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Research report

Neuronatin mRNA: alternatively spliced forms of a novel brain-specific mammalian developmental gene *

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Abstract

Neurogenesis begins with the closure of the neural tube around mid gestation and continues in the rat for about two weeks postnatally. Therefore, we investigated the role of neuronatin, a novel cDNA that we cloned from neonatal rat brain (Joseph et al., *Boichem. Biophys. Res. Commun.,* 201 (1994) 1227-1234), in brain development. Further studies described in the present manuscript, lead to the identification of two alternatively spliced forms of neuronatin mRNA, α and β. with the same open reading frame. Neuronatin-α encoded a novel protein of 81 aa, and the β-form encoded 54 aa. Both forms were identical, except that the α-form had an additional 81 bp sequence inserted into the middle of the coding region. On Northern analyses, neuronatin mRNA was relatively selective for the brain. It first appeared at E11-14, a time when the neural tube has closed and neuroepithelial proliferation initiated, became pronounced at E16-19 with a surge in neurogenesis, and declined postnatally to adult levels with the completion of neurogenesis. In order to determine whether there were other forms of neuronatin mRNA, and to study the expression of the α and β forms separately during development, reverse transcriptase-polymerase chain reaction was carried out using primers flanking the coding region of the α and β forms. The RT-PCR results clearly indicated that there were only two forms of neuronatin. The β-form first appeared at E11-14, whereas the α-form was present even earlier at E7-10. Together, these findings indicate that the two forms of neuronatin mRNA is regulated differently during brain development. The appearance of the β-form at mid-gestation, coinciding with the onset of neurogenesis, may suggest that alternative splicing of neuronatin mRNA has relevance in brain development.

Keywords: Neuronatin; Development; Brain; Gene; Neuroepitheliurn: Proliferation: Commitment; Differentiation; Migration

1. Introduction

Although development is a continuum, there are certain stages when the changes are more pronounced. The development of the brain begins with neural induction during gastrulation leading to the formation of the neural plate. Once the neural plate closes to form the neural tube at about embryonic (E) day 10 in the rat, the neuroepithelial cells of the ventricular zone begin to proliferate [6,15] and generate waves of stem cells, which following clonal expansion, stop dividing and become committed neuroblasts or gliablasts [19]. Subsequently, these cells migrate along supporting radial glial fibers into the surrounding tissue and become layered in an inside-out manner to form the cortical mantle [3,13,14,16,17]. Although, it appears that neurons and glia arise from a common precursor in the neuroepithelium, more recent studies suggest they originate from distinct stem cells [20]. In any event, the surge of neuroepithelial proliferation and commitment to a neuronal or glial fate results in a rapid increase in brain mass that continues into the early postnatal period.

The molecular events that initiate neuroepithelial proliferation and their subsequent commitment as neuroblasts or gliablasts are undoubtedly complex and may involve multiple interacting signals. Nevertheless, we proceeded to investigate the role of neuronatin, a novel cDNA that we recently cloned from postnatal (P) day 3 [7] rat brain, in development. In our original report, we noted that neuronatin cDNA, identified a mRNA species of about 1.2 kb which was selectively more abundant in neonatal rat brain compared to the adult brain. As rat brain development extends into the postnatal period, we reasoned that neuronatin may be involved in development. In the present study, we have characterized the two alternatively spliced

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forms of neuronatin mRNA, and investigated their role in rat brain development.

2. Materials and methods

2.1. Fischer 344 rats

Healthy F344/N rats (Harlan Sprague-Dawley, Indianapolis, IN), an inbred strain [5]. were used in all experiments. In order to study embryonic development, pregnant animals at different stages of gestation timed from the appearance of the vaginal plug were sacrificed. Embryos were removed following decapitation of the mother. Early- (E7-10). mid- (E11-14) and late- (E16-19) gestation embryos were studied. Postnatally, P3, P7 and P14 pups, and adult animals were studied. In the case of E7-10, whole embryos were used, and at E11-14, the head region was used for RNA extraction. By E16-19, the coverings of the brain having developed, allowed dissection to remove the brain. Animals were decapitated, brain quickly removed and placed in liquid nitrogen. In experiments to study expression of neuronatin mRNA in different tissues, the heart, liver and kidneys were removed from P3 pups.

