Introduction

At the early stage of neural development, the neural tube is made up of multipotent neuroepithelial cells that can generate oligodendrocytes, astrocytes and neurons irrespective of their dorsal or ventral origin (Chandran et al., 1998). In dissociated or explant cultures, oligodendrocytes and other ventral derivatives can be induced from both dorsal and ventral neuroepithelial cells by the ventral midline signal protein sonic hedgehog (SHH; Trousse et al., 1995; Poncet et al., 1996; Pringle et al., 1996; Roelink et al., 1995), and inhibited by dorsal signals, notably the bone morphogenetic proteins (BMPs; Zhu et al., 1999). Thus, the developmental fate of the multipotent neuroepithelial cells depends on their interactions with environmental signals. During normal development, oligodendrocytes appear to originate from the ventral neural tube (Warf et al., 1991; Noll and Miller, 1993; Ono et al., 1995; Orentas and Miller, 1999; Richardson et al., 2000), which is directly exposed to a gradient of SHH protein produced in the notochord and the floor plate (Echelard et al., 1993; Roelink et al., 1995). SHH is both necessary and sufficient to induce oligodendrocyte differentiation (Trousse et al., 1995; Poncet et al., 1996; Pringle et al., 1996) and blocking SHH signaling in vivo and in vitro can completely block the induction of oligodendrocytes from the ventral spinal cord (Orentas et al., 1999; Nery et al., 2001).

Although it is generally accepted that oligodendrocytes are derived from the ventral neuroepithelium in response to SHH signals, the exact domain of their origin is still controversial and the molecular control of oligodendrogenesis remains to be defined. Richardson and colleagues have suggested that expression of the gene for platelet-derived growth factor receptor α (PDGFRα) defines the oligodendroglial precursor as a single column of cells in the ventral neural tube along the entire rostrocaudal axis. In contrast, no effect is seen on astrocytic differentiation. Interestingly, absence of Nkx2.2 expression leads to a ventral expansion of the Olig1/Olig2 expression in neuroepithelial cells into the Nkx2.2 domain and a consequent increase in the production of Olig1/Olig2-positive and platelet-derived growth factor receptor α-positive oligodendrocyte progenitors. These results strongly suggest that Nkx2.2 regulates the differentiation and/or maturation, but not the initial specification, of oligodendrocyte progenitors. Consistent with this suggestion, overproduction of Nkx2.2 protein in fibroblast cells can induce gene expression from the proteolipid protein promoter.

Summary

Oligodendrocytes are derived from glial precursors that arise from the ventral neural tube early in development. In the developing chicken CNS, oligodendrocyte progenitors selectively express Nkx2.2 homeodomain transcription factor, raising the possibility that Nkx2.2 may directly regulate oligodgliogenesis. In this study, we have examined Nkx2.2 expression in rodent glial precursors and studied the effect of a loss of Nkx2.2 on oligodendrocyte and astrocyte differentiation. We show that Nkx2.2 is also expressed in mammalian oligodendrocyte progenitors and that the differentiation of MBP-positive and PLP-DM20-positive oligodendrocytes is dramatically retarded in Nkx2.2-null mutants along the entire rostrocaudal axis. In contrast, no effect is seen on astrocytic differentiation. Interestingly, absence of Nkx2.2 expression leads to a ventral expansion of the Olig1/Olig2 expression in neuroepithelial cells into the Nkx2.2 domain and a consequent increase in the production of Olig1/Olig2-positive and platelet-derived growth factor receptor α-positive oligodendrocyte progenitors. These results strongly suggest that Nkx2.2 regulates the differentiation and/or maturation, but not the initial specification, of oligodendrocyte progenitors. Consistent with this suggestion, overproduction of Nkx2.2 protein in fibroblast cells can induce gene expression from the proteolipid protein promoter.

Key words: Oligodendrocytes, Nkx2.2, Expression pattern, Differentiation, Mutation, Rat, Mouse

Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor

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Introduction

At the early stage of neural development, the neural tube is made up of multipotent neuroepithelial cells that can generate oligodendrocytes, astrocytes and neurons irrespective of their dorsal or ventral origin (Chandran et al., 1998). In dissociated or explant cultures, oligodendrocytes and other ventral derivatives can be induced from both dorsal and ventral neuroepithelial cells by the ventral midline signal protein sonic hedgehog (SHH; Trousse et al., 1995; Poncet et al., 1996; Pringle et al., 1996; Roelink et al., 1995), and inhibited by dorsal signals, notably the bone morphogenetic proteins (BMPs; Zhu et al., 1999). Thus, the developmental fate of the multipotent neuroepithelial cells depends on their interactions with environmental signals. During normal development, oligodendrocytes appear to originate from the ventral neural tube (Warf et al., 1991; Noll and Miller, 1993; Ono et al., 1995; Orentas and Miller, 1999; Richardson et al., 2000), which is directly exposed to a gradient of SHH protein produced in the notochord and the floor plate (Echelard et al., 1993; Roelink et al., 1995). SHH is both necessary and sufficient to induce oligodendrocyte differentiation (Trousse et al., 1995; Poncet et al., 1996; Pringle et al., 1996) and blocking SHH signaling in vivo and in vitro can completely block the induction of oligodendrocytes from the ventral spinal cord (Orentas et al., 1999; Nery et al., 2001).

