Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons

Yoshinobu Sugitani,1 Shigeyasu Nakai,1 Osamu Minowa,1,2 Miyuki Nishi,1 Kou-ichi Jishage,1 Hitoshi Kawano,3 Kensaku Mori,4 Masaharu Ogawa,5 and Tetsuo Noda1,2,6,7,8

1Department of Cell Biology, JFCR–Cancer Institute, Tokyo 170-8455, Japan; 2Mouse Functional Genomics Research Group, RIKEN Genomic Sciences Center, Kanagawa 244-0804, Japan; 3Department of Developmental Morphology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183-8526, Japan; 4Department of Physiology, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan; 5Laboratory for Cell Culture Development, Brain Science Institute, RIKEN, Saitama 351-0198, Japan; 6Department of Molecular Genetics, Tohoku University School of Medicine, Miyagi 980-8575, Japan; 7Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Saitama 332-0012, Japan

Formation of highly organized neocortical structure depends on the production and correct placement of the appropriate number and types of neurons. POU homodomain proteins Brn-1 and Brn-2 are coexpressed in the developing neocortex, both in the late precursor cells and in the migrating neurons. Here we show that double disruption of both Brn-1 and Brn-2 genes in mice leads to abnormal formation of the neocortex with dramatically reduced production of layer IV–II neurons and defective migration of neurons unable to express mDab1. These data indicate that Brn-1 and Brn-2 share roles in the production and positioning of neocortical neuron development.

Received January 22, 2002; revised version accepted May 23, 2002.

The mature neocortex is organized into six cell layers, each of which contains neurons with similar morphologies, molecular properties, and projection patterns. The development of this neocortical structure depends on a highly ordered pattern of neuronal production and migration. Cortical neurons that comprise each layer are sequentially produced in the ventricular zone of the dorsal telencephalon [Angevine and Sidman 1961; Takahashi et al. 1999]. Although the regulatory factors that function in this sequential production of a variety of layer-specific neurons have not been identified in mammals, in Drosophila the successive production of different types of cells from neuroblasts has been found to require a temporally stereotyped pattern of expression of a set of transcription factors including the Drosophila POU transcription factors Pdm1 and Pdm2 [Isshiki et al. 2001]. In mammals, newly produced neurons leave their birthplace, migrate toward the cortical surface, and form cortical layers in an inside-out pattern with respect to their time of birth [Angevine and Sidman 1961; Rakic 1972]. Recent genetic studies have identified large numbers of functional molecules involved in the migration/positioning of neocortical neurons (for review, see Rice and Curran 1999).

Brn-1 and Brn-2, members of the mammalian class III POU transcription factor family, are prominently expressed in the embryonic brain, including the neocortex [He et al. 1989]. Each single mutant, however, shows abnormalities only in limited brain regions. In Brn-2 mutant neonates, neuronal loss was observed only in the hypothalamic supraoptic and paraventricular nuclei, where Brn-1 is not expressed [Nakai et al. 1995; Schonemann et al. 1995]. In Brn-1 mutants, remarkable changes in brain morphology were observed only in the hippocampus, where Brn-2 expression is barely detectable (data not shown). In the neocortex, where both Brn-1 and Brn-2 are expressed, no overt developmental defects were seen in either single mutant. These observations suggest functional complementation between Brn-1 and Brn-2 in neocortical development.

Results and Discussion

To explore their possible overlapping functions in neocortical development, we generated Brn-1/Brn-2 double homozygous mutants by intercrossing double heterozygotes that were healthy and fertile, with no apparent phenotype. Double homozygous mutants were born at the expected Mendelian ratio (76 double homozygous mutants among 1192 pups), but all of them died within 1 hr after birth. In contrast to the limited abnormalities in Brn-1−/− or Brn-2−/− single mutants, Brn-1/Brn-2 double mutants suffered severe, broad brain defects. The olfactory bulb showed hypoplasia [Fig. 1A,B], and the cerebellum was less foliated, with loosely packed Purkinje cells [Fig. 1C,D]. The neocortex was severely affected; its thickness was markedly reduced, and the stratification of the cortical neurons appeared to be disorganized [Fig. 1E,F].

