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Expression and distribution of NBCn2 (Slc4a10) splice variants in mouse brain: cloning of novel variant NBCn2-D

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Abstract

The *SLC4A10* gene, which is highly expressed in the mammalian brain, contains two known alternative splicing units, inserts A and B, and is theoretically capable of producing four NBCn2 splice variants: NBCn2-A, -B, -C, and -D. By immunoprecipitation and western blotting, a previous study showed the putative NBCn2-D to be expressed predominantly in the subcortex (SCX) and medulla (MD) of mouse brain. However, no evidence has been provided, in any species, for the existence of a full-length transcript encoding NBCn2-D. In the present study, we report for the first time the cloning of the full-length cDNAs encoding NBCn2-D from mouse SCX and MD. Based on the frequency of bacterial colonies obtained after PCR, we conclude that in SCX, the NBCn2-A transcript is the dominant transcript, whereas in MD, NBCn2-B is dominant. NBCn2-D is the least abundant transcript in each of these two brain regions. An analysis based upon the present PCR data as well as the previous immunoprecipitation/western-blot data suggests the following prevalence of NBCn2 variants in total mouse brain: NBCn2-A (~83%), NBCn2-B (~10%), NBCn2-C (~5%), and NBCn2-D (~2%). We also estimate the prevalence of each variant in each of five brain regions (i.e., cerebral cortex, SCX, cerebellum, hippocampus, and MD). We hypothesize that the expression of different NBCn2 splice variants is characteristic of specific tissue/cells.

Keywords

bicarbonate transporter; alternative splicing; NCBE; central nervous system

1. Introduction

The regulation of intracellular pH (pH_i) as well as the pH of the extracellular fluid is vital for the normal physiology of the central nervous system. The Na-coupled HCO₃⁻ transporters

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(NCBTs) of solute carrier 4 (SLC4) family are widely expressed in the central nervous system, and play important roles in the regulation of pH_i of neurons (Jacobs et al., 2008; Schwiening and Boron, 1994) and astrocytes (Bevensee et al., 1997), as well as in the formation of cerebrospinal fluid (Jacobs et al., 2008; Praetorius et al., 2004; Praetorius and Nielsen, 2006). In mammals, five NCBTs have been identified (for review, see Boron et al., 2009), including the two electrogenic Na/HCO_3 cotransporters NBCe1 (SLC4A4, see Romero et al., 1997) and NBCe2 (SLC4A5, see Sassani et al., 2002; Virkki et al., 2002), the two electroneutral Na/HCO_3 cotransporters NBCn1 (SLC4A7, see Choi et al., 2000; Pushkin et al., 1999) and NBCn2 (SLC4A10, aka NCBE, see Parker et al., 2008b; Wang et al., 2000), and the electroneutral Na -driven Cl/HCO_3 exchanger NDCBE (SLC4A8, see Grichtchenko et al., 2001).

First cloned from an insulinoma cell line, NBCn2 was initially characterized as a Na^+ -driven Cl/HCO_3 exchanger and thus designated as NCBE (Wang et al., 2000). However, based on measurements of surface $[\text{Cl}^-]$ transients and ^{36}Cl fluxes on *Xenopus* oocytes expressing SLC4A10, Parker et al (Parker et al., 2008b) provided evidence that human SLC4A10 normally performs electroneutral Na/HCO_3 cotransport with Cl/Cl self-exchange. Nevertheless, Parker did observe Na -driven Cl/HCO_3 exchange activity with SLC4A10, but only under the non-physiological condition in which extracellular Cl^- is absent. Thus Parker et al (Parker et al., 2008b) proposed that SLC4A10 is an electroneutral Na/HCO_3 cotransporter, NBCn2.

By northern blotting (Parker et al., 2008b; Wang et al., 2000) or RT-PCR analysis (Damkier and Praetorius, 2007), investigators have demonstrated that NBCn2 transcripts are predominantly expressed in the brain. In the choroid plexus, NBCn2 is expressed in the basolateral membrane of epithelial cells (Chen et al., 2008; Jacobs et al., 2008; Liu et al., 2010; Praetorius et al., 2004), consistent with the hypothesis that NBCn2 is involved in the formation of cerebrospinal fluid, which contains high concentration of HCO_3^- . NBCn2 protein is detected both in cultured and freshly dissociated hippocampal neurons (Chen et al., 2008), as well as in sections of many brain regions (Jacobs et al., 2008; Liu et al., 2010). Interestingly, Liu et al demonstrated that NBCn2 is expressed in specific neuron populations in specific medullary nuclei (Liu et al., 2010).

