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The cloning and characterization of TOAD-64: a gene expressed transiently during neuronal differentiation in the mammalian nervous system

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ABSTRACT

The Cloning and Characterization of TOAD-64: A Gene Expressed Transiently During Neuronal Differentiation in the Mammalian Nervous System

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During the development of the mammalian central nervous system, a pool of morphologically homogeneous, mitotically active progenitor cells in the neural tube gives rise to the enormously diverse population of postmitotic cells that assume the properties of neurons or glia. Postmitotic neurons elaborate processes that are involved in the migration of neurons to their adult positions as well as the elaboration of axonal and dendritic arbors that will ultimately form specific synaptic contacts. The generation of postmitotic neurons from precursor cells is a central differentiative event in development, but molecular markers of this event are few.

Using 2D gel electrophoresis, proteins that are developmentally regulated over the course of neurogenesis were identified. One of these, TOAD-64 (Turned On After Division, 64kDa) is expressed by neurons immediately after their birth, and is dramatically down-regulated in the adult.

The expression of the TOAD-64 protein and gene is coincident with initial neuronal differentiation and down regulated when the majority of axon growth is complete. TOAD-64 is up-regulated following neuronal induction of P19 and PC12 cells; it is re-expressed in adult spinal motor neurons following axotomy of the sciatic nerve; and is present on the lamellipodia and filopodia of growth cones.

The gene encoding TOAD-64 shows sequence homology to the *unc-33* gene of *C. elegans*, mutations in which lead to aberrations in axon outgrowth. A chick homolog (CRMP-62) of TOAD-64 has also recently been cloned, and appears to mediate growth cone collapse in response to collapsin, suggesting a similar role for TOAD-64 in the rat.

Taken together these results suggest that TOAD-64 may be a central element in the processes underlying axonal outgrowth and pathfinding.

The Cloning and Characterization of TOAD-64: A Gene Expressed Transiently During Neuronal Differentiation in the Mammalian Nervous System

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PREFACE

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The majority of this work has been previously published and is appropriately cited throughout the dissertation.

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CHAPTER 1

INTRODUCTION

One of the fundamental tasks that we have as developmental neurobiologists, is to gain an understanding of the basic molecular and cellular strategies used by the developing nervous system to generate the complex anatomical diversity that defines the mature cerebral cortex. This task is daunting and includes understanding, (a), the production of phenotypically diverse neurons and glia from a morphologically homogeneous pool of precursor cells, (b), how these cells are organized into functional units, and (c), how cells initiate and alter their connectivity through the formation and reorganization of specific axonal and synaptic contacts.

Many of these developmental events have been carefully investigated and described, such as the time course of neuro- and gliogenesis (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1974; Luskin and Shatz, 1985), the commitment of precursor cells to specific cell fates (Sanes et al., 1986; Turner and Cepko, 1987; Luskin et al., 1988; Price and Thurlow, 1988; Wetts and Fraser, 1988; Walsh and Cepko, 1990; Kornack and Rakic, 1995), and certain aspects of cell migration (Rakic, 1972; Sidman and Rakic, 1973; Walsh and Cepko, 1988; Austin and Cepko, 1990; Hatten, 1990; Rakic,

1990). However, an understanding of the molecular components that underlie these processes is incomplete. My work has involved the isolation and characterization of a gene whose expression is correlated with the period of neurogenesis, neuronal migration, and axonogenesis in the embryonic rat cerebral cortex.

Cortical cells are generated by mitotic progenitors in two proliferative layers deep in the brain, the ventricular zone and the subventricular zone (Boulder Committee, 1972). Neuronal birth refers to the final mitotic division resulting in a daughter cell (pre-migratory neuron) no longer able to re-enter the cell cycle (Berry and Rogers, 1965; Frederikson and McKay, 1988). In the rat, the vast majority of cerebral cortical neurons are born between embryonic day 14 (E14) and E21. ^{3}H-Thymidine birthdating studies have shown that precursors sequentially produce neurons destined for the six layers of the cortex in an inside-first, outside-last manner (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1974). Thus neurons destined for layer VI have their peak of generation on E15/16, while neurons destined for layer II are born from E18-E21 (peak E20) (Berry and Rogers, 1965, Miller, 1986; Miller, 1990).

The correlation between the birthday of a neuron and its ultimate laminar destination has raised the question of whether cell birthday determines cell fate. Is cell fate determined by an intrinsic developmental clock, or can environmental cues (such as cell-cell or cell-substrate

interactions) regulate a young neuron's migration to its correct laminar position. Transplantation studies of progenitor cells at different phases in the cell cycle indicate that early in the cell cycle (S-phase), the age of the host determines where the daughter will migrate, suggesting that neuronal cell fate can be altered by environmental cues. However if transplanted later in the cell cycle, daughter cells migrate appropriate to their birthday (intrinsic cues) (McConnell and Kaznowski, 1991). The nature of these cues are unknown.

After neurons are born in the ventricular and subventricular zones, they migrate in a largely radial fashion through the intermediate zone into the growing cortical plate (Berry and Rogers, 1965; Hicks and D'Amato, 1968). The mechanism and pattern of migration has been controversial since the time of Cajal (1911), and continues to be a source of debated work today. The work by Rakic (1972) indicating that neurons migrate along radial glial cells into the developing cerebral cortex has largely been supported by more recent *in vitro* studies showing neurons attaching and migrating along glial processes (Gasser and Hatten, 1990; Hatten, 1990). Retroviral lineage tracing studies have shown widespread scattering of clonally related cells suggesting tangential in addition to radial patterns of migration (Walsh and Cepko, 1988; Austin et al., 1990). Recent experiments in the developing primate by Kornack and Rakic (1995) suggest that non-radial distribution patterns of

clonally related cells in the cortex may reflect distinct modes of cell division in the ventricular zone.

Early neuronal differentiation begins embryonically, soon after migration is completed, and includes the development of axons and the elaboration of primitive dendritic trees. However, much of the cytological and molecular differentiation that a neuron undergoes as it acquires its mature phenotype begins in the embryonic period, but slowly continues after birth and into the early postnatal period (Stanfield and O'Leary, 1985; Parnavelas et al., 1988; Hockfield et al., 1990; Schlaggar and O'Leary, 1991; Yamagata et al., 1993). These features include axon elongation, dendritic arborization, and synaptogenesis; and the ability to respond to neighboring cells and the extracellular environment by the expression of molecules such as receptors, ion channels, neurotransmitters, cell adhesion molecules, and other as yet undefined components that all comprise a neuron's mature form.

The events described above: neurogenesis, neuronal migration, and early differentiation, are not distinct steps during corticogenesis, but overlap spatially and temporally during development. It is easier however, to think in discrete steps when trying to imagine the unique cellular and molecular mechanisms that may underlie these processes.

To identify proteins that may participate in these developmental and differentiative events, two-dimensional gel electrophoresis was previously used to compare the change in

rate of accumulation of membrane associated proteins expressed by progenitor cells to those expressed by postmitotic neurons. This was done by analyzing metabolically labeled crude membrane preparations from the rat neocortex at embryonic day 14 (E14), E17, and E21; embryonic day 14 (E14), when the neocortex contains largely progenitor cells, E17, when a large number of the cells are postmitotic neurons, and at E21, when the neocortex contains the mature complement of neurons (Geschwind and Hockfield, 1989).

The ages of the animals used in these studies span a period of substantial morphogenetic changes in the developing cerebral cortex. At E14, the neural tube consists of a relatively homogeneous band of pseudostratified, mitotic progenitor cells. Between the ages of E14 and E17, cortical neurons are born and begin their migration through the intermediate zone and into the enlarging cortical plate. At E21, the vast majority of neurons have exited the cell cycle and ventricular zone, and the complex laminated structure of the neocortex is beginning to be revealed. Therefore, these ages span the period of neurogenesis, neuronal migration and early neuronal differentiation in the rodent cerebral cortex. The rationale of this analysis was that proteins expressed by neurons and not by progenitor cells should increase in their rate of synthesis between E14 and E21, and that proteins expressed by progenitor cells should decrease.

Ten proteins that are up-regulated greater than 3-fold between E14 and E21 were identified. One of these, TOAD-64 (Turned On After Division of M_r 64kDa) was selected for further study. TOAD-64 was up-regulated nearly 7-fold during late embryonic cortical development, and represented an abundant amount (approximately 0.1%) of embryonic brain protein. Further analyses of different ages and tissues showed that TOAD-64 is down-regulated between the day of birth and adulthood and suggested that the expression of TOAD-64 might be restricted to neural tissue (Geschwind, 1991, Minturn et al., 1995b). Furthermore, the regulation of the accumulation rate of TOAD-64 made it a good candidate for expression by early postmitotic neurons, and motivated its subsequent anatomical and molecular analysis.

Using sequence information from gel purified TOAD-64 protein, polyclonal antisera were generated. The antisera enabled me to examine the tissue specificity and temporal regulation of the protein during normal development and following nerve injury using both Western blotting and immunohistochemical techniques. In addition, the antisera allowed further biochemical characterization of the protein including subcellular localization to particular cell membrane fractions.

The peptide sequence information also allowed me to construct oligonucleotide probes for cDNA library screening and design primers for polymerase chain reaction (PCR) experiments which enabled me to clone and sequence the gene

that encodes TOAD-64. Once I had cDNA sequence information, I was able to further characterize the expression and regulation of TOAD-64 by analyzing its mRNA expression in tissues and cell lines using Northern and *in situ* hybridization. Ultimately, these data will provide an important basis for investigating the functional role of TOAD-64. For example, the sequence can be used to identify homologous genes in other species or within a gene family in the rat, and perform knockout experiments using antisense oligonucleotides.

Together, the biochemical and molecular experiments described in the following chapters have allowed us to characterize a protein that was originally identified as a spot on a two-dimensional gel. Our results suggest that TOAD-64 is a previously unreported gene expressed exclusively by neurons in the developing and regenerating nervous system; and may be a central component in the early molecular and cellular interactions that contribute to the generation of the anatomical complexity found in the mature mammalian central nervous system.

CHAPTER 2

EARLY POSTMITOTIC NEURONS TRANSIENTLY EXPRESS TOAD-64, A NEURAL SPECIFIC PROTEIN

SUMMARY

In previous work from our laboratory, two-dimensional gel electrophoresis was employed to compare proteins expressed at embryonic day 14 (E14), E17, and E21 in the developing rat; times chosen in order to identify proteins underlying the morphogenetic changes in the early periods of cerebral corticogenesis. Here we describe the initial biochemical and immunohistochemical characterization of one of these proteins, TOAD-64 (Turned On After Division of M_r 64kDa), using polyclonal antisera to two synthetic peptides derived from purification and partial sequence of the protein. This analysis reveals that TOAD-64 is a 64,000 Da protein that increases in abundance over the period of corticogenesis and then subsequently decreases to very low levels in the adult. TOAD-64 is neural specific and is expressed by post-mitotic neurons as they begin their migration out of the ventricular zone into the developing cortical plate. It is expressed in advance of most other neuronal proteins. Mitotically active progenitor cells do not express TOAD-64. Therefore, this protein is a marker for

postmitotic cells that have made a commitment to a neuronal phenotype.

INTRODUCTION

During embryonic development, the neural tube generates an enormously diverse population of terminally differentiated neurons and phenotypically distinct glia from a mitotically active, morphologically homogeneous pool of precursor cells (Turner and Cepko, 1987; Luskin et al., 1988; Price and Thurlow, 1988; Galileo et al., 1990). The generation of post-mitotic neurons from progenitor cells is a central differentiative event in development, but molecular markers of this event are few. The progenitor cells express the intermediate filament proteins vimentin (Cochard and Paulin, 1984) and nestin (Hockfield and McKay, 1988; Lendahl et al., 1990), neither of which is expressed by post-mitotic neurons. Surprisingly few proteins have been identified that mark the onset of the terminal differentiation into a neuronal phenotype. Neurons can be identified by the expression of a number of proteins, including neurofilaments and microtubule associated protein II (Nakahira et al., 1990; Miura et al., 1990; Besnard et al. 1991), but these proteins are not expressed until after postmitotic neurons have left the proliferative zone and begin to elaborate axons and dendrites. To understand the molecular events that determine that a cell becomes a neuron, our goal has been to identify proteins that are expressed coincident with the commitment to a neuronal phenotype.

We have previously identified fifteen proteins by 2D-gel electrophoresis whose synthesis is regulated during the period of corticogenesis in the rat (Geschwind and Hockfield, 1989). One of these proteins, TOAD-64 has been chosen for further characterization based on subsequent 2D-gel analysis that revealed the protein was up-regulated nearly 7-fold between E14 and E21, and markedly decreased in the adult (Geschwind, 1991).

This chapter describes the initial characterization of the TOAD-64 protein using polyclonal antisera raised against two peptide sequence fragments obtained from tryptic digestion of purified protein. This biochemical and histochemical analysis reveals that TOAD-64 is a neural specific, developmentally regulated protein expressed soon after neurons leave the cell cycle, and migrate from their birthplace in the ventricular zone to the developing cortical plate.

The extremely early expression, the relative abundance of the protein in newly born neurons, as well as the restriction in expression to the period of initial neuronal differentiation, suggest that TOAD-64 may be a key structural protein for early neuronal function.

MATERIALS AND METHODS

Protein purification and antibody generation

Preparative isoelectric focusing (IEF) gels loaded with 300 mg of protein from E21 cortex were run as previously described in the first and second dimensions (Geschwind and Hockfield, 1989). A spot corresponding to TOAD-64 (according to pH and molecular weight) was excised from 22 gels and electroeluted in 50 mM ammonium bicarbonate buffer containing 0.02% SDS using a BioRad 442 electroelutor (Geschwind, 1991). The electroeluted protein was subjected to amino acid composition analysis by the W.M. Keck protein sequencing facility (Yale). The purified protein was then cleaved with cyanogen bromide and trypsin, yielding approximately 8mg of protein (Stone et al., 1989; Aebersold et al., 1987). Peptide fragments were purified by HPLC and sequenced by the W.M. Keck Foundation biotechnology resource laboratory at Yale (Stone et al., 1989). Five peptide fragments ranging from 7 to 18 amino acids in length were sequenced. From the sequence information, two peptides were synthesized (peptide A: YDGPVFDLTTTPK, and peptide B: IAVGSDSDLVIWDPDAVK), conjugated to thyroglobulin using glutaraldehyde (Protein and Nucleic Acid Chemistry Center, Woods Hole Oceanographic Institution), and injected into rabbits to generate polyclonal antisera (antisera to TOAD-64a from peptide A and antisera to TOAD-64b from peptide B) (Pocono Hill Rabbit Farm and Laboratory, Canadensis PA).

Tissue Extraction and Protein Determination

Tissues from euthanized animals were dissected and used immediately or stored frozen at -70°C. Fresh or frozen tissues were homogenized in a Teflon-glass Dounce homogenizer at a concentration of 0.2 g (wet weight) of tissue / ml of Tris buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4). A cocktail of protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleamide, 5 mM e-amino-n-caproic acid, 5 mg/ml leupeptin and 5 mg/ml pepstatin A) were used in all buffers. Protein concentrations were determined by the method of Bradford (Bradford, 1976) using the Bio-Rad dye reagent kit and BSA as a standard.

Western blot analysis

Samples to be analyzed by immunoblotting were combined with gel loading buffer (20 mM Tris-Cl, pH 6.8, 3% SDS, 10% glycerol, 0.01% bromophenol blue) and b-mercaptoethanol (0.05%), boiled for 5 min and electrophoresed by SDS-PAGE minigel (10%) in 50 mM Tris base, 0.38 M glycine, 0.2% SDS (Laemmli, 1970). The proteins were electrophoretically transferred to nitrocellulose overnight at 50 mA in 25 mM Tris, 0.192 M glycine, 0.01% SDS, and 20% methanol (Towbin et al., 1979). The blots were blocked in Blotto (5% nonfat dry milk in TBS) for 2 hr and incubated in primary antibody (diluted in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 0.2% Tween 20, and 0.2% sodium azide) overnight at room temperature. The blots were washed

and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:7000) for 2 hr at room temperature. Immunoreactive bands were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Immunoprecipitation of TOAD-64

Antibody to TOAD-64 or preimmune serum was incubated 4-6 hr with protein A-agarose (Pharmacia /LKB) at 4°C. Following washes of the beads in TBS containing 0.1% Tween 20 (TBST), soluble fractions of postnatal day 12 (P12) cortical homogenates were incubated with the beads overnight at 4°C. The beads were washed in TBST, boiled in gel loading buffer, and analyzed by western blotting with biotinylated antibodies. Antibodies to peptides TOAD-64a and TOAD-64b were biotinylated with N-hydroxysuccinimide biotin (Sigma) (Harlow and Lane, 1988). Following biotinylation, antibodies were dialyzed against TBS and stored at 4°C until used.

Immunohistochemistry

Timed pregnant, Sprague-Dawley rats (E1=plug date) were obtained from Charles River Laboratory Raleigh, NC. Animals, ages E17 to adult, were perfused transcardially with 0.1 M phosphate buffer (PB) pH 7.4, then 4% paraformaldehyde (PFA) in PB. Whole brains were dissected out and postfixed in 4% PFA for 24 hr at 4°C, then equilibrated in 30% sucrose in PB. The E17 brains were embedded in gelatin (15%)-albumin (4%),

postfixed in 4% PFA and re-equilibrated in 30% sucrose. Coronal and parasagittal sections were cut at 30 μm on a cryostat and collected as free-floating sections in PB for immunohistochemistry. E12 animals were removed individually into buffered 4% PFA and fixed for 24 hr at 4°C. Twelve micron nonembedded horizontal sections were cut on a cryostat and thaw mounted directly onto gelatin coated slides. Tissue sections were incubated in either rabbit anti-TOAD-64 (1:2000), mouse monoclonal Rat-401 (undiluted) (Hockfield and McKay, 1985), mouse anti-PCNA (1:100) (Boehringer Mannheim, clone PC10), mouse anti-MAP2 (1:500) (Sigma), or mouse anti-ß-tubulin (TuJ1, 1:1000; generously provided by Dr. Anthony Frankfurter, University of Virginia) (Moody et al., 1987; Lee et al., 1990b) at room temperature overnight. The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (1:200, Cappel) or goat anti-mouse IgG (1:250, Cappel), FITC-conjugated goat anti-rabbit IgG (1:200, Southern Biotechnology Associates, Inc.), or Texas Red-conjugated goat anti-mouse IgG (1:200, Southern Biotechnology Associates, Inc.). All antibodies were diluted in DMEM with 5% fetal calf serum (FCS), 0.2% sodium azide, and 2% Triton X-100. HRP was localized using a 0.7 mg/ml solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.003% H_2O_2 in PB. For double label experiments using HRP-conjugated antibodies, the tissue was incubated sequentially in the primary antibodies, and the first antibody was visualized using 0.2% nickel sulfate enhanced DAB resulting in a

black/blue reaction product. Sections were dehydrated in graded ethanols, equilibrated in xylenes, and coverslipped with Permount (Fisher Scientific). Fluorescent labeled sections were mounted in Aqua-Poly Mount (Polysciences, Inc.).

RESULTS

Purification of TOAD-64

TOAD-64 has an isoelectric point of 6.4 and an apparent molecular mass of 64,000 Da on the gel system used for the initial two-dimensional PAGE study (Geschwind and Hockfield, 1989). The protein was purified (Geschwind, 1991), digested and separated by HPLC, and five well defined peaks were chosen for sequencing (Aebersold et al., 1987). The sequences of the five peptide fragments of TOAD-64 were used to search the PIR, Genbank and SwissProt databases using TFASTA analysis (Pearson and Lipman, 1988) and no significant homology to any other protein was found (Geschwind, 1991).

Antibodies generated to two different TOAD-64 peptides recognize a single protein.

