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Neuraxin, a novel putative structural protein of the rat central nervous system that is immunologically related to microtubule-associated protein 5

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During screening of a rat spinal cord λgt11 cDNA library with poly- and monoclonal antibodies against the postsynaptic glycine receptor a cDNA was isolated which covers an open reading frame encoding a protein of calculated mol. wt 94 kd. Sequence analysis identified a novel type of neuron-specific protein (named neuraxin) which is characterized by an unusual amino acid composition, 12 central heptadecarepeats and putative protein and/or membrane interaction sites. The gene encoding neuraxin appears to be unique in the haploid rat genome and conserved in higher vertebrates. Northern blot and *in situ* hybridization revealed neuraxin mRNA to be expressed throughout the rodent central nervous system (CNS). In spinal cord, neuraxin transcripts were abundant in motoneurons which also expressed glycine receptor subunit mRNA. A bacterial fusion protein containing ~90% of the neuraxin sequence was found to specifically bind tubulin. Polyclonal neuraxin antibodies cross-reacted with microtubule-associated protein 5 (MAP5), and a monoclonal antibody against MAP5 recognized the neuraxin fusion construct. Based on these data we suggest that neuraxin is related to MAP5 and may be implicated in neuronal membrane-microtubule interactions.

Key words: central nervous system/*in situ* hybridization/microtubule-associated protein/neuraxin/rat/repeat structure

Introduction

Neurons are highly differentiated cells which display an elaborate regionalization of cytoskeletal and cell surface components throughout their various dendritic, axonal and nerve terminal regions. Morphological and biochemical studies indicate that extensive membrane-cytoskeleton interactions underlie the selective localization of different membrane components to postsynaptic sites, the nodes of Ranvier and the presynaptic membrane. For example, ankyrin and spectrin are crucial for sodium channel topography in brain neuronal membranes (Srinivasan *et al.*, 1988). Microtubules have been implicated in synaptic transmission (Wooten *et al.*, 1975) and tubulin has been identified in presynaptic membranes (Gozes and Littauer, 1979). Subsynaptic filaments and receptor-associated proteins, like the recently cloned 43 kd *v*-protein (Frail *et al.*, 1987), are thought to cause the clustering and immobilization of nicotinic acetylcholine receptors at the motor endplate (Cartaud *et al.*, 1981; Burden *et al.*, 1983; Froehner, 1986). A similar role has been proposed for the 93 kd polypeptide associated with the inhibitory glycine receptor which mediates postsynaptic inhibition in spinal cord and other brain regions (Betz, 1987; Schmitt *et al.*, 1987; Betz and Becker, 1988; Langosch *et al.*, 1988). Many other yet unknown membrane-associated and structural proteins are probably required for maintaining the elaborate surface architecture of neuronal cells.

To elucidate the structure of the 93 kd polypeptide of the postsynaptic glycine receptor complex further, we have made an attempt to isolate corresponding cDNAs by screening a rat spinal cord expression library with poly- and monoclonal antibodies raised against purified rat glycine receptor

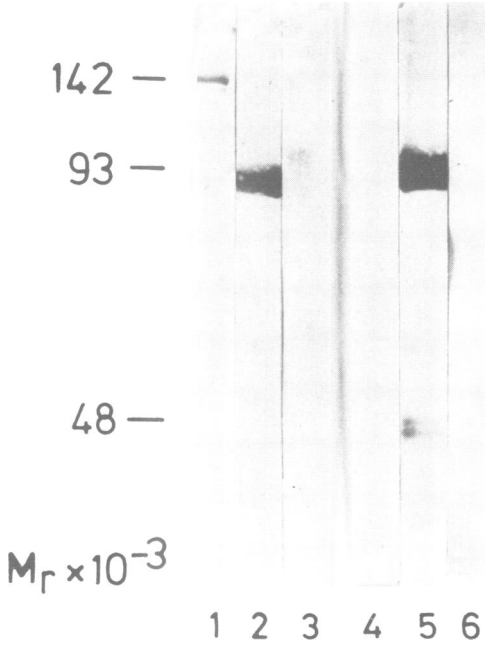
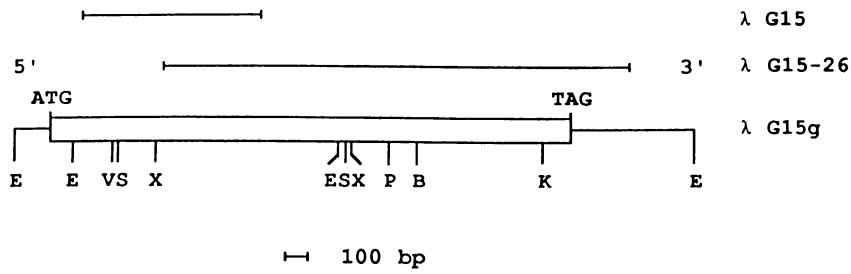


Fig. 1. Immunological characterization of the fusion protein of clone G15. Bacterial proteins were separated on a 7.5% SDS-polyacrylamide gel. Western blotting with glycine receptor mAbs-9b (**lanes 1-3**) and 7a (**lanes 4-6**) was performed as described in Materials and methods. Lanes 1 and 4 contained 30 μg of protein of lysate prepared from *E.coli* Y1089 infected with clone G15, lanes 3 and 6 30 μg of protein of lysate prepared from *E.coli* Y1089 infected with the empty λgt11 vector (negative control) and lanes 2 and 5 1 μg of affinity-purified rat glycine receptor. Sizes of stained bands are given on the left. All other mAbs tested gave a negative immunoreaction with the fusion protein like that shown for mAb-7a.

