GROWTH-ASSOCIATED MESSENGER RIBONUCLEIC ACID EXPRESSION IN A MODEL OF SUCCESSFUL CENTRAL NERVOUS SYSTEM REGENERATION

by

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B.Sc, The University of British Columbia, 1987

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Zoology)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

APRIL 1992

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ABSTRACT

Recent experiments examining the development and plasticity of the chick embryonic spinal cord have described its inherent ability to recover from injury suffered prior to embryonic day (E)13. Severing the spinal cord on or before E12 resulted in complete anatomical and functional recovery, which defined the permissive period for repair. Transections performed on E13 or later resulted in paraplegia characteristic of the restrictive period for repair. Previous studies have described the expression of growth-associated proteins (GAPs) such as GAP 43 and α-tubulin which were expressed at high levels during axon extension, then down-regulated at the time of target contact. These proteins were also expressed during abortive attempts at regeneration, and characterized a genetic growth program which was recruited after injury.

This study examined the neuronal response to injury as reflected by changes in growth-associated gene expression in the hindbrain measured using Northern blotting. Levels of mRNA for GAP 43 and total α-tubulin during normal development were found to peak at E10-12, the period of maximal outgrowth of brainstem-spinal projections. Complete spinal cord transections performed on E11 (successful repair) or E14 (unsuccessful repair) did not detectably alter total α-tubulin mRNA levels. GAP 43 mRNA levels were not detectably altered after E11 transection. In contrast, transection on E14 (unsuccessful repair) resulted in a maintained increase in GAP 43 mRNA levels at least until 7 days post-transection, the longest survival period studied.

Northern blotting was likely not sensitive enough to detect the full complement of changes which occurred after injury. However, transection during the restrictive period for repair resulted in a maintained increase in GAP 43 mRNA expression. These data suggested that some brainstem-spinal

projection neurons injured on E14 retained the inherent ability to re-express at least part of the axonal growth program, indicated by the appropriate re-expression of GAP 43 mRNA. This suggests that the presence of inhibitory influences (or the absence of facilitatory influences) after E13 may have prevented the re-growth of axons and the re-formation of appropriate synapses.

TABLE OF CONTENTS

ABSTRACT ii

TABLE OF CONTENTS iv

LIST OF TABLES v

LIST OF FIGURES vi

ACKNOWLEDGEMENTS vii

INTRODUCTION 1

MATERIALS AND METHODS 14

RESULTS 24

DISCUSSION 40

CONCLUSIONS 49

REFERENCES 52

LIST OF TABLES

1. Sample Sizes used for the developmental study and the transection study 15

2. Analysis of Variance of log-transformed GAP 43 expression levels after transection.. 33

LIST OF FIGURES

1. Construction of the GAP 43 cDNA clone 20

2. Quantification of RNA amounts loaded in lanes 25

3. Typical Northern blots of GAP 43 and total α-tubulin mRNA levels during development 28

4. Densitometric analysis of GAP 43 mRNA levels during development 29

5. Typical developmental changes in GAP 43 mRNA levels from a selected blot 30

6. Densitometric analysis of total α-tubulin mRNA levels during development 31

7. Changes in GAP 43 expression after E11 transection 36

8. Changes in GAP 43 expression after E14 transection 37

9. Changes in GAP 43 expression after transection superimposed upon typical developmental expression levels 39

ACKNOWLEDGEMENTS

I dedicate this thesis to my wife Soiban who was largely responsible for maintaining normalcy in my non-scientific life (heaps of understanding, moral support, help in the library, warm dinners, and clean laundry), and without whom I would probably still be writing this. To a lesser degree, this also applies to the rest of my family, whom I think still remember who I am.

Special thanks to John Steeves for supporting my efforts in these endeavours. Thanks are also deserved by Sohail Hasan for assistance with the transections. I have a great number of colleagues throughout the department, besides everyone in the Steeves lab, whose helpful hints and equipment loans were instrumental in the success of these experiments, and I thank them all.

