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# Dynamic Expression of De Novo DNA Methyltransferases Dnmt3a and Dnmt3b in the Central Nervous System

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To explore the role of DNA methylation in the brain, we examined the expression pattern of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the mouse central nervous system (CNS). By comparing the levels of Dnmt3a and Dnmt3b mRNAs and proteins in the CNS, we showed that Dnmt3b is detected within a narrow window during early neurogenesis, whereas Dnmt3a is present in both embryonic and postnatal CNS tissues. To determine the precise pattern of Dnmt3a and Dnmt3b gene expression, we carried out X-gal histochemistry in transgenic mice in which the lacZ marker gene is knocked into the endogenous *Dnmt3a* or *Dnmt3b* gene locus (Okano et al. [1999] Cell 99:247–257). In *Dnmt3b-lacZ* transgenic mice, X-gal-positive cells are dispersed across the ventricular zone of the CNS between embryonic days (E) 10.5 and 13.5 but become virtually undetectable in the CNS after E15.5. In *Dnmt3a-lacZ* mice, X-gal signal is initially observed primarily in neural precursor cells within the ventricular and subventricular zones between E10.5 and E17.5. However, from the newborn stage to adulthood, Dnmt3a X-gal signal was detected predominantly in postmitotic CNS neurons across all the regions examined, including olfactory bulb, cortex, hippocampus, striatum, and cerebellum. Furthermore, Dnmt3a signals in CNS neurons increase during the first 3 weeks of postnatal development and then decline to a relatively low level in adulthood, suggesting that Dnmt3a may be of critical importance for CNS maturation. Immunocytochemistry experiments confirmed that Dnmt3a protein is strongly expressed in neural precursor cells, postmitotic CNS neurons, and oligodendrocytes. In contrast, glial fibrillary acidic protein-positive astrocytes exhibit relatively weak or no Dnmt3a immunoreactivity in vitro and in vivo. Our data suggest that whereas Dnmt3b may be important for the early phase of neurogenesis, Dnmt3a likely plays a dual role in regulating neurogenesis prenatally and CNS maturation and function postnatally. © 2005 Wiley-Liss, Inc.

**Key words:** DNA methylation; neural development; transgenic mice; neural precursor cells; cortical neurons; astrocyte; oligodendrocyte

DNA methylation in vertebrate animals is an epigenetic modification in which a methyl group is covalently coupled to the C-5 position of the cytosine residue at the CpG dinucleotide sites. In adult mammalian cells, approximately 70% of CpGs in the genome are methylated. The adult methylation pattern in a particular cell is established through waves of demethylation and de novo methylation during embryonic development; consequently, different methylation patterns can occur in different developmental stages as well as in different types of cells and tissues (Jaenisch, 1997; Jaenisch and Bird, 2003). For example, the total methylation level in the developing cortex undergoes biphasic changes by a first wave of transient reduction during late-gestation stages, then rising to the adult level during the first week of postnatal development (Tawa et al., 1990). It has been postulated that dynamic changes in DNA methylation may serve as a key mechanism in regulating tissue- and cell type-specific gene expression in development (Teter et al., 1994; Condorelli et al., 1997; Mostoslavsky et al., 1998; Barresi et al., 1999; Boatright et al., 2000).

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that include the maintenance enzyme Dnmt1 and de novo methyltransferases Dnmt3a and Dnmt3b (Bestor, 2000; Robertson and Wolffe, 2000). Other family member genes, including Dnmt2 and Dnmt3L, have been identified based on sequence similarity (Okano et al., 1998b; Yoder and Bestor, 1998; Aapola et al., 2001). The function of Dnmt2 is still unclear, whereas Dnmt3L, which lacks conserved methylation catalytic domain, has been demonstrated to interact with de novo methyltransferases and to play an important role in genomic imprinting and targeting methylation to the dis-

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persed retrotransposon elements (Bourc'his et al., 2001; Hata et al., 2002; Bourc'his and Bestor, 2004).

The essential role of DNA methylation and Dnmts in embryonic development has been demonstrated by targeted mutation of *Dnmt1* or both *Dnmt3a/3b* genes, which results in substantial demethylation of the genome and early embryonic lethality (Li et al., 1992; Lei et al., 1996; Okano et al., 1999). Compared with Dnmt1−/− embryos, single-knockout mice lacking either Dnmt3a or Dnmt3b alone exhibit a relatively moderate but specific demethylation pattern (Okano et al., 1999). Although the cause of the lethality in Dnmt3a−/− and Dnmt3b−/− mutant mice is still unclear, gene knockout studies clearly showed that de novo DNA methylation activity is indispensable for embryonic development and animal viability.

Recent studies also imply an important role for DNA methylation in the development and function of the central nervous sytem (CNS). Human genetic studies have revealed that abnormal DNA methylation is associated with several mental retardation disorders, including ICF (for immunodeficiency, centromere instability, and facial anomaly), fragile X, and ATRX(Alpha-Thalassemia Retardation X-linked) syndromes; moreover, mutations of methyl-CpG binding protein 2 (MeCP2) cause Rett syndrome (Robertson and Wolffe, 2000). We found, in a mouse model in which the Dnmt1 enzyme is exclusively deleted in nestin-positive CNS precursor cells (Fan et al., 2001), that DNA hypomethylation disrupts vital CNS functions at birth and induces postnatal cell death of CNS neurons. More recently, we and others have shown that depolarization-induced expression of brain-derived neurotrophic factor (BDNF) in cultured cortical neurons is correlated with the dissociation of the MeCP2-histodeacetylase-mSin3A repression complex and a decrease in DNA methylation in the BDNF gene regulatory region (Martinowich et al., 2003; W.G. Chen et al., 2003). These findings suggest that dynamic changes in DNA methylation occur in postmitotic neurons and that methylation-related chromatin remodeling is involved in neuronal maturation and synaptic plasticity.

Previous studies have examined the expression pattern of Dnmt1 mRNA and protein in the mammalian nervous system (Goto et al., 1994; Brooks et al., 1996; Trasler et al., 1996; Inano et al., 2000). High levels of Dnmt1 mRNA are detected in both dividing neural precursor cells and postmitotic neurons (Goto et al., 1994), which is consistent with the important role of Dnmt1 in the developing brain. However, the expression pattern of de novo methyltransferases Dnmt3a and Dnmt3b in the nervous system has not been well studied. Using transgenic mice in which the lacZ transgene is knocked into *Dnmt3a* and *Dnmt3b* gene loci, Okano et al. (1998a) showed that Dnmt3b, and to a much lesser extent Dnmt3a, is expressed in the neural tube in mouse embryos between embryonic days (E) 7.5 and 9.5. Northern blot analysis showed that Dnmt3a mRNA is present in the embryonic and adult nervous system (Okano et al., 1998a; Xie et al., 1999). However, the subcellular localization and tissue distribution of Dnmt3a in the CNS are unknown. We, therefore, carried out a detailed study by examining the expression pattern of Dnmt3a and Dnmt3b in the developing and mature CNS. Our results show that Dnmt3a and Dnmt3b exhibit distinct expression patterns in the embryonic and adult CNS, suggesting differential involvement of Dnmt3a and Dnmt3b in neural development and function.

## MATERIALS AND METHODS

### Transgenic Mice

The generation of *Dnmt3a-lacZ* and *Dnmt3b-lacZ* mice has been described by Okano et al. (1999). Heterozygous *Dnmt3a-lacZ* or *Dnmt3b-lacZ* (+/lacZ) mice were bred overnight and checked for plugs on the following morning. Noon of that day was designated as E0.5. The protocol for using transgenic mice in this project was approved by the UCLA Institutional Animal Research Committee.

### Histological Preparation of Brain Samples

Timed-pregnant mice were euthanized with anesthesia, followed by cervical dislocation. The embryos were dissected on ice in phosphate-buffered saline (PBS). For embryos younger than E15.5, whole embryos were fixed in 4% paraformaldehyde (PFA)/PBS (pH 7.4) overnight and cryoprotected with 30% sucrose/PBS. For E15.5 and older embryos, brains were dissected out for fixation and cyroprotection for frozen sectioning. Newborns and adult mice were cardioperfused with 2% PFA/PBS, and brains were dissected out for 2-hr postfixation and cyropreservation in 30% sucrose/PBS overnight. Samples were subsequently embedded in O.C.T. medium (Tissue-Tek), and sections were cut at 10 μm thickness with a Leica Cryostat and stored at −80°C until use.