Although it is generally accepted that oligodendrocytes are derived from the ventral neuroepithelium in response to SHH signals, the exact domain of their origin is still controversial and the molecular control of oligodendrogenesis remains to be defined. Richardson and colleagues have suggested that expression of the gene for platelet-derived growth factor receptor α (PDGFRα) defines the oligodendroglial precursor as a single column of cells in the ventral neural tube along the entire rostrocaudal axis (Pringle and Richardson, 1993; Yu et al., 1994). Cell isolation experiments have suggested that PDGFRα-expressing cells are indeed oligoglial precursors (Ellison and de Vellis, 1994; Grinspan and Franceschini, 1995) and that A2B5 immunoreactivity coupled with PDGFRα expression defines an oligodendroglial precursor (Hall et al., 1996). Recent studies have shown that two additional glial genes, Olig1 and Olig2, are also expressed in an overlapping
domain (Lu et al., 2000; Zhou et al., 2000), suggesting that Pdgfra/Olig1/Olig2 expression defines a glial precursor cell domain. This domain has been subsequently mapped to the ventral border of the Pax6 expression domain but dorsal to the Nkx2.2 expression domain (Ericson et al., 1997; Sun et al., 1998; Lu et al., 2000; Zhou et al., 2000).

However, our recent studies in chicken embryos have shown that oligodendrocytes might also be generated from the Nkx2.2 domain of the ventral neuroepithelium in both the spinal cord and the forebrain (Xu et al., 2000). In the developing chicken spinal cord, Nkx2.2 expression is initially confined to a narrow stripe of neuroepithelium that flanks the floor plate but ventral to the Pdgfra/Olig1/Olig2 domain. Starting at embryonic day (E) 6, Nkx2.2-positive cells migrate radially and tangentially into the surrounding gray and white matter regions where they actively proliferate. Double labeling experiments demonstrate that a majority of the Nkx2.2-positive migratory cells co-express oligodendrocyte markers. These results strongly suggest that oligodendrocytes can also be generated from the Nkx2.2-positive neuroepithelium and that Nkx2.2 may directly control oligodendrocyte specification and differentiation.

To investigate the role of Nkx2.2 in oligodendrocyte lineage specification and differentiation, we have examined the expression of Nkx2.2 in mouse oligodendrocyte progenitors and investigated the development of oligodendrocytes in the Nkx2.2-null mutants (Sussel et al., 1998). We show that Nkx2.2 is also expressed in oligodendrocyte progenitor cells in mouse spinal cord. In Nkx2.2 knockout mice, the number of myelin basic protein (MBP)-positive and proteolipid protein (PLP-DM-20)-positive oligodendrocytes is dramatically reduced and delayed in both the spinal cord and the brain. However, the number of Pdgfra-positive and Olig1/Olig2-positive oligodendrocyte progenitors is slightly increased, and the Olig1/Olig2 expression in neuroepithelial cells also expands ventrally into the Nkx2.2 domain. These results confirm that Nkx2.2 controls the identity of the ventral neuroepithelium and provide direct evidence that the Nkx2.2 transcription factor regulates oligodendrocyte differentiation.

MATERIALS AND METHODS

Materials

A2B5 hybridoma cells were obtained from ATCC. Anti-GFAP polyclonal antibody was obtained from Chemicon. The Alexa-488- or Alexa-594-conjugated secondary antibodies were obtained from Molecular Probes.

In situ RNA hybridization

Spinal cord and brain tissues from mouse embryos and postnatal pups were fixed in 4% paraformaldehyde at 4°C overnight. Tissue preparation and in situ hybridization with digoxigenin-labeled riboprobes were performed as previously described (Cai et al., 1999), according to Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993) with minor modifications.

Isolation and characterization of A2B5 cells by immunopanning and immunofluorescence

Spinal cords from E14 rats were dissected and dissociated by EDTA-trypsin. NCAM cells were depleted by immunopanning. Cells in supernatant were cultured in NEP basal medium with 35 ng/ml basic fibroblast growth factor (bFGF) and 10 ng/ml PDGF for 2 days. A2B5 cells were purified by immunopanning and cultured in NEP basal media with 35 ng/ml bFGF and 10 ng/ml PDGFA. After one day in culture, bFGF was withdrawn and T3 was added. Two days later, cells were stained with anti-A2B5 supernatant (1:1) for an hour and then FITC-anti-mouse IgM (1:100) for 15 minutes at room temperature. Then the cells were fixed by 2% PFA. Cells were preblocked in blocking buffer for 1 hour and then incubated in anti-Nkx2.2 supernatant overnight (100%) at 4°C. Cy3-anti-IgG, Fc specific, (1:100), was used to stain the second day.

Nkx2.2 expression in oligodendrocyte progenitors was further confirmed in A2B5 cell line (P6) cells, which were cultured in NEP basal media with 35 ng/ml bFGF and 10 ng/ml PDGFA for 2 days. The bFGF concentration was reduced to 10 ng/ml and cells were cultured for 4 more days before being fixed and stained by anti-Nkx2.2 and DAPI.