INTRODUCTION

It is well established in the literature that the central nervous system (CNS) of adult higher vertebrates is incapable of responding to injury with a regenerative response culminating in the restoration of function (Ramon y Cajal, 1928; Barnard and Carpenter, 1950; Bjorklund et al, 1971). This is in marked contrast to the peripheral nervous system which is proficient at re-innervating targets after injury in the same higher vertebrates (Ramon y Cajal, 1928; Hall, 1989). This dichotomous response to injury has been the focus of a great many studies attempting to elucidate the factors responsible for allowing neurons to, or inhibiting them from re-connecting with their targets.

This is not to say that the adult mammalian CNS is incapable of growth or plasticity in response to injury. Neurons within the CNS demonstrated non-regenerative terminal sprouting after injury (Crutcher and Marfurt, 1988) but this was usually insufficient to restore lost function. Given the correct environment, such as a peripheral nerve graft, CNS neurons of mammals have demonstrated a robust growth response (Tello, 1911; Richardson et al, 1980; Weinberg and Raine, 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982; Aguayo et al, 1983; Richardson et al, 1984; Vidal-Sanz et al, 1987; Carter et al, 1989). However, when this growth was re-directed into the adult CNS environment, it was aborted within close proximity to the graft/CNS border (David and Aguayo, 1981). Under the right conditions then, some mammalian CNS neurons demonstrated the ability to regenerate.

It was clear that all adult mammalian CNS neurons did not enjoy this capacity for regeneration. The studies quoted above did not claim to rescue 100% of axotomized neurons with the peripheral nerve graft (Richardson et al, 1982; Villegas-Perez et al, 1988). Studies using the sensitive technique of *in situ*

hybridization to examine neuronal growth-associated gene expression in the rubrospinal system (neurons previously thought incapable of regeneration) have demonstrated a sub-population of neurons capable of mounting a regenerative response qualitatively similar to that seen in the peripheral nervous system (Tetzlaff et al, 1991). Thus these neurons appeared to be capable of regeneration yet were unable to functionally re-connect with appropriate targets. The CNS is a tissue of unimaginably complex connections, trophism, extracellular and intracellular factors which orchestrate its functions. Clearly there were many elements to consider when trying to determine why CNS neurons failed to successfully regenerate.

There were yet other examples of regeneration observed in the **developing** CNS of higher vertebrates. The ultimate goal of regeneration, re-connecting a neuron with its targets, was similar to that of a developing nervous system which built these connections *de novo*. Initially, the developing CNS provided a permissive environment for neuronal growth, and therefore increased the likelihood that it would be capable of a successful regenerative response. This hypothesis has been tested in several experimental paradigms. Destruction of the ceruleo-cerebellar projection showed a critical developmental period for successful regeneration, beyond which repair ultimately failed (Schmidt et al, 1980), as did the retinotectal pathway projecting through the superior brachium in neonate hamsters (So et al, 1981). A high level of plasticity in the corticospinal tract of the neonatal rat has also been confined to a critical period in development (Bernstein and Stelzner, 1983). Studies on neonatal cortical lesions in the rat by Kolb and Whishaw (1989) also described a developmental window during which sparing of function is greatest. These observations set a precedent for developmental "windows" during which propensity for CNS repair was increased.

The experiments described here endeavoured to combine the sensitivity of molecular biological techniques with the study of regeneration in the embryonic CNS to examine gene expression in brainstem-spinal locomotor projection neurons after spinal cord injury in the developing chick embryo. The embryonic chicken was an ideal model for studies of CNS regeneration for several reasons. The embryo was readily accessible and much more amenable to experimental manipulation than a mammalian embryo, as maternal complications were avoided. There were striking similarities in the development and organization of avian and mammalian locomotor systems, allowing direct comparisons to be made. Most importantly, the embryonic spinal cord demonstrated a critical period for successful repair of damage, allowing the comparison of regenerative responses in the permissive and restrictive periods for repair as defined below.