### Cultures of CNS Precursor Cells and Cortical and Cerebellar Neurons

E11.5 mouse CNS tissues were dissected and mechanically dissociated with Pasteur pipets. Dissociated cells were plated on polyornithine (PO; 10 μg/ml)- and fibronectin (10 μg/ml)-coated coverslips with serum-free DMEM/F12 medium supplemented with B27, penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively; Invitrogen, La Jolla, CA). Cultures were fed with 10 ng/ml basic fibroblast growth factor (bFGF) at the time of plating and were fixed after 4 days in vitro with 4% PFA/PBS. For cortical neuronal cultures, E17.5–18.5 cortices were dissected and dissociated with 0.1% trypsin/PBS (Worthington) and dissociated with a fire-polished glass pipet. Cortical dissociates were cultured on PO/laminin (both at 10 μg/ml in PBS)-coated coverslips with DMEM plus 10% fetal bovine serum (FBS; Gibco Life Technology, Grand Island, NY). For cerebellar cultures, P5/6 mouse cerebelli were dissected out in HHGN solution [Hank's balanced salt solution (HBSS) 500 ml; 2.5 mM Hepes, pH 7.3; 35 mM glucose; 4 mM $NaHCO_3$]. Cerebelli were isolated and dissociated mechanically by trituration with a flame-polished pasture pipet. Dissociated cells were plated onto PO (15 μg/ml in PBS)- and laminin (10 μg/ml in PBS)-coated coverslips in 24-well plates (Costar, Cambridge, MA) or in six-well plates in cerebellum culture

medium [BME medium (Sigma, St. Louis, MO) supplemented with 24 mM KCl, 10% FBS, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1% glucose, and 10 μg/ml insulin]. AraC (Sigma; 10 μm final concentration) was added every other day. All cultures were either fixed with 4% PFA/PBS for 20 min at room temperature for immunocytochemistry or harvested for Western blot analysis of proteins.

### X-Gal Staining

Slides were air dried at room temperature for 1 hr, washed once in PBS and twice in X-gal wash solution [0.1 M sodium phosphate buffer, pH 7.4; 2 mM $MgCl_2$, 5 mM ethylene glycol tetraacetic acid (EGTA), 0.01% sodium deoxycholate, 0.02% Nonidet P-40], and stained overnight at 37°C in X-gal staining solution (X-gal wash solution plus 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal). After a brief rinse in water, slides were counterstained with nuclear fast red (Sigma), dehydrated in an ethanol series (75%, 95%, and 100% ethanol), and then cleared in xylene and mounted with Permount.

### Immunohistochemistry

Coverslips of CNS precursor cells and cortical and cerebellar cultures were washed three times in PBS and then permeablized for 1 hr in PBS with 5% normal goat serum, 0.5% Triton X in a humidified chamber. The coverslips were incubated overnight at 4°C in primary antibodies diluted in blocking solution [monoclonal mouse anti-Dnmt3a and Dnmt3b antibodies from Imgenex or Abgent (San Diego, CA; 1:400 dilution), polyclonal rabbit anti-Dnmt3a (1:1,500) and Dnmt3b (1:2,500) (Chen et al., 2002), and polyclonal rabbit anti-MAP2 from Penisula Labs (1:1,000), rabbit anti-myelin basic protein (MBP; 1:500, gift of Dr. David R. Colman, Mount Sinai School of Medicine), rabbit antiglial fibrillary acidic protein (GFAP) from Dako (Carpinteria, CA; 1:500), rabbit antinestin (1:50; gift of Dr. Ron McKay, NIH), monoclonal mouse anti-NeuN (1:100; Chemicon, Temecula, CA) or GFAP (1:300; Sigma)]. After three washes in PBS, they were incubated in flurochrome-conjugated secondary antibodies for 1 hr at room temperature (Cy2-conjugated goat anti-rabbit or mouse, 1:300; Cy3-conjugated goat anti-rabbit or mouse, 1:500; both from Jackson Immunoresearch, West Grove, PA), with protection from light. DAPI counterstaining of cell nuclei was carried out in the middle of final washes of coverslips with PBS. The coverslips were mounted with 5% n-propylgallate (Sigma) in 50% glycerol. and immunostaining was observed with an Olympus fluorescent microscope connected to a CCD camera for photography.

### Western Blot Analysis

The protein samples were harvested from either freshly dissected brain tissues or cerebellar cultures by using 1× sodium dodecyl sulfate (SDS) Laemmli buffer [62.5 mM Tris-HCl, pH 6.8; 2% (w/v) SDS; 10% glycerol, and 0.01% (w/v) bromophenol blue, supplemented with 0.01% of concentrated β-mercaptoethanol (Sigma)]. Samples were sonicated and denatured at 95°C for 10 min. After centrifugation, the supernatants were collected and stored at −20°C for future PAGE gel separation and blot analysis as described in detail previously (Fan and Katz, 1993). The primary antibodies used were mouse monoclonal anti-Dnmt3a or anti-Dnmt3b (1:250; Abgent), polyclonal rabbit anti-Dnmt3a (1:3,000) and Dnmt3b (1:5,000) (Chen et al., 2002). GAPDH protein was probed with a monoclonal antibody from Abcam (1:5,000) as a loading control.

### Northern Blot Analysis

Total RNAs were extracted from brain samples with Trizol (Invitrogen) according to the manufacturer's instruction and subjected to fractionation by 1.2% formaldehyde agarose gel. After capillary transfer of RNAs from agarose gel onto a Zetabond nylon membrane, the RNA blot was sequentially hybridized with Dnmt3b, Dnmt3a, and GAPDH probes by using the standard Quickhyb protocol (Strategene, La Jolla, CA). Kodak X-ray films were used for autoradiography of hybridization signals.

## RESULTS

### Comparison of Dnmt3a and Dnmt3b mRNA and Protein Levels in the Developing CNS

Previous studies have demonstrated that Dnmt3a and Dnmt3b are constitutively expressed in embryonic stem (ES) cells and in early embryos. The Dnmt3a mRNAs are detected at relatively constant levels in various embryonic and adult tissues, including the brain. In contrast, Dnmt3b is down-regulated both during embryogenesis and during in vitro differentiation of ES cells (Okano et al., 1998a; Xie et al., 1999).

To compare the relative abundance of Dnmt3a and Dnmt3b mRNAs in the mouse cortex, we carried out Northern blot analysis with newborn and adult cortical samples. As shown in Figure 1A, although both Dnmt3a and Dnmt3b mRNAs can be detected in the newborn cortex, levels of Dnmt3b mRNA were significantly lower than those of Dnmt3a. Both Dnmt3a and Dnmt3b mRNAs contain two splicing variants with different lengths of 3′UTR, resulting in a longer transcript form of 9.5 kb and a shorter isoform at 4.4 kb. We noticed that the 9.5-kb longer form of Dnmt3a mRNA is the predominant transcript in both newborn and adult cortex, whereas the two isoforms of Dnmt3b were approximately at the same level. The level of Dnmt3a mRNA (9.5 kb isoform) remains high in adult cortex as assayed by Northern blot analysis (Fig. 1B). In contrast, Dnmt3b mRNA in the adult cortex cannot be detected with our Northern blot method (data not show).

To examine the levels of Dnmt3a and Dnmt3b proteins in the CNS, we harvested whole CNS or cortical tissues from E11.5 to postnatal day (P) 14. Figure 1C shows that Dnmt3a proteins can be detected clearly in E11.5–15.5 CNS as two isoforms (approximately 120 and 72 kDa). The longer form is the full-length Dnmt3a1, whereas the short form is Dnmt3a2, which is highly expressed in undifferentiated ES cells (Chen et al., 2002). Whereas the full-length Dnmt3a1 proteins were present in the CNS from early embryonic stages into young adulthood (E11.5–P14), the short-isoform Dnmt3a2 is detected only between E11.5 and P7 and is no longer detectable from P14 onward. In

