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Expression of a conserved cell-type-specific protein in nerve terminals coincides with synaptogenesis

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ABSTRACT **Contact of axons with target territories results in the formation of synapses, specific junctional complexes that may represent a final stage of neuronal maturation. Synaptosomal-associated protein 25 (SNAP-25) is a component of particular nerve terminals recently identified in rodent brain. To evaluate the structure and regulation of molecular components of the synapse, we investigated the expression of SNAP-25 in the developing chicken nervous system. Analysis of SNAP-25 cDNA clones demonstrated that the chicken homologue is identical in amino acid sequence to the mouse protein. In chicken retina and neural tube, the onset of SNAP-25 mRNA and protein expression was found to correspond to the time of synaptogenesis. These results suggest that SNAP-25 plays a role in the physiology of mature nerve terminals and that its expression may be regulated by specific cell–cell interactions occurring during synapse formation.**

At the nerve terminal, specialized vesicles, cytoskeletal elements, and other specific proteins interact to organize the structure and function of the presynaptic compartment of the synapse (1, 2). Recent evidence suggests that the differential expression of presynaptic vesicle-associated proteins contributes to the formation of synapses with various molecular phenotypes (3, 4). Given that synapses are assembled when growing axons reach their target territory and neurons receive their own afferences, the expression of some elements of the synaptic machinery may be induced by the onset of these cell–cell interactions. As a first step in evaluating this hypothesis, we have examined the structure and expression of synaptosomal-associated protein 25 (SNAP-25),§ a protein component of subsets of nerve terminals, in the developing chicken nervous system.

SNAP-25 is a 25-kDa protein originally identified with a cDNA clone corresponding to a mRNA abundantly expressed in brain but not nonneuronal tissues of rodents (5). *In situ* hybridization and ultrastructural immunocytochemistry have shown that SNAP-25 mRNA is differentially expressed by subsets of neurons and that the protein is localized in particular nerve terminals (6, 7). Fractionation of synaptosomal components has indicated that SNAP-25 is associated with detergent-solubilized membrane complexes of the axoplasmic matrix, although not primarily with synaptic vesicles, suggesting a role in the cytoskeletal architecture of the nerve terminal. The precise nature of the association of SNAP-25 with synaptosomal membranes, as well as the function of the protein, is yet unknown. Although analysis of the polypeptide sequence indicates that SNAP-25 is not an integral membrane protein, several regions of the protein have been identified that may mediate interactions with other cellular components (6). Herein, we report that the chicken and mouse SNAP-25 are identical in amino acid sequence, indicating a high degree of evolutionary constraint throughout the structure of the protein. Moreover, we show that, in the retina and neural tube, SNAP-25 gene expression is induced after initial neurite extension at the time of synaptogenesis. These studies define, therefore, a uniquely well-characterized system to evaluate the influence of target-derived signals in neuronal maturation.

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MATERIALS AND METHODS

Animals. Fertile White Leghorn chicken eggs were kept at 38°C and 60% relative humidity. The stage of embryonic development was determined according to criteria described by Hamburger and Hamilton (8). Hatchlings were sacrificed when 2 days old.

Isolation and Sequence of Retina cDNA Clones. A cDNA library of 8×10^5 recombinants was generated from poly(A)$^+$ RNA of pooled embryonic day (E) 15 chicken retinas in the λGem-4 vector (Promega) as described (9). The library was screened with a ^{32}P-labeled 880-base-pair (bp) fragment, comprising the entire coding region of the mouse SNAP-25 (clone p8.52, ref. 6), and washed at low stringency (2× SSC/0.2% SDS at 42°C; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Isolated phage DNA was converted into plasmid by digestion with appropriate restriction enzymes and recircularization. Both strands of cDNA were sequenced, after alkali denaturation, by the dideoxynucleotide chain-termination method (10).