The hypoplastic neocortex could be caused by reduced cell proliferation or accelerated cell death during embryonic corticogenesis. Because there was no evidence of increased apoptosis in Brn-1/Brn-2 double mutant cortex from embryonic day 14.5 (E14.5) to postnatal day 0 (P0, data not shown), we examined the proliferation of cortical progenitor cells by bromodeoxyuridine (BrdU) labeling. In mice, most cortical plate neurons are produced in the ventricular zone (VZ) or in the subventricular zone (SVZ) from E12.5 to E16.5 [The Boulder Committee 1970; Takahashi et al. 1999]. Up to E13.5, there was no significant difference in the number of BrdU-labeled cells in the VZ of the double mutant embryos, compared with wild-type [E12.5: 100.0% ± 1.8% of wild-type, E13.5: 100.8% ± 2.2% of wild-type, Fig. 2A,A′]. Reduced
cell proliferation in the VZ was observed at E14.5 and thereafter in Brn-1/Brn-2 mutant neocortex. [E14.5: 63.4% ± 2.6% of wild-type; E16.5: 60.2% ± 3.4% of wild-type, Fig. 2B,C]. Reduction in the number of BrdU-labeled cells was particularly severe in the cortical SVZ in the double mutant [E16.5: 15.1% ± 2.5% of wild-type; Fig. 2C]. Despite the hypoplasticity of the Brn-1/Brn-2 deficient cortex, expression of GAD67 and calbindin appeared to be unaffected in the E19.0 neocortex [Fig. 3J; data not shown], suggesting intact generation and migration of the cortical interneurons, most of which are derived from the ganglionic eminence [Anderson et al. 1997]. These results indicate that Brn-1 and Brn-2 share an essential role in the proliferation of cortical precursor cells within the VZ/SVZ from E14.5 onward, and that the reduction in subsequent cortical cell production could result in the hypoplastic neocortex seen in the double mutant neonate. Analysis of the temporal expression pattern for Brn-1 and Brn-2 proteins in the developing wild-type neocortex revealed that their expression in the VZ is initiated at ~E14.5 and is prominent thereafter in the VZ/SVZ [Fig. 2D–I], with a pattern that corresponds with the period of reduced cell proliferation in the neocortex of double mutant embryos. These results suggest that Brn-1 and Brn-2 may function in the proliferation of late cortical progenitor cells in a cell-autonomous manner.

Lineage analyses and birthdating studies suggest that common cortical precursor cells first produce neurons of layer VI and then layer V at E15.5 and, even later, generate neurons destined for layers IV–II at E15.5–E17.0 by successive cell division [Luskin et al. 1988; Takahashi et al. 1999]. From the late embryonic neurogenesis stage, glial progenitor cells also proliferate and increase their numbers [Berman et al. 1997], differentiating into astrocytes or oligodendrocytes during a postnatal stage. The finding that Brn-1 and Brn-2 function in cell proliferation, specifically at the late neurogenesis stage, prompted us to examine whether Brn-1 and Brn-2 function in the production of upper-layer neurons and/or in the generation/expansion of glial progenitor cells. We assessed the formation of each cortical layer and the status of gliogenesis in the double mutant cortex at E19.0 or E18.5, using the following markers for different layers and glial progenitors: Tbr-1 for layer VI, subplate and SVZ [Fig. 3A]; Wnt7b for layer VI [data not shown; Rubenstein et al. 1999], ER81 for layer V [Fig. 3C]; RORB for layer IV [Fig. 3E; Weimann et al. 1999], mSorLA or Svet1 for layers II/III and SVZ cells [Fig. 3G; data not shown; Hermans-Borgmeyer et al. 1998; Tarabykin et al. 2001],
we labeled E12.5, E13.5, and E14.5 embryos, stages during which layer VI–V neuronogenesis is at a peak, with BrdU and examined the localization of BrdU-positive cortical neurons in E19.0 embryos. If the abnormal lamination is caused by cell fate defects, BrdU-labeled neurons should appear in comparable positions in the wild-type and Brn-1/Brn-2 mutant cortices. Conversely, if neuronal migration is affected, neurons labeled at the same time should occupy different positions in wild-type and mutant mice. In E19.0 wild-type cortex, cells born on E12.5 occupied the SP and the deepest part of layer VI (Fig. 4A), and most of the cells at E13.5 predominantly occupied layer VI above the E12.5-born cohort (Fig. 4B). The relative positions of E13.5-born to E12.5-born neurons in the Brn-1/Brn-2-deficient cortex at E19.0 (Fig. 4D,E) were comparable with those in their wild-type littermates (Fig. 4A,B). The positioning of E14.5-born neurons, however, was significantly altered. E14.5-born cells in wild-type cortex occupied layers V and IV in a superficial region of the CP (Fig. 4C), whereas those in Brn-1/Brn-2-deficient cortex remained in the intermediate zone (IZ), beneath the cohort of E12.5-born cells (Fig. 4F). Together with the abnormal localization of the layer V neurons in the IZ of Brn-1/Brn-2 mutant cortex (Fig. 3D), these BrdU neural birthdating experiments suggest abnormal migration of the layer V neurons born after E13.5 in Brn-1/Brn-2 mutant cortex (Fig. 4K,L).

