CHAPTER-III: RESULTS

A. Experience with Subtraction Library

1. Screening of the Library & Analysis of Clones
2. Comments on the Subtracted Library

B. Differential Display & cDNA Fragments

1. Isolation of cDNA Fragments
2. Cloning of cDNA Fragments
3. Sequencing of cDNA Fragments
4. Comments on Differential Display

C. The cDNA Sequence of *Neuronatin*

1. Screening of the Rat Brain eDNA Library & Sequencing of *Neuronatin*

D. GenBank Analysis of *Neuronatin*

1. Relationship of *Neuronatin* to *Calbindin-d28k*
2. Analysis of *Calbindin-d28k* sequence, #M27839
3. Analysis of *Calbindin-d28k* sequence, *EST #06992*
4. The Significance of the Homology

E. The cDNA Fragments & *Neuronatin*

1. Comments about *JT- 1* and *Neuronatin*

F. *Neuronatin* mRNA Expression

1. Expression in Tissues
2. Expression of Spliced Forms during Development

G. Analysis of the Deduced Protein

H. Role of *Neuronatin* in Neuronal Growth

1. *Neuronatin* mRNA Expression in Neuronal Cell Lines
2. NGF Down-Regulates *Neuronatin* mRNA

IIIA. EXPERIENCE WITH SUBTRACTION LIBRARY

IIIA-1. Screening of the Subtracted Library & Analysis of the Clones

The experiments to isolate genes selectively expressed in the developing brain began with an attempt to prepare a library enriched for neonatal rat brain as described in **SECTION-IIC**. The library was prepared by hybridizing neonatal brain cDNA with an excess of adult brain mRNA and removing the hybrids. Unhybridized cDNA fragments, specific for the neonatal brain, were cloned into the unique Pst-1 site of pBR322 vector, and small scale plasmid preparations made by the alkaline lysis method (Sambrook et al 1989). Twenty four clones *(L (large) 1 to 24; L-1 to L-24)* were isolated for screening. In order to amplify the cDNA inserts in these clones, primers were synthesized to hybridize the pBR322 sequence on either side of the Pst-1 site.

Up primer: 5'-TGC-GCA-ACG-TTG-TTG-3' (homologous to 3588-3062 nt of pBR322).
Down primer: 5'-ATA-CCA-AAC-GAC-GAG-3'(complementary to 3631-3645 nt of pBR322).

Before setting up the reaction, the plasmid preparation was freed of contaminating RNA by treating with RNase, phenol/chloroform extracted, ethanol precipitated, washed, air dried and dissolved in 20 μl of 1x TE. Five μl of the purified DNA from clones *L-1* to *L-24* were used as template for PCR amplification with the pBR322 primers shown above. Other constituents in the reaction mixture were 100 μM dNTP (all four), 0.4 μM up primer, 0.4 μM down primer

and 2.5 u Taq DNA polymerase. The samples were denatured at 94°C for 5 min and amplified for 40 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 52°C for 2 min, and extension at 72°C for 3 min. Thereafter, extension was continued at 72°C for 10 min, and reactions maintained at 4°C. Samples were electrophoresed on 1.2% agarose using 1x Tris-acetate buffer and photographed with UV after staining with ethidium bromide (**FIGURE-III-1**). The presence of inserts in clones *(L-3, L-4, L-6, L-7, L-8, L-9 & L-17)* were confirmed using the same PCR conditions (**FIGURE-III-2**). Of these, the insert in *L-8* was about 350 bp and that in *L-17* about 500 bp. These inserts were cut out of the gel, random prime labelled and used for Northern analysis. Unfortunately, both clones were nonspecific, i.e., the mRNA expression was not different between the *adult* and *neonatal* rat brain (**FIGURE-III-3**).

IIIA-2. Comments on the Subtraction Library

Although, much experience was gained from this effort, the results of the subtracted library were extremely disappointing. The results indicated that the library was nonspecific which may have arisen because of the original low-ratio (1: 1) of driver mRNA *(adult)* to tracer cDNA *(neonate)* that was used. To improve the enrichment of neonatal sequences, it would be necessary to consider using a 30:1 ratio. An alternative approach was investigated instead using differential display (I thank Sandra Rempel, Ph.D. for informing me of this technique). The advantage of the differential display technique was that it would allow visualization of the cDNA fragments expressed in both the neonatal and adult brain, and bands from either group could be selected. Therefore, it was decided to proceed with differential display.

SUBTRACTION LIBRARY: SCREENING OF 24 CLONES

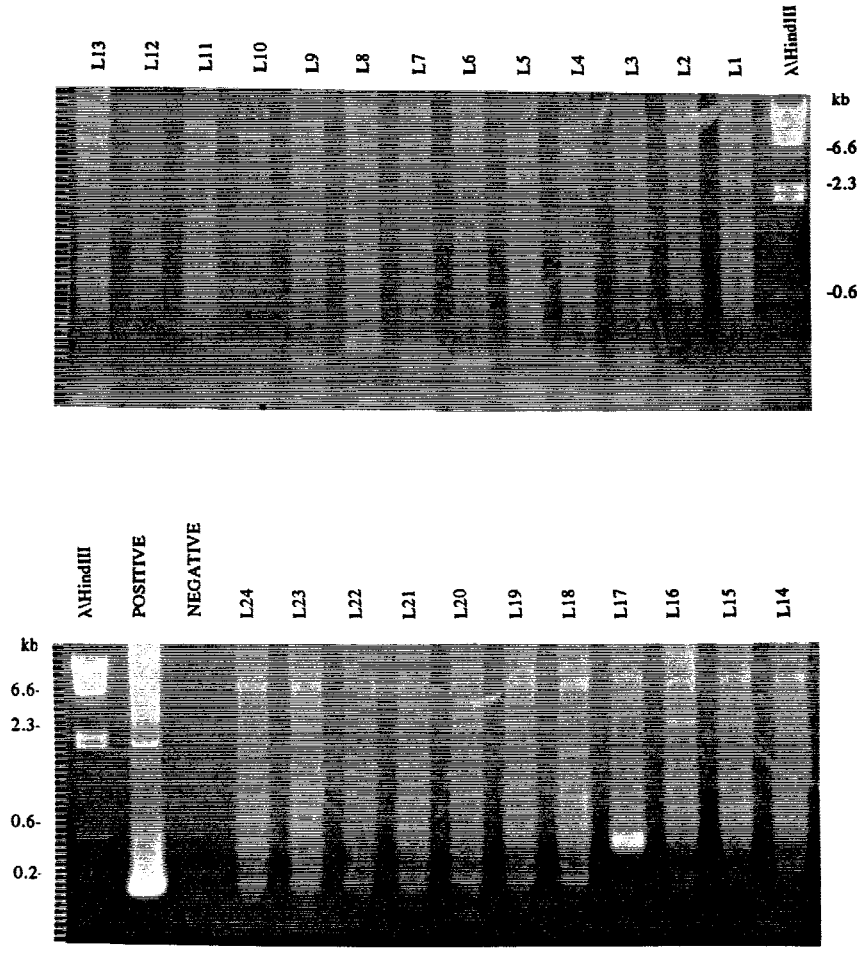


FIGURE-III-1: Screening of clones from the subtraction hybridization library, enriched for neonatal brain. The subtracted cDNA was cloned into the Pst-1 site of pBR322 by GC-tailing, transformed in competent HB101, and selected using Tetracycline. In order to PCR amplify the cloned product, pBR322-specific primers on either side of the Pst-l site were used. A total of 24 colonies *(L-1 to L-24)* were studied. Negative and positive controls were also included. The negative sample did not contain any template. In the positive sample, *β-amyloid precursor protein* template and primers were used, and as noted previously (Joseph et al 1993), a 0.2 kbp product was amplified. Inserts were visible in clones, *L-3, L-4, L-6, L-7, L-8, L-9 & L-17.* The standard marker was a HindIII digest of λ-DNA. kb = kilo base pairs.

SUBTRACTION LIBRARY: SCREENING OF SELECTED CLONES

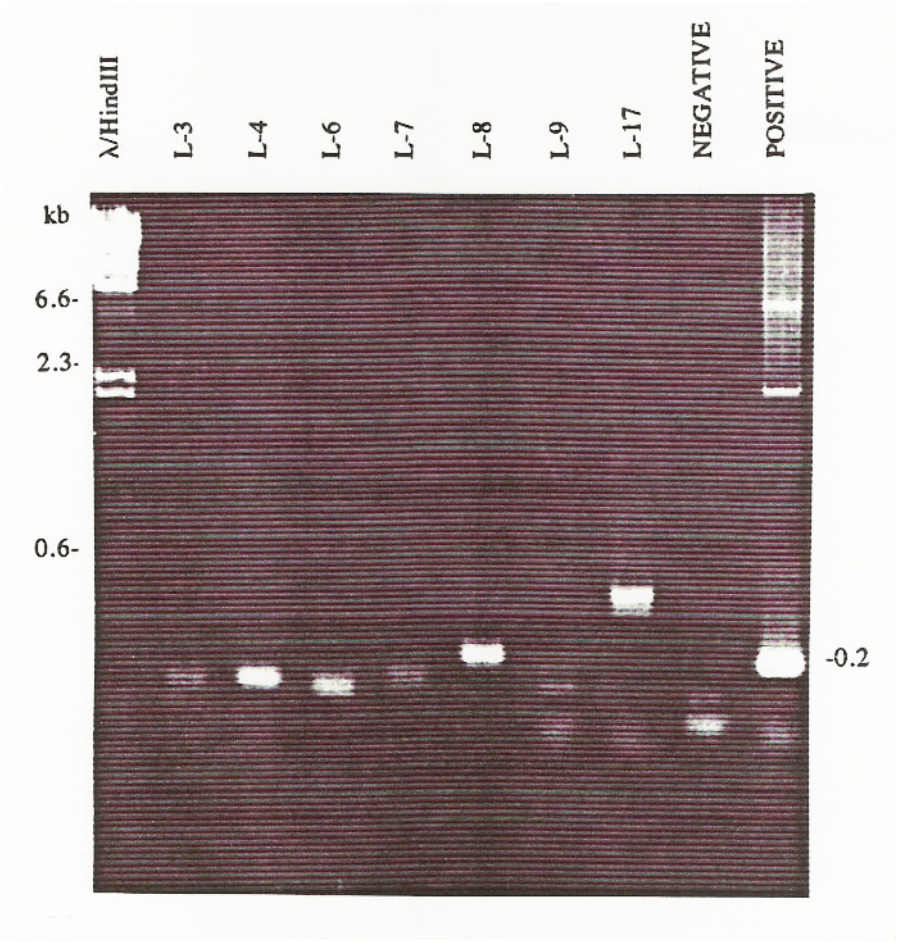


FIGURE-III-2: Screening of selected clones from the subtraction hybridization library. Based on screening 24 colonies *(L-1 to L-24*, certain clones *(L-3, L-4, L-6, L-7, L-8, L-9 & L-17)* were noted to contain inserts. These 7 clones were again PCR amplified using pBR322 primers on either side of the Pst-1 cloning site. The clones, *L-8* and *L-17* contained the largest inserts. Negative and positive controls were also included. The negative sample did not contain any template. In the positive sample, *β-amyloid precursor protein* template and primers were used, and, as was noted previously (Joseph et al 1993), a 0.2 kbp product was amplified. The standard marker was a HindIII digest of λ-DNA. kb = kilo base pairs.

SUBTRACTION LIBRARY: NORTHERN BLOTTING WITH *L-8* & *L-17*

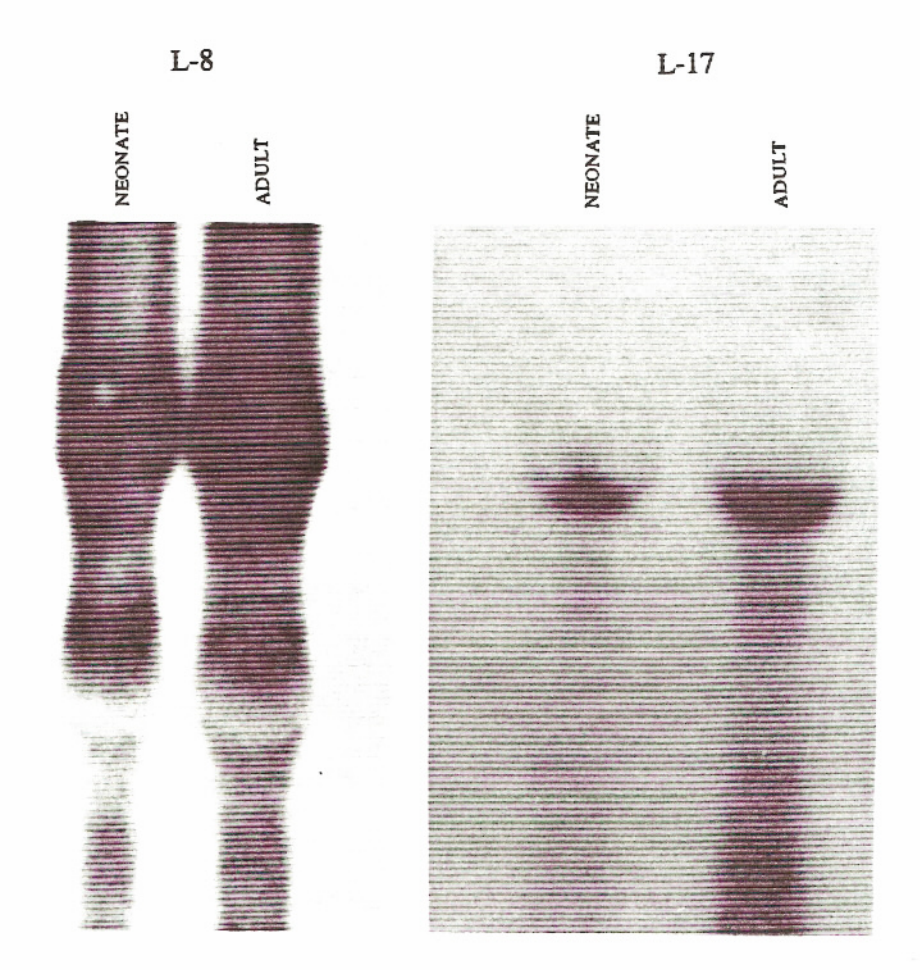


FIGURE-III-3: Northern blotting using probes from the subtraction library. The probes used were derived from clones L-8 and L-I 7, and the membrane contains RNA extracted from neonatal and adult rat brain. The pattern of hybridization seen with both probes was not specific for the neonatal brain.

IIIB. DIFFERENTIAL DISPLAY & cDNA FRAGMENTS

IIIB-1. Isolation of Differentially Expressed cDNA Fragments

As an alternative to the subtraction library, differential display was utilized to isolate genes selectively expressed in the developing rat brain. In this technique, poly(A) mRNA sequences are reverse transcribed and amplified using degenerate primers, and the resulting cDNA bands separated by gel electrophoresis. Brain RNA samples prepared from neonatal (3 days), adult (3 months) and aged (33 months) male rats were used. cDNA bands selectively expressed in the neonatal (N), young adult (Y) and aged (A) brain were identified. A total of 35 cDNA fragments *(JT-I to JT-35)* that were differentially expressed in the neonatal or adult rat brain were extracted from the gels. The selection of bands for study was arbitrary and dependent on the expression pattern seen on the autoradiogram. Although, the expressions of most of the isolated cDNA bands were increased in the neonatal brain, some were increased in the young adult and aged brain. The autoradiograms of each of the four gels used in this study are shown below:

JT-I to JT-5: GEL-1 **(FIGURE-III-4)**

JT-6 to JT-I5: GEL-3 **(FIGURE-III-5)**

JT-16 to JT-27: GEL-4 **(FIGURE-III-6)**

JT-28 to JT-35: GEL-S **(FIGURE-III-7)**

(The first set of samples was electrophoresed on two separate gels, GEL-1 and GEL-2. The patterns were identical).

DIFFERENTIAL DISPLAY GEL-1: cDNA FRAGMENTS *JT-1* TO *JT-5*

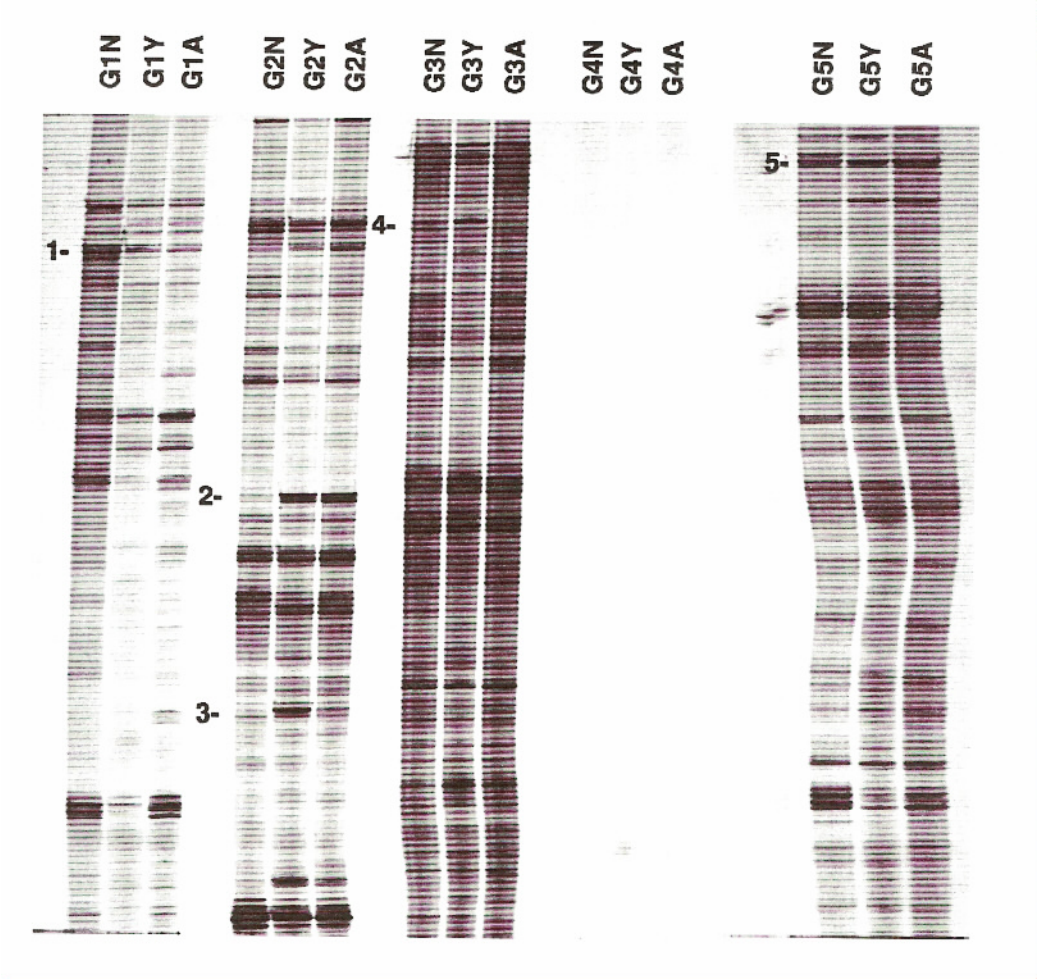


FIGURE-III-4: Differential display (Gel-1) from which cDNA fragments *JT-I* to *JT-5* was isolated. The 3'-primer used in this gel was T_{12}MG (G). The 5'-primers used were OPA-1 (1), OPA-2 (2), OPA-3 (3), OPA-4 (4) and OPA-5 *(5)*. N = *neonate* (3 days old), Y = *young adult* (3 months old) and A = *aged* (33 months old) rat brain. The location of the cDNA bands, *JT-1* to *JT-5* are indicated on the gel. For example, the first lane, G1N, indicates that the primers, T_{12}MG and OPA-1 were studied in neonatal brain.