Synthetic peptides, 13 and 18 amino acids in length, representing two of the obtained sequences (TOAD-64a and TOAD-64b) were conjugated to bovine thyroglobulin and used to raise polyclonal antisera (Figure 1). Western blot analysis of E17 rat brain homogenates with both antisera to TOAD-64a and TOAD-64b revealed a single immunoreactive band with an apparent molecular weight of 64,000 Da (Figure 2A), corresponding to the apparent molecular weight of TOAD-64 on 2-D gels. Each antiserum specifically recognized the peptide to which it was generated, and did not cross react with the other peptide. This was demonstrated by incubating each

antiserum with the appropriate or inappropriate peptide before using the antiserum on western blots (Figure 2A). Incubation with the peptide to which an antiserum was generated blocked the appearance of the 64 kDa immunoreactive band, whereas incubation of the antiserum with the inappropriate peptide had no effect on the appearance of the 64 kDa band. Antisera to peptide TOAD-64a recognized an antigen in tissue sections of embryonic rat cortex (described further below) and this immunohistochemical staining with antisera to peptide TOAD-64a was also specifically blocked with the TOAD-64a peptide, but not the TOAD-64b peptide (data not shown). Antisera to peptide TOAD-64b did not show any specific immunoreactivity on tissue sections.

Immunoprecipitation experiments demonstrate that peptides TOAD-64a and TOAD-64b were derived from the same protein from rat cortex. A postnatal day 12 (P12) rat cortical homogenate was immunoprecipitated with antiserum TOAD-64a or non-immune serum. The immunoprecipitated material was blotted and then stained with biotinylated antiserum TOAD-64a or TOAD-64b (Figure 2B). The 64 kDa antigen immunoprecipitated with antiserum TOAD-64a was recognized by both antisera. Both antisera occasionally show a doublet. The lower band of the doublet appears to be a proteolytic fragment of the 64 kDa band, in that its intensity increases with the time that the homogenates are stored. Neither antibody recognized an antigen in non-immune immunoprecipitates. Thus, both antisera to TOAD-64a and TOAD-

64b recognize the same 64 kDa protein from rat cortex. Because antisera to peptide TOAD-64a specifically recognized its antigen in both biochemical and immunohistochemical assays, it was used for the further characterization of TOAD-64.

The expression of TOAD-64 is neural specific.

Two-dimensional gel studies suggested that TOAD-64 was enriched in neural tissue (Geschwind and Hockfield, 1989), however differences in the constellations of protein spots on 2D-gels from different tissues makes this kind of analysis inconclusive. Antisera to TOAD-64 have now permitted a more definitive examination of tissue specificity. On western blots of cortex, spinal cord, liver, lung, heart, kidney, and striated muscle from P1 animals, the antisera detects a 64 kDa band that is present only in neural tissue (Figure 3A). Ten micrograms of P1 cortical homogenates yielded a strong signal, while even 25 mg of non-neural tissue did not produce a detectable immunoreactive band.

TOAD-64 is expressed during embryonic and early postnatal development and is down-regulated in the adult.

Two-dimensional gel analyses of coomassie blue and silver stained proteins suggested that the amount, as well as the rate of synthesis, of TOAD-64 increased during development. Antisera have now permitted a more precise analysis of protein levels over the course of cortical

development. Western blots of cortical homogenates from E14, E21, P5, P12, P19, P34 and adult rats show that TOAD-64 increases in abundance between E14 and E21 (Figure 3B). From E21 to P12 the amount of TOAD-64 remains roughly constant. A decline is seen after P12, so that by adulthood TOAD-64 is almost undetectable.

TOAD-64 is expressed by post-mitotic neurons.

During the early stages of corticogenesis the cortical anlage contains three cell types: mitotic precursor cells that reside in the ventricular zone (VZ), radial glial cells with processes that span the width of the cortex, and post-mitotic neurons that are found primarily in the marginal and intermediate zones and in the developing cortical plate.

At E14 (Figure 4A), antisera to TOAD-64 stains a subpopulation of cortical cells, located in the primordial plexiform layer. (Cortical layers are as described in Kageyama and Robertson, 1993.) Many of the antibody-positive cells have processes (Figure 4B). In sharp contrast to the labeling in the superficial layers of the cortex, the ventricular zone is unstained (Figure 4A). By E17 (Figure 4C), a large number of antibody-positive cells have accumulated in the developing cortical plate, as well as in the subplate and intermediate zone. As observed at E14, at E17 the ventricular zone remains relatively free of antibody-positive cells. The few immunoreactive cells in the ventricular zone have long processes (Figure 4D), suggestive

of migrating neurons. While some of these cells are oriented radially to the pial surface, a large number of labeled cells in the VZ and IZ are oriented obliquely or laterally. At later stages (P0, Figure 4E), the immunoreactivity in the cortical plate, subplate, and intermediate zone is more diffuse than at earlier ages. In the subventricular zone individual antibody-positive cells with the characteristic fusiform shape of early post-mitotic neurons can still be identified (Figure 4F). The ventricular zone at P0 remains largely free of antibody-positive cells. The intensity of staining with antisera to TOAD-64 declines dramatically over the first postnatal week, so that by P5 little staining is detected. The expression of TOAD-64 in the cortical plate becomes diffuse and is not clearly associated with distinct cellular profiles. The reduction in immunohistochemical detection of TOAD-64 in the first postnatal week, when it can still be detected biochemically, could be due to the very diffuse distribution of the protein in the enlarging postnatal cortex. This difference may also reflect a limitation in antigen accessibility or a movement of the protein from a membrane associated form to a soluble form and a loss of histological detection under these fixation conditions.

The morphology and distribution of cells stained with TOAD-64 antisera suggested that they are neurons. To confirm this identification we compared staining patterns of antisera to TOAD-64 to that of other markers of early cortical cells.

One of the earliest markers for post-mitotic neurons is neuron-specific class III ß-tubulin, recognized by the TuJ1 antibody (Lee et al., 1990a; Lee et al., 1990b; Easter et al., 1993). Easter and colleagues have demonstrated in the mouse, that TuJ1 immunoreactive cells first appear near the neural folds, in the neural plate (at the level of the caudal border of the optic evagination) at E8.5, prior to neural tube closure. They suggest that these cells may be the first neurons of the CNS, or neural crest cells about to exit the plate (Easter et al., 1993).

At E12 in the rat brain, when only a small number of neurons have been generated, double immunofluorescent labeling demonstrates that all cells stained with the TuJ1 antibody also express TOAD-64 (Figure 5). The presumptive neuroepithelial cells (unlabeled cells in figure 5) do not express the TOAD-64 protein, demonstrating that TOAD-64 is expressed only by cells that we detect as neurons by TuJ1 immunoreactivity (the earliest neuronal marker we have available). The coincident labeling of cells with antisera to TOAD-64 and the TuJ1 antibody was apparent throughout the embryonic period of neurogenesis as demonstrated at E17 (Figure 6), indicating that TOAD-64 is expressed by all neurons during the period of corticogenesis. The protein recognized by the antisera to TOAD-64 is expressed earlier than we can detect labeling for a number of other neuronal proteins, including the enzyme neuron specific enolase and the low and medium molecular weight neurofilament subunits

(NF) (data not shown). Therefore, TOAD-64 is expressed in post-mitotic neurons as early as class III ß-tubulin, and in advance of several other neuronal markers.

To determine whether TOAD-64 is expressed by radial glial cells or neural progenitor cells, double labeling experiments were performed with TOAD-64 antisera and monoclonal antibody Rat-401, which recognizes an intermediate filament protein, nestin, present only in radial glial cells and undifferentiated progenitor cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988). Radial glial processes spanning the width of the cortex are stained with Rat-401 (Figure 7A, brown), but not with antisera to TOAD-64 (Figure 7A, black). Rat-401 also labels a band of cells along the ventricular surface that are negative for TOAD-64. At higher magnification, individual Rat-401 positive processes are visible in the cortical plate and are not stained by antisera to TOAD-64. In the same sections, neurons with radially-oriented leading processes present in the cortical plate as well as horizontally-oriented Cajal-Retzius cells in layer I express TOAD-64 but do not express nestin (Figure 7B, black). There were no cells found that expressed both proteins.

The mutually exclusive expression of nestin and TOAD-64 indicates that TOAD-64 is not expressed by progenitor cells. To confirm that mitotic cells do not express TOAD-64 we examined the expression of TOAD-64 in cells that express the proliferating cell nuclear antigen (PCNA) using double label immunohistochemistry. PCNA is identical to cyclin and the

auxiliary protein of DNA polymerase-δ. It labels cells during the G1 and S phase of the cell cycle, making it an ideal marker for proliferating cells (Miyachi et al., 1978; Mathews et al., 1984; Bravo et al., 1987). At E17 in the rat, PCNA (Figure 7C, black) is restricted to cell nuclei in the proliferative ventricular zone and expression abruptly ends at the border of the subventricular zone (SVZ). Cells that express TOAD-64 (Figure 7C, brown) are abundant in the developing cortical plate, intermediate zone, and the subventricular zone. A small number of cells in the ventricular zone express TOAD-64. However, observation at higher magnification shows that all TOAD-64 positive cells are PCNA negative (unstained nuclei, Figure 7D). The TOAD-64 positive cells in the ventricular zone are oriented either radially or horizontally and extend long leading and trailing processes characteristic of migrating neurons at the light microscopic level. Throughout the cortical proliferative zones examined, we have never observed a cell expressing both TOAD-64 and PCNA.

DISCUSSION

This chapter describes the localization, neural specific expression, and temporal regulation of the TOAD-64 protein. TOAD-64 was first identified by two-dimensional gel analysis as a protein that showed a marked increase in its rate of accumulation over the period of early corticogenesis in the developing rat. The migration pattern of TOAD-64 by two-dimensional gel electrophoresis and the partial sequence of peptides obtained from gel purified protein indicated that it has not been previously identified. While two-dimensional gels permitted the identification of proteins that were candidates for early expressed neuronal proteins, the analysis was limited to the capacity and resolution of the two-dimensional gel system. For example, it was not possible to determine definitively if TOAD-64 was neural specific because the constellation of proteins is so different in different tissues. Most importantly, two-dimensional gels do not permit localization of protein at the single cell level; so while it was clear that TOAD-64 was developmentally regulated, it was not possible to determine which cell class in the developing CNS expressed the protein. The generation of specific antisera allowed us to confirm our previous results from two-dimensional gels and to extend them to the resolution of single cells.

Here we have used polyclonal antisera generated to synthetic peptides to show that TOAD-64 is not expressed

outside of the nervous system, that it is expressed by post-mitotic neurons, and that it is among the very earliest markers for neurons after they have completed their terminal mitosis and are beginning to elaborate axons during their initial differentiation. The expression of TOAD-64 shows a marked decrease in the early postnatal period, and it is present at very low abundance in the adult. The increase in TOAD-64 expression coincident with the early stages of rat corticogenesis suggests an important role in cortical development, and the transient nature of its expression suggests a role in a short-lived event in neuronal differentiation.

Throughout our studies, we have used the TuJ1 antibody, which recognizes neuron specific class III ß-tubulin, to detect the earliest generated neurons in the rat brain. This antibody has been demonstrated by others to be expressed by neurons very soon after their terminal mitoses during initial process formation in the mouse brain (Easter et al., 1993), and in initial neuroepithelial differentiation of murine ovarian teratomas (Caccamo et al., 1989a,b). However, we can not definitively determine at what point in neuronal differentiation TuJ1 detects neurons. While we believe that TOAD-64 and TuJ1 are labeling all early postmitotic neurons when they begin their migration into the developing cortical plate, we have not examined the specific ultrastructural correlates of this stage in a neuron's differentiation, and are limited by our currently available tools. The TuJ1

antibody (and now TOAD-64) is currently the only available marker for the detection of very early postmitotic neurons.

TOAD-64 is one of the earliest expressed neuronal proteins.

The immunobiochemical and immunohistochemical data presented here show that TOAD-64 is one of the earliest expressed neuron specific proteins reported to date. Our immunohistochemical results indicate that TOAD-64 is expressed by all cortical neurons including the earliest born Cajal-Retzius cells (see figure 7A), but it is not expressed in radial glial cells or mitotic precursor cells, suggesting a role in the earliest differentiative events of neurogenesis. A number of other neuron specific proteins are also expressed after the final mitosis, including the three neurofilament subunits (Cochard and Paulin, 1984; Kaplan et al., 1990), α-tubulin (Tα1) (Miller et al., 1987), and MAP 2 (Matus, 1988; Riederer and Matus, 1985; Crandall and Fischer, 1989). We do not detect these neuronal markers in the developing cortex as early as we detect TOAD-64 and class III ß-tubulin. Studies in cell culture may be useful in determining at higher resolution the temporal order of expression of these various neuronal proteins.

TOAD-64 is not expressed by mitotic precursor cells.

TOAD-64 is not expressed until after a cell has completed its final mitosis, which distinguishes it from a number of other early expressed proteins which are expressed

by mitotic as well as by post-mitotic cells. Cell cycle regulatory proteins such as the retinoblastoma susceptibility gene, RB, as well as its inactivator, the cell cycle-related kinase cdc2, are expressed early in neurogenesis and have been postulated to play a role in neuronal differentiation (Okano et al., 1993); however, these proteins are detected in neuronal precursors and glia, as well as in committed neurons. In addition, there are large regional differences within the brain in the expression of these proteins implying a role in differentiation that reflects location rather than basic cell type.

The commitment to some aspects of phenotype may occur prior to the completion of neurogenesis, implying that fate commitment occurs before the final cell division from which a neuron is generated (McConnell, 1992; Reh, 1992). For example, studies indicate that the decision by a neuron to express early neuronal markers, such as LAMP and PC3.1 occurs prior to the completion of neurogenesis (Barbe and Levitt, 1991; Ferri and Levitt, 1993; Arimatsu et al., 1992; Arimatsu et al., 1994), but these proteins are expressed by only a subpopulation of neurons. This class of proteins may play a role in determining areal features of neuronal identity, such as laminar location or connectivity, but are not likely to participate in the general morphological and physiological changes that accompany the acquisition of a neuronal phenotype, including the ability to extend processes, migrate

to adult positions, form synapses, and maintain an excitable membrane.

TOAD-64 immunoreactivity is detected as early as E12. Another neuronal protein, class III ß-tubulin (Moody et al., 1987; Lee et al., 1990a; Lee et al., 1990b) is also expressed very early in neural development. Easter and colleagues have shown that class III ß-tubulin is expressed during initial tract formation in the embryonic mouse (Easter et al., 1993). In double labeling experiments we show here that at E12 in the rat, all cells that are positive for class III ß-tubulin are also positive for TOAD-64, indicating that all neurons express TOAD-64. Class III ß-tubulin may not be specific for post-mitotic cells: it was recently reported that cells that are still undergoing mitosis express the protein (Hall et al., 1993). In our experiments using an antibody to PCNA to label mitotic cells, we have never detected a mitotic cell that expresses TOAD-64. These data indicate that TOAD-64 represents a class of protein that is expressed after the final mitosis.

The expression of TOAD-64 is transient.

The appearance of TOAD-64 in conjunction with the initial differentiation of neurons places it in a relatively small class of "early onset" genes of neuronal differentiation. Other proteins that fall into this class are neurotransmitters such as catecholaminergic and serotonergic neuronal markers (Lauder and Bloom, 1975;

Wallace and Lauder, 1983) and markers for the endorphin and enkephalin systems (Bayon et al., 1979), as well as the enzyme neuron-specific enolase (Marangos et at., 1980), and the neuron specific cytoskeletal elements class III ß-tubulin, α-internexin, α-tubulin (Tα1), microtubule associated proteins and neurofilaments (Moody et al., 1987; Kaplan et al., 1990; Miller et al., 1987; Matus, 1988; Crandall and Fischer, 1989; Riederer and Matus, 1985; Cochard and Paulin, 1984). A major difference between TOAD-64 and all of the other proteins is that the expression of TOAD-64 decreases in the second postnatal week, while the other proteins persist in the adult. A number of genes are expressed transiently in the developing mammalian brain, including Int-1 (Wnt-1) (Wilkinson et al., 1987), and some of the mouse homologs of homeobox (*En-1, En-2, Tes-1, Tes-2, Otx1, Otx2, Emx1, and Emx2*) and achaete scute genes (*MASH-1 and MASH-2*) (Davis and Joyner, 1988; Porteus et al., 1991; Porteus et al., 1992; Simeone et al., 1992; Johnson et al., 1990), however most of these are expressed in progenitor cells. The products of these genes are restricted to the nucleus and may play a central role in regulating the expression of structural genes including developmentally regulated proteins such as TOAD-64.

Many neuronal proteins are first detected predominantly or entirely during the early postnatal period, for example Thy-1, synapsin I, DARPP-32, and 1B236 (Barclay, 1979; DeGennaro et al., 1983; Lewis et al., 1983; Lenoir et al.,

1986). These proteins most likely are involved in regulating later stages of neuronal maturation, and again, their expression tends to persist into adulthood, indicating that constitutive expression is important for the maintenance of a differentiated neuronal phenotype. There is, in addition, another class of proteins whose expression is regulated by neuronal activity and requires that intercellular connectivity be at least in place, if not mature, such as the Cat-301 proteoglycan and cyclooxygenase (Hockfield et al., 1990; Yamagata et al.,1993).

At present we can only speculate about possible functions of TOAD-64. The timing of expression is consistent with a role in the initial morphological and physiological changes that a cell undergoes as it makes the decision to become a neuron, such as migration or axonal and growth cone extension. Preliminary studies indicate that TOAD-64 is a phosphoprotein (Geschwind, 1991; Minturn et al., 1995), suggesting a role in signal transduction pathways.

Surprisingly, few brain specific proteins have been identified that are expressed by neurons immediately following their final mitosis. Our previous study found that fewer than 3% of membrane associated proteins showed a change of more than 3-fold during the period of cortical neurogenesis; only one-third of these were up-regulated (Geschwind and Hockfield, 1989), which may explain why so few markers of early post-mitotic neurons have been reported.

The studies presented in this chapter extend a previous two-dimensional gel analysis of proteins regulated during mammalian corticogenesis. We have characterized one up-regulated protein by protein purification, amino acid sequence analysis and antibody generation. There has been only a small number of reports of the successful characterization of novel proteins identified on two-dimensional gels (Skene and Willard, 1981; Benowitz and Lewis, 1983; Yamakuni et al., 1984; Kennedy et al., 1988; Barzilai et al., 1989; Winsky et al., 1989; Drescher et al., 1995). However, this approach, while technically demanding, represents one of the few strategies to identify new proteins, or families of proteins, expressed in a spatially or temporally differential manner. In our hands, this strategy has permitted the identification of a novel neural specific protein. The age of onset and the short-lived expression of TOAD-64 make it one of the few proteins expressed coincident with the earliest events in the acquisition of neuronal phenotype. Molecular characterization of TOAD-64 should allow a better understanding its function in early neural development.

Figure 1. TOAD-64 peptide sequences

Peptide sequences obtained from purified TOAD-64 were glutaraldehyde conjugated to thyroglobulin and injected into rabbits to generate polyclonal antisera. Antisera to TOAD-64a was raised against peptide A; antisera to TOAD-64b against peptide B.

TOAD-64 PEPTIDES USED FOR RABBIT IMMUNIZATION

TOAD-64A: YDGPVFDLTTTPK

TOAD-64B: IAVGSDSDLVIWDP(D)AVK

Figure 2. Polyclonal antisera generated to two synthetic peptides (64a and 64b) obtained from amino acid sequence analysis of TOAD-64 recognize a single protein. **(A)** Antisera to 64a *(lane A)* and 64b *(lane C)* detect a band of 64 kDa on western blots of rat E17 cortical homogenates. Incubation of each antisera with the peptide to which it was generated blocked the appearance of the 64 kDa band. In *lane B,* antisera to TOAD-64a was incubated with peptide TOAD-64a, and in *lane D,* antisera to TOAD-64b was incubated with peptide TOAD-64b. Incubation of the antisera with the inappropriate peptide did not block the appearance of the immunoreactive band (data not shown). **(B)** Immunoprecipitation of rat cortical homogenates demonstrate that both antisera recognize the same protein. P12 cortical homogenates *(lanes A and D)* or homogenates immunoprecipitated with antiserum TOAD-64a *(lanes B and E)* or non-immune serum *(lanes C and F)* were blotted to nitrocellulose and stained with biotinylated antisera to TOAD-64a *(A-C)* or TOAD-64b *(D-F)*. Both antisera to TOAD-64a and TOAD-64b recognize a 64 kDa antigen immunoprecipitated with antiserum TOAD-64a (arrow). Neither antibody detected an antigen in the non-immune immunoprecipitates.