A CRITICAL PERIOD FOR SPINAL CORD REGENERATION IN THE EMBRYONIC CHICK

Recent experiments have studied the development and plasticity of avian descending brainstem-spinal projection neurons involved in the control of locomotion. These brainstem-spinal projections originated from the red nucleus, vestibular nuclei, pontine and medullary reticular formation regions (Lawrence and Kuypers, 1968a,b; Armstrong 1986; Garcia-Rill, 1986; Jordan 1986; McClellan, 1986; Garcia-Rill and Skinner, 1987a,b; Steeves et al, 1987; Grillner and Dubuq, 1988; Webster and Steeves, 1988). A large proportion of brainstem locomotor output was directed to the spinal cord by reticulospinal pathways originating in the pons and medulla (Peterson, 1984; Steeves and Jordan, 1984; Armstrong, 1986; Garcia-Rill, 1986; Steeves et al, 1987). By studying the effect of complete high thoracic spinal cord transection on these pathways at different developmental

stages, a critical period for spinal cord repair has been defined in the chick embryo (Hasan et al, 1991; 1992)

This critical period for repair has been characterized using a combination of anatomical and physiological techniques on embryos which received complete high thoracic spinal cord transections (Valenzuela et al, 1990; Hasan et al, 1991; 1992). Anatomical recovery was assessed after transection by restricted injection of a neuroanatomical retrograde tracing chemical into the lumbar cord. If the spinal cord transection was performed prior to embryonic day (E)13, the distribution and number of retrogradely labelled brainstem-spinal neurons was identical to sham-operated or unoperated controls. Transections performed on embryos aged E13 or older resulted in reduced retrograde labelling of brainstem nuclei (Hasan et al, 1991).

The functional recovery from mid-thoracic transection was assessed by behavioural observation of the motor function of post-hatching chicks, and by focal electrical stimulation of brainstem locomotor regions (Valenzuela et al, 1990; Hasan et al, 1991). Leg and wing electromyographic recordings were used to monitor brainstem-evoked motor activity both *in ovo* (E18-E20) and after hatching. In comparison to sham- and un-operated control animals, complete functional recovery of motor function was evident for chicks with spinal cords transected prior to E13. These findings were consistent for all methods of assessment (behavioural observation or brainstem stimulation), therefore embryonic spinal cord was capable of complete functional recovery from transections performed prior to E13. The quality of spinal cord repair diminished with transection at later stages of development (E13 or older), resulting in paraplegia as in adult spinal cord-injured animals. These findings were reminiscent of the critical periods for repair seen in the other developing systems mentioned previously (Schmidt et al, 1980; So et al, 1981; Bernstein and Stelzner, 1983; Kolb and Whishaw, 1989).

One possible criticism of these observations arose from attributing the repair to the descent of late-developing pathways. However, studies of the ontogeny of brainstem-spinal projection systems refuted this claim, as these pathways were found to be constructed early in development. Reticular neurons in the hindbrain were among the earliest to become post-mitotic, the earliest becoming post-mitotic at stage 4 (Sechrist and Bronner-Fraser, 1991; staging according to Hamburger and Hamilton, 1951). The earliest developing projections have reached the lumbar cord by E5 (Windle and Austin, 1936; Okado and Oppenheim, 1985), and synaptogenesis occurred soon after the arrival of supraspinal projections (Shiga et al, 1991). All descending supraspinal pathways were largely complete by E11-12 (Okado and Oppenheim, 1985). These findings, combined with the physiological and anatomical evidence presented above, indicated that spinal repair was dependent on regeneration of axotimized projections as opposed to the subsequent descent of late developing pathways.

Data from double retrograde labelling experiments supported the involvement of regeneration in the recovery process as opposed to subsequent development. In these experiments, brainstem spinal projections were retrogradely labelled on E10, a transection performed on E12, then a second dye injected caudal to the transection site several days later, labelling those projections which have grown through the transection site. The presence of both retrograde dyes within the same neuron supported the hypothesis that regeneration had occurred (Hasan et al, 1992). These data clearly defined a permissive period for successful spinal cord regeneration (prior to E13), and a restrictive (unsuccessful) period (post-E13).

These results, along with descriptions from other developing systems listed previously, suggested that for functional regeneration to occur in developing (or mature) CNS, there must be a permissive environment and the ability on the part

of the neurons to regenerate. During these "critical developmental periods" clearly the first criterion was met. But what of the second? Were all developing neurons capable of repairing injury? One approach to addressing this question was to examine molecular events occurring during normal axonogenesis, then compare them to events which occurred after injury. The underlying assumption was that rebuilding an axon involves virtually the same mechanisms which supported the original outgrowth. There was a great deal of evidence in the literature which suggested that cellular events occurring during regeneration were essentially a recapitulation of developmental processes (Price and Porter, 1972; Holder and Clark, 1988). The following pages describe some molecular events occurring during development, and what is known of their recapitulation during regeneration.