Correct neuronal migration requires both radial glial fibers as guiding scaffolds for migrating neurons (Rakic 1972) and Cajal-Retzius neurons that play a key role in neuronal lamination by producing the secreted Reelin protein (Ogawa et al. 1995; Rice and Curran 1999). The alignment and density of radial glial fibers, labeled with antibodies against B-FABP or Nestin, were not altered (Fig. 3N,R). Furthermore, neither the number of Cajal-Retzius neurons nor their immunolabeling intensity for Reelin was changed in the Brn-1/Brn-2-deficient cortex (Fig. 3P). In fact, Cajal-Retzius neurons in the wild-type cortex expressed neither Brn-1 nor Brn-2 at E16.5 and E18.5 (M–P) and E16.5 (Q,R) cortices of wild-type [I,M,O,Q] and Brn-1/Brn-2 mutant [I,N,P,R]. Scale bar: (A–N) 100 µm, (O,P) 20 µm, (Q,R) 50 µm.

Figure 3. Loss of upper-layer neurons and altered positioning of cortical neurons in Brn-1/Brn-2 mutant neocortex. In situ hybridization using Tbr-1 (A,B), ER81 (C,D), RORβ (E,F), mSorLA (G,H), Olg-1 (K,L), riboprobes on coronal sections of E19.0 wild-type (A,C,E,G,K) and Brn-1/Brn-2 mutant (B,D,F,H,L) cortices. In wild-type cortex, Tbr-1-positive layer VI, ER81-positive layer V, RORβ-positive layer IV, and mSorLA-positive layer II/III neurons are ordered from deep to superficial (A,C,E,G). In Brn-1/Brn-2 mutant cortex, however, the majority of the ER81-positive neurons are found beneath the Tbr-1-positive layer, with a few ER81-positive neurons detected in the superficial region within the cortical plate (B,D), but the numbers of layer IV or layer II/III neurons positive for RORβ or mSorLA are drastically reduced (F,H), although mSorLA expression is found in the SVZ with a similar pattern of Tbr-1 expression in the SVZ (H,B). Immunostaining against GAD67 (red) (I,J), B-FABP (red) (M,N), Reelin (green) and CR-50 (red) (O,P), and Nestin (brown) (Q,R) on sagittal (M,N,Q,R) and coronal (I,J,O,P) sections of E19.0 (I,J), E18.5 (M–P) and E16.5 (Q,R) cortices of wild-type [I,M,O,Q] and Brn-1/Brn-2 mutant [I,N,P,R]. Scale bar: (A–N) 100 µm, (O,P) 20 µm, (Q,R) 50 µm.

