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MOLECULAR CLONING OF A NOVEL mRNA (NEURONATIN) THAT IS HIGHLY EXPRESSED IN NEONATAL MAMMALIAN BRAIN+

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As differential gene expression governs the progression of development into senescence, we attempted to define the genes that are selectively expressed during postnatal brain development. A cDNA fragment selectively expressed in neonatal rat brain was identified by differential display and used to screen a cDNA library prepared from the same mRNA sample. The full length cDNA, neuronatin, was 1195bp long and coded for a novel protein of 81 amino acids. The cDNA detected an mRNA species of similar size that was highly expressed in rat neonatal and human fetal brain. The deduced protein exhibited a hydrophobic N-terminal and hydrophilic C-terminal, suggesting that it is membrane bound and might function in signal transduction. The selective expression of this novel mRNA in late fetal and early postnatal brain development, and loss of expression in adulthood and senescence, suggests that downregulation of neuronatin may be involved in terminal brain differentiation.

Normal brain development predictably leads to senescence manifesting as progressive failure of function, cellular degeneration and death. The consequences of aging are most devastating when brain function begins to fail. Although, environmental factors may be contributory, development is primarily genetically determined. The onset of senescence is species specific and its course not significantly prolonged even under optimal environmental conditions (1). Nevertheless, with increasing age, cellular macromolecules progressively accumulate

+The GenBank Accession number for neuronatin is U08290.

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errors, such as mutations (2), telomere shortening (3), DNA hypomethylation (4), impaired DNA repair (5), protein cross linking (6) and decreased protein degradation (7). However, the mechanism by which these changes may be involved in senescence remain unclear. Cells in culture also exhibit senescence, for example, human diploid fibroblasts show growth failure after a certain number of population doubling levels (8). The inevitable progression of development into senescence is likely to involve changes in gene expression manifesting either as upregulation or downregulation of genes, or both. Therefore, we attempted to define the genes that are uniquely expressed during brain development and senescence. We report the experiments leading to the identification of a novel mRNA species that is selectively expressed in fetal and neonatal human and rat brain.

MATERIALS AND METHODS

Fisher 344 Rats: Healthy male Fisher 344N/NIA rats, an inbred strain (9), obtained from the National Institute of Aging, Bethesda, MD were studied at 3 days (neonate), 3 months (young adult) and 33 months (aged adult) of age. At 3 months of age, the rat is fully mature and at 33 months it is senescent (10).

Differential Display: Animals were decapitated, brain quickly dissected out, immersed in liquid nitrogen, homogenized and total RNA extracted by acid guanidinium thiocyanate-phenol-chloroform method (11). The methods used were as previously described (12,13,14). Total RNA (50µg) was treated with DNase—I RNase—free (20u), human placental RNase Inhibitor (80u), 10mM Tris HC1, 1.5mM $MgCl_2$, 50mM KC1, incubated at 37°C for 30 mm, phenol:chloroform extracted and ethanol precipitated. DNase-I treated RNA (2kg) was reverse transcribed using 5′-T_{12}MG—3′ (2.5DM) as the 3′-primer (M=mixture of G,A and C), dNTP (20.µM), MMLV-Superscript-II (300u) and MMLV buffer (lx) (Life Technologies, Grand Island, NY): the preparation was incubated at 35°C for 60 mm and then at 95°C for 5 mm (13). Thereafter, the reaction was PCR amplified with a degenerate lO—mer sequence (CAGGCCCTTC (Operon Technologies, Alameda, CA) in a concentration of o.5µM. Also included in the reaction were ^{5}S-adATP (1386 Ci/mmol, 0.5µM) and Tag DNA polymerase (2.5u) (Boehringer Mannheim, Indianapolis, IN), in a total volume 20µl. The preparation was denatured at 94°C for 5 mm., and then cycled 40 times. Each cycle consisted of denaturation at 94°C for 30 secs, annealing at 40°C for .2 mm, and extension at 72°C for 30 secs. Thereafter, the samples were extended at 72°C for an additional 5 mm, and then held at 4°C. Loading buffer was added and the samples electrophoresed on 6% acrylamide DNA sequencing gels

under denaturing conditions. The gel was dried at 80°C for 45 mm. and exposed (X-OMAT-AR, Eastman Kodak, Rochester, NY) overnight at —70°C. One cDNA fragment was seen to be strongly expressed only in the neonatal bramn. This cDNA fragment was cut out of the gel, phenol-chloroform extracted, ethanol precipitated and PCR amplified using the same set of primers and conditions used in the first amplification reaction for differential display. The presence of amplified PCR products was confirmed on 1.5% agarose gels.

