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FULL SEQUENCE OF NEUROCALCIN, A NOVEL CALCIUM-BINDING PROTEIN ABUNDANT IN CENTRAL NERVOUS SYSTEM¶

Katsuo Okazaki, Masato Watanabe, Yuhko Ando, Masatoshi Hagiwara†, Motomu Terasawa and Hiroyoshi Hidaka*

Department of Pharmacology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan

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SUMMARY: We determined the cDNA sequence for neurocalcin, a novel calcium-binding protein in bovine brain. This clone (pCalN) has 582 nucleotides in the open reading frame including the termination codon TGA, 11 nucleotides of the 5' leader and 1251 nucleotides of the 3' noncoding region. The deduced amino acid sequence revealed that neurocalcin is composed of 193 amino acids, has a molecular mass of 22,284 daltons, and contains three putative calcium-binding sites (EF-hand motifs). By Northern blot analysis, 3.8kbp mRNA was detected in brain. The deduced amino acid sequence had a strong homology to visinin (46.5%) and recoverin (51.6%) in retina, suggesting that neurocalcin may play a visinin- or recoverin-like role in brain.

Many members of the calcium-binding proteins to date have been reported. These proteins are subdivided into two groups with structural features, the EF-hand protein family and the annexin protein family (1). The EF-hand proteins exhibit general structural principls, which bind calcium selectively with high affinity (2). Calmodulin is present in all cells, but most other calcium-bnding proteins are expressed in a cell-type specific manner. The exact functions of these proteins remain unknown.

We have been studying calcium-binding proteins, such as calvasculin (3), calgizzarin (4, 5), calcyclin (6) and S100P (7) using newly synthesized calmodulin

¶Sequence data from this article have been deposited with the EMBL/Genbank Data Libraries under Accession No. D10884.

† Present address: Department of Anatomy, Nagoya University School of Medicine.

* To whom correspondence should be addressed.

Abbreviations used: Tricine; Tris(hydroxymethyl)methylglycine, SDS; sodium dodecyl sulfate, PAGE; polyacrylamide gel electrophoresis, PCR; polymerase chain reaction, bp; base pairs.

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antagonists. Neurocalcin was found as one of calcium binding proteins from a bovine brain with an affinity column of a calmodulin antagonist, W77; ((S)-P-(2-aminoethyloxy)-N-[2-(4-benzyloxy-carbonylpiperazinyl)-1-(P-methoxybenzyl)ethyl]-N-methylbenzene sulfonamide dihydrochloride), coupled with Sepharose. Neurocalcin can be separated as a 23k dalton protein by Tricine-SDS PAGE. Details of the methods for purification will be reported elsewhere, by which at least four isoproteins were identified in the process. Homologic investigations of NBRF-PDB (release 69) and SWISS-PROT database (release 19), with partial peptide sequences of each isoform revealed that neurocalcin was a novel protein. Abundant amounts of neurocalcin can be found in the nervous system, but the function of this particular calcium-binding protein is still unclear. In this study, we attempted to characterize the primary structure of this protein to clarify the function of neurocalcin. We isolated and sequenced a cDNA clone encoding neurocalcin from a bovine brain cDNA library using authentic PCR-amplified cDNA as a probe, and examined the characteristics of the primary structure of neurocalcin.

Materials and Methods

Materials. [α-^{32}P]dCTP (110TBq/mmol) was purchased from Amersham Japan. T_4 DNA polymerase, Taq polymerase (Ampli Taq), restriction endonucleases and other modifying enzymes were obtained from Takara Shuzo. Oligonucleotides were synthesized on a Model 392A DNA synthesizer (Applied Biosystems Inc.). A bovine brain λgt10 library was purchased from Clontech. All other chemicals and reagents were of analytical grade and were obtained from commercial suppliers.