2.2. RNA extraction

Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method [4,8]. The tissue was crushed in a mortar in the presence of liquid nitrogen; 10 ml of denaturing solution (4 M guanidinium thiocyanate. 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was added, homogenized using a glass-teflon homogenizer (Eherbach, Ann Arbor, MI) and kept on ice. Thereafter, 0.2 M sodium acetate (pH 4.0), 10 ml of water saturated phenol and 2 ml of chloroform-isoamyl alcohol (49:1) were added, vortexed, kept on ice for 15 mm and centrifuged (10,000 x g for 20 min) at 4°C. The aqueous phase was precipitated with an equal volume of isopropanol, the resulting pellet suspended in 0.3 ml of denaturing solution, transferred to a micro-centrifuge tube and precipitated again with isopropanol. The final RNA pellet was washed with 75% ethanol and dissolved in 0.5% SDS.

2.3. Library screening and sequencing

Differential display and library construction were carried out as previously described [7]. Briefly, total RNA samples from neonatal (P3), adult (3 months) and aged (33 months) male F344 rat brain were reverse transcribed using anchored 3-primers and degenerate 5-primers, and separated on denaturing polyacrylamide sequencing gels. Thirty-five cDNA fragments present only in the neonatal or adult brain were extracted, PCR amplified, and used in Northern blotting experiments. Only 7 of the 35 fragments were confirmed to be differentially expressed on Northern blotting. When these 7 fragments were cloned and sequenced, only *5* had novel sequences. These *5* fragments were pooled and used to screen the rat brain λgt11 cDNA library that we prepared.

2.4. Northern blotting

The procedure for the Northern blots was as previously described [8]. Briefly, 15 μg of total RNA containing 0.1 μg/ml of ethidium bromide was eleetrophoresed on denaturing formaldehyde gels. The amount of RNA was estimated to be comparable by UV photography (and later by glyceraldehyde 3-phosphate dehydrogenase (G3PDH) hybridization), and transferred to nylon by overnight capillary action. The membrane was cross-linked, baked, and prehybridized in a solution containing 1% bovine serum albumin. 7% SDS, 0.5 M sodium phosphate (pH 7.0) and 1 mM EDTA for 2 h. Hybridization was continued overnight at 65°C in the same solution to which was also added 1 x 106 cpm/ml of random prime labeled probe. The membrane was washed (1% SDS, 40 mM Na_3PO_4 (pH 7.0), 1 mM EDTA) at room temperature for 30 min and at 65°C for 30 min (X2), and exposed to film for 6 h.

2.5. Reverse-transcriptase-polymerase chain reaction (RT-PCR.)

In order to study the expression of the two alternatively spliced forms of neuronatin cDNA during brain development, we synthesized primers designed against the neuronatin cDNA sequence (see Fig. 1). The 5'-primer was homologous to positions -40 to – 15 of the cDNA sequence, 5'-GCGAACCCTTGCTCTCGACCACCCAC-3'; the 3-primer was complementary to positions 652-671. 5'-CCCACTAGTTTTCTTAACCC-3. The predicted sizes of the products was 710 for the β-form. and 791 for the α-form (including the 81 bp sequence (box in Fig. 1)).

For cDNA synthesis, total RNA extracted as described above from E7-10, E11-14, E16-19, P7 and P14 rats were reverse transcribed using a modification of previously described methods [9]. Initially, reactions containing RNA (5 μg), RNase inhibitor (40 U, Promega). oligo-dT (0.5 μg, BRL), made up to 10 μl with DEPC-treated water was heated to 70°C for 10 min, and then chilled on ice. Thereafter, final concentrations of DTT (10 μM), dNTP (125 μM), RT-MMLV (10 U, BRL) and RT-buffer (1 X, BRL) were added in a total volume of 20 μl, and the reactions incubated at 37°C for 60 min. Thereafter, the RT-enzyme was inactivated by heating to 94°C for 5 min and chilled on ice. Aliquots (4 μl) of the cDNA reaction was used for PCR amplification. The final concentration of the ingredients of the PCR reaction were, dNTP (100 μM), 5-primer (0.4 μM), 3-primer (0.4 μM), *Taq* DNA polymerase (5 u, Promega), Tris-HCI (10 mM), $MgCl_2$ (1.5 mM), KCl *(50* mM) and gelatine *(5* μg), in a total

volume of 50 μl. The samples were denatured at *95°C'* for 2 min, and then amplified for 40 cycles. Each cycle consisted of denaturation at 95°C for 30 s, annealing at 62°C for 1 min, and extension at 72°C for 2 min. The samples (30 μl per well) were electrophoresed using 1 X Tris acetate buffer, on 1% agarose containing ethidium bromide. A 100 bp ladder (BRL) was used as the DNA size marker. The gel was photographed under UV light.

