The RNA-binding protein HuD regulates neuronal cell identity and maturation


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Neural Hu proteins (HuB/C/D) are RNA-binding proteins that have been shown to induce neuronal differentiation activity when overexpressed in immature neural progenitor cells or undifferentiated neuronal tumors. Newly generated HuD-deficient mice exhibited a transient impaired-cranial-nerve-development phenotype at an early embryonic stage. Adult HuD-deficient mice exhibited an abnormal hind limb reflex and poor rotarod performance. Analysis of neurosphere formation revealed that the number and self-renewal capacity of the neural stem/progenitor cells were increased in HuD-deficient mice. HuD-deficient primary neural spheres also generated a smaller number of neurons. Cohort analysis of the cellular proliferative activity by using BrdUrd and iododeoxuridine labeling revealed that the number of differentiating quiescent cells in the embryonic cerebral wall was decreased. Long-term administration of BrdUrd revealed that the number of slowly dividing stem cells in the adult subventricular zone was increased in the HuD-deficient mice. Taken together, the results suggest that HuB is required at multiple points during neuronal development, including negative regulation of proliferative activity and neuronal cell fate acquisition of neural stem/progenitor cells.

ELAV | Hu | neural stem cell

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u proteins have been identified as the target antigens of autoantibodies appearing in the sera of patients with paraneoplastic encephalomyelitis (1). By molecular cloning, four members of the Hu protein family have been identified as RNA-binding proteins that resemble the Drosophila ELAV protein (1–4). These mammalian Hu/ELAV proteins, with the exception of HuA (HuR), are widely expressed in both early postmitotic and mature neurons; HuA (HuR), however, is expressed ubiquitously. All members of the Hu family proteins contain three RNA-recognition motifs, and their structures are highly conserved. Previous reports have suggested the binding of Hu proteins to several putative target mRNAs, both in vitro and in vivo. Because most of the target mRNAs have AU-rich elements in their untranslated regions, Hu proteins possibly regulate the stability or translational efficiency of their target mRNAs. Most of these putative target genes are involved in cellular proliferation [p21 (5), p27 (6), c-fos, and N-Myc (7)] or are important in the formation of neurites [Neurofilament-M (8), GAP-43 (9), and tau (10, 11)].

Thus, Hu proteins have been considered to be involved in the differentiation and/or maintenance of neurons. Previously, we reported, by a gene transfer experiment conducted with the electroporation method, that overexpression of HuB/HuC induces neuronal differentiation in PC12 cells and in the periventricular immature neural stem or progenitor cells of embryonic mice (12). HuD has also been shown to have similar functions in PC12 cells (13). It has been also reported that antisense-mediated knockdown of HuC results in impaired spatial learning performance in mice, with concomitant down-regulation of GAP-43 expression (14). These findings indicate the possible involvement of the Hu proteins in the sprouting and regeneration of neurons. In this paper, we report on the phenotype of mice with targeted disruption of the HuD gene, whose expression commences even earlier than that of HuC, another member of the same family (4), and continues to be seen even in mature neurons.

Materials and Methods

Generation of HuD-Deficient mice. HuD-deficient mice were generated by a method similar to one described previously (15). Briefly, a 2.5-kb fragment containing intronic sequences upstream and a 6.1-kb fragment containing intronic sequences downstream of the second exon that contains the second RNA-recognition motifs (25–265 bp of cDNA) were inserted into the target plasmid vector to delete a 1.0-kb genomic DNA fragment and induce a frame shift. RT-PCR analysis was performed by using the primers 5’-AGAAGGGAATGCACGTTTTTTTTT-3’ (exon 1) and 5’-TGAATTCCTTGGGTCACTA-3’ (exon 2), or 5’-TATGACCAAGGAATATCA-3’ (exon 2) and 5’-TGGTCTTGAGGACCACTA-3’ (exon 3).

Immunoblotting was performed with the same method as described (12). Anti-HuD monoclonal antibody 16C12 (Clonegene, Hartford, CT) was used in 1:500 dilutions. Anti-HuB/C/D human serum (a gift from Robert Darnell, The Rockefeller University, New York) was used in 1:2,000 dilutions.