The very high level expression of NBCn2 in brain suggests that the transporter plays critical roles in the central nervous system. Indeed, in humans, disruption of *SLC4A10* gene by genomic translocation is associated with partial epilepsy, mental retardation, and cognitive impairment (Gurnett et al., 2008). A deletion mutation in *SLC4A10* gene was detected in a pair of identical twins concordant for autism-spectrum disorders (Sebat et al., 2007). Moreover, in mice, knocking out *slc4a10* results in a substantial decrease in the size of brain ventricles and an increase in epilepsy threshold (Jacobs et al., 2008).

Like the genes encoding other NCBTs, the mammalian *SLC4A10* gene is capable of producing multiple splice variants. In the mammals so far examined, the *SLC4A10* gene contains 27 known exons for the coding region of NBCn2 (Fig. 1A). In transcripts of *SLC4A10*, two major alternative splicing units have been identified, inserts A and B. Insert A corresponds to the entire exon 8, which is 90 nucleotides (nt) in length, whereas insert B corresponds to the entire exon 26, which is 39-nt in length. Insert B contains a stop codon, resulting in a short cytosolic carboxy terminus (Ct) in variants containing this insert, and a long Ct—which contains a PDZ-binding motif—in variants lacking the insert. In addition to inserts A and B, a minor variation (i.e., omission of Ala^{256}) has been reported in some NBCn2 variants (Giffard et al., 2003), see Fig. 1B).

In theory, based upon the presence/absence of these two major alternative splicing units, the mammalian *SLC4A10* gene is capable of producing four splice variants: NBCn2-A, -B, -C, and -D (Fig. 1B). The NBCn2-A was first cloned from mouse insulinoma (Wang et al., 2000). Choi et al cloned the human ortholog of the mouse NBCn2-A from brain and kidney, and named the human clone NCBE-A (Choi et al., 2002). They also cloned NBCn2-B (named NCBE-B) from the same organs (Choi et al., 2002). In 2003, Giffard et al cloned NBCn2-B (rb1NCBE) and NBCn2-C (rb2NCBE) from rat brain (Giffard et al., 2003). Although a partial NBCn2-D-like cDNA—with insert A but no insert B—from rat brain has been cloned (Giffard et al., 2003), the full-length cloning of the fourth variant, NBCn2-D, had never been reported.

In a previous study, Liu et al (Liu et al., 2010) developed three polyclonal antibodies: (1) anti-ABCD directed against the extreme N-terminus and recognizing all four known, potential NBCn2 variants; (2) anti-BD directed against insert A and recognizing NBCn2-B/D; and (3) anti-CD directed against the long Ct, thus recognizing NBCn2-C/D. Using these three antibodies with a combination of immunoprecipitation and western blotting, Liu et al demonstrated the presence in mouse brain of a protein that contains insert A but not insert B—presumably NBCn2-D—though they did not clone the full-length cDNA. Moreover, Liu et al demonstrated that the putative NBCn2-D is predominantly expressed in subcortex (SCX) and medulla (MD). Here we report the first cloning—from mouse SCX and MD—of the full-length cDNA encoding NBCn2-D. In addition, using a combination of (a) the new data on the frequency distribution of NBCn2 PCR products from SCX and MD and (b) the earlier immunoprecipitation/western-blot data (Liu et al., 2010) on tissue from five mouse brain regions, we estimate the prevalence of each of the four NBCn2 variants in each brain region.