Dissociated cell culture of rat embryonic spinal cord

E14 rat spinal cords were dissected and dissociated by EDTA-trypsin. Cells were cultured in NEP basal with 35 ng/ml bFGF for 5 days. Then cells were fixed with 2% PFA. Cells were blocked in blocking buffer for 1 hour and then incubated in anti-Nkx2.2 (100%) supernatant overnight at 4°C. Cells were stained by anti-mouse-IgG (H+L) 568 (Alexa) (1:500) on the next day. Cells were then re-fixed in 2% paraformaldehyde for 1 minute. Anti-β III tubulin (1:100) was added and incubated overnight at 4°C. Cells were stained by FITC-anti-mouse IgG2b (1:100) on the second day.

Transient transfection assay

The following constructs were used to transiently transfect NIH 3T3 cells using FuGene 6 (Boehringer Mannheim): (1) pEGFP alone as a control; (2) pLNCX-NKX2.2/FLAG alone; (3) PLP-GFP alone; (4) GFAP-GFP alone; (5) pLNCX-NKX2.2/FLAG and PLP-GFP; and (6) pLNCX-NKX2.2/FLAG and GFAP-GFP. Each plasmid (1 µg) was transfected into each dish according to the manufacturer’s protocol. Cells were fixed in 2% paraformaldehyde for 30 minutes at room temperature 2-3 days after transfection and stained with either the anti-FLAG tag (1:1000; Sigma) or anti-NKX2.2 (1:1; DSHB) overnight at 4°C followed by TRITC IgG1 (1:200; Southern) or Alex fluor 568 (1:500; Molecular Probes), respectively. The number of GFP-positive and Nkx2.2-positive cells was counted from 50 random fields under a Nikon fluorescent microscope.

RESULTS

Nkx2.2-expressing cells migrate into the gray and white matter of the developing mouse spinal cord

We recently demonstrated that Nkx2.2 was selectively expressed in the migratory oligodendrocyte progenitors in the chicken embryos (Xu et al., 2000). To investigate whether Nkx2.2 is also expressed in mouse oligodendrocyte progenitors, we examined the spatiotemporal pattern of Nkx2.2 expression at various embryonic and postnatal stages of mouse spinal cords using in situ RNA hybridization. Attempts to use immunohistochemistry were not successful because the anti-Nkx2.2 monoclonal antibody did not work well on mouse spinal cord sections.

As reported previously, before embryonic day 10.5 (E10.5), Nkx2.2 transcription in the spinal cord is restricted to a narrow ventromedial column flanking the floor plate (Fig. 1A). At E13.5, a small number of Nkx2.2-positive cells start to migrate dorsally (arrow) and ventrally (arrowhead) (Fig. 1B). By E15.5, Nkx2.2 signal can be clearly detected in the ventral white matter (arrowhead, Fig. 1C). At postnatal day 0 (P0), the Nkx2.2-expressing cells are detected throughout the entire
spinal cord, including the lateral and dorsal white matter (Fig. 1D). The expression of Nkx2.2 in the white matter continues to accumulate during postnatal development (Fig. 1E-H). The dispersion of Nkx2.2-positive cells in the white matter resembles that seen for Nkx2.2-positive oligodendrocyte progenitors in the embryonic chicken spinal cord (Xu et al., 2000). In fact, closer examination of Nkx2.2-positive cells under high magnification in the white matter (Fig. 1G,H) revealed that they had an oligodendrocyte-like morphology with numerous branching processes. Interestingly, the number of Nkx2.2-positive cells in the perinatal mouse spinal cord appears to be smaller than that observed in the embryonic chicken spinal cord at equivalent stages (chicken E14; Xu et al., 2000; Richardson et al., 1997).

**Nkx2.2 is selectively expressed in mammalian oligodendrocyte progenitor cells**

To determine if Nkx2.2 was expressed by oligodendroglial precursors, we isolated A2B5 immunoreactive glial restricted precursor (GRP) cells from E13.5 rat spinal cord as previously described (Rao and Mayer-Proschel, 1997). The A2B5-positive GRP cells are oligodendroglial progenitor cells that are able to generate astrocytes and oligodendrocytes in clonal culture (Raff et al., 1983; Rao et al., 1998; Rao and Mayer-Proschel, 1997). When GRP cells were stained with an antibody to Nkx2.2, a fraction (~33%, n=67) of the A2B5-positive cells were clearly Nkx2.2 immunoreactive (Fig. 2D, data not shown). Staining was localized to the nucleus as would be expected of a transcription factor and no staining was seen in controls where the primary antibody was omitted or in Nkx2.2 knockout mouse tissue. The expression of Nkx2.2 in oligodendroglial progenitors was further confirmed with staining of an immortalized GRP cell line that can differentiate into oligodendrocytes and astrocytes but not into neurons (Fig. 2A-C). In contrast, when mixed cultures of E14 rat or mouse spinal cord cells were stained, few β-III tubulin-positive neurons (Fig. 2E) or GFAP-positive astrocytes (Fig. 2F) were Nkx2.2 immunoreactive. Thus, the majority of Nkx2.2 staining that was seen in vivo (Fig. 1) could be attributed to Nkx2.2 expression by oligodendroglial precursors.