MOLECULAR CORRELATES OF AXONOGENESIS

Studies of axonogenesis have described many measurable events which resulted in the formation of functionally appropriate synapses between a neuron and its target. The construction of neurites began with the elaboration of structures called growth cones at the leading edge of the neurite process. The growth cone was thought to mediate interactions with the extracellular environmental cues which result in the directed extension of a neurite (Goldberg and Burmeister, 1989; Lipton and Kater, 1989; Gordon-Weeks, 1991; Strittmatter and Fishman, 1991).

Axonogenesis required the accelerated synthesis of many structural components, such as α- and β-tubulin for the synthesis of microtubules. Microtubules were directly involved in axonal transport and growth cone motility, and provided structure to the developing axon (Yamada et al, 1970; Bamburg et al,

1986; Bray, 1987;). Other proteins whose presence correlated with axonogenesis but whose functions were not so nearly well defined such as the growth-associated protein (GAP) 43, were also expressed at high levels during axonogenesis (Kalil and Skene, 1986; McGuire et al, 1988; Dani et al, 1991) and were expressed in a developmentally regulated manner (Jacobson et al, 1986).

GAP 43

GAP 43 was originally identified by Skene and Willard (1981a,b) as one of several proteins travelling via fast axonal transport in growing and regenerating axons. Subsequent isolation and purification revealed its true molecular weight to be approximately 24kD, but its highly basic structure resulted in anomolous migration on SDS-PAGE gels, hence the misnomer (Benowitz and Routtenberg, 1987). This protein was simultaneously described by several other groups examining different aspects of neuronal function, and not until amino acid sequence data was available did they realize all were studying one and the same protein. Hence the literature contains references to pp46 (Meiri et al, 1986), F1 (Lovinger et al, 1985), B-50 (Dokas et al, 1990), and P-57 (Masure et al, 1986). It has also been proposed that it be referred to as neuromodulin (Liu and Storm, 1990).

A review of its structure and cellular distribution provides some clues for the reported function of GAP 43. It was highly enriched in growth cones of developing or regenerating axons (Meiri et al, 1986; 1988; Moya et al, 1989), but not dendrites (Goslin et al, 1988, 1990). With the correct isolation procedure, it purified with cytoskeletal elements (Allsop and Moss, 1989), although a cytoplasmic pool has also been proposed (Estep et al, 1990). In the growth cone it has been localized to the cytoplasmic side of the plasma membrane (Van Lookeren Compagne et al, 1989; Gorgels et al, 1989) and in the adult brain it occupied a

similar site in presynaptic terminals of highly plastic cortical areas (Gispen et al, 1985).

GAP 43 has been implicated to play a role in neurotransmitter release and long-term potentiation (LTP; Lovinger et al, 1985; Nelson and Routtenberg, 1985; Routtenberg and Lovinger, 1985; De Graan et al, 1990). As a substrate of protein kinase C, it was one of the major proteins whose phosphorylation correlated with the onset of LTP. It was not surprising that areas of the brain which continued to express GAP 43 in the mature CNS included the hippocampus and associative areas of cortex (Neve et al, 1988; Benowitz et al, 1988; de la Monte et al, 1989; Benowitz et al, 1990).

Studies of the structure of GAP 43 revealed a potential palmitylation site near the amino terminus which may allow interaction with the growth cone membrane via insertion of the fatty acyl tail into the lipid bilayer (Skene, 1989). The 10 N-terminal amino acids were thought to be sufficient to target the protein to axonal growth cone membranes (Zuber et al, 1989). However, recent work indicated they may be necessary, but not sufficient to target protein products to the axonal growth cone membrane (Liu et al, 1991). The molecule also had a calmodulin binding site at residues 43-51 with an adjacent phosphorylation site at serine residue 41. Phosphorylation at this site has been shown to block the calmodulin binding activity of GAP 43, thus it may be a modulator of intracellular Ca^{++} signalling by acting as a calmodulin "sponge" (Skene, 1990). Other phosphorylation sites have also been described, but their functional significance was unknown (Apel et al, 1991). GAP 43 also stimulated the binding of GTP-γ-S to G_o, another way in which it may interact with second messenger systems (Strittmatter et al, 1990).