2. Results

As noted in the Introduction, Liu et al had previously demonstrated that the expression of a fourth NBCn2 splice variant, presumably NBCn2-D, and showed that, in mouse brain, putative NBCn2-D protein is predominantly expressed in SCX and MD (Liu et al., 2010). We therefore chose these two mouse brain regions as the tissue source in our attempt to clone NBCn2-D cDNA. By nested RT-PCR, we obtained DNA products of ~3.5 kb from MD (Fig. 2A) and SCX (Fig. 2B). The PCR products were restriction-digested and subcloned into the pGH19 vector. We performed colony PCR with the primers flanking insert A to examine the presence vs the absence of insert A of an NBCn2 clone. Similarly, we performed colony PCR with the primers flanking insert B to examine the presence vs the absence of insert B of an NBCn2 clone. The upper panel of Fig. 2C shows a representative detection pattern of insert A for four colonies that represent all four NBCn2 variants. The lower panel of Fig. 2C shows a representative detection pattern of insert B for the same four colonies. Note that insert A was present only in colonies representing NBCn2-B and NBCn2-D, whereas insert B was present only in colonies representing NBCn2-A and NBCn2-B. The four NBCn2 variants in Fig. 2C were finally verified by full-length cDNA sequencing.

Fig. 3A summarizes the distribution of the four NBCn2 variants—as determined using primers flanking inserts A and B—identified among 34 consecutive clones from SCX and 44 consecutive clones from MD¹. We identified all four insert-A/B combinations—presumably representing four NBCn2 variants (i.e., NBCn2-A, -B, -C, and -D)—in both brain regions. Note that even though western blotting previously showed that NBCn2-D is most abundant in SCX and MD vs other regions of mouse brain (Liu et al., 2010), the

¹In making the assignments NBCn2-A, -B, -C, and -D, we are assuming that these are the only four possible NBCn2 variants.

abundance of the NBCn2-D transcript is much lower than that of the other variants even in SCX (1 out of 34 total NBCn2 colonies) and MD (4 out of 44 total NBCn2 colonies).

As shown graphically in Fig. 3B, the most abundant NBCn2 transcript in SCX of mouse brain appears to be NBCn2-A, which accounts for 76% ($23/33 \cong 76\%$) of all cDNAs. NBCn2-B is less abundant, accounting for 15% ($5/33 \cong 15\%$). NBCn2-C accounts for 6% ($2/33 \cong 6\%$), and NBCn2-D accounts for only 3% ($1/33 \cong 3\%$).

In MD of mouse brain (Fig. 3C), NBCn2-B appears to be the dominant NBCn2 transcript, accounting for 52% ($23/44 \cong 52\%$). NBCn2-A and -C are less abundant, accounting for 20% ($9/44 \cong 20\%$) and 18% ($8/44 \cong 18\%$), respectively. NBCn2-D accounts for only 9% ($4/44 \cong 9\%$).

As mentioned in the Introduction, besides inserts A and B, a third minor variation exists in *SLC4A10* gene transcripts: the omission of Ala²⁵⁶ (for review, see Boron et al., 2009). This variation arises from alternative splicing utilizing a cryptic splicing-donor site three nucleotides downstream from the 5'-end of exon 7 (Fig. 4). In the present study, in addition to the normal NBCn2-C with Ala²⁵⁶ (2 clones with full-length sequences), we also identified—for the first time the mouse—NBCn2-C', which is identical to NBCn2-C but lacking Ala²⁵⁶ (1 clone with a full-length sequence). Moreover, we identified—for the first time in any species—NBCn2-D with Ala²⁵⁶ (1 clone with a full-length sequence) and NBCn2-D' (2 clones with full-length sequences), which is identical to NBCn2-D but lacking Ala²⁵⁶. A search in Genbank shows that the omission of Ala²⁵⁶ also occurs in mouse NBCn2-A, as well as in rat NBCn2-B and -C (see legend of Fig. 4). Transcripts lacking this Ala have yet to be reported in humans.

3. Discussion

3.1. Estimation of the fractional distribution of NBCn2 splice variants in mouse-brain regions

In the previous paper—working with the polyclonal antibodies anti-ABCD (recognizing all four NBCn2 variants), anti-BD (recognizing NBCn2-B/D), and anti-CD (recognizing NBCn2-C/D), Liu et al studied the distribution of NBCn2 variants in five mouse brain regions: cerebral cortex (CX), SCX, cerebellum (CB), hippocampus (HC), and MD, the combination of all which represents the entire brain. (Liu et al., 2010). A combination of western blotting and immunoprecipitation followed by western blotting yielded the relative protein distribution data that we replot here as Fig. 5A-D. For example, they found that the presumable NBCn2-D is predominantly expressed in SCX and MD (Fig. 5D). However, Fig. 5D reports the fraction of total NBCn2-D protein that is present in each brain region (i.e., the sum of each group of 5 bars is 100%). The purpose of the present analysis—the starting point for which is Fig. 5—is to arrive at an estimate of the relative protein distribution for each NBCn2 variant in each brain region, relative to total-brain NBCn2 protein (i.e., NBCn2-A + NBCn2-B + NBCn2-C + NBCn2-D).