A



B

Fig. 2. Characterization of G15 cDNAs. (**A**) Restriction map of clones analyzed. The box indicates the large ORF encoding neuraxin. ATG, translation start site; TAG, translation stop site. Only major restriction sites are indicated (E, *Eco*RI; S, *Sst*I; K, *Kpn*I; V, *Eco*RV; X, *Xho*I; P, *Pvu*II; B, *Bam*HI). (**B**) cDNA and deduced amino acid sequence of clone G15g. Numbering of bases starts at the first nucleotide, that of amino acids at the potential start site of the neuraxin ORF. The central heptadecarepeats (left) and the three potential α-helices of the basic domain (right) are boxed.

preparations (Pfeiffer, 1983; Pfeiffer *et al.*, 1984). During this work, we identified a cDNA which encodes a novel brain-specific protein for which we propose the name neuraxin (for being localized along the entire neuraxis). This protein shows features typical of structural proteins; it binds to tubulin and is immunologically related to microtubule-associated protein 5 (MAP5). We suggest that this protein may be implicated in neuronal membrane–cytoskeleton interactions.

Results

Isolation of neuraxin cDNA

Our initial goal was the isolation of a cDNA encoding the 93 kd polypeptide which colocalizes and copurifies with the postsynaptic glycine receptor of mammalian spinal cord (Pfeiffer *et al.*, 1982; Triller *et al.*, 1985; Altschuler *et al.*, 1986; Betz, 1987; Schmitt *et al.*, 1987; Betz and Becker, 1988; Becker *et al.*, 1989). A randomly primed λgt11 cDNA library from spinal cord of 20-day-old rats was therefore screened for expression of fusion proteins reacting with poly- and monoclonal antibodies raised against affinity-purified rat glycine receptor preparations. From 1.2×10^6 recombinants, 13 phages were isolated which bound a polyclonal receptor antiserum. Western blot analysis confirmed the presence of immunoreactive sequences in the corresponding LacZ fusion proteins (not shown). When probed with glycine receptor monoclonal antibodies (mAbs) 1a, 2b, 4a, 7a and 9b, the fusion protein of clone G15 stained with mAb-9b (Figure 1). This antibody reacts with the 93 kd polypeptide band in affinity-purified glycine receptor preparations (Pfeiffer *et al.*, 1984). Also, a fraction of polyclonal antibodies against the affinity-purified receptor, bound and eluted from the G15 fusion protein and immobilized on nitrocellulose, was capable of re-staining the 93 kd polypeptide in a 'retroblot' (not shown). Antigenic determinants thus appeared to be shared by the G15 fusion protein and the 93 kd polypeptide of affinity-purified glycine receptor preparations.

Sequencing the 700 bp G15 cDNA insert revealed a continuous ORF throughout its entire length. A λgt10 cDNA library prepared from rat spinal cord poly(A)$^+$ RNA of 3-week-old rats using oligo(dT)$_{18}$ as a primer [6×10^5 recombinants (Grenningloh *et al.*, 1987)] was therefore screened with the G15 probe to isolate additional overlapping cDNA clones. A restriction map of the clones analyzed is presented in Figure 2A; the longest, G15g, was 3418 bp in size and contained an ORF of 2589 bp. The first ATG start codon was found at positions 174–176 (Figure 2B). The four preceding bases are AGAA, a sequence which fulfils the criteria of an eukaryotic translation start site (Lütcke *et al.*, 1987). A TAG stop codon is found at positions 2763–2765. The following 3′ untranslated region has a length of 653 bp and does not contain a common polyadenylation signal.

Table I. Predicted amino acid composition of neuraxin

Amino acid	Mol %
Trp	0.3
Tyr	4.2
Phe	1.6
Ile	3.0
Leu	4.5
Val	4.3
Met	2.0
Pro	9.3
Cys	1.4
Ala	5.9
Gly	4.8
Ser	14.2
Thr	9.9
Asp	6.6
Glu	10.1
Asn	2.0
Gln	3.4
His	2.0
Lys	7.5
Arg	3.1

Protein structure of neuraxin

The ORF of clone G15g encodes a protein (neuraxin) containing 863 amino acids with a calculated mol. wt of 94 368 daltons and a theoretical isoelectric point of 4.6. Its amino acid composition is rather unusual (Table I). The hydroxylated residues serine and threonine constitute 24%, the charged amino acids aspartate, glutamate, lysine, arginine and histidine 29.3% and proline 9.3% of the deduced polypeptide sequence. Most amino acids are equally distributed through the protein, exceptions being tyrosine and

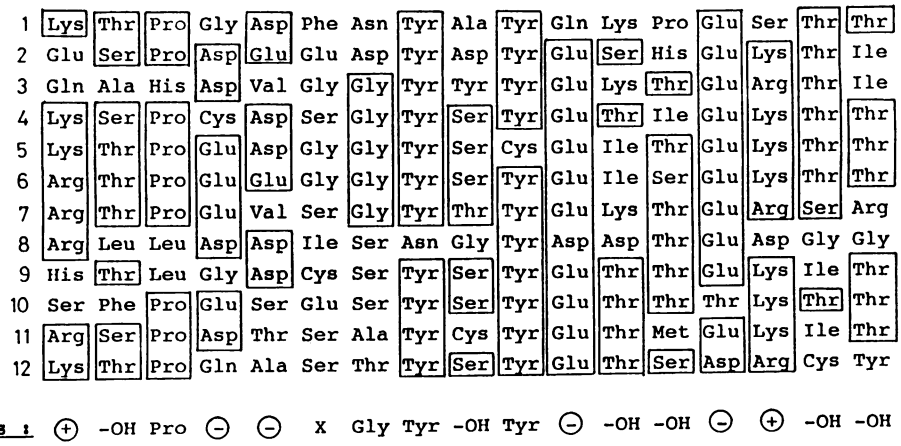


Fig. 3. Alignment of the central heptadecarepeat region of neuraxin. All 12 repeats between amino acids 258 and 461 and the deduced consensus sequence are shown. Identical or isofunctional residues are boxed.