Whole-Mount Immunohistochemistry. Whole-mount immunohistochemistry of the embryonic mice was performed as described (16).

Analysis of Motor Functions. Mice of each genotype that were 20–26 weeks old were used for the analysis. Each mouse was placed on a 3.5-cm-diameter rod covered with rubber to evaluate its rotarod performance. The mice were left on the rod for 1 min for habituation. The rotarod was then rotated at 20 rpm, and the performance of the mice was measured in terms of latency (time successfully spent on the rotating rod without falling off). Six trials were conducted for each individual. Mice that stayed on the rotarod for 300 s were considered complete responders, and their latencies were recorded as 300 s.

Cell Culture and Immunochemistry. Cells from the ganglionic eminence or cerebral cortex of embryonic day (E)14.5 embryos

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Abbreviations: BrdUrd, iododeoxuridine; SVZ, subventricular zone; En, embryonic day n; P, proliferative; Q, quiescent.

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were cultured as described (17). For the differentiation assays of the neurospheres, neurospheres were plated as described (15). Subsequently, triple-label immunostaining was performed to examine their differentiation into neurons, astroglia, and oligodendroglia, as described (18).

**Cohort Analysis of Cellular Proliferation in the Embryonic Cerebral Wall.** This experimental concept was identical to one described previously (19), except that iododeoxuridine (IdUrd) was used in place of tritiated thymidine. (Also see Fig. 6, which is published as supporting information on the PNAS web site.) Proliferating cells of the embryonic cerebral wall were exposed sequentially, by i.p. injection, to the S phase marker IdUrd (50 mg/g of body weight; Sigma) and BrdUrd (50 mg/g of body weight; Sigma). Briefly, IdUrd was injected at 8:00 a.m. in both (P + Q) protocols. Then BrdUrd was injected at 10:00 a.m., 1:00 p.m., 4:00 p.m., and 7:00 p.m. in the protocol for Q. In the protocol for P + Q, BrdUrd was injected only at 10:00 a.m. Animals in both protocols were killed and fixed at 10:30 p.m. (14.5 h from initial IdUrd injection). These protocols yielded separate values for the number of Q cells [N(Q)] and P + Q cells [N(P + Q)] in 2 h (8:00–10:00 a.m.) cohort (19) (for details, see Fig. 6 legend). Immunohistochemical staining was performed on 4-μm paraffin-embedded coronal sections. Anti-BrdUrd labeling was performed as described (19). Anti-IdUrd staining was performed by using the anti-IdUrd monoclonal antibody, IU4 (Caltag, South San Francisco), which reacts with both BrdUrd and IdUrd. For visualization of the IdUrd-positive cells, Vectastain-AP and the alkaline phosphatase substrate kit (Vector Laboratories) were used. Blue cells without horseradish peroxidase signals represented the cells showing positive labeling for IdUrd but not for BrdUrd (Q cells).

**Detection of Apoptosis.** Evaluation of apoptosis was performed in E14.5 4-μm paraffin-embedded sections by using Apoptag (Inter-gen, Purchase, NY).

**Long-Term BrdUrd Labeling of Slowly Dividing Cells.** BrdUrd (1 mg/ml) was given, mixed with drinking water, to 20- to 24-week-old adult HuD−/− mice and their littermates for 4 weeks, followed by a 1-week BrdUrd free-chase period to wash out rapidly dividing cells. Four-micrometer-thick paraffin-embedded sections were immunostained as described for the embryonic sections. The number of BrdUrd-positive cells was counted in one-half of five independent sections for each frontal level.

**Statistical Analysis.** The statistical significance of differences was analyzed by Student's t test (n > 3, each group). All values were expressed as mean ± SEM. Asterisks on the error bars denote statistical significance (P < 0.05).

