial decrease with Cdk5−/− cells (Fig. 7Be). The reduction in the number and morphological complexity of O4+ cells in these Cdk5−/− cultures likely reflects the overlapping expression of O4 and MBP with the extended culture period. Taken together these results suggest that lack of Cdk5 activity effects the maturation of oligodendrocytes and their ability to myelinate on schedule.

Discussion

The molecular mechanisms mediating the development of oligodendrocytes are highly complex with multiple growth factors and extracellular signals regulating the induction, proliferation, migration, differentiation of OPCs and maturation of oligodendrocytes. How these signals are integrated within the cells to influence their biology is not well understood. Here we show that the intracellular signaling molecule Cdk5 plays an important role in regulating the appearance of oligodendrocytes at a specific stage in their development. In Cdk5 null animals during embryonic development the number and distribution of mature oligodendrocytes is significantly reduced in the spinal cord. In age-matched WT animals MBP+ cells are localized in the ventral midline of the spinal cord as well as more laterally while in Cdk5 null animals they are restricted to midline regions. Furthermore, while the number and distribution of Olig2+ progenitor cells is relatively unaffected, the number of MBP+ cells is significantly reduced in Cdk5 null animals throughout development. The reduction in mature oligodendrocytes is not a reflection of reduced proliferation by OPCs since the proportion of BrdU+ cells was not significantly different between Cdk5−/− and wild type animals. Consistent with these data, conditional deletion of Cdk5 from olig1+ cells resulted in reduced expression of MBP and PLP in postnatal animals although there was no obvious effect in the number of PDGFαR+ cells or their proliferation in either white or gray matter. The reduction in myelin gene expression is reflected in a reduction in the level of myelination in both the spinal cord and the optic nerve that remains pronounced until at least the second postnatal week. Analysis of the expression and function of Cdk5 in cells of the oligodendrocyte lineage revealed its expression in both early and late stages of the oligodendrocyte lineage. Both kinase inhibition and RNAi-induced knockout down of Cdk5 impaired the biochemical and morphological maturation of oligodendrocytes, while over expression of Cdk5 resulted in more robust oligodendrocyte maturation. Morphological analyses of cultured oligodendrocytes demonstrated a reduction in the number of primary processes and branching in the absence of Cdk5 that correlated with reduced expression of MBP. Thus, in the oligodendrocyte lineage the primary role of Cdk5 appears to be to modulate maturational and morphological changes in oligodendrocytes rather than the cell division kinetics of the OPC population.

One common linkage between many of the biological phenomena impacted by Cdk5 in neuronal cells is a connection to cell polarity and motility (Morfini et al., 2004). Previous in vitro studies suggested that Cdk5 modulates OPC migration (Miyamoto et al., 2008), however the distribution of OPCs in the developing spinal cord was not significantly different in transgenic animals lacking Cdk5 indicating that it is not essential for OPC dispersal in vivo. Rather we propose that a dysregulation of cell polarity and motility events underlie the disruption of maturation of oligodendrocytes devoid of Cdk5. Several lines of evidence are consistent with this hypothesis. First in Cdk5 null animals the appearance of MBP+ cells in the embryonic spinal cord is reduced and more restricted than in wild type littermates, implicating a maturational defect. Second, the reduction in the complexity of Cdk5 null oligodendrocytes and the reduced length of their processes compared to wild type cells suggests that oligodendrocyte process extension is compromised in the absence of Cdk5. This is reminiscent of the lack of axonal growth seen in neurons lacking Cdk5 (Fang et al., 2011; Hahn et al., 2005). One potential mechanism for the modulation of oligodendrocyte process development seen in the absence of Cdk5 may be phosphorylation of the paxillin by Cdk5 that influence paxillin’s association with the focal adhesion kinase (FAK) in an oligodendrocyte cell line (Miyamoto et al., 2007). Whether similar signaling pathways are operative in primary oligodendrocytes is currently unclear. Finally, the reduction in myelination in Cdk5 null animals may also reflect the reduction in the cellular polarity or motility of the myelinating processes. Indeed, the mechanics of the initiation of myelination are still not well understood, although it is clear they reflect the polarization of oligodendrocytes, the compartmentalization of the membrane domains and the motility of the myelinating process (Kippert et al., 2007).

While alterations in the morphology and myelination of Cdk5 null oligodendrocytes are consistent with changes in cell motility, the reduction in expression of MBP is less easily explained. It may be, however that the reduction in MBP expression is also a direct effect of modulation of intracellular motile activity in the absence of Cdk5. Earlier studies demonstrated that the mRNA for MBP is not located in the cytoplasm adjacent to the oligodendrocyte nucleus, but rather is transported to the terminal of the processes where it is translated into protein that is locally inserted into the membrane (Boccaccio et al., 1999; Boccaccio and Colman, 1995). The mechanisms of mRNA transport have been extensively studied and the data suggest a hypothesis in which clusters of mRNAs are transported along microtubules through the activity of kinesins and dyneins (Carson et al., 1998; Carson et al., 1997; Shan et al., 2003). This activity is likely a consequence of cell polarization and has recently been shown to be regulated both by adapter proteins such as heterogeneous nuclear ribonucleoprotein (hnRNP) (White et al., 2012). This intracellular transport, similar to fast axonal transport, (Drake et al., 1985) also occurs in neurons where it targets dendritic RNAs to post synaptic sites. In neurons, retrograde transport of proteins important in polarity and migration such as LIS1 and NDEL1 is dependent on the activity of Cdk5 and this related intracellular transport is completely blocked in cultured neurons treated with a dominant negative Cdk5 (Pandey and Smith, 2011). Based on these data we propose that the disruption in the maturation of oligodendrocytes in the absence of Cdk5 is largely a reflection of perturbed transport and targeting of mRNAs including MBP that are essential for directed oligodendrocyte precursor migration, process outgrowth and myelination.

Recent studies have begun to reveal the distinct roles for different intracellular signaling pathways in the maturation of oligodendrocytes and myelination. For example, over expression of AKT1 in oligodendrocytes and their precursors via the PLP promoter results in exuberant production of myelin in the CNS that ultimately results in axonal compression and death suggesting that signaling through the AKT pathway selectively regulates the extent of myelination in oligodendrocytes rather than in cell death as it does in many other cell types (Narayan et al., 2009; Tyler et al., 2009). Consistent with a role for AKT signaling in modulating myelination inhibition of mTOR by rapamycin treatment results in...
a blockade in oligodendrocyte development (Gomez et al., 2011; Guardiola-Diaz et al., 2012). By contrast, inhibition of the ERK pathway alters the timing of myelination in the CNS (Pyfe-Marichic et al., 2011) and possibly more importantly reduces the thickness of the myelin sheaths that develop around large diameter axons. Like Cdk5 neither the AKT nor ERK pathways appear to be essential for the proliferation of OPCs. Rather OPC proliferation may be mediated predominantly by p38 kinase (Chew et al., 2010). Further studies are required to define the interactions between AKT and ERK signaling and Cdk5.

In conclusion our studies demonstrate an important role for Cdk5 in the development of oligodendrocytes and myelination. Several intracellular pathways converge on Cdk5 and future studies will be directed at defining which of these pathways are essential for the timely myelination and myelin repair after injury.

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References


CONCLUSION

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