Isolation of neurocalcin cDNA clone. A cDNA library synthesized from the mRNA of bovine brain using a DNA synthesis kit (Pharmacia P-L Biochemicals, Inc.) according to the manufacturer's instructions was used as a template for synthesizing the authentic cDNA probe of neurocalcin. Two PCR primers, For and Rev, were designed according to the peptide sequence of neurocalcin. PCR was performed 25 times in a 100μl reaction volume containing 10μl of the double-stranded cDNA library solution, 10pmol of the primers and reagents as described in the amplification protocol of Perkin-Elmer/Cetus. One amplification cycle consisted of 1 min-denaturation at 94°C, 1 min-annealing at 37°C, and 2 min-polymerization at 72°C in a thermocycler (Perkin-Elmer/Cetus). The PCR product was cloned into pUC8 and used as the authentic cDNA probe. About 2.4 x 10^5 plaques from a bovine brain λgt10 cDNA library were screened using the authentic cDNA probe which had been labeled by a multi-priming method (8). Hybridization was performed overnight at 50°C. The final wash was made up of 0.2 x SSC (20 x SSC; 3M NaCl, 0.3M sodium citrate) containing 0.1% SDS at 50 °C.

DNA sequencing. The nucleotide sequences were determined by the dideoxy chain termination method (9) as modified by Hattori and Sakaki (10) with [α-^{32}P] dCTP and the Sequenase Ver.2 kit (United States Biochemical Corp.). The double strand plasmid was first sequenced from both strands using two vector primers which flanked the cDNA insert. The complete sequence was obtained by custom primer-directed DNA sequencing using specific primers complementary to internal cDNA sequence.

RNA blotting and hybridization. Total RNAs were extracted from bovine brain, liver, kidney, spleen, testis and heart by the guanidium isothiocyanate/cesium chloride method (11). Twenty μg of total RNA was denatured and electrophoresed in a formaldehyde-containing agarose gel. The RNA was transferred onto a Hybond-N (Amersham Japan) and hybridized in a solution containing 6 x SSC, 1 x Denhalt (5 x Denhalt; 1% bovine serum albumin, 1% polyvinylpyrollidone, 1% Ficoll 400), 0.1% SDS and 200μg/ml heat denatured salmon testis DNA at 55°C with [α-^{32}P] dCTP-

labeled cDNA fragments of 20ng. Finally, the filter was washed in 0.2 x SSC containing 0.1% SDS at 55°C and exposed to an imaging plate. The image was visualized for analysis using Bioimage analyzer (Model Fujix BAS2000, Fuji Film Corp).

Sequence analysis. The nucleotide and the deduced amino acid sequences of neurocalcin were screened for homology with known sequences using the EMBL-GDB (release 28), the SWISS-PROT data base at the European Molecular Biology Laboratory (release 19), the GenBank (release 69) at the IntrelliGenetics. Inc., and the NBRF-PDB (release 29) at National Biochemical Research Foundation.

Results and Discussion

We obtained an authentic cDNA probe of neurocalcin by the PCR technique. PCR amplification was performed using two synthetic primers (For and Rev) synthesized according to the partial amino acid sequence which is conserved in all isoproteins (Fig. 1A upper and middle lines). The PCR product was subjected to electrophoresis and a 59 bp band of an expected size was isolated from 4% Nu-Sieve (FMC) agarose gel. The fragment was cloned into pUC8 plasmid, identified by sequencing, and used as the authentic probe (Fig. 1A lower line).

About 2.4 x 10^5 plaques from a bovine brain λgt10 cDNA library was screened with the authentic probe. One positive clone with a molecular size of about 1.9 kbp was isolated, but a nucleotide sequence revealed that this clone was incomplete since the open reading frame encoding neurocalcin was not preceded by a 5' untranslated region or initiation codon. We rescreened 2.4 x 10^5 plaques of a bovine brain cDNA from a library purchased from Clontech using the first cDNA insert as a probe. Another positive plaque was obtained and purified. The insert was thus amplified as a blunt-end product by the PCR method using two λgt10 primers purchased from Takara Shuzo and cloned into pUC8 by HincII digestion (pCalN).

Fig. 1B shows the nucleotide and the deduced amino acid sequences for neurocalcin. The cDNA of pCalN consisted of 1,844 nucleotides including the coding region of 582 nucleotides, 11 nucleotides of the 5' leader, and 1,251 nucleotides of the 3' noncoding region. The nucleotide in positions -9, -4, -3, +4 are C, C, A, G around the presumed initiation codon for pCalN all met Kozak's criteria (12) designating residue 1 as the translation initiation site. Calculated molecular mass of 22,284 corresponding to the open reading frame of the 193 amino acids is well in accord with that of native protein judged by Tricine SDS-PAGE. This similarity strongly suggests that ATG is the probable translation initiation site. The amino acid sequence from the amplified PCR product (Fig. 1A, the middle line) was found in that of pCalN except for RE, which was replaced to KA (Fig. 1B, under line). There is a posibility of a technical artifact during cDNA cloning, while another close related clone may exist.