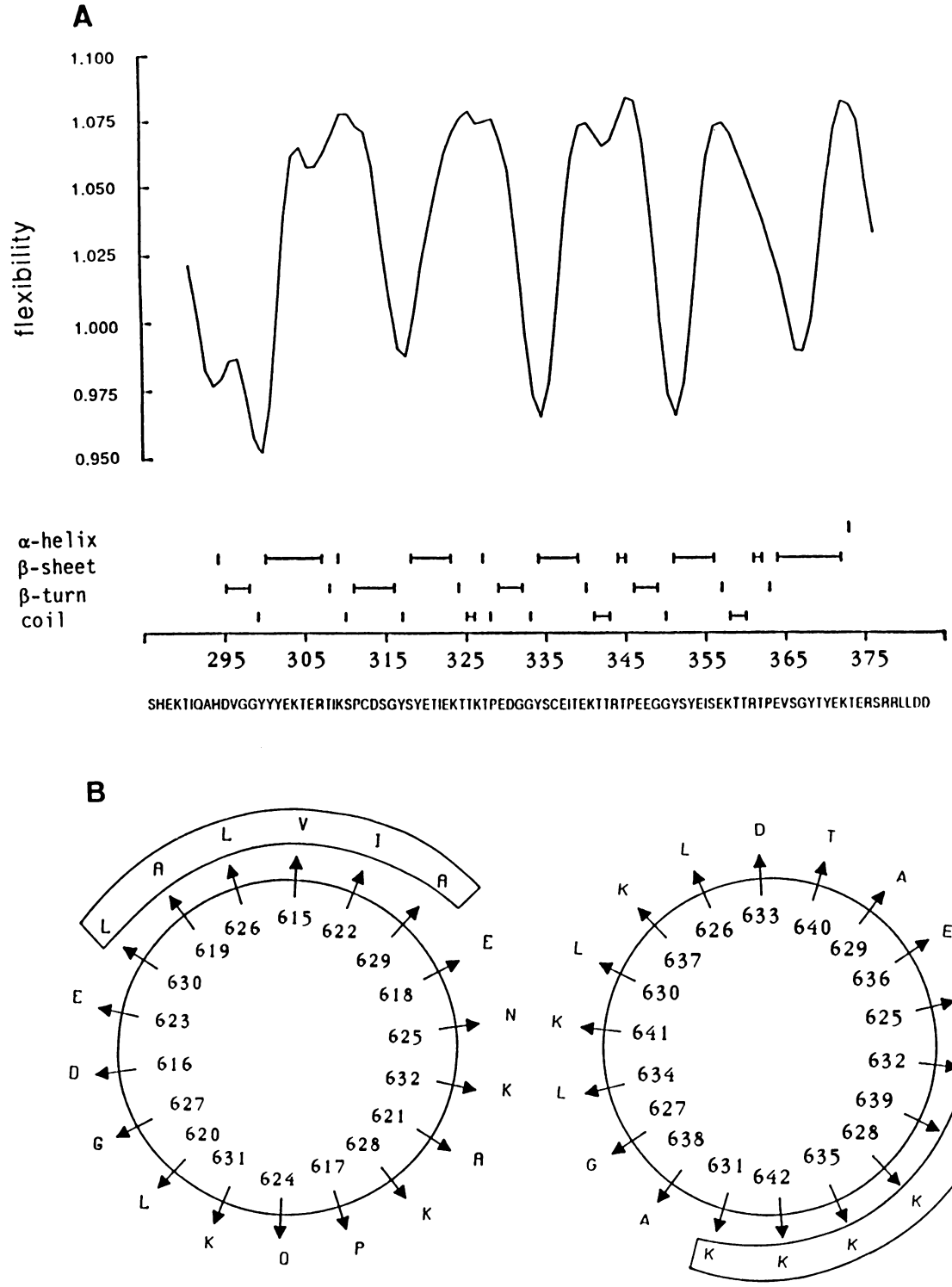


Fig. 4. Structural predictions for the central repeat region and the basic tail domain of neuraxin. (**A**) Presumptive local flexibilities and secondary structures of repeats 3–7 (amino acids 292–376). Directly above the amino acid sequence, the most probable secondary structures predicted by the Robson and Suzuki (1976) algorithm are indicated by bars. Superimposed is the corresponding flexibility plot calculated according to Karplus and Schulz (1985). Flexibility maxima coincide with K/R-T-P-E, and minima with Y-S-Y-E, motifs. (**B**) Helical wheel plot for helix 1 of the basic tail region (amino acid positions 615–632, left; and 625–642, right). Six hydrophobic residues on one side of the anterior and six lysines on the opposite side of the posterior parts of the helix are boxed.

basic residues. Of 36 tyrosines, 24 are located between positions 258 and 461. A cluster of basic residues is found between positions 628 and 741; 28% of all amino acids in this region are lysine or arginine. In accordance with the high content of charged and hydrophilic amino acids, hydropathy analysis according to Kyte and Doolittle (1982) revealed a highly hydrophilic amino acid sequence without any significantly hydrophobic regions (not shown).

Dot matrix analysis uncovered a prominent repetitive sequence in the central region of neuraxin. Between amino acid positions 258 and 461, a motif of 17 residues is repeated 12 times (Figure 3). Within this motif, two tetrapeptide