**The differentiation of oligodendrocytes is severely retarded in the Nkx2.2 mutant spinal cord**

The selective expression of the Nkx2.2 gene in at least a subset of oligodendrocyte progenitors suggests an important role for Nkx2.2 in the control of oligodendrocyte development. To investigate this possibility, we studied the differentiation of oligodendrocytes in the Nkx2.2-null mutants by examining the expression and distribution of two myelin-specific proteins, MBP and PLP-DM20. In the wild-type mice, expression of MBP in the spinal cord could be detected as early as E15.5 in the ventral white matter (Fig. 3A) and gradually increased thereafter (Fig. 3B-E). However, in the Nkx2.2 mutants, no MBP expression was observed until E18.5 and P0 (Fig. 3A–C). At P4 and P8, only a small number of MBP-positive oligodendrocytes were found in the white matter region, but not in the gray matter (Fig. 3D–E). Thus, in the mutant spinal cord, the number of MBP-positive oligodendrocytes in the spinal cord is greatly reduced when compared with their wild-type littermates. The further development of MBP-positive and PLP-positive oligodendrocytes in the postnatal Nkx2.2 mutants can not be assessed because the Nkx2.2 mutants survive no more than 8 days, owing to severe diabetes (Sussel et al., 1998). The retarded differentiation of oligodendrocytes in the mutants does not appears to be a simple developmental delay, as no oligodendrocytes are observed in the gray matter of the mutant spinal cords at any stages. Interestingly, expression of MBP in dorsal root ganglia is not affected in the Nkx2.2 mutants.
(asterisks, Fig. 3A,A'), indicating the specific effect of the Nkx2.2 mutation on the differentiation of oligodendrocytes, but not Schwann cells.

Defective differentiation of spinal oligodendrocytes was further verified by the abnormal expression of another myelin-specific protein, PLP-DM20. PLP-DM20 expression in the spinal cord is slightly later than MBP and can only be detected by in situ analysis at around E18.5 in wild-type embryos (Fig. 3F-I). In parallel to the reduction of MBP expression, expression of PLP-DM20 is also severely reduced and delayed in the Nkx2.2 mutants (Fig. 3F'-I'). Like MBP-positive oligodendrocytes, only a small number of PLP-DM20-positive...

Fig. 3. Retarded differentiation of MBP-positive and PLP-positive oligodendrocytes in Nkx2.2 mutant spinal cords. Transverse sections from various stages of wild-type (A-I) and mutant (A'-I') littermates were hybridized with MBP (A-E) or PLP-DM20 (F-I) riboprobes by in situ hybridization. Note the reduced and delayed expression of MBP and PLP in the white matter and the lack of MBP-positive and PLP-positive cells in the gray matter of the mutant spinal cords.
Nkx2.2 regulates oligodendrocyte differentiation

Oligodendrocytes are observed in the mutants in the peripheral white matter, but not in the gray matter.

Development of brain oligodendrocytes is also affected by the Nkx2.2 mutation

Previous studies have suggested that oligodendrocytes in the rostral regions of the neural tube might also arise from the ventral CNS, similar to those in the spinal cord (Pringle and Richardson, 1993; Spassky et al., 1998; Perez Villegas et al., 1999). We have recently shown that in the developing chicken brain, Nkx2.2-positive oligodendrocyte progenitors migrate into all parts of the embryonic brains, including hindbrain and forebrain at later stages (Xu et al., 2000). To study the effects of Nkx2.2 mutation on the brain oligodendrocyte development, we examined the expression of MBP in the hindbrain/midbrain tissues at E17.5 (Fig. 4A,B) and in the forebrain at postnatal day 7 (P7; Fig. 4C-F). At E17.5, many MBP-positive oligodendrocytes can be detected in the ventral half of the medulla in the wild-type animals (Fig. 4A). In the mutants, only a small number of oligodendrocytes are found in the ventromedial white matter (Fig. 4B).

Oligodendrogenesis in the forebrain is much later than that in the spinal cord and hindbrain. Thus, MBP-positive oligodendrocytes in the forebrain can be detected only in postnatal animals. In cross sections of the P7 wild-type forebrain, many MBP-positive cells (Fig. 4C) and PLP-positive cells (Fig. 4E) are found in association with the corpus callosum and anterior commissure. A few oligodendrocytes are scattered in the surrounding cortical and striatal regions. In the mutants, no clearly labeled MBP-positive or PLP-positive cells were observed throughout the entire brain. Because the mutant animals did not survive more than postnatal day 8, it is not clear at present whether MBP-positive oligodendrocytes can be generated at later stages in the mutant forebrain as in the mutant spinal cord.

Nkx2.2 mutation does not affect astrocyte development

As oligodendrocytes and astrocytes can be generated from common glial precursor cells, at least in vitro (Rao et al., 1998), we investigated the development of astrocytes by immunostaining embryonic spinal cord with an anti-GFAP polyclonal antibody. The earliest expression of GFAP could be detected at around E15.5 in the ventral white matter regions (Fig. 5A-B). By E18.5, strong GFAP immunoreactivity was observed in the entire white matter (Fig. 5C,D). No significant difference between GFAP staining in the wild-type and mutant embryos was detected at either stage (Fig. 5).