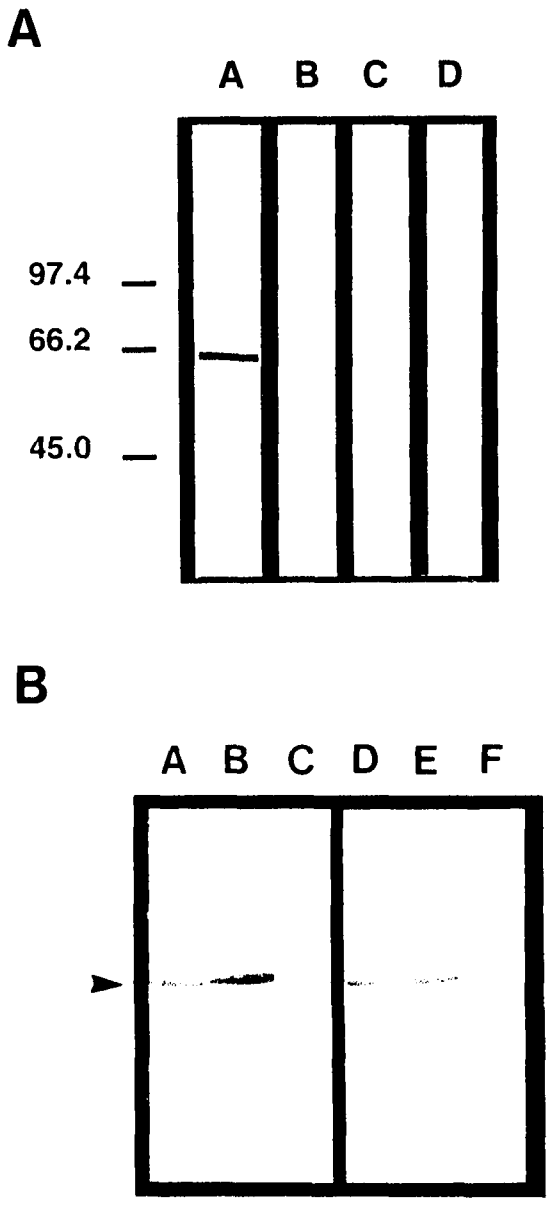


Figure 3. TOAD-64 is nervous system specific and is developmentally regulated during corticogenesis. **(A)** Western blots of P1 rat homogenates from various tissues stained with antiserum TOAD-64a demonstrate that TOAD-64 is neural specific. Twenty-five micrograms of protein per lane was loaded (except the first lane, as indicated), and an immunoreactive band of 64 kDa was detected in the neural tissue, but not in the non-neural tissue. As little as 10 μg of protein from a cortical homogenate produced a strongly immunoreactive band. **(B)** Western blots of cortical homogenates from a range of embryonic and postnatal ages demonstrate that TOAD-64 can be detected as early as E14, increases between E14 and E21, and declines after P12. A steady decline in TOAD-64 expression is seen after the second postnatal week, so that by adulthood, almost no immunoreactivity is detectable (10μg of protein loaded per lane).

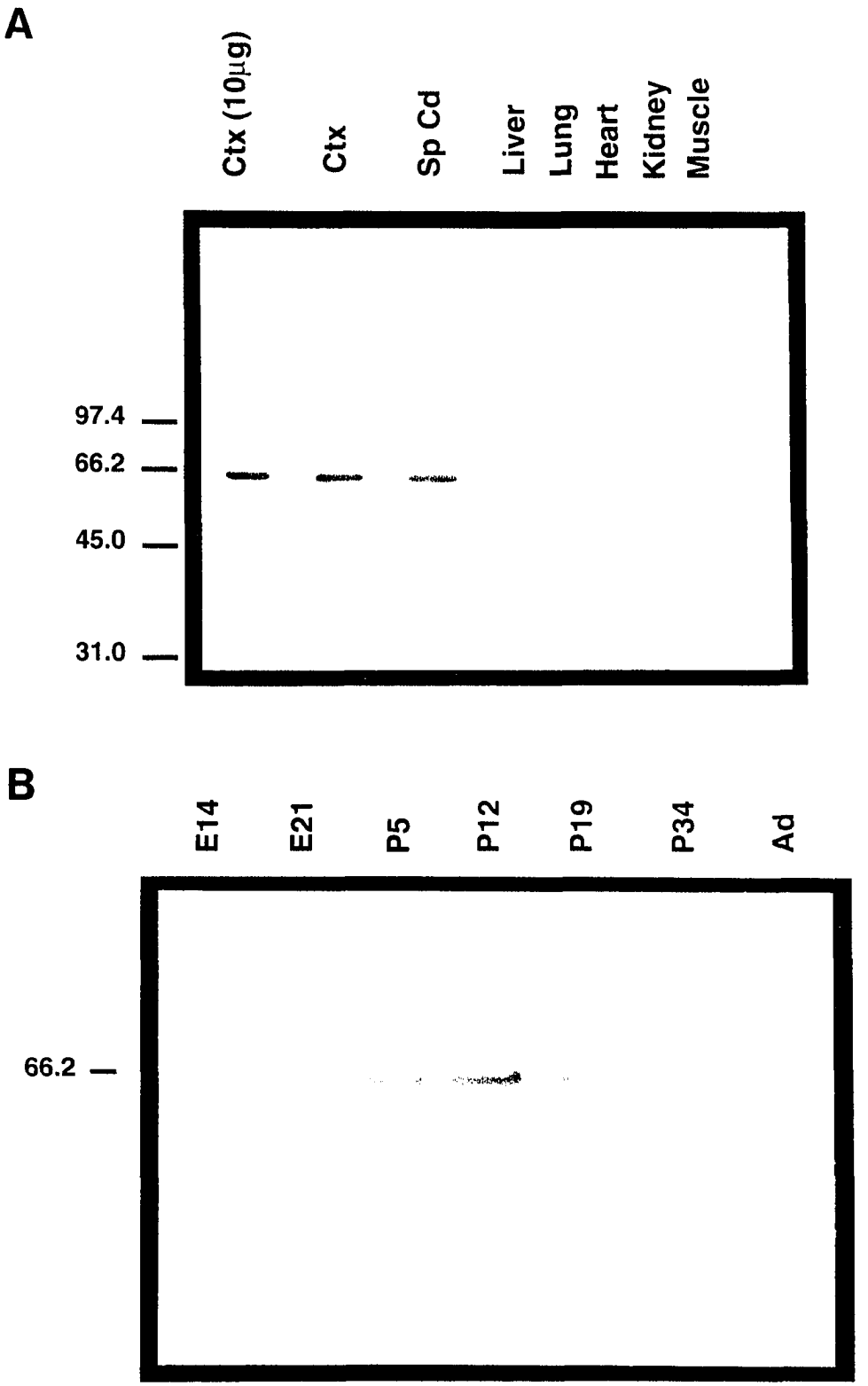


Figure 4. TOAD-64 is expressed by post-mitotic neurons in the developing cortex.

Parasagittal sections at low and high magnification from E14 **(A,B)** coronal sections from E17 **(C,D)**, and parasagittal sections from P0 **(E,F)** rats were incubated with antisera to TOAD-64a and visualized with HRP-conjugated secondary antibodies. **(A)** At E14, antisera to TOAD-64a stains a small population of cells and processes in the primordial plexiform layer (PPL). **(B)** At higher magnification, the antibody positive cells have darkly stained cell bodies and extend processes. Very little staining is detected in the cell dense ventricular zone (VZ). **(C)** At E17 a large number of TOAD-64 positive cells have accumulated in the developing cortical plate (CP), subplate (SP), intermediate zone (IZ), and subventricular zone (SVZ). **(D)** Similar to earlier ages, at E17 the majority of cells in the ventricular zone (VZ) are unstained; however, the few antibody positive cells in the VZ have long radially and horizontally oriented processes suggestive of migrating neurons. **(E)** By P0 the immunoreactivity in the developing cortical plate (CP), subplate (SP), and intermediate zone (IZ) is diffuse and is not as clearly associated with cell bodies and processes as at the earlier ages. **(F)** The ventricular zone (VZ) still remains largely unstained but for a few lightly stained cells bearing processes and the fusiform shape characteristic of post-mitotic neurons (arrowhead). Scale bar: 60 μm for A and C, 125 μm for E, 30 μm for B, D, and F.

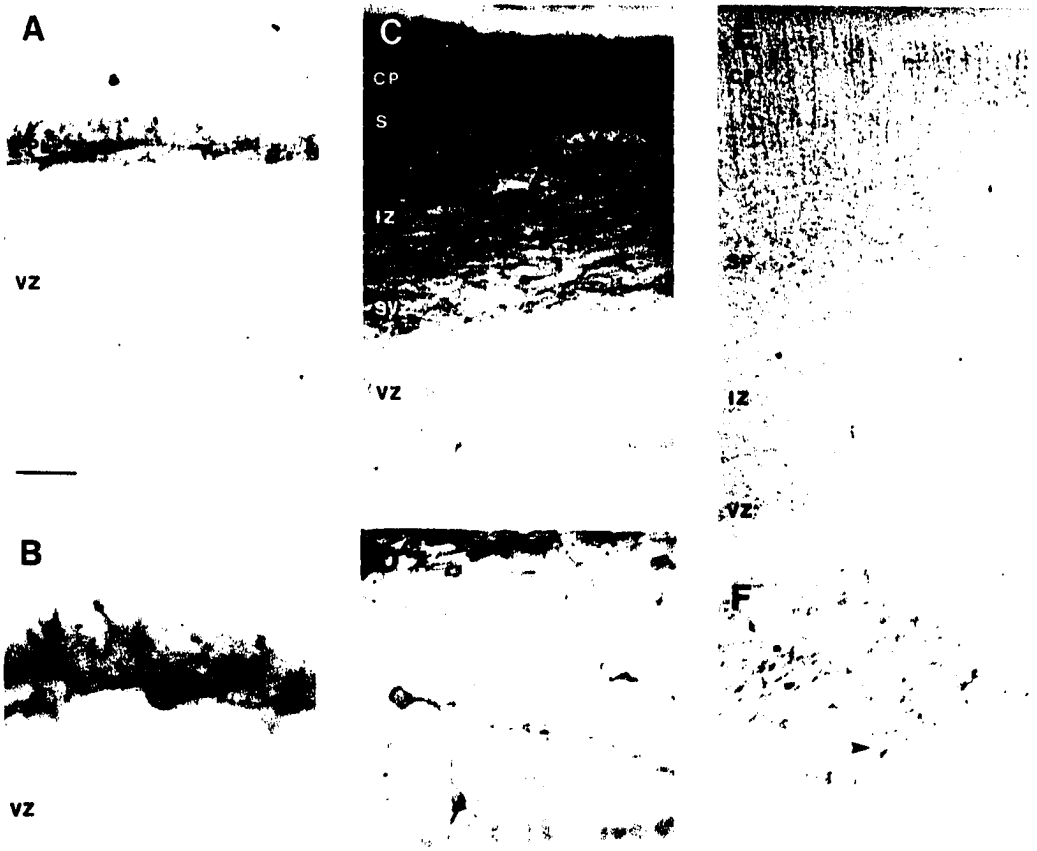
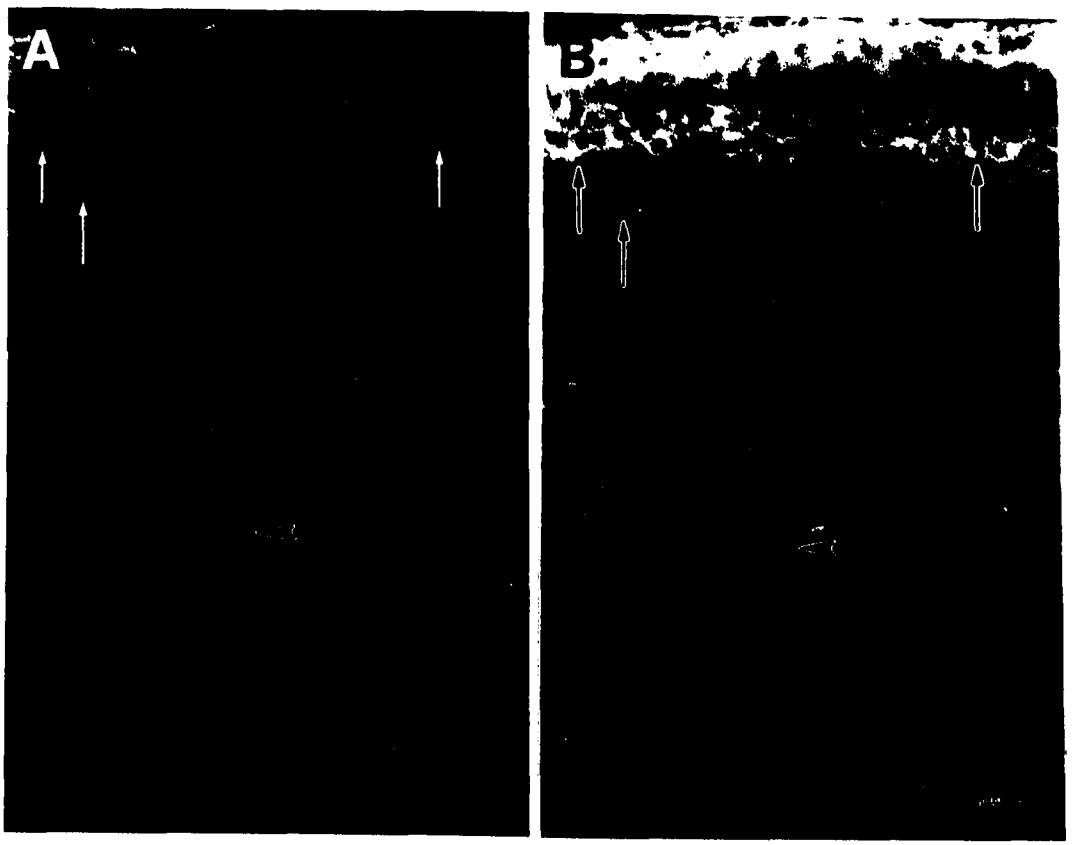


Figure 5. TOAD-64 is expressed by neurons very early in their development.

Horizontal sections of the brain from E12 rats were double labeled with antisera to TOAD-64 (using an FITC-conjugated secondary antibody) **(A)** and the TuJ1 antibody to neuron-specific class III ß-tubulin (using a Texas Red-conjugated secondary antibody) **(B)** At E12, when only a few of the earliest generated neurons have been born and reside in the marginal zone, all cells stained with the TuJ1 antibody also express TOAD-64. To control for specificity of staining, experiments in which one of the primary antibodies was omitted while both secondary antibodies remained, demonstrated no inappropriate cross-reactivity between primary and secondary antibodies. In addition, there was no "bleed through" signal between fluorescent labels. Scale bar, 25 µm.

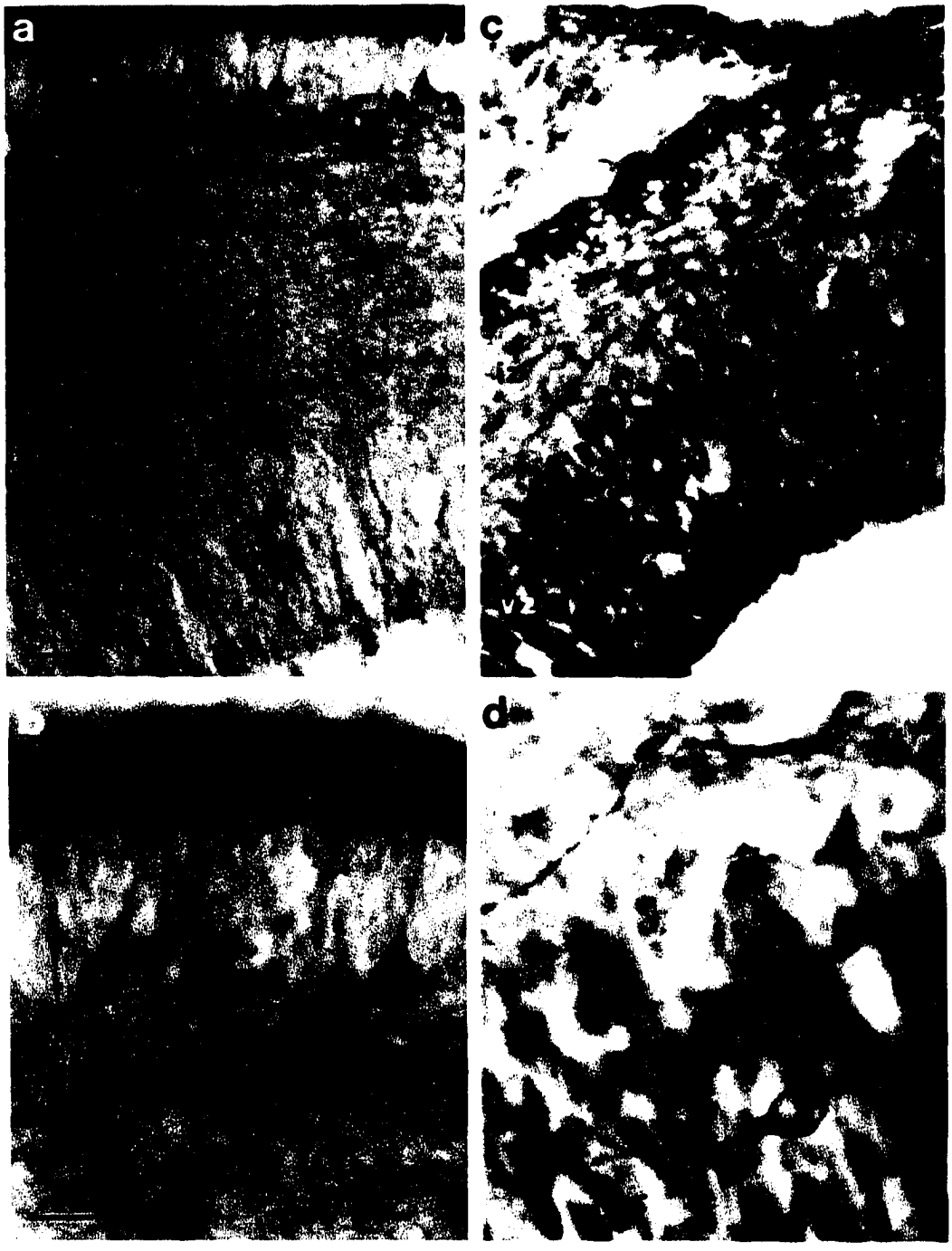
Figure 6. Neurons co-express TOAD-64 and TuJ1 throughout the period of cortical neurogenesis.

Coronal sections from E17 rats were double labeled with antisera to TOAD-64 (using an FITC-conjugated secondary antibody) **(A)**, and a monoclonal antibody to neuron specific class III ß-tubulin (TuJ1, using a Texas Red-conjugated secondary antibody) **(B)**. At E17, neurons throughout the cortical plate, intermediate zone, and subplate express both the TOAD-64 protein and neuron specific ß-tubulin (matching arrows in **A** and **B**; cells that cannot be lined up in figure, are in different planes of focus, and double-labeled when viewed under the microscope). This demonstrates that throughout the period of cortical neurogenesis, all neurons that we can detect (by staining with TuJ1 antibody) are expressing the TOAD-64 protein.



of the cortical sections in figures A and C, are due to the regions photographed. **A** and **B** are from the ventrolateral region of the cortex, while sections in **C** and **D** are more dorsomedial. Scale bars: 25 μm for A and C, 10 μm for B and D.