DIFFERENTIAL DISPLAY GEL-3: cDNA FRAGMENTS *JT-6* TO *JT-15*

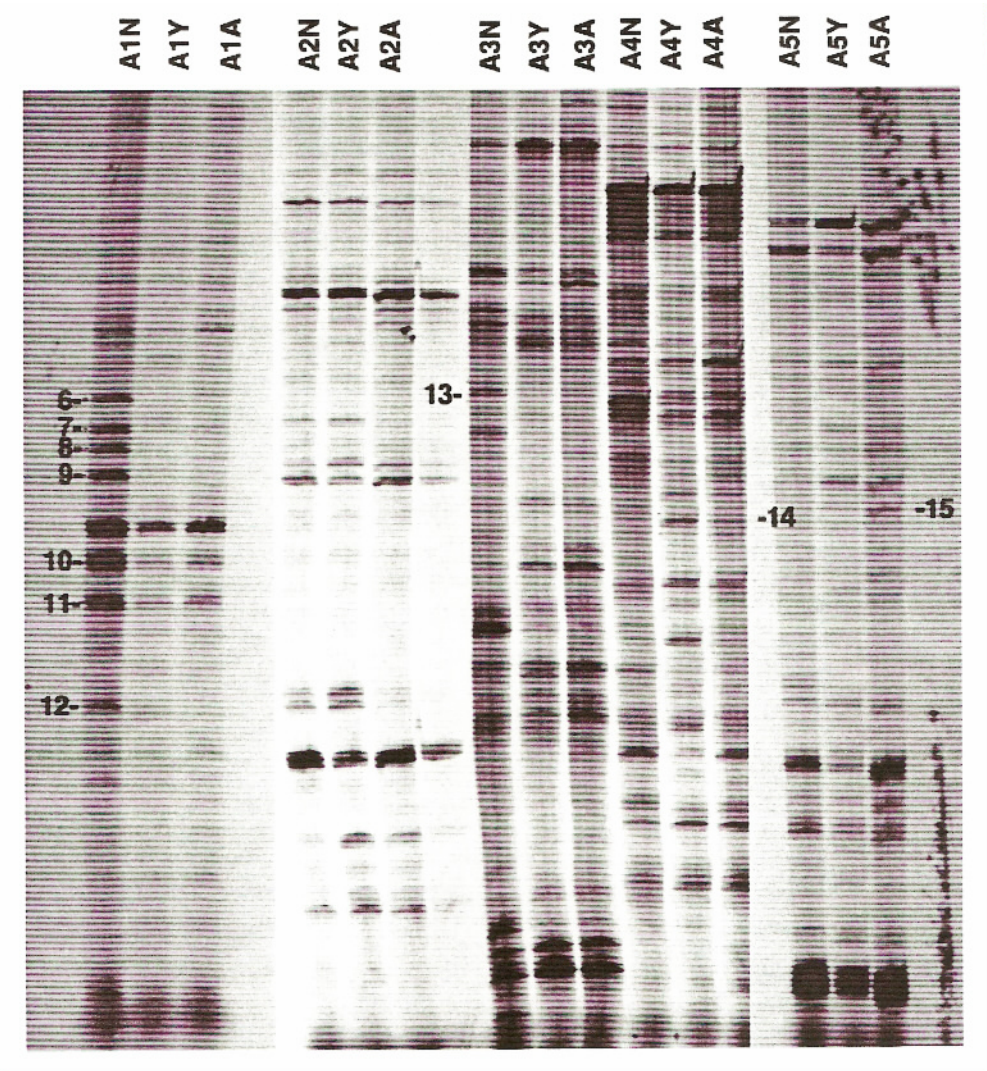


FIGURE-III-5: Differential display (Gel-3) from which cDNA fragments *JT-6 to JT-15* were isolated. The 3'-primer used in this gel was $T_{12}MA$ (A). The 5'-primers used were OPA-1 (1), OPA-2 (2), OPA-3 (3), OPA-4 (4) and OPA-5 (5). N = *neonate*, Y = *young adult* and A = *aged* rat brain. The location of the cDNA bands, *JT-6 to JT-15* are indicated.

DIFFERENTIAL DISPLAY GEL-4: cDNA FRAGMENTS *JT-16* TO *JT-27*

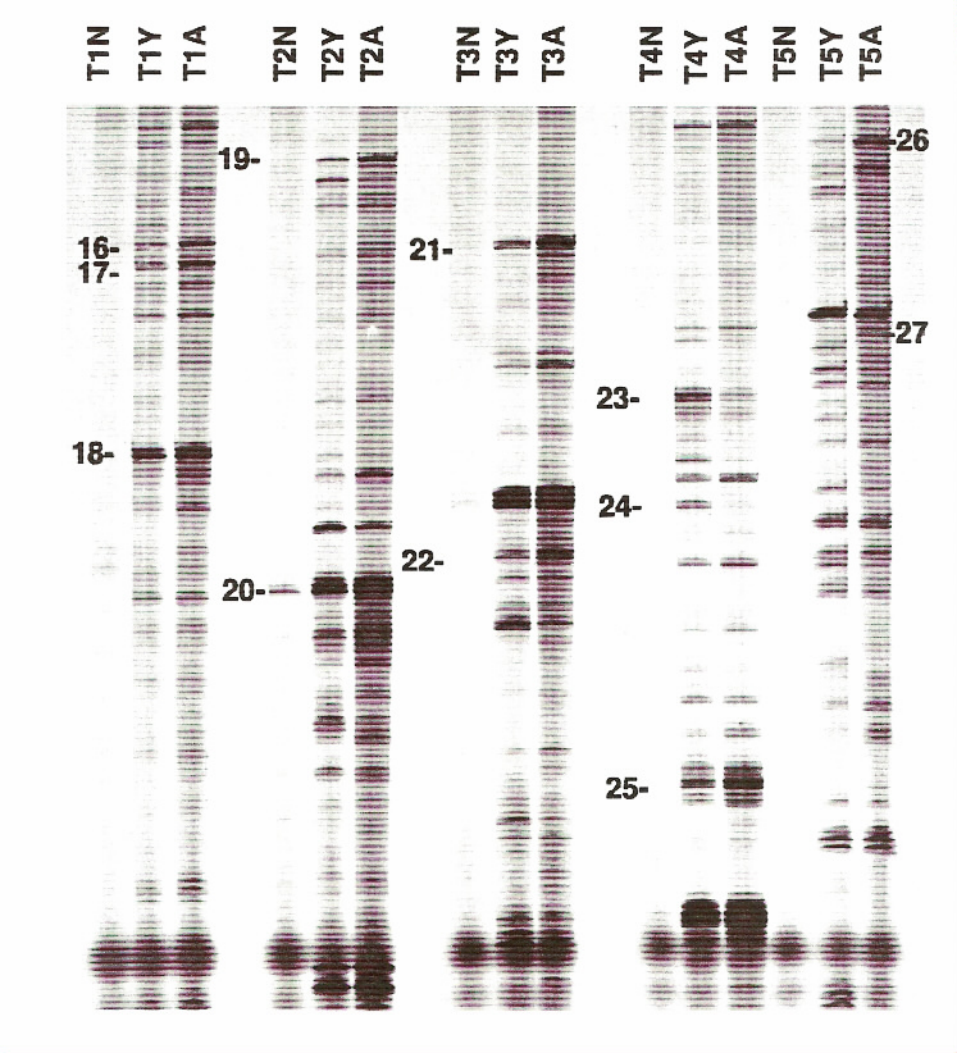


FIGURE-III-6: Differential display (Gel-4) from which the cDNA fragments *JT-16* to *JT-27* were isolated. The 3'-primer used in this gel was $T_{12}MT$ (T). The 5'-primers used were OPA-1 (1), OPA-2 (2), OPA-3 (3), OPA-4 (4) and OPA-5 *(5)*. N = *neonate,* Y = *young adult* and A = *aged.* The location of the cDNA bands, *JT-16* to *JT-27* are indicated.

DIFFERENTIAL DISPLAY GEL-5: cDNA FRAGMENTS *JT-28* TO *JT-35*

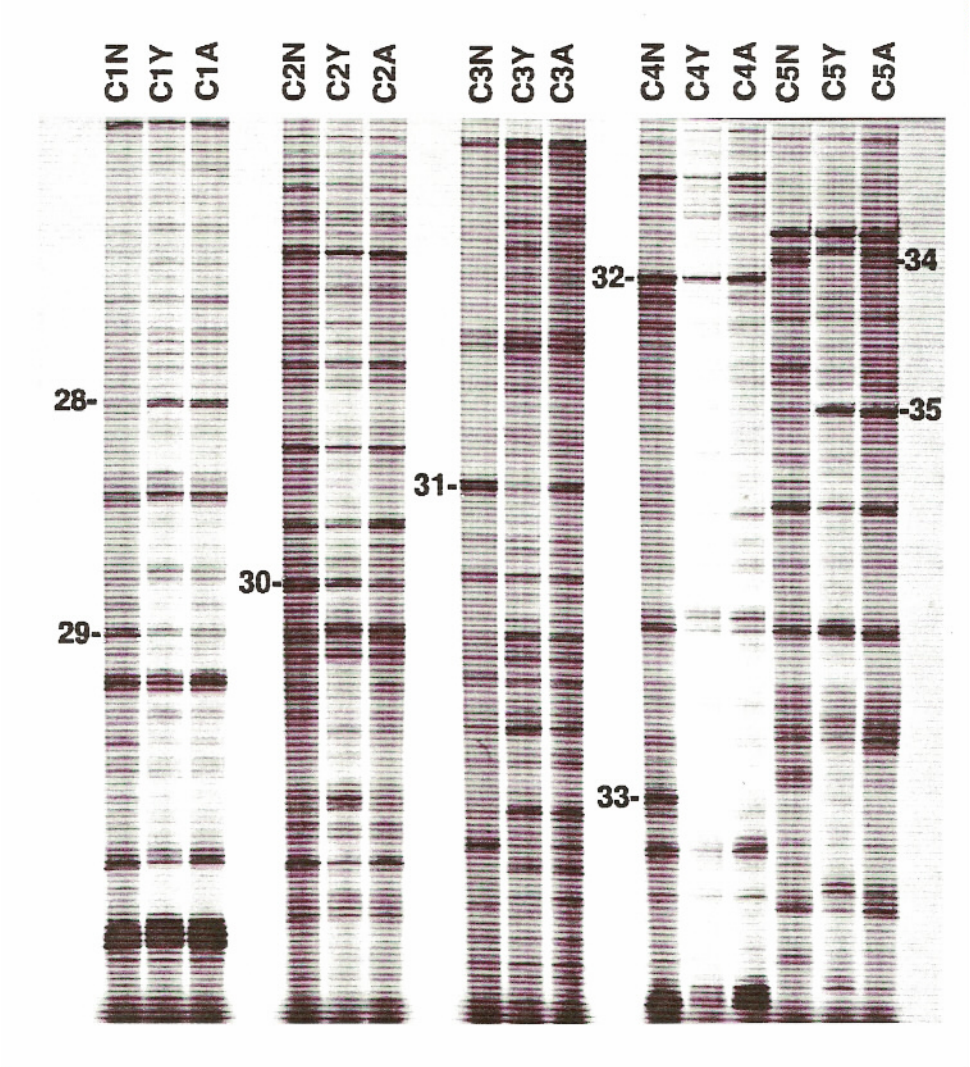


FIGURE-III-7: Differential display (Gel-5) from which the cDNA fragments *JT-28* to *JT-35* were isolated. The 3'-primer used in this gel was T_{12}MC (C). The 5'-primers used were OPA-1 (1), OPA-2 (2), OPA-3 (3), OPA-4 (4) and OPA-5 *(5).* N = *neonate,* Y = *young adult* and A = *aged.* The location of the cDNA bands, *JT-28* to *JT-35* are indicated

The primers used and the expression pattern of the 35 cDNA fragments are shown (**TABLE-III-1**). The cDNA fragments were PCR amplified using the same primer combinations as that used in the differential display reactions, and used as probes for hybridization with RNA from *neonatal, young adult* and *aged* rat brain in **35** separate Northern blotting experiments. However, only seven of these 35 cDNA fragments *(JT-1, JT-4, JT-8, JT-10, JT-18, JT-30 & JT-33)* were confirmed on Northern blotting to be differentially expressed **(FIGURE-III-8).**

THE cDNA FRAGMENTS: PRIMERS & EXPRESSION PATTERN

NUMBER	3'-PRIMER	5'-PRIMER	NEONATE	YOUNG	AGED
JT-1	T_{12}MG	caggcccttc (OPA-1)	+ + + +	+	+
JT-2	T_{12}M0	tgccgagctg (OPA-2)	+	+ + + +	+ + + +
JT-3	T_{12}MG	tgccgagctg (OPA-2)	-	+ + + +	+ + +
JT-4	T_{12}MG	agtcagccac (OPA-3)	+	+ + + +	+
JT-5	T_{12}MG	aggggtcttg (OPA-5)	+ +	+ +	+ + + +
JT-6	T_{12}MA	caggcccttc (OPA-1)	+ + +	-	+
JT-7	T_{12}MA	caggcccttc (OPA-1)	+ + +	-	+
JT-8	T_{12}MA	caggcccttc (OPA-1)	+ + +	-	+
JT-9	T_{12}MA	caggcccttc (OPA-1)	+ + +	-	+
JT-10	T_{12}MA	caggcccttc (OPA-1)	+ + + +	+	+ +
JT-11	T_{12}MA	caggcccttc (OPA-1)	+ + + +	+	+ +
JT-12	T_{12}MA	caggcccttc (OPA-1)	+ +	-	-

THE cDNA FRAGMENTS: PRIMERS & EXPRESSION PATTERN

NUMBER	3'-PRIMER	5'-PRIMER	NEONATE	YOUNG	AGED
JT-13	T_{12}MA	agtcagccac (OPA-3)	+ +	-	-
JT-14	T_{12}MA	aatcgggctg (OPA-4)	+	+ +	-
JT-15	T_{12}MA	aggggtcttg (OPA-5)	+	+	+ +
JT-16	T_{12}MT	caggcccttc (OPA-1)	-	+	+ + +
JT-17	T_{12}MT	caggcccttc (OPA-1)	-	+	+ + +
JT-18	T_{12}MT	caggcccttc (OPA- 1)	-	+ + +	+ + + +
JT-19	T_{12}MT	tgccgagctg (OPA-2)	-	+	+ + +
JT-20	T_{12}MT	tgccgagctg (OPA-2)	+	+ +	+ + + +
JT-21	T_{12}MT	agtcagccac (OPA-3)	-	+ +	+ + + +
JT-22	T_{12}MT	agtcagccac (OPA-3)	-	+	+ + +
JT-23	T_{12}MT	aatcgggctg (OPA-4)	-	+ +	+
JT-24	T_{12}MT	aatcgggctg (OPA-4)	-	+	-

THE cDNA FRAGMENTS: PRIMERS & EXPRESSION PATTERN

NUMBER	3'-PRIMER	5'-PRIMFR	NEONATE	YOUNG	AGED
JT-25	$T_{12}MT$	aatcgggctg (OPA-4)	-	+ +	+ + +
JT-26	TJ_2MT	aggggtcttg (OPA-5)	-	+	+ + + +
JT-27	$T_{12}MT$	aggggtcttg (OPA-5)	-	-	+
JT-28	$T_{12}MC$	caggcccttc (OPA-1)	+	+ + +	+ + +
JT-29	$T_{12}MC$	caggcccttc (OPA-1)	+ +	+	+
JT-30	$T_{12}MC$	tgccgagctg (OPA-2)	+ + + +	+ +	+
JT-31	$T_{12}MC$	agtcagccac (OPA-3)	+ + + +	+	+ +
JT-32	$T_{12}MC$	aatcgggctg (OPA-4)	+ + + +	+ +	+ + +
JT-33	$T_{12}MC$	aatcgggctg (OPA-4)	+ +	-	-
JT-34	$T_{12}MC$	aggggtcttg (OPA-5)	+ +	-	+
JT-35	$T_{12}MC$	aggggtcttg (OPA-5)	+	+ + + +	+ + + +

TABLE-III-1: The cDNA fragments *JT-1* to *JT-35*, primers used for isolation and their expression pattern. Expression is rated on the following arbitrary scale: (-) = no expression, (+) = trace, (+ +) = mild, (+ + +) = moderate, (+ + + +) = abundant.

DIFFERENTIALLY EXPRESSED cDNA FRAGMENTS

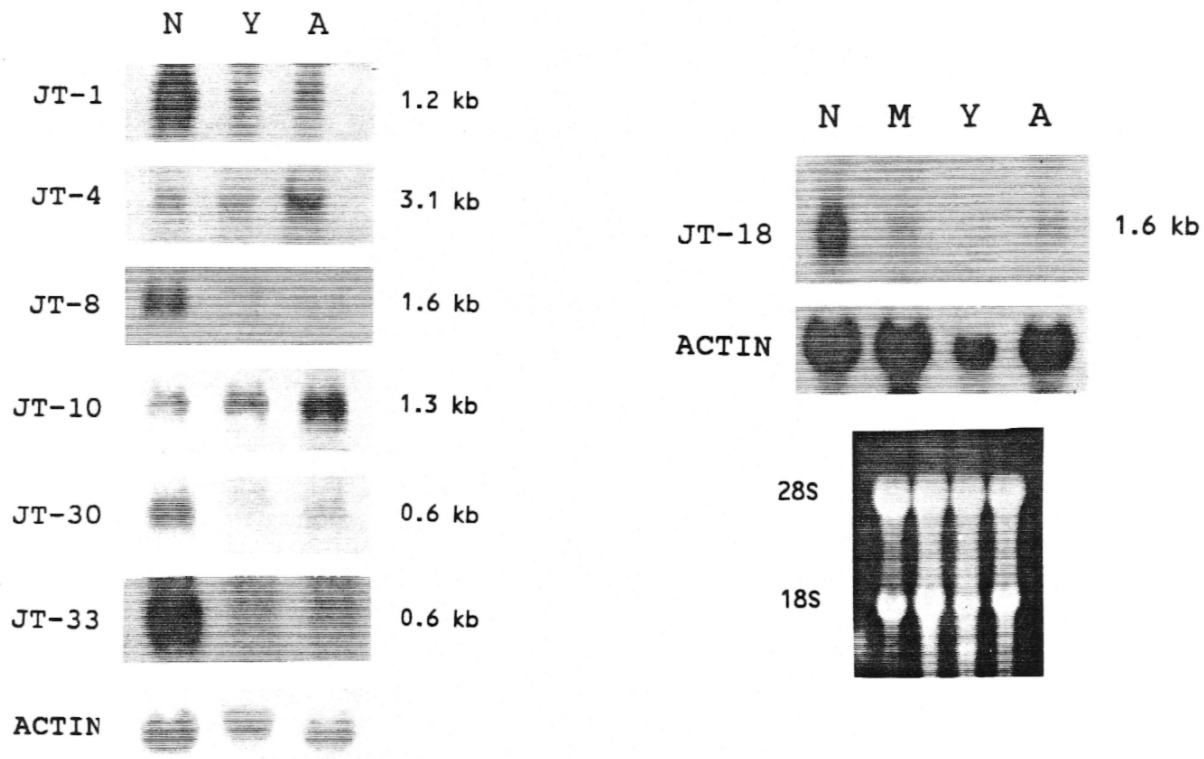


FIGURE-III-8: Northern blotting of the cDNA fragments confirmed to be differentially expressed. Of the 35 separate Northern blots carried out using the probes *JT-I* to *JT-35,* only seven *(JT-1, JT-4, JT-8, JT-10, JT-I8, JT-30 & JT-33)* were confirmed to be differentially expressed. The blots contained RNA (20 μg) extracted from rat brain, N = *neonate* (3 days old), Y = *young adult* (3 months), A = *aged* (33 months), M = mother rat. The sizes of mRNA are indicated in kilobases. Representative ethidium bromide stained gels photographed with UV light are shown. The location of 28S and 18S RNA are indicated.