**Results**

**Targeted Disruption of the HuD Gene.** Targeted disruption of the HuD locus in ES cells was performed and confirmed as described in Fig. 1 A and B. Interbreeding of the heterozygous mutant (HuD+/−) mice yielded homozygous mutant (HuD−/−) pups with the expected Mendelian ratio, indicating that HuD may not be essential for embryonic viability. We confirmed the absence of HuD mRNA (Fig. 1C) in adult homozygous mouse brains by RT-PCR. To demonstrate the absence of the HuD protein in HuD−/− mice, we performed immunoblotting of brain lysates with anti-HuD-specific monoclonal antibody 16C12 (Fig. 1D Upper Left) and anti-HuB/C/D serum (Fig. 1D Lower Left). Liver lysates of wild-type mice were also characterized with these antibodies as negative controls (see Fig. 1D legend). In HuD−/− mice, the band corresponding to HuD (indicated by the double arrowhead, Fig. 1D Upper Left) was absent in the immunoblot with the anti-HuD antibody. The results of the immunoblot with anti-HuB/C/D serum are shown in Fig. 1D Lower. The level of expression of the band indicated by the single asterisk was not significantly changed in the HuD−/− brain, whereas the expression of the band indicated by the single arrowhead decreased. Taken together, these results show that expression of HuD protein is missing, and expression of HuB/C proteins is unlikely to be strongly affected in HuD−/− mice, although we cannot exclude the possibility of attenuated expression of other splice variants of HuB/HuC by this immunoblot.

**Neural Development and Motor Functions in HuD-Deficient Mice.** We first visualized and evaluated the midembryonic nervous system development of HuD−/− animals by using whole-mount immu-
nostaining of Neurofilament-M at the stage of E10.5, when HuD-positive mature neurons normally extend neurites toward the ventral neural tube (Fig. 2 A–D). We observed that the neurite extension of several cranial nerves [glossopharyngeal nerve (IX), hypoglossal nerve (XII), trigeminal nerve (V), and acousticofacial (VII/VIII) nerves] was impaired in most HuD–/– embryos (Fig. 2 A and C). However, no such developmental delay of the nervous system was observed in later-stage embryos. In E14 and E16 embryonic sections, the expression of neuroepithelial markers (Nestin or Musashi-1) in the VZ remained undisturbed (data not shown). No morphological abnormalities of the central nervous system were observed in E14 embryos (Fig. 7 A and B, which is published as supporting information on the PNAS web site), indicating that the above-mentioned impaired-cranial-nerve-development phenotype appeared only transiently. The apparent masking of the cranial-nerve-phenotype in the later-stage embryos could perhaps be explained by the sequential onset of expression of the other Hu proteins in the developing nervous system (4).

HuD–/– pups were indistinguishable from their wild-type littermates during the first several postnatal weeks, in terms of both their size and appearance. There were no significant differences between adult HuD–/– mice and their wild-type littermates with respect to the structure of the brain as evaluated by histological analysis. The structures of the cerebral cortex, cerebellum, and hippocampus were indistinguishable between HuD–/– mice and their wild-type littermates (Fig. 7 C–F). Although we could not find any significant morphological abnormalities between the two groups of mice in the adult spinal cord either, including the dorsal root ganglia (data not shown), by 4–8 weeks postnatally, 70–80% of the HuD–/– animals showed a consistently abnormal clasp reflex of the hind limbs upon being suspended by the tail, whereas age-matched wild-type animals extended their limbs normally (Fig. 2 E and F). This clasp reflex is seen in mutant mice with cortical (20) and basal ganglia defects (21) and indicates the presence of motor/sensory defects in adult HuD-deficient mice. To determine whether HuD deficiency affected the sensory and/or motor functions of the mature nervous system, we conducted rotarod analysis to evaluate motor coordination in HuD–/– adults. (Fig. 2G). Six trials were performed for each individual, and the duration for which the mice could stay on an accelerating rotarod without falling was measured. It was found that the duration for which HuD–/– mice could stay on the rotarod without falling was significantly shorter than that of their wild-type littermates. When the test was repeated, although the performance of wild-type mice improved significantly after a few trials, HuD–/– mice continued to perform very poorly even after several trials. In addition, the reproductive performance of the HuD–/– mice was noted to be poor as compared with that of their wild-type littermates.