Figure 7. The expression of TOAD-64 is restricted to post-mitotic cells. Coronal sections from E17 rats were double labeled with TOAD-64 antisera and monoclonal antibody Rat-401 **(A,B)**, or TOAD-64 antisera and an antibody to the proliferating cell nuclear antigen (PCNA) **(C,D)**. PCNA is expressed in the nuclei of cells during the G1 and S phase of the cell cycle. **(A)** Rat-401 *(brown in A and B)*, which labels the intermediate filament protein nestin, stains radial glial processes that span the width of the cortex and a band of undifferentiated precursor cells that lie along the ventricular surface. Antisera to TOAD-64 *(black in A and B)* stains neurons in the developing cortical plate and the horizontally oriented Cajal-Retzius cells in layer I. **(B)** At higher magnification, individual Rat-401 positive processes (arrows) can be seen spanning the width of the developing cortical plate adjacent to antisera to TOAD-64 positive neurons (arrowheads). No cells expressing both proteins were detected. **(C)** At E17, PCNA positive cells are restricted to the proliferative ventricular zone (VZ) *(black)*. Neurons that express TOAD-64 are abundant in the developing cortical plate (CP) and intermediate zone (IZ) *(brown)*. **(D)** A small number of cells in the VZ express TOAD-64, and at higher magnification it can be seen that the cells stained with antisera to TOAD-64 are not stained with PCNA *(brown cells with unstained nuclei)*. In extensive examination of the cortical proliferative zones no TOAD-64-positive cell was ever observed to express PCNA. The differences in the width.



CHAPTER 3

THE TOAD-64 GENE IS EXPRESSED EARLY IN NEURONAL DIFFERENTIATION IN THE RAT, AND IS RELATED TO *unc-33*, A *C. elegans* GENE INVOLVED IN AXON OUTGROWTH

SUMMARY

The previous chapter described biochemical and immunohistochemical evidence that TOAD-64 is expressed immediately after neuronal birth and is dramatically down-regulated in the adult, leading us to believe that it may play a role in events that underlie early neuronal differentiation. In order to gain better access to the function of TOAD-64, it was important to know its molecular identity. In this chapter I report the full coding sequence of the TOAD-64 gene. Northern and *in situ* hybridization analyses show that TOAD-64 mRNA is neural specific and developmentally regulated in parallel with the protein. TOAD-64 is up-regulated following neuronal induction of P19 and PC12 cells, and is re-expressed in regenerating nerves following sciatic nerve lesions in the adult rat. The gene shows homology to the *unc-33* gene from *C. elegans*, mutations in which lead to aberrant axon outgrowth, and to a collapsin response gene from chick, CRMP-62. The data presented in

this chapter suggest that TOAD-64 is likely to play a central role in axonal outgrowth and pathfinding.

INTRODUCTION

During development of the nervous system, postmitotic neurons elaborate processes that are involved in the migration of neurons to their adult positions, processes that contribute to the formation of intricate, characteristic axonal projections and dendritic arbors, and instill the capacity of neurons to respond to a variety of extracellular signals. These extracellular signals include not only neurotransmitters, but also trophic factors and guidance cues.

As described in Chapter 1, TOAD-64 was identified by two-dimensional PAGE as a protein whose rate of synthesis was dramatically up-regulated during the period of neurogenesis in the rat neocortex. Further analysis (as described in the previous chapter) revealed that TOAD-64 was expressed immediately after neuronal birth and down-regulated in the second postnatal week to nearly undetectable levels in the adult; timing coincident with initial neuronal differentiation and down- regulation when the majority of axon growth is completed. The neural specificity and timing of expression suggested that TOAD-64 might play a role in axon outgrowth or neuronal migration. Three additional lines of evidence examined in this chapter support this possibility: TOAD-64 is up-regulated following neuronal induction of P19 and PC12 cells, paralleling the onset of neuritogenesis in these cells; the protein is found in the

lamellipodia and filopodia of growth cones; and axotomy of the sciatic nerve induces re-expression in adult animals.

The full length sequence of a cDNA encoding TOAD-64 is described in this chapter, also the pattern of expression of TOAD-64 mRNA, *in vivo* and in two cell lines. TOAD-64 is homologous to the *C. elegans unc-33* gene, mutations in which lead to aberrant patterns of axon outgrowth (Hedgecock et al., 1985; Li et al., 1992). TOAD-64 also shows substantial homology to a newly cloned gene from chicken nervous system, CRMP-62, (Collapsin Response Mediator Protein of 62kD) (Goshima et al., 1995). Molecular and electrophysiological experiments suggest that CRMP-62 is an intracellular component mediating the cellular response to collapsin, a protein that causes the growth cones of extending axons to collapse (Luo et al., 1993).

The gene homologies and the mRNA expression patterns described in this chapter, further support our hypotheses for the role of TOAD-64 in early neural differentiative events including the guidance of axons to their targets.

MATERIALS AND METHODS

PCR Amplification

Degenerate DNA oligomer primers were designed based on peptide sequences obtained from microsequencing of purified TOAD-64 protein. The only primer combination that resulted in a single major PCR product was 5'-GA(C/T)(C/T)TIGT(G/T/C)AT(A/C/T)TGGGA(C/T)CC-3' (72-fold degenerate, 5'primer) and 5'-GTC(G/A)AAIACIGG(A/C/G/T)CC(G/A)TC(G/A)TA-3' (32-fold degenerate, 3' primer). First strand cDNA, prepared using polyA RNA isolated from postnatal day 5 (P5) rat cortex and random primers, was used as a template for PCR. The PCR conditions were as follows: 94°C, 1 min; 49°C, 2 min; 72°C, 2 min; for 35 cycles. PCR products were analyzed by agarose gel electrophoresis, and a major band [280 base pair (bp)] was isolated and cloned into the TA cloning vector pCRII (Invitrogen). The insert was sequenced using the vector M13 and T7 primer sites.

Isolation of cDNA Clones

An embryonic day 18 (E18) rat brain library (generously provided by J. Boulter, The Salk Institute) in the vector λZAP was plated on E. coli (strain BB4) at a density of 40,000 phage per 150 mm plate, and 400,000 clones screened according to standard techniques (Sambrook et al., 1989). Briefly, nitrocellulose filters were prehybridized at 65°C for 4-5 hours in 6X SSC (1X= 0.15 M sodium chloride, 0.015 M

sodium citrate), 0.1% SDS, 1X Denhardt's (0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), and 100 μg/ml salmon sperm DNA. Hybridization was carried out in the same solution with the addition of 5 x 10^5 cpm/ml of radiolabeled probe at 65°C for 20 hr. Filters were washed once in 2X SSC, 0.1% SDS, and twice in 0.2X SSC, 0.1% SDS, at 65°C for 20 min. For the initial screening, filters were probed with the 280bp PCR product radiolabeled ([^{32}P]-dCTP, Amersham) by random priming (Feinberg and Vogelstein, 1983) (NEBlot Kit, New England Biolabs, Inc., Beverly, MA). To obtain the 5' end of the gene, an additional 200,000 clones were screened with a 230bp region amplified from the 5'-most end of the initial clones.

DNA Sequencing and Analysis

DNA sequence was obtained by the dideoxy chain termination method using the Sequenase kit (Sanger et al., 1977) (United States Biochemical Corp., Cleveland, OH). Bluescript SK/T7 primers or cDNA specific 20-mers were used. Sequence was verified from overlapping clones or by sequencing both strands of DNA. Sequence compressions were resolved using dITP nucleotides and terminal deoxynucleotidyl transferase in the sequence reaction mix. Sequence alignments and analyses were performed using the University of Wisconsin Genetics Computer Group (GCG) software.

Northern Analysis

Total RNA (25µg) was denatured in 2.2 M formaldehyde, 50% formamide, 1x MOPS (3-(N-morpholino) propanesulfonic acid) buffer at 65°C for 15 min. The RNA was electrophoresed on a 1.0% agarose-formaldehyde gel with 1x MOPS buffer at 50V with buffer recirculation, and blotted to Zetaprobe (BioRad Labs., Hercules,CA). Blots were prehybridized at 65°C for 30 min in 7% SDS, 1% BSA, 0.5 M phosphate buffer (pH 6.8) (PB), and 1 mM EDTA. Hybridization was carried out overnight in the same buffer containing 1-5 x 10^6 cpm/ml [^{32}P]dCTP-labeled cDNA. Blots were washed twice in 5% SDS, 0.5% BSA, 40 mM PB, 1 mM EDTA, and once in 1% SDS, 40 mM PB, 1mM EDTA at 65°C for 20 min (Church and Gilbert, 1984) and exposed to film (Hyperfilm, Amersham) at -70°C. The ubiquitously expressed, non-developmentally regulated gene cyclophilin (Lenoir et al., 1986; Danielson, 1988) was used to determine equal loading of lanes.

In Situ Hybridization

In situ hybridization was carried out as described by Jaworski et al. (1994). Briefly, twelve to fourteen µm thick fresh-frozen sections were thaw-mounted onto gelatin-coated slides and postfixed in 0.1 M phosphate buffered 4% paraformaldehyde. Sections were probed with [^{35}S]CTP (New England Nuclear, Boston, MA) labeled antisense or sense cRNA transcribed *in vitro* from a 280bp PCR product inserted into the vector pCRII (Invitrogen). Following hybridization and washing, initial localization of probe was determined by

exposing the slides to film (Hyperfilm, Amersham) for 48 hr. Autoradiograms were used as negatives for prints. For higher resolution, the slides were exposed to Kodak NTB-2 liquid emulsion, developed after 2-5 days, and counterstained with cresyl violet. Neurofilament-middle (NF) antisense (Martin et al., 1992) was used as a positive control.

Immunohistochemistry

Sprague-Dawley rats (Charles River Laboratory, Raleigh, NC) ages E17 to adult were perfused transcardially with 0.1 M sodium phosphate (pH 7.4) (PB), then 4% paraformaldehyde (PFA) in PB. Whole brains or spinal cords were dissected out and postfixed in 4% PFA for 24 hr at 4°C, then equilibrated in 30% sucrose in PB. Coronal sections of E17 brain were cut at 30 μm, and transverse sections of adult spinal cord cut at 50 μm on a cryostat and collected as free-floating sections in PB for immunohistochemistry. Tissue sections were incubated in rabbit anti-TOAD-64 (1:2000) (Minturn et al., 1994) at room temperature overnight. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (1:200, Cappel). HRP was localized using a 0.7 mg/ml solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.003% H_2O_2 in PB. Sections were dehydrated in graded ethanols, equilibrated in xylenes, and coverslipped with Permount (Fisher Scientific). Sections retrogradely labeled with Fast Blue were mounted in Aqua-Poly Mount (Polysciences, Inc.).

Cell Culture

Culture and induction of P19 cells was as previously described (McBurney et al., 1988). P19 cells (ATCC) were maintained in alpha modified MEM supplemented with 2.5% FCS and 7.5% newborn calf serum in 100 mm tissue culture plates. Cells adhering to the plates were treated with 0.05% trypsin/0.5 mM EDTA DPBS without Ca^{++} or Mg^{++}, triturated with a flame constricted pipette in complete medium and plated into bacteriological culture dishes at a density of 1 x 10^5 cell/ml. For neural induction the culture medium was supplemented with 1μM all trans-retinoic acid (day 0). Four days after induction the floating clumps of cells were treated with trypsin, dispersed by trituration and plated onto culture dishes at a density of 5 x 10^4 cells/cm^2 in medium lacking inducer. Cells were collected and RNA prepared (as described above) at 2, 4, 6, 8, and 11 days following induction or at 4 and 11 days without induction.

PC12 cells were plated on polyornithine/laminin treated 100 mm tissue culture dishes at a density of 75 cells/mm^2 in DMEM/10% FCS. Twenty-four hours after plating the medium was replaced with medium containing 100 ng/ml NGF. Cells were harvested for either RNA or protein before NGF induction and at 1, 3, 5, and 7 days following induction.

Primary neuronal cultures were made of rat sensory ganglia. Dorsal root ganglia (DRG) dissected from E16 Sprague-Dawley rat embryos, were collected in DMEM. Following washes in Ca^{2+}/Mg^{2+}-free DPBS, ganglia were treated

with 0.05% trypsin in Ca^{2+}/Mg^{2+}-free DPBS for 30 minutes at 37°C. After washes in DMEM supplemented with 10% FCS (Hyclone), 2mM glutamine and penicillin-streptamycin, cells were dissociated by trituration using a fire-polished Pasteur pipette. Cells were plated onto flame sterilized, polyornithine/laminin (Sigma) coated, round cover glasses, at a density of 90 cells/mm^2 in medium supplemented with 50 ng/ml 2.5S NGF (Collaborative Research). After 20 hr at 37°C and 5% CO_2, 4% phosphate-buffered paraformaldehyde/0.2% glutaraldehyde was added to the cultures in a volume equal to that of the culture medium. After 10 min the cells were washed extensively with 0.1 M phosphate buffer. Cultures were incubated for 2 hr with DMEM supplemented with 5% FCS, 0.1% glycine, 0.1% lysine, and 0.2% Triton X-100.

Immunohistochemistry was performed as described above, using either rabbit antisera to TOAD-64 or an antibody to class III ß-tubulin (Lee et al., 1990ab; Easter et al., 1993; Minturn et al., 1995b), generously provided by A. Frankfurter, Univ. of Virginia. Antibody binding was visualized with species specific secondary antibodies and DAB with 2% $NiSO_4$ for HRP in single-labeling studies or with FITC or Texas Red for double-labeling studies.

Sciatic Nerve Lesion

Adult Sprague-Dawley rats weighing 150-200 g were obtained from Charles River Laboratory (Raleigh, NC). Rats were anesthetized with 400 mg/kg chloral hydrate, and the

right sciatic nerve was exposed at the level of the midshaft femur. The nerve was crushed with forceps and injected (proximal to the crush) with one microliter of the retrograde tracer Fast blue (1% solution in PB). Survival times ranged from 4 hr to 2 weeks after surgery, at which time the animals were deeply anesthetized and perfused transcardially with 0.1 M PB followed by 4% paraformaldehyde in PB. The spinal cords were removed at the level of the lumbar enlargement and processed for immunohistochemistry as described above. The motor neurons of the anterior horn of the spinal cord labeled with Fast blue were visualized using epifluorescence microscopy with a UV filter. Appropriate control experiments were performed to insure that the Fast blue tracer did not interfere with our antibody detection of TOAD-64 protein.

Tissue Extraction and Western Analysis

Tissues from euthanized animals were dissected and used immediately or stored frozen at -70°C. Fresh or frozen tissues were homogenized in a Teflon-glass Potter-Elvehjem homogenizer at a concentration of 0.2 g (wet weight) of tissue / ml of Tris buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4) containing a cocktail of protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleamide, 5 mM e-amino-n-caproic acid, 5 µg/ml leupeptin and 1 µg/ml pepstatin A). Whole homogenate samples were taken directly from this preparation. Supernatant (S) and pellet (P) fractions were prepared from the homogenates

as follows. The samples were centrifuged at 100,000 x g for 1 hr and the supernatant (S fraction) was saved. The resulting pellet was rehomogenized with PBS and again separated into pellet and supernatant fractions by centrifugation. For differential extractions this pellet (from the second centrifugation) was homogenized with either 0.1 M Na_2CO_3 pH11, PBS, or 2 M NaCl in PBS, 1% Triton X-100 in PBS, 10 mM EDTA in PBS or 2 M urea in PBS. The samples were centrifuged as above, the supernatants saved and the pellets washed with the appropriate buffer. Protease inhibitors were used in all buffers. Protein concentrations were determined by the method of Bradford (Bradford, 1976) using the Bio-Rad dye reagent kit and BSA as a standard.

Samples to be analyzed by immunoblotting were combined with gel loading buffer (20 mM Tris-Cl, pH 6.8, 3% SDS, 10% glycerol, 0.01% bromophenol blue) and b-mercaptoethanol (0.05%), boiled for 5 min and electrophoresed by SDS-PAGE minigel (10%) in 50 mM Tris base, 0.38 M glycine, 0.2% SDS (Laemmli, 1970). The proteins were electrophoretically transferred to nitrocellulose overnight at 50 mA in 25 mM Tris, 0.192 M glycine, and 20% methanol (Towbin et al., 1979). The blots were blocked in Blotto (5% nonfat dry milk in TBS) for 2 hr and incubated in primary antibody (diluted in Dulbecco's modified Eagle's medium supplemented with 5% FCS, 0.2% Tween 20, and 0.2% sodium azide) overnight at room temperature. The blots were washed and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG or goat

anti-mouse IgG (Promega) for 2 hr at room temperature. Immunoreactive bands were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

RESULTS

Molecular Cloning and Sequence Analysis of TOAD-64

The TOAD-64 protein is expressed at its highest levels during the first postnatal week. To clone the gene encoding the TOAD-64 protein, randomly primed cDNA from postnatal day 5 (P5) rat cortex was used as a template for PCR amplification. Degenerate oligonucleotide primers were based on 5 peptide sequences obtained from microsequencing of purified TOAD-64 protein (peptide A: GAPLNSIXQG; peptide B: NHQSVAE, peptide C: YDGPVFDLTTTPK, peptide D: IAVGSDSDLVIWDPDAVK, peptide E: ALHVDIT). The primers were designed to encode only a portion of each peptide, so that a correct PCR product would be expected to contain the remainder of the peptide sequence. Of twelve possible primer combinations, one combination (representing peptide D and peptide C) amplified a single major band of approximately 280bp, while the others amplified multiple minor bands or generated no PCR product. The major 280bp PCR product was cloned into pCRII (Invitrogen) and sequenced. The predicted amino acid sequence from the region internal to both of the primers matched that of the original peptide fragments from the TOAD-64 protein. To obtain full length sequence, the 280bp PCR product was used to screen an E18 rat brain lambda ZAP II cDNA library. A total of 4.0×10^5 recombinant plaques were screened, resulting in 15 positive clones. The insert size of each of the clones was determined and

redundant clones were eliminated by constructing a map of each clone using PCR with vector- and TOAD-specific primers. To obtain the 5' end of the gene, an additional 200,000 recombinants were screened with a PCR product comprising the most 5' end of the first set of clones. The composite sequence of TOAD-64 was obtained from four of these overlapping clones. The complete TOAD-64 coding sequence is 1716bp (Figure 8). The first in-frame methionine is preceded by a translational start consensus sequence, as described by Kozak (Kozak, 1984). An in-frame termination codon is found 30bp upstream of the translation initiation site. The TOAD-64 gene encodes a 572 amino acid long protein. Analysis of the deduced amino acid sequence shows no signal sequence or likely transmembrane domain, consistent with a cytoplasmic location for the protein. The sequence also has several (S/T) X (R/K) consensus sites for potential protein kinase C phosphorylation, as well as a potential tyrosine kinase phosphorylation site, (K) XXX (D) XX (Y), at Tyr-479 (Pearson and Kemp, 1991), suggesting that TOAD-64 is a phosphoprotein.

Several converging lines of evidence indicate that the gene we have cloned encodes the TOAD-64 protein. First, the isolated gene encodes a protein with a predicted molecular mass of 62,364 daltons and an isoelectric point (pI) of 6.34. These values are remarkably similar to the molecular mass (64 kDa) and pI (6.4) of TOAD-64 (protein 310) on two-dimensional SDS-PAGE (Geschwind and Hockfield, 1989). Second, 4 of the original 5 peptides obtained by amino acid sequence analysis are represented in the cDNA

sequence (see Figure 8). And third, synthetic peptides generated from a region of the sequence (residues 381-399) that was not obtained in the original amino acid sequence analysis was used to immunize rabbits. Antisera to this new sequence recognizes TOAD-64 protein immunoprecipitated by one of the original antisera to TOAD-64 (data not shown). Together these data confirm that the isolated gene encodes the TOAD-64 protein.

Sequence similarity analysis using the GenBank and EMBL databases at both the nucleic acid and amino acid levels indicate that TOAD-64 is a previously unreported gene. The TOAD-64 gene product is homologous to the *unc-33* gene product of *C. elegans*. Over the entire length of the proteins, TOAD-64 and *unc-33* show 34% identity, and as much as 79% similarity (Figure 9A). *C. elegans* mutants in *unc-33* have pronounced abnormalities in axonal arborizations, presumably due to errors in axonal branching and guidance during axonogenesis (Hedgecock et al., 1985; Li et al., 1992).

Substantial amino acid homology also exists between TOAD-64 and 6 sequences isolated from a human fetal brain library using a random cloning strategy (Adams et al., 1993). Three of these map to overlapping stretches of the TOAD-64 gene, 2 map to a region in the 5' end, and one to a region in the 3' end of the gene. Over very long stretches, there is close to 100% identity between the TOAD-64 gene and the human fetal brain sequences (Figure 9B-E). The overlap and degree of similarity between these six gene fragments and the TOAD-

64 gene suggests that all six fragments represent multiple sequences from a single gene or a gene family. These human fetal brain sequences have not been characterized, but it can be predicted that they represent the human homolog(s) of rat TOAD-64. The very high degree of conservation between rat and human makes it likely that TOAD-64 has a highly conserved, essential function.