Estimation of fractional distribution of NBCn2 variants in mouse medulla—

Note that, in the medulla, the sum of all NBCn2 splice variants accounts for ~6% of the total NBCn2 protein expressed in mouse brain (Fig. 5A, bar “MD”). Based on our cloning data summarized in the lower row of Fig. 3A and in Fig. 3C, we know that NBCn2-A represents 9 of 44 consecutive cDNA clones or ~20% of all NBCn2 clones from the medulla. If we assume that—for each NBCn2 variant in mouse medulla—the fraction of total colonies is proportional to the relative level of transcript, and that this is proportional to the relative level of protein, then we can estimate that NBCn2-A in medulla accounts for ~1.2% ($6\% \times 20\% = 1.2\%$) of the total NBCn2 protein in mouse brain. Similarly, we can estimate that

medullary NBCn2-B accounts for ~3.1% ($6\% \times 52\% = 3.1\%$), medullary NBCn2-C accounts for ~1.1% ($6\% \times 18\% = 1.1\%$), and medullary NBCn2-D accounts for ~0.5% ($6\% \times 9\% = 0.5\%$) of the total NBCn2 protein in mouse brain.

Estimation of fractional distribution of NBCn2-B/D and -C/D in mouse brain—

Summing up the estimated amounts of medullary NBCn2-B (3.1%) and NBCn2-D (0.5%), we estimate that the medullary NBCn2-B/D antibody signal corresponds to ~3.6% of the total NBCn2 brain protein. If we rescale the NBCn2-B/D in Fig. 5B so that the bar “MD” has a value of 3.6%, then each new bar—shown in Fig. 6A—represents the fraction of NBCn2-B/D in total brain NBCn2 protein. We can apply the same approach to Fig. 5C, where the medullary NBCn2-C/D accounts for ~1.6% ($1.1\% + 0.5\% = 1.6\%$) of the total-brain NBCn2 proteins. After we rescale Fig. 5C so that the “MD” bar has a value of 1.6%, each new bar in Fig. 6B represents the fraction of NBCn2-C/D in the total-brain NBCn2 protein.

Estimation of fractional distribution of NBCn2-D in mouse brain—As noted above, NBCn2-D in the medulla accounts ~0.5% of total NBCn2 protein expressed in mouse brain. If we rescale Fig. 5D so that the “MD” bar has value of 0.5%, then each rescaled bar represents NBCn2-D as a percentage of total-brain NBCn2 (Fig. 7A). Integrated over all five regions (i.e., the 5 bars in Fig. 7A), NBCn2-D accounts for ~1.9% of the total-brain NBCn2 protein, and is thus the least abundant NBCn2 variant in mouse brain.

Estimation of fractional distribution of NBCn2-B in mouse brain—If we subtract each bar in Fig. 7A (percentage of total-brain NBCn2 represented by NBCn2-D) from the corresponding bar in Fig. 6A (percentage of total brain NBCn2 represented by NBCn2-B + NBCn2-D), we obtain estimates of the percentage of total-brain NBCn2 represented by NBCn2-B (Fig. 7B). Integrated over the five brain regions, NBCn2-B accounts for ~10.3% of total-brain NBCn2 protein—the second most abundant NBCn2 variant in mouse brain.

Estimation of fractional distribution of NBCn2-C in mouse brain—Following the same approach as in the previous paragraph, if we subtract each bar in Fig. 7A (percentage of total brain NBCn2 represented by NBCn2-D) from the corresponding bar in Fig. 6B (percentage of total brain NBCn2 represented by NBCn2-B + NBCn2-D), we obtain estimates of the percentage of total-brain NBCn2 represented by NBCn2-C (Fig. 7C). Summed over all five regions, NBCn2-C accounts for ~4.6% of total-brain NBCn2—the second least abundant NBCn2 variant in mouse brain.