The deduced amino acid sequence of neurocalcin resulted in three putative EF-hand motifs (#74-85, #110-121, #160-171 amino acid positions) (Fig. 2). The EF hand was first observed in the crystal structure of parvalbumin (13). This basic functional and evolutionarily conserved domain consists of 29 amino acids arranged in

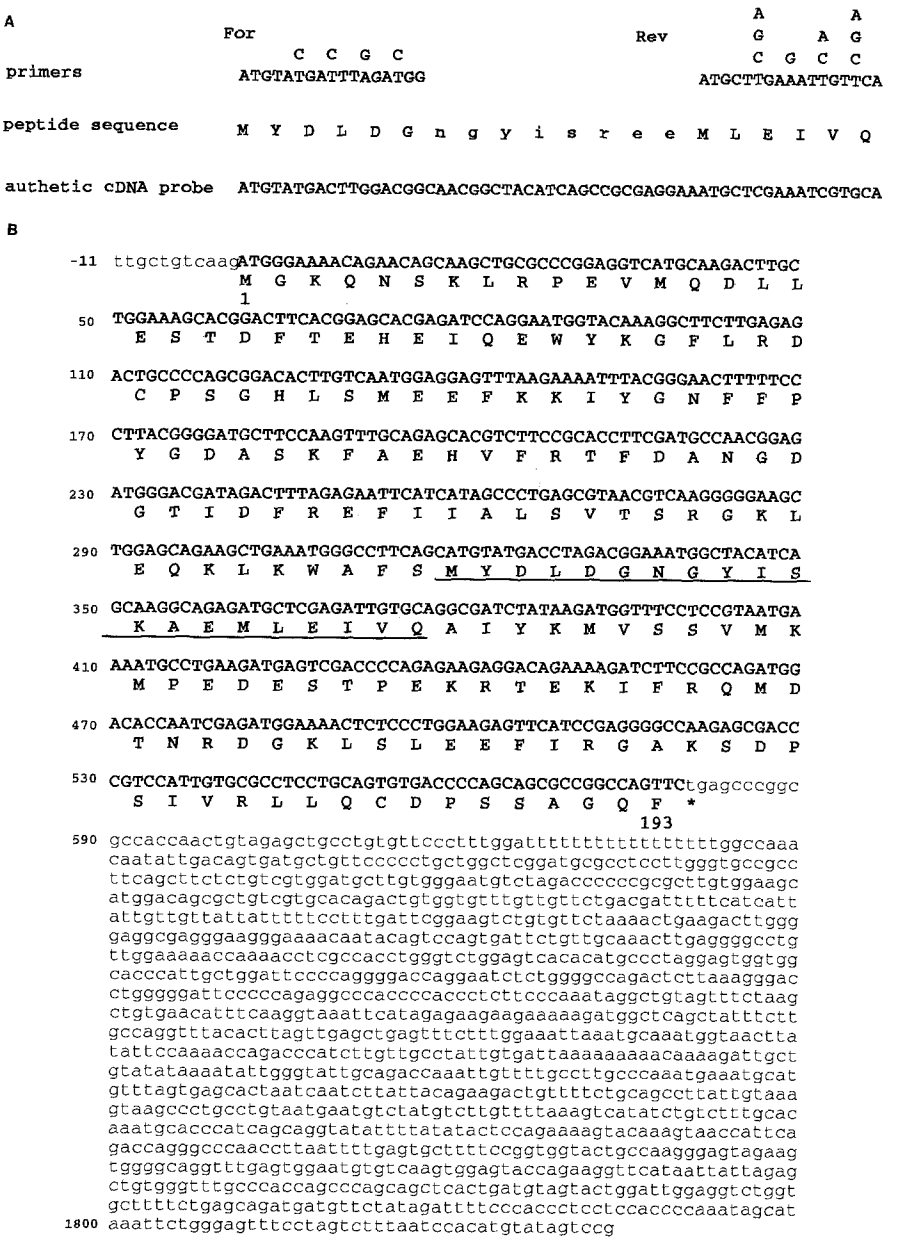

Fig. 1A. Strategy for neurocalcin cDNA.
Upper line indicates the primers synthesized by a DNA synthesizer Model 392A. Forward primer (For) is a 17-mer olignucleotide corresponding to MYDLDG while the reverse primer (Rev) is a 17-mer anti-oligonucleotide corresponding to MLEIVQ. Nucleotide sequence from the amplified PCR product is shown in the lower line. Middle line indicates the peptide sequence derived from Edman degradation (Capital letters) and the deduced amino acid sequence from the PCR product (lower case letters).

Fig. 1B. The nucleotide sequence of cDNA for neurocalcin and the deduced amino acid sequence. The nucleotide sequence is numbered starting with the initiation of this clone (left side) and the predicted amino