Immunocytochemical and Immunoblot Analysis. A rabbit polyclonal antibody directed against a synthetic peptide representing the 12 carboxyl residues of the mouse SNAP-25 polypeptide was used (6). For immunocytochemistry the antibody was affinity purified to the immunizing peptide. Embryonic tissue was immersion fixed for 2 hr with 4% (wt/vol) paraformaldehyde in 0.4 M sodium phosphate (pH 7.4) and hatched animals were perfused with the same fixative and then postfixed 2 hr. Tissues were cryoprotected in graded series of sucrose solutions and 20-μm cryostat sections were immunostained with secondary antibodies conjugated to fluorescein (Boehringer Mannheim) or by using an avidin–biotin-peroxidase complex kit (Vector Laboratories). Controls included omission and blockade (with the synthetic peptide) of the primary antibody and a reaction for endogenous peroxidase activity. Protein extracts (20 μg from each developmental stage) were separated by electrophoresis on 12.5% polyacrylamide gels containing SDS and electrophoretically transferred to nitrocellulose. The blots were blocked by a 30-min incubation in 5% (wt/vol) nonfat dried milk in Tris-buffered saline (11) and incubated with SNAP-25

Abbreviations: SNAP-25, synaptosomal-associated protein 25; E, embryonic day; NF, neurofilament.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M57957).

antisera at a 1:500 dilution for 3 hr at room temperature. Bound antibodies were detected by an avidin–biotin-peroxidase reaction.

Hybridization Analysis. Total cytoplasmic RNA (4 μg) from E9 and E15 retinas was separated by electrophoresis on a formaldehyde/agarose gel, transferred to nylon membranes, and hybridized with a random-primed ^{32}P-labeled purified cDNA insert of plasmid 4.1c comprising 300 bp of open reading frame and 250 bp of 3′ untranslated region from the chicken SNAP-25 mRNA. For *in situ* hybridization, sections were prepared as described for immunocytochemistry and hybridized with ^{35}S-labeled antisense or sense complementary RNA probes generated using the murine 8.52 cDNA plasmid as template and T7 or T3 polymerase, respectively. The probes were hydrolyzed to fragments 100–200 nucleotides long by treatment with 0.2 M NaOH at 0°C for 20 min and used for *in situ* hybridization as described (6).

RESULTS

Cloning of Chicken SNAP-25 mRNA. Initial analysis indicated that antibodies raised against the carboxyl-terminal sequence of mouse SNAP-25 recognized a protein of similar molecular weight on immunoblots of extracts of E15 chicken retina. A mouse cDNA probe comprising the coding region of SNAP-25 mRNA, therefore, was used to screen a cDNA library prepared from poly(A)$^+$ RNA of E15 chicken retina. Two clones, 4.1c and 4.1d, containing cDNA inserts of 550 and 980 bp, were isolated. The 4.1c cDNA hybridized to a mRNA of ≈2200 nucleotides on Northern blots of total cytoplasmic RNA of E15 chicken retina and adult mouse brain (data not shown). The nucleotide sequences of clones 4.1c and 4.1d were determined and assembled into a composite sequence of 908 nucleotides. This sequence contained an open reading frame of 618 nucleotides with 91% identity to the coding region of mouse SNAP-25 (Fig. 1). The remaining 9% difference resulted in synonymous codons, predicting a chicken protein of 206 residues identical in sequence to the mouse protein.

Antisera raised to the carboxyl terminus of the predicted protein sequence was used to survey SNAP-25 expression in the central and peripheral nervous systems of the hatchling chicken. The pattern of immunoreactivity indicated that SNAP-25 was expressed predominantly in synaptic regions by subsets of neurons (to be described in detail elsewhere), consistent with data reported for rodent brain (6, 7). In particular, these results demonstrated that SNAP-25 was abundantly expressed in chicken retina and spinal cord.