3. Results

The novel cDNA fragments isolated by differential display from neonatal rat brain were used to probe the λgt11 rat brain cDNA library that we prepared. A total of nine positive plaques were identified. These plaques were isolated, purified and parallel samples digested with EcoRI and *Not*I *(EcoRI* arms containing nested *Not*I sites were used to construct the library). Both *EcoRI* and *Not*I digestion released inserts of the same size. Of the nine plaques that were isolated, the inserts in four were between 1.2 and 1.3 kbp, one about 2 kbp, and the remaining four were less than 0.5 kbp. The clones in the 1.2-1.3 kbp range corresponded in size to the mRNA species detected on Northern blotting using the cDNA fragments. These four clones were sequenced in both directions using the fMol™ Sequencing System (Promega, Madison, WI) with custom made primers. All four clones had identical *5'*- and 3-untranslated regions, translation initiation (GAAC-CATGG) [10]. stop (TGA) and termination (AATAAA) signals [21]. Two of these four clones were identical including having a coding region of 243 nt, whereas the remaining two clones were also identical with each other with a coding region of 162 nt. Therefore, we were able to confirm the cDNA sequences for the longer, neuronatin-α. and shorter, neuronatin-β, forms by sequencing two separate clones for each (Fig. 1). Although we used a pooled sample of five novel fragments to screen the library, all the clones identified were of neuronatin.

The restriction map generated for neuronatin-α was confirmed by digestion with *Eco*RI, *Not*I, *Nco*I and *Xho*I. There were no sites for *Eco*RI and *Not*I, *Xho*I had one site resulting in two fragments of *556* and 639 bp, and *Not*I had three sites resulting in four fragments, 40. 122, 471 and 562 bp. The difference between the two forms of neuronatin was confined to a portion of the coding region.

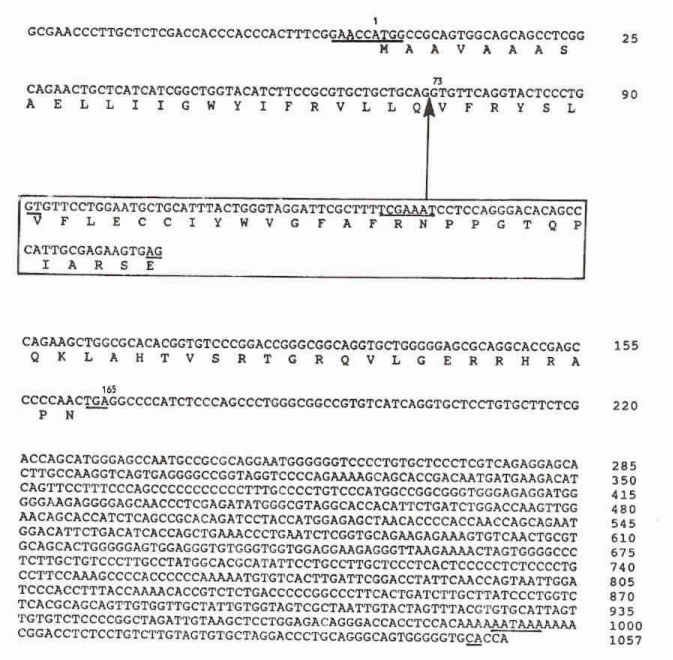

Fig. 1. The cDNA sequences for the two alternatively spliced forms of neuronatin. Bascd on the cDNA sequence, neuronatin-α has three regions.. possibly exons, consisting of 72, 81 and 90 nucleotides. Neuronatin-β was identical to neuronatin-α except that it lacked the 81 bp middle region (box). Neuronatin-α contained the 81 bp middle region which exhibited prototypical features of an intron including consensus GT-AG and a branch site, TCGAAAT, located 33 nt upstream of the 3'-end (underlined). Also underlined are the translation start (GAACCATGG), stop (TGA) and termination (AATAAA followed by CA) signals. The poly(A) tail begins at position 1057. For the RT-PCR study, primers flanking the coding region were synthesized, the 5'-primer was homologous to sequences -40 to -15, and the 3'-primer was complementary to sequences 652-671.