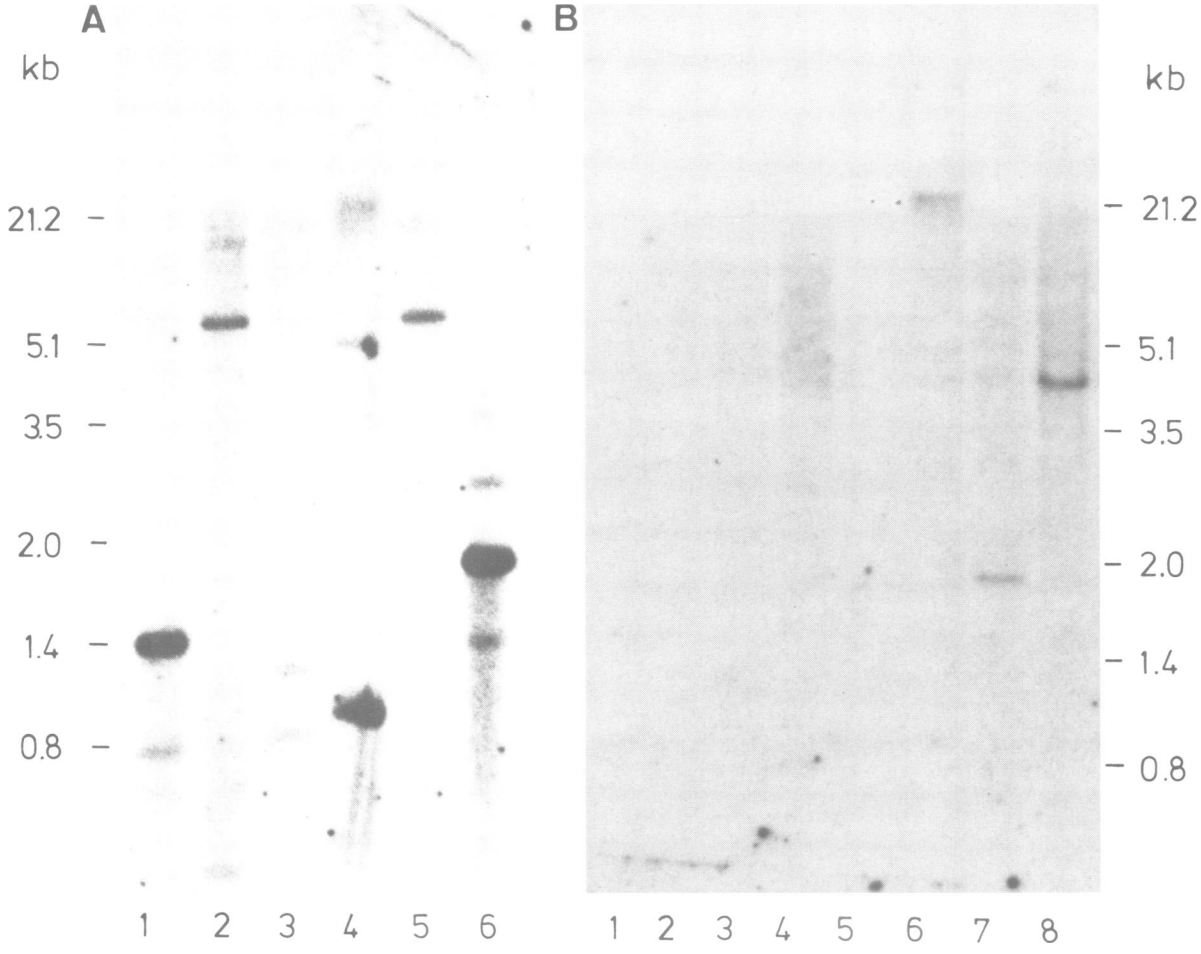


Fig. 5. Southern blot analysis of neuraxin genomic sequences. Genomic DNA (10 μg) digested with different restriction enzymes was separated on 0.9 or 1% agarose gels and hybridized with the 1323 bp *Eco*RI fragment of clone G15g. (**A**) Hybridization of differently restricted rat genomic DNA under stringent conditions. **Lane 1**, *Eco*RI; **lane 2**, *Eco*RV; **lane 3**, *Sst*I; **lane 4**, *Xho*I; **lane 5**, *Hin*dIII; **lane 6**, *Bam*HI. (**B**) Low-stringency hybridization with *Eco*RI restricted genomic DNA from different organisms. **Lane 1**, yeast; **lane 2**, *Dictyostelium discoideum*; **lane 3**, *Drosophila melanogaster*; **lane 4**, sea urchin; **lane 5**, *Xenopus laevis*; **lane 6**, chicken; **lane 7**, mouse; **lane 8**, human.

sequences, K/R-T-P-E and Y-S-Y-E, are particularly well conserved. Repeats 4–7 are highly homologous (82–88% identical or isofunctional amino acids) whereas repeats 1–3 and 8–12 exhibit a lower number of conserved residues (41–71%).

Secondary structure prediction according to the algorithm of Robson and Suzuki (1976) indicated the potential existence of regularly alternating β-turn and β-sheet structures within the central repeat region of neuraxin (Figure 4A). Here, the motif Y-E-X-T/S corresponds to β-sheet and E-D/E-G to β-turn structures. Flexibility calculations (Karplus and Schulz, 1985) suggest that alternating rigid and flexible regions correlate with the repetitive sequence elements Y-S(T,Y)-Y-E and K/R-T-P-E respectively (Figure 4A). Thus repeats 3–7, and probably the other repeats as well, most probably form an antiparallel β-sheet structure which on one side exposes the highly conserved tyrosine residues.

The basic tail region of neuraxin is also likely to contain ordered secondary structures. Amino acid positions 615–642, 687–697 and 704–726 are all predicted with high probability to form α-helices (Figure 2B; data not shown). The first of these helices exhibits a particular arrangement of its side chains in a helical wheel plot: its anterior part is characterized by six hydrophobic residues clustered on one side of the α-helix, where six lysines are located on the opposite side more posteriorly (Figure 4B).

Neuraxin is distinct from the 93 kd polypeptide of the glycine receptor

As described above, the fusion protein of clone G15 bound both polyclonal glycine receptor antiserum and mAb-9b, but none of the other glycine receptor-specific monoclonal antibodies. Since this fusion protein contained only a minor portion of the G15g cDNA coding sequence, a fragment of clone G15g covering ~90% of the neuraxin ORF (amino acids 42–826) was subcloned in the pEX 34b expression vector (Strebel *et al.*, 1986). After purification, the respective MS2 polymerase–neuraxin fusion product was used for assaying immunological cross-reactivity with all available glycine receptor mAbs.

Except for mAb-9b, none of the other monoclonals bound to the MS2 polymerase–neuraxin fusion construct (data not shown). In particular, mAb-7a, which has been used to immunochemically define and localize the 93 kd polypeptide of the postsynaptic glycine receptor complex (Pfeiffer *et al.*, 1984; Triller *et al.*, 1985; Altschuler *et al.*, 1986; Schmitt *et al.*, 1987; Becker *et al.*, 1988), did not exhibit any reactivity. Similarly, two rabbit and two mouse antisera raised against the original LacZ fusion protein of clone G15 strongly stained the fusion protein produced with the G15g insert on Western blots, but not the 93 kd polypeptide of synaptic membrane fractions nor affinity-purified glycine receptor preparations (not shown).