Expression of SNAP-25 in Chicken Retina. The vertebrate retina is highly stratified with alternating cellular and synaptic layers that display distinct spatio-temporal gradients of differentiation (see refs. 12 and 13). In the retina of E16 embryos and hatchlings, SNAP-25 immunoreactivity was found predominantly in the inner plexiform layer, where amacrine cells make synapses, and in axons of the ganglion cells (Fig. 2*a*). In contrast, staining of the outer plexiform layer, largely occupied by terminal fields of photoreceptor and horizontal cells, was much less intense. Within the cell body layers, immunoreactivity was variable in intensity, outlining the perikarya of the majority of neurons in the amacrine cell layer and some neurons of the ganglion cell layer but not in the photoreceptor, horizontal, and bipolar cell layers. Thus *in situ* hybridization in adult retina demonstrated much higher levels of SNAP-25 mRNA expression in amacrine and ganglion cell layers compared with photoreceptor, horizontal, and bipolar cells (Fig. 2 *b* and *c*). These data indicate that SNAP-25 is differentially expressed by subsets of mature neurons in the chicken retina.

Synaptogenesis is initiated at E11 to E12 at ganglion cell terminals (14) and in the inner plexiform layer where, in the central retina, the density of synapses reaches adult levels at E16 (15). In protein extracts of retina from E7 to E15 embryos, SNAP-25 was first clearly detectable at E11 followed by a marked increase during the subsequent 4 days of development (Fig. 2*d*). Northern blot analysis of total RNA of E9 and E15 retina demonstrated that SNAP-25 mRNA also increased markedly during this same period (Fig. 2*e*). This was confirmed by immunocytochemical studies in the central retina where SNAP-25 immunoreactivity was barely detectable before E10 and increased to an adult-like distribution in E16 embryos (data not shown).

Expression of SNAP-25 by Motor Neurons and Dorsal Root Ganglia Neurons. *In situ* hybridization of coronal sections of the spinal cord clearly demonstrated the expression of SNAP-25 mRNA in mature motor neurons of hatchlings (Fig. 3 *e* and *f*). Immunoreactive protein, however, was not detected in perikarya or axons at this stage (data not shown), consistent with localization of the protein at nerve terminals (6, 7). In the rat, transient accumulation of SNAP-25 in fiber tracts has been observed during brain maturation (16). Although similar translocation of SNAP-25 was apparent in chicken spinal cord, ganglion cell axons remain immunore-

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ATGGCGGAAGACGCGGATATGCGCAATGAGTTGGAAGAAATGCAGCGGCGTGCTGACCAGCTTGCTGATGAATCCCTCGAGAGTACCCGT   90
  C G   A C   T   AC   G G     A AG     G     G   G A C
METAlaGluAspAlaAspMetArgAsnGluLeuGluGluMetGlnArgArgAlaAspGlnLeuAlaAspGluSerLeuGluSerThrArg
           10                  20                  30

CGAATGCTGCAGCTTGTTGAAGAGAGTAAAGATGCTGGTATCAGGACTTTGGTTATGTTGGATGAGCAAGGAGAACAACTGGAACGCATT  180
 C     G C       C         C
ArgMetLeuGlnLeuValGluGluSerLysAspAlaGlyIleArgThrLeuValMetLeuAspGluGlnGlyGluGlnLeuGluArgIle
           40                  50                  60

GAAGAGGGGATGGACCAAATCAATAAGGACATGAAAGAAGCAGAAAAGAATTTGACGGACCTAGGAAAATTCTGTGGTCTTTGTGTCTGT  270
  G A         T                                       C G     G
GluGluGlyMetAspGlnIleAsnLysAspMetLysGluAlaGluLysAsnLeuThrAspLeuGlyLysPheCysGlyLeuCysValCys
           70                  80                  90

CCATGTAACAAGCTTAAATCAAGTGATGCTTACAAAAAAGCCTGGGGCAATAATCAGGATGGCGTTGTAGCCAGCCAGCCTGCACGTGTT  360
 C           C                       A A G           C   G
ProCysAsnLysLeuLysSerSerAspAlaTyrLysLysAlaTrpGlyAsnAsnGlnAspGlyValValAlaSerGlnProAlaArgVal
          100                 110                 120

GTAGATGAACGGGAGCAGATGGCCATCAGTGGTGGCTTTATCCGCAGGGTAACAAACGATGCCCGGGAAAACGAAATGGATGAGAACCTT  450
 G           C                       T       A   A
ValAspGluArgGluGlnMetAlaIleSerGlyGlyPheIleArgArgValThrAsnAspAlaArgGluAsnGluMetAspGluAsnLeu
          130                 140                 150