Table 1
Comparison of the extent of homology between the cDNA sequences of neuronatin-α and rat calbindin-D28k isolated by Lomri et al. [12]

	Neuronatin-α	Calbindin-D28k
Locus	RNU08290	RATCALBDF
GenBank accession no.	U08290	M27839
Total cDNA length (bp)	1195	3352
Location of coding region	41–283	628–1413
Homology:		
Region of interest	1–1177	2160–3352
No match	1–208	2160–2380
Additions		2390 (+C)
	242 (+G)	
	401 (+G)	
	434 (+G)	
	437 (+C)	
	438 (+C)	
		2623 (+G)
		2626 (+C)
		2642 (+C)
		2796 (+A)
		2821 (+C)
		2881 (+G)
Substitutions	810 (T)	2985 (G)
	811 (G)	2986 (T)
	1146 (G)	3321 (C)

Neuronatin-α had an additional 81 bp sequence inserted into the coding region without altering the reading frame (Fig. 1). This additional stretch of cDNA had the prototypical features of an intron [11], including the consensus sequences CT at the 5-end, AG at the 3-end, and the branch site, TCGAAAT, located 33 nt upstream of the 3'-end. Based on the cDNA sequence, it appears that neuronatin-α had three regions, possibly exons, the first extending from positions 1 to 72 (72 nt, 24 aa), the third from 73 to 162 (90 nt, 30 aa); the middle region was inserted between positions 72 and 73 and consisted of the 81 bp sequence coding for 27 aa. However, pending information of the genomic structure, this analysis based on the cDNA sequences must remain tentative.

GenBank analysis [2] using the BLAST server [1] revealed that the 3'-untranslated region of neuronatin cDNA matched with part of thc 3'-end of rat calbindin-D28k isolated by Lomri et al (LOCUS: RATCALBDF. GenBank #M27839) [12] (Table 1). However, the entire 5'-end and first 208 nt (out of 243 nt) of the coding region of neuronatin were nonhomologous and unrelated to calbindin-D28k. Although the rat calbindin-D28k clone isolated by Lomri et al. exhibited significant homology with calbindin-D28k present in other species, neuronatin did not match with any of the several other forms of calbindin-D28k present in GenBank.

Northern hybridization revealed that both forms of neuronatin cDNA hybridized to a mRNA species of about 1.2 kb, therefore, subsequent studies were carried out using neuronatin-α as the probe. Because of the homology between the α and β forms, the α-probe would also hybridize to the β-form. Therefore, the results of the Northern blotting experiments would reflect the combined expression of neuronatin-α and $-\beta$ mRNA. Neuronatin mRNA was relatively selectively expressed in the brain and not in the heart, kidney or liver, the three other tissues studied (Fig. 2). Next, the expression of neuronatin during different stages of brain development was investigated. Northern blotting was carried out using RNA extracted from E7-10, E11-14, E16-19, P3, and adult (3 months) male and female rats. Neuronatin was not expressed in the early embryonic period, It first appeared in the mid embryonic period, and its expression became abundant later at E16-19. Postnatally, its expression declined such that only minimal levels were present in adulthood (Fig.3).

In order to determine the relative importance of neuronatin-α and -β during development, and in order to exclude other spliced forms of this gene, we carried out RT-PCR with primers flanking the coding regions of neuronatin-α and -β. On the basis of the location of the primers (see Fig.1), we would predict that the α-form would generate a product of 791 bp, and the β-form 711 bp. The findings reveal that of the two forms, it is the β-form that appears at E11-14 (Fig.4). Although the