IIIB-2. Cloning and Sequencing of the cDNA Fragments

The seven cDNA fragments *(JT-1, JT-4, JT-8, JT-10, JT-18, JT-30 & JT-33)* isolated from the differential display gels, and confirmed to be differentially expressed on Northern analysis, were ligated into pCR^{TM} (Invitrogen, San Diego, CA) by TA-cloning (Clark 1988). The cloned vector was used to transform TA One Shot^{TM} competent cells. White colonies were selected for plasmid preparation from plates containing Kanamycin and Bluo-gal (Life Technologies).

The inserts were amplified using SP6 and T7 primers that recognized sequences on either side of the cloning site. The reaction included 1 μg plasmid template, 0.4 μM T7 primer, 0.4 μM SP6 primer, 100 μM dNTP (all four), 1x buffer and 2.5 u Taq polymerase. The reactions were denatured at 94°C for 5 min, and amplified for 35 cycles. Each cycle consisted of denaturation at 94°C for 1 min 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 reactions maintained at 4°C. Twenty five μl of the sample was loaded on 1.5% agarose gel containing 0.1 μg/ml ethidium bromide, electrophoresed using 1x Tris-acetate buffer and photographed with UV light. In the absence of an insert, the predicted size of the amplified product was 188 bp, the length of the vector sequence between the SP6 and T7 sites. Clones containing inserts *(JT-1, JT-4, JT-8, JT-10, JT18, JT-30 & JT-33)* exhibited larger sized products (**FIGURE-III-9; TABLE-III-2**). Based on the PCR results, the sizes of the cDNA inserts are shown.

PCR AMPLIFICATION OF THE cDNA FRAGMENTS

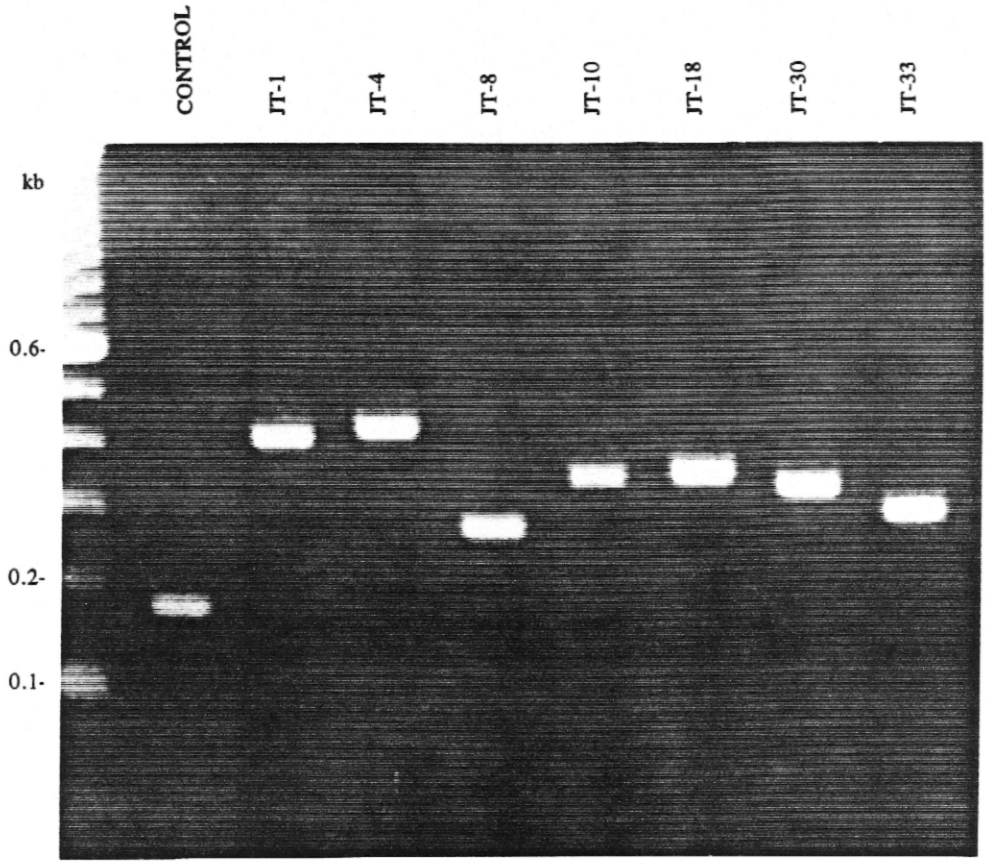


FIGURE-III-9: PCR amplification of the differentially expressed cDNA fragments. The cDNA fragments, *JT-J*, *JT-4*, *JT-8*, *JT-10*, *JT-18*, *JT-30 & JT-33*, confirmed to be differentially expressed on Northern blotting, were TA-cloned into pCR^{TM} and sequenced using T7 or SP6 primers located on either side of the cloning site. A 100 bp DNA ladder was the marker used. kb = kilo base pairs.

SIZES OF THE cDNA FRAGMENTS

NUMBER	VISUALIZED SIZE (bp)	SIZE OF CONTROL (bp)	ESTIMATED SIZE OF cDNA FRAGMENT (bp)
JT-1	400	188	212
JT-4	400	188	212
JT-8	250	188	62
JT-10	350	188	162
JT-18	350	188	162
JT-30	300	188	112
JT-33	250	188	62

TABLE-III-2: The sizes of the differentially expressed cDNA fragments shown in FIGURE-III 9. In the absence of any insert, the size of the product was estimated to be 188 bp, the region between T7 and SP6 sites in the pCR^{TM} vector. The estimated size of the cDNA fragments was the difference between the visualized size and the control size.

IIIB-3. Sequencing of *JT-1, JT-4, JT-8, JT-1O, JT-18, JT-30 & JT-33*

Double stranded sequencing of the cloned cDNA inserts were carried out using Sequenase Version 2.0 (United States Biochemicals, Cleveland, Oil). Five μg of template was denatured, annealed with T7 or SP6 primers, labelled with ^{35}S-dATP and extended by the chain termination method (Sanger et al 1977). Only partial sequencing of each of these fragments was carried out. As would be expected, the partial sequences of all seven cDNA fragments contained one of the two primers used in the differential display reaction (**FIGURE-III-10**).

The seven cDNA sequences *(JT-1, JT-4, JT-8, JT-10, JT-18, JT-30 & JT-33)* were compared for homology in the GenBank database both in the forward and inverse orientations (Benson et al 1993). Five of these cDNA fragments, *JT-1, JT-4, JT-8. JT-10 & JT-30,* had novel nucleotide sequences without homology in the database. Of these, three *[JT-I (U05219), JT-8* (U05225) and *JT-30* (U05222)] were selectively expressed in the neonatal brain, and, two *[JT-4* (U05224) and *JT-10* (U05220)] were mainly expressed in the aged brain. These findings are consistent with the earlier findings seen on differential display. The GenBank accession numbers are shown in parenthesis. Clones *JT-I8* (U05221) and *JT-33* (U05223) exhibited homology to existing GenBank sequences.

The *JT-18* sequence exhibited 81% homology to *F_1-ATP synthase β-subunit* (**FIGURE-III-11**). *F_1-ATP synthase β-subunit* is a part of the catalytic region of the *F_0F_1-ATP synthase* enzyme complex, essential for ATP synthesis (Boyer 1989). Although *F_1-ATP synthase* activity

THE PARTIAL cDNA SEQUENCES

JT-1 (U05219)

Primers Used: T_{12}MG/CAGGCCCTTC (OPA- 1)
Complementary: CNA_{12}/GAAGGGCCTG
Estimated Product Size: 212 bp

ACACTGAGCATGAGAGATGTGTATCCTTTAGCCACAGCTCCAGAGCGAGGCTAAGG
AGCTTGGCTTTGCTTTCTGTTGTCGTCTGTTGTGCCACCC**CCAAAAAAAAAAAA**

JT-4 (U05224)

Primers Used: T_{12}MG/AGTCAGCCAC (OPA-3)
Complementary: CNA_{12}/GTGGCTGACT
Estimated Product Size: 212 bp

AGTCAGCCACTATGAGGAGGGTCGGGAAGAATTTGCGTTTTCAGTGGAAACA
AGTGCGTACTCTATCGATGACTGTGATTGACTCGATCT

JT-8 (U05225)

Primers Used: T_{12}MA/CAGGCCCTTC (OPA-1)
Complementary: TNA_{12}/GAAGGGCCTG
Estimated Product Size: 62 bp

ACATTAAGGAATAATTTTTCCAATTGTTAAAAACAGTTTGAAGCGGTTCCTACTGGG
GCTCGTGGG**GAAGGGCCTG**

JT-10 (U05220)

Primers Used: T_{12}MA/CAGGCCCTTC (OPA-1)
Complementary: TNA_{12}/GAAGGGCCTG
Estimated Product Size: 162 bp

TGATCGTCAGCTGCATGAATGACAGACTGTCATGACTATCCCTGTCGACTGCACCAG
TCTCGCCCTCTCACGTCCTTGGCGCTATGAGGGCACATGTTGAATCACAGTAAATTA
TTTGATGG**TCAAAAAAAAAAAA**

THE PARTIAL cDNA SEQUENCES

JT-18 (U05221)

Primers Used: T_{12}MT/CAGGCCCTTC (OPA-1)
Complementary: ANA_{12}/GAAGGGCCTG
Estimated Product Size: 162 bp

CTCTGGGGCTCGTTTGCCTGTGGCCACAGAGCCTTGATTGAAGATGTGATGTTCTCTC
TGAAGAGTATTTAAAGTTTTCAATAAAGTATATACCC**ACAAAAAAAA**

JT-30 (U05222)

Primers Used: T_{12}MC/TGCCGACTG (OPA-2)
Complementary: GNA_{12}/CAGTCGGCA
Estimated Product Size: 112 bp

TGTACACTTAAGTTTTCTGTACATAAATATAATTACAAAGTTATCTTCCTTCTTTGGT
TCTTTGGGTTGCTGGTC**GGAAAAAAAA**

JT-33 (U05223)

Primers Used: T_{12}MC/AATCGGGCTG (OPA-4)
Complementary: GNA_{12}/CAGCCCGATT
Estimated Product Size: 62 bp

GGGCTGCCAAATTTCACCGGTTTGCCCAGGGATATTATAGAAAATTATTTGTATGAT
GATGAAATAAACACACCCGGC

FIGURE-III-10: Partial sequences of the differentially expressed cDNA fragments. *JT-1, JT-4, JT-8, JT-10, JT-18, JT-30 & JT-33* were the cDNA fragments sequenced. The primers used to isolate the fragment, and the complementary sequence of the primers, are shown. As only partial sequencing was carried out, the location of one primer was identified in each sequence, and this is underlined. The GenBank accession numbers are shown in parenthesis.

HOMOLOGY OF *JT-18* TO *F_1-ATP SYNTHASE β SUBUNIT*

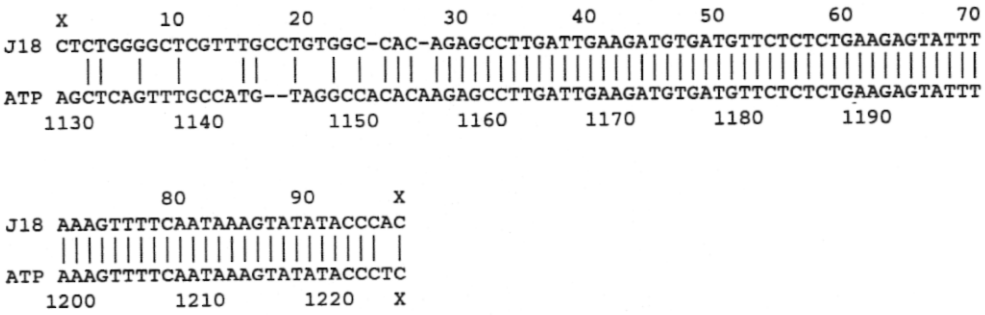


FIGURE-III-11: Homology of *JT-18* to *F_1-ATP synthase β subunit.* The *JT-18 (J18)* cDNA sequence exhibited 81% homology with *F1-ATP synthase β subunit (ATP)* (Locus: RATATPASEF).

may decrease during senescence (Guerrieri et al 1992), it is unclear whether there is a mechanistic relationship (Valearce et al 1988; Izquierdo et al 1990). The *JT-33* sequence showed 89% homology with *thymosin β-10* gene (**FIGURE-III-12**). *Thymosin β-10 is* an actin binding protein that is known to be strongly expressed during embryonic brain development (Hall et al 1991; Lin & Morrison-Bogorad 1991; Lugo et al 1991). In the Northern hybridization studies described earlier, both *JT-18* and *JT-33* were selectively expressed in the neonatal brain. This difference in expression was particularly evident when compared to aged brain samples (**FIGURE-III-8**).

IIIB-4. Comments on Differential Display

Using the differential display technique, five cDNA fragments without homology in the GenBank database were isolated. As these fragments represent only partial sequences of the 3'-untranslated regions of mRNAs, it was necessary to isolate their full length sequences. At this stage in the experimentation, it did not matter which full length sequence was isolated, as they were all confirmed to be differentially expressed. Therefore, the five "novel" sequences *(JT-1, JT-4, JT-8, JT-10 & JT-30)* were pooled, and the mixture used to probe the rat brain library that was prepared. In order to maximize the chances of obtaining the full length sequence of the fragments, the library was constructed by mixing mRNA from *neonatal, young adult* and *aged* rat brain, the same samples that were used for the differential display reactions.

HOMOLOGY OF *JT-33* TO *THYMOSIN β-l0*

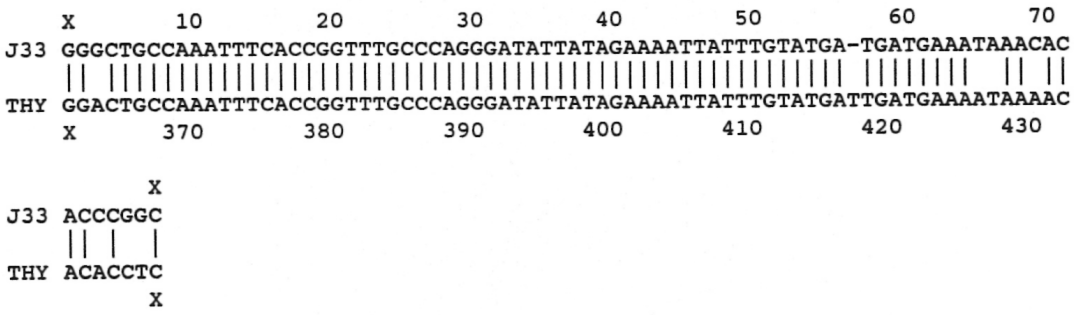


FIGURE-III-12: Homology of *JT-33* to *thymosin β-10*. The *JT-33 (J33)* cDNA sequence exhibited 89% homology with rat *thymosin β-10* mRNA *(THY)* (Locus: RATTHYB10).

IIIC. THE cDNA SEQUENCE OF *NEURONATIN*

IIIC-1. Screening of the Library and Isolation of *Neuronatin*

The novel cDNA fragments *(JT-J, JT-4, JT-8, JT-J10 & JT-30)* were pooled and used to screen a rat brain cDNA library prepared by mixing mRNA from neonatal, young adult and aged rat brain as described in **SECTION-IIE**. The pooled probe was labeled with ^{32}P-dCTP by the random primer method to an activity of 10^8-10^9 cpm/μg DNA. The labeled probe was used to screen about 25000 plaques at 73°C in a solution containing 3x SSC, 1 M NaCl, 50x Denharts, 10% SDS and 5 mg/ml salmon sperm DNA. Hybridization was continued overnight, the membranes washed in a solution containing 0.1% SDS and 0.2x SSC at room temperature for 2 hours and exposed to film for about 16 hours. Nine plaques showed strong hybridization. These nine plaques *(#P-2 to P-10)* were used to prepare DNA, and PCR amplified using λgt11 forward and reverse primers that hybridized to vector sequences on either side of the cloning site. Also included as a negative control was a tenth plaque *(P1)* that did not show hybridization. Of these, four plaques *(#P-3, P-4, P-8 & P-9)* generated products of about 1 kb, corresponding to the size of the mRNA species detected in the Northern blot using the partial cDNA sequences (**FIGURE-III-8**). Two of these plaques *(P-3 & P-8)* were smaller, and the other two *(P-4 & P-9)* were larger.

These four clones *(P-3, P-4, P-8 & P-9)* were sequenced in both directions using the *fMol*^{TM} *Sequencing System* (Promega, Madison, WI). All four clones had identical 5'- and 3'-

untranslated regions, and the same type and location of translation initiation (GAACCATGG) (Kozak 1991), stop (TGA) and termination (AATAAA) signals (Wahle & Keller 1992). Two of these four clones were identical, including having a coding region of 162 nucleotides, similarly, the remaining two clones were also identical with each other with a coding region of 243 nucleotides. Therefore, it was possible to confirm the cDNA sequences for the longer, *neuronatin-α,* and shorter, *neuronatin-β,* isoforms by sequencing two separate clones for each form of *neuronatin (nnn)* **(FIGURE.-III-13)**. As these novel mRNA isoforms were isolated from neonatal rat brain, they were named *neuronatin* (neuronal-neonatal). The restriction map for *neuronatin- α* was generated, and some sites are shown **(FIGURE-III-14).** This was confirmed using four enzymes, EcoRI and NotI did not digest *neuronatin-α,* NcoI generated four fragments, and digestion with XhoI yielded two fragments of 556 and 639 bp.