Olg-1 for oligodendrocyte progenitors [Fig. 3K, Lu et al. 2000; Zhou et al. 2000], B-FABP/BLBP for immature astrocytes and radial glial cells [Fig. 3M, Feng et al. 1994; Kurtz et al. 1994], and CR-50 for Cajal-Retzius neurons in the marginal zone [Mizutani et al. 2003, Ogawa et al. 1995; D’Arcangelo et al. 1997]. The marker studies indicated that the initial step of gliogenesis seemed to be unaffected in Brn-1/Brn-2 mutant neocortex [Fig. 3L,N], whereas the numbers of RORβ-positive, mSorLA-positive, or Svet1-expressing or Svet1-expressing SVZ cells lining the entire surface of the enlarged lateral ventricles of the mutant brains [Fig. 3F,H, data not shown]. These results suggest that Brn-1 and Brn-2 are essential for proper production of neocortical neurons destined for layers VI–II.

Molecular marker analysis also revealed abnormal layering of the remaining cortical neurons in Brn-1/Brn-2-deficient neocortex, in which the majority of ER81-positive layer V neurons, normally laminated above the Tbr-1-positive or Wnt7β-positive layer VI [Fig. 3A,C, data not shown], were found beneath the Tbr-1-positive or Wnt7β-positive layer (Fig. 3B,D, data not shown). It has been well documented that the laminar structure of the neocortex is built by migration of successively produced neurons in an inside-to-outside fashion, such that neurons born earlier reside in deeper layers, and those born later occupy more superficial layers within the cortical plate (CP) between the MZ and the subplate (SP). Thus, the largely inverted texting pattern of layer V and VI neurons in Brn-1/Brn-2 mutant cortex can be caused by either abnormal cell migration or cell fate defects such that the timing of layer VI and layer V neuronogenesis is inverted. To distinguish between the two possibilities,

Sugitani et al.
Doublecortin are also thought to affect neuronal migration in the developing cortex. Among all these tested genes, only mDab1 expression was clearly affected in the Brn-1/Brn-2 double mutant cortex at E16.5 (Fig. 5A–F) and E15.5–E16.5 (Fig. S5A,E) in E19.0 wild-type (A–C) and Brn-1/Brn-2 mutant (D–F) sagittal cortical sections. BrdU-positive nuclei [brown] were detected by immunohistochemistry. [Right panels] Bar graphs showing the radial distribution of heavily labeled cells (first generation at time of BrdU injection) (G–L) and lightly labeled cells [the majority of them are second and perhaps third generation cells from subsequent progenitor cell divisions] (G′–L′) in E19.0 wild-type (G–G′, L–L′) and Brn-1/Brn-2 mutant (J–J′, K–K′) neocortex. In Brn-1/Brn-2 mutants, most E14.5 BrdU-labeled cells (L′) occupy the deepest positions. The subpopulation of E13.5 lightly labeled cells is also shifted to deeper positions (K′). (2–4) Layer II–VI, (5) Layer V, (6) Layer VI. For other abbreviations, see Fig. 1. Scale bar: 80 µm.

Figure 4. Abnormal migration of Brn-1/Brn-2 mutant neurons. (Left panels) Distribution of cells labeled with BrdU during E12.5 (A, D), E13.5 (B, E), or E14.5 (C, F) in E19.0 wild-type (A–C) and Brn-1/Brn-2 mutant (D–F) sagittal cortical sections. BrdU-positive nuclei [brown] were detected by immunohistochemistry. [Right panels] Bar graphs showing the radial distribution of heavily labeled cells (first generation at time of BrdU injection) (G–L) and lightly labeled cells [the majority of them are second and perhaps third generation cells from subsequent progenitor cell divisions] (G′–L′) in E19.0 wild-type (G–G′, L–L′) and Brn-1/Brn-2 mutant (J–J′, K–K′) neocortex. In Brn-1/Brn-2 mutants, most E14.5 BrdU-labeled cells (L′) occupy the deepest positions. The subpopulation of E13.5 lightly labeled cells is also shifted to deeper positions (K′). (2–4) Layer II–VI, (5) Layer V, (6) Layer VI. For other abbreviations, see Fig. 1. Scale bar: 80 µm.
mammalian neocortex with its great diversity of cortical neurons.