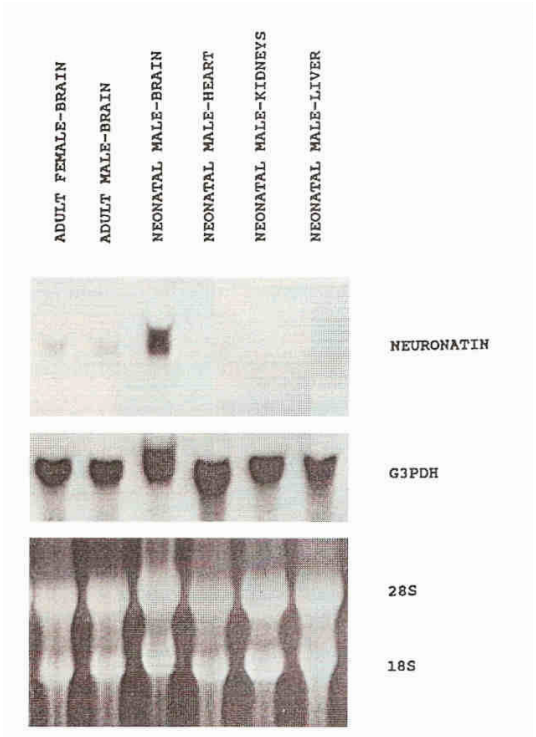

Fig. 2. Northern blot study to compare neuronatin mRNA expression in different tissues. Total RNA was extracted from P3 rat brain, heart, liver and kidneys; and from adult rat brain. Following hybridization with neuronatin-α (top panel), the blot was stripped and re-hybridized with human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) (middle panel). The elhidium bromide-stained gel, photographed prior to transfer. is also shown (bottom panel).

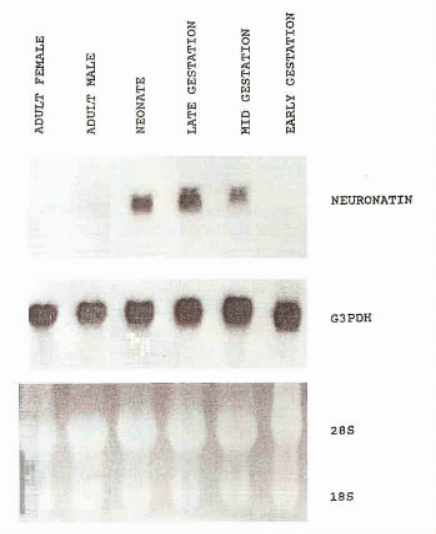

Fig. 3. Northern blot study of neuronatin expression during embryonic and postnatal rat brain development. Total RNA was extracted from E7-11), E11-14, El6-l9, P3, adult male and female rats, and processed for northern blotting using neuronatin-α as the probe (top panel) The blot was also labeled with G3PDH (middle panel). The ethidium bromide stained gel is also shown (bottom panel).

α-form was not observed on Northern blotting at E7-11), it was seen on RT-PCR to be present at this developmental stage. Apart from the predicted bands for neuronatin-α and

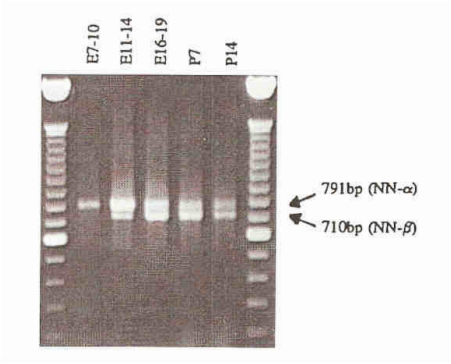

Fig.4. RT-PCR study of neuronatin-α and β mRNA during rat development. As described in Section 2. primers flanking the coding region were designed to amplify products that would he specific for the α and β forms. The predicted size for neuronatin-α was 791 bp and that for neuronatin-β, 710 hp. RNA extracted from E7-10, E11-14. E16-19, P7 and P14 rat were used. The appearance of the β-form at E11-14 coincided with a period in development when neurogenesis has been initiated.

-β, no other products were amplified, indicating that there are no other forms of neuronatin mRNA during brain development.

4. Discussion

Although originally isolated from neonatal rat brain, the studies described in this paper reveal that neuronatin mRNA is abundant and differentially expressed during brain development. There are two forms of mRNA, α and β, which appear to be alternatively spliced. The shorter β-form first appears in mid gestation, a time when the neural tube has closed and neuroepithelial proliferation and commitment to a neuronal or glial fate are taking place. On the other hand. neuronatin-α mRNA, although not detected on Northern analysis, was observed on RT-PCR to he present even earlier in gestation. Nevertheless, the abundance of the α-form was also increased in mid-gestation. Maximum levels of neuronatin mRNA were seen late in gestation; thereafter, its expression decreased to baseline levels in the adult brain. This close temporal relationship between the mRNA expression pattern of neuronatin and brain development raises the possibility that the two phenomena are related.