A recently reported gene isolated from the developing chicken nervous system, CRMP-62 (Collapsin Response Mediator Protein of 62kD) (Goshima et al., 1995) is the chicken homolog of rat TOAD-64. The chicken sequence shares 97% predicted amino-acid identity with TOAD-64 (Figure 10). Interestingly, this gene was isolated in a *Xenopus laevis* oocyte expression system designed to identify molecules that participate in the collapsin-induced growth cone collapse signaling pathway. There is *in vitro* evidence that the collapse of growth cones is mediated by G protein coupled receptors (Igarashi et al., 1993), and this premise was used in the initial expression screen of injected chick RNA (identifying mediators of pertussis toxin-sensitive inward currents). This newly reported data further confirms our suggestion that TOAD-64 could be a central element in the complex machinery that contributes to axonal outgrowth and pathfinding in the developing nervous system.

TOAD-64 mRNA is nervous system specific and is developmentally regulated.

Two different partial sequences from the TOAD-64 gene, representing bases 530-1215 and 1220-1533, were used to study the regulation and distribution of TOAD-64 mRNA. Northern analysis shows that a single band at 4.5 kb representing the TOAD-64 mRNA is developmentally regulated (Figure 11A). TOAD-64 mRNA is detected in brain at E17. The levels increase up to postnatal day 5 (P5), and subsequently decline to an almost undetectable level in the adult. No TOAD-64 message is detectable in non-neural tissues (Figure 11B). The pattern of regulation of the TOAD-64 mRNA parallels that previously described for the TOAD-64 protein (Minturn et al., 1994).

In situ hybridization confirms both the developmental regulation and the neural specificity of TOAD-64 mRNA (Figure 12). TOAD-64 mRNA is abundant throughout the neuraxis at E14. Hybridization signals are high in both the central (CNS) and peripheral nervous system. In the peripheral nervous system, sensory and autonomic ganglia express TOAD-64. No hybridization is detected outside of the nervous system. In the CNS, all regions of the brain and spinal cord express TOAD-64, however the distribution within each area indicates that not all CNS cells are TOAD-64 positive (see below). As development proceeds, hybridization remains high through the first postnatal week, but then declines to an almost undetectable level in most areas of the brain by postnatal day 14 (P14). The only area of the CNS in which we detect TOAD-64 mRNA in adults is the hippocampal formation (not well demonstrated in figure 12E). Antibody to TOAD-64 shows that the adult dentate gyrus contains a small

population of antibody positive cells, whose position and morphology matches that of the hilar dentate neurons that are born in adult rats (Bayer, 1982). Antibody staining also reveals TOAD-64 expression in the primary afferents to the olfactory bulb in adult animals, axons from the olfactory sensory neurons that continue to be generated in the adult (Graziadei and Monti-Graziadei, 1978). While we have not examined the nasal sinuses in adult animals, in embryos TOAD-64 mRNA is present in the olfactory epithelium (Figure 13A).

In the previous chapter we showed that the TOAD-64 protein is first expressed by neurons shortly after they have completed their final mitosis (Minturn et al., 1994). It was important to determine if this pattern of protein expression reflected transcriptional or translational regulation. *In situ* hybridization shows clearly that TOAD-64 mRNA, like TOAD-64 protein, is not detected in the ventricular zone of the developing brain. For example, in the cortex at E17, the highest level of hybridization is seen in the cortical plate (Figure 13B), with far lower levels of hybridization in the ventricular and subventricular zones, similar to the distribution of the protein (Figure 13D). These results demonstrate that the restricted expression of the protein to newly born neurons is likely to be controlled at the level of gene transcription.

TOAD-64 is expressed by P19 and PC12 cells coincident with neuronal differentiation.

In the developing brain TOAD-64 is expressed only by cells that have undergone a commitment to a neuronal phenotype. To address the possible association of TOAD-64 expression with neuronal differentiation, we examined the expression of TOAD-64 in two cell lines that can be induced to assume a neuronal phenotype, the embryonal carcinoma cell line, P19, and the rat pheochromocytoma cell line, PC12.

P19 cells are developmentally pluripotent (Edwards and McBurney, 1983). P19 cells exposed to retinoic acid (at greater than $3x10^{-7}M$) develop neural properties: by six days after exposure to retinoic acid approximately 85% of cells express neuronal markers (McBurney et al., 1988; Staines et al., 1994). We examined undifferentiated and retinoic acid differentiated P19 cells by northern analysis (Figure 14A). Prior to neural induction, TOAD-64 mRNA is not expressed, but exposure to retinoic acid induces the expression of TOAD-64 mRNA.

PC12 cells are an adrenal chromaffin-derived tumor cell line (Tischler, 1975; Greene and Tischler, 1976), that, when grown in the absence of nerve growth factor (NGF), proliferate and do not assume neuronal properties. When NGF is added to the culture medium, PC12 cells differentiate into cells with neuronal properties, including the cessation of mitotic activity, the extension of neurites, and the expression of genes associated with mature neurons (Tischler, 1975; Stein et al., 1988; Vetter and Betz, 1989; Sano et al., 1990;). TOAD-64 expression was assayed in uninduced and NGF-

induced PC12 cells. In contrast to P19 cells, PC12 cells, in the absence of any induction by NGF, express TOAD-64 mRNA (Figure 14B). Furthermore, there is no apparent up-regulation of mRNA following NGF induction. The disparity between the behavior of TOAD-64 mRNA in these two cell types prompted us to examine the expression of TOAD-64 protein in PC12 cells (Figure 14C). In the absence of NGF, little TOAD-64 protein is detected. After NGF addition, the amount of TOAD-64 increases at 24 hours and continues to increase up to 5 days. These results suggest that the TOAD-64 protein is involved in the neuronal differentiation of PC12 cells, perhaps the elaboration of neurites. However, in PC12 cells, in marked contrast to both P19 cells and to neurons *in vivo*, TOAD-64 protein expression is not transcriptionally regulated.

TOAD-64 is present in elongating axons in development and in growth cones in culture

The expression of TOAD-64 during the initial outgrowth of processes is not restricted to neuritogenesis of cells *in vitro*. In the developing rat spinal cord, axons of the dorsal root express TOAD-64 during their initial entry into the dorsal horn, and as they extend in the dorsal funiculus prior to growing into the spinal cord gray matter (Figure 15). To determine whether TOAD-64 might have a role in the mechanisms by which axons advance toward their targets, we examined the distribution of the TOAD-64 protein in neurons

in primary culture. In cultures of dorsal root ganglion cells or cortical neurons grown on a polyornithine-laminin substrate, growth cones can be easily identified after 20 hours in culture. Immunohistochemical analysis shows that TOAD-64 is expressed in neuron cell bodies and processes (Figure 16A). TOAD-64 immunoreactvity is present along the full length of the axon into the growth cone. In the growth cone immunoreactivity is most intense at the center of the growth cone, but it also extends through the lamellipodia to the edges of the lamellipodial veil (Figures 16B,C; 17A,C). Often a region of particularly intense immunoreactivity for TOAD-64 is observed in the filopodial extensions from the growth cone. The distribution of TOAD-64 in the growth cone is different from the cytoskeletal protein, class III ß-tubulin (Figures 16D,E; 17B,D), which does not extend into the lamellipodia or filopodia.

TOAD-64 is re-expressed following peripheral axotomy

The expression of TOAD-64 in elongating axons in the developing spinal cord, as well as in extending neurites in primary neuronal cultures and cell lines suggested a role for this protein in axon extension. This led us to examine TOAD-64 expression during axon regeneration after peripheral axontomy in adult animals. Following lesions of the sciatic nerve, sciatic motor neurons with cell bodies in the spinal cord can regenerate axons toward their target muscles. Expression of the TOAD-64 protein by the sciatic motor

neurons was studied following a sciatic nerve crush. In normal adult animals, there is no detectable TOAD-64 expression by motor neurons. Following sciatic nerve crush, TOAD-64 protein is expressed in sciatic motor neurons (Figure 18B). Neuronal cell bodies of the crushed axons can be identified by the retrograde tracer Fast blue, which was injected into the crushed nerve proximal to the injury. The unlesioned, contralateral, motor neurons do not express the protein (Figure 18A). A time course of the re-expression of TOAD-64 following nerve injury reveals expression of TOAD-64 protein as early as 1 day after nerve crush.

TOAD-64 is a membrane associated protein.

While the sequence of TOAD-64 does not contain a transmembrane domain, biochemical experiments indicate that TOAD-64 has a membrane associated form. Two-dimensional gel analyses showed that TOAD-64 partitions approximately equally between a soluble fraction, which contains cytoplasmic constituents, and a particulate fraction, which contains membrane and cytoskeletal elements. Western blot analysis of a P12 cortical homogenate using antisera to TOAD-64 provides further evidence that TOAD-64 has both soluble and particulate forms (Figure 19A).

To determine the mechanism of association with the pellet fraction, further extractions were performed (Figure 19). Extraction of the pellet with PBS or with 2 M NaCl releases only 20% of the TOAD-64 protein from the particulate

fraction. TOAD-64 is not bound to the insoluble fraction through Ca^{2+}-dependent mechanisms, because extraction of the particulate fraction with EDTA also releases only 20% of the protein. In contrast, treatment of the particulate fraction with 2 M urea releases 50% of the protein, and 90% of the protein is extracted with 1% Triton X-100. Efficient extraction with Triton makes it unlikely that TOAD-64 is associated with the particulate fraction by binding to the Triton insoluble cytoskeleton. The fact that over 90% of the TOAD-64 protein can also be extracted with Na_2CO_3 indicates that it is either a peripheral membrane protein or trapped within vesicles. Both the amino acid sequence deduced from the nucleic acid sequence, which does not contain a putative transmembrane domain, and the immunohistochemical localization of the protein to the cytoplasm (Minturn et al., 1994, 1995), further indicate that TOAD-64 is an intracellular protein. Together these data show that the particulate form of TOAD-64 is likely to be tightly associated with a membrane protein, and not itself a transmembrane protein.

DISCUSSION

We report here the full length sequence and expression pattern of a gene, TOAD-64, that is initially transcribed at the earliest stages in neuronal differentiation. Neither the TOAD-64 protein nor its mRNA is expressed by mitotically active progenitors in the ventricular zone, but both are expressed by postmitotic neurons. TOAD-64, is one of the few known genes that is turned on coincident with the very earliest events in neuronal differentiation. The regulation of TOAD-64 in parallel with axon elongation in several different paradigms, together with its localization to lamellipodia and filopodia of growth cones, suggests a role for this protein in axonogenesis or the navigation of growth cones.

The TOAD-64 gene encodes a deduced protein with a molecular mass of 62,364 and pI of 6.34, almost identical to the estimates for the protein from two-dimensional gels (Geschwind and Hockfield, 1989). At the time of our initial studies, the TOAD-64 gene was not identical to any gene in the databases, but showed significant homology to the *C. elegans unc-33* gene. *Unc-33* was originally isolated in a screen for motorically uncoordinated mutants. Further histological analysis revealed that all neurons in *unc-33* mutants have defective patterns of axon outgrowth (Hedgecock et al., 1985; Li et al., 1992). In *unc-33* mutants, axons terminate prematurely, have abnormal branch points and follow inappropriate routes (Hedgecock et al.,

1985; Li et al., 1992; Siddiqui and Culotti, 1991; Desai et al., 1988). Based on axonal morphology, it was predicted that *unc-33* would encode a cytoskeletal protein, such as a microtubule associated protein (Hedgecock et al., 1985), but subsequent sequence analysis revealed that, like TOAD-64, *unc-33* showed no homology to any protein already in the database (Li et al., 1992). The *unc-33* product is predicted to have several potential phosphorylation sites, consistent with the fact that the TOAD-64 gene sequence contains consensus sites for serine, threonine and tyrosine phosphorylation. In addition, *unc-33* lacks a transmembrane region or signal peptide, as does TOAD-64, based on sequence information and biochemical studies. The major difference between *unc-33* and TOAD-64 is that *unc-33* has three transcripts, while we have detected only one for TOAD-64. The size of the largest predicted *unc-33* protein, 90kDa, is significantly larger than TOAD-64, however, the *unc-33* protein(s) itself has not been isolated or characterized. Nevertheless, the sequence and predicted structural similarities between the *unc-33* gene product and TOAD-64 suggest that they are related, and perhaps homologous proteins from evolutionarily distant species. This provides support for the possibility that TOAD-64 may, like *unc-33*, play a critical role in axon outgrowth in the rodent brain.

A chick homolog of TOAD-64 has been recently cloned and characterized using an expression system designed to identify elements of the cellular response to collapsin (Goshima et al., 1995). Collapsin is a protein that causes growth cones

to collapse in an *in vitro* assay, and is likely to play an important role in axon navigation (Raper and Kapfhammer, 1990; Luo et al., 1993). Expression of the chick homolog of TOAD-64, called CRMP-62 (Collapsin Response Mediator Protein of 62kD) in oocytes causes a pertussis toxin (PTX)-sensitive inward current in response to soluble collapsin suggesting a G protein-coupled receptor mediated event. This same signaling pathway has been suggested as mediating dorsal root ganglia (DRG) growth cone collapse in response to solubilized embryonic brain membranes, CNS myelin, and the NI-35 protein of myelin (Igarashi et al., 1993). In addition, introduction of anti-CRMP-62 antibodies into chick dorsal root ganglion neurons blocks collapsin-induced growth cone collapse. These data are highly suggestive for the role of TOAD-64/CRMP-62 as a central element in the signaling cascade that transduces extracellular guidance cues to the extending axon.

Evidence that led to the suggestion that TOAD-64 might play a role in early neuronal differentiation, such as in cell migration or axon outgrowth, was suggested in experiments demonstrating the extremely early expression of TOAD-64 in developing neurons (discussed in Chapter 2) (Minturn et al., 1995b). Antibodies showed that the TOAD-64 protein is first expressed by neurons shortly after the final mitosis, at a time when neurons are just beginning their migration out of the ventricular zone. While we have no direct evidence for the expression of TOAD-64 in pre-migratory neurons, the expression of TOAD-64 in non-process

bearing cells in E12 rat brains that also express neuron specific tubulin (see Figure 5) suggests TOAD-64 is expressed at least coincident with initial process formation and cell migration. TOAD-64 expression precedes that of several neuronal markers, including neurofilament and MAP2. Double labeling with an antibody to class III ß-tubulin showed that all postmitotic neurons express TOAD-64. The *in situ* hybridization analyses presented here indicate that the TOAD-64 gene, like the protein, is not expressed in the region occupied by progenitor cells, but is first detected outside of the ventricular zone. Unlike most previously described neuronal genes and proteins, TOAD-64 mRNA is down-regulated to an almost undetectable level in most regions of the adult brain. Expression declines during the second postnatal week, coincident with the end of the major period of axon growth. The data on the expression of TOAD-64 mRNA demonstrate that the regulation of TOAD-64 protein in the developing brain is at the level of gene transcription.

TOAD-64 expression in cell lines further underscores its regulation in parallel with neuronal differentiation. TOAD-64 mRNA is not detected in the embryonal carcinoma cell line, P19, prior to the induction of a neuronal phenotype. Induction of neuronal properties by retinoic acid induces the expression of TOAD-64 mRNA. In PC12 cells, neuronal differentiation apparently increases the rate of translation or the stability of the TOAD-64 protein without altering the constitutive expression of TOAD-64 mRNA. This mechanism of

protein regulation is similar to that observed for other neuronal proteins in PC12 cells (Sharma et al., 1993). Although the mechanism of regulation of protein levels is different in P19 and PC12 cells, it appears likely in both cases that TOAD-64 protein plays a role in the acquisition of a neuronal phenotype, potentially in mediating neurite outgrowth.

A role in neurite outgrowth is also suggested by the localization of TOAD-64 to the lamellipodia and filopodia at the advancing edge of growth cones. Moreover, further evidence supporting such a role for TOAD-64 comes from our experiments on axon regeneration. Re-expression during axon regeneration would be predicted for a protein required for axon outgrowth or navigation. During development, TOAD-64 is expressed at a high level in the spinal cord. In the normal adult spinal ventral horn, as in most other areas of the adult CNS, there is little detectable TOAD-64. Motor neurons with axons in the sciatic nerve re-express TOAD-64 as they regenerate to find targets following a sciatic nerve lesion. Protein up-regulation during axon regeneration has also been demonstrated for GAP-43 (Skene and Willard, 1981; Skene, 1989), a protein known to be associated with axonal elongation. Also consistent with a role in axonogenesis is the expression of TOAD-64 in areas of the adult brain which have the capacity for ongoing neurogenesis and axon growth.

A membrane-associated protein expressed during axonal extension and localized at lamellipodia would be optimally

situated to participate in the signal transduction processes that permit growing axons to choose correct routes and targets. Sequence analysis shows that the protein lacks a signal sequence or a transmembrane domain. The sequence data dictate that the TOAD-64 protein is cytoplasmic, however, extraction experiments indicate that the protein has both a soluble and membrane associated form. The mechanism by which TOAD-64 associates with the membrane is not apparent from the sequence alone. Biochemical experiments show that conditions that extract transmembrane proteins and proteins associated with them can release TOAD-64 from a crude membrane preparation. It is therefore likely that TOAD-64 binds non-covalently with high affinity to a membrane protein, possibly a collapsin-binding protein. While the definitive demonstration of the function of TOAD-64 still lies before us, the data presented in this dissertation and previously (Geschwind and Hockfield, 1989; Minturn et al 1995a,b) suggest that TOAD-64 could be a central element in the complex machinery underlying axonal outgrowth and target recognition.

Figure 8. cDNA and deduced amino acid sequences of TOAD-64. The coding sequence for TOAD-64 is 1716bp, which encodes a 572 amino acid protein. The deduced protein contains several potential protein kinase C phosphorylation sites and a tyrosine kinase phosphorylation site (dotted underlines). The four peptide sequences obtained by microsequence analysis of purified TOAD-64 protein are underlined. These sequence data are available from the EMBL Nucleotide Database under accession number Z46882.