Recent elegant work by Meiri et al (1991) using antibodies to phosphorylated and non-phosphorylated isoforms of GAP 43 indicated that

phosphorylation had the effect of stabilizing the growth cone cytoskeleton. Thus it likely acted as a part of the physiological "stop signal" when the appropiate cue was received from the environment (such as contact with target cells). These results, although exciting, were still preliminary and much work must be done to determine the molecular interactions which produced this phenomenon.

In spite of the work quoted above, it was still not clear how all of the observations could be coalesced into a working model for the actual function of GAP 43. Taken together, these observations have been proposed to indicate a role in signal transduction modulation (perhaps extracellular cues) and/or integration with growth cone cortical cytoskeletal movement. It was clear, however, that the correlation with axon outgrowth could be exploited for studying development and regeneration in the nervous system (Benowitz et al, 1990).

TUBULIN

During the neuronal process extension stage of neuronal development, the assembly of microtubules was required to provide the structure of the neurite (Daniels, 1972). Microtubules were assembled from the α- and β-tubulins (and the microtubule-associated proteins), both of which have several isotypes derived from different genes and different post-translational modifications (Cleveland and Sullivan, 1985). Functional α-tubulin proteins arose from at least five genes in an eight member gene family in the chicken (Pratt et al, 1987; Pratt and Cleveland, 1988; Valenzuela et al, 1981) producing five distinct polypeptide isotypes which are then subjected to post-translational modification. The expression of at least four of these genes has been detected in the chick CNS (Pratt and Cleveland, 1988).

Up-regulation of α-tubulin expression in the CNS during development has been described in the chick and rat (Bamburg et al, 1973; Bhattacharya et al, 1987; Miller et al, 1987b). Differential developmental expression of α-tubulin isotypes has been described in the rat CNS (Miller et al, 1987a). Ta1 tubulin (Tα1, a neuron specific isoform) was expressed at high levels during embryogenesis, correlating with the period of axon extension, and was then rapidly down-regulated when axonal growth was complete. This contrasted with T26 tubulin which was constitutively expressed (ie. not developmentally regulated, Miller et al, 1987b). This phenomenon has not been well studied in the chick, and it was unknown if a similar situation exists.

Tubulin mRNAs were subject to a rapid autoregulatory mechanism whereby the presence of excess depolymerized tubulin subunit protein resulted in the degradation of tubulin mRNA (Cleveland, 1988). The mechanism appeared to involve binding of unpolymerized β-tubulin subunits to four amino-terminal amino acids of the nascent β-tubulin polypeptide which resulted in cleaveage of the mRNA under translation (Cleveland, 1988; Cleveland, 1989) Hence cells only produced as much tubulin protein as was needed at any time due to the translational control imposed by excess unpolymerized tubulin subunits. Thus the resultant degradation of tubulin mRNA by this mechanism dictated that the levels of intact tubulin mRNA detected by Northern blotting reflected the cellular need for tubulin.

The observations described above for GAP 43 and tubulin provided insight into the developmental machinery required for axonogenesis. An understanding of these mechanisms underlying normal growth and development was necessary for unravelling the processes occurring during successful and unsuccessful CNS regeneration. In light of the CNS/PNS dichotomous response to injury, a

description of what was known about growth-associated gene expression during regeneration follows.

GENETIC GROWTH PROGRAMS

An examination of developmentally regulated mRNA from the nervous system provided one aspect of the description of a neuronal genetic "growth program" which can be re-activated in response to injury (Hoffman and Cleveland, 1988; Miller and Geddes, 1990). During PNS development and repair, events such as axonal elongation were monitored by examining the expression of specific mRNAs for growth associated proteins such as GAP 43, and α-tubulin. This recapitulation of developmental processes (Holder and Clark, 1988) during regeneration has been described in numerous studies of the molecular response to neuron injury.