The only difference between the two forms of *neuronatin* was confined to a portion of the coding region. *Neuronatin-α* had an additional 81 bp sequence inserted into the coding region. It is of interest to note that this additional stretch of cDNA had the prototypical features of an intron (Lewin 1994), including the consensus sequences GT at the 5'-end, AG at the 3'-end, and the branch site, TCGAAAT, located 33 nucleotides upstream of the 3'-end. Although, exhibiting characteristics of an intron, and absent in *neuronatin-β,* this segment appeared to be the middle exon of *neuronatin-α.* Therefore, *neuronatin-α* may possess three exons, the first exon from position 1 to 72 (72 nt, 24 aa); the third exon from position 73 to 162 (90 nt, 30 aa); and the second (middle) exon was inserted between positions 72 and 73, and consisted of the 81 bp sequence coding for 27 amino acids.

THE cDNA SEQUENCE OF ***NEURONATIN***

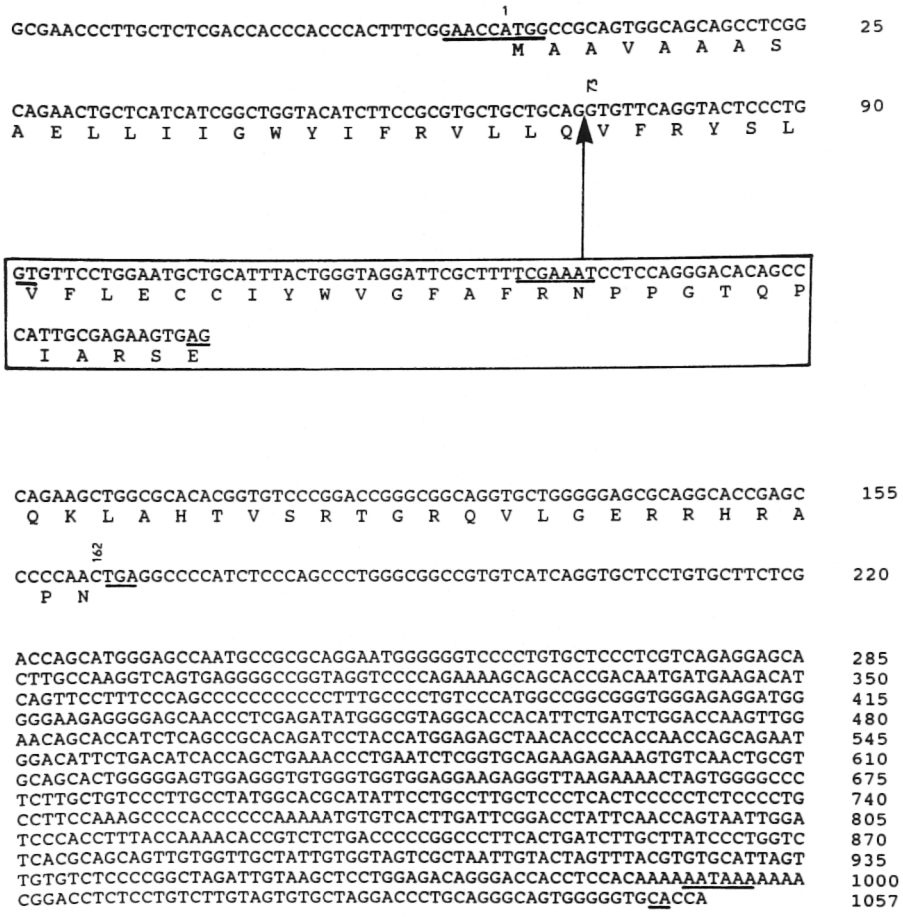


FIGURE-III-13: The *neuronatin* cDNA sequence. The cDNA sequences for the two alternatively spliced forms of *neuronatin*, *α* and *β*, are shown. Based on the cDNA sequence, *neuronatin-α* has three exons consisting of 72, 81 and 90 nucleotides. *Neuronatin-β* was identical to the *α*-form, except that it lacked the 81 bp middle exon (box). *Neuronatin-α* contained the 81 bp middle exon which exhibited prototypical features of an intron including consensus GT-AG, and a branch site, TCGAAAT, located 33 nucleotides upstream of the 3'-end (underlined). Also underlined are the translation start (GAACCATGG), stop (TGA) and termination (AATAAA followed by CA) signals. The numbering begins with the methionine start codon. The GenBank accession number for *neuronatin-α* is U08290, and for *neuronatin-β* U09785.

THE RESTRICTION MAP OF *NEURONATIN*

ENZYME SITES

AluI:	218 642 687 1082
ApaI:	796
BamHI:	925
BcgI:	77
BglI:	51
BsaI:	995
BscBI:	38 270 290 354 379 380 427 437 575 793 794 927 1097 1153
BstBI:	155
DdeI:	284 614
DpnI:	587 627 927 974
EcoRII:	118 164 303 985 1084
FokI:	545
HaeIII:	45 289 312 428 516 794 963
HincII:	724
HindII:	724
HinfI:	145 698 899
HpaII:	236 241 429 517 960 1067
MaeI:	786 1037 1071 1148
MaeII:	1044
MaeIII:	891
MboI:	585 625 925 972
MboII:	86 477 552 723 781
NaeI:	518
NcoI:	40 511 633
PstI:	112 214 1160
PvuII:	687
RsaI:	90 204 1035
Sau961:	238 288 378 426 435 591 792 793 904 962 1096 1124 1151
SduI:	278 329 390 407 796 1176
SpeI:	785 1036
TaqI:	16 155 340 557
XhoI:	556
XhoII:	625 925
XmaIII:	310

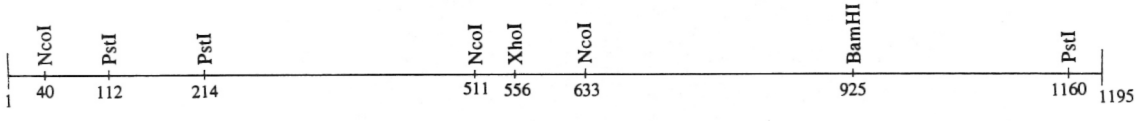


FIGURE-III-14: Some restriction enzyme sites for *neuronatin-α* (GenBank #U08290).

Although, *neuronatin-β* does not have the middle exon, it maintained the same open reading frame as that in *neuronatin-α*, suggesting that the two forms are alternatively spliced. The GenBank accession number for *neuronatin-α* is U08290, and that for *neuronatin-β* is U09785 (Joseph et al 1995a).

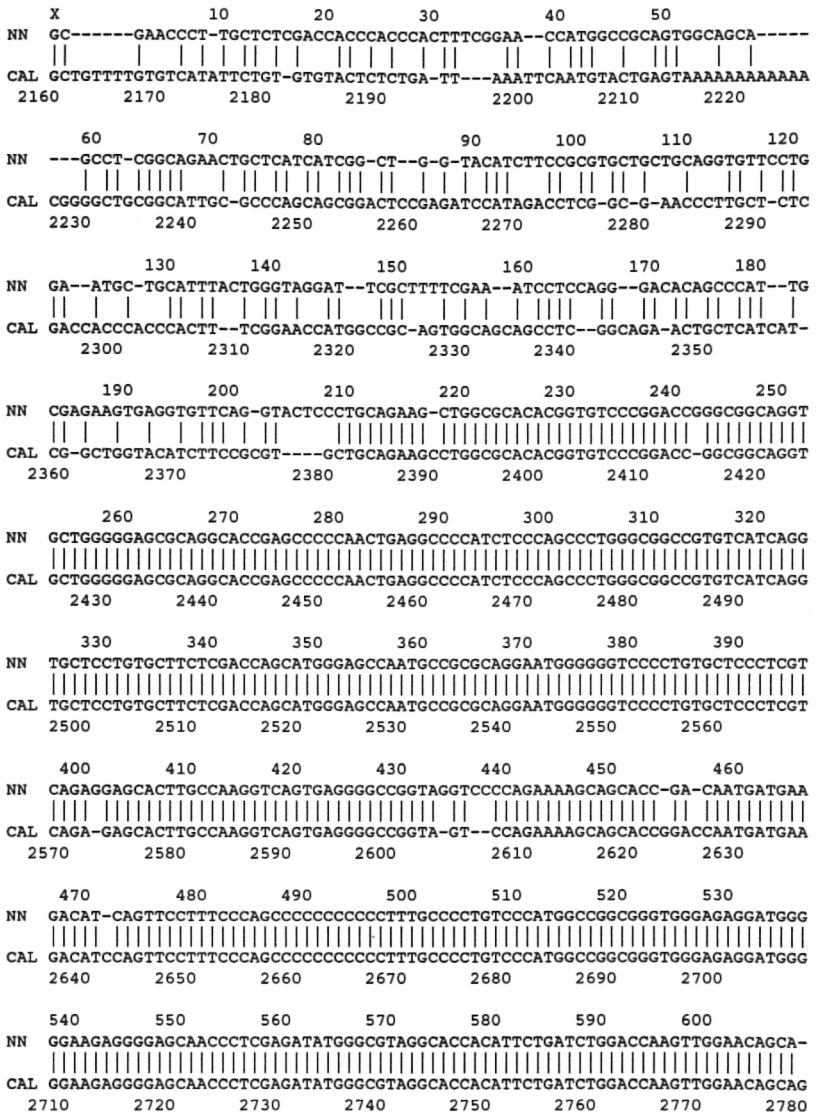
IIID. GENBANK ANALYSIS OF *NEURONATIN*

IIID-1. The Relationship of *Neuronatin* to *Calbindin-d28k*

Neuronatin-α cDNA was compared for homology in the GenBank database using Intelligenetics and the BLAST server (Altschul et al 1990). When the full length of *neuronatin-α* was analyzed, there were no completely homologous sequences in the database. However, there was 88% homology to rat *calbindin-d28k* (Locus: RATCALBDF, GenBank #M27839) (Lomri et al 1989). The region of homology involved mainly the 3'-untranslated region of *neuronatin-α* and part of the 3'-untranslated region of the Lomri et al rat *calbindin-d28k.* The entire 5'-untranslated region and the first 208 nucleotides (out of 243) of the deduced coding region of *neuronatin-α* were non-homologous and unrelated to *calbindin-d28k* (**FIGURE-III-15** and **TABLE-1II-3**).

Importantly, the 81 bp putative middle exon of *neuronatin-α* had no homology to any form of *calbindin-d28k,* including the Lomri et al sequence, or with any other sequence in the GenBank database (**Search done on June 30, 1994**). Furthermore, the coding regions for *neuronatin-α* and *calbindin-d28k* were separated by 746 bp, and there was no homology between *neuronatin-a* and any of the several other forms of *calbindin-d28k* present in GenBank. In contrast, the Lomri sequence was highly homologous with the other forms of *calbindin-d28k.* In conclusion, *neuronatin* and *calbindin-d28k* are shown to be two different genes.

COMPARISON OF *CALBINDIN-D28K* TO *NEURONATIN*



COMPARISON OF *CALBINDIN-D28K* TO *NEURONATIN*

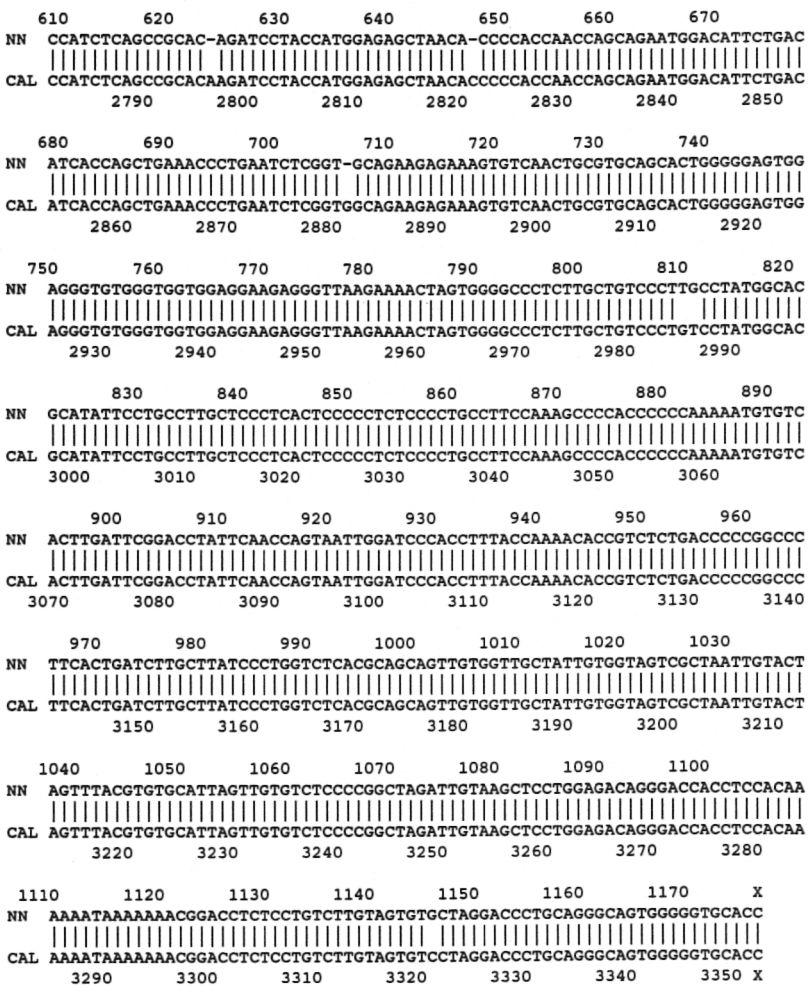


FIGURE-III-15: Comparison of *calbindin-d28k* and *neuronatin* cDNA sequences. GenBank analysis of *neuronatin* revealed no completely homologous sequence. However, the 3'-untranslated region of *neuronatin-α (NN)* matched with part of the 3'-untranslated region of one form of *calbindin-d28k* (Locus: RATCALBDF, GenBank #M27839) *(CAL).* The details of the homology are provided in TABLE-III-3. The entire 5'-untranslated region, and the first 208 nucleotides (out of 243) of the coding region of *neuronatin-α* were not homologous to *calbindin-d28k.* When a separate GenBank analysis was carried out with only the 81 bp middle exon of *neuronatin,* no match was identified in the database.

DIFFERENCES BETWEEN *CALBINDIN-D28K* AND *NEURONATIN*

	Neuronatin-a	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)

TABLE-III-3: Comparison of *calbindin-d28k* and *neuronatin* cDNA sequences. Note that the coding regions of *neuronatin* and *calbindin-d28k* are separated by 746 bp. It is inferred that *neuronatin* is not a homolog of *calbindin-d28k.*

IIID-2. The Calbindin-d28k Sequence: RATCALBDF

Lomri et al (Locus: RATCALBDF, GenBank #M27839) used a mixture of three 20-mer synthetic oligos, S_1, S_2 and S_3, derived from the cDNA sequence of rat *spot-35* cDNA (Yamakuni et al 1987), a calcium binding protein *(calbindin-d28k)*, to screen a commercially obtained rat brain cDNA library (Clontech, Palo Alto, CA). One positive clone, *pC28,* was isolated and sequenced. The total length of *pC28* was 3370 nucleotides (3352 without the poly(A) tail). The longest open reading frame was 783 nucleotides encoding 261 amino acids, with homology to *spot-35.* The 5'-UTR extended 525 nucleotides (A_1-fragment) upstream from the 5'-end of *spot-35* cDNA. The 3'-UTR extended 1145 nucleotides (A_2-fragment) downstream from the 3'-end of *spot-35* cDNA. Therefore, the following segments of *pC28* were studied:

1-525: A_1-fragment
526-2226: A_4-fragment
2227-3370: A_2-fragment

A_3-fragment consisted of the A_4 plus A_2-fragments.

The 526-2226 fragment was homologous to *spot-35* cDNA and *calbindin-d28k,* and on Northern analysis hybridized to three mRNA species, a major species at 1.9 kb, and, two minor species at 2.8 and 3.2 kb. Lomri et al proceeded to determine whether the A_1 and A_2-fragments were spliced forms of *calbindin-d28k.* The A_1-fragment hybridized a 1.1 kb mRNA which was not detected when *spot-35* cDNA was used as the probe. Therefore, Lomri et al reasoned that A_1-fragment was a cloning artifact resulting from an unrelated piece of cDNA being ligated to

the 5'-end of the cDNA during library construction.

Next they investigated the A_2-fragment. Based on the observation that chicken *calbindin-d28k* had three mRNA species, 3.1, 2.8 and 2.0 kb, resulting from the presence of three polyadenylation signals in the 3'-UTR (Flunziker 1986; Hunziker & Schrickel 1988), Lomri et al predicted that full length *pC28* cDNA should be a copy of the longest *calbindin-d28k* mRNA. To test this hypothesis they used the A_2-fragment as a probe in Northern analysis. Only a 1.3 kb mRNA was detected which was not the size of the rat *spot-35* mRNA (Yamakuni et al 1987). Therefore, *pC28* cDNA was not a copy of the largest *calbindin-d28k* mRNA. As this 1.3 kb mRNA species detected by the A_2-fragment was found only in the nervous system, the suggestion was made that it may have resulted from alternative splicing occurring only in the nervous system. However, this too was deemed unlikely for several reasons. First, another *pC28* fragment named A_3 (which is a cDNA copy of the mature mRNA) hybridizes not only to *spot-35* mRNA but also to the smaller 1.3 kb mRNA. Second, the A_2-fragment hybridizes only the 1.3 kb mRNA. In the case of alternative splicing, the A_2 probe should hybridize the larger *spot-35* mRNA (3.2 kb).

Therefore, Lomri et al inferred that the A_2-fragment may have also resulted from a similar cloning artifact. It is generally believed that cloning artifacts are rare; therefore, it is surprising that two such events should have occurred in *pC28*. Hence, Lomri et al advises the need to carry out both sequencing and Northern analyses to characterize all cDNA clones isolated from libraries. The possibility that the A_1 and A_2-fragments were novel genes was not

considered. The full length of *pC28* sequence is retrieved from the GenBank database as rat *calbindin-d28k* (#M27839).