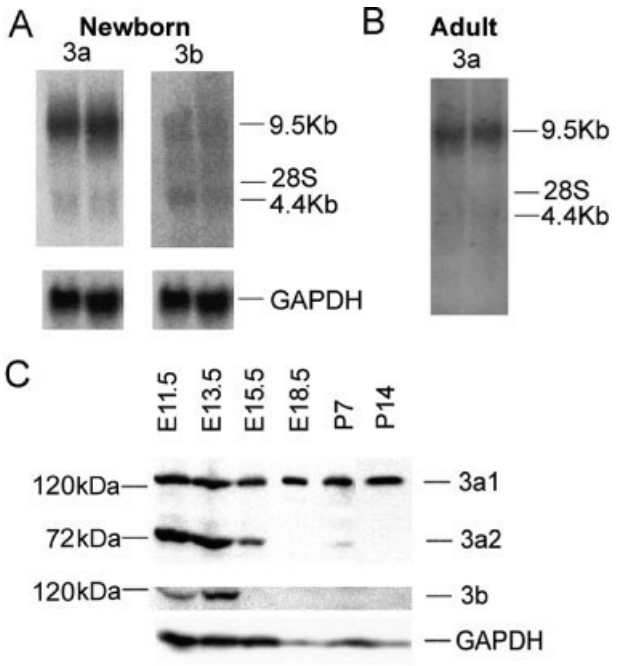


Fig. 1. Northern and Western blot analysis of Dnmt3a and Dnmt3b mRNA and protein in mouse CNS. Twenty micrograms of total RNA from newborn (**A**) and adult (**B**) mouse cortices was fractionated in formaldehyde-agarose gel and transferred to a nylon membrane (Zetabond). Each lane represents the RNA sample from one animal. The blots were hybridized with Dnmt3b, Dnmt3a, and GAPDH cDNA probes as shown. **C:** Western blot analysis of Dnmt3a and Dnmt3b proteins in the developing brain. Whole mouse brains from E11.5 and E13.5 embryos and cortices of E15.5, E18.5, P7 and P14 animals were dissected and lysed in 1× SDS sample buffers for gel electrophoresis and blotting as described in Materials and Methods. Protein membrane was sequentially blotted with monoclonal antibodies against Dnmt3a, Dnmt3b, and GAPDH.

contrast, Dnmt3b protein was detected only in E11.5–13.5 CNS but was below the detection threshold in Western blot analysis from E15.5 onward. Our results for Dnmt3a and Dnmt3b protein expression in the CNS are consistent with the analysis of differential expression of Dnmt3a and Dnmt3b mRNA in the newborn and adult cortex (Fig. 1), suggesting that Dnmt3a and Dnmt3b may play distinct roles in neural development.

### Immunocytochemical Localization of Dnmt3a Proteins in Neural Precursor Cells, Postmitotic Neurons, Astrocytes, and Oligodendrocytes

Although the data showed that Dnmt3a and Dnmt3b proteins were detected at different levels during embryogenesis and in adulthood, the cell types that express these enzymes and their subcellular localization within CNS cells are unclear. To identify precisely the CNS cell types that express Dnmt3a and Dnmt3b, we decided to perform double-labeling immnuocytochemistry on dissociated cell cultures by using antibodies against Dnmt3a and Dnmt3b in combination with antibodies against specific markers for neural precursor cells (nestin), postmitotic neurons (MAP2 and NeuN), astrocytes (GFAP) and oligodendrocyte [myelin basic protein (MBP)]. For this purpose, we carried out short-term dissociated cell cultures (one or two days in vitro) of E11.5 CNS, E17.5 cerebral cortex, and P6 cerebellum containing a variety of CNS cell types, including neural precursor cells and immature CNS neurons (in E11.5 CNS); postmitotic cortical neurons and astrocytes (in E17.5 cortex); and postnatal cerebellar granule neurons, astrocytes, and oligodendrocytes (in P6 cerebellum). Although Dnmt3b antibodies are useful for Western blot analysis, they did cross-react with other unknown antigens in the CNS and failed to show a specific pattern in all the cell cultures tested. Nevertheless, we did succeed in carrying out Dnmt3a immunostaining either with dissociated neural cell cultures (Fig. 2) or with brain sections (see Figs. 3, 4). Dnmt3a immunoreactivity was detected exclusively in nuclei of various types of CNS cells (Figs. 2–4), consistently with previous studies showing nuclear localization of Dnmt3a in cultured cell lines and in early embryonic cells in vivo (Margot et al., 2001; Watanabe et al., 2002). In the dissociated cell cultures of E11.5 CNS, strong Dnmt3a signal was detected predominantly in nestin-positive neural precursor cells, which constitute approximately 85–90% of cells in culture (Fig. 2A). Among nestin-positive precursor cells, the intensity of Dnmt3a immunoreactivity varies, suggesting a heterogeneous expression of Dnmt3a protein in these cells. Compared with many neural precursor cells exhibiting strong staining of Dnmt3a, postmitotic MAP2-positive neurons, which make up the remaining 10–15% cells in the same E11.5 CNS culture, showed relatively weak to moderate signals for Dnmt3a (Fig. 2B). This result raises the possibility that levels of Dnmt3a protein in the newly born immature neurons may be initially lower than the levels in mitotic neural precursor cells.

In E17.5 cortical cell cultures and P6 cerebellar cultures in which postmitotic neurons are more enriched, double-labeling immunocytochemistry showed that Dnmt3a protein was easily detected in the nuclei of all MAP2-positive neurons (Fig. 2C,E) Moreover, the intensity of Dnmt3a staining in these postmitotic neurons appears to be more intense that that in E11.5 CNS neurons. In contrast to neuronal cells, astroglial cells in both E17.5 cortical and P6 cerebellar cultures, which were positive for GFAP marker, were either weakly or negatively stained with Dnmt3a antibodies (Fig. 2D,F). It is still unclear whether the low or absent Dnmt3a expression in astrocytes is due to down-regulation of Dnmt3a expression in these cells or whether Dnmt3a levels in astrogliogenic precursor cells are already very low, subsequently generating astrocytes that contain little Dnmt3a expression. Another very interesting finding in P6 cerebellar cultures is the observation that 100% of oligodendrocytes ($n = 172$), as identified by MBP marker (Fig. 2G), were all strongly positive for

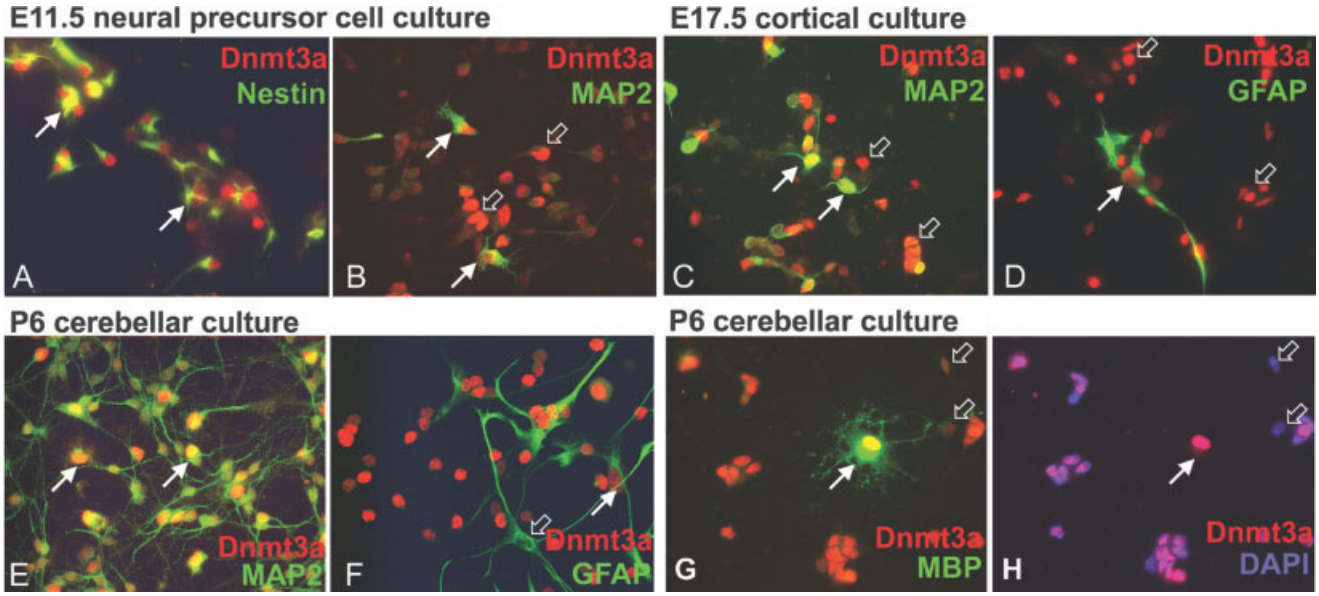


Fig. 2. Cellular localization of Dnmt3a immunoreactivity in CNS precursor cells, cortical and cerebellar neurons, astrocytes, and oligodendrocytes. E11.5 CNS precursor cells after 1 DIV (**A,B**), E17.5 cortical cell cultures (1 DIV; **C,D**), and P6 cerebellar cell cultures (2 DIV for **E,F**; 1 DIV for **G,H**) were doubly labeled with monoclonal antibodies against Dnmt3a in combination with rabbit polyclonal antibodies against nestin, MAP2, GFAP, or MBP. DAPI counterstaining was used to show nuclear morphology (H). The solid arrows point to cells positive for double staining. The open arrows indicate cells with strong Dnmt3a staining but that are negative for MAP2 staining (B,C) or GFAP staining (D). Note that the open arrow in F points to a GFAP-positive but Dnmt3a-negative astrocyte. Open arrows in G and H indicate two cells that are negative for the oligodendrocyte marker MBP and show virtually no Dnmt3a signal.