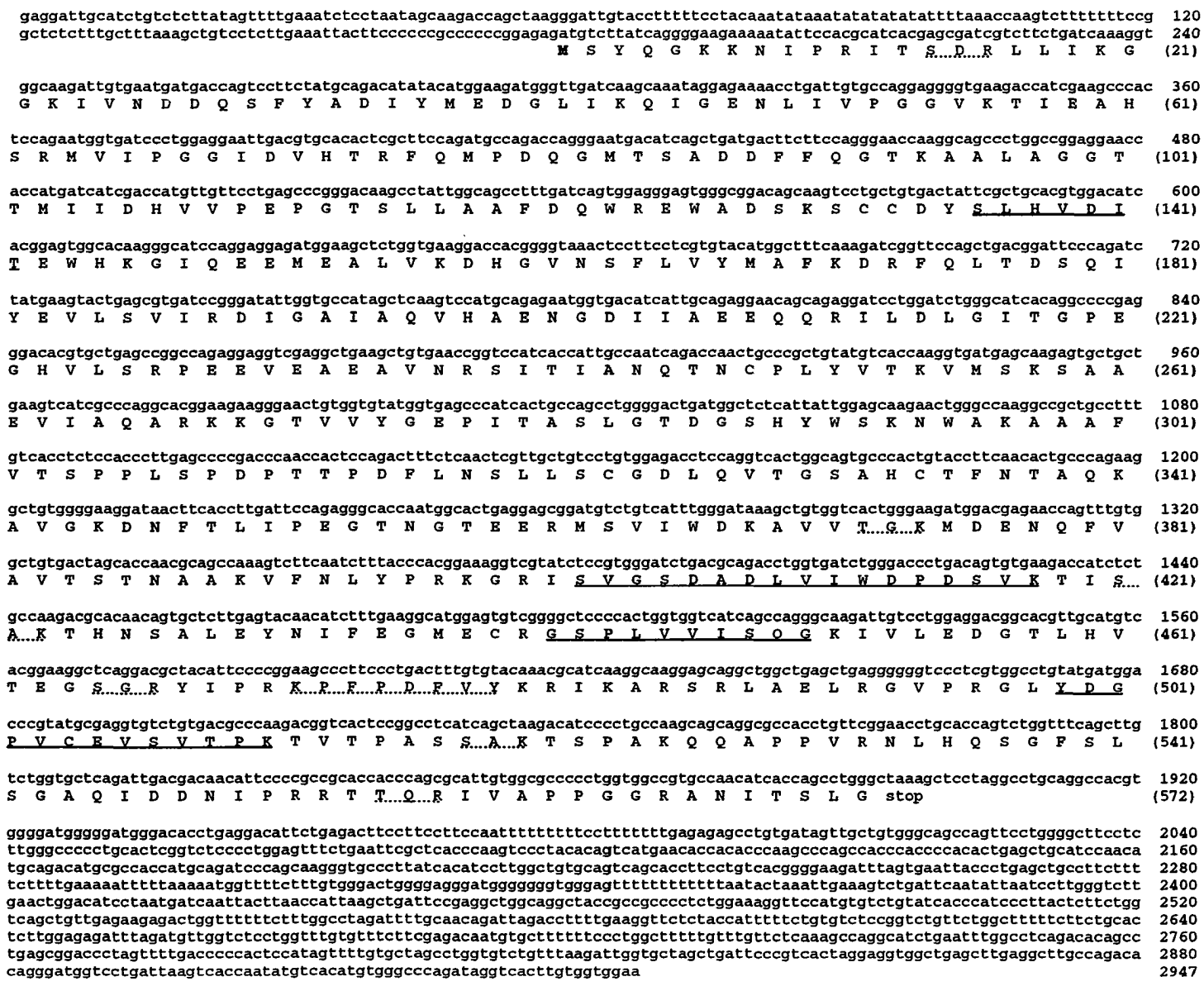
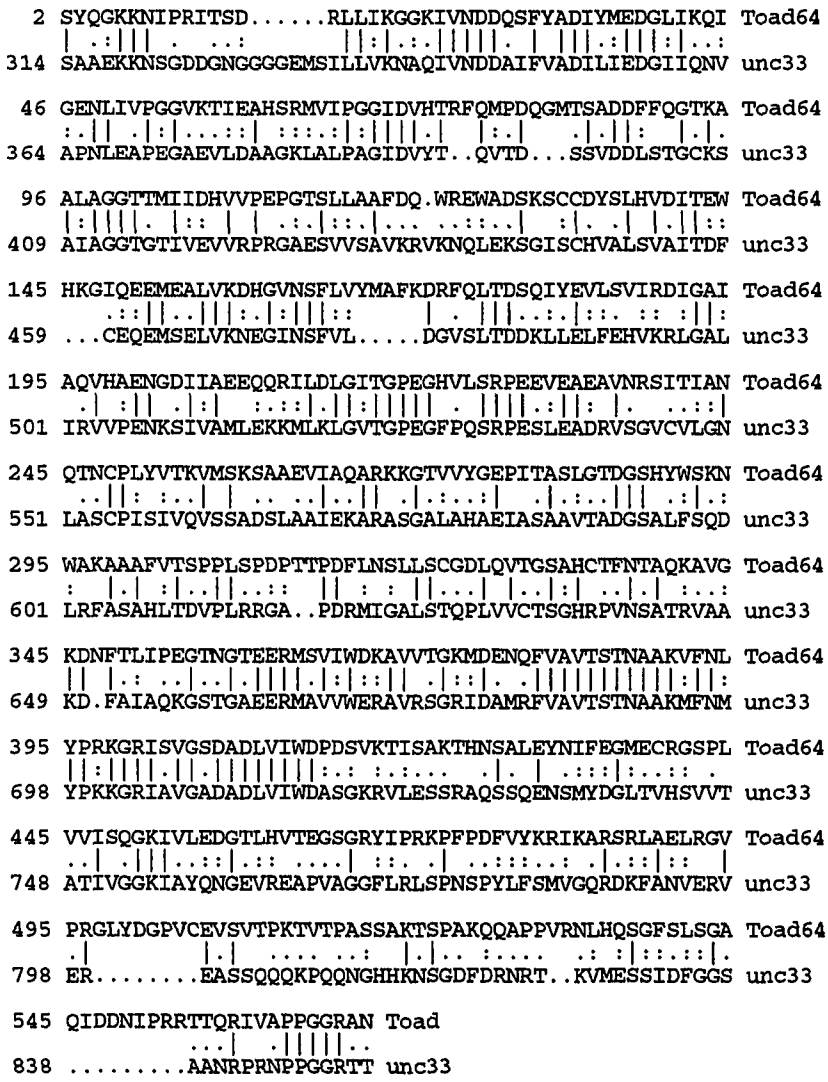


Figure 9. The predicted amino acid sequence of TOAD-64 is homologous to the *C. elegans unc-33* gene (A) and to several human fetal brain cDNAs (B-E). **(A)** The predicted amino acid sequences for TOAD-64 and *unc-33* (Li et al., 1992) are shown aligned. Solid lines between the two sequences represent identical sequence and dots indicate conserved substitutions. The two proteins are 34% identical over the entire coding region and possess many conserved substitutions. **(B and C)** The first 114 coding amino acids of TOAD-64 are nearly identical to human sequences T07524 (EMBL accession number) and T06278. The initiating methionine is indicated in bold. **(D and E)** The homology between TOAD-64 and the human clones continues through the length of the protein, and ranges between 74-95% identity. The human sequence, T08129, shown in (D) is also found in sequences T06728 and T09404.

TOAD-64 x Unc-33



TOAD-64 x Human

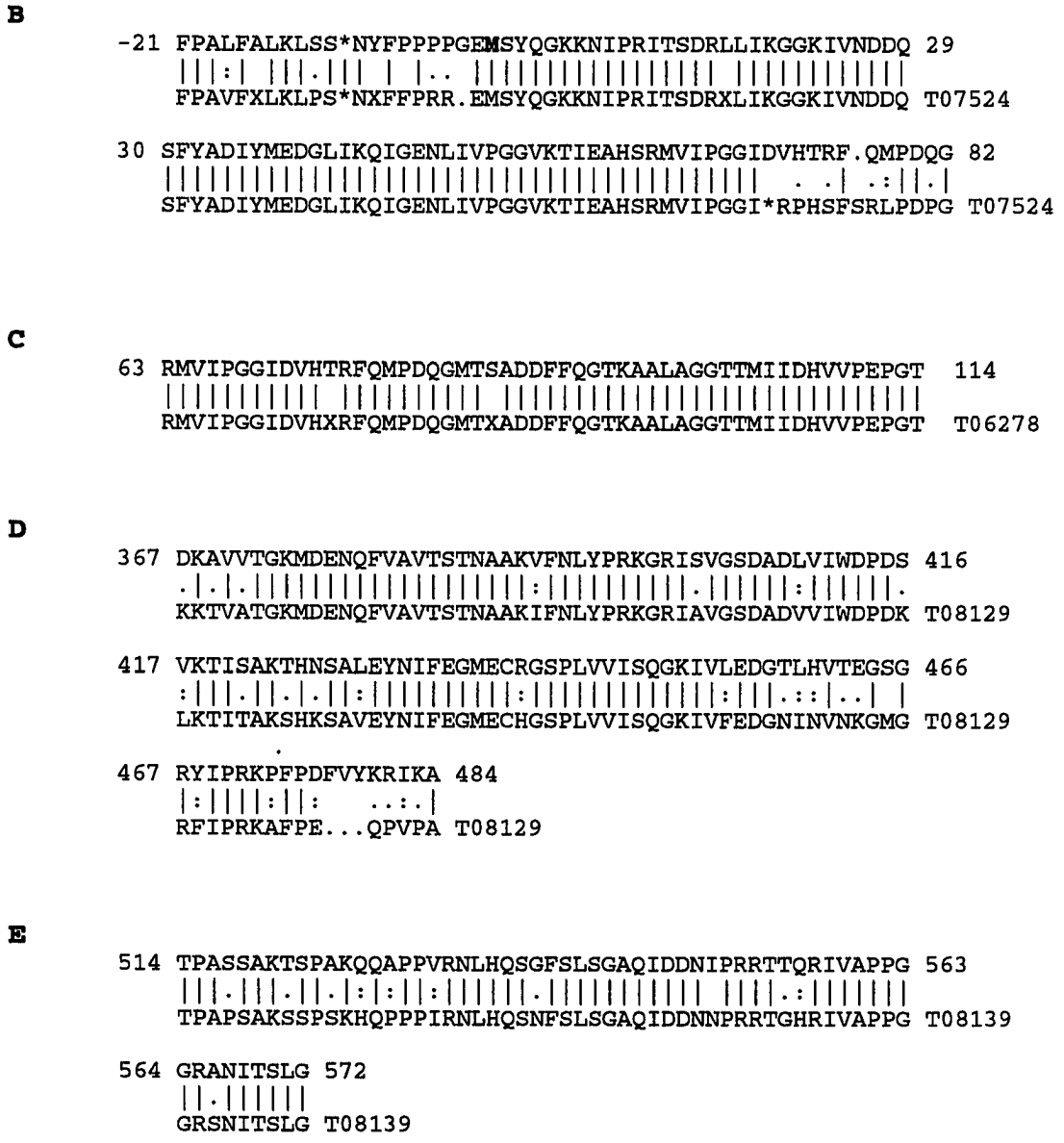


Figure 10. The predicted amino acid sequence of TOAD-64 is homologous to the chick collapsin response mediator protein (CRMP-62).

The predicted amino acid sequences for TOAD-64 and CRMP-62 (Goshima et al., 1995) are shown alligned. Identical residues are alligned with solid lines between them. The proteins are 97% identical over the entire coding region.

TOAD-64 X CRMP-62

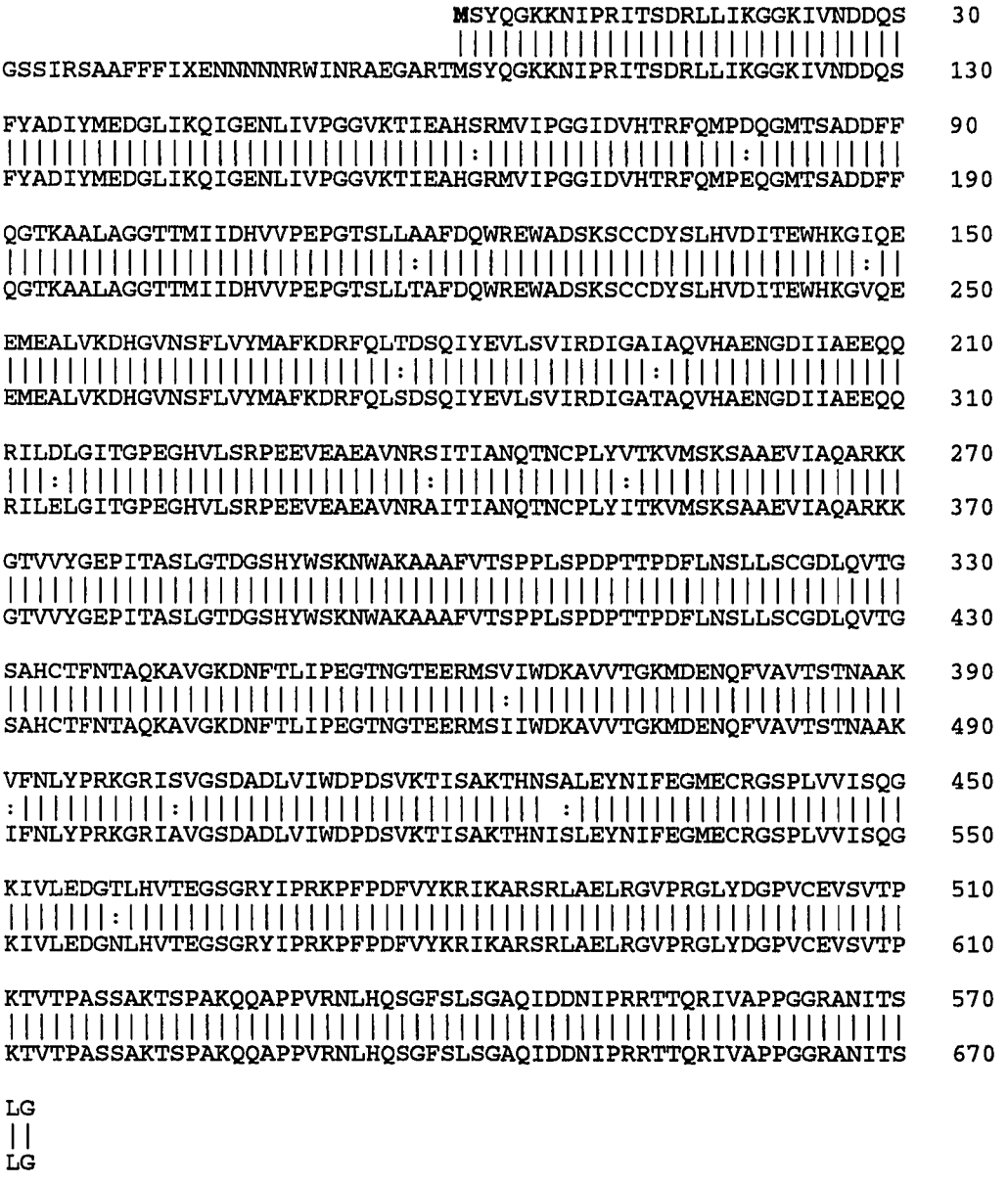


Figure 11. TOAD-64 mRNA is neural specific and developmentally regulated.

Total RNA (25μg) from E17, P0, P5, P14, P21, and adult rat cortex **(A)** and from P21 rat liver, kidney, and spleen **(B)** were probed for TOAD-64 mRNA. A single 4.5 kb transcript is detected in the cortex at E17, continues to be expressed during the first postnatal week (P5), and subsequently declines during the second postnatal week to a level that is nearly undetectable in the adult. **(B)** TOAD-64 mRNA is not detected in non-neural tissues. Even after over exposure, no TOAD-64 mRNA was detected in liver, kidney, or spleen. Both filters were simultaneously hybridized with a probe for the non-developmentally regulated gene, cyclophilin (cyc), to verify equal RNA loading levels.

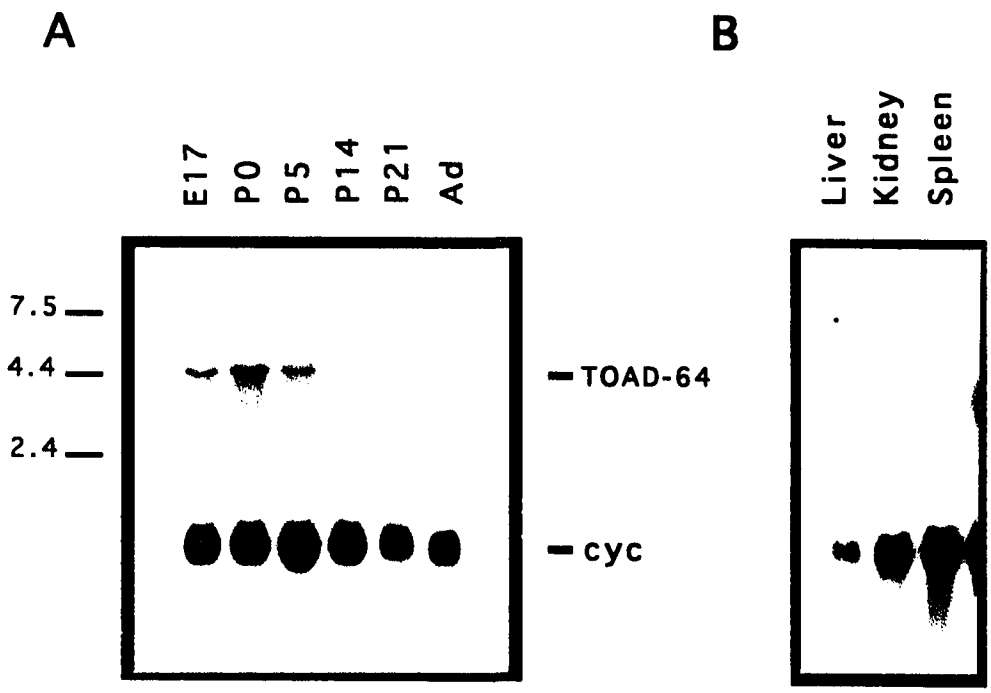


Figure 12. In situ hybridization confirms the neural specificity of TOAD-64 and demonstrates the broad distribution of TOAD-64 in the developing brain.

Parasagittal sections from E14 (A), P0 (B), P5 (C), P14 (D), and P21 (E) rats were hybridized to a ^{35}S-labeled cRNA probe for rat TOAD-64 and the middle subunit of neurofilament (F) as a control. **(A)** At E14, TOAD-64 mRNA is detected throughout the developing brain and spinal cord (sc), and in the dorsal root ganglia (arrow) of the peripheral nervous system. **(B)** At P0 TOAD-64 hybridization is detected throughout the brain, with a particularly high level of signal in the neocortex (c). The hybridization signal in the neocortex is non-uniform; labeling is high in the developing cortical plate and far lower in the ventricular zone (arrow) (see also **figure 8**). **(C)** At P5, hybridization is still high in the neocortex as is labeling in the olfactory bulb (o) and pyriform cortex (p). **(D)** At P14, hybridization throughout the brain is reduced. **(E)** By P21, the expression of TOAD-64 mRNA is almost undetectable. **(F)** At P21, neurofilament mRNA is abundantly expressed in differentiated neurons throughout the CNS. Scale bar, 1.3 mm.

Figure 13. In the developing cortex, TOAD-64 is expressed in the cortical plate, which contains postmitotic neurons, but not in the mitotic cells of the ventricular zone.
Parasagittal sections of E17 rat embryos were hybridized with TOAD-64 antisense cRNA probe **(A,B)**, and stained with cresyl violet **(C)**, or, sections were stained with TOAD-64 antiserum **(D)**. **(A)** A view of the whole embryo demonstrates the restriction of TOAD-64 mRNA expression to the CNS. Hybridization is abundant in the spinal cord (sc), midbrain (m), and neocortex (c). Signal is also detected in the olfactory epithelium (arrow). **(B)** An emulsion dipped section viewed under dark-field illumination shows that in the E17 cortex, hybridization signal is most abundant in the cortical plate (cp). The ventricular zone (v), though very cell dense (compare with panel C), has a far lower density of silver grains. **(C)** Cresyl violet staining shows the relative cell densities of each layer of the E17 neocortex. **(D)** Antibody staining of the cortex shows that the TOAD-64 protein has a very similar distribution to that of the mRNA. Antibody staining is largely restricted to the intermediate zone and the developing cortical plate. The ventricular zone is largely unstained, with the exception of a few process-bearing cells (arrows) that appear to be migrating into the intermediate zone (i). Scale bar (A) 2.25 mm, (B) 75 μm.

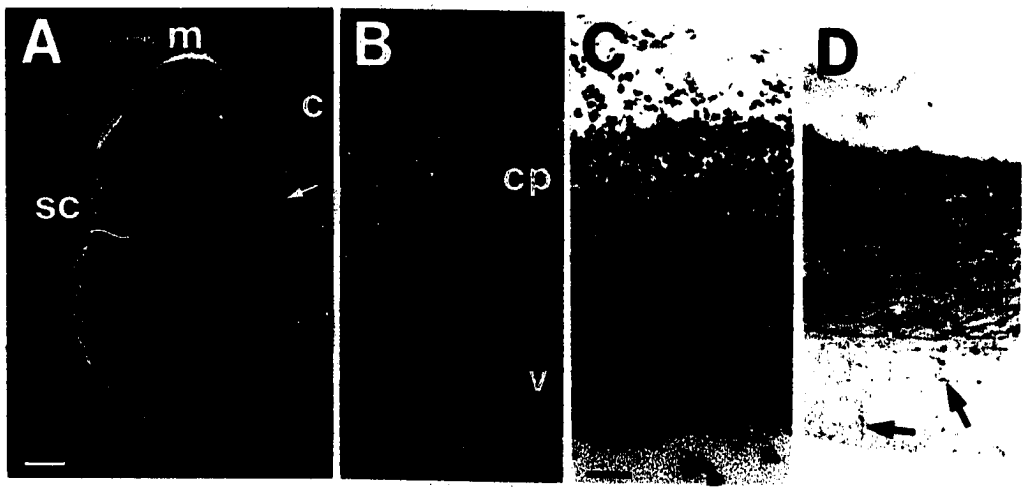


Figure 14. TOAD-64 is regulated in parallel with neuronal differentiation of P19 and PC12 cells.