acid at initiation (under the sequence). The translated region is shown in upper case letters and the 5'-, 3'- noncoding regions are shown in lower case letters. The predicted amino acid sequence is shown below the nucleotide sequence in a one-letter code. Under line indicates the animo acid sequence corresponding to the deduced amino acid sequence from the PCR product. *, termination codon.

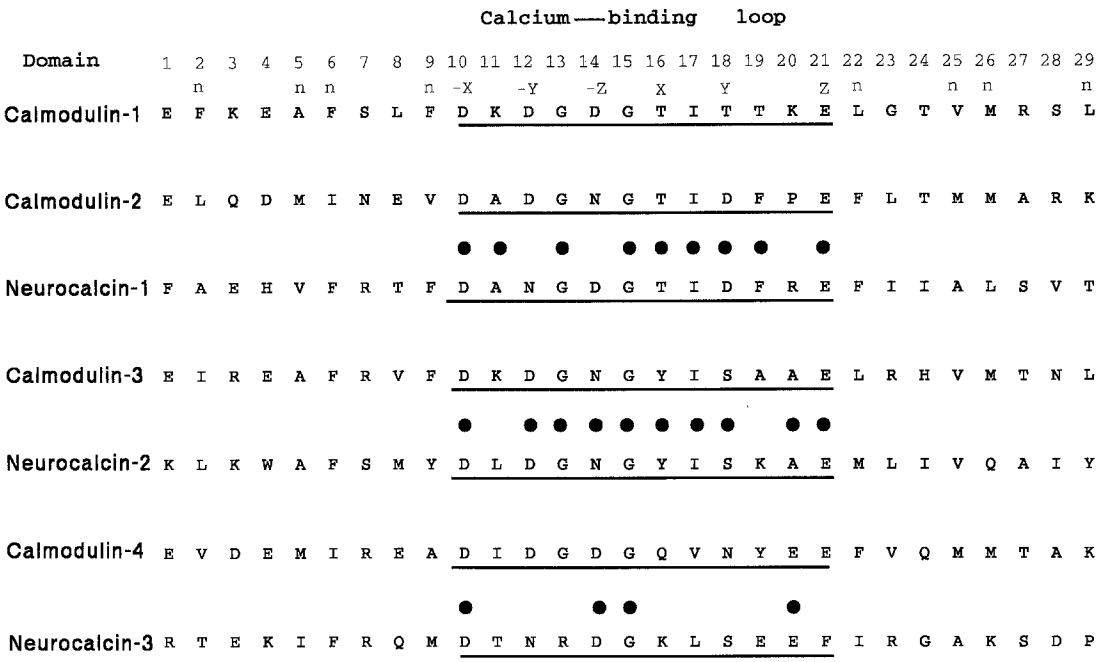

Fig. 2. EF-hand motifs compared between calmodulin and neurocalcin. Numbers on the right next to characters of calmodulin and neurocalcin represent the calcium-binding domain. Lower lines show the calcium-binding loops. Identical amino acid residues in calcium-binding loops are indicated by closed circles. The first and second domains of neurocalcin had a high identity with the second and third domains of calmodulin, respectively. See ref. (14) for the source of the sequence for calmodulin. The hydrophobic aspect of helix (residues 2,5,6,9 and 22,25,26,29) are listed under "n". Calcium ligands are provided at the octahedral vertices -X, -Y, -Z, X, Y, Z.