Dnmt3a immunostaining (Fig. 2G,H). This raises possibility that Dnmt3a is required for the differentiation and function of oligodendrocytes postnatally.

To determine the expression pattern of Dnmt3a during brain development, we performed Dnmt3a immunostaining with brain sections at different developmental stages from E10.5 to adulthood. Consistent with the localization of Dnmt3a in neural precursor cells in the early neurogenic phase, Dnmt3a immunohistochemistry experiments with brain sections showed that Dnmt3a signal was detected primarily in ventricular and subventricular zones of cerebral cortex at E13.5 (Fig. 3A–D). At this stage, only sporadic Dnmt3a-positive cells were present in the layer of cortical plate where newly born postmitotic CNS neurons reside (Fig. 3A–D). In contrast, at E17.5 and the newborn stage (P0), cortical neuronal cell layers (I–VI) exhibited strong Dnmt3a immunostaining (Fig. 3E–J). Consistently with the detection of Dnmt3a mRNA in postnatal and adult cortex, Dnmt3a-positive neurons are abundant in both P14 and adult cortex (Fig. 3K–P). Nevertheless, compared with that in newborn and P14 cortex, the density of Dnmt3a-positive neurons in adult cortex (Fig. 3M–P) appeared to be lower, suggesting a potential down-regulation of Dnmt3a protein in a subpopulation of adult cortical neurons. Judging from the nuclear morphology on DAPI counterstaining, a vast majority of Dnmt3a-positive cells in the cerebral cortex appears to be neuronal cells. This conclusion was supported by double-labeling experiments with antibodies against Dnmt3a and a neuronal marker, NeuN, on newborn (data not shown), P14 (Fig. 4A–F), and adult (Fig. 4M–P) brain sections. We found that, in P14 cerebral cortex, 100% of NeuN-positive neurons were positive for Dnmt3a (Fig 4A–F, solid arrows). A few nonneuronal cells in the cortical layer were either Dnmt3a positive (Fig. 4A–F, open arrow) or Dnmt3a negative (Fig. 4A–F, arrowheads). This result is consistent with the observation that MAP2-positive neurons in E17.5 cortical and P6 cerebellar cultures are all Dnmt3a positive. In contrast, GFAP-positive astroglial cells in the layer of white matter (WM) are either moderately stained (Fig. 4G–L, solid arrows) or negative for Dnmt3a signals (Fig. 4G–L, open arrows), similar to what we observed in in vitro cultures (Fig. 3D,F). In adult cortex, although many NeuN-positive neurons retained Dnmt3a immunoreactivity (Fig 4M–P, solid arrows), a portion of NeuN positive neurons exhibited weak or undetectable levels of Dnmt3a signal (Fig. 4M–P, open arrows), suggesting a down-regulation of Dnmt3a expression in a subset of CNS neurons.

It is also worth pointing out that Dnmt3a protein expression in postmitotic CNS neurons appears to be heterogeneous both in vivo and in vitro. In cultured E17 cortical neurons and P6 cerebellar granule neurons, different subsets of MAP2-positive neurons showed either strong or relatively weak Dnmt3a immunoreactivities

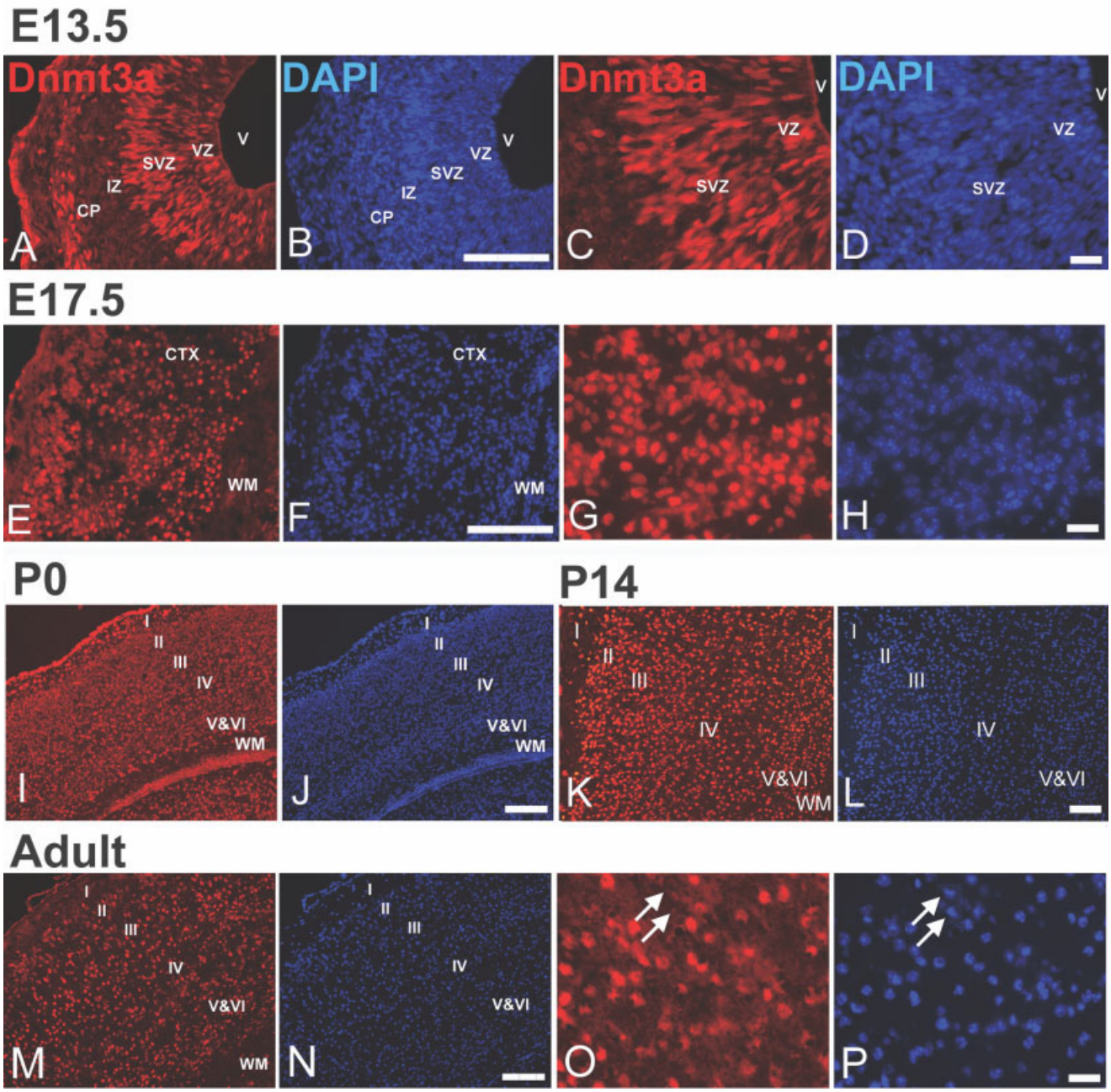


Fig. 3. Immunohistochemical localization of Dnmt3a protein in the cerebral cortex from E13.5 to adulthood. Brain sections from E13.5 (**A–D**), E17.5 (**E–H**), P0 (**I,J**), P14 (**K,L**), and 4-month-old adult wild-type mice (**M–P**) were stained with a rabbit polyclonal Dnmt3a antibody (in red). The sections were counterstained with DAPI (in blue), showing cortical cell nuclei in different layers of cortex. The arrows in O and P show virtually no staining for Dnmt 3a in two cortical cells, whereas adjacent neurons were positive for Dnmt3a. CP, cortical plate; I–VI, cortical layers I–VI; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Scale bars = 200 μm in A,B,E,F,I–N, 40 μm in C,D,G,H,O,P.

(Fig. 2C,E). Similarly, different subpopulation of NeuN-positive neurons exhibited different intensities of Dnmt3a staining in P14 and adult cortex in vivo (Fig. 4A–F,M–P). Such a heterogeneity of Dnmt3a expression in postmitotic neurons might partially reflect the complex nature of the generation and differentiation of a vast amount of neuronal subtypes in the CNS. Taken together, the detection of Dnmt3a expression in a majority of CNS precursor cells as well as postmitotic neurons in vitro and in vivo is consistent with the notion that Dnmt3a may be actively involved in regulating both neurogenesis and adult neuronal function in the CNS.