Estimation of fractional distribution of NBCn2-A in mouse brain—Finally, if we subtract the sum of the corresponding bars in Fig. 7A, B, and C (percentage of total-brain NBCn2 represented by NBCn2-D + -B + -C) from the corresponding bar in Fig. 5A (total brain NBCn2), we obtain estimates of the percentage of total-brain NBCn2 represented by NBCn2-A (Fig. 7D). We see that NBCn2-A is by far the dominant variant in every brain region other than medulla (where NBCn2-B is dominant), and accounts for ~83.4% of total NBCn2 expression in mouse brain.

Fig. 8A summarizes the fractional distribution of each NBCn2 variant, relative to total-brain NBCn2 protein. The four bars, which sum to 100%, show that global abundance of NBCn2-A in brain is four-fold greater than all other variants combined.

In Fig. 8B, the hatched bars are a re-plot of the clone data in Fig. 3B. The stippled bars are a re-plot of the SCX data in Fig. 7A-D. The close agreement between hatched and stippled bars indicates that our analysis is internally consistent. The extremely low relative abundance of NBCn2-D could explain why this variant has never been previously cloned.

Of course, the accuracy of the foregoing analysis depends on a combination of cloning data (which reflects the relative abundance of mRNA of different NBCn2 variants), western-blotting/immunoprecipitation data (which represents the relative abundance of proteins of different NBCn2 variants), and the assumption that the two are approximately proportional to one another for the four NBCn2 variants. These assumptions are probably not too unreasonable inasmuch as (a) the open reading frames are of similar length for all variants (minimizing the bias of PCR), and (b) all variants have the same 5'- and 3'-UTRs (minimizing variability in the initiation of transcription and transcript stability). Although only semiquantitative, our approach may be the most reliable option for estimating the distribution for NBCn2 variants. It would be almost impossible to develop an antibody capable of specifically recognizing a particular NBCn2 variant. Moreover, even if we had a series of four variant-specific antibodies, it would be difficult to compare their signals quantitatively.

3.2. Physiological implications

As mentioned in the Introduction, the transcripts of both NBCn2-A and -C omit exon 8 (i.e., insert A; see Fig. 1A), resulting in a protein 30 residues shorter than NBCn2-B and -D (see Fig. 1B). It appears that the mouse brain preferentially splices exon 8 out of *slc4a10* transcripts, inasmuch as our estimate indicates that exon 8 is absent in 83.4% (NBCn2-A) + 4.6% (NBCn2-C) $\cong$ 88% of NBCn2 protein. Conversely, we suggest that the mouse brain preferentially splices exon 26 into *slc4a10* transcripts, inasmuch as exon 26 appears to be present in 83.4% (NBCn2-A) + 10.3% (NBCn2-B) $\cong$ 94% of NBCn2 protein. Thus, the rarest NBCn2 splice variant in mouse brain appears to be NBCn2-D, which results from two low-probability events: simultaneously splicing-in exon 8 and splicing-out exon 26.

The likely physiological consequences of the alternative splicing of NBCn2 transcripts are differences in: (1) functional activity and (2) interaction with binding partners. Regarding the functional activity of NCBT variants, a well studied example is rat NBCe1, the product of *slc4a4*. NBCe1 has three reported variants (for review, see Boron et al., 2009): NBCe1-A, -B, and -C. NBCe1-A has a unique short Nt (41 aa), whereas NBCe1-B/C has a unique long Nt (85 aa). The unique short Nt of NBCe1-A has stimulatory effect on the activity of NBCe1, whereas the long Nt of NBCe1-B/C is inhibitory (McAlear et al., 2006). Truncation of the unique Nt portion of NBCe1-A substantially lowers the activity of NBCe1-A. Conversely, truncation of unique portion Nt portion of NBCe1-C greatly enhances the transporter activity (McAlear et al., 2006). The unique portion of the long Nt of NBCe1-B/C contains a binding site for IRBIT, an IP_3 receptor binding protein released with inositol 1,4,5-trisphosphate (IP_3); the binding of IRBIT strongly increases the activity of NBCe1-B expressed in *Xenopus* oocytes (Shirakabe et al., 2006).

A second example of diverse functional activity among splice variants is human NDCBE, the produce of *SLC4A8* (Parker et al., 2008a). Here, the alternative splicing of exon 22 can yield either a relatively long cytoplasmic Ct (NDCBE-A, terminating in 66 unique residues) or a relatively short one (NDCBE-B, terminating in 17 unique residues). The unique 17 residues of NDCBE-B contains an element that reduces functional expression in oocytes. In the case of NBCn2, it will be instructive to see whether the 30-aa insert encoded by insert A (exon 8) or the alternative Ct defined by insert B (exon 26) also have functional consequences.