a helix-loop-helix conformation. Five of these, X,Y,Z, -X and -Z, usually have oxygen-containing side chains: Asp(D), Asn(N), Ser(S), Thr(T), Glu(E), or Glu(Q). The oxygen at position 16(-Y) comes from the main chain and can be supplied by any amino acids. Asp(D) is usually found at position 10, and Glu(E) is often found at position 21. Gly(G) at position 15 permits a sharp bend in the calcium binding loop. Ile(I), Leu(L), or Val(V) at position 17 contributed to the hydrophobic core of the molecule (14). Three domains of neurocalcin satisfies this criteria (Fig. 2). Many of calcium binding proteins with EF-hand have two or four domains. Parvalbumin, visinin, and recoverin are the members of three EF hand group. Neurocalcin is a new member of three EF-hand group, and the amino acid sequence of calcium binding loop is homologous with calmodulin but not with parvalbumin. The first and second motifs had a close homology to the socond and third motifs of calmodulin, respectively (15). Therefore, neurocalcin may bind more than two, probably three, moles of Ca^{2+} per mole of protein. Visinin and recoverin lack the EF-hand motif in the C-teminal domain, while neurocalcin appears to degenerate in the N-teminal domain like parvalbumin. Therefore, these findings are interesting in the aspect of the evolution of EF-hand proteins.

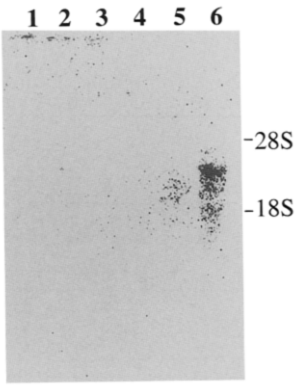

Fig. 3. Northern blot analysis of Neurocalcin in bovine tissue. Total RNAs were isolated from heart (lane 1) , kidney (lane 2), testis (lane 3), spleen (lane 4), liver (lane 5), and brain (lane 6) of bovine. Positions of 28S and 18S ribosomal RNAs are shown on the right side as molecular markers. The 3.8kbp band was detected in lane 6.

By Northern blot analysis using the entire insert of pCalN as a probe, mRNA of neurocalcin was detected in brain, but not in liver, kidney, spleen, testis or heart (Fig. 3). The 3.8 kbp band detected suggests that the pCALN did not contain the full length of neurocalcin mRNA. It is also presumed that pCalN contained neither a polyadenylation signal (AATAAA) nor poly $(A)^+$ tail.

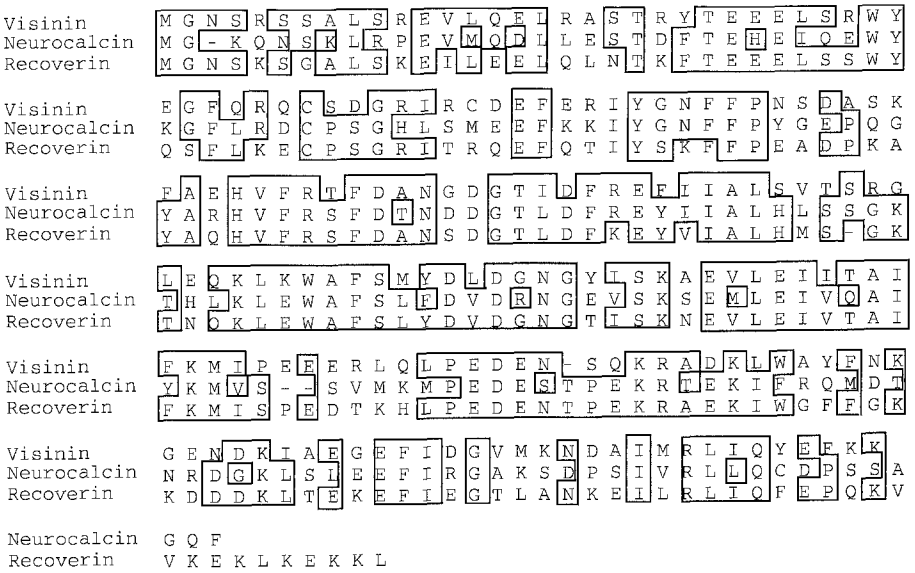

Fig. 4. Homology for the amino acid sequence of recoverin, visinin and neurocalcin. The amino acid sequences of recoverin and visinin were aligned with that of neurocalcin. Identical residues are boxed. Neurocalcin exhibited a 46.5% homology to visinin and 51.6% to recoverin.