Nkx2.2 mutation does not affect neuregulin expression in the ventral spinal cord

Neuregulin 1 (Nrg1), an extracellular signal expressed in the ventral spinal cord, has been shown to regulate oligodendrocyte proliferation, differentiation and survival in culture (Vartanian et al., 1994; Canoll et al., 1996). Mutation of Nrg1 completely blocks the generation of oligodendrocytes from spinal cord neuroepithelium (Vartanian et al., 1999). To test the possibility that Nkx2.2 mutation affects oligodendrocyte differentiation by altering the Nrg1 expression, we examined the expression of Nrg1 in both wild-type and mutant spinal cord from E10.5 to E15.5, the critical time window for oligodendrogenesis in the spinal cord. Consistent with earlier studies (Vartanian et al., 1999), Nrg1 expression was mainly observed in motoneurons (Fig. 6A-C). However, we did not detect a change in Nrg1 expression in Nkx2.2 mutants when compared with the wild-type (Fig. 6D).
Nkx2.2 mutation leads to ventral expansion of Olig1/Olig2 expression in the spinal neuroepithelium and increased production of Olig1/Olig2-positive and Pdgfra-positive oligodendrocyte progenitors

The dramatic reduction of oligodendrocyte formation in Nkx2.2 mutants could be due to a defective production of oligodendrocyte progenitors in the mutants. Thus, we examined the expression of three candidate oligodendrocyte progenitor markers: Olig1, Olig2 and Pdgfra, in the Nkx2.2 mutant spinal cords at various embryonic stages (Fig. 7). Olig1 and Olig2 are two bHLH genes that are initially expressed in the ventral spinal neuroepithelium dorsal to the Nkx2.2 domain (Lu et al., 2000; Zhou et al., 2000). At E13.5, the Olig1-positive and Olig2-positive oligodendrocyte progenitor cells spread to the surrounding gray and white matter (Fig. 7A,C). In the Nkx2.2 mutants, expression of Olig1 and Olig2 in the ventricular zone is expanded ventrally into the Nkx2.2 domain (Fig. 7B,D). The ventral expansion of the Olig1/Olig2-positive neuroepithelium was confirmed by double in situ labeling with Olig2 and Shh, a floor plate marker. In the wild-type embryos, Olig2 and Shh expression was separated by the Nkx2.2 domain (arrow in Fig. 7G), while in the Nkx2.2 null mutants, the Olig2 expression is ventrally expanded into the Nkx2.2 domain and thus directly flanks the Shh-expressing floor plate (Fig. 7H).

The ventral expansion of Olig1/Olig2 gene expression indicates that the identity of the Nkx2.2 domain is re-specified to the Olig1/Olig2 domain. This ventral expansion of the Olig1/Olig2 domain is accompanied by a significant increase of Olig1-positive (52%, Fig. 7A,B) and Olig2-positive (34%, Fig. 7C,D) oligodendrocyte progenitors in the surrounding regions at E13.5. The increased number of Olig2-positive oligodendrocyte progenitors was also observed in E15.5 embryos (55%, Fig. 7E,F).

Pdgfra is another well-characterized marker of oligodendrocyte progenitors whose expression is also initially observed in the ventral neuroepithelium at E13.5, similar to that of Olig1 and Olig2 (Fig. 8A,B). The Pdgfra-positive cells subsequently spread dorsally and laterally into the entire spinal cord (Fig. 8C-F). The appearance of Pdgfra-positive cells was not delayed, and the number of Pdgfra-positive cells was not reduced in the Nkx2.2 mutants. Instead, there is a significance increase (30-50%) in the number of Pdgfra-positive progenitors throughout embryonic development, possibly owing to the ventral expansion of the Olig1/Olig2-positive neuroepithelium from which most Pdgfra-positive cells arise.

Nkx2.2 mutation does not reduce Olig1/Olig2-positive and Pdgfra-positive oligodendrocyte progenitors in the brain regions

To study whether the production of Pdgfra-positive and Olig2-positive oligodendrocyte progenitors is affected by the Nkx2.2 mutation in the brain regions, we first examined the expression of Pdgfra (Fig. 9A,B) and Olig2 (Fig. 9C,D) in the E17.5 hindbrain/midbrain tissues. At this stage, similar numbers of Pdgfra-positive and Olig2-positive oligodendrocytes are distributed throughout the entire hindbrain and midbrain region.
Nkx2.2 regulates oligodendrocyte differentiation

in both normal and mutant animals (Fig. 9A-D). Examination of Pdgfra expression in the P6 forebrain sections revealed a somewhat normal number and distribution of oligodendrocyte progenitors in the forebrain (data not shown). Taken together, these results indicated that the generation of Pdgfra-positive and Olig2-positive oligodendrocyte progenitors in the brain regions is neither delayed nor reduced in the mutants.

Nkx2.2 can drive gene expression from the PLP promoter

As loss of Nkx2.2 function leads to a delay and reduction in the appearance of oligodendrocytes, we next investigated whether Nkx2.2 could directly modulate the expression of oligodendrocyte-specific genes. To answer this question, we transiently co-transfected NIH 3T3 cells with both a full-length, FLAG-tagged Nkx2.2 expression vector and a PLP-GFP promoter reporter construct wherein a fragment of the PLP promoter drives GFP (green fluorescence protein) expression. This promoter fragment had been previously used to drive appropriate expression of lacZ in transgenic mice (Spassky et al., 1998). Furthermore, analysis of the PLP promoter sequence identified several consensus Nkx2.2-binding sites (J. L. and M. Rao, unpublished; Watada et al., 2000).