Northern Blotting: Initially, the partial sequence extracted from the differential display gel, was used as a probe in northern blots containing brain RNA from neonatal, young adult and aged rat brain. Once the full length cDNA sequence was determined, this was also used in two sets of northern blots, one containing rat brain RNA and the second containing human fetal and aged brain. The procedure for the northern blots was as previously described (15). Briefly, 15kg of total RNA containing 0.lµg/m1 of ethidium bromide was electrophoresed on denaturing formamide gels. The amount of RNA applied across the lanes was estimated to be comparable by UV photography. The gel was transferred onto nitrocellulose by overnight capillary transfer. The membrane was cross-linked, baked, and prehybridized in a solution containing 1% bovine serum albumin, 7% SDS, 0.5M sodium phosphate pH7.0 and 1mM EDTA for 2 hours. Hybridization was continued overnight in the presence of $1x10^6$ cpm/inl of random prime labelled probe, at 65°C. The membrane was washed (1% SDS, 40mM Na_3PO_4 pH7.0, 1mM EDTA) at room temperature for 30 mm, and then twice at 65°C for 30 mm. The washed membrane was autoradiographed for about 6 hours.

Cloning and sequencing of the cDNA Fragment: The cDNA fragment isolated from the differential display gel was cloned into pCR (Invitrogen, San Diego, CA) as described (16). The cloned vector was used to transform TA One Shot competent cells. White colonies were selected for plasmid preparation from plates containing Kanamycin and Bluo-gal (Life Technologies, Grand Island, NY). The inserts were amplified using primers across the cloning site. The reaction included the plasmid template (1μg), T7 primer (0.4μM), SP6 primer (0.4μM), dNTP (100μM), buffer (lx) and Tag polyinerase (2.5u). The reactions were denatured at 94°C for 5 mm. Thereafter, samples were amplified for 35 cycles. Each cycle consisted of denaturation at 94°C for 1 mm., annealing at 62°C for 2 min. and extension at 72°C for 3 min. This was followed by extension at 72°C for 5 min, and samples held at 4°C. 25µl of the sample was loaded on 1.5% agarose gel containing 0.1µg/ml. ethidium bromide, electrophoresed using Tris-acetate buffer (lx), and photographed using UV light. In the absence of an insert, the predicted size of the amplified product was 188bp. The clone containing the insert exhibited a larger sized product estimated to be about 300bp using a lOObp DNA ladder (Life Technologies, Grand Island, NY) that was run simultaneously. Double stranded sequencing of the cloned cDNA insert was carried out using Sequenase Version 2.0 (United states Biochemicals, Cleveland, OH). Five µg of template was denatured, annealed with T7 or SP6 primers, labelled with ^{35}S-dATP and extended by the chain termination method of Sanger (17). The presence of the anchored 3'—primer was used to locate the cloned insert. The partial sequence (96nt) was unique with no homology in GenBank (18). This sequence was used to isolate the full length sequence from the rat brain cDNA library we constructed.

Construction of Fisher Rat Brain cDNA Library: We prepared a cDNA library using a mixture of RNA from neonatal, young adult and aged Fisher 344 rat brain. The overall scheme was as previously described (19). High quality total RNA (1mg) was separated by the single step acid guanidinium thiocyanate-phenolchloroform method (11). Through all the steps of the library construction, products were monitored by UV spectrophotometry, DNA dipstick (Invitrogen, San Diego, CA), or by ^{32}P-dCTP incorporation. Total brain RNA was treated with RNase-free DNase—I (lu/32 µg of RNA) in order to digest contaminating genomic DNA. DNase—free RNA was applied to oligo-dT cellulose column (Pharmacia, Piscataway, NJ). mRNA, eluted by 0.5M NaCl in lx TE (10mM Tris HC1, 1mM EDTA), represented 2.7% of the total RNA pool. 4µg of mnRNA was used to synthesize cDNA using 200u of Superscript—Il MMLV reverse transcriptase, 0.5/µg of oligo-dT_{12-18}, in 80µl of 50mM Tris, pH 8.3 buffer. All steps upto this stage used dH_2O treated with 0.02% DEPC and autoclaved, and contained 2u/µl of RNase inhibitor. The resulting RNA-DNA hybrids were converted to ds—cDNA in lOOµl of 20mM Tris pH 7.5, 4u of RNase-H and 46u of DNA polymerase. ds-cDNA was blunt ended using mung bean nuclease (43u/200µl of reaction mixture), 30mM sodium acetate pH 5.2 and 1mM Zn^{2+} as an activator. For high efficiency cloning of blunt ended ds—CDNA, EcoRI adapters with a nested NotI site (Life Technologies, Grand Island, NY) were ligated to the ends using 20u of T4 Ligase, 66mM Tris pH 7.5, in a SOM[1] reaction volume. NotI recognizes an 8—base site which rarely exists in ds— cDNA (about one site per 65kb) increasing the chances of excising the intact insert after cloning. The excess adapters and smaller cDNA fragments (<500bp) were removed by cDNA fractionation column (Life Technologies). ds-cDNA with ligated Ec0RI(NotI)-adapters were cloned into the single EcoRI site located at the 3'-end of LacZ gene in λgtll. To minimize background, the dephosphorylated λgt11 EcoRI arms (Life Technologies) were ligated with phosphorylated EcoRI (NotI) -adapter-ds-cDNA. Phosphorylation of EcoRI(NotI)—adapter—ds-cDNA was carried out with 40u of T4 nucleotide kinase, 66mM Tris pH 7.5, 1mM ATP, in 250µl reaction volume. Finally, the recombinant λgtll phage were packaged in vitro using λ Packaging System (Life Technologies), and used to infect E.coli Y1090(R^-M^-ΔLa cU169). The efficiency was about 3.5 x 10^5 pfu/µg cDNA. More than 90% of the plaques were detected by blue/white selection to contain recombinant phage.