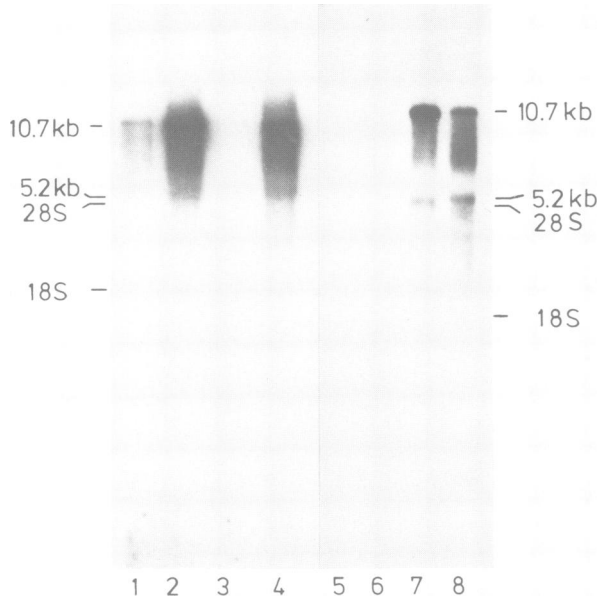


Fig. 6. Tissue distribution of neuraxin mRNA revealed by Northern blotting. Poly(A)$^+$ RNA (5 μg per lane) isolated from different rat tissues was electrophoresed, blotted and hybridized with the 1323 bp *Eco*RI fragment of clone G15g as described in Materials and methods. **Lanes 1–4**, poly(A)$^+$ RNA isolated from 3-day-old rats; **lanes 5–7**, poly(A)$^+$ RNA isolated from 40-day-old rats. Lane 1, spinal cord; lane 2, cerebrum; lanes 3 and 5, liver; lanes 4 and 7, cerebellum; lane 6, spleen; lane 8, medulla oblongata and brain stem.

Conservation of the neuraxin gene in higher vertebrates

Southern blot analysis of rat genomic DNA cleaved with different restriction enzymes revealed only few hybridizing bands when probed with the 1323 bp *Eco*RI fragment of clone G15g (Figure 5A). The following major fragments were observed: *Eco*RI, 1323 bp; *Eco*RV, 6000 and 13 000 bp; *Sst*I, 1135 and 925 bp; *Xho*I, 1000 and 5000 bp; *Hin*dIII, 7000 bp; and *Bam*HI, 2000 bp. The neuraxin gene thus most probably exists only once in the haploid rat genome. In addition, some weakly hybridizing fragments were seen (*Eco*RI, 875 bp. *Bam*HI, 1400, 2600 and 3700 bp); these may correspond to short exonic regions or related sequences.

Hybridization of the 1323 bp *Eco*RI restriction fragment of clone G15g to *Eco*RI digested genomic DNA from chick, mouse and human suggested conservation of neuraxin sequences in higher vertebrates (Figure 5B). No significant cross-hybridization was detected with yeast, *Dictyostelium*, sea urchin, *Drosophila* or *Xenopus laevis* genomic DNA.

Nervous system-specific expression of neuraxin mRNA

Northern blots of poly(A)$^+$ RNA prepared from different rat tissues at various postnatal stages indicated that neuraxin transcripts are found exclusively in nervous tissues, i.e. spinal cord, brain stem, cerebellum and cerebrum (Figure 6). Liver, spleen, kidney, heart or muscle did not contain any detectable amounts of this mRNA (Figure 6; data not shown). The size of the major transcript in all nervous tissues was 10.7 kb. In addition, a 5.2 kb species was revealed upon prolonged exposure.

In situ hybridization confirmed the nervous system-specific expression of the neuraxin gene. Figure 7 (A and B) shows the light microscopy and film autoradiograph of entire longitudinal sections through a newborn mouse hybridized with the 1323 bp ^{32}P-labeled *Eco*RI fragment of clone G15g. Only brain and spinal cord are labeled. Furthermore, when longitudinal sections of spinal cord of 20-day-old rats were hybridized with ^{3}H-labeled G15g cDNA, silver grains were abundant over large motoneurons (Figure 7C; data not shown). The same cells also hybridized to the insert of clone GR-2 (Figure 7D) which encodes the strychnine binding 48 kd subunit of the glycine receptor (Grenningloh *et al.*, 1987). Neuraxin transcripts are thus localized in neurons and, in spinal cord, codistribute with the 48 kd subunit mRNA.

Neuraxin is a tubulin binding protein

The MS2 polymerase–neuraxin fusion protein described above was subjected to SDS–PAGE, blotted onto nitrocellulose and overlaid with a buffer containing tubulin. Bound tubulin was then detected by means of mAbs directed against tubulin. Under these conditions, tubulin was bound to the 94 kd fusion protein, while no interaction was seen with the empty MS2 polymerase fragment which did not contain the neuraxin sequence (Figure 8).

Neuraxin is immunologically related to MAP5

On Western blots, polyclonal antibodies generated against a peptide representing the central repeat structure of neuraxin (amino acids 337–354) were found to cross-react with a microtubular protein (reviewed in Olmsted, 1986; Matus, 1988) from rat brain that has identical electrophoretic mobility to MAP5 (Figure 9A, lane 5). Similar results were obtained using polyclonal antibodies directed against the original LacZ fusion protein of clone G15 (Figure 9A, lane 6). On the other hand, mAb-9b originally used for isolating clone G-15 failed to react with MAP5 (Figure 9A, lane 7). The structural similarity of neuraxin and MAP5 was also demonstrated by the ability of another mAb, directed against MAP5, to react weakly with the MS2 polymerase–neuraxin fusion protein (Figure 9B). The same antibody did not interact with a 93 kd protein in taxol-precipitated cytoplasmic rat brain microtubules, suggesting either low abundance or even absence of neuraxin from cytoplasmic microtubules (not shown). The observation that the neuraxin-specific mAb-9b did not bind to rat brain MAP5 suggests that both proteins are related, but not identical.