An example of this was the up-regulation of α-tubulin mRNA in response to axotomy of facial and hypoglossal nerves (Pearson et al, 1988; Miller et al, 1989). Again, Tα1 was rapidly (within 4 hrs) upregulated in neurons of the facial nucleus in response to injury whereas T26 tubulin mRNA levels remained unchanged (Miller et al, 1989). As expected, there were parallel changes in the level of tubulin protein synthesis in response to injury (Tetzlaff et al, 1988). The level of Tα1 (and total α-tubulin) mRNA was subsequently down-regulated at the time of target contact (Miller et al, 1989). Similarily, severing PNS axons initiated regenerative mechanisms including the re-expression of GAP 43 at high levels (Meiri et al, 1988; Van der Zee et al, 1989; Hoffman, 1989; Tetzlaff et al, 1991) which resulted in regrowth of the axon.

Less was known about the expression of these molecules in the CNS in response to injury. Initially, it was thought that GAP 43 re-expression only

appeared at high levels during successful regeneration of the axon (Skene and Willard, 1981a,b; Skene, 1984; Kalil, 1988) and therefore would only be re-expressed in PNS after injury. A similar re-induction of GAP 43 and structural element mRNAs was seen in the rubrospinal and retino-tectal pathway after injury, and when allowed to grow into a peripheral nerve graft (Tetzlaff et al, 1991; Doster et al, 1991). Recent reports, however, have indicated that GAP 43 and total α-tubulin were re-expressed after injury with or without regeneration (Bisby, 1988; Doster et al, 1991; Tetzlaff et al, 1991), and were subsequently down-regulated after successful re-contact with appropriate targets.

The pattern of expression of these mRNAs likely formed a part of the genetic "growth program" which resulted in the successful linking of neuron and target during development. This "growth program" was also recruited after CNS injury, even when regeneration was unsuccessful (Doster et al, 1991; Tetzlaff et al, 1991). GAP 43 and total α-tubulin mRNA levels remained low in most areas of the adult CNS unless the appropriate signal for re-expression, such as injury to the axon, was received. The fact that high levels of expression of these mRNAs was required during attempted growth, taken together with their low levels of expression in the adult CNS, indicated that they may be excellent markers of the capacity for CNS repair. However, no studies have been done on GAP expression in a successful, functionally regenerating CNS model.

The experiments described here provided for the first time an examination of neuronal growth-associated mRNA expression in the successfully regenerating embryonic CNS. This model also allowed comparison of the successful response to injury suffered during the permissive period for repair with the response during the restrictive (unsuccessful) period for repair. This allowed the correlation of the regenerative responses seen at the molecular level with the

physiological success or failure of spinal cord repair in the chick embryo. The experiments were designed to address the following hypotheses:

1) The normal developmental expression levels of GAP 43 and total α-tubulin mRNA in pons and medulla will follow the classic growth-associated pattern of expression and temporally correlate with previous anatomical data delineating the temporal development of brainstem-spinal axonal projections.

2) Previous anatomical and physiological data demonstrated successful repair of brainstem-spinal projections after E11 transection, therefore, complete thoracic spinal cord transection on E11 will result in a transient re-expression of total a-tubulin and GAP 43 mRNA, followed by a return to control levels.

3) Complete thoracic spinal cord transection on E14 does not result in anatomical or functional recovery, therefore E14 transection will result in a maintained re-expression of GAP 43 and total α-tubulin mRNA levels.

Briefly, GAP 43 and total α-tubulin mRNA expression in the hindbrain of the chicken embryo peaked at E10-12, correlating with the time of maximal axonal outgrowth of brainstem-spinal projections. There was no detectable change in total α-tubulin mRNA levels after either permissive or restrictive period spinal cord transection. Transection during the permissive period for repair did not result in a transient re-expression of GAP 43. However, transection during the restrictive period for repair did result in a maintained increase in GAP 43 mRNA levels.

MATERIALS AND METHODS

Animals

Fertilized White Leghorn chicken eggs (*Gallus Domesticus*) were obtained from B&J Farm (Surrey, B.C.). The eggs were stored at 10°C for no longer than two weeks before incubation at 38°C with high humidity to allow germination to continue. All animals used in this study were staged at the time of sacrifice according to Hamburger and Hamilton (1951).