IIID-3. The Calbindin-d28k Sequence: EST #06992

On GenBank analysis, the *Expressed Sequence Tag (EST #06992),* isolated by Adams et al (GenBank #T09099) is also retrieved as being 97% homologous to the Lomri et al *calbindin-d28k* sequence (Locus RATCALBDF; GenBank #M27839) (Adams et al 1993). This homology led Adams et al (1993) to conclude that *EST #06992* was the human homolog of *calbindin-d28k.* However, on further analysis it is determined that the region of homology between *EST #06992* and that of *calbindin-d28k* is confined to the A_2-fragment, between nucleotides 2318 and 2389. However, *EST #06992* also exhibited homology to *neuronatin-α* **(FIGURE-III-16).** Although, *EST #06992* is only 414 bp long and not characterized, it appears to be a homologue of *neuronatin* and not *calbindin-d28k.*

IIID-4. The Significance of the Homology

Although, *neuronatin* and *calbindin-d28k* are different genes, the question remains as to the relevance of the observed homology. The 3'-untranslated region of *neuronatin* is nearly totally homologous to the A_2-fragment of the Lomri et al sequence. Although, the 81 bp middle exon of *neuronatin* was absent in the Lomri sequence, part of the coding region also showed some homology. Therefore, the A_2-fragment of the Lomri et al sequence is most likely not a

COMPARISON OF *EST #06992* AND *NEURONATIN*

CAGCGGACTCCGAGACCAGCGGATCTCGGCAAACCCTCTTTCTCGACCACCCACCTA

CCATTCTTGGAACC***ATGGCGGCAGTGGCGGCGGCCTCGGCTGAACTGCTCATCATCG***

GCTGGTACATCTTCCGNGTGCTGCTGCAGGTGTTCAGGTACTCCCTGCAGAAGCTGGC

ATACACGGTGTCGCGGACCGGGCGGCAGGTGTTGGGGGAGCGCAGGCAGCGAGCCCC

CAACTGAGGCCCCAGCTCCCAGCCCTGGGCGGCCGTATCATCAGGTGCTCCTGTGCA

TCTNGGCCAGCACGGGAGCCAGTNCCGCGCAGGAATNTGGGGTCCCCTGTGTTCCCT

CGCCAGAGGAGNCACTTTGCAAGGTCAGTTGAGGGGCCAGTAGACCCCCGGAGAAGC

AGTAACCGACAATT

FIGURE-III-16: The sequence *of EST #06992* (GenBank #T09099) with its region of homology to *neuronatin* cDNA. *EST #06992* is *a* 414 bp expressed sequence tag isolated by Venter and colleagues as being a homolog of *calbindin-d28k* (Locus: RATCALBDF, GenBank #M27839). The region *of* homology with *neuronatin* is underlined.

part of *calbindin-d28k,* and instead, it is a homologue of *neuronatin.* This conclusion would mean that *EST #06992* (T09099) is also not *calbindin-d28k,* but instead a part of the cDNA of human *neuronatin.*

IIIE. THE cDNA FRAGMENTS *& NEURONATIN*

The next issue was to determine which of the five cDNA fragments *(JT-I, JT-4, JT-8, JT-10 & JT-30)* hybridized *neuronatin* cDNA. For this purpose, each of the cDNA fragments were random prime labeled and used to carry out five separate Southern blots. Each blot contained DNA from the nine positive clones *(P-2 to P-10)* obtained when the rat brain library was screened with the *mixed probe.* These were the clones used to sequence *neuronatin* cDNA. The clones, *P-2* to *P-10,* hybridized strongly to the *mixed probe.* Clone *P-1* did not hybridize the *mixed probe* and is included as a negative control.

When tested individually, the *JT-J* sequence hybridized to all nine positive clones *(P-2* to *P-10),* but not *P-1,* the negative control (**FIGURE-III-17**)**.** The remaining four cDNA fragments *(JT-4, JT-8, JT-10 & JT-30)* did not hybridize any of the clones. Therefore, *JT-I* is the cDNA fragment that lead to the isolation of *neuronatin.* Thereafter, λgt11 forward and reverse primers, flanking the cloning site, were used to amplify the inserts in these 10 clones, and Southern analysis carried out using *JT-1* as the probe (**FIGURE-III-18**). As would be expected based on the results shown in **FIGURE-III-17**, *JT-I* hybridized to products from clones *P-2 to P-10,* but not *P-1.* This confirms that *JT-1* is the cDNA fragment that hybridized *neuronatin.* Based on size comparison to the mRNA species, clones *P-3* and *P-8,* when sequenced, lead to the identification of *neuronatin-β* cDNA, and clones *P-4* and *P-9* contained *neuronatin-α* cDNA.

JT-1 HYBRIDIZES PLAQUES *P-2* TO *P-10*

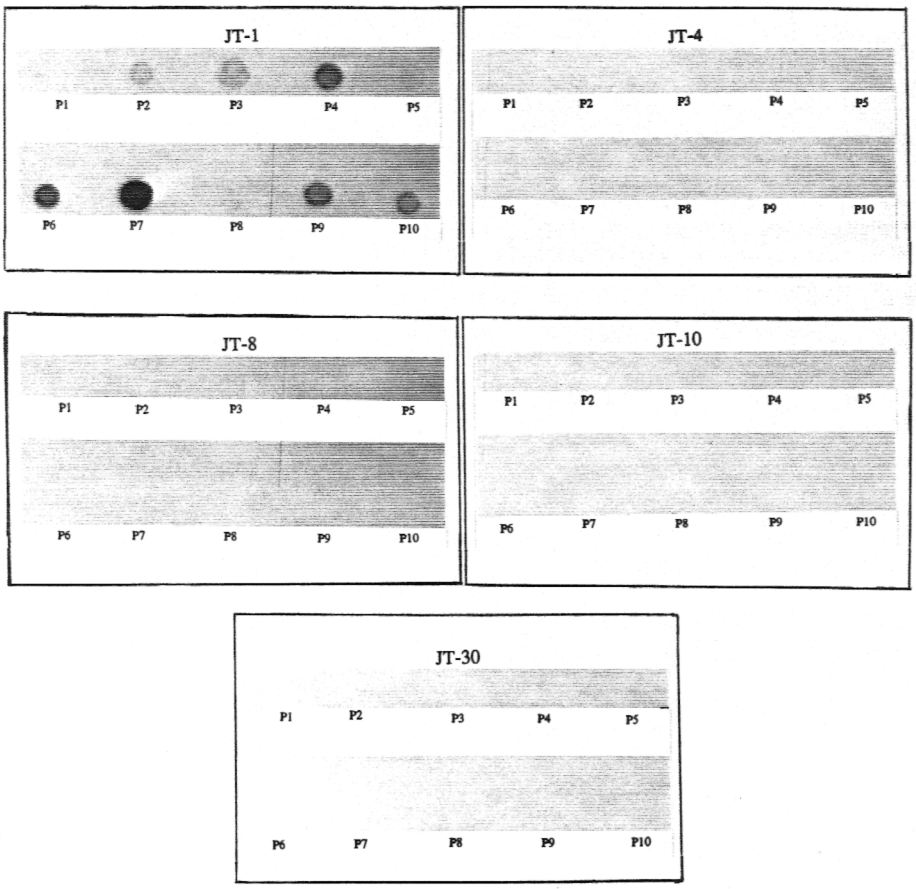


FIGURE-III-17: Determination of the cDNA fragment that hybridizes *neuronatin.* Separate Southern blots were carried out using the five novel cDNA fragments *(JT-1, JT-4, JT-8, JT-10* & *JT-30)* as probes. When the rat brain cDNA library was screened with a mixture of these five cDNA fragments, nine positive plaques *(P-2 to P-10)* were isolated. These nine plaques, *P-2 to P-10,* together with a negative plaque *(P-1),* were placed on five separate membranes, and hybridized to each of the five probes. Only *JT-1* hybridized to plaques *P-2 to P-10,* none of the other probes hybridized. The findings indicate that *JT-1* is the cDNA fragment that leads to the isolation of *neuronatin.*

JT-1 HYBRIDIZES PCR PRODUCTS OF PLAQUES *P-2* TO *P-10*

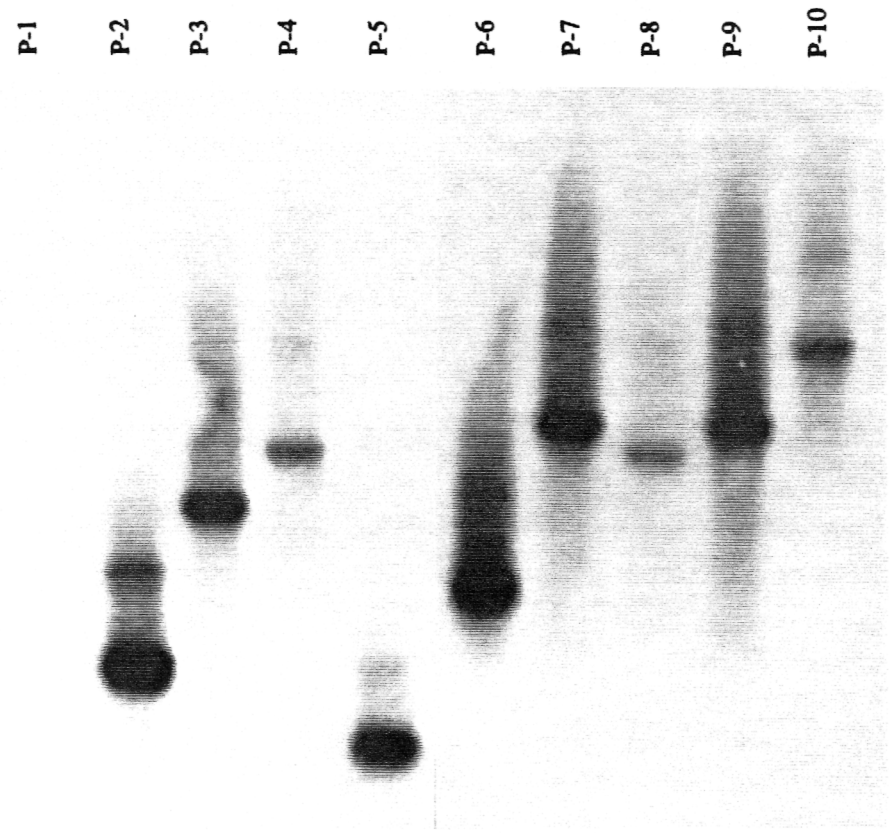


FIGURE-III-18: *JT-I* hybridizes PCR products derived from plaques *P-2* to *P-10,* studied by Southern blotting. PCR amplification was carried out using DNA from plaques *P-1 to P-10* as template, and primers flanking the cloning site of λgt11. The membrane was hybridized with *JT-1*. As noted in FIGURE-III-17, *JT-1* hybridized with *P-2* to *P-10,* but not with *P-1,* the negative control. *P-4* and *P-9* were used to sequence *neuronatin-α,* and *P-3* and *P-8* were used to sequence *neuronatin-β.*

These results would imply that the *JT-I* sequence should be homologous to *neuronatin* cDNA. To investigate this, the *JT-I* clone was fully sequenced. *JT-1* cDNA fragment was 224 bp long, and the two primers used for its isolation in the differential display reaction, $T_{12}GG$ at the 3'-end and CAGGCCCTTC (OPA-1) at the 5'-end, were located. Surprisingly, however, there was no homology between the sequences of *JT-1* and *neuronatin.* This simply did not appear right. On the one hand, the *JT-I* probe hybridized strongly to *neuronatin* cDNA, on the other hand, the sequences did not exhibit homology!

For a long time the basis for this incompatibility was not understood. However, based on an analysis of the 3'-untranslated region of *neuronatin,* the reason seems apparent. The 3'-untranslated region of *neuronatin* cDNA actually contains sequences that will hybridize the same primers, $T_{12}GG$ and CCAGGCCCTTC (OPA-1), as that used for generating the *JT-1* cDNA fragment (**FIGURE-III-19**). Only the *A* in the OPA-1 sequence is replaced by *C* in *neuronatin.* The primer $T_{12}GG$ is located at the 3'-end of *neuronatin* and the primer CCAGGCCCTTC is located at position 838 bp of the *neuronatin* cDNA sequence (GenBank #U09785). Therefore, the predicted product sizes using these primers would be 231 bp when *neuronatin* cDNA is used as template and 224 bp when *JT-1* is used as template.

To confirm this, a PCR reaction was carried out under conditions identical to that used in the original differential display reaction. The same primers, $T_{12}MG$ (M = U + A + C) and CAGGCCCTTC (OPA-1) were used in two separate reactions, one containing *JT-1* as template, and the other containing *neuronatin-α* cDNA as template. The PCR conditions, identical to that

COMPARISON OF *JT-1 & NEURONATIN* SEQUENCES

Neuronatin cDNA SEQUENCE

```
GCGAACCCTTGCTCTCGACCACCCACCCACTTTCGGAACC-ATG-GCC-GCA-GTG-GCA-GCA-
                                           M   A   A   V   A   A

GCC-TCG-GCA-GAA-CTG-CTC-ATC-ATC-GGC-TGG-TAC-ATC-TTC-CGC-GTG-CTG-
 A   S   A   E   L   L   I   I   G   W   Y   I   F   R   V   L

CTG-CAG-GTG-TTC-CTG-GAA-TGC-TGC-ATT-TAC-TGG-GTA-GGA-TTC-GCT-TTT-
 L   Q   V   F   L   E   C   C   I   Y   W   V   G   F   A   F

CGA-AAT-CCT-CCA-GGG-ACA-CAG-CCC-ATT-GCG-AGA-AGT-GAG-GTG-TTC-AGG-
 R   N   P   P   G   T   Q   P   I   A   R   S   E   V   F   R

TAC-TCC-CTG-CAG-AAG-CTG-GCG-CAC-ACG-GTG-TCC-CGG-ACC-GGG-CGG-CAG-
 Y   S   L   Q   K   L   A   H   T   V   S   R   T   G   R   Q

GTG-CTG-GGG-GAG-CGC-AGG-CAC-CGA-GCC-CCC-AAC-TGAGGCCCCATCTCCCAGCCC
 V   L   G   E   R   R   H   R   A   P   N

TGGGCGGCCGTGTCATCAGGTGCTCCTGTGCTTCTCGACCAGCATGGGAGCCAATGCCGCGCAGG
AATGGGGGGTCCCCTGTGCTCCCTCGTCAGAGGAGCACTTGCCAAGGTCAGTGAGGGGCCGGTAG
GTCCCCAGAAAAGCAGCACCGACAATGATGAAGACATCAGTTCCTTTCCCAGCCCCCCCCCCTT
TGCCCCTGTCCCATGGCCGGCGGGTGGGAGAGGATGGGGGAAGAGGGGAGCAACCCTCGAGATAT
GGGCGTAGGCACCACATTCTGATCTGGACCAAGTTGGAACAGCACCATCTCAGCCGCACAGATCC
TACCATGGAGAGCTAACACCCCACCAACCAGCAGAATGGACATTCTGACATCACCAGCTGAAACC
CTGAATCTCGGTGCAGAAGAGAAAGTGTCAACTGCGTGCAGCACTGGGGGAGTGGAGGGTGTGGG
TGGTGGAGGAAGAGGGTTAAGAAAACTAGTGGGGCCCTCTTGCTGTCCCTTGCCTATGGCACGCA
TATTCCTGCCTTGCTCCCTCACTCCCCCTCTCCCCTGCCTTCCAAAGCCCCACCCCCCAAAAATG
TGTCACTTGATTCGGACCTATTCAACCAGTAATTGGATCCCACCTTTACCAAAACACCGTCTCTG
ACCCCC*GGCCCTTCACTGATCTTGCTTATCCCTGGTCTCACGCAGCAGTTGTGGTTGCTATTGTG
GTAGTCGCTAATTGTACTAGTTTACGTGTGCATTAGTTGTGTCTCCCCGGCTAGATTGTAAGCTC
CTGGAGACAGGGACCACCTCCACAAAAAATAAAAAAACGGACCTCTCCTGTCTTGTAGTGTGCTA
GGACCCTGCAGGGCAGTGGGGGTGCACCAAAAAAAAAAAAAAAAAAA
```

FULL JT-1 SEQUENCE

```
CAGGCCCTTC GAGCATCAAA CCTACTGTCA GCGCACGCTG AGAGAAATCC AGTCTTGCTG
CGATTCCGCC ATGAGAATGT CATAGGCATC CGAGGCATCC TCAGAGCACC CACCCTGGAA
GCCATGAGAG ATGTGTATCC TTTAGCCACA GCTCCAGAGC GAGGCTAAGG AGCTTGGCTT
TGCTTTCTGT TGTCGTCTGT TGTGCCACCC CCAAAAAAAA AAAA
```

```
PRIMERS USED:      TTTTTTTTTTTTMG  (M=G+A+C)
                   CAGGCCCTTC (OPA-1)

PREDICTED PRODUCT SIZES USING THE ABOVE PRIMERS:

                   Neuronatin:          231 bp
                   JT-1:                224 bp
```

FIGURE-III-19: Comparison of the *JT-1* and *neuronatin* sequences. The location of the two primers used to identify *JT-I* and *neuronatin* are indicated. The 3'-primer was T_{12}MG, and the 5'-primer was CAGGCCCTTC (OPA-1). The complementary sequence to the 3'-primer, CCA_{12}, was seen at the 3'-end of both *JT-1* and *neuronatin* cDNA. Regions homologous to the 5'-primer were seen at the 5'-end of *JT-1*, and 220 nucleotides proximal to the 3'-end of *neuronatin.* The predicted product sizes using these primers were 224 bp with *JT-1*, and 231 bp with *neuronatin.*

used for differential display consisted of denaturation at 94°C for 30 secs, annealing at 40°C for 2 min, extension at 72°C for 30 secs, for 40 cycles; followed by extension at 72°C for 5 min. Twenty μl of each sample was applied to a 1.5% agarose gel, stained with ethidium bromide and photographed with UV light (**FIGURE-III-20**). As predicted, both *JT-1* and *neuronatin* generated products of comparable sizes. The results were comparable to the pattern seen on the differential display gel used to generate *JT-1* (**FIGURE-III-4**). The additional larger band seen in the sample using *neuronatin* cDNA as template was also observed on differential display.

IIIE-1. Comments about *JT-1* & *Neuronatin*

By an unusual coincidence, the differential display primers used to generate *JT-l* also amplified a product of comparable size from *neuronatin* cDNA. Therefore, it appears that the *JT-1 sample* used for library screening and Southern blotting also contained the *neuronatin* product. In hindsight, the *JT-1* product was prepared by PCR amplification using T_{12},MG and CAGGCCCTTC as primers, and a *single* band was cut out of the gel and purified. This band was TA cloned and sequenced. On the assumption that this was a single product, only one clone was fully sequenced, and indeed, this clone exhibited the presence of the T_{12}GG primer at the 3'-end, and the CAGGCCCTTC (OPA-1) primer at the 5'-end. Hence, there was no reason to believe that there was anything unusual. Furthermore, the *JT-I* sample hybridized on Southern blotting to *neuronatin* cDNA under high stringency conditions.