GAGCAGGTCAGCGGCATCATTGGCAACTTACGTCACATGGCCCTGGACATGGGCAACGAGATCGACACACAGAATCGCCAGATTGACAGG  540
   G     C A C C   T     A     T   T   C
GluGlnValSerGlyIleIleGlyAsnLeuArgHisMetAlaLeuAspMetGlyAsnGluIleAspThrGlnAsnArgGlnIleAspArg
          160                 170                 180

ATCATGGAGAAGGCTGATTCCAATAAAACCAGGATTGATGAAGCCAACCAGCGTGCAACAAAGATGCTCGGCAGTGGTTAA          621
          C   C   A     A       G A
IleMetGluLysAlaAspSerAsnLysThrArgIleAspGluAlaAsnGlnArgAlaThrLysMetLeuGlySerGly***
          190                 200
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FIG. 1. Comparison of the open reading frame nucleotide sequence and encoded protein of chicken and mouse SNAP-25. Only the nucleotides that are different are indicated for the mouse (middle row). All nucleotide substitutions produce synonymous codons and the two open reading frames predict the same amino acid sequence. The positions of hydrophobic residues within the amino-terminal domain that may form an amphipathic helix (positions 7–36), the clustered cysteine residues (positions 84–92), and the carboxyl-terminal dodecapeptide used to raise antisera are in boldface type.