Discussion

The neuraxin cDNA isolated in this work encodes a novel neuron-specific protein of rat CNS that is immunologically related to MAP5, a major associated protein of brain microtubules (Matus, 1988). As implicated from its identification, neuraxin appears to be associated with affinity-purified preparations of the postsynaptic glycine receptor. It is, however, not identical to the previously described 93 kd receptor-associated polypeptide despite its reaction with polyclonal antibodies against purified rat glycine receptor preparations and mAb-9b, which stains the 93 kd polypeptide band on Western blots (Pfeiffer *et al.*, 1984). Both our immunological experiments as well as recent peptide sequence analysis of the glycine receptor 93 kd polypeptide (P.Prior and G.Multhaup, unpublished data) clearly distinguish the two proteins. The presence of 'common' immunogenic epitopes recognized by both polyclonal glycine

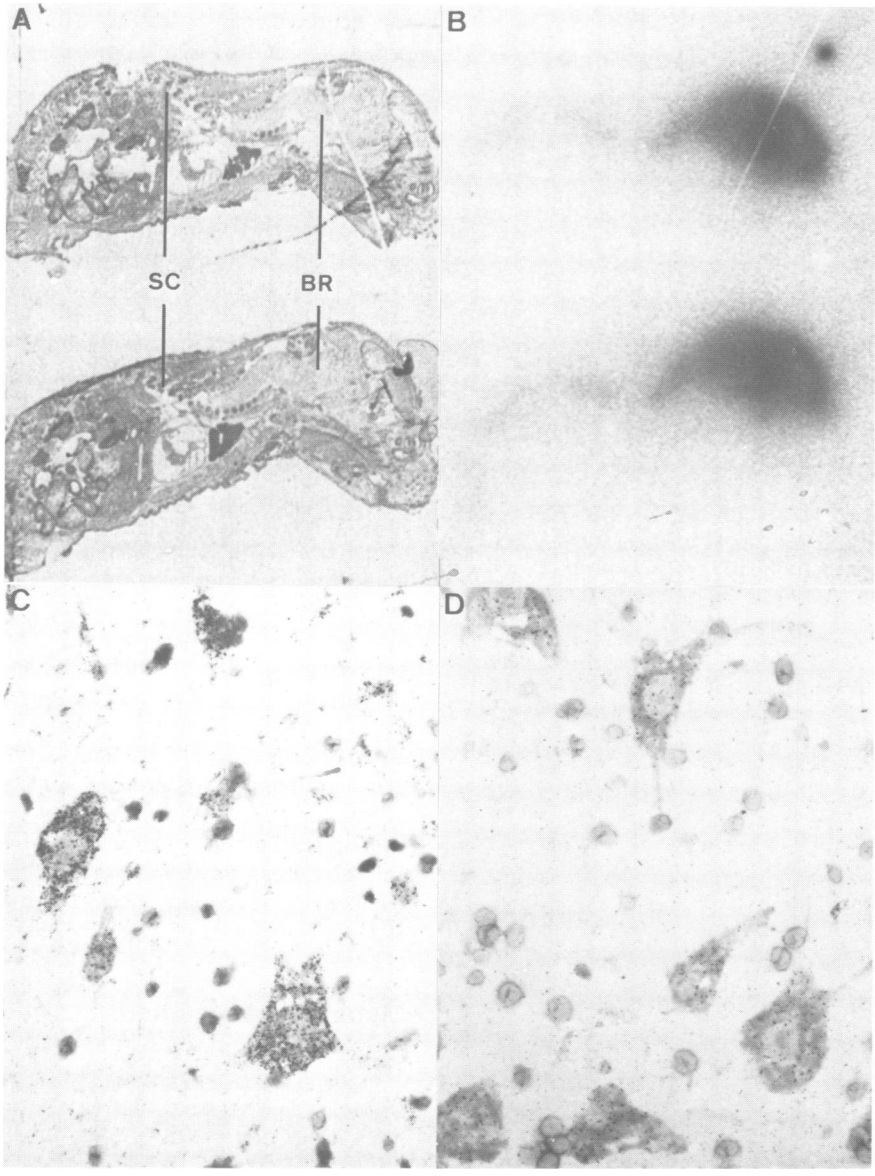


Fig. 7. Localization of neuraxin transcripts by *in situ* hybridization. (A) and (B), entire sections of newborn mice hybridized to the ^{32}P-labeled 1323 bp *Eco*RI fragment of clone G15g. (**A**) Giemsa stain; (**B**) corresponding film autoradiograph (exposure time 25 h). Note localization of label over the CNS (BR, brain; SC, spinal cord). (**C**) Localization of neuraxin and (**D**) of glycine receptor 48 kd subunit transcripts in rat spinal cord. Longitudinal frozen sections obtained from a 20-day-old rat were hybridized to the ^{3}H-labeled 1323 bp *Eco*RI fragment of clone G15g (C) and clone GR-2 (D; see Grenningloh *et al.*, 1987) as described in Materials and methods. After exposure to Kodak NTB-2 emulsion for 2 weeks, the sections were stained with Giemsa. Magnification 400-fold.

receptor antisera and mAb-9b thus probably results from copurification of neuraxin (or fragments thereof) with receptor components. Indeed, a marked heterogeneity of the 93 kd band is revealed upon two-dimensional gel electrophoresis; at least four or five distinct spots have been visualized recently in the 93 kd mol. wt region (P.Prior and B.Schmitt, unpublished data).