Screeninci of Library: Using the unique partial cDNA fragment, we screened about 25,000 plaques. The probe was labelled with ^{32}PdCTP by the random primer method to about 10^8-10^9 cpm/µg DNA.

Nine plaques showed strong hybridization (at 73°C). These nine plaques were purified, DNA prepared and digested in separate reactions with Ec0RI and NotI. Both these restriction enzymes released single fragments suggesting the absence of these restriction sites within the insert. One plaque released an insert of about 1.2—1.3kb, corresponding to the size of the mRNA species detected in the northern blot using the partial cDNA sequence. This clone was fully sequenced.

Sequencing of Full-Length cDNA: The clone isolated from the library and predicted to contain the full length cDNA was sequenced using cycle sequencing with the fmol™ sequencing System (Promega, Madison, WI). Sequencing was carried out in both directions using a total of 9 primers, five from one end and four from the other. Primers were custom made (DNA International, Lake Oswego, OR).

RESULTS AND DISCUSSION

Total RNA was extracted from neonatal, young adult and aged rat brain and processed for differential display. A cDNA fragment selectively expressed in neonatal brain was identified (Figure-1). This cDNA fragment was extracted from the gel, PCR-amplified and confirmed on northern blot to be selectively expressed in the neonatal brain (Figure—2). The mRNA was estimated to be about 1.2kb. The cDNA fragment was cloned and sequenced. As there was no homology for this sequence in GenBank, we used this cDNA fragment to screen a λgtll cDNA library prepared from the same sample of brain RNA. Of the nine positive clones identified, one clone contained an insert matching the size of the detected mRNA species. This insert was fully sequenced in both directions.

The full length sequence was 1195bp long and had no homology in GenBank (Figure—3). Although, the 3'-untranslated region of our

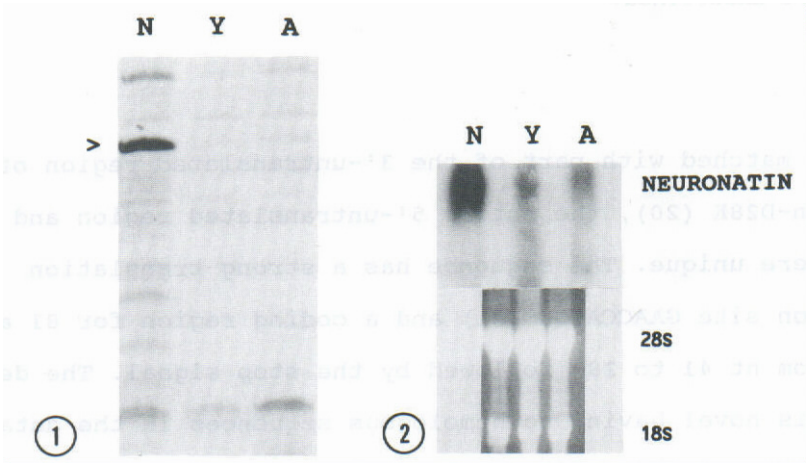

Figure 1. Differential display of brain cDNA from healthy 3 day old (neonate (N)), 3 month old (young adult (Y)) and 33 month old (aged adult (A)) male Fisher 344 rats. The cDNA fragment (arrowhead) selectively expressed in neonatal brain is shown.

Figure 2. Northern hybridization of brain RNA from neonatal (N), young adult (Y), and aged adult (A) rats using the cDNA fragment extracted from the differential display gel. The ethidium bromide stained gels pictured under UV light prior to transfer is also included to show that similar amounts of RNA were applied. The estimated size of the mRNA derived using RNA standards was about 1.2kb.