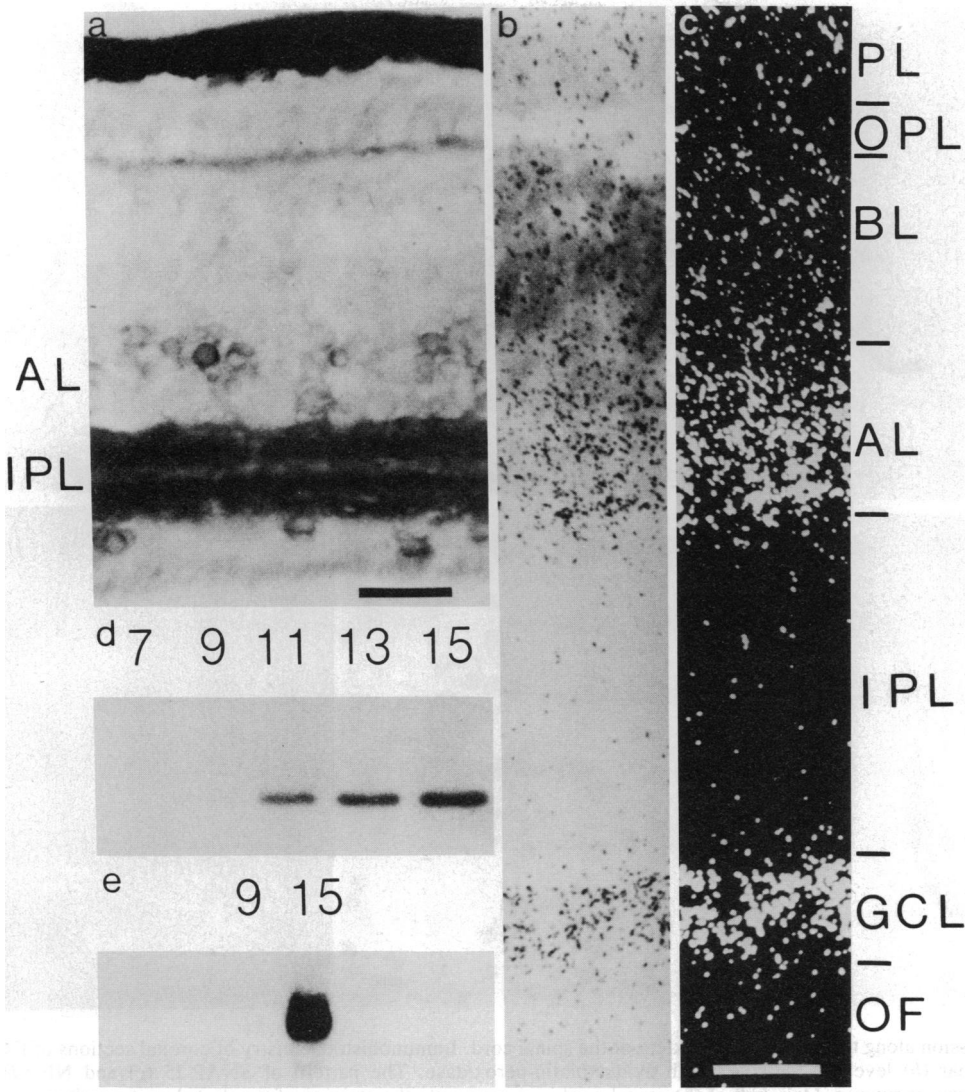

FIG. 2. Localization of SNAP-25 mRNA in the retina. Immunostaining of embryonic retina at E16 by using avidin–biotin-peroxidase complex is shown in *a*. Bright-field (*b*) and dark-field (*c*) photomicrographs demonstrate *in situ* hybridization with an antisense complementary RNA probe to a transverse section of a hatchling retina. After development, the sections were Nissl-stained to indicate perikarya. PL, photoreceptor layer; OPL, outer plexiform layer; HL, horizontal cell layer; BL, bipolar cell layer; AL, amacrine cell layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OF, optic fibers layer. (Bar = 20 μm.) (*d* and *e*) Immunoblots of retina protein extracts prepared from E7, -9, -11, -13, and -15 staged embryos and Northern blots of RNA extracts of E9 and E15 retina, as indicated.

active in adult retina (data not shown). The reason for these differences is yet unclear but may be related to variations in abundance of SNAP-25 in various neuronal populations.

In the chicken, the neural tube develops following a rostro-caudal gradient of differentiation where, at E3 to E4, development at the brachial level appears advanced by ≈12 hr compared with the lumbar level. Axons of the brachial plexus enter their target region, the fore limb, at E3.5 (17), whereas axons from the lumbar plexus enter the hind limb and make functional connections only at E4 (18, 19). At the brachial level, SNAP-25 was first detected at E4 localized in the neuropil around motor neurons and in motor axons (Fig. 3*a*). By E6 most tracts within the spinal cord showed immunoreactivity and spinal nerve branches could readily be identified in the wing bud (data not shown). To relate more precisely the onset of SNAP-25 expression with the time of synaptogenesis, serial sections along the rostro-caudal axis of the developing neural tube of E4 embryos were examined. Comparison of various levels showed a clear gradient of SNAP-25 expression. At the brachial level most motor axons were labeled (Fig. 3*a*); in contrast, SNAP-25 expression at the lumbar level appeared just initiated, with only few of the outgrown motor axons very weakly labeled (Fig. 3*b*).

Sensory neurons of the dorsal root ganglia establish contacts with peripheral and central targets at about E8, several days after motor neurons were located at the same level of the neural tube (20, 21). At the brachial level, although dorsal root ganglia fibers were visualized as early as E4, SNAP-25 immunoreactivity was not detected until E7 to E8, when both central and peripheral branches exhibited immunoreactivity (data not shown).

Comparison with Neurofilament (NF) Proteins. The previous results indicated that induction of SNAP-25 expression occurs after initial axonal elongation. To compare the developmental regulation of SNAP-25 with a structural component of growing and mature axons, the expression of the major cytoskeletal NF proteins was examined with a monoclonal antibody (22) that recognized all three subunits of NF protein in chicken retina (data not shown). In neural tube and retina, the appearance of NF immunoreactivity in axons preceded SNAP-25 by several days. Striking examples were peripheral and central fibers of the dorsal root ganglia, where NF protein was detected clearly at E4, in contrast to SNAP-25 seen only after E7 (for a comparison at E6, see Fig. 3 *c* and *d*). Within the spinal cord, the distribution of NF and SNAP-25 were clearly different: NF staining was enriched in white matter, in comparison to an abundance of SNAP-25 immunoreactivity in the neuropil surrounding the cell bodies of the grey matter, consistent with the accumulation of SNAP-25 at axonal terminals.