Several features of the predicted primary sequence of neuraxin characterize it as a putative structural protein. Firstly, in common with other structural proteins like collagens (Bornstein and Sage, 1980), neuraxin has an unusually high content of proline and hydroxylated amino acid residues. Secondly, its middle portion displays a highly regular repetitive region of 12 well-conserved heptadecapeptides. Similarly repeated motifs have also been found in other protein components of the cyto- and membrane skeletons, i.e. clathrin (Kirchhausen *et al.*, 1987), the middle mol. wt neurofilaments (Zopf *et al.*, 1987), spectrin (Birkenmeier *et al.*, 1985) and the microtubule-associated tau protein (Lee *et al.*, 1988), and in surface antigens of *Plasmodium falciparum* (Godson, 1985). Interestingly, the repeat region of neuraxin is predicted to possess an alternating β-turn/β-sheet structure potentially capable of forming an antiparallel β-sheet containing 24 conserved tyrosine residues. Similarly folded domains have also been proposed for other structural proteins, including the adenovirus fiber protein (Green *et al.*, 1983). We therefore suggest that neuraxin may have a structural role in central neurons. Its basic domains predicted to form α-helical regions qualify as potential sites for protein and/or membrane interaction. Furthermore, the many hydroxylated amino acid side chains might represent candidates for extensive covalent

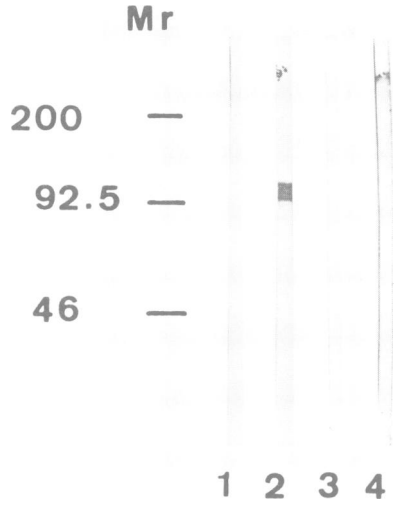


Fig. 8. Tubulin binding to neuraxin. The MS2 polymerase−neuraxin fusion protein was resolved on a 10% SDS−polyacrylamide gel, electroblotted onto nitrocellulose, stained with Ponceau S to verify transfer of proteins, and overlaid with PEM buffer in the absence (**lane 1**) and presence of tubulin (**lane 2**). Bound tubulin was visualized with a mixture of mAbs against α- and β-tubulin. **Lanes 3** and **4** contained the MS1 polymerase fragment without neuraxin insert; lane 3 was overlaid with PEM buffer alone and lane 4 with PEM buffer containing tubulin.

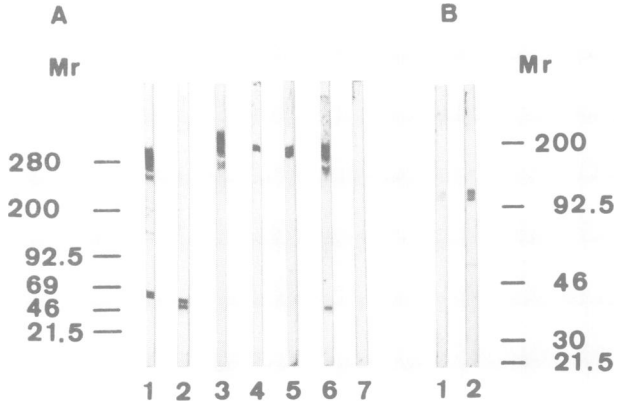


Fig. 9. Immunological cross-reactivity of neuraxin with MAP5. (**A**) Rat brain microtubular proteins were resolved by 5−10% SDS−PAGE, transferred to nitrocellulose and reacted with the following antibodies: **lane 1** mAb/c recognizing MAPs 2a, b and c; **lane 2**, mAb-tau1, recognizing τ-proteins; **lane 3**, mAb-MAP1a and **lane 4**, mAb-MAP5, staining the respective microtubule-associated proteins; **lane 5**, polyclonal antibodies against a peptide representing amino acids 337−354 of neuraxin; **lane 6**, polyclonal antibodies against the LacZ fusion protein of clone G15; **lane 7**, mAb-9b. (MAb-tau1 was used at a dilution of 1:100 and mAb-9b at 1:5 dilution. All other antibodies were diluted 1000-fold.) (**B**) The MS2 polymerase−neuraxin fusion protein was separated by 10% SDS−PAGE, transferred onto nitrocellulose and reacted with mAb-MAP5 (dilution 1:1000, lane 1) or mAb-9b (dilution 1:5, lane 2).

modification, e.g. phosphorylation. For other neuronal structural components, including the MAPs and the neurofilament protein family, such modifications are known to regulate their interactions with cytoskeletal elements during localization into specific compartments of the nerve cell (Lee *et al.*, 1986; Peng *et al.*, 1986; Matus, 1988).

A structural role of neuraxin is also suggested by its efficient binding of tubulin, the dominant cytoskeletal protein in brain tissue (Olmsted, 1986). Interaction between tubulin and neuraxin may be mediated by the central heptadecapeptide repeat region, a hypothesis which appears attractive in light of the immunological cross-reactivity of neuraxin and MAP5 demonstrated in Western blots. MAP5, as other MAPs, promotes tubulin polymerization *in vitro*; it is specifically expressed in neurons and particularly abundant during development, and therefore has been implicated in neurite outgrowth (Riederer *et al.*, 1986; Matus, 1988). The relationship between MAP5 and neuraxin is presently not clear, but may involve conserved structural elements in different gene products as well as alternative RNA splicing of a single primary transcript similar to that proposed for MAP2 and MAP2c (Garner and Matus, 1989).

What then may be the physiological function of neuraxin? The tissue specific expression of neuraxin mRNA in all segments of the CNS as well as the conservation of neuraxin genomic sequences in higher vertebrates point to the importance of this protein in a variety of neurons. Also, in spinal cord localization of neuraxin transcripts by *in situ* hybridization coincides with that of 48 kd glycine receptor subunit mRNA. Neuraxin thus may be associated with elements of central synapses and contribute to interactions between postsynaptic membrane components (e.g. the 93 kd polypeptide of the glycine receptor) and the microtubular system. Due to its widespread distribution in the CNS, such a role of neuraxin may be more general and involve various neuronal membrane proteins, including other receptor systems and ion channels. This view is consistent with recent data indicating that microtubule binding proteins play a central role as mediators between the different structural components of neurons, including proteins of the membrane skeleton (reviewed in Matus, 1988).