PCR AMPLIFICATION OF *JT-1 & NEURONATIN*

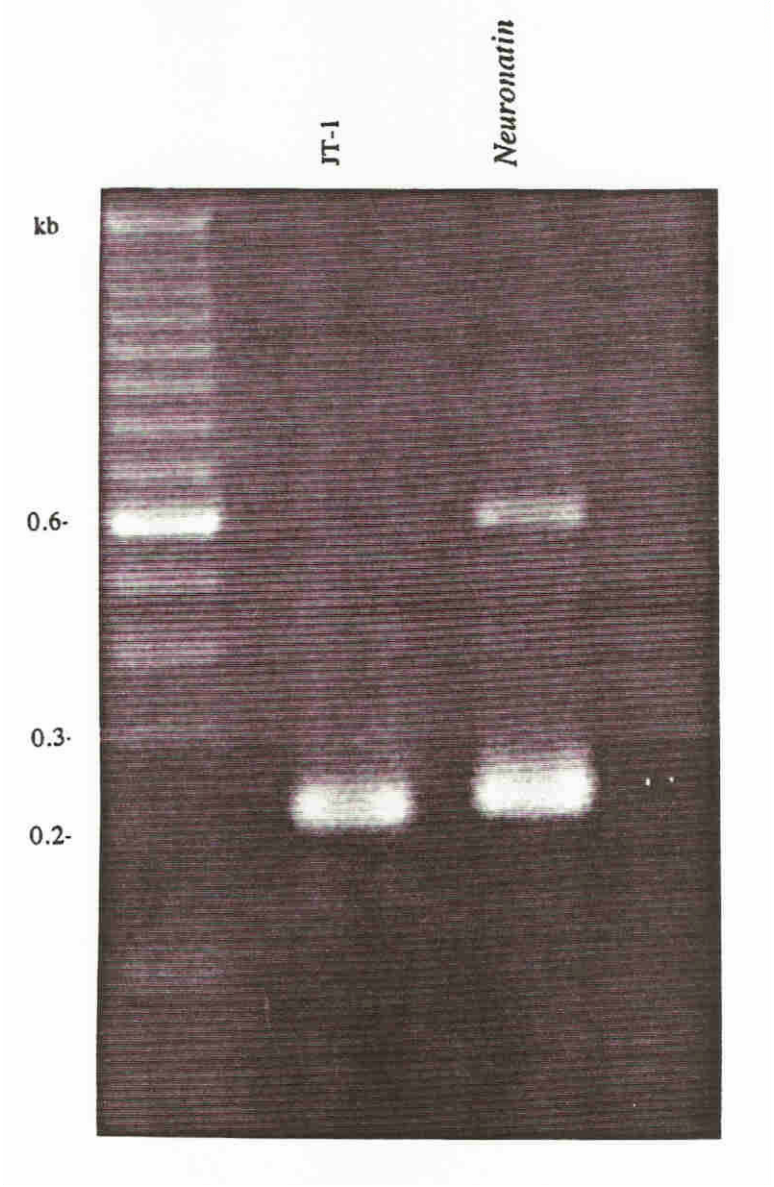


FIGURE-III-20: Comparison of the PCR products of *JT-I* and *neuronatin.* Amplification of was carried out using conditions identical to that used for differential display. The primers used to isolate *JT-I* on differential display were T_{12}MG and OPA-l. These same primers were used in two separate PCR reactions containing *JT-1* or *neuronatin* as templates. The predicted product sizes using these primers were 224 bp with *JT-1,* and 231 bp with *neuronatin.* Lane-1 is a DNA standard.

It is only when the *JT-1* sequence was noted not to match with the *neuronatin* cDNA sequence that a mystery arises. It is now apparent that the *neuronatin* product was (all along) present in the *purified JT-1 sample.* This explains the strong hybridization of *JT-1* to *neuronatin* cDNA, and is the reason that the *JT-1* sequence is not homologous with *neuronatin.* Paradoxically, this unusual occurrence may have actually helped in the identification of *neuronatin* as a novel gene. In hindsight, if instead of the *JT-1* clone that was sequenced, the clone homologous to the A_2-fragment had been sequenced, the high homology to *calbindin-d28k* would have dampened the effort needed to characterize this gene.

IIIF. *Neuronatin* mRNA EXPRESSION

IIIF-1. *Neuronatin* mRNA Expression Pattern

Northern blotting experiments revealed that both forms of *neuronatin* hybridized to a mRNA species of about 1.2 kb, therefore, subsequent studies were carried out using *neuronatin-α* as the probe. When studied in three day old rats, *neuronatin* was expressed only in the brain, and not in the heart, lung, liver, kidney, muscle, skin or spleen **(FIGUIRE-III-21).**

Thereafter, the expression of this gene during different stages of brain development was investigated. Northern blotting was carried out using RNA extracted from rat embryos in the early (E7-10), mid (E11-14) and late (E16-19) embryonic stages of development, postnatal (P)-3 days (P3), P7, P14, P28 and P56 days. As the brain is not developed early in embryogenesis, E7-10 embryos were studied whole, and the cephalad half of E11-14 embryos were used for RNA extraction. From E16-19 onwards, it was possible to dissect the brain out for study. *Neuronatin* was not expressed in the early embryonic period, it first appeared in mid embryonic stages of development, and its expression became abundant later at E16-19, a time in development when there is a marked increase in brain weight due to rapid neuroepithelial cell proliferation and neuroblast commitment. Postnatally, the expression of *neuronatin* declined by the second postnatal week such that only minimal levels were present in adulthood (**FIGURE-III-22**).

NEURONATIN mRNA IN RAT TISSUES

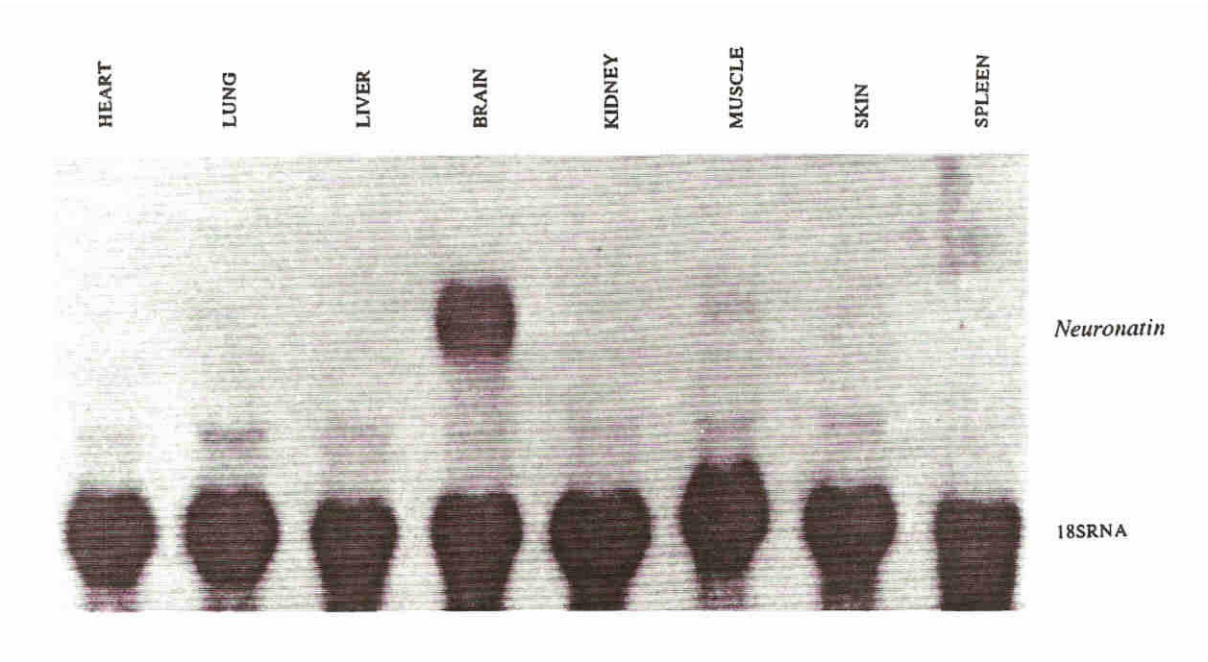


FIGURE-III-21: *Neuronatin* mRNA expression in different tissues of the neonatal rat. RNA samples, extracted from the heart, lung, liver, brain, kidney, muscle, skin and spleen of postnatal day-3 rats were used to carry out Northern blotting. After hybridization with *neuronatin*, the blot was stripped and rehybridized with *I8SRNA* probe to control for the amount of RNA applied.

NEURONATIN mRNA DURING RAT DEVELOPMENT

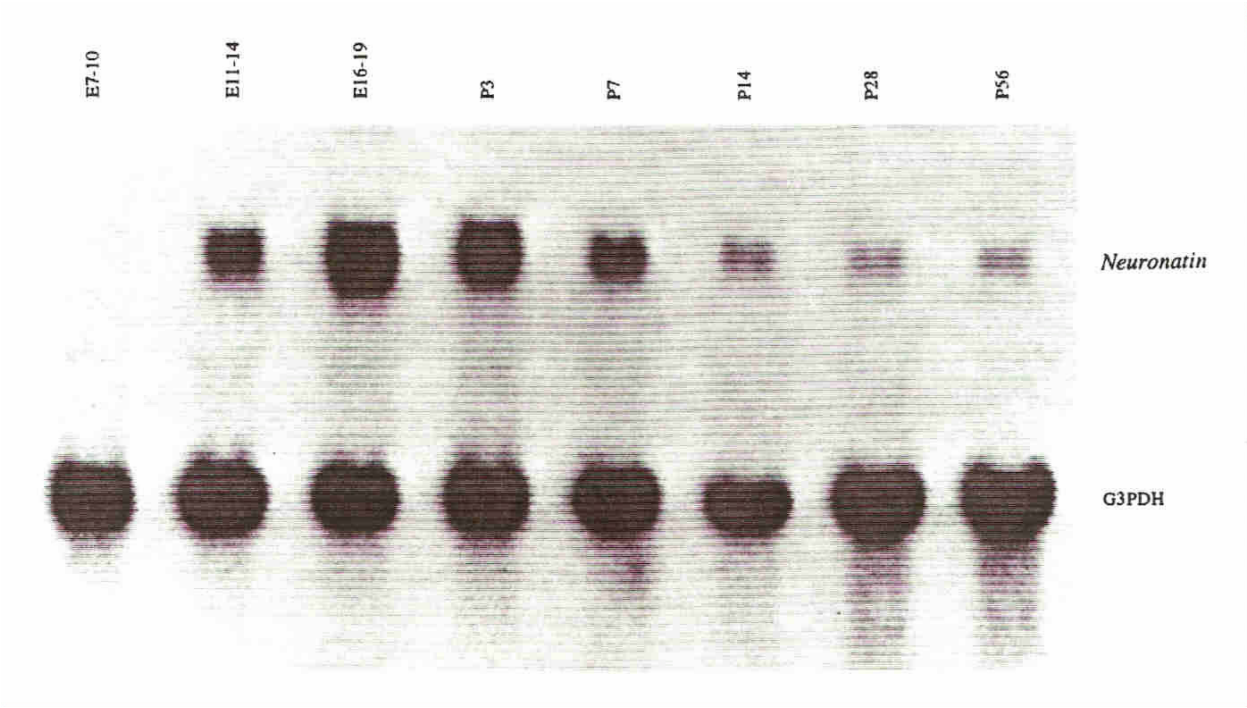


FIGURE-III-22: *Neuronatin* mRNA expression during rat brain development. RNA was extracted from the brains of embryonic (early (E7-10), mid (E11-14) & late (E16-19) and postnatal (P3, P7, P14, P28 & P56) rats. In the case of E7-10, the embryo was used whole, and at E11-14, the upper halves of the embryos were used. From E16-19 onwards, it was possible to separate out the brain for RNA extraction. Following hybridization with *neuronatin,* the blot was stripped and rehybridized with *glyceraldehyde 3-phosphate dehvdrogenase (G3PDH)* as a control.

In another experiment, *neuronatin* mRNA expression was studied in E13-15 and E16-18 day old embryos, and in an adult mother rat; sister embryos from each of the two litters used in this experiment were photographed to show the rapid increase in head (and brain) size during this three day period (**FIGURE-III-23**).

GROSS APPEARANCE & *NEURONATIN* mRNA

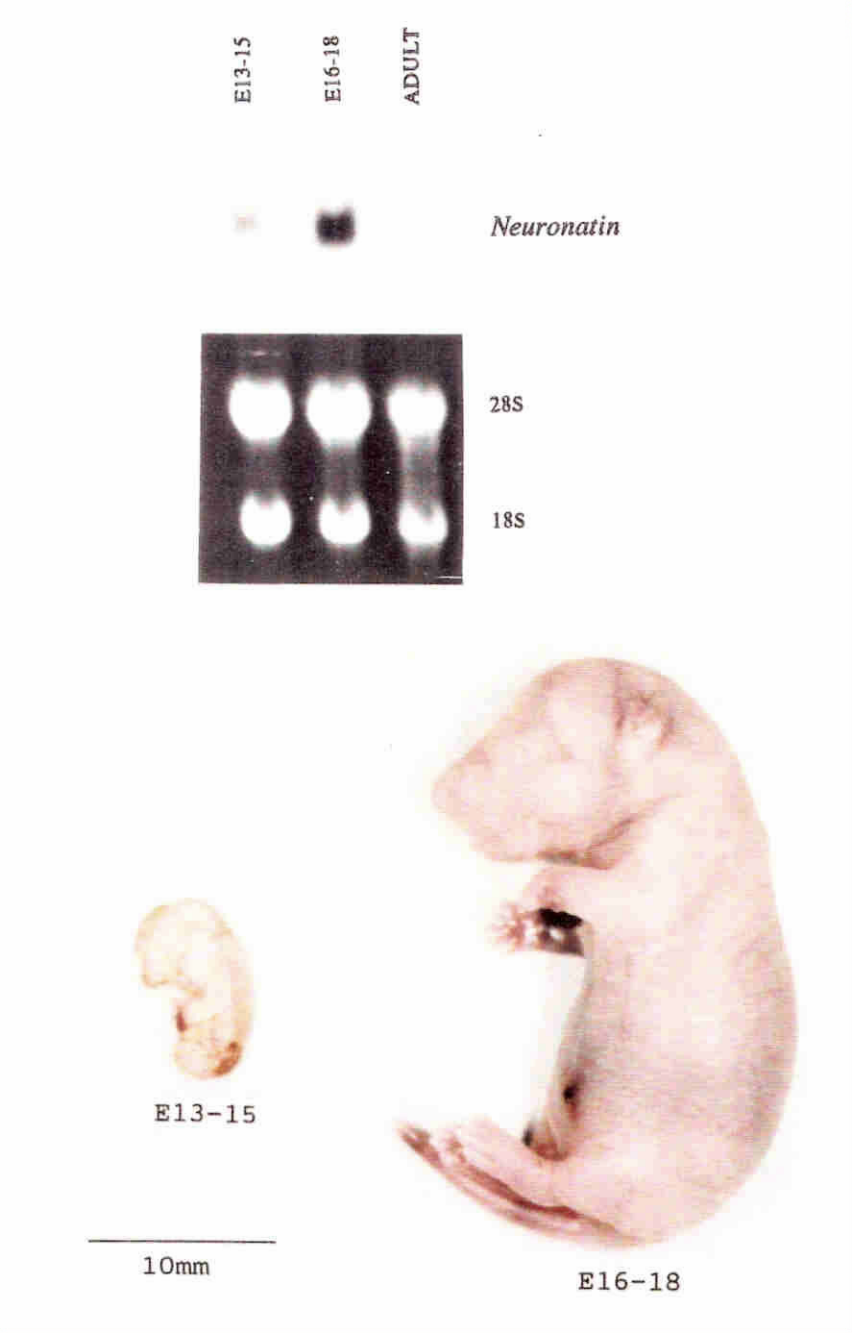


FIGURE-III-23: Gross appearance and *neuronatin* mRNA expression in embryonic and adult rats. Total RNA samples, extracted from E13-15, E16-18 and adult rat brain were used to carry out Northern blotting with *neuronatin* cDNA probe. A sister pup from each of the two litters used in this experiment was photographed.

IIIF-2. Expression of *Neuronatin* mRNA Isoforms During Development

In order to study the expression of the two alternatively spliced forms of *neuronatin* mRNA during brain development, primers were designed to hybridize the *neuronatin* cDNA sequence (**FIGURE-III-13**).

5'-Primer:

5' -GCG-AAC-CCT-TGC-TCT-CGA-CCA-CCC-AC-3' (homologous to positions -40 to -15)

3'-Primer:

5' -CCC-ACT-AGT-TTT-CTT-AAC-CC-3' (complementary to positions 652 to 671)

The predicted product sizes were 710 bp for the β-form; and 791 bp for the α-form (including the 81 bp sequence (box in **FIGURE-III-13**)).

For cDNA synthesis, total RNA extracted from E7-10, E11-14, E16-19, P7 and P14 rats were reverse transcribed using a modification of previously described methods (Kawasaki 1990). Initially, reactions containing 5 μg RNA, 40 u RNase inhibitor, 0.5 μg oligo-dT_{12-18}, made up to 10 μl with diethylpyrocarbonate-treated water was heated to 70°C for 10 min, and then chilled on ice. Thereafter, final concentrations of 10 μM DTT, 125 μM dNTP (all four), 10 u RT-MMLV and 1x RT-buffer were added in a total volume of 20 μl, and the reactions incubated at 37°C for 60 min. The RT-enzyme was then inactivated by heating to 94°C for 5 min and

chilling on ice. Four μl aliquots of the cDNA reaction were used for PCR amplification. The final concentration of the ingredients of the PCR reaction were, 100 μM dNTP, 0.4 μM 5'-primer, 0.4 μM 3'-primer, 5 u Taq DNA polymerase, 10 mM Tris HC1, 1.5 mM $MgCI_2$, 50 mM KCI and *5* μg gelatine, in a total volume of 50 μl. The samples were denatured at 95°C for 2 min and amplified for 40 cycles. Each cycle consisted of denaturation at 95°C for 30 secs, annealing at 62°C for 1 min, and extension at 72°C for 2 min. The samples (30 μl per well) were electrophoresed using 1x Tris acetate buffer on 1% agarose containing ethidium bromide. A 100 bp ladder (Life Technologies) was used as the DNA size marker. The gel was photographed with UV light.

The findings reveal that of the two forms, it is the β-form that appears at E11-14 (**FIGURE-III-24**). Although, the α-form was not observed on Northern blotting at E7-10, it was seen on RT-PCR to be present at that earlier developmental stage as well. Besides the predicted bands for *neuronatin-*α and β, no other products were amplified, indicating that there are no other forms of *neuronatin* mRNA expressed during brain development.