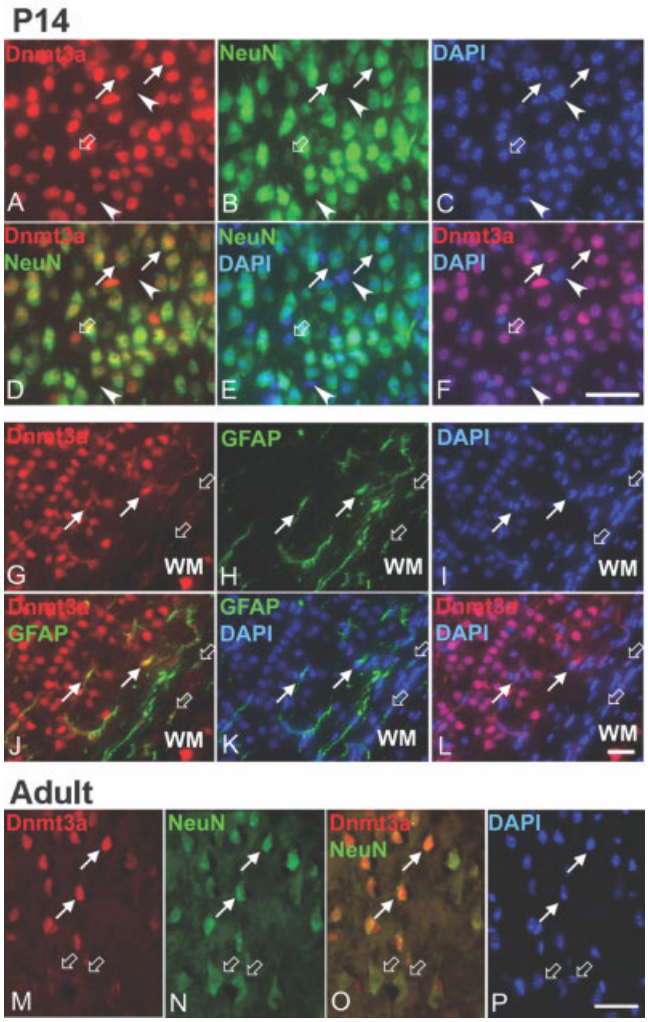


Fig. 4. Dnmt3a protein is strongly detected in postmitotic neurons but less so in astroglial cells in the CNS. Brain sections from P14 (**A–L**) and 4-month-old (**M–P**) wild-type mice were doubly labeled with antibodies against Dnmt3a and NeuN (A–F showing layer IV of cerebral cortex at P14; M–P showing layer III/IV in adult cingulate cortex) or Dnmt3a and GFAP (G–L, cortical layer V/VI and white matter at P14). A and M, B and N, and C and P show single staining patterns of Dnmt3a, NeuN, and DAPI, respectively. D–F and O show overlay double-staining pattern of Dnmt3a, NeuN, and DAPI. In A–F, solid arrows point to cells positive for both Dnmt3a and NeuN. Open arrows indicate cells with strong Dnmt3a staining but that are negative for NeuN. Arrowheads point to nonneuronal cells negative for both Dnmt3a and NeuN. G–I show single staining patterns with Dnmt3a, GFAP, and DAPI, respectively. J–L show overlay staining pattern of Dnmt3a, GFAP, and DAPI. In G–L, solid arrows show the cells positively stained for both Dnmt3a and GFAP, whereas the open arrows indicate GFAP-positive astrocytes negative for Dnmt3a. Solid arrows in M–P point to cells with both Dnmt3a and NeuN staining, whereas open arrows indicate NeuN-positive cells without Dnmt3a staining. Scale bars = 40 μm.

### Determination of Dnmt3a and Dnmt3b Gene Expression Patterns in the Embryonic CNS by Using *Dnmt3a-lacZ* and *Dnmt3b-lacZ* Transgenic Mice

As an alternative approach to determine the expression pattern of Dnmt3a and Dnmt3b in the developing CNS, we decided to examine the expression pattern of a lacZ transgene that has been knocked into the *Dnmt3a* (*Dnmt3a-lacZ*) or *Dnmt3b* (*Dnmt3b-lacZ*) gene locus (Okano et al., 1999). With these two lines of *Dnmt3a-lacZ* and *Dnmt3b-lacZ* transgenic mice, we performed X-gal staining to detect lacZ protein localization on the CNS sections from E10.5 to adult stages. *Dnmt3a-lacZ* or *Dnmt3b-lacZ* mice are maintained as heterozygous mice because the insertion of the lacZ transgene disrupts Dnmt3a and Dnmt3b gene transcripts, leading to the death of *Dnmt3b lacZ/lacZ* (–/–) mice by E15.5 and *Dnmt3a lacZ/lacZ* (–/–) animals at 3–4 weeks postnatally. We compared the X-gal staining pattern between heterozygous and homozygous *Dnmt3-lacZ* littermates and found in general a similar staining pattern between these two genotypes. However, both *Dnmt3a-lacZ* and *Dnmt3b-lacZ* staining did exhibit a threshold- and dosage-dependent pattern; in fact, X-gal signals in homozygous lacZ mice are robust and more apparent than those in heterozygous animals. Because we did not find any obvious morphological defect in homozygous *Dnmt3a-lacZ* and *Dnmt3b-lacZ* mice compared with heterozygous mice, we have chosen to show pictures of homozygous lacZ staining patterns whenever possible. As shown in Figure 5A,B, at E10.5, X-gal signals in the cortex of *Dnmt3b-lacZ* heterozygous (Fig. 5A) and homozygous (Fig. 5B) mice were quite similar, although the staining in *Dnmt3b-lacZ* homozygous mice were more intense. At this stage, X-gal-positive cells were evenly distributed across the cortical ventricular layer, where most cells are proliferating neural precursor cells. Because this lacZ reporter transgene lacks nuclear localization signal (Okano et al., 1999), the staining signals were punctuated in the cytoplasm. Similarly, X-gal staining in E10.5 *Dnmt3a-lacZ* homozygous mice was also detected in a dispersed pattern along the ventricular zone. Furthermore, X-gal staining in *Dnmt3b-lacZ* mice (Fig. 5A,B,D,E) appeared to be more intense than that in *Dnmt3a-lacZ* mice at this stage (Fig. 5C,F), which is consistent with the possibility that levels of Dnmt3b gene expression are higher than those of Dnmt3a in the cortex at E10.5.

We found that at E13.5 Dnmt3b X-gal signal was decreased throughout the entire CNS (Fig. 5G,I), whereas the intensity of Dnmt3a X-gal signal was significantly increased in the ventricular and subventricular zones (Fig. 5H,J). In the cortical plate, only weak Dnmt3a signals were observed (Fig. 5H,J), which is consistent with our findings with Dnmt3a immunohistochemistry (Fig. 3A–D). At E17.5, we cannot detect any Dnmt3b-lacZ signals in heterozygous mice (data now shown; also,

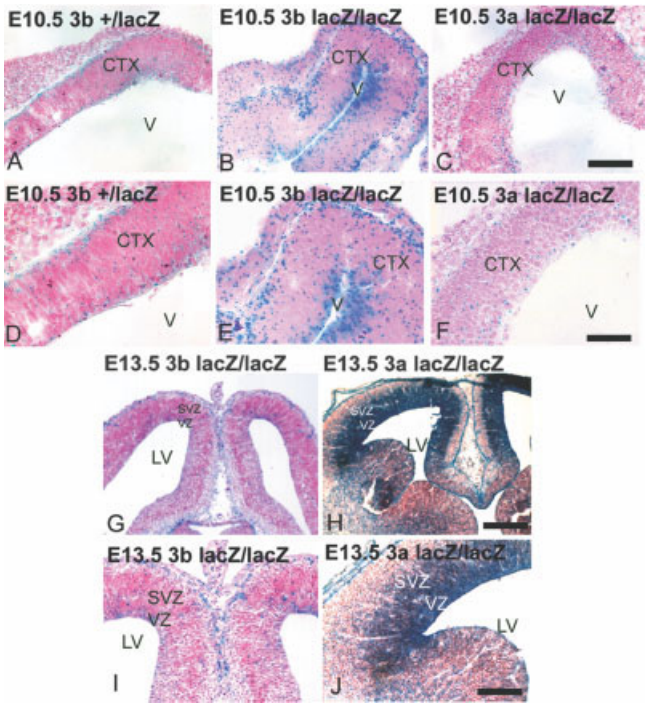


Fig. 5. Gene expression pattern of Dnmt3a and Dnmt3b in E10.5 and E13.5 cerebral cortex as revealed by lacZ marker gene expression. Brain sections from E10.5 (**A–F**) and E13.5 (**G–J**) were processed for X-gal histochemical staining to visualize the expression pattern of Dnmt3b-positive (A,B,D,E,G,I) and Dnmt3a-positive (C,F,H,J) cells. A and B compare the X-gal staining in the cortices of Dnmt3b-lacZ heterozygous (+/lacZ; A) and homozygous (lacZ/lacZ; B) mice. C shows X-gal staining of Dnmt3a-lacZ homozygous (lacZ/lacZ) mice, whereas E10.5 Dnmt3a-lacZ heterozygous exhibited much weaker staining (data not shown). D–F show portions of A–C at higher magnification, respectively. G and H show the X-gal staining in E13.5 Dnmt3b lacZ/lacZ and Dnmt3a lacZ/lacZ homozygous mice, respectively. I and J show portions of G and H at higher magnification. CTX, cortex; LV, lateral ventricle; SVZ, subventricular zone; V, vesicle; VZ, ventricular zone. Scale bars = 100 μm in A–C,I,J, 50 μm in D–F, 200 μm in G,H.