DISCUSSION

The studies reported here define two distinctive features of SNAP-25: the extreme conservation of its primary structure and its expression during development coincident with syn-

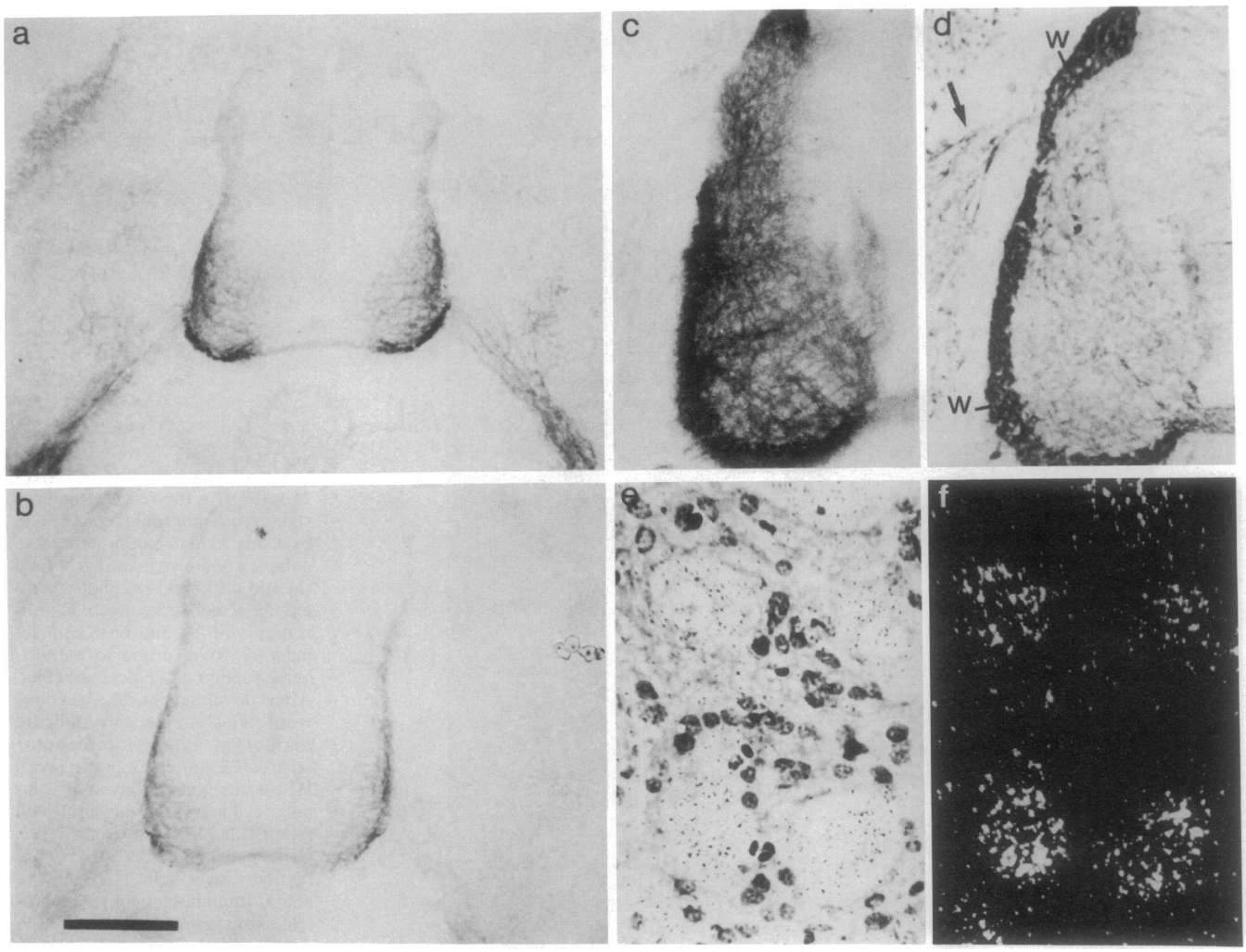

FIG. 3. Gradient of SNAP-25 expression along the rostro-caudal extent of the spinal cord. Immunohistochemistry of coronal sections of E4 embryos at the brachial (*a*) and lumbar (*b*) levels as detected with avidin–biotin-peroxidase. The pattern of SNAP-25 (*c*) and NF (*d*) immunoreactivity in spinal cord at E6 was compared. Only half of the spinal cord is shown; note the staining of the dorsal root ganglion in *d* (arrow). Bright (*e*)- and dark (*f*)-field photomicrographs show *in situ* hybridization of motor neurons in the spinal cord of a hatchling chicken with an antisense RNA probe. W, white matter. (Bar: *a*, *b*, *c*, and *d*, 100 μm; *e* and *f*, 20 μm.)

aptogenesis. Both of these properties have important implications for the possible function(s) of this nerve-terminal protein.

Absolute conservation of protein structure between mammals and birds is rare: of >90 proteins characterized in both classes, only calmodulin, muscle α-actin, ubiquitin, and some histones are identical (R. Doolittle, personal communication); in contrast, other well conserved proteins, such as cytochrome *c* and glyceraldehyde-3-phosphate dehydrogenase, exhibit 92–93% identity between chicken and rat (23, 24). The evolutionary conservation of the primary structure of SNAP-25, therefore, indicates that the three-dimensional structure of the protein cannot tolerate any changes, possibly because the protein engages in multiple interactions with other cellular components. Other proteins thought to have a role in the structure or function of the presynaptic terminal and for which amino acid sequence is available also have been found to be highly conserved, although not to the extent of SNAP-25. For example, the synaptic vesicle-associated protein synaptophysin is highly conserved but does not display complete sequence identity, even among mammalian species (25, 26). Similarly, the low molecular weight vesicle-associated membrane protein (VAMP) or synaptobrevin, which shows considerable conservation from mammalian species to *Torpedo* and *Drosophila*, differs at two residues between cow (27) and rat (28). The sequence of the SNAP-25 polypeptide contains several regions that may mediate interactions with cytoskeletal and membrane components of the nerve terminal (6), including a region of the amino terminus (residues 7–36) that may form an amphipathic α-helix that could mediate membrane associations and a cluster of four cysteine residues (positions 85–92) that could provide sulfhydryl bonds for intermolecular linkage or binding of metal ions.