NEURONATIN SPLICED FORMS & DEVELOPMENT

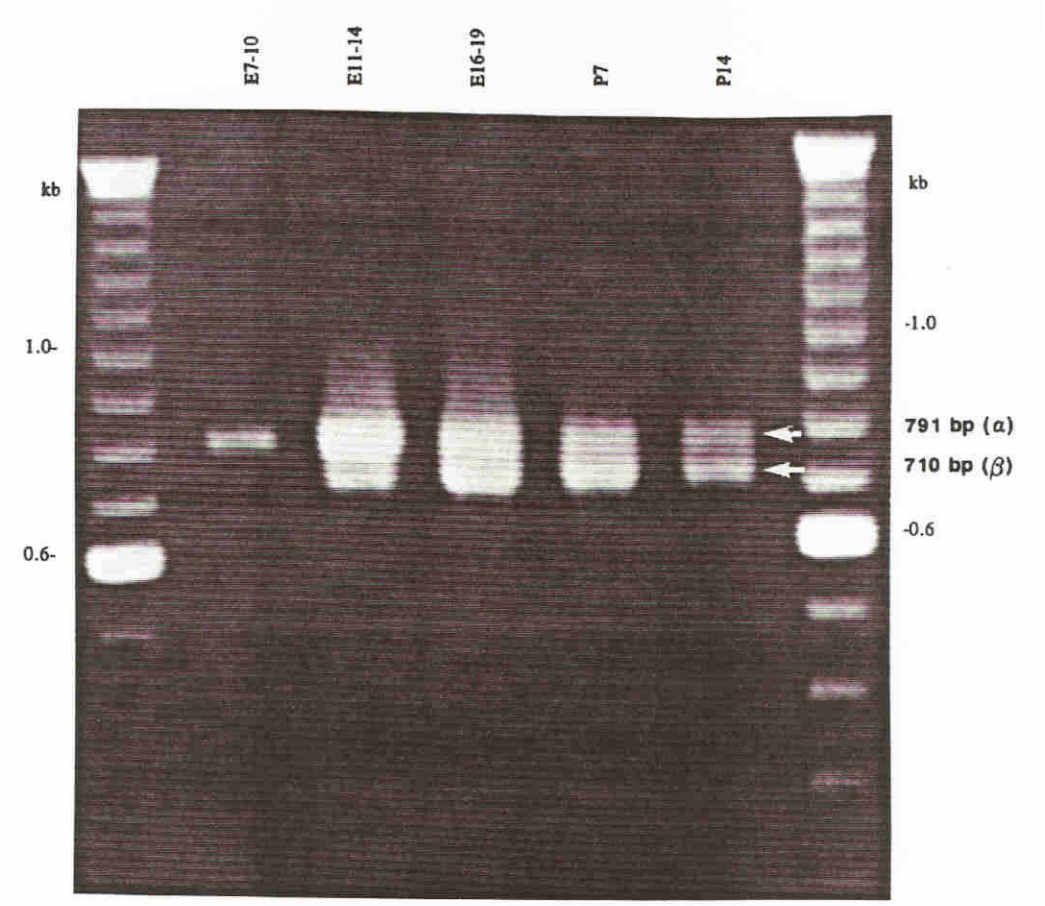


FIGURE-III-24: Differential expression of *neuronatin* isoforms during development. Reverse transcriptase-PCR study of *neuronatin-a* and *β* mRNA during rat development. Primers flanking the coding region of both *neuronatin-a* and *β* were synthesized (see **FIGURE-III-13).** The *5'*-Primer was homologous to sequences -40 to -15, and the 3'-Primer was complementary to sequences 652-671. The predicted size for the *a*-form was 791 bp, and that for the *β*-form 710 bp. RNA extracted from E7-10, El 1-14, E16-19, P7 and P14 rat brain were used. Although, the *a*-form was present even at E7-10, *neuronatin-β* appeared only at E11-14, coinciding with a period in development when neurogenesis has been initiated.

IIIG. ANALYSIS OF THE DEDUCED PROTEIN

The deduced gene product for *neuronatin-α,* based on its cDNA sequence, was a novel polypeptide of 81 amino acids with a molecular weight of 9.1 kDa. *Neuronatin-β* encoded 54 amino acids of 6.2 kDa. The hydrophobicity plot of *neuronatin-β* cDNA indicated the presence of two distinct domains, a hydrophobic domain at the N-terminal and a hydrophilic domain at the C-terminal (Kyte & Doolittle 1982) (**FIGURE-III-25**). In the case of *neuronatin- α,* the 81 bp middle exon is neither hydrophilic nor hydrophobic. Therefore, further protein analysis was carried out only with the *β*-form.

The hydrophobic domain contains 23 amino acid residues. This is a suitable size to form a transmembrane *α*-helix structure (Tanford 1978; Adams & Rose 1985). When *neuronatin* cDNA was fused to LacZ, the expressed fusion protein was noted to be anchored to the *E.coli* cytoplasmic membrane, suggesting that this protein is membrane bound. However, more experiments are required to evaluate the precise cellular location of the protein. The C-terminal domain of *neuronatin* is hydrophilic and highly basic, and is 20% (6 of 30 amino acids) constituted by arginine residues.

The primary amino acid sequence of *neuronatin* showed about 50% homology with two known proteins, *PMP1* which is a subunit of H^{+}*-ATPase* (Navarre et al 1992ab) and *phospholamban* which is a subunit of Ca^{2+}*-ATPase* (Tada 1991). The secondary structural organization of these three polypeptides were also similar, each consisting of two

HYDROPHOBICITY OF *NEURONATIN* PROTEIN

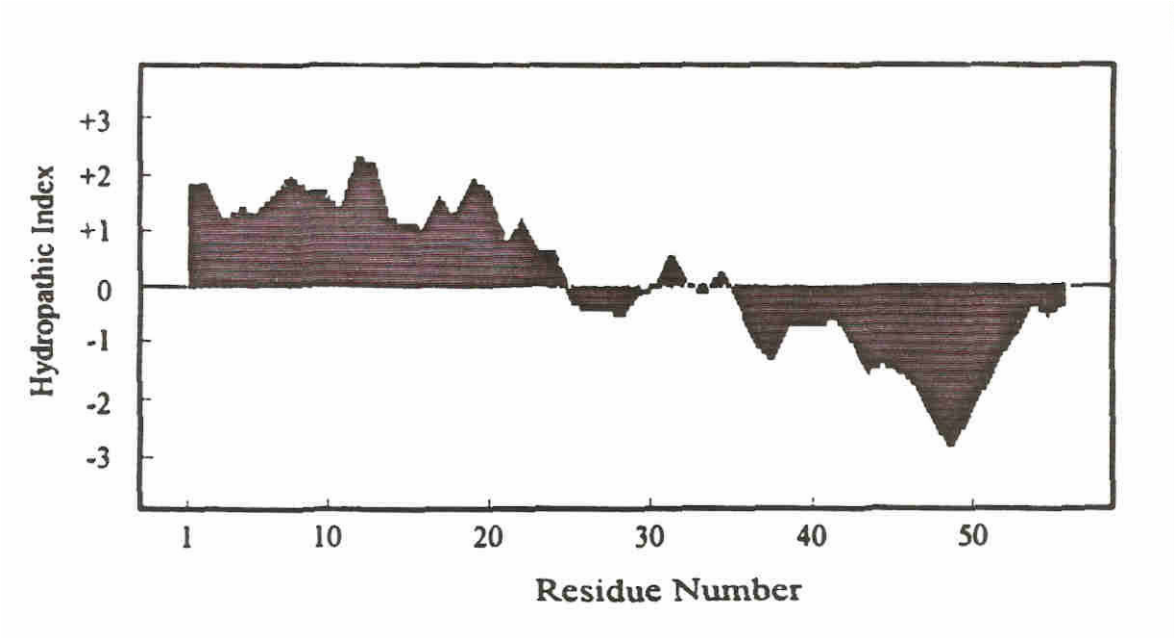


FIGURE-III-25: Hydrophobicity plot of the deduced protein for *neuronatin-β*. The N-terminal half (corresponding to exon-1) of the protein is hydrophobic, and the C-terminal half (corresponding to exon-3) is hydrophilic. Exon-2, present only in *neuronatin-α*, was neutral. The hydrophohicity profile was determined using the method of Kyte and Doolittle.

domains, a hydrophobic transmembrane domain and a charged hydrophilic domain **(FIGURE-III-26**). The structural similarity between *neuronatin* and these subunits of cation-translocating channels suggests that *neuronatin* may also function in a similar manner to regulate ion channels.

The amino acid sequence of the 81 bp middle exon, present in *neuronatin-α,* exhibited 40% identity with *periplasmic hydrogenase* (Stokkermans et al 1989) (**FIGURE-III-27**). When related amino acids were taken into consideration, the extent of homology increased to 54%. This electron transfer enzyme is an *iron-sulphur hydrogenase* which 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 sulphate reducer *Desulfovibrio vulgaris (Hildenborough).* Nevertheless, the region of homology included conserved cysteines involved in the functional coordination of the catalytic iron-sulphur cluster.

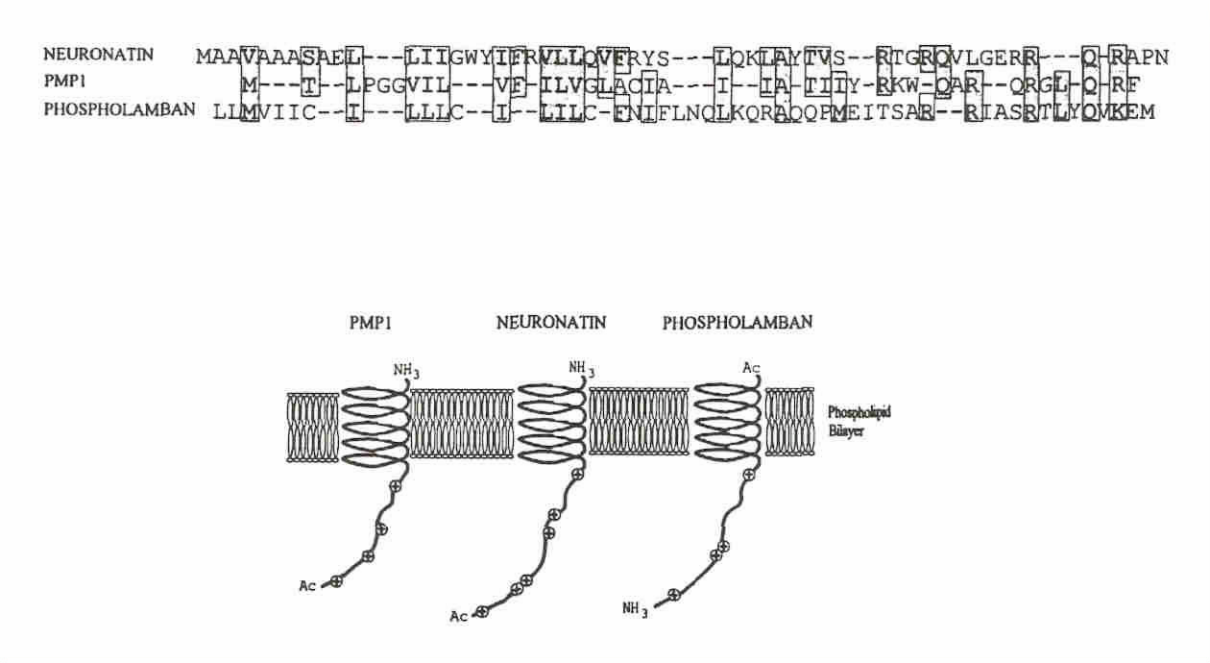


FIGURE-III-26: Amino acid sequence alignment of *PMP1, phospholamban* and *neuronatin* protein sequences (top panel). The left side of the alignment is predicted to be the α-helix domain, it begins with the N-terminal for *neuronatin* and *PMP1,* and with the C-terminal for *phospholamban.* The predicted secondary structures of the three polypeptides are also shown (bottom panel).

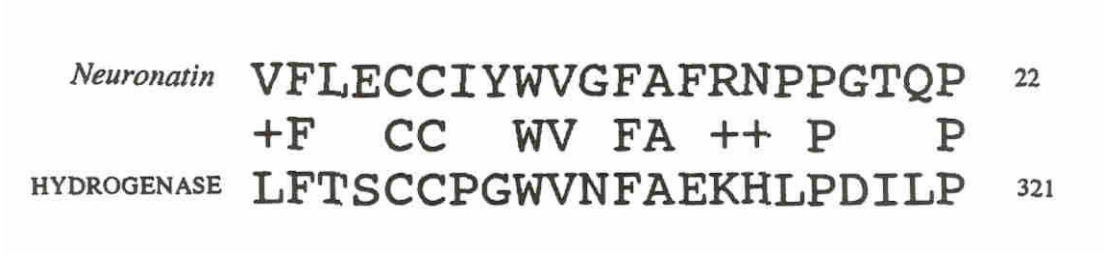


FIGURE-III-27: The deduced protein of exon-2 and homology to periplasmic hydrogenase. When the 27 amino acid sequence of the middle exon of *neuronatin-α* was searched in GenBank using the BLAST server, there was 40% identity with *periplasmic hydrogenase* (GenBank #S13526). This increased to 54% when related amino acids were taken into consideration.

IIIH. ROLE OF *NEURONATIN* IN NEURONAL GROWTH

IIIH-1. Expression of *Neuronatin* mRNA in Neuronal Cell Lines

In order to determine whether *neuronatin* was expressed in neurons or glia, neuronal and glial cell lines were studied. *Neuronatin* was expressed in PC12 and NCB2O, both neuronal cell lines, but not in the rat glial cell lines, 9L and a primary glial line **(FIGURE-III-28).** In this experiment it was also noted that *neuronatin* mRNA levels were down-regulated following treatment (for a few hours) with *NGF*. This finding, together with the fact that *NGF* treatment stops the growth of PC12 cells and induces them to extrude neurites, raises the possibility that *neuronatin* may be involved in PC12 growth and differentiation. Furthermore, this preliminary observation was supportive of the *in vivo* observation that *neuronatin* mRNA is abundant in the relatively undifferentiated developing brain, but not in the differentiated adult brain.

IIIH-2. NGF Down-Regulates *Neuronatin* mRNA Expression

The differential expression of *neuronatin* mRNA during development suggested that this gene was involved in neuronal growth. Therefore, the preliminary findings noted in the PC12 experiment described above was investigated further by studying the effect of *NGF* on the expression of *neuronatin* mRNA. When *NGF* (200 ng/ml) was applied for 7 days, there was downregulation of *neuronatin* mRNA expression (**FIGURE-III-29**).

NEURONATIN mRNA IN NEURONAL CELL LINES

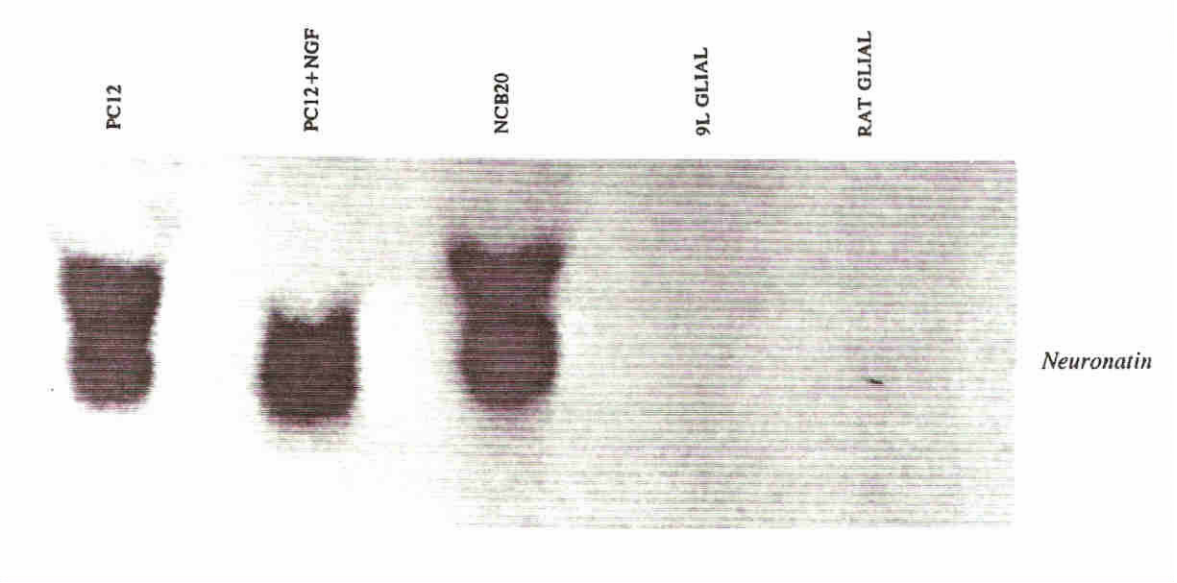


FIGURE-III-28: *Neuronatin* mRNA expression in neuronal cell lines. Northern blotting was carried out in neuronal and glial cell lines. PCI2 (rat pheochromocytoma), NGF-treated (few hours) PC12 cells, and NCB2O (Chinese hamster cortical neurons x rat neuroblastoma) cells were the neuronal-type cell lines studied. In order to evaluate for expression in glia, the 9L cell line and a primary rat glial cell line were studied in separate blots (a representation is shown). *Neuronatin* was expressed in neuronal cell lines, but not in glial lines.

EFFECT OF *NGF* ON *NEURONATIN* mRNA

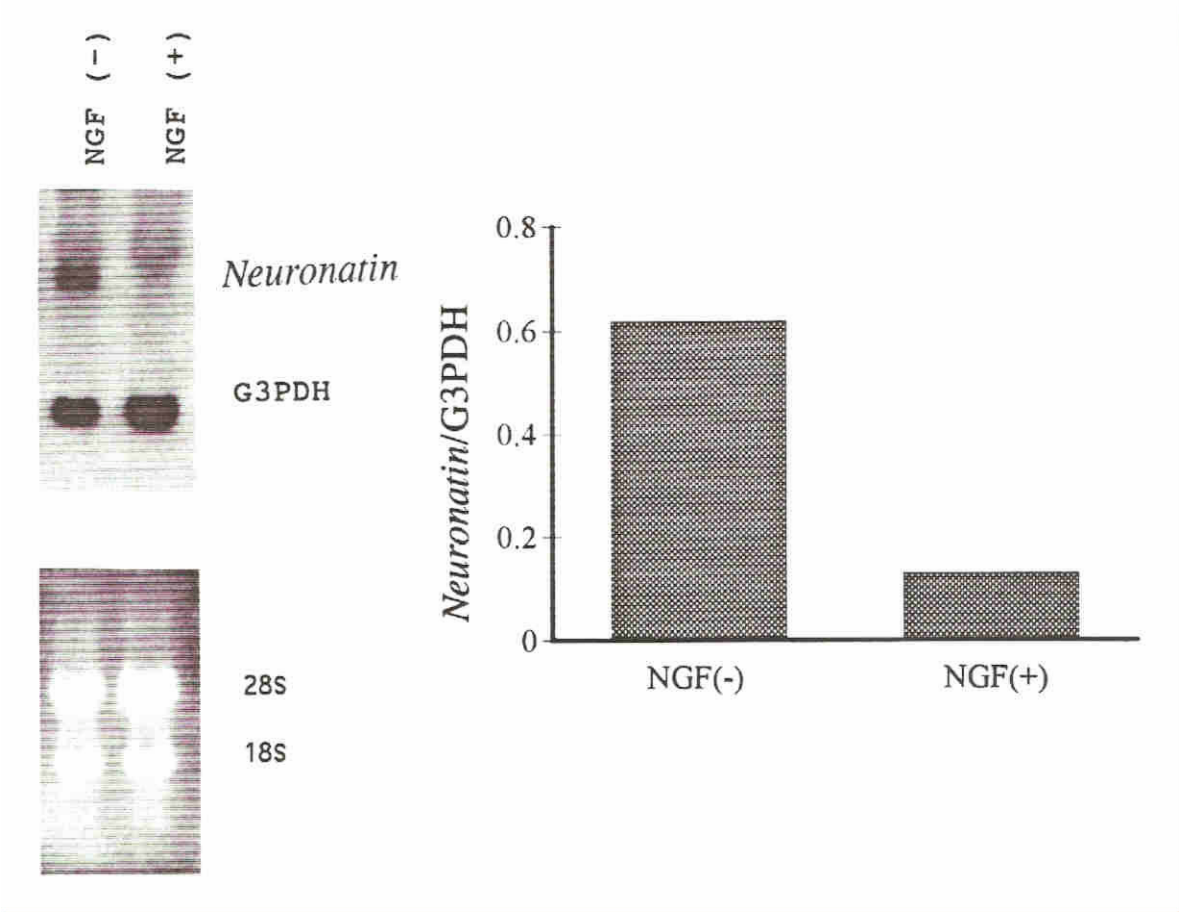


FIGURE-III-29: Effect of *NGF* on *neuronatin* mRNA expression, studied by Northern blotting. Comparison of undifferentiated PC12 cells (*NGF* (-)) to that following treatment with *NGF* (200 ng/ml) for 9 days (*NGF* (+)). *Neuronatin* mRNA expression is downregulated following *NGF*-treatment. The densitometric evaluation using G3PDH expression to control for the amount of mRNA applied is shown. The ethidium bromide stained gel pictured before transfer is also shown.