**Materials and methods**

**Histology and immunohistochemistry for calbindin, BrdU, and Nestin**

Fixed samples in Bouin’s fixative were dehydrated and embedded in paraffin blocks, from which 5–8-µm serial sections were cut. Hematoxylin and eosin (HE) staining was performed following standard protocols. For immunohistochemistry, the following antibodies were used: anti-Calbindin [a gift of M. Watanabe, Hokkaido University, Japan], anti-BrdU [Becton Dickinson], anti-Nestin [a gift of Y. Tomooka, Science University of Tokyo, Japan], and anti-Pax6 [a gift of N. Osumi, Tohoku University, Japan]. The Vectastain ABC kit (Vector Laboratories) was used for detection. The sections were counterstained with hematoxylin.

**BrdU-labeling analysis**

For the cell proliferation assay, we injected pregnant mice intraperitoneally with BrdU (50 mg/kg) 1 h before death. BrdU-positive cells were visualized as described above. Three embryos for each genotype were analyzed at the indicated stages, and 10 sagittal sections at the level of the olfactory bulb for each embryo were used. The fraction of BrdU-positive cells in the VZ was determined by dividing the number of BrdU-positive nuclei by the total number of the nuclei identified in units of the 200-µm-wide VZ. For the assay in the SVZ, because of the difficulty in distinguishing SVZ cells from postmitotic cells, BrdU-positive SVZ cells were counted in the same units as the assay in the VZ. For birthdating analysis to determine the distributions of the cells labeled with BrdU (30 mg/kg) in the E19.0 neocortical wall, parasagittal sections at the level of the accessory olfactory bulb were used. At the level, 500-µm-wide radial stripes in the medial portions were divided into 40-µm-deep bins (20 horizontal bins in wild-type cortex and 14 bins in mutant cortex, respectively), and the position of each heavily and lightly labeled cell was assigned to a bin to generate histograms of the number of labeled cells against depth. Data from five sections from each of two to three littersmates were averaged to give the histograms.

**Immunofluorescence, apoptosis assay, and RNA in situ hybridization**

Fixed samples with 4% paraformaldehyde in PBS were embedded in OCT compound, and serial sections (6–30 µm) were cut cryostat and immunostained with the following primary antibodies: anti-GAD (Chemicon, 1:1500 dilution), anti-B-FABP (a gift of F. Spener, University of Münster, Germany), anti-Brn-2 (1:800 dilution), anti-Nestin (a gift of Y. Tomooka, Science University of Tokyo, Japan), anti-BrdU (Beckton Dickinson), anti-CSPGs (Sigma, 1:600 dilution), and anti-Brn-2 (1:800 dilution). Anti-BrdU rabbit polyclonal antibodies were raised against the C terminus of BrdU (amino acids 422–433). Western blot analysis and immunostaining confirmed the specificity of the antibodies. Apoptosis in the cortex at E14.5–P0 was assayed by using a TUNEL assay kit (Onco). RNA in situ hybridization was performed by modified protocols as described earlier [Minowa et al. 1999]. Riboprobes were synthesized using the following murine cDNAs: mDab1 (30–464), p35 (1142–1791), Tbr-1 (2219–2600), Tbr-1 (2307–2956), Wnt7b (1138–1449), Svet1 (2622–3243), and tailless (729–1410).

**RT-PCR analysis**

Total RNA (7.5 µg), extracted from dissected E16.5 dorsal cortices of each embryo, were reverse-transcribed with an oligo(dT) primer (Invitrogen), and 1/20 of each RT reaction was subjected to PCR amplification using specific primer pairs. The β-actin gene was used as control. The primer pairs used were mDab1, 5'-GGGCGTGGAGGCCTGGTTGGAGTCGC-3', 5'-CCTTATCATGCAGTGAGACACTGAATG-3'; p35, 5'-TCGCCGTCGTTGACCACTCATCTTCCG-3', 5'-AAACAGGTACTCAAGCGACCCAGG-3', 5'-CTACTGAGGAGACCTGCTTTCC-3'; Tbr-1, 5'-CTCACTGAGGAGACCTGCTTTCC-3', 5'-TCACGTACCCCAACCGACTCTTCG-3'; and tailless, 5'-CCTCCAAACCGCCGACTCTTGCG-3'. The PCR
products obtained were subjected to electrophoresis, and the intensities of each amplified band were analyzed by densitometry. The PCR products for mDab1, p55, CDK5, and β-actin were transferred to nylon-based membranes and hybridized with the following 32P-labeled oligonucleotides specific for each cDNA: mDab1, 5’-AAGCTCAAGGTCAGGATCGCAGC-3’, H11032, for relative quantitation of serially diluted wild-type RT-products. The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