Upon contact with their target cells, growing axons induce specific events that contribute to the maturation of the postsynaptic compartment of the synapse (29–31). Similarly, phenotypic differentiation can be determined by interactions between neurons and their targets (21, 32); however, a specific role for the target cell in the functional differentiation of the nerve terminal has yet to be demonstrated. A first step in testing this hypothesis is an accurate description of the spatio-temporal patterns of expression of gene products that are likely to play a role in the function of nerve terminals. As described in the retina and in both motor and sensory peripheral fibers, SNAP-25 is expressed late during neuronal differentiation—after the stages of proliferation, migration, and initial process outgrowth—when neurons establish contacts with their targets and start to form synapses. This late pattern of expression is also exhibited by other proteins involved in synapse function, such as synaptophysin (33) and synapsin I (34), and contrasts with that of axonal components

such as the growth-associated protein GAP-43 (35) or microtubule-associated protein 5 (36), whose early expression has been related to a role in axonal growth or plasticity.

After synaptogenesis, neuronal populations are known to become dependent on interactions with their targets for survival and further differentiation (21, 37, 38). Whether target-derived signals play a role in the induction of SNAP-25 remains to be demonstrated; however, lesions of postsynaptic cells show that expression of SNAP-25 is maintained independent of synaptic activity in mature neurons (7). Other events occurring at the time of target contact may be involved in the regulation of SNAP-25 expression, including the receipt of afferents and the increase of electrical activity. Alternatively, the expression of SNAP-25 and other synaptic proteins may reflect an endogenous program of differentiation independent of cell–cell interactions. These hypotheses may be distinguished by lesion and blockade of activity in the experimental models of developing retina and neural tube in the chicken embryo.

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