Thereafter, the effect of *NGF* was compared to that of other inducing agents. *NGF* (200 ng/ml) was applied for different time periods (1,3,7 and 9 days), and compared with the effects of *basic Fibroblast Growth Factor (bFGF)* (100 ng/ml), *Transforming Growth Factor (TGFβ1)* (1 ng/mI), 12-O-tetradecanoylphorbol-13-acetate *(TPA)* (100 nM) and dexamethasone (10 μM), all applied for 7 days (**FIGURE-III-30**). *Neuronatin* mRNA, abundant in undifferentiated PC12 cells, was significantly reduced at 24 hours after the addition of *NGF,* and remained suppressed even at 9 days. Although less potent than *NGF, bFGF* also causes transient differentiation of PC12 cells and as may be predicted based on the *NGF* results, caused some decrease in *neuronatin* mRNA levels. On the other hand, *TGFβI*, *TPA* and dexamethasone, agents that do not induce neuronal differentiation, were not associated with the suppression of *neuronatin* mRNA levels. Furthermore, *Epidermal Growth Factor (EGF),* which unlike *NGF* is known to enhance PC12 proliferation (Huff et al 1981), did not downregulate *neuronatin* mRNA levels even when applied in a concentration of 200 ng/ml for periods ranging from 6 hours to 8 days (data not shown).

The effect of adding and removing *NGF* was then investigated. Treatment with *NGF* for 3 days, followed by its removal for 7 days, returned *neuronatin* mRNA levels to baseline (**FIGURE-III-31**).

NGF & OTHER AGENTS ON *NEURONATIN* mRNA

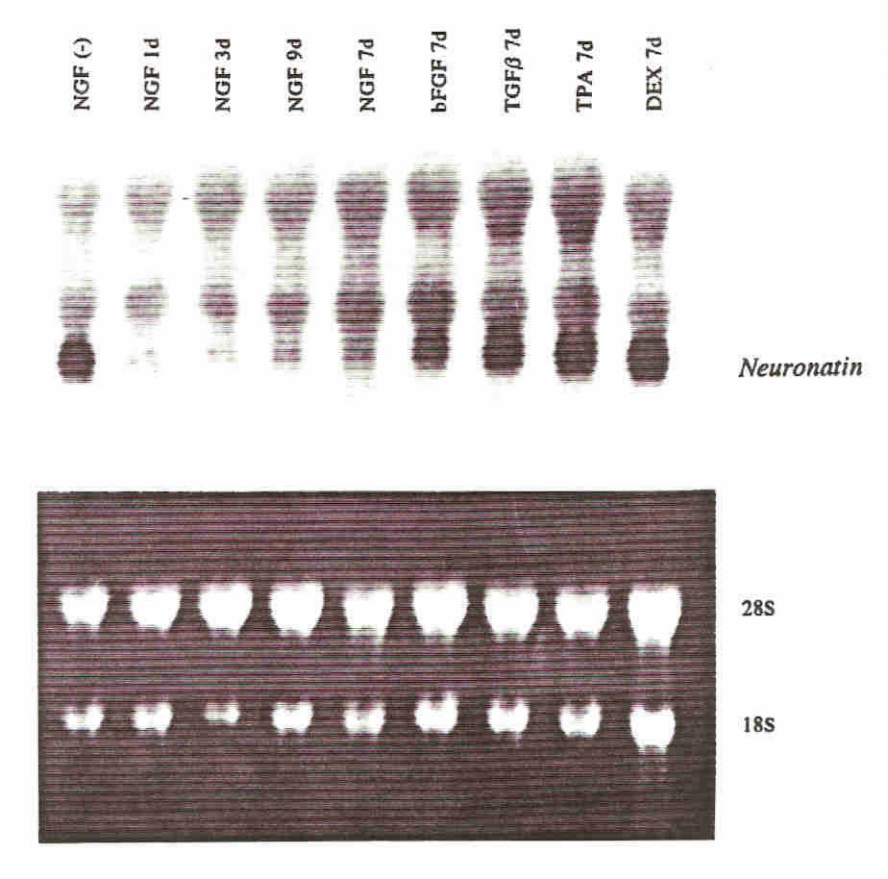


FIGURE-III-30: The effects of *NGF* and other inducing agents on *neuronatin* mRNA levels. *NGF* (200 ng/ml) was applied for 1,3,7 and 9 days; and parallel samples were treated for 7 days with *bFGF* (100 ng/ml), *TGFβ1* (1 ng/ml), *TPA* (100 nM) and Dexamethasone (Dex) (10 μM). NGF-treatment, resulting in the cessation of proliferation and the commitment of PC12 cells to a differentiated fate, was associated with the downregulation of *neuronatin* mRNA at 24 hours, that persisted even when studied at 9 days. The differentiating effect of *bFGF* was less prominent than *NGF*, and as would be predicted on the basis of the *NGF* results, *neuronatin* mRNA was only partially downregulated. *TGFβ1, TPA* and dexamethasone do not induce neuronal differentiation, and indeed, these agents do not suppress *neuronatin* mRNA. The ethidium bromide gel pictured before transfer is also shown.

ADDITION & REMOVAL OF *NGF* ON *NEURONATIN* mRNA

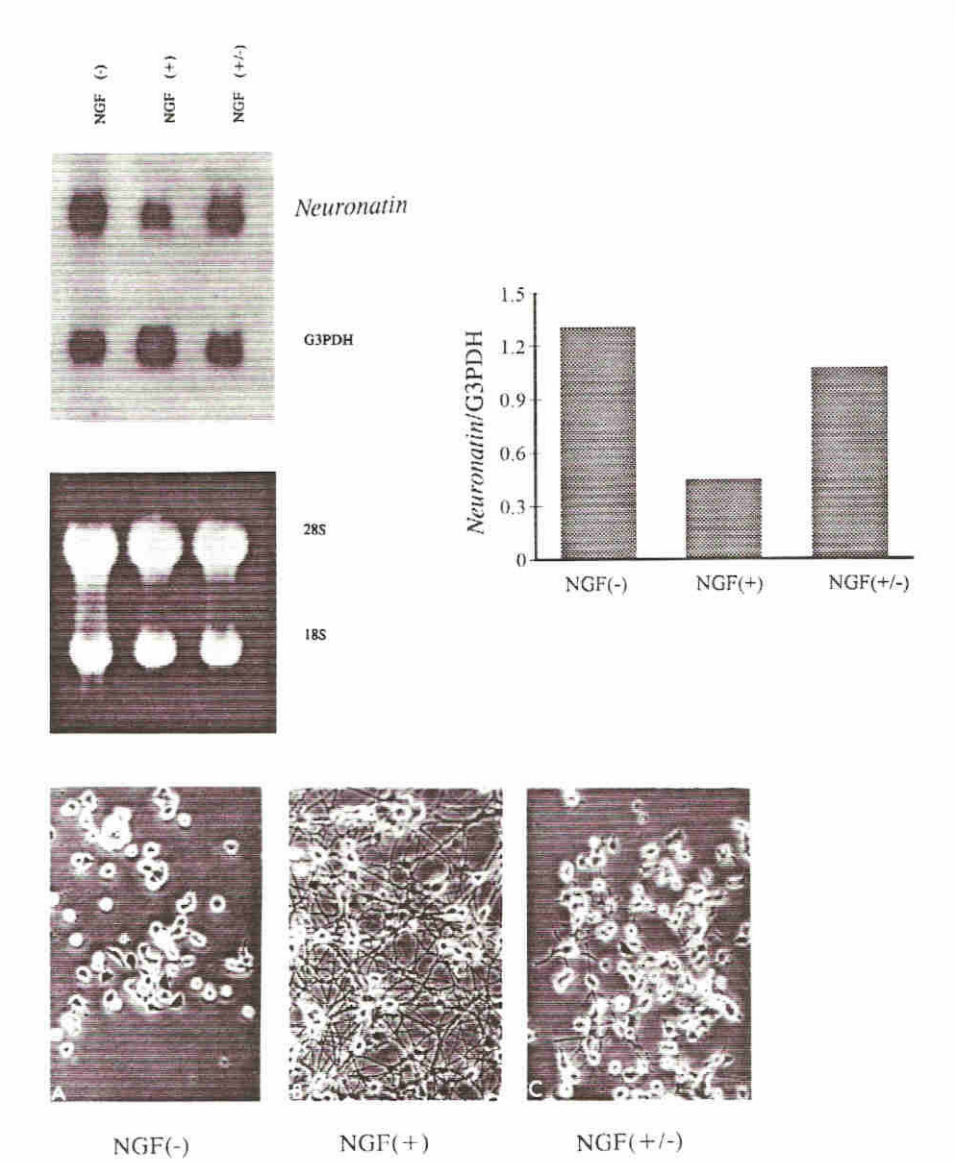


FIGURE-III-31: The effect of addition and removal of *NGF* on *neuronatin* mRNA expression. Lane-1, undifferentiated PC 12 cells *(NGF* (-)); Lane-2, *NGF* for 2 days (*NGF* (+)); Lane-3, *NGF* for 2 days, and then *NGF* removed for 7 days (*NGF* (+/-)). *NGF* was applied in a concentration of 200 ng/ml in all samples. *NGF* downregulates *neuronatin* mRNA. Removal of *NGF* allows *neuronatin* mRNA to return to baseline. The densitometric evaluation using G3PDH to control for the amount of mRNA is shown. Photomicrographs of representative PC12 cells are also shown, undifferentiated (A); *NGF* (200 ng/ml) for 7 days (B); and, *NGF* for 7 days followed by its removal for 7 days (C).

In order to determine whether the effect of *NGF* involved protein and RNA synthesis, the effect of *NGF* in the presence and absence of cyclohexiniide, a protein synthesis inhibitor, and Actinomycin-D, a RNA synthesis inhibitor were studied. Pre-treatment of PC12 cells with either cycloheximide or Actinomycin D did not prevent the effect of *NGF* on *neuronatin* mRNA expression (**FIGURE-III-32**). These findings suggest that the effect of *NGF* on *neuronatin* mRNA expression is not dependent on protein and RNA synthesis.

Finally, RT-PCR was carried out to study changes in the spliced forms of *neuronatin* mRNA before and after *NGF* application. The primers and conditions used for RT-PCR were identical to that described (**FIGURE-III-24**). Only the α-form of *neuronatin* mRNA was expressed in PC12 cells, and this was downregulated following treatment with *NGF* (200 ng/ml) (**FIGURE-III-33**).

PROTEIN & RNA SYNTHESIS INHIBITION & *NEURONATIN* mRNA

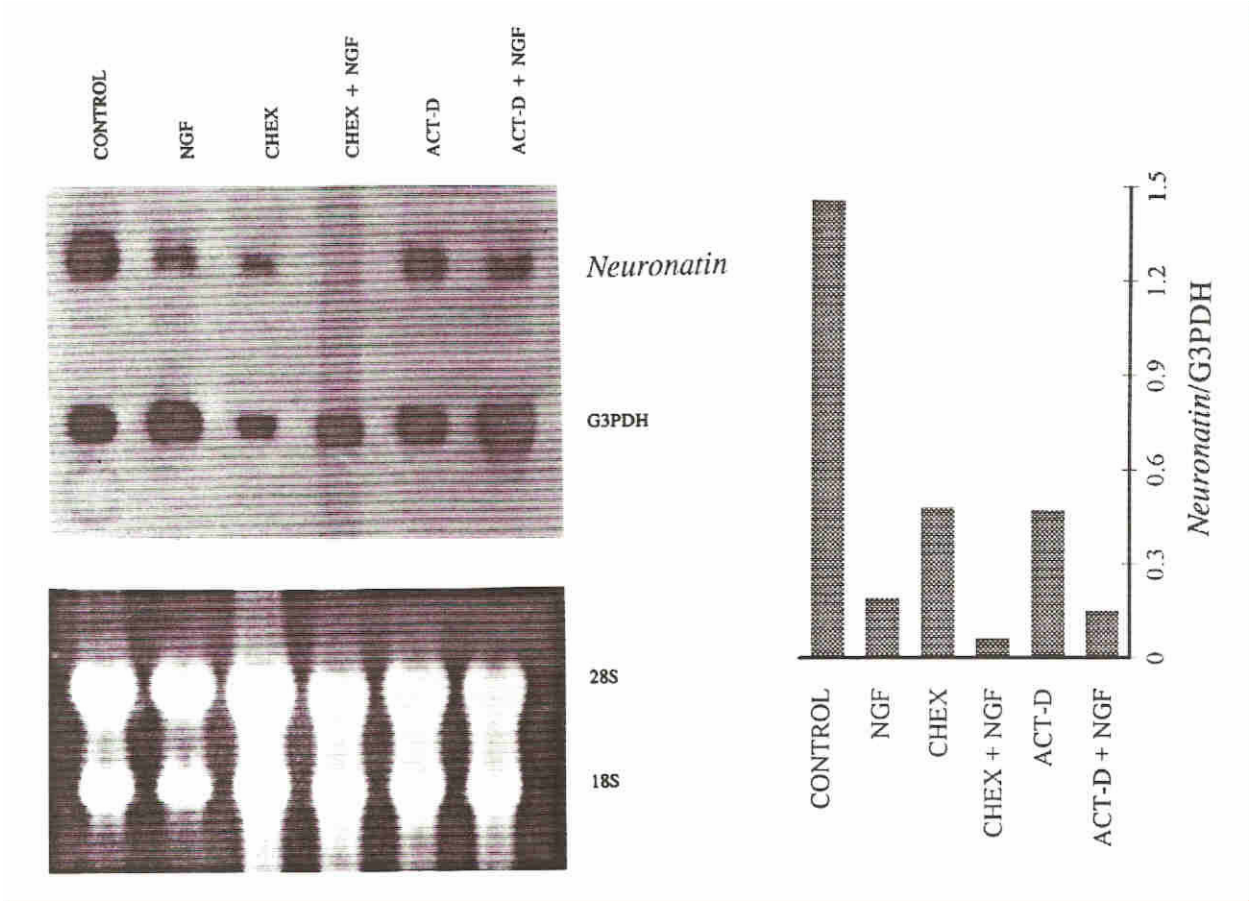


FIGURE-III-32: Effect of protein and RNA synthesis inhibitors on *neuronatin* mRNA expression. Protein synthesis was inhibited with cycloheximide (10 μg/ml), and RNA synthesis with Actinomycin-D (1 μg/ml). Lane-1 (CONTROL), undifferentiated PC12 cells; Lane-2, *NGF;* Lane-3, cycloheximide (CHEX); Lane-4, cycloheximide plus *NGF;* Lane-5. ActinomycinD (ACT-D); Lane-6, Actinomycin-D plus *NGF*. *NGF* was applied in a concentration of 200 ng/ml; all treatments were for 2 days. Although. cycloheximide and Actinomycin-D by themselves downregulated *neuronatin,* the effect of *NGF* was still visible in these samples as well. The densitometric assessment using G3PDH to control for the amount of mRNA applied is also shown.

NEURONATIN ISOFORMS IN PC12 CELLS

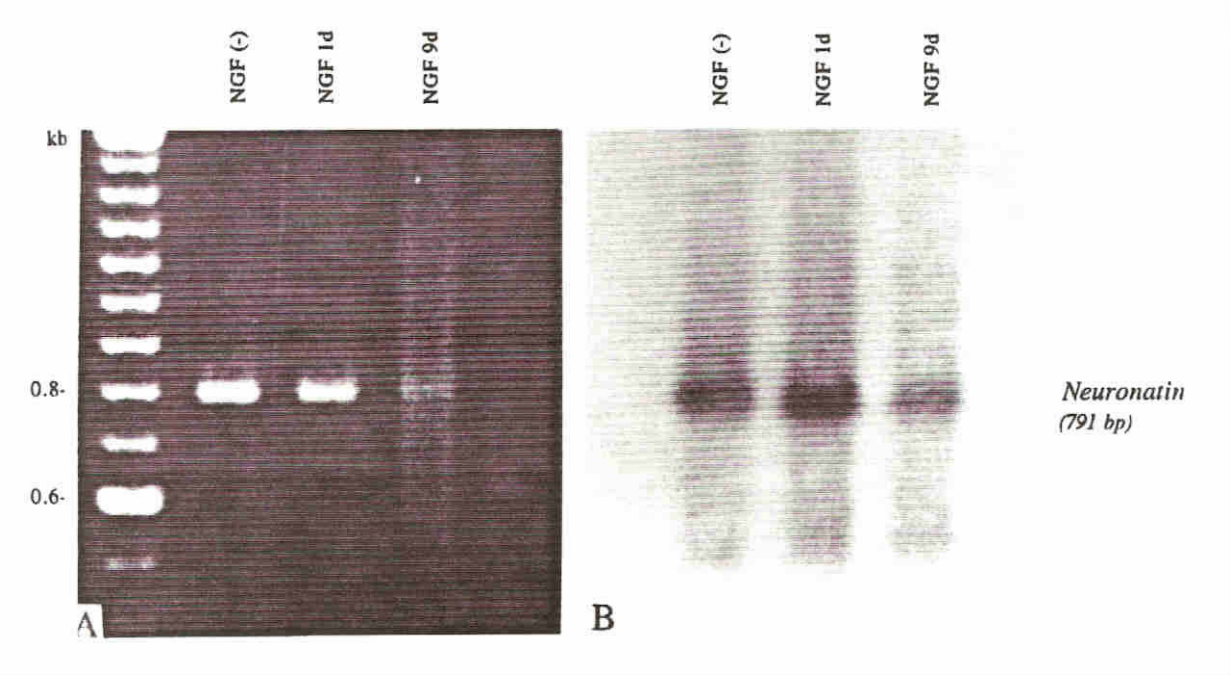


FIGURE-III-33: Study of the expression of *neuronatin-α* and *β* isoforms in PC12 cells. RT-PCR was carried out using primers that flank the coding regions of both forms as described in FIGURE-III-24. The predicted product size for the *α*-form was 791 bp, and that for the *β*-form 710 bp. (A). Ethidium bromide stained gel, Lane-1, undifferentiated PC12 cells; Lane-2, *NGF* (200 ng/ml) applied for 1 day; Lane-3, *NGF* (200 ng/ml) for 7 days. (B). Southern blot of the gel shown in (A) using *neuronatin* cDNA as the probe. Only the *α*-form of *neuronatin* is expressed in PC12 cells.