no homozygous Dnmt3b–/– embryos can be recovered after E15.5). In contrast, Dnmt3a expression was clearly detected in most CNS regions at E17.5 with the intensity of X-gal staining as depicted in Figure 6A–D. Indeed, many CNS regions, including the olfactory bulb, striatum, hippocampus, cerebellum, and cortex, showed strong X-gal staining signals. In addition to strong X-gal signals in ventricular and subventricular zones, lacZ staining was very intense in the striatum (Fig. 6A–C), raising the possibility that Dnmt3a is of particular importance for the maturation of striatal neurons. Overall, the results of Dnmt3a-lacZ expression with X-gal staining in neural precursor cells and differentiating neurons are very consistent with the immunohistochemical examination of Dnmt3a protein expression in the embryonic CNS (Figs. 2, 3).

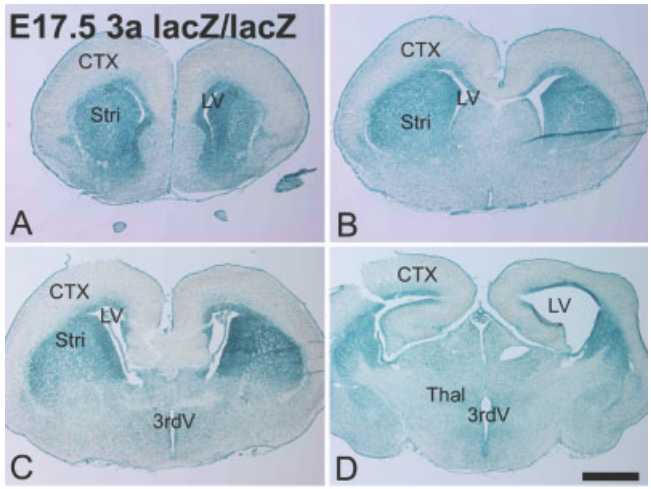


Fig. 6. Dnmt3a gene expression pattern in the forebrain of E17.5 Dnmt3a-lacZ homozygous mice. Coronal serial brain sections from E17.5 Dnmt3a-lacZ homozygous mice were stained with X-gal. Panels from cortex (**A–D**), striatum (A–C), thalamus (D) are shown. 3rd V, third ventricle; CTX, cortex; LV, lateral ventricle; Stri, striatum; Thal, thalamus. Scale bar = 600 μm.

## Dynamic Regulation of Dnmt3a Expression in Postnatal and Adult CNS In Vivo and In Vitro

By Western blot analysis and immunostaining, we have detected Dnmt3a in cortices during early postnatal and juvenile stage and in adulthood (Figs. 1C, 2–4). To examine the expression pattern of Dnmt3a in postnatal and adult CNS, we carried out X-gal staining with serial brain sections from postnatal to adult Dnmt3a-lacZ transgenic mice. X-gal staining can be easily detected in the entire CNS in either heterozygous (data not shown) or homozygous Dnmt3a-lacZ mice between P0 and P21 (Fig. 7), which covers a critical period for neuronal maturation in the CNS. Interestingly, the intensity of X-gal staining was significantly increased in many neuron-enriched regions, including olfactory bulb, cortex, striatum, hippocampus, and cerebellum, between P0 and P21, suggesting that Dnmt3a protein level in individual CNS neurons is increased during the period of postnatal maturation.

To examine directly whether Dnmt3a expression increases in postmitotic neurons during maturation, we compared Dnmt3a protein levels in postnatal cerebellar neuronal cultures at different time points. Cultured cerebellar granule neurons undergo a process of neuronal maturation that mimics neuronal differentiation in vivo (Diaz et al., 2002). As can be seen in Figure 8, we found that Dnmt3a levels are significantly increased during the first 6 days in cerebellar cultures with either wild-type or *Dnmt3a-lacZ* (+/–) heterozygous mice; furthermore, comparison of wild-type and heterozygous proteins showed a clear dosage effect of Dnmt3a gene expression. The intensity of Dnmt3a immunoreactivities in cultured cerebellar neurons was also increased over the 6-day pe-

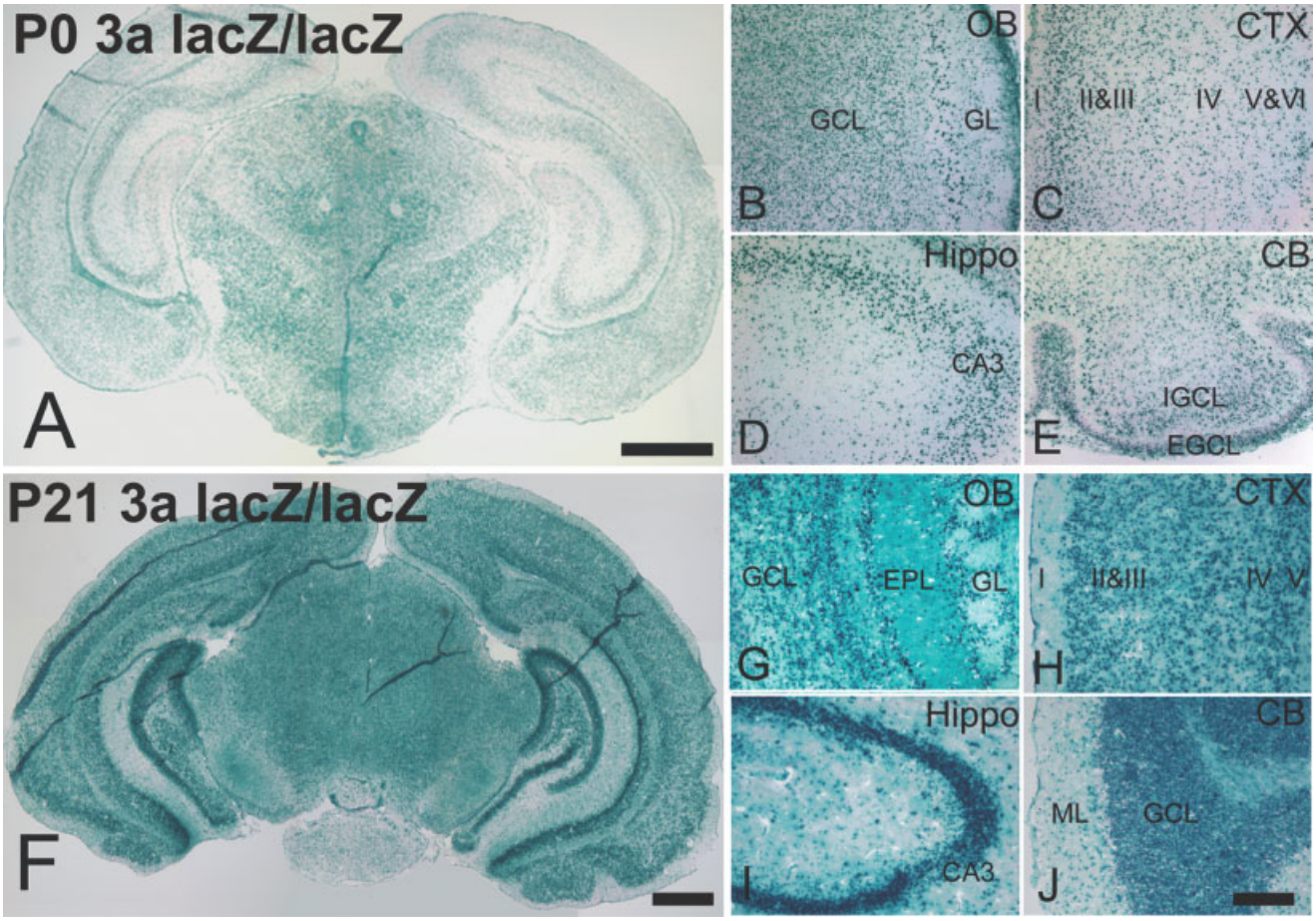


Fig. 7. Expression pattern of Dnmt3a in the CNS in newborns and at young adulthood. Coronal brain sections from Dnmt3a homozygous mice at newborn (P0; **A–E**) and young adult (P21; **F–J**) stages were processed for X-gal histochemical staining. Stronger X-gal staining was observed in P21 CNS compared with newborn (P0) CNS. CB, cerebellum; CTX, cortex; EGCL, external granule cell layer; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; Hippo, hippocampus; I–VI, cortical layers I–VI; IGCL, internal granule cell layerl ML, molecular layer; OB, olfactory bulb. Scale bars = 1 mm in A,F, 150 μm in B–E,G–J.