(A) Total RNA (40 μg/lane) from control, uninduced (C; lanes 6,7) or retinoic acid induced P19 embryonal carcinoma cells (RA; lanes 1-5) was probed for TOAD-64 mRNA expression. TOAD-64 mRNA is detected 4-6 days post-induction (PI) with retinoic acid (lanes 2 and 3), but not in controls (lanes 6 and 7). **(B)** Total RNA (35 μg/lane) from PC12 pheochromocytoma cells probed for TOAD-64 mRNA shows that prior to (lane 1) or in the days following induction with NGF (PI; lanes 2-5) TOAD-64 mRNA is constitutively expressed. **(C)** In contrast to the mRNA, western blots of protein from uninduced (lane 1) or NGF-induced (lanes 2-5) PC12 cells show that prior to induction little TOAD-64 protein is detected. Three days following NGF-induction (lane 3) TOAD-64 protein is detected on western blots and increases gradually thereafter (lanes 4 and 5).

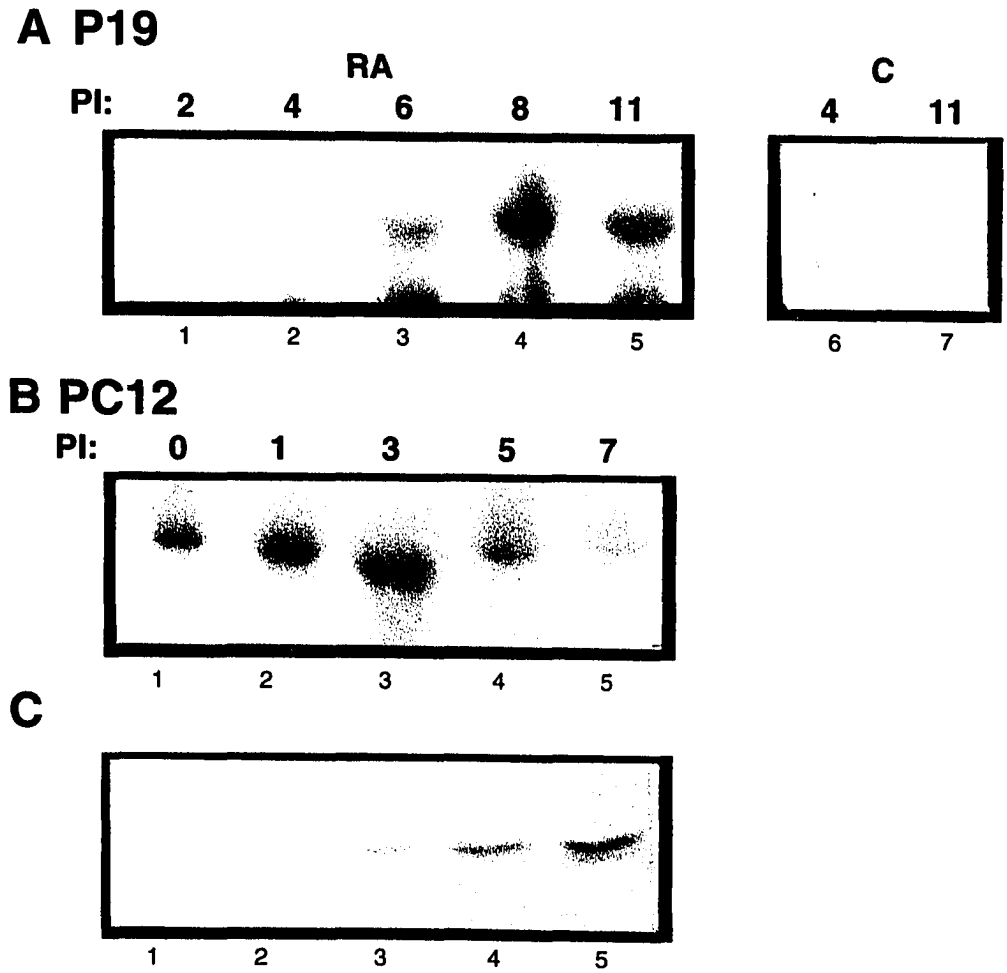


Figure 15. In the developing spinal cord, TOAD-64 is expressed in elongating axons of the dorsal root. Horizontal sections at low and high magnification from E14 **(A,B)**, and E20 **(C,D)** rat spinal cord were incubated with antisera to TOAD-64 and visualized with HRP-conjugated secondary antibodies. **(A and B)** At E14, TOAD-64 is expressed throughout the length of the axons comprising the dorsal root (arrowheads) as it enters the dorsal funiculus of the spinal cord. **(B)** At higher magnification, individual axons can be visualized as they grow ventrally into the spinal cord grey matter (small arrowheads). **(C and D)** At E20, immunoreactivity is present along the elongating dorsal root (arrowheads), and is also present in both the cell bodies and the axons within the dorsal root ganglia (DG, arrows). (MZ= marginal zone; DG= dorsal root ganglia).

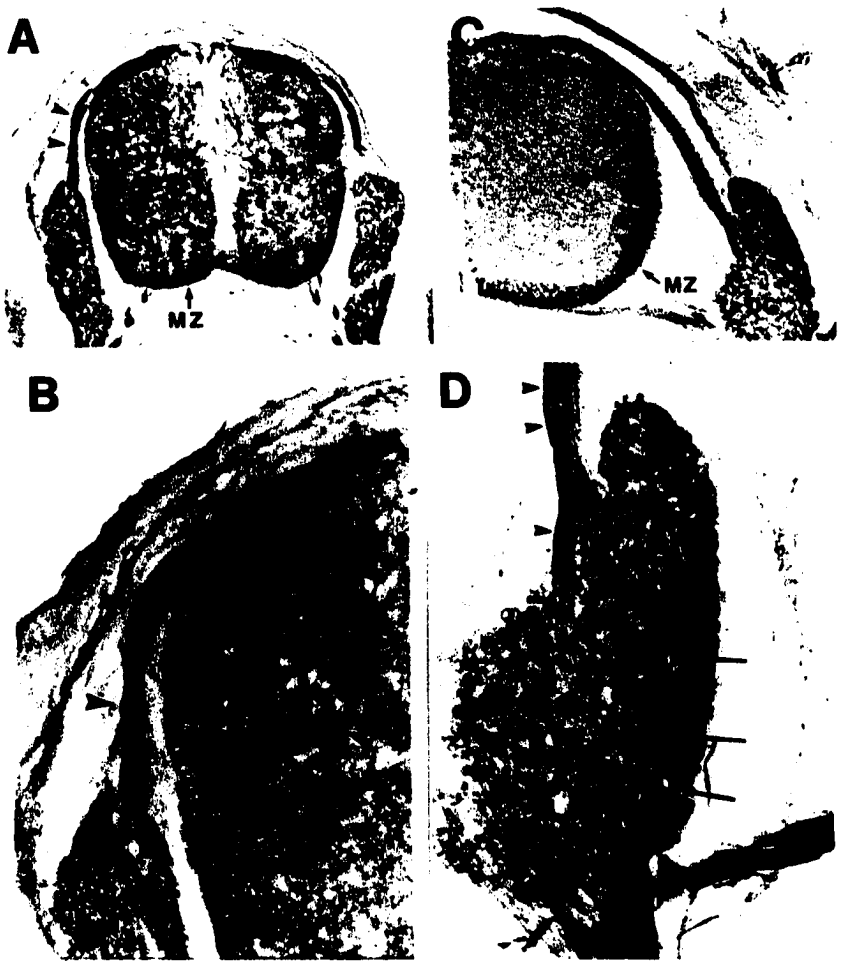


Figure 16. TOAD-64 is present in the lamellipodia of growth cones in primary neuronal cultures.

(A) In primary cultures of rat dorsal root ganglion neurons, TOAD-64 is present in the cell bodies and processes of neurons. **(B and C)** TOAD-64 immunoreactivity is detected throughout the growth cone. The darkest staining for TOAD-64 is seen in the distal part of the neurite, just as the neurite gives rise to the growth cone (* B,C). TOAD-64 immunoreactivity extends into the growth cone itself, with staining present at the leading edge of the lamellipodia (curved arrow in C). Even many of the fine filopodia that arise from the growing tip of the neurite contain TOAD-64 protein (arrowheads). **(D and E)** In contrast to the distribution of TOAD-64 in the growth cone, immunoreactivity for class III ß-tubulin is restricted to the axon and only the most proximal portion of growth cones (* D,E). Class III ß-tubulin is not observed either in the distal part of the growth cone (curved arrow in E) or in the filopodia (arrowheads in D). Scale bar = 28µm in A, 7µm for B-E..

Figure 17. Double labeling shows that TOAD-64 is present in lamellipodia and filopodia of growth cones. Primary cultures of dorsal root ganglion neurons were stained for TOAD-64, visualized with FITC **(A and C)**, and for class III ß-tubulin, visualized with Texas red **(B and D)**. The same field is shown under FITC optics for TOAD-64 in **(A)** and under Texas red optics for ß-tubulin in **(B)**. Similarly, the same field is shown in C and D. **(A and C)** TOAD-64 immunoreactivity is detected throughout the neurite and into the growth cone. Intense staining for TOAD-64 is seen in the distal part of the neurite, just as the neurite gives rise to the growth cone (open arrows). TOAD-64 immunoreactivity extends into the growth cone itself, with staining often present at the leading edge of the lamellipodia (curved arrows). Many of the fine filopodia that arise from the growing tip of the neurite contain TOAD-64 protein (arrowheads). **(B and D)** In contrast to the distribution of TOAD-64 in the growth cone, immunoreactivity for ß-tubulin is restricted to the neurite and only the most proximal portion of growth cones (open arrows). No filopodia were seen to stain for ß-tubulin. Scale bar = 12µm.

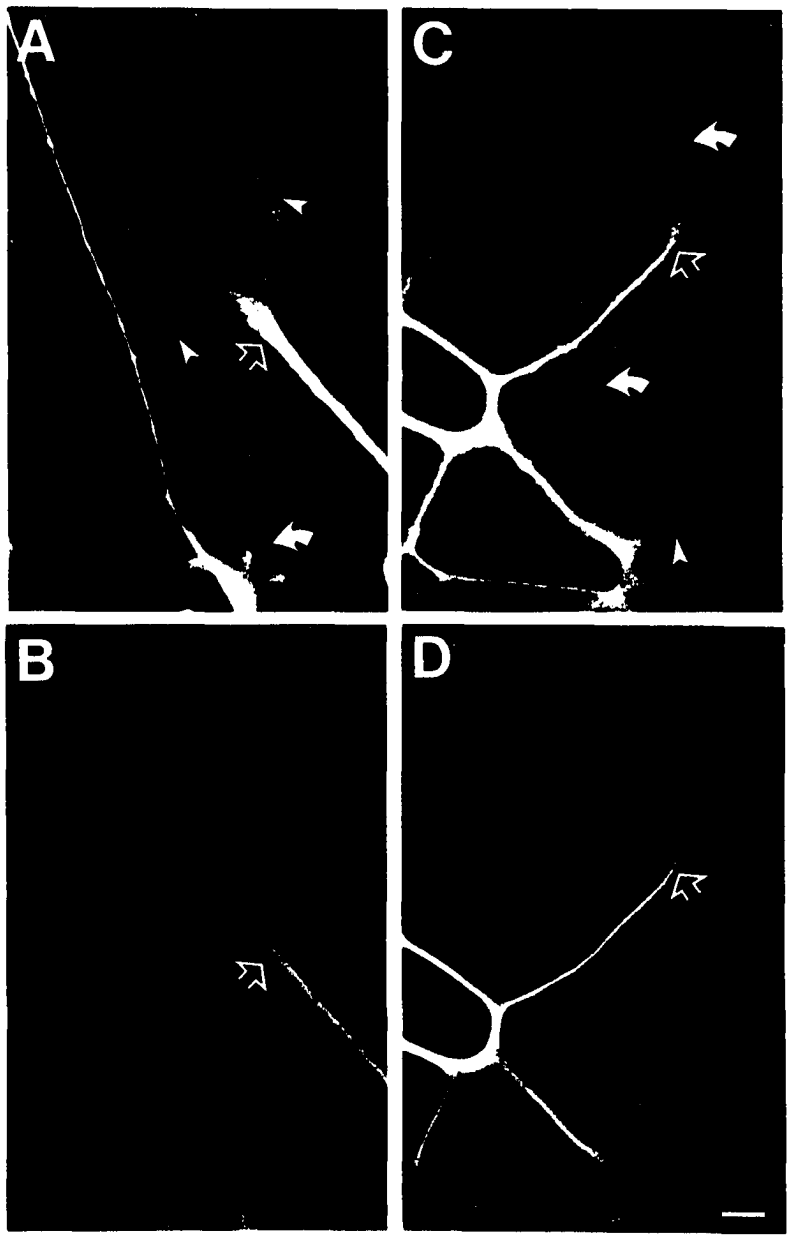


Figure 18. In adult animals, TOAD-64 is re-expressed in spinal cord motor neurons following a peripheral axotomy. Five days following sciatic nerve crush, TOAD-64 is detected ipsilateral to the lesion **(B)**, but not contralateral to the lesion **(A)**. **(A)** If the sciatic nerve is intact, antisera to TOAD-64 does not stain motor neurons in the ventral horn of the spinal cord. **(B)** The sciatic motor neurons on the side of the nerve crush show staining with TOAD antisera, indicating a re-expression of the protein after injury, coincident with axon regeneration. wm = white matter; gm = gray matter; Scale bar, 150μm.

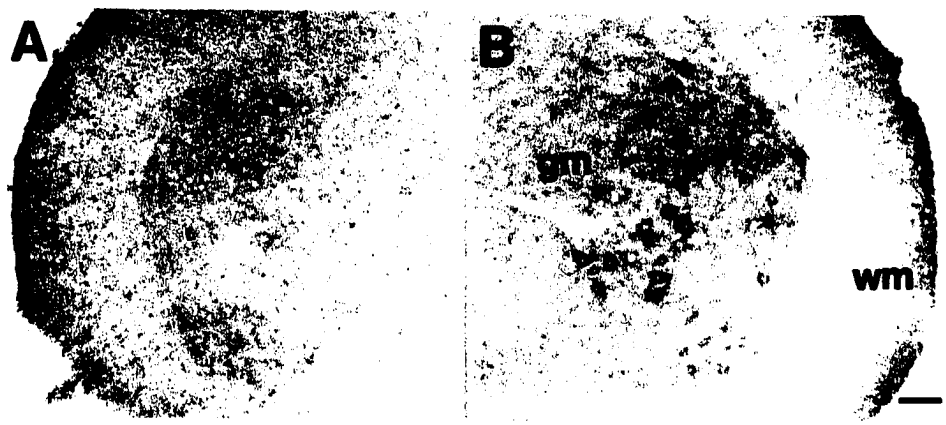
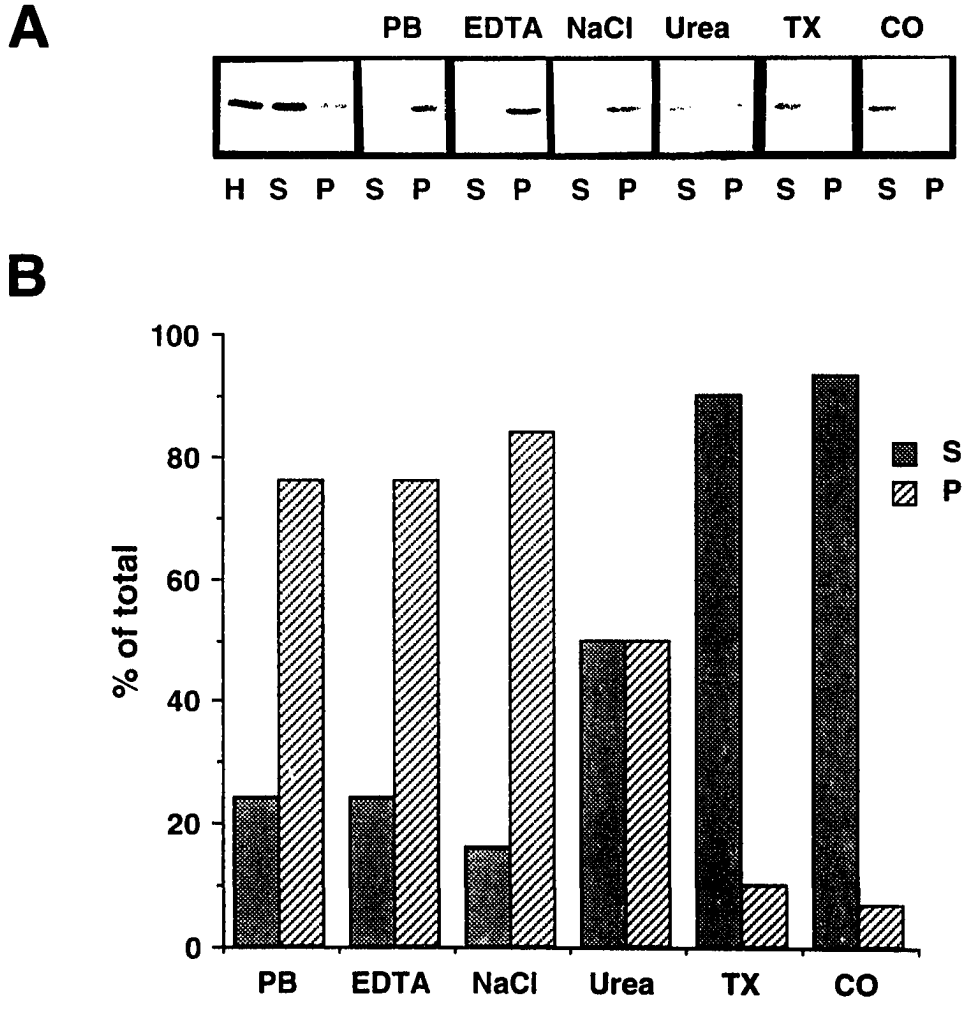


Figure 19. Extraction conditions for TOAD-64 demonstrate that it is membrane associated.

(A) Homogenates from P12 rat cerebral cortex (H) were separated into soluble (S) and pellet (P) fractions by centrifugation at 100,000 x *g* for 1 hour. The pellet fraction was rehomogenized in PBS and divided into six separate aliquots that were recentrifuged. After resuspending the aliquots in either 0.1M Na_2CO_3 pH 11 (CO), PBS (PB), PBS + 2M NaCl (NaCl), PBS + 10mM EDTA (EDTA), PBS + 2M urea, or PBS + 1% Triton X-100 (TX), soluble and pellet fractions were once again generated. Immunoblots of the protein extracts were then probed with the antibody to TOAD-64. **(B)** The relative amount of TOAD-64 in the soluble or pellet fractions was quantified using densitometry.



CHAPTER 4

GENERAL DISCUSSION

The experiments described here extend previous work from this laboratory using two-dimensional gel electrophoresis to identify proteins whose rate of synthesis was up- or down-regulated during the period of neurogenesis in the embryonic rat cortex. From this initial data, TOAD-64, a neural specific, developmentally regulated gene expressed in early post-mitotic neurons has been cloned, and its mRNA and protein expression have been characterized in the developing rat nervous system. The sequence of the TOAD-64 gene has not been previously described. At the time of our initial studies, the sequence most similar to TOAD-64 was the *unc-33* gene from the nematode *C. elegans*, with 34% identity and as much as 79% similarity over segments of the protein. Mutations in the *unc-33* gene lead to uncoordinated movement in the nematode, and misguided axonal outgrowth; suggesting a possible role for TOAD-64 in the mechanics of axon outgrowth and/or growth cone navigation in the rat. The developmental time course of expression and localization of TOAD-64 to filopodia and lamellipodia of growth cones, the re-expression of the protein in regenerating nerves, and its persistent expression in areas of the adult brain that have the capacity

for ongoing neurogenesis and axon growth supported our hypothesis.