By homology search, no identical nucleotide or amino acid sequence could be found for neurocalcin in the databases, suggesting that neurocalcin is a novel protein. The deduced amino sequence of neurocalcin is unique with its strong homology to recoverin (46.5%) and visinin (51.6%), which are rich in retina (Fig4). Recoverin is a 26kd calcium-binding protein in the outer rod segments of retina which acts as a modulator of photoreceptor guanylate cyclase (16). Visinin is also a 24kd calcium-binding protein with three EF-hand loops in the retinal cone cells (17). It is possible that neurocalcin may play a visinin- or recoverin-like role in brain. In a personal communication from Walsh, partial amino acid sequences of 21k-CaBP from bovine brain reported by him and his colleagues (18), have a close homology to neurocalcin. Recoverin-like Ca^{2+}-binding protein (P23K) was also reported from Takamatsu et al (19). P23K has a strong homology of more than 90% to neurocalcin, and might be a member of neurocalcin family.

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References

1. Heizmann, C.W., and Hunziker, W. (1991) Trends Biochem. Sci., **16**, 98-103.
2. Kretsinger, R.H (1980) CRC Cirt. Rev. Biochem. **8**, 119-174.
3. Watanabe, Y., Kobayashi, R., Ishikawa T., and Hidaka, H. (1992) Arch. Biochem. Biophys., **292**, 563-569.
4. Todoroki, H., Kobayashi, R., Watanabe, M., Minami, H., and Hidaka, H. (1991) J. Biol. Chem., **288**, 18668-18673.
5. Watanabe, M., Ando, Y., Todoroki, H., Minami, H., and Hidaka, H. (1991) Biochem. Biophys. Res. Commun., **181**, 644-649.
6. Tokumitsu, H., Kobayashi, R., and Hidaka, H. (1991) Arch. Biochem. Biophys., **288**, 202-207.
7. Emoto, Y., Kobayashi, R., Akatsuka, H., and Hidaka, H. (1992) Biochem. Biophys. Res. Commun. **182**, 1246-1253.
8. Feinberg , A.P., and Vogelstein, B. (1983) Anal. Biochem., **132**, 6-13.
9. Sanger, F., Coulson, A.R., and Barrel, B.G. (1980) J. Mol. Biol., **143**, 161-178.
10. Hattori, M., and Sakaki, Y. (1986) Anal. Biochem., **152**, 232-238.
11. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1987) Molecular cloning, A Laboratory Manual /2nd ed. Cold Spring Habor Laboratory, Cold Spring Habor, NY.
12. Kozak , M. (1987) Nucleic Acid Res., **12**, 857-872.
13. Moews, P.G. and Kretsinger, R.H. (1975) J. Mol. Biol., **91**, 201-228.
14. Moncrief, N.D, Kretsinger, R.H., and Goodman, M. (1990) J. Mol. Evol., **30**, 522-562.
15. Watterson, D.M., Scharief, F., and Vanaman, T.C. (1980) J. Biol. Chem., **255**, 962-975.
16. Yamagata, K., Goto, K., Kuo, C.H., Kondo, H., and Miki, N. (1990) Neuron, **2**, 469-476.
17. Dizhoor, A.M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K.A., Philipov, P.P., Hurley, J.B., and Stryer, L. (1991) Sience, **251**, 915-918.
18. Walsh, M.P ., Valentine, K.A., Ngai, P.K., Carruthers, C.A., and Hollenberg, M.D. (1984) Biochem. J., **224**, 117-127.
19. Takamatsu, K., Noguchi, T., Hirano, S., and Uyemura, K. (1992) Neurochem. Res. Suppl.,in press.