As far as binding partners are concerned, the Ct end of both NBCn2-C and -D contains a PDZ-binding motif "ETCL", whereas the short Ct of NBCn2-A and -B does not. Rat NBCn2-C (rb2NCBE) has a greater functional expression than rat NBCn2-B (rb1NCBE) in transfected mouse fibroblast 3T3 cells (Giffard et al., 2003). Lee et al demonstrated that NBCn2-C (rb2NCBE) interacts with ezrin binding protein 50 (EBP50, see Lee et al., 2006),

a PDZ adaptor protein that mediates the interaction between membrane proteins and the cytoskeleton (for review, see Fehon et al., 2010). Moreover, the authors found that disruption of the cytoskeleton by cytochalasin B substantially reduced the activity of NBCn2-C in 3T3 cells (Lee et al., 2006). Taken together, these observations suggest that the cytoskeleton plays critical roles in regulation of NBCn2-C activity mediated via PDZ-domain scaffold proteins.

Lee et al also found that NBCn2-C activity is increased by inhibiting protein kinase A (PKA), and decreased by activating PKA with either forskolin or 8-Br-cAMP (Lee et al., 2006), suggesting that PKA downregulates NBCn2-C activity. In the renal proximal tubule, the PKA-mediated down-regulation of NHE3 activity requires EBP50 (for review, see Weinman et al., 2005), which forms complex with PKA (for review, see Cornez and Tasken, 2010). Thus, it is possible that the PDZ-binding motif of NBCn2-C and possibly -D (but not -A or -B) interacts with EBP50, which in turn binds PKA, thereby reducing NBCn2 activity.

We have already noted that the mouse brain is heavily biased toward including exon 26, and thus eliminating the PDZ-binding motif from the vast majority of NBCn2 proteins (i.e., NBCn2-A and NBCn2-B). Thus, it is likely that only a small minority of NBCn2 proteins (i.e., NBCn2-C and NBCn2-D) in mouse brain is subject to regulation by the PKA-PDZ-domain-scaffold protein pathway. It will be interesting to see if the expression of different NBCn2 splice variants is characteristic of particular cell types.

3.3. Summary and conclusion

In the present study, we report the first cloning of NBCn2-C cDNA from mouse, and the first cloning of NBCn2-D cDNA from any species. We estimate the relative abundance of each NBCn2 variant in five brain regions, concluding that—integrating over the whole mouse brain—NBCn2-A is by far the most prevalent variant (>80%), whereas NBCn2-D is the most rare (~2%). Thus, our data provide a valuable starting point for understanding the physiological roles of NBCn2 variants in the brain. It would be valuable to know whether specific NBCn2 variants are associated with specific cell types.

4. Experimental procedures

4.1. Tissue collection and RNA preparation

C57BL/6J mice were purchased from the Animal Center at Wuhan University (Wuhan, Hubei Province, China). All procedures involving the care and usage of mice have been approved by the Committee on Research Animal Care at Huazhong University of Science and Technology. We dissected the brain tissue from mice 2-3 months old as previously described (Liu et al., 2010), and isolated the subcortex (i.e., basal ganglia, diencephalon, and midbrain) and medulla (pons and medulla oblongata). The tissues were immediately frozen in liquid nitrogen, and stored at -80°C until used for RNA preparation.

Total RNA was prepared from mouse tissues with TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Briefly, ~200 mg mouse tissue was placed in 2 ml of TRIzol[®] reagent and homogenized in a glass homogenizer. The homogenate was centrifuged at $12,000\text{ g} \times 5\text{ min}$ at 4°C . The aqueous phase was extracted with chloroform, and then centrifuged at $12,000\text{ g} \times 15\text{ min}$ at 4°C . The RNA was precipitated with isopropyl alcohol, collected by centrifugation at $7,500\text{ g} \times 5\text{ min}$ at 4°C , washed with 75% ethanol, and stored at -80°C until use.

cDNA was synthesized with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) according to manufacturer's instructions.