Analysis of the Neuronal Cell Lineage in HuD–/– Embryos. We then examined the role of HuD in neuronal cell lineage development by using the neurosphere assay, which is a selective culture system for neural stem/progenitor cells (18). The ganglionic eminence or cerebral cortex from E14.5 HuD–/– embryos, as well as their littermates as controls, was dissociated into single cells and cultured in the presence of EGF and FGF type 2 to generate neurospheres. The number of neurospheres formed under this condition is believed to reflect the number of spheres forming multipotent neural stem cells within the dissociated cell population. As shown in Fig. 3A, the number of primary neurospheres formed from the HuD–/– embryonic ganglionic eminence was 1.5-fold higher in

![Fig. 2. Histological analysis of the HuD-deficient mouse embryos. (A–D) Lateral views of E10.5 embryos of HuD–/– mice (A and C) and their wild-type littermates (B and D) stained with 2H3 antineurofilament-M antibody. In the wild-type embryos, axons of the glossopharyngeal nerve (IX) and hypoglossal nerve (XII) are seen extending from the caudal hindbrain and rostral spinal cord. On the other hand, the axons of these cranial nerves are not visualized in the HuD–/– embryos. Development of other cranial nerves, including the trigeminal (V) and acousticofacial (VII/VIII) nerves, also seems to be impaired in the HuD-deficient embryos as compared with that in their wild-type littermates. (E and F) The hind-feet-clenching phenotype in adult HuD–/– mice at 24 weeks of age. The HuD–/– mice displayed hind-feet-clenching behavior when picked up by the tail from 4 to 8 weeks of age (E). In wild-type littermates, however, the angles of the hind feet were close to a right angle (F). (G) Rotarod analysis. HuD–/– and their wild-type littermates at 20–26 weeks of age were used for the analysis. In HuD–/– mice, improvement of retention time by learning could scarcely be seen. The retention time was significantly shorter in the HuD–/– mice than in the wild-type littermates in the fifth and sixth trials.](image-url)
HuD—/— mice than in their wild-type littermates. The number of primary spheres forming cells was also increased in the HuD—/— embryonic cerebral cortex. The number of secondary neurospheres generated from the subcloning of a single primary neurosphere may be considered as an estimate of the extent to which the initial primary neurosphere-forming stem cell undergoes symmetric divisions (18). The number of secondary neurosphere colonies was also significantly increased in E14.5 HuD—/— brains as compared with that in the brains of their wild-type controls (Fig. 3B), suggesting that the sphere-forming cells derived from E14.5 HuD—/— brains exhibited enhanced self-renewal capacity.

Next, we tested the multipotency of the neural stem cells by the differentiation assay; each neurosphere can be induced to differentiate into TuJ1-expressing neurons (N), GFAP-expressing astrocytes (A), and/or O4-expressing oligodendrocytes (O). After allowing differentiation to take place for 72 h, the cell sheets generated from the neurospheres were fixed and triple-immunostained by using anti-TuJ1, anti-GFAP, and anti-O4 antibody (Fig. 3C). Then, each sphere was analyzed for NAO expression. The number of spheres that generated neurons was significantly decreased to 0.8-fold in the HuD—/— embryos (Fig. 3D), whereas the number of spheres that generated oligodendrocytes and astrocytes remained unchanged (Fig. 3D). These observations indicate that the neural stem cells in E14.5 HuD—/— embryos have increased self-renewal ability and reduced ability to differentiate into neuronal progeny.