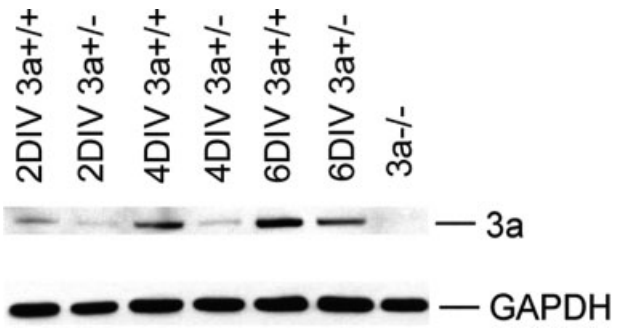


Fig. 8. Increased expression of Dnmt3a proteins during neuronal maturation in vitro. Western blot of the protein samples from P6 cerebellar cultures after 2, 4, and 6 DIV. The Dnmt3a protein levels are significantly increased in both wild-type (+/+) and Dnmt3a-lacZ heterozygous (+/–) cerebellar cultures during the first 6 days in vitro.

riod of cell culture, which is consistent with elevated Dnmt3a protein levels in more mature cerebellar neurons in vitro (data not shown).

Because homozygous Dnmt3a-lacZ mice die after 3–4 weeks postnatally, we can only examine adult Dnmt3a expression pattern by using *Dnmt3a-lacZ* heterozygous mice. In a 6-month-old *Dnmt3a-lacZ* heterozygous mouse, X-gal-positive cells were detected in most CNS regions examined, including cortex, hippocampus, thalamus (Fig. 9A), as well as in brainstem and cerebellum. Relatively stronger signals were detected in neuron-enriched structures, such as olfactory bulb, striatum, and hippocampus (Fig. 9B–G). In contrast, astroglia-enriched regions, such as internal capsules and corpus callosum, exhibited relatively weak staining, consistent with relatively low or no expression of Dnmt3a in astroglial cell populations (data not shown). Furthermore, the general intensity of X-gal staining in the adult CNS appears to be weaker than that in early postnatal CNS, suggesting a

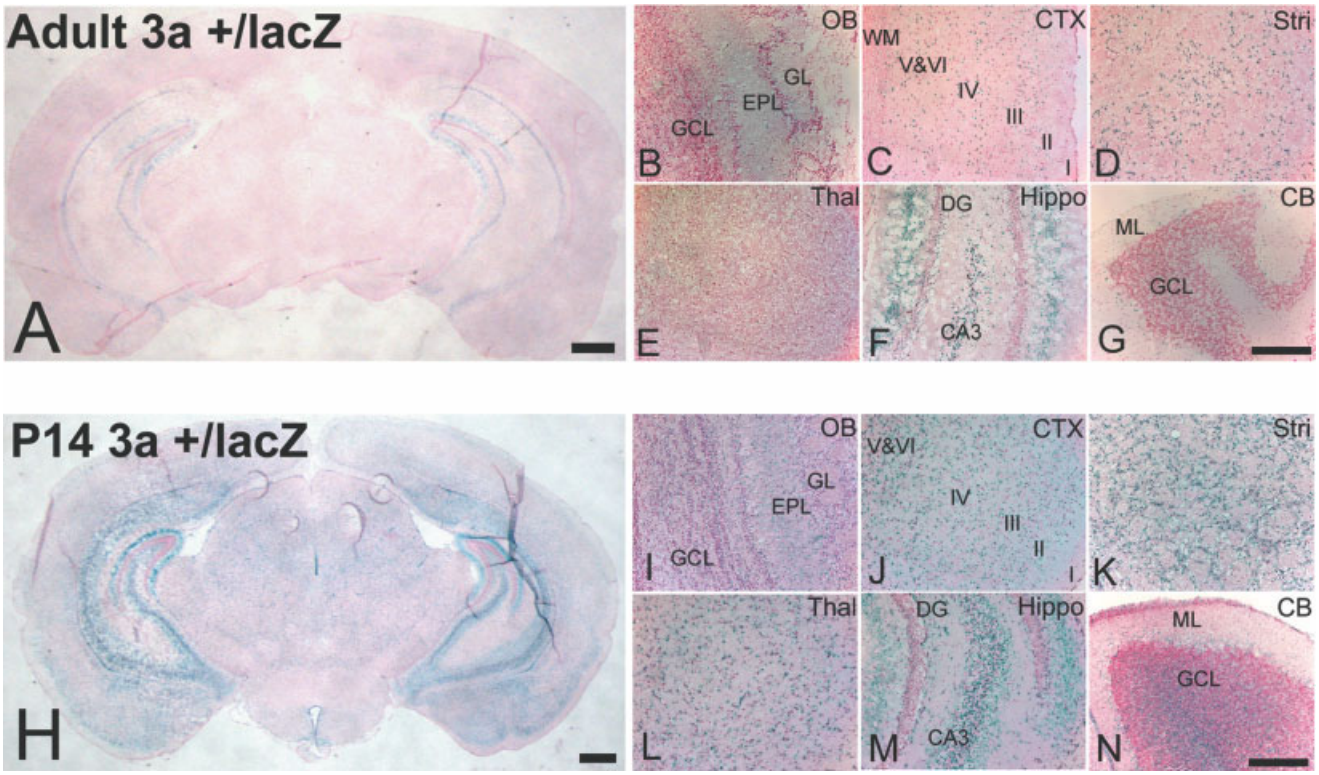


Fig. 9. Comparison of Dnmt3a gene expression pattern in juvenile and adult CNS. **A–G:** Rostral to caudal brain sections from a 6-month-old adult Dnmt3a-lacZ heterozygous mouse were processed for X-gal histochemical staining. **H–N:** A P14 juvenile Dnmt3a-lacZ heterozygous mouse was processed with the exact same procedure side-by-side with the adult mouse shown in A–G. The forebrain coronal sections show cortex, hippocampus, thalamus, lateral ventricle, and third ventricle in P14 (H) and adult (A). The higher magnifications of olfactory bulb (OB), cortex (CTX), striatum (Stri), thalamus (Thal), hippocampus (Hippo), and cerebellum (CB) are shown in B–G for adult and in I–N for P14. DG, dentate gyrus; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; I–VI, cortical layers I–VI; ML, molecular layer; WM, white matter. Scale bars = 1 mm in A,H, 180 μm in B–G,I–N.

potential down-regulation of Dnmt3a expression after the period of neuronal maturation. In fact, we saw clearly that X-gal signal in adult mice was weaker than that in P14 mice at all the corresponding CNS regions compared with the X-gal staining pattern from the P14 *Dnmt3a-lacZ* heterozygous mouse (Fig. 9H–N) that was processed side by side with the adult *Dnmt3a-lacZ* heterozygous mouse. To quantitate the difference in the levels of Dnmt3a proteins in early postnatal and adult CNS, we performed Western blot analysis of Dnmt3a protein in four CNS regions in wild-type mice at P2, P14, and adulthood. As shown in Figure 10, Dnmt3a protein was detectable in the adult olfactory bulb, cortex, striatum, and cerebellum but at a much lower level than that in newborn (P2) and juvenile mice (P14; Fig. 10). Overall, our findings suggest that Dnmt3a proteins in CNS neurons exhibit a biphasic expression pattern, in that Dnmt3a expression is initially at a relatively low level in newly born postmitotic neurons, becomes significantly increased during early postnatal stages, reaches peak levels during juvenile stages and young adulthood, and afterwards decreases to a low level in adulthood.