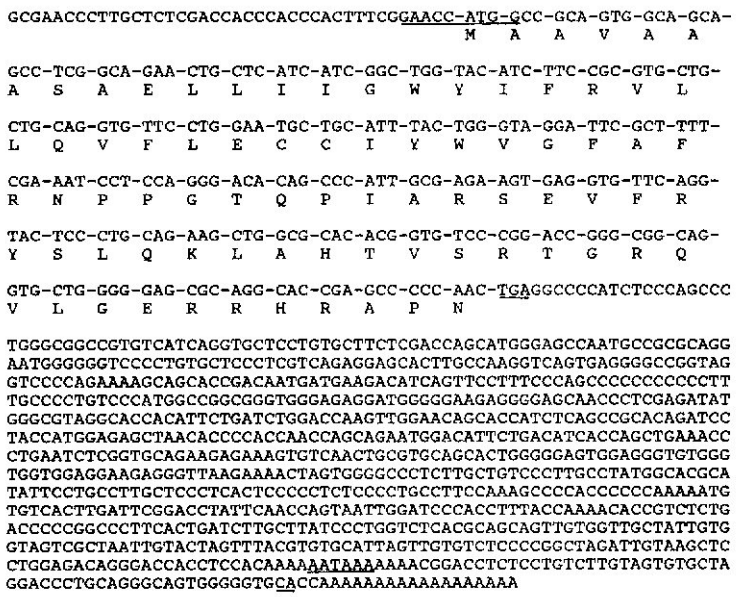

Figure 3. Full length cDNA sequence for neuronatin. The unique cDNA fragment identified on differential display to be selectively expressed in neonatal brain was used to screen a λgtll cDNA library we constructed using Fisher rat brain. Of the 9 positive plaques identified, one clone had an insert of about 1.2kb, closely matching the size of mRNA detected on northern. This clone was fully sequenced in both directions. The mRNA had a total length of l195b with prototypical translation initiation and termination sites. The translation start site (GAACCATGG), coding stop signal (TGA), mRNA termination signals (AATAAA and CA) are underlined.

sequence matched with part of the 3′—untranslated region of calbindin—D28K (20), the entire 5′-untranslated region and coding region were unique. The sequence has a strong translation initiation site GAACCATGG (21) and a coding region for 81 amino acids from nt 41 to 283 followed by the stop signal. The deduced protein is novel having no homologous sequences in the database. The 3′-untranslated region was 912 nt long with the prototypical termination signals AATAAA located at position 1112, CA at position 1174 followed by the poly(A) tail beginning at position 1178 (22). The full length sequence, designated neuronatin, was used in northern blotting to confirm selective expression in neonatal brain (Figure—4). In addition, in northern blots using

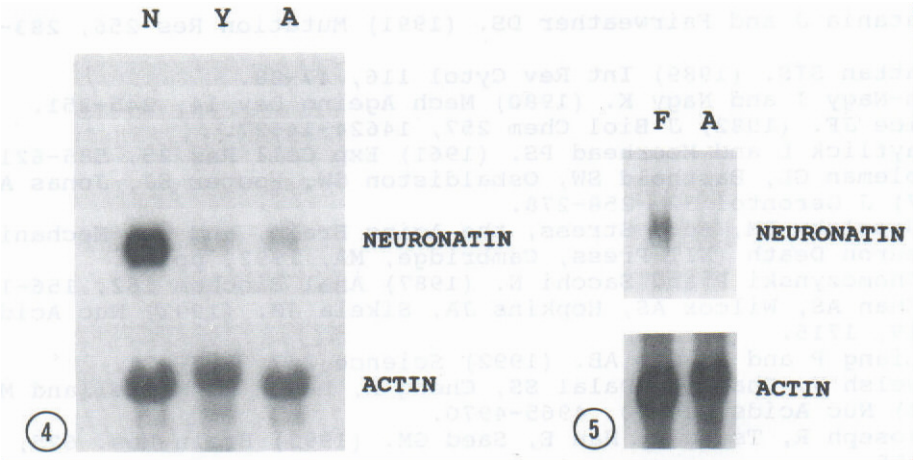

Figure 4. Northern blotting of neonatal rat brain (N), young adult brain (Y) and aged rat brain (A) using the full length sequence for neuronatin as the probe.

Figure 5. Northern blotting of human fetal (F) (18—24 weeks old) and human aged (A) brain (60 years old) with full length sequence for neuronatin.

RNA from human brain, neuronatin was expressed in human fetal brain aged 18-24 weeks (Figure-5). When tested for hydropathy (23), the protein was highly hydrophobic from residues 1 to 36 and hydrophilic from residues 74 to 81 suggesting that the N-terminal may be membrane bound whereas the C—terminal was located in the cytoplasm. Such a protein may be involved in signal transduction. The abundant expression of neuronatin mRNA during late fetal and early postnatal periods of mammalian brain development, and downregulation during adulthood and senescence, suggests a role in brain differentiation.

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