Subsequent cloning and characterization of the chicken homolog of TOAD-64 (97% identity, named CRMP-62) by Goshima and colleagues here at Yale (Goshima et al., 1995) have put forth additional evidence that the TOAD-64 gene is involved in specific guidance mechanisms of developing axons. Their data indicate that the CRMP-62 (TOAD-64) protein is an intracellular component of the signal transduction pathway by which neuronal growth cones receive inhibitory cues from collapsin. Collapsin is a secreted protein in the embryonic chick brain that can steer and induce the collapse of sensory ganglion growth cones but not retinal ganglion growth cones (Luo et al., 1993; Fan and Raper, 1995). The collapsin binding receptor that would likely mediate the signal from this external guidance cue (collapsin) to the intracellular TOAD-64 protein, has not yet been identified.

The idea that axons may be guided by extracellular cues to their distant targets to establish predictable patterns of connectivity is not new. Ramón y Cajal first proposed chemotropism- guidance directed by a gradient of a soluble factor diffusing from a target- as a mechanism of axon guidance in the 1890's (Ramón y Cajal, 1892); however, it has taken nearly 100 years to identify the first target-derived chemotropic molecules for developing axons in vertebrates (Kennedy et al., 1994; Serafini et al., 1994). Only recently too are we beginning to appreciate the role of neurite

outgrowth inhibitors and chemorepulsive factors in axon guidance.

In the last twenty years, advances in molecular biological and *in vitro* culture techniques have resulted in rapid progress in identifying and understanding the nature and mechanisms of axon guidance (Goodman and Shatz, 1993; Luo and Raper, 1994; Dodd and Schuchardt, 1995; Kennedy and Tessier-Lavigne, 1995; Keynes and Cook, 1995a,b). Here I will review some of the better characterized candidate molecules involved in these processes, with an emphasis on repulsive and inhibitory signals; and finally discuss how the regulation of a molecule such as TOAD-64 may play an important role in determining the trajectories of growth cones during development.

Guidance cues in the developing nervous system are thought to be provided by immobilized (cell surface or extracellular matrix bound) and diffusible molecules, and can be loosely categorized as factors that promote cell and growth cone adhesion; molecules that act as diffusible chemoattractants; molecules that render substrates non-permissive; and molecules that inhibit growth cone motility.

The Role of the Growth Cone in Axon Guidance

The suggestion that extracellular guidance cues define the pathways that lead elongating axons to their appropriate targets has been widely accepted since the identification and localization of many of the extracellular matrix molecules.

However, it is the ability of the growth cone at the distal tip of the growing axon to interpret and transmit these signals into cytoskeletal rearrangements that will ultimately, physically direct the outgrowth of an axon and determine the initial morphology of the developing neuron.

The cytomechanics of growth cone motility are thought to be due to the distribution of F-actin (filamentous actin) in the peripheral lamellipodial region of the growth cone. The lamellipodium is an extended thin veil at the leading edge of the growth cone that excludes most organelles. The actin filaments are organized into filopodia (dense unidirectional actin filaments) extending from the leading edge embedded within lamellipodia, which themselves have a more randomly oriented network of cross-linked actin. Microtubules, while restricted to the proximal growth cone, are believed to interact with actin via unidentified binding protein(s) to mediate force transduction between the two cytoskeletal domains (Mitchison and Kirschner, 1988; Smith, 1988; Lin et al., 1994).

Distinct processes contribute to growth cone motility. These include: the assembly of F-actin monomers at the leading edge of the growth cone and proximal disassembly of F-actin due to depolymerization, resulting in a constant retrograde flow of F-actin without net movement of the growth cone. In order to achieve net advance or retraction of the growth cone, polymerization has to exceed depolymerization, protrusion has to exceed retraction or vice versa (Smith,

1988; Lin et al., 1994; Luo and Raper, 1994; Tanaka and Sabry, 1995).

The stabilization of lamellar protrusion by cytoskeletal-substrate interactions has been suggested as a mechanism for net forward extension. This is most likely mediated by specific cell surface receptors that link extracellular adhesion molecules to the intracellular actin filaments, either directly or by "clutch proteins" that engage or disengage the F-actin network to regulate net forward growth (Lin et al., 1994; Luo and Raper, 1994). Mechanisms that regulate growth cone retraction may act to interfere with these linkage mechanisms, or to destabilize the infrastructure of the growth cone, as has been demonstrated in the net loss of F-actin in the growth cone by contact with the repulsive extracellular cue, collapsin (Fan et al., 1993).

Adhesion Molecules

Cell adhesion molecules (CAMs) have been implicated as axon guidance molecules by early studies *in vitro* showing that differential adhesion can play a major role in growth cone guidance (Letourneau, 1975). Many of these CAMs have now been cloned and characterized, and a number are expressed in the developing nervous systems of both vertebrates and invertebrates. The majority of these neural CAMs are members of two different gene families, the immunoglobulin superfamily and the cadherin family (reviewed in Jessell,

1988; Rutishauser and Jessell, 1988; Edelman and Crossin, 1991). An example of a well studied CAM is TAG-1, a member of the immunoglobulin/fibronectin type III (Ig/FNIII) superfamily that has been shown to promote neurite outgrowth and adhesion *in vitro* (Furley et al., 1990). TAG-1 is transiently expressed on the axons of commissural neurons in the rat spinal cord as they extend through the neuroepithelium and approach the floor plate early in development (Furley et al., 1990; Dodd et al., 1988). Once these axons cross the floor plate and turn rostrally to project toward the brain, TAG-1 disappears from their surface and is replaced by a distinct glycoprotein, L1 (a molecule shown to promote axon fasciculation by homophilic binding). The transition from TAG-1 to L1 expression coincides with the change in trajectory and the onset of fasciculation of these axons. Thus, the spatial regulation of these CAMs may aid in the directed guidance of these commissural axons.

In the chick embryo, axonin-1 (homologous to TAG-1) is expressed on commissural interneurons before and after they cross the floor plate. Injection of anti-axonin-1 antibodies *in ovo* causes defasiculation of axons and failure to cross the floor plate (Stoeckli and Landmesser, 1995). This phenotype can be reproduced by injection of anti-Nr-CAM antibodies (an Ig/FNIII CAM expressed on floor plate cells). This suggests that heterophillic adhesion/interaction between growth cones (expressing axonin-1) and floor plate cells

(expressing Nr-CAM) modulate axonal guidance (Stoeckli and Landmesser, 1995).

While the expression of these CAMs on restricted subsets of axons in a developmentally regulated manner suggest a likely role for axon extension and specific cell-cell interactions, it is clear that cell adhesion is just one of several mechanisms that allow growth cones to find and recognize correct paths and targets.

Diffusible Chemoattractants

Chemotropism and the regulation of axon guidance by positive cues was discussed as early as the 1890's by Ramón y Cajal, and experimentally examined by Sperry in the 1960's when his "chemoaffinity hypothesis" was examined (Sperry, 1963).

Direct evidence for the existence of chemotropic factors secreted by target tissues has been examined *in vitro* by the coculture (in collagen gel matrices) of specific neurons and source tissues in a number of systems, the best characterized being the guidance of commissural axons to the floor plate of the spinal cord (Tessier-Lavigne et al., 1988). The recent identification of the netrins as floor plate-derived molecules with chemoattractant and outgrowth-promoting activity by Serafini et al. (1994), has already advanced our understanding of this developmental model.

Netrin-1 (the better characterized of the two molecules) is a secreted protein whose mRNA is expressed in the floor

plate after closure of the neural tube, and maintained at high levels throughout the period of commissural axon growth (Kennedy et al., 1994). In an *in vitro* assay in which recombinant netrin-1 is secreted by COS cell aggregates and presented to dorsal spinal cord explants, commissural axons turn and project toward the COS cells, in the same way as they would towards floor plate explants (Kennedy et al., 1994; Serafini et al., 1994).

Netrin-1 is homologous to *unc-6*, a *C. elegans* gene which is required for circumferential guidance of growth cones and migrating mesodermal cells in the nematode (Hedgecock et al., 1990). Experiments in the nematode have led to a model in which UNC-6, present in a ventral-to-dorsal gradient, acts as an attractant for cells and axons migrating ventrally, and as a repellent for cells and axons that migrate dorsally (Hedgecock et al., 1990; Culotti, 1994). This model was tested by Colamarino and Tessier-Lavigne (1995a,b) in the rat using recombinant netrin-1 and floor plate explants. In both cases, they found that a specific group of dorsally migrating axons (cranial nerve IV) was repelled by floor plate cells and COS cells secreting netrin-1 suggesting that this molecule has repellent as well as attractant activities.

The demonstration of a guidance molecule with dual effects is not new (e.g. extracellular matrix molecule tenascin, Spring et al, 1989; myelin-associated glycoprotein, Mukhopadhyay et al., 1994); however, it alters our thinking

about distinct categories of guidance cues, and suggests that the burden of distinguishing a positive versus a negative signal lies with the growth cones (and their expression of receptors and intracellular signal transduction molecules such as TOAD-64).

Chemorepulsion as a mechanism for axon guidance

Positive influences have long been considered the major mechanisms by which the rate and direction of growth cone extension is controlled. Recently however, there has been a growing appreciation that growth cone navigation also depends on negative influences. These repulsive or inhibitory cues appear to modulate the speed and direction of neurite outgrowth by two principal mechanisms: interference with contact dependent axon growth, and interference with normal growth cone motility.

Molecules that inhibit axon/neurite extension

A limited number of substrates allow growth cones to advance. Proper guidance appears to operate by contact with appropriate cues in a cell-cell or cell-substrate dependent manner. Substances have been identified which interfere with this contact and thus inhibit neurite/axon growth. A number of these growth-inhibitory molecules have been found to be myelin-associated. Two of these, NI-35 and NI-250, identified by Caroni and Schwab (1988a,b) are associated with oligodendrocyte membranes and CNS myelin, and possess neurite

growth-inhibitory properties *in vitro*. Interestingly, the IN-1 antibody, which recognizes both of these molecules has been shown to neutralize their inhibitory effect *in vitro* and *in vivo*, and render a once non-permissive substrate (CNS white matter) into a substrate that allows neurite growth and local sprouting (Caroni and Schwab, 1988a; Schnell and Schwab, 1990). Recently, it has been suggested that NI-35 inhibition of axon outgrowth and neuronal regeneration is mediated by growth cone collapse (Bandtlow et al., 1993; Igarashi et al., 1993). The collapse is mediated by a G protein-coupled receptor in a manner similar to the proposed CRMP-62 (TOAD-64) signaling cascade in response to collapsin (Igarashi et al., 1993; Goshima et al., 1995).

Another myelin-derived inhibitor of neurite growth is the myelin-associated glycoprotein (MAG),once thought only to play a role in the signaling for the initiation of myelination. It has been shown biochemically to be a dominant growth inhibitor in CNS myelin preparations (McKerracher, et al., 1994), and may have bifunctional outgrowth activity (both an inhibitor and a promoter) in a developmentally and spatially regulated fashion (Mukhopadhyay et al., 1994).

In addition to inhibitors that are immobilized on surfaces (discussed above), there is evidence for diffusible factors that make substrates non-permissive and inhibit neurite outgrowth. Much in the same manner as the netrins have been studied, Pini and colleagues have used explant

cocultures to identify diffusible chemorepulsive activity in the embryonic rat in both the olfactory system and the ventral spinal cord (Pini, 1993; Fitzgerald, et al., 1994). Axons from explants turned and grew away from explants of tissues that they similarly avoid *in vivo* without obvious collapse of their growth cones.

Growth inhibitors that act as "antagonists" by specifically binding to growth-promoting substrate molecules or their receptor counterparts on cell surfaces are seen as potentially important directional cues because they inhibit axon extension without interfering with growth cone motility (discussed below). The extracellular matrix molecule janusin (of the J1 family) has been proposed to act in this manner by binding, specifically, to the cell adhesion molecule F3/F11 and blocking its normally growth promoting activity (Pesheva et al., 1993).

A novel case of receptor-ligand interactions that may result in axon guidance by repulsion, is the recent identification of a receptor tyrosine kinase (RTK) ligand named RAGS with growth cone collapsing activity expressed in the tectum of the developing chick (Drescher et al., 1995;). This discovery is made even more interesting by the finding that another RTK ligand (ELF-1) and its receptor (Mek4) are expressed in complementary graded fashions in the tectum and retinal ganglion cells respectively (Cheng et al., 1995); implicating a role for RTKs as positional labels, possibly

directing the ingrowing axons by repulsion and growth cone collapse.

The creation of "exclusion zones", by the various inhibitory mechanisms described above, to repel incoming axons appears to be an important way in which the developing nervous system guides growth cones to their proper targets.

Inhibition of growth cone motility

The extending axon relies on the growth cone for receiving, integrating, and ultimately responding to the signals in its immediate environment. On permissive substrates, growth cones are believed to advance by protrusion of the leading edge and stabilization (by adhesion) of this protrusion to permissive substrate molecules (Mitchison and Kirschner, 1988; Smith, 1988). When a growth cone encounters an inhibitory environment, adhesion to the substrate can be disrupted (e.g. by tenascin or janusin) or its motility can be inhibited resulting in paralysis and collapse. Evidence suggests that a large number of inhibitory cues exert their effect by growth cone collapse (e.g. collapsin, NI-35, RAGS).

The data presented in the previous chapters indicate that TOAD-64 is present in the filopodia and lamellipodia of growth cones at a time in the developing cortex when axons are extending toward their targets guided by extracellular cues. The ability of the chick homolog (CRMP-62) of TOAD-64 to mediate collapsin induced growth cone collapse *in vitro*

suggests that TOAD-64 is involved in either the signaling or motile machinery that enables growth cones to actively respond to their immediate environment.

Collapsin induced growth cone collapse is thought to be due to a net loss of F-actin at the leading edge of the growth cone without affecting microtubular organization, and appears to be a receptor coupled G-protein mediated event (Fan et al., 1993; Goshima et al., 1995). The increase in intracellular Ca^{2+} due to G protein activation may alter the dynamics of actin polymerization (Cohan et al., 1987). Other examples of molecules thought to inhibit growth cone motility by transient increases in intracellular Ca^{2+} are the neurotransmitters serotonin, glutamate and dopamine (Haydon et al., 1984; Mattson et al., 1988; Lankford et al., 1988).

Chick collapsin, a member of the semaphorin family (Kolodkin et al., 1993), was one of the first cloned and characterized proteins found to induce paralysis of growth cones (Luo et al., 1993). Another family member, identified in a monoclonal antibody screen looking for proteins expressed on subsets of axon pathways, is semaphorin I (Sema I, formerly fasciclin IV), a transmembrane molecule in insects that has been implicated in guiding pioneer axons in the grasshopper limb bud (Kolodkin et al., 1992). Subsequent cloning based on a conserved sequence region (the N-terminal region, 500aa "sema" domain) has yielded a diverse family of molecules in grasshopper, drosophila, chick, mouse, and human, with the apparent conserved function of navigating

axon extension by localized repulsion (Kolodkin et al., 1993; Fan and Raper, 1995; Luo et al., 1995; Püschel et al., 1995).

The ability of the collapsin/semaphorins to guide specifics axons in a spatially and temporally regulated manner has been examined by a number of recent experiments. The five newly identified murine semaphorins and five chick collapsins are expressed in the embryonic nervous system with transcript levels regulated at different stages of development; and anatomically they are expressed in spatially distinct but overlapping domains (Luo et al., 1995; Püschel et al., 1995). At a functional level, the murine semaphorin III/collapsin (Sema III) molecule expressed in the ventral spinal cord, has been shown to specifically repel NGF-responsive axons from explant DRGs, with little effect on the ingrowing NT-3 responsive axons (Messersmith et al., 1995). This group also showed Sema III mRNA exists as a gradient in the ventral spinal cord; suggesting regulation of this signal on two levels: that of the ligand expression (Sema III), and that of the receptor for this ligand (presumably a Sema III receptor expressed only by the NGF-responsive neurons). Our evidence that regulation may also occur at the level of the intracellular signaling molecules (e.g. TOAD-64) only increases the complexity of this system.

Our data indicate that the regulation of TOAD-64 expression is limited to developmental and regenerative periods of neurogenesis and axonogenesis. It is an abundant protein in the embryonic rat CNS and is expressed by all

postmitotic neurons during the period of corticogenesis, including neurons that have been shown not to respond to collapsin (e.g. retinal ganglion cells, data not shown). This suggests that the function of TOAD-64 may not be restricted to a single extracellular signal, but is a more central element in the complex machinery underlying axonal navigation. Recently isolated human homologs sharing substantial sequence identity with TOAD-64 reveal two distinct human cDNAs (see Figure 9 for partial human sequence)(Goshima et al., 1995), suggesting there may be additional members of the TOAD/CRMP family in the rat. Further analysis of proteins that interact with TOAD-64, and the identification of additional family members will enable us to elucidate the complex mechanisms which contribute to axonal outgrowth and pathfinding.

FUTURE DIRECTIONS

Since the initial cloning of TOAD-64, rapid progress has been made in identifying other *unc-33* gene family members. The cloning and initial characterization of three additional rat family members (Strittmatter, unpublished data), and the identification of two murine homologs (Byk et al., 1996; S. Tontsch, unpublished) suggests that the role and regulation of TOAD-64 is not as simple as initially imagined. It will be important to examine, with specific probes, the distribution and temporal regulation of these new family

members to determine if there is any overlapping expression, and if any of these new proteins are responsive to collapsin family members. With the recent data suggesting a role for this protein in the collapsin/semaphorin response pathway, (Goshima et al., 1995), and the rapid addition of members to the collapsin/semaphorin family (Kolodkin et al., 1993; Luo et al., 1995; Püschel et al., 1995), it will be important to identify Unc-33/TOAD-64 homologs in the grasshopper and Drosophila, where a number of semaphorin mutants have been previously characterized. This will allow the generation of double knockouts, and the ability to more easily determine which Unc-33-like proteins are sensitive to which semaphorin molecules.

One caveat in all of these studies, is the fact that we have not yet identified the transmembrane receptor(s) that couples the extracellular ligand (collapsin) to the intracellular signalling cascade (TOAD-64). In order to fully understand the function of these proteins, it will be critical to identify not only the receptor, but other intracellular proteins that interact with TOAD-64 in differentiating neurons. One method that we have begun to use to determine the protein-protein interactions of TOAD-64 is the yeast two hybrid system (Fearon et al., 1992).

Another approach to understanding the function of TOAD-64, is to look for alterations in neuronal development in the absence of TOAD-64 expression. This "knockout" can be approached in a number of ways. An initial approach is to

use antisense oligonucleotides to decrease the expression of TOAD-64 protein in primary neuronal cultures. Our initial observation in these experiments has been the potentiation of axonogenesis, and the presence of "normal" appearing neurites even when cultures are exposed to recombinant collapsin (Fryer, Minturn, and Zhang, unpublished observations). This suggests a release from inhibition of neurite extension in the absence of TOAD-64. One difficulty with these experiments is the abundance of the TOAD-64 protein in these cells, the lack of certainty that we achieve a complete knockout, and the toxic effects of the oligonucleotides. A way to bypass these problems is to use a technique designed by Dan Jay at Harvard University that achieves complete and specific protein inactivation in a discrete intracellular domain (15Å) while the remainder of the cell is healthy and functioning normally. The technique relies on antibodies to the protein of interest and laser inactivation, and is named CALI (Chromophore-assisted laser inactivation). It has been used successfully by Jay and colleagues to identify the role of specific proteins in directed growth cone motility (Beermann and Jay, 1994; Chang et al., 1995; Jay, unpublished observations). A collaboration with the Jay laboratory is underway, and initial experiments are in progress. And finally, the generation of null mutations in transgenic mice should provide significant information about the function of the specific TOAD-64 like proteins.

Through the apparent conservation of function across such diverse species as *C. elegans*, chick, mouse, and the rat, and the increasing numbers of molecules identified within this gene family, the tools to continue future investigations of the molecular mechanisms that contribute to precise patterns of neuronal connectivity appear limitless.

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