Neuronatin-α exhibited partial homology to the 3'-un translated region of the rat calbindin-D28k clone isolated by Lomri et al. [12]. There was no homology between neuronatin-α cDNA and the forms of calbindin-D28k cDNA isolated by other investigators. As would be expected, the rat calhindin-D28k isolated by Lomri et al. was highly homologous with calbindin-D28k derived from other species. Review of the region of overlap between neuronatin-α cDNA and the calbindin-D28k sequence of Lomri et al. revealed that the coding region of calbindin-D28k and the region of interest with neuronatin-α were separated by 746 hp. Furthermore, even within the region of the homology between neuronatin-α cDNA and the 3'-end of rat calbindin-D28k, the entire 5'-end and most of the coding region (208 nt out of 243) of neuronatin-α did not match. The observed homology involved mainly the 3'-untranslated region of neuronatin-α, and part of the 3'-untranslated region of calbindin-D28k. Therefore, neuronatin-α and calbindin-D28k are different and unrelated. The explanation for the homology seen with the Lomri et al. sequence is unclear. Conceivably, as speculated by Lomri et al., cloning artifacts may have resulted in unrelated pieces of cDNA becoming ligated to their clone. The deduced protein sequence for neuronatin-α was also novel, however, the amino acid sequence for the 81 bp middle sequence exhibited 40% identity with periplasmic hydrogenase [18]. When related amino acids were taken into consideration, the homology increased to 54%. This electron transfer enzyme is an iron-sulfur hydrogenase that catalyzes the oxidation and production of molecular hydrogen. The significance of this homology is uncertain as

hydrogenases are known to be present only in microorganisms such as the anaerobic sulfate reducer *Desulfovibrio vulgaris* (Hildenborough). Nevertheless, the region of homology included conserved cysteines involved in the functional coordination of the catalytic iron-sulfur cluster.

Neuronatin has two alternatively spliced forms which were confirmed by sequencing two clones for each. Both had the same open reading frame with neuronatin-α encoding 81 aa and neuronatin-β 54 aa. The only difference between the α and β forms of neuronatin was the presence in the α-form of an additional 81 nt sequence encoding 27 aa located in the middle of thc coding region. Tentatively, based on the cDNA sequences, it is suggested that neuronatin-α has three exons, the first containing 72 nt encoding 24 aa. the second containing 81 nt encoding 27 aa, and the third with 90 nt encoding 30 aa. As neuronatin-β contains only the first and third exons, it appears to be derived from the α-form by the splicing out of the middle exon. Definitive proof will require the elucidation of the gene structure of neuronatin.

In order to compare the expression of neuronatin-α and -β during development, we synthesized primers flanking their coding regions and carried out RT-PCR. The prediction would be that neuronatin-α will generate a product of 791 bp, and neuronatin-β, a product of 710 bp. And, if there were any other spliced forms of neuronatin, additional products would be generated. The results indicate that only products corresponding in size to the α and β forms are amplified. In the absence of a third product, we believe that there are no other spliced forms of neuronatin mRNA. The β-form appeared at E11-14 and correlated with a time in gestation when the neural tube has closed and neuroepithelial proliferation and their commitment to a neuronal or glial fate are manifest. Although RT-PCR, known to be more sensitive than Northern analysis, detected the presence of the α-form at E7-10, the expression of the α-form also increased later in development. In any event, it appears that the splicing mechanism that generates the β-form becomes active during neurogenesis. The significance of this observation, and its importance in neuroepithelial proliferation and the generation of post-mitotic brain cells need to be determined. It may be speculated that the removal of the middle exon may be important to function of this gene.

We do not yet know the function of neuronatin. The available data suggest that neuronatin mRNA is abundant and expressed in almost a precise relationship to brain development. Assuming that this relationship is mechanistic, one may speculate that neuronatin is involved in neuroepithelial stem cell proliferation or their commitment to a post-mitotic fate. A clearer picture may emerge when the growth characteristics of sister cell lines created to express and not express neuronatin mRNA are compared. The selective expression of neuronatin in the brain during development raises the fascinating possibility that the promoter sequence driving the expression of this gene could also be used to drive the expression of other beneficial genes during brain development. Furthermore, as neuronatin mRNA is also expressed in human fetal brain [7], these findings may have relevance to both normal and abnormal human brain development.

In conclusion, neuronatin is a novel mRNA that is selectively expressed during mammalian brain development. There are two forms, α and β, that are identical, except for the presence of an additional 81 bp sequence within the coding region of the α-form. The brain-specific nature of expression, its abundance and close correlation with brain development suggests that neuronatin may be functionally involved in mammalian brain development.

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