Animals aged E4-E14 were sacrificed by rapid decapitation without anaesthetic, as the pain pathways resulting in the conscious perception of pain were not functional until later in development. Animals E15 or older were anaesthetized by the application of 0.05ml of Sodium Pentobarbital (MTC Pharmaceuticals) to the air-sac membranes (embryo weight 30-50g) prior to decapitation. Animals younger than E11 required tissue sample pooling prior to RNA isolation as follows: E10 - 2 embryos; E9 - 3 embryos; E8 - 4 embryos; E6 - 6 embryos; E4 - 10 embryos.

The experimental animals which received transections were divided into two groups: the first received complete mid- to high-thoracic spinal cord transections on E11, the second received transections on E14. The young age of the transected animals abrogated the use of anaesthetic during the procedure. Transections were performed by first making a small window in the eggshell, peeling away the shell membrane. A small hole was then made in the chorion, and the embryo secured around the neck with a bent glass probe. A sharpened pair of forceps were used to sever the spinal column, and a complete transection was ensured by passing a pin, calibrated to the depth of the cord for that embryonic age, through the transection site. The window was sealed with

paraffin and a glass cover slip, and the egg returned to the incubator for 1, 3, 5 or 7 days post-transection (DPT) survival periods. A 2DPT group was also included for the E11 transection study. GAP 43 and total α-tubulin expression was then measured using Northern blotting as described below, to examine the normal developmental expression and compare transected animal groups with age-matched control group expression levels with sample sizes as indicated in Table I.

DEVELOPMENTAL STUDY		TRANSECTION STUDY		
Age at Sacrifice	Sample Size	Treatment Group	Sample Size	# of Controls
E4	3*	E11T12	7	6
E6	3*	E11T13	5	4
E8	4*	E11T14	6	6
E9	2*	E11T16	6	4
E10	7*	E11T18	4	4
E11	2			
E12	7	E14T15	9	6
E13	2	E14T17	8	7
E14	7	E14T19	8	5
E15	2	E14T21	5	5
E16	5			
E17	3			
E18	3			
E19	3			
E21	3			

Table I: Sample sizes used for the developmental study and the transection study. Transected animal groups are coded by the age when transected (ie. E11T) followed by the age at sacrifice. * indicates number of pooled tissue groups as outlined above.

RNA Isolation

All chemicals and reagents were from the Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The procedures described below were adapted from Miller (1989). The pons and medulla (cerebellum excluded) were rapidly dissected out of the embryos in ice-cold phosphate buffered saline (PBS, 10mM $NaPO_4$ pH 7.4, 7.5% NaCl) and pooled as described above if younger than E11. The tissue was rinsed several times with PBS, then placed in 250ml ice-cold lysis buffer (20mM Tris[hydroxymethyl]aminomethane hydrochloride buffer pH 8.0, 0.2M NaCl, 20mM $MgCl_2$) for homogenization with manually operated teflon-coated micro-homogenizers. The homogenate was spun in an Eppendorf microfuge at 4°C (16000g) and the pellet discarded. An equal volume of "second buffer" (2% SDS, 0.1M NaCl, 40mM EDTA) was added to each sample followed by proteinase K (Boehringer Mannheim) to a final concentration of 200 µg/ml, the digestion allowed to proceed for twenty minutes at ambient temperature. The samples were extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, BDH), then precipitated overnight at -20°C by addition of one tenth sample volume of 3.0M sodium acetate and 3 sample volumes of 95% ethanol (BDH).

The following day, the precipitate was spun in a microfuge at 4°C (16000g) for twenty minutes, the supernatant discarded and the pellet allowed to dry for 10-15 minutes at ambient temperature. The pellet was resuspended in 50µl TE buffer (10mM Tris[hydroxymethyl]aminomethane hydrochloride buffer pH 8.0, 1mM EDTA) and re-precipitated by addition of 5µl 3.0M sodium acetate and 150µl 95% ethanol and stored at -20°C for at least one hour, then the precipitation protocol was repeated once. After the final precipitation, the RNA

was resuspended in 20-40µl of TE, depending upon the yield as estimated by the final pellet size.