4.2. Cloning of full-length NBCn2 cDNA and identification of NBCn2 variants

We performed nested RT-PCR for cloning of full-length cDNA encoding NBCn2 from total RNA of mouse brain. We performed the first round RT-PCR with gene-specific forward/reverse primers GSP-n2-F1 (5'-AATACTAAGCAGAGCGAGTGCCCG-3') and GSP-n2-R1 (5'-GAGGCTGAACACAGAAATAGAATGAAGGCTTGC-3'), and second round PCR with primers GSP-n2-F2 (5'-actactcccgGGCTGAGTGGAAGACACTGAAGACACTGC-3') and GSP-n2-R2 (5'-actactgcggccgcGTATGAAGGTGGGATGGGAGAGAGGG-3'). The PCR reactions (cycling parameters: 10 sec at 98 °C, 5 sec at 60 °C, 255 sec at 72 °C for 30 cycles) for amplifying the full-length NBCn2 were performed with PrimeSTAR™ HS DNA polymerase (Cat# DR010S, TaKaRa Biotechnology Co., Ltd., Dalian, China). For convenience in cloning the PCR products into vectors, we introduced an XmaI site into GSP-n2-F2 and a NotI into GSP-n2-R2.

Both the PCR product and the *Xenopus* vector pGH19 were double digested with XmaI and NotI. The two DNA fragments were ligated and the constructs were transformed into *E. coli*. A single bacterial colony was chosen and PCR reactions—using Taq DNA polymerase (Cat# ET101, TIANGEN Biotech Co., Ltd., Beijing, China)—were performed to examine the presence/absence of inserts A and B, which allowed us to identify the NBCn2 variant. The presence or absence of insert A was determined by PCR with primers GSP-n2-FA (5'-TGAGTTGAGAAGCTGCATCC-3') and GSP-n2-RA (5'-ACTCCAGTTCTCCTACCAAG-3'). Similarly, the presence or absence of insert B was determined by PCR with primers GSP-n2-FB (5'-CAGTACAACCTCCCACTGGAG-3') and GSP-n2-RB (5'-CATAGACAAGTCTCCCTGTC-3').

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DISCLOSURES

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Fig. 1. Structure of mouse *slc4a10* gene and diagram of NBCn2 splice variants

A, Structure of mouse *slc4a10* gene. The mouse *slc4a10* gene contains 27 exons in the coding regions. Both exon 8 (Insert A or InsA) and exon 26 (Insert B or InsB) can be alternatively spliced-in or -out. Exon 13 (indicated by arrow) is partly in the putative Nt and partly in the putative transmembrane domain TMD of the protein. Exon 23 (indicated by arrow) is partly in the putative TMD and partly in the putative Ct of the protein. **B**, Structural diagram of NBCn2 splice variants. Both NBCn2-C and -D contain a PDZ-binding motif at the end of Ct. In addition to the two major variations represented by the presence/absence of insert A and the short/long Ct (determined by the presence/absence of insert B), a third minor variation is the deletion of Ala²⁵⁶ (indicated by arrow) in the putative Nt. The omission of Ala²⁵⁶ occurs in all four NBCn2 variants of various species. The numbers 3 and 21 indicate the number of unique amino-acid residues in the extreme Ct of NBCn2-A/B and NBCn2-C/D.

Fig. 2. Amplification of full-length cDNA encoding mouse NBCn2

A, Cloning of full-length NBCn2 cDNA from subcortex (SCX). B, Cloning of full-length NBCn2 cDNA from medulla (MD). C, Identification of NBCn2 variants by colony PCR. The bands at ~3.5 kb in panel A and B were excised from the gel, DNA was recovered, double-digested, subcloned into vector pGH19, and transformed into bacteria. Single colonies were selected for PCR to examine the presence/absence of inserts A and B (i.e., to identify the NBCn2 variant). A band of 518bp was obtained in the presence of insert A, whereas a band of 428bp was obtained in the absence of insert A. A band of 253bp was obtained in the presence of insert B, whereas a band of 214bp was obtained in the absence of insert B.

A Distribution of colony numbers from specific brain regions

	NBCn2-A	NBCn2-B	NBCn2-C	NBCn2-D	Total
SCX	25	6	2	1	34
MD	9	23	8	4	44

Fig. 3. Distribution of colony numbers of NBCn2 variants in subcortex (SCX) and medulla (MD)
 A, Number of colonies of each NBCn2 variant identified from SCX and MD using approaches like that shown in Fig. 2C. B, Fractional distribution of NBCn2 variants in SCX. The sum of all bars is 100%. C, Fractional distribution of NBCn2 variants in MD.