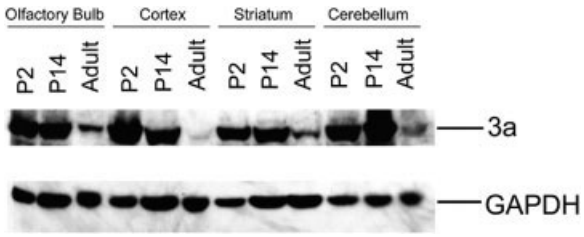


Fig. 10. Western blot analysis of Dnmt 3a proten levels in olfactory bulb, cortex, striatum, and cerebellum at newborn (P2), juvenile (P14), and adult ages. Olfactory bulb and cortical, striatal, and cerebellar tissues were freshly dissected from wild-type P2, P14, and 4-month-old adult mice and immediately lysed in 1× SDS sample buffer for Western blot analysis. Protein membrane was sequentially blotted with monoclonal antibodies against Dnmt3a and GAPDH.

## DISCUSSION

In this study, we have demonstrated that expression of Dnmt3a and Dnmt3b mRNAs and proteins is dynamically regulated in the embryonic and adult CNS. Consis-

tently with previous studies showing Dnmt3b expression in early stages of embryogenesis (Okano et al., 1999; Watanabe et al., 2002), Dnmt3b expression in the developing CNS is detected within a narrow window of early neurogenesis (E11–15), suggesting that Dnmt3b is important mainly in the early phase of neural development. In contrast, Dnmt3a expression in the CNS is initially high in neural precursor cells at the ventricular and subventricular zones and remains relatively low in cortical plate, where the first wave of postmitotic CNS neurons reside. However, Dnmt3a expression in postmitotic CNS neurons is significantly increased during early postnatal stages, coinciding with postnatal maturation of the CNS. Thus, Dnmt3a is likely involved in regulating both neurogenesis and neuronal maturation during embryonic and postnatal stages, respectively.

Dynamic regulation of DNA methylation in the CNS has been implicated in regulating neuronal and glial differentiation. Teter et al. (1994) first demonstrated that several CpG sites in the rat astrocyte marker gene GFAP undergo a demethylation and remethylation process during embryonic and postnatal stages. Takizawa et al. (2001) further showed that demethylation of the CpG site within the binding element of the signal transducer and activator of transcription (STAT) transcriptional activator is required for the binding of STAT3 to the GFAP promoter and the activation of GFAP gene transcription. We recently found that, in Dnmt1−/− CNS (Fan et al., 2001), demethylation triggers precocious astroglial differentiation, confirming that DNA methylation is one of the key mechanisms regulating the timing of glial cell differentiation (Martinowich et al., 2002). Therefore, dynamic regulation of de novo DNA methyltransferases in CNS precursor cells may contribute to the precise regulation of DNA methylation on the genes that are critical for neuronal and glial differentiation. Whether Dnmt3a and Dnmt3b deficiency would affect the program of neurogenesis and gliogenesis is currently under investigation.

Although both Dnmt3a and Dnmt3b enzymes are characterized as de novo DNA methyltransferases, recent studies showed that Dnmt3a and Dnmt3b, in concert with Dnmt1, also play a role in the maintenance of DNA methylation (Liang et al., 2002; T. Chen et al., 2003). Expression of the Dnmt1 gene in the CNS is detected in both embryonic and adult CNS at a relatively constant level (Goto et al., 1994; Brooks et al., 1996), which is consistent with a major role as a maintenance enzyme that preferentially adds methyl groups to hemimethylated DNA. In contrast, Dnmt3a and Dnmt3b can methylate DNA in both unmethylated and hemimethylated states, which would be particularly important for postmitotic neurons that no longer undergo active DNA synthesis for cell division. In this regard, the presence of Dnmt3a in postmitotic CNS neurons suggests that Dnmt3a may play an active role in regulating DNA methylation pattern and silencing neuronal gene expression in response to environmental cues. Whether differential expression patterns of Dnmt3a and Dnmt3b are correlated with dynamic regulation in DNA methylation levels and patterns in the developing and adult CNS remains to be seen.

Epigenetic mechanisms involving DNA methylation-related chromatin remodeling have been shown to mediate both activity-dependent neuronal gene expression (Martinowich et al., 2003; W.G. Chen et al., 2003) and the influence of maternal behavior on perinatal programming of the HPA axis in rat pups (Weaver et al., 2004). Our previous work showed that depolarizing conditions in cultured cortical neurons lead to reduced DNA methylation levels in the BDNF promoter compared with that in control cultures. Furthermore, methylation levels are correlated with levels of BDNF gene expression (Martinowich et al., 2003). In consideration of the presence of high levels of Dnmt3a and Dnmt1 in postmitotic neurons, it is possible that depolarizing conditions also block the action of Dnmt3a and Dnmt1 activity on the BDNF promoter, whereas, in control cultures, Dnmt3a (and also Dnmt1) may play a role in maintaining or even increasing DNA methylation in the BDNF promoter when neuronal activity is suppressed. Similarly, it has been shown that increased pup licking and grooming (LG) and arched-back nursing (ABN) by rat mothers lead to a lower level of DNA methylation on a glucocorticoid receptor gene promoter in the hippocampus of their pups (Weaver et al., 2004). Interestingly, increased methylation in glucocorticoid receptor gene promoter occurs during the first week of postnatal life in poorly nursed rat pups. We speculate that the methylation increase in the pups of low-LG-ABN mothers could be mediated by Dnmt3a, potentially in concert with Dnmt1. Although little is known about how methylation is regulated in CNS neurons, our data suggest that Dnmt3a is a major player if de novo DNA methylation activity is required for neuronal gene regulation.

During early postnatal stages, in parallel with the process of neuronal maturation, expression of Dnmt3a appears to reach peak levels. This was observed both in cultured cerebellar neurons during the culture period and in different CNS regions in vivo at the juvenile age. Increased Dnmt3a expression may be a necessary step to boost the levels of DNA methylation in the CNS during this critical period. It has been reported that, during this period, expression of other methylation-related proteins, such as MeCP2, is also significantly increased (Cassel et al., 2004; Mullaney et al., 2004). It is possible that Dnmt3a, through its de novo DNA methylation activity, actively takes part in the suppression of superfluous gene expression during the critical window of neuronal maturation in the brain.

Levels of Dnmt3a protein in the adult CNS appears to be down-regulated compared with those in embryonic and early postnatal CNS. We noticed that down-regulation of the shorter isoform Dnmt3a2 in the CNS occurs during embryonic stages, whereas the longer isoform of Dnmt3a1 protein is reduced only after young adulthood. The mechanism underlying the down-regulation of Dnmt3a1 and Dnmt3a2 protein remains unclear. In an experimental paradigm of in vitro differen-

tiation of mouse embryonic stem (ES) cells, Chen et al. (2002) showed that the Dnmt3a2 isoform is significantly down-regulated in differentiated cells compared with undifferentiated ES cells, suggesting that Dnmt3a2 might be required only for immature embryonic cells or precursor cells. With regard to the observed down-regulation of Dnmt3a in the adult CNS, Dnmt3a protein (primarily Dnmt3a1 isoform) is detected predominantly in neuronal cells postnatally, so the possibility remains that the apparent decrease in Dnmt3a protein level observed via immunoblotting could be partially due to a dramatic increase in the number of astroglia, which express relatively lower levels of Dnmt3a. Nevertheless, Dnmt3a immunostaining in the adult cortex showed clearly that a subpopulation of cortical neurons exhibits weak or no staining of Dnmt3a, supporting the notion that down-regulation of Dnmt3a expression occurs at least in a subpopulation of adult CNS neurons. Interestingly, DNA methylation levels in aged animals are reduced compared with those in young animals (Wilson et al., 1987). It is tempting to speculate that the decreased Dnmt3a expression in elderly animals is one of the factors contributing to the gradual decline of DNA methylation level in the CNS. Such a hypothesis could be tested with conditional knockout mice, in which Dnmt3a is exclusively knocked out in postmitotic CNS neurons, possibly leading to a further reduction of DNA methylation levels with age. Conversely, transgenic mice engineered to either maintain or increase the neuronal expression of Dnmt3a could potentially reveal a prevention of the decline of DNA methylation in aged animals.

In conclusion, we have demonstrated that Dnmt3a and Dnmt3b are differentially expressed in CNS precursor cells, astrocytes, oligodendrocytes, and postmitotic neuronal cells. The results of this study will help us to understand how the de novo DNA methyltransferases Dnmt3a and Dnmt3b play disctinct roles in early neurogenesis as well as in CNS maturation and function.

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