To investigate the in vivo kinetics of neurogenesis (production of postmitotic neurons) in the HuD-deficient embryonic cerebral wall, we performed sequential labeling for S phase markers by using IdUrd and BrdUrd, as described in previous reports, except that IdUrd was used in the place of tritiated thymidine (19). Using BrdUrd-only cumulative labeling, we could not observe any visible differences in the cell cycle length of the proliferative cells in the embryonic pseudostratified ventricular epithelium (PVE) between HuD—/— and wild-type mice (data not shown). Therefore, we concluded that 14.5 h of continuous BrdUrd exposure as described (22) was sufficiently long to cover the duration of the total cell cycle length (Tc); the S-phase length was then subtracted from Tc to calculate the percentage of mitotically Q cells, which represents the percentage of postmitotic neurons in this region. The distribution of Q cells and P + Q cells in the HuD—/— and wild-type embryos is shown in Fig. 4A and B. In the case of the wild-type mice, the Q cells were mainly localized at a distance of 80–100 μm from the lateral ventricular surface, which corresponds to the position at which the cells leave the cell cycle and migrate out from the PVE (19). In the HuD—/— embryos, the number of Q cells (Fig. 4B) in these regions was decreased as compared with that in the wild-type embryos. In contrast, the number of P + Q cells (Fig. 4B) was not changed. Total numbers of P and P + Q cells in intermediate zone (bin6–16) are shown in Fig. 4C. The ratio of Q cells is calculated as N(Q)/N(P + Q). This value (Q fraction) represents the ratio of cells exit from cell cycles during G1 phase. A decreased Q fraction in HuD—/— embryos suggests that the number and ratio of cells leaving the cell cycle are decreased, and that the P population in the developing cerebral wall is increased in the HuD—/— embryos and further suggests that in the absence of HuD, some of the neural stem/progenitor cells or committed neuronal progenitor cells fail to leave the cell cycle, resulting in reduced production of postmitotic neurons.

Macrocephaly has been described in several mutant mice lacking negative regulators of the cell cycle: Mice deficient in PTEN, a negative regulator of the cell cycle in neural stem cells (23), as well as those deficient in the cyclin-dependent kinase inhibitor p27(Kip1), a putative target of the Hu proteins (6, 24), show the macrocephaly phenotype in common, and these phenotypes are compatible with that predicted by theoretical simulation in the mathematical model of neocortical histogenesis (22). However, we found that the actual size of the postnatal brain was not larger in the
HuD-/- mice. This discrepancy may be accounted for by increased apoptosis within the progenitor population. To investigate this possibility, we evaluated the status of apoptosis in the HuD-/- embryonic cerebral cortex (Fig. 4D). The number of apoptotic cells, as detected by the TUNEL method, was 2.2-fold higher in the ventricular zone, but not in the intermediate zone and cortical plate, of the HuD-/- as compared with that in their wild-type littermates. These data suggest that at least some of the cells that fail to leave the cell cycle undergo apoptosis, possibly due to their inability to undergo appropriate neuronal differentiation.

The Number of Slowly Dividing Stem Cells Is Increased in the SVZ of Adult HuD-Deficient Mice. It has been reported that several neurogenic regions are present in the adult mouse brain. Slowly dividing cells present in the adult SVZ are believed to exhibit neural stem-cell activities (25). To label slowly proliferating cells in vivo, adult HuD-/- and WT mice were administered BrdUrd continuously for 4 weeks, followed by a BrdUrd-free chase period of 1 week. For quantification of the labeled cells, we subdivided the lateral ventricle into three distinct coronal levels (Fig. 8). Cells labeled with BrdUrd were visualized by immunohistochemical staining by using anti-BrdUrd antibody (Fig. 5A and B). BrdUrd-positive cells were present within the SVZ along the lateral ventricular wall. At all of the coronal levels analyzed (SVZ1, -2, and -3), the total number of BrdUrd-positive cells was 1.5- to 2.0-fold higher in the HuD-/- mice than in their wild-type littermates (Fig. 5C).

These results taken together suggest that the neural stem/progenitor cells in both fetal (embryonic periventricular and embryonic ganglionic eminence cells; Figs. 3 and 4) and adult (SVZ cells in the adult; Fig. 5) brains exhibit increased self-renewal
activity in HuD−/− mice. Disruption of the HuD gene had similar positive effects on the cellular P behavior of the neural progenitor/stem cells derived from these three regions.