Materials and methods

Antisera

The mAbs and the polyclonal rabbit serum against rat glycine receptor used in this study have been described previously (Pfeiffer, 1983; Pfeiffer *et al.*, 1984). mAb/c, recognizing MAP2a, b and c, and mAb-MAP5 were obtained from H.Langbeheim, Sigma Immunochemicals, mAb-tau1 from L.Binder and mAb-MAP1a from Amersham. Polyclonal antisera against the β-galactosidase fusion protein of the λgt11 clone G15 (purified by preparative gel electrophoresis) and a peptide corresponding to amino acids 337−354 of neuraxin (kindly synthesized by R.Pipkorn) were raised in rabbits and mice using standard procedures (see Grenningloh *et al.*, 1987; Schloß *et al.*, 1988). The peptide was synthesized with an additional C-terminal cysteine residue and coupled to keyhole limpet hemocyanin according to Green *et al.* (1982). Antibodies cross-reacting with *Escherichia coli* proteins were removed from the antisera by adsorption with protein extract from *E.coli* strain Y1089 immobilized on Sepharose CL-4B (Pharmacia).

Construction of the λgt11 cDNA library

Total RNA was isolated from spinal cord of 20-day-old rats by LiCl precipitation of guanidinium isothiocyanate homogenates according to Cathala *et al.* (1983). Poly(A)$^+$ RNA was enriched using an oligo(dT) cellulose column (Aviv and Leder, 1972). First cDNA strands were synthesized from 5 μg poly(A)$^+$ RNA using 50 nmol pd(N)$_6$ (Pharmacia) as a primer and second strands using RNase H as described (Gubler and Hoffmann, 1983). The double-stranded cDNA was briefly digested with S1 nuclease, end-polished with the Klenow fragment of *E.coli* polymerase I and *Eco*RI linkers were added (Maniatis *et al.*, 1982). After digestion with *Eco*RI, the cDNA was purified over a Sepharose CL-4B column and ligated to *Eco*RI digested and dephosphorylated λgt11 arms (Promega). The ligation mixture was packaged using commercially available packaging extract (Promega) and the resulting phage particles used to infect *E.coli* strain Y1090. Thus, a library of 6×10^6 independent recombinants was established (average length of inserts ~500 bp).

Immunological screening of the library and analysis of the fusion proteins produced by individual recombinants were carried out according to the

protocol of Huynh *et al.* (1985) using the Proto-Blot detection system (Promega).

Gel electrophoresis and Western blotting

Electrophoresis by SDS–PAGE was performed according to Laemmli (1970). Protein bands in gels were visualized by silver staining (Merill *et al.*, 1982). Transfer of proteins from gels to nitrocellulose was performed as described (Towbin *et al.*, 1979). The Western blots were blocked for 20 min in Tris-buffered saline (TBS), pH 8.0, 3% (w/v) BSA and 0.1% (w/v) Nonidet P-40. Blots were then incubated with first antibody diluted in the same solution for 1 h at room temperature. After washing in TBS, TBS/NP-40 and TBS again (each wash for 10 min), antigenic sites were revealed using the Proto-Blot detection system (Promega).

DNA sequence analysis

The insert of clone G15g was sequenced in both directions after subcloning overlapping short fragments in M13 mp18/19 using the chain termination method (Sanger *et al.*, 1977). For computer analysis of DNA and deduced amino acid sequences the BSA program library of the German Cancer Research Center, Heidelberg, was used.

Construction of expression plasmid

To obtain a fusion protein encompassing residues 42–826 of the neuraxin sequence and four additional amino acids derived from the polylinker region, a 2.2 kb *Kpn*I fragment was cut from a pUC18 construct containing the two large *Eco*RI fragments of clone λG15g by exploiting a second *Kpn*I site in the 5′ polylinker of the plasmid. The fragment was end-polished with T4 DNA polymerase and subcloned into the *Eco*RI site (blunt-ended by fill-in reaction) of the expression plasmid pEX34b (Strebel *et al.*, 1986). Expression of the fusion protein was performed as described (Zabeau and Stanley, 1982).

Southern and Northern blot analyses

Genomic DNA was digested with restriction endonucleases, electrophoresed, transferred to nitrocellulose and hybridized with the nick-translated 1323 bp *Eco*RI fragment of clone G15g under conditions of high and low stringency as previously described (Grenningloh *et al.*, 1987).

Poly(A)$^+$ RNA samples were isolated from different rat tissues as described under construction of the λgt11 cDNA library. About 5 μg of the respective RNA were electrophoresed through a 1% agarose gel in 3.7% formaldehyde, 20 mM morpholine propane sulfonic acid, 50 mM sodium acetate and 10 mM EDTA, pH 7.0. RNA was transferred to Hybond N (Amersham) and hybridized with the nick-translated 1323 bp *Eco*RI fragment of clone G15g as described (Grenningloh *et al.*, 1987).

In situ hybridization

In situ hybridizations were carried out essentially as described by Hafen *et al.* (1983). DNA fragments were labeled by random priming (Feinberg and Vogelstein, 1983) using either [^{32}P]dCTP or a mixture of all four ^{3}H-labeled deoxyribonucleotides.

Tubulin overlay

MS2 polymerase–neuraxin fusion protein and control construct were electrophoretically separated and transferred onto nitrocellulose membranes as described above. After blocking with 5% (w/v) fat-free milk powder in PBS for 30 min and equilibration in PEM buffer (0.1 M PIPES–NaOH, 1 mM EGTA and 1 mM $MgSO_4$, pH 6.6) containing 1 mM GTP and 1 mM dithiothreitol, strips containing the electroblotted proteins were overlaid with a solution of 4.6 μg/ml polymerization-competent phosphocellulose-purified bovine tubulin in PEM buffer containing 1 mM GTP and 1 mM dithiothreitol and incubated for 1.5 h at 37°C. Taxol was added to 20 μM and incubation continued for 30 min at 37°C. Unbound tubulin was removed by three washes with PBS for 5 min each. A mixture of mAbs against α- and β-tubulin (Amersham) at a dilution of 1:1000 in PBS was applied for 1 h at 37°C. Bound antigen–antibody complexes were revealed using biotinylated goat anti-mouse IgG and peroxidase-conjugated extravidin (BioMakor, Israel).

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