Fig. 4. Alternative splicing of exon 7

The open triangles indicate the boundaries of intron 6-7, whereas the solid triangle indicates the cryptic splicing donor site of exon 7. Splicing to exon 7 utilizing this cryptic splicing site results in deletion of Ala²⁵⁶ in the gene products. Exclusion of Ala²⁵⁶ has been identified in all four NBCn2 variants, including mouse NBCn2-A (accession# AAH39226.1), rat NBCn2-B (accession# AAO59640.1 and NP_835193.1), rat NBCn2-C (accession# AAO59639.1), our novel mouse NBCn2-C (accession# HQ831517), and our novel NBCn2-D (ADT62122.1). Ala²⁵⁶ may also be present in all four variants, including human NBCn2-A (accession# NP_071341.2), human NBCn2-B (accession# NP_001171486.1), mouse NBCn2-A (accession# BAB17922.1), our novel mouse NBCn2-C (accession# ADJ94893.1) and novel mouse NBCn2-D (accession# HQ825316).

Fig. 5. Regional distribution of NBCn2 variants

A, NBCn2-A/B/C/D. B, NBCn2-B/D. C, NBCn2-C/D. D, NBCn2-D. In each case, we show the percent of the total indicated NBCn2 variants in the cortex (CX), subcortex (SCX), cerebellum (CB), hippocampus (HC), and medulla (MD) of mouse brain. The bar graphs are a re-plot of data from a previous paper (Liu et al., 2010). Within each panel, the five bars sum to 100%.

Fig. 6. Estimate of fraction of NBCn2-B/D or NBCn2-C/D in total brain NBCn2 protein

From the frequency distribution of colonies for each NBCn2 variant in medulla (Fig. 3C) and the fraction of total NBCn2 in MD (6% in Fig. 5A), we estimate that NBCn2-B/D in MD accounts for 3.6% of the total NBCn2 expressed in mouse brain. Because of rounding errors, the actual figure is nearly 3.7%. We created panel A by rescaling Fig. 5B so that the bar “MD” has a value of 3.6%. Thus the pattern in panel A is an estimate of the regional fraction of NBCn2-B/D in the total NBCn2 expressed in mouse brain. Using a similar approach, we created panel B by rescaling Fig. 5C so that the bar “MD” has a value of 1.6%. The sum of the 5 bars in panel A is the fraction of total brain NBCn2 that is either NBCn2-B or -D. Similarly, the sum of the 5 bars in panel B is the fraction of total brain NBCn2 that is either NBCn2-C or -D.

Fig. 7. Estimate of regional fraction of each NBCn2 variant in total brain NBCn2

A, NBCn2-D. B, NBCn2-B. C, NBCn2-C. D, NBCn2-A. We created panel A by rescaling Fig. 5D so that the bar “MD” has a value of 0.5%, the fraction of total-brain NBCn2 that is NBCn2-D from the MD. We created panel B by subtracting each bar in Fig. 7A from the corresponding bar in Fig. 6A. Using a similar approach, we created panel C by subtracting each bar in Fig. 7A from the corresponding bar in Fig. 6B. Finally, we created panel D by subtracting the sum of the bars of each region in Fig. 7A-C from the corresponding bar in Fig. 5A. The 20 bars in panels A-D sum to 100%.

Fig. 8. Estimate of fraction of each NBCn2 variant in total brain or just the subcortex
 A, Total brain. The bar for “NBCn2-A” is the sum of all five bars in Fig. 7D, and represents the fraction of total-brain NBCn2 that is NBCn2-A. Similarly, the bars for “NBCn2-B”, “-C”, and “-D” are the sum of all five bars in Fig. 7B, Fig. 7C, and Fig. 7A, respectively. The four bars in panel A sum to 100%. B, Subcortex. The hatched bars of each pair are a re-plot of Fig. 3B, and represent the distribution of colony numbers. We created the stippled bars by transforming each “SCX” bar in Fig. 7A–D into a percent of the sum of the four “SCX” bars. Thus, the four bars in panel B also sum to 100%. The two bar patterns are well-matched.