Discussion

Interestingly, we found that the ratio of neuron-producing neurospheres is decreased in HuD−/− mice. This could be interpreted as suggesting that HuD is required for (i) neuronal commitment of neural stem/progenitor cells, (ii) selective survival of neuronal committed progenitor cells, and/or (iii) production of postmitotic neurons from neuronal progenitor cells. The increased self-renewal activity of neural stem/progenitor cells observed in HuD−/− mice (Fig. 3) might lend the strongest support to the first possibility above. However, this issue should be investigated further in depth, because the latter two indicate the possible roles of HuD in committed neuronal progenitor cells. A previous study characterizing the expression of Hu proteins in subependymal zone cells of the adult songbird forebrain indicates that Hu proteins begin to be expressed in the neuronal committed progenitor cells only after the S phase, during either the premitotic G2 phase of the progenitor cells or the early G0/G1 phase of the daughter cells (26). Furthermore, it was reported that HuD mRNA was expressed mainly in the intermediate and outer ventricular zones of the cortex, rather than in the cortical plate in E14.5 mice (4). In the spinal cord of stage 17 chick embryos, HuD mRNA was shown to be mainly localized in the ventral immature neuronal progeny, where the immunoreactivity of the 16A11(HuB/HuC/HuD) monoclonal antibody has not yet been seen (27). Taken together, it might be feasible to suggest that HuD commences its expression in proliferative neuronal progenitor cells and drives them to exit the mitotic cycle. We also found that the percentage of spheres that generate oligodendrocytes and astrocytes was not significantly changed in HuD−/− mice, indicating that HuD may not be involved in glial cell fate determination, and that its roles may be restricted to only the neuronal committed lineage.

The transcripts of p27 and p21, both cyclin-dependent kinase (CDK) inhibitors, have been reported as the binding targets of the Hu proteins. They are known to be involved in the acquisition of postmitotic phenotypes in the cells of neuronal lineages. Although overexpression of the Hu proteins in SH-SY5Y or PC12 cells induced the expression of CDK inhibitor proteins and growth arrest (W-A. and H.O., unpublished results), we could not detect any difference in the expression pattern between the levels of the CDK inhibitors between HuD−/− mice and their wild-type littermates. It should be further investigated whether they are regulated by the Hu proteins and induce cell cycle exit of neuronal progenitors in vivo. Although HuD seems to be down-regulated in postmitotic mature neurons, it might still be required for the acquisition of the fully differentiated characteristics and various functions of mature neurons, including neurite extension and expression of neuronal markers. The mRNA of several neuronal markers, including tau, GAP-43, and Neuroserpin, which are involved in the structure (e.g., neurite extension) and function of neurons, has also been reported to bind between their mRNA and Hu proteins (9, 10, 28).

In this HuD−/− model, we examined the expression of GAP-43 and p27 protein by immunoblotting (Fig. 9, which is published as supporting information on the PNAS web site). We also examined the expression of tau mRNA by using real-time quantitative PCR in E17 brain (data not shown). Although we could not detect any significant difference in the expression of these molecules between HuD+/+ and HuD−/− mice, our observations do not directly deny the previous results of the study on GAP-43, p27, and tau. Even if not up-regulated, other Hu protein might have compensated the loss of HuD because of the functional redundancy of Hu-family proteins. Impaired cranial nerve development phenotype in E10.5 embryos (Fig. 2 A–D), impaired rotator performance (Fig. 2G), and abnormal claspers reflex in the HuD−/− mice might lend support to such possibilities.

Conclusion

Based on the present loss-of-function studies, we suggest that HuD may be involved at multiple stages during neuronal development: (i) HuD negatively regulates the proliferation of neural stem/progenitor cells, (ii) HuD promotes the exit of neuronal committed progenitor cells from the cell cycle, and (iii) HuD promotes the differentiation of postmitotic neurons. It is of note that although the time of onset of the expression is, to some extent at least, specific for each of the HuB/C/D proteins (4), members of the Hu-family proteins showed marked similarities in their primary structures, expression profiles, and functions, as revealed by their misexpression studies. Thus, partially redundant functions of the different Hu proteins might mask the actual phenotype of the HuD-deficient mice in a developmental-stage-dependent manner (Fig. 2). Because our HuD-deficient mice were generated by an irremovable PGK-neocassette, and we cannot completely exclude the altered expression of neighbor
good genes (29), future studies of multiple and conditional targeted disruptions using Cre-loxP system with a reversible neocassette may reveal the in vivo functions of Hu proteins in greater detail.

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