When the Nkx2.2-FLAG construct was transfected into 3T3 cells, the Nkx2.2 protein can be clearly detected in both nuclei and cytoplasm with anti-Flag or anti-Nkx2.2 antibody (Fig. 10A), but no GFP-positive cells were observed. When the PLP-GFP construct was transfected alone, virtually no GFP expression was detected (Fig. 10B¢). Cells were also negative for immunostaining with anti-Flag or anti-Nkx2.2 (data not shown). However, when both constructs were co-transfected into 3T3 cells, GFP expression was observed (Fig. 10B,D). Induction was seen 48 hours after transfection and increased over the next 24 hours. Nkx2.2 staining on the co-transfected
dishes clearly demonstrated GFP expression in the Nkx2.2-expressing cells.

Because GFAP expression was not perturbed in the Nkx2.2 mutant animals, we assumed that Nkx2.2 did not regulate GFAP expression or astrocyte differentiation. To confirm this, we performed the same set of experiments as above, except with a GFAP-GFP reporter construct. When both Nkx2.2-FLAG and GFAP-GFP were co-transfected into the 3T3 cells, no GFP expression was observed, whereas numerous Nkx2.2-positive cells were (Fig. 10C,D). Transfection of the GFAP-GFP construct alone also displayed no GFP-positive cells (Fig. 10C').

These transient transfection assays demonstrate that Nkx2.2 can drive gene expression from the PLP promoter but not from the GFAP promoter in transient transfection assays, suggesting that Nkx2.2 expression in glial precursors can drive expression of oligodendrocyte-specific genes.

DISCUSSION

Lineage relationship of Nkx2.2-positive and Olig1/Olig2/Pdgfra-positive oligodendrocyte progenitors in mouse spinal cord

We have demonstrated that Nkx2.2 is expressed in oligodendrocyte progenitors in the developing mouse spinal cord both in vivo and in vitro, similar to our previous findings in the embryonic chicken CNS (Xu et al., 2000). Starting at E13.5, expression of Nkx2.2 can be found in oligodendrocyte progenitor cells that spread into adjacent gray and white matter of the spinal cord (Fig. 1). By postnatal day 8, the Nkx2.2-positive cells with characteristic oligodendrocyte morphology are observed throughout the entire spinal cord. Expression of Nkx2.2 appears to be restricted in oligodendrocyte progenitors but not in neurons or astrocytes, based on double immunostaining in dissociated spinal cord culture and isolated GRP cells (Fig. 2).

Interestingly, although Nkx2.2 is specifically expressed in oligodendrocyte progenitor cells, the expression pattern of Nkx2.2 is not identical to that of Olig1/Olig2 and Pdgfra in the developing mouse spinal cord. For example, at E13.5, Nkx2.2 expression is restricted to a small cluster of cells immediately adjacent to the ventral ventricular zone, whereas the Olig1/Olig2-positive cells and Pdgfra-positive are already dispersed into the dorsal and lateral spinal cord (Figs 1, 8, 9). Expression of Nkx2.2 in the dorsal and lateral white matter is not apparent until postnatal day 0

![Fig. 9. Normal production of the Pdgfra-positive and Olig2-positive oligodendrocyte progenitors in the Nkx2.2 mutant brain regions. (A-D) Sagittal sections of the E17.5 wild type (A,C) and Nkx2.2 mutants (B,D) were hybridized with riboprobes for Pdgfra (A,B) or Olig2 (C,D). The number of Olig2-positive and Pdgfra-positive oligodendrocyte progenitors appears not to be reduced.](image)