Acknowledgments
We thank M. Watanabe, Y. Tomooka, F. Spener, and N. Osumi for their gifts of rabbit polyclonal antibodies; and K. Okubo, T.M. Jessell, S.K. McConnell, and D.H. Rowitch for their gifts of the doublecortin, ER81, the RORβ, and the Olig-1 probes.

References
Anderson, S.A., Eisenstat, D.D., Shi, L., and Rubenstein, J.L. 1997. Interneuron migration from basal forebrain to neocortex: Dependence on DLx genes. Science 278: 474–476.

Angeline, J.B. and Sidman, R.L. 1961. Autoradiographic study of cell migration during histogenesis of the cerebral cortex in the mouse. Nature 192: 766–768.

Berger, N.E., Johnson, J.K., and Klein, R.M. 1997. Early generation of glia in the intermediate zone of the developing cerebral cortex. Brain Res. Dev. Brain Res. 101: 149–164.

The Boulder Committee. 1970. Embryonic vertebrate central nervous system: Revised terminology. Anat. Rec. 166: 257–261.

D’Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., and Curran, T. 1997. Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. J. Neurosci. 17: 23–31.

Feng, L., Hatten, M.E., and Heintz, N. 1994. Brain lipid-binding protein [BLBP]: A novel signaling system in the developing mammalian CNS. Neuron 12: 895–908.

Frantz, G.D. and McConnell, S.K. 1996. Restriction of late cortical to tangential progenitors to an upper-layer late. Neuron 17: 55–61.

He X., Treacy, M.N., Simmons, D.M., Ingraham, H.A., Swanson, L.W., and Rosenfeld, M.G. 1989. Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature 340: 35–41.

Hermans-Borgmeyer, I., Hampe, W., Chinkin, B., Mether, A., Nykjaer, A., Susen, U., Fenger, U., Herbarth, B., and Schaller, H.C. 1998. Unique expression pattern of a novel mosaic receptor in the developing cerebral cortex. Mech. Dev. 70: 65–76.

Ishiki, T., Pearson, B., Hollbrook, S., and Doh, C.Q. 2001. Drosophila neuroblastas sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell 106: 511–521.

Kurtz, A., Zimmer, A., Schnuriger, F., Bruni, G., Spener, F., and Muller, T. 1994. The expression pattern of a novel gene encoding brain-fatty acid binding protein correlates with neuronal and glial cell development. Development 120: 2637–2649.

Luo, Q.R., Yuk, D., Albert, J.A., Zhu, Z., Pawlitzky, I., Chan, J., McManon, A.P., Sules, C.D., and Rowitch, D.H. 2000. Sonic Hedgehog: Regulated olodendrocyte lineage genes encoding HhLH proteins in the mammalian central nervous system. Neuron 25: 317–329.

Luskin, M.B., Pearlman, A.L., and Sanes, J.R. 1988. Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a recombinant retrovirus. Neuron 3: 635–647.

Mathis, J.M., Simmons, D.M., He, X., Swanson, L.W., and Rosenfeld, M.G. 1992. Brain 4: A novel mammalian POU domain transcription factor exhibiting restricted brain-specific expression. EMBO J. 11: 2551–2561.

McConnell, S.K. and Kaznowski, C.E. 1991. Cell cycle dependence of laminar determination in developing neocortex. Science 254: 282–285.
Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons

Yoshinobu Sugitani, Shigeyasu Nakai, Osamu Minowa, et al.

Genes Dev. 2002, 16: 
Access the most recent version at doi:10.1101/gad.978002

Supplemental Material
http://genesdev.cshlp.org/content/suppl/2002/07/30/16.14.1760.DC1

References
This article cites 27 articles, 11 of which can be accessed free at:
http://genesdev.cshlp.org/content/16/14/1760.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.