![Fig. 10. Nkx2.2 can drive the expression of GFP under the control of the PLP promoter. NIH 3T3 cells were transiently transfected with Nkx2.2 alone (A), PLP-GFP alone (B'), GFAP-GFP alone (C'), Nkx2.2 and PLP-GFP (B) and Nkx2.2 and GFAP-GFP (C). Few, if any, cells are GFP positive in cells transfected with Nkx2.2 alone, PLP-GFP alone or GFAP-GFP alone. In the presence of Nkx2.2 (stained in red), the number of GFP-positive cells (stained in green, indicated by arrows) is dramatically increased by co-transfection with PLP-GFP, but with not GFAP-GAP. (D) The sum of GFP-positive cells in various experiments. In the Nkx2.2/PLP-GFP co-transfection experiment, the vast majority of cells that were GFP positive were also Nkx2.2 positive (n=244, blue bar), although a small number of cells were Nkx2.2/GFP positive (n=8, red bar). For each transfection experiment, 50 random fields of cells were counted for GFP-positive cells.](image)
Pdgfra progenitor cells co-express, Third that in vitro (data not shown), consistent with the in vivo observation (Fruttiger et al., 1999). Second, the percentage of development of MBP-positive/PLP-positive oligodendrocytes cells in PDGF-A knockout mice dramatically suppresses the Pdgfra oligodendrocyte progenitor cells in rodents. Immunodepletion positive cells appear to represent the only population of spinal oligodendrocyte progenitor cells. It is conceivable that the Nkx2.2-positive oligodendrocyte progenitors may be derived from the Nkx2.2 domain (p3 domain; Briscoe et al., 2000), while the Olig1/Olig2-positive oligodendrocyte progenitors originate from the more dorsally located motoneuron domain (pMN domain; Briscoe, et al., 2000). However, our recent studies demonstrate that Nkx2.2 oligodendrocyte progenitors co-express Pdgfra and Olig2 in postnatal mouse spinal cord (Y. Q. and M. Q., unpublished). Thus, we favor the alternative possibility that Nkx2.2 is upregulated in Olig1/Olig2-positive/Pdgfra-positive oligodendrocyte progenitors after they emigrate into the surrounding spinal cord parenchyma. In other words, Nkx2.2, Olig1/Olig2 and Pdgfra are expressed in the same population of oligodendrocyte progenitor cells but at different time windows. There are several lines of evidence to support this proposal. First, Pdgfra-positive/Olig1/Olig2-positive cells appear to represent the only population of spinal oligodendrocyte progenitor cells in rodents. Immunodepletion of Pdgfra-positive cells completely eliminates oligodendrocyte cells (Hall et al., 1996). Similarly, reduction of Pdgfra-positive cells in Pdgf-A knockout mice dramatically suppresses the development of MBP-positive/PLP-positive oligodendrocytes (Fruittiger et al., 1999). Second, the percentage of Nkx2.2-positive cells in immunopurified A2B5-positive cells and dissociated spinal cord culture appears to increase with time in vitro (data not shown), consistent with the in vivo observation that Nkx2.2 expression increases in postnatal animals (Fig. 1). Third, as discussed above, Nkx2.2-positive oligodendrocyte progenitor cells co-express Pdgfra (Xu et al., 2000) and Olig2 in both chicken and mouse (data not shown). In P1 mouse spinal cord, nearly every Nkx2.2-positive cell expresses Olig2, whereas many Olig2-positive progenitors express no or little Nkx2.2 (Y. Q. and M. Q., unpublished). Therefore, Nkx2.2 appears to be upregulated in Olig1/Olig2-positive progenitors in the developing mouse CNS. As a matter of fact, the Nkx2.2 domain and Olig1/Olig2 domain start to merge together soon after oligodendrocyte progenitor cells are produced, as the Olig1/Olig2 domain gradually ‘sinks’ into the Nkx2.2 domain in both chicken (H. F. and M. Q., unpublished) and rat (Woodruff et al., 2001), suggesting that Nkx2.2 and Olig1/Olig2 could be co-expressed in some oligodendrocyte precursor cells, even before they start to migrate away from the ventricular zone. However, the precise lineage relationship of the Nkx2.2-positive, Olig1/Olig2-positive and Pdgfra-positive oligodendrocyte progenitors requires further molecular and immunological characterization and in vivo lineage analysis using transgenic approaches.

**Nkx2.2 activity is required for the normal development of oligodendrocytes in both the spinal cord and the brain**

Persistent expression of Nkx2.2 in oligodendrocyte progenitors strongly suggests that Nkx2.2 may directly control oligodendrocyte development. Consistently, Nkx2.2 is required for the normal differentiation of oligodendrocytes. In the mutant spinal cord, the production of the MBP-positive and PLP-DM20-positive differentiated oligodendrocytes in the mutants is drastically reduced; those that are detected are significantly delayed. For example, MBP-positive oligodendrocytes can be detected in the wild type as early as E15.5, whereas they can not be seen in the mutants until E18.5 (Fig. 3). A similar defect in oligodendrocyte development was also observed in the brain (Fig. 4), in agreement with our recent observations that Nkx2.2 is also expressed in brain oligodendrocytes in embryonic chicken CNS (Xu et al., 2000). The ventral origin of brain oligodendrocytes was previously proposed based on the dorsal spreading of the Pdgfra-positive and O4-positive oligodendrocyte progenitors from the ventral diencephalon and rhombencephalon (Pringle and Richardson, 1993; Ono et al., 1997; Perez Villegas et al., 1999; Spassky et al., 1998), and was further confirmed by the absence of Pdgfra-positive progenitors in the forebrain of the Nkx2.1 mutants (Nery et al., 2001).

The defective oligodendrocyte differentiation in the Nkx2.2 mutants is not caused by the reduced production or proliferation of oligodendrocyte progenitor cells, as the number of Olig-positive/Pdgfra-positive progenitors is significantly increased in the mutant spinal cord and brain throughout embryogenesis (Figs 7-9). The enhanced generation of oligodendrocyte progenitors appears to result from a ventral expansion of the Olig1/Olig2-positive domain into the Nkx2.2 domain. Previous studies have demonstrated that Nkx2.2 directly controls the identity of p3 ventral neuroepithelium (Briscoe et al., 1999; Briscoe et al., 2000). Consistently, in the Nkx2.2 mutants, expression of the Olig1/Olig2 genes in the ventricular neuroepithelial cells is ventrally expanded into the Nkx2.2 domain, suggesting that the p3 domain is transformed into the pMN domain, and Nkx2.2 is not required for the initial specification of oligodendrocyte lineage.

The increased production of Olig1/Olig2-positive/Pdgfra-positive progenitors would predict an increase in oligodendrocyte number in the Nkx2.2 mutants, given that the Olig1/Olig2 genes regulate the expression of some oligodendrocyte-specific genes (Lu et al., 2000; Zhou et al., 2000). In contrast, we observed a significant reduction in the number of differentiated MBP-positive/PLP-positive oligodendrocytes throughout the entire CNS of the Nkx2.2 mutants (Figs 3, 5), indicating that expression of Olig1/Olig2 is not sufficient for the normal differentiation of oligodendrocytes in vivo. Nkx2.2 may be required downstream of, or co-operates with, Olig1/Olig2 in controlling oligodendrocyte differentiation.

To this end, it is not clear at what stage oligodendrocyte differentiation is blocked in the mutants, owing to the lack of molecular or immunological probes that can determine intermediate stages between Olig/Pdgfra and MBP expression in mouse tissue sections. The undifferentiated progenitor cells did not appear to undergo apoptosis, as we detected no significant differences in cell death in the normal and mutant spinal cords by TUNEL labeling (data not shown). The cells also did not appear to switch their fates into astrocytes, as we did not see a premature expression or increase in the domain.
of GFAP expression. Nor was there an obvious specific increase in levels of a neuronal marker in the white matter (data not shown). Given the increase in Olig1/Olig2 and Pdgfra expression throughout embryogenesis, we believe that cells that do not differentiate persist at least till P1 and may be lost later. However, in vivo lineage analysis in the Nkx2.2 knockout animals is needed to confirm this hypothesis.

**Nkx2.2 has a direct effect on oligodendrocyte lineage development**

The mechanism that underlies the defective oligodendrogenesis in the Nkx2.2 knockout animals remains unknown at this stage. The simplest explanation for the reduction and delay of oligodendrocytes in the Nkx2.2 mutants is a general developmental delay of the CNS development. However, no discernible differences in size and shape of the neural tube can be observed between the wild-type and Nkx2.2 mutant embryos. The development of astrocytes and motoneurons is not delayed by the Nkx2.2 mutation (Fig. 4; Briscoe et al., 1999), and the production of Pdgfra-positive and Olig1/Olig2-positive oligodendrocyte progenitor cells is neither delayed nor reduced in the mutant embryos (Fig. 4). Thus, the oligodendrocyte phenotype is cell type specific and stage dependent, instead of a general developmental perturbation.

An alternative explanation is that the defective oligodendrocyte development is a secondary defect in the production of extracellular signals. Previous studies have demonstrated that oligodendrocyte progenitor cell specification, proliferation and differentiation are regulated by a variety of extracellular signal molecules, particularly SHH (Trousse et al., 1995; Ponec et al., 1996; Pringle et al., 1996), PDGF (Barres and Raff, 1994; Calver et al., 1998) and neuregulin (Canoll et al., 1996). Inactivation of the genes for these proteins leads to deletion or severe reduction of oligodendrocytes (Orentas et al., 1999; Fruttiger et al., 1999; Vartanian et al., 1999). However, expression of Shh (Briscoe et al., 1999; Fig. 7G-H) and neuregulin (Fig. 6) is not affected in the Nkx2.2 mutants. Expression of PDGF also appears to be normal based on the uncompromised production of Pdgfra-positive oligodendrocyte progenitors in the mutant CNS (Figs 8, 9; Calver et al., 1998). Nevertheless, it should be noted that Nkx2.2 mutants display defective pancreatic β-cell differentiation and consequent insulin production (Sussel et al., 1998), raising the possibility that insulin deficiency might be responsible for the defective oligodendrocyte development. Although insulin is known to be an important extracellular signal for neuronal culture in vitro (Robinson et al., 1994), there is no in vivo evidence that insulin influences the early development of the CNS (Bruning et al., 2000). To address this possibility directly, we examined the differentiation of MBP-positive oligodendrocytes in the spinal cords of both the insulin receptor knockout (Accili et al., 1996), and the insulin 1 and insulin 2 double knockout (Duvillie et al., 1997). We found no defects in oligodendrocyte differentiation in these mutants (M. Q. and R. Schechter, unpublished). More directly, a similar reduction of MBP-positive/PLP-positive oligodendrocytes in the mutants was observed in spinal cord explants that were isolated from E13.5 and cultured in vitro for 6 days (data not shown). These results indicate that insulin and in vitro culture conditions cannot rescue defective oligodendrocyte differentiation in the mutant cord.

Although we cannot formally rule out the possibility that Nkx2.2 might regulate oligodendrocyte differentiation through an unidentified intercellular signaling molecule, we propose that Nkx2.2 has a direct role in the control of expression of myelin structure genes and oligodendrocyte differentiation. It is plausible that the Nkx2.2 homeodomain transcription factor may directly bind to the promoters of structural myelin genes and subsequently regulate their expression. In support of this hypothesis, Nkx2.2 expression is gradually increased in oligodendrocyte progenitors during midgestation and postnatal spinal cord (Figs 1, 2), preceding the expression of MBP and PLP. More directly, consensus binding sites for Nkx2.2 are found in PLP and MBP promoters (J. L. and M. Rao, unpublished), and overexpression of Nkx2.2 transcription factor can induce gene expression from the PLP promoter in transient transfection assays (Fig. 10). Intriguingly, deletion of the Nkx2.2 only leads to a reduction and delay, but not a complete inhibition, of transcription of MBP and PLP in the developing spinal cord (Fig. 3B), implying that Nkx2.2 may have a partially redundant function with other transcription factors such as Olig1/Olig2 or Sox10 that are co-expressed in oligodendrocyte progenitors. It is possible that Nkx2.2 may enhance or modulate the activities of these transcription factors. It would be of interest to determine how the Nkx2.2 and Olig1/Olig2 transcription factors interact genetically or biochemically in orchestrating oligodendrocyte development.

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