The concentration of each sample was determined by spectrophotometric analysis of a 4µl aliquot diluted to 1ml in water. The absorbance at 260nm and 280nm was measured with a Beckman DU 64 spectrophotometer to determine the concentration (A_{260} x 10) and purity (A_{260} / A_{280} ratio), then each sample diluted as required with TE to a final concentration of 3µg/4.7µl. Samples with an A_{260} / A_{280} ratio of less than 2 were subject to re-purification.

The RNA was stored for no longer than two weeks at -20°C before proceeding to the electrophoresis procedure. If it was necessary to store samples for longer periods of time, the isolation procedure was halted during one of the precipitation steps, and continued when the sample was needed.

Denaturing Gel Electrophoresis

Samples were size separated in standard formaldehyde denaturing agarose gels. Samples were applied to wells in the gel to produce blots of a developmental series, or blots with transected animal samples and their age-matched controls. Each lane of the developmental series blots represented RNA isolated from a single animal, with the exception of samples from animals younger than E11, which represented RNA isolated from pooled tissue as described above. Each lane of the transection blots represented RNA isolated from a single animal.

The gels were prepared using MOPS buffer (20mM 3-[N-Morpholino]propane-sulfonic acid pH 7.4, 5mM sodium acetate, 1mM EDTA) with 1.1M formaldehyde, and agarose added to 1.5%. The running buffer was also MOPS buffer with 1.1M formaldehyde. A 4.7µl (3µg) aliquot of each sample

was diluted with 14µl "mix" (50% formamide, 1.1M formaldehyde and MOPS buffer, final concentrations). Samples were heated at 65°C for 10 minutes, then placed on ice. Two microlitres of loading dye were added to each sample prior to loading the gel. The loading dye was made by mixing 6X loading dye (15% Ficoll type 400, 0.25% Bromophenol Blue) and ethidium bromide (10mg/ml) in a 10:1 ratio. Typically, gels were run at 60V (constant voltage conditions) for 4-5 hrs with a Hoeffer submarine gel unit powered by a GPS 200 power supply (Pharmacia), then photographed with UV transillumination (FisherBiotech FBTI 816) using type 57 Polaroid film.

After equilibrating the gel in 10X SSC (1X SSC is 0.15M NaCl, .015M sodium citrate) for 30-60 min., a standard capillary transfer apparatus was used to transfer the RNA from the gel to Nytran membrane (0.45µm pore size, Schleicher and Schuell). Using 10X SSC as the transfer buffer, the gel was placed upside down supported on plexiglass above a 10X SSC buffer reservoir on Whatman #3 filter paper wicks arranged to draw fluid from the buffer reservoir. A piece of Nytran cut to the size of the gel and pre-soaked for 5 min. in distilled water was layered on top of the gel, followed by 3 layers of #3 filter paper pre-soaked in distilled water and a stack of paper towels topped with a small glass weight. The entire set-up was draped in Saran Wrap (Safeway) to prevent evaporation.

The transfer was allowed to proceed overnight, then the Nytran and the gel were viewed under UV transillumination to ensure that the transfer was complete. The blots were stained using 0.02% methylene blue in 0.3M sodium acetate for 60-75 seconds, and destained with three rapid rinses in ice-cold 2X SSPE (1X SSPE is 0.15M NaCl, 10mM $NaPO_4$ pH 7.4, 2.5mM EDTA) then immediately photographed with type 57 polaroid film. Inspection of the methylene blue stained blots revealed lanes which appeared over- or under-

loaded based on the intensity of the stained ribosomal RNA bands. Samples from these blots were re-run with slightly adjusted amounts of RNA loaded in lanes judged to be unequal on the initial blot. This was repeated until blots were obtained which demonstrated equal amounts of RNA in each lane as judged by methylen blue staining. The blots were baked at 80°C under vacuum for one hour then stored in a sealed plastic bag at -20°C until used for hybridizations.

Northern Hybridizations

Plasmids containing cDNAs for chicken GAP 43 (kind gift from Dr. Larry Baizer; Baizer et al, 1990) and rat α-tubulin (kind gift from Dr. Freda Miller; Miller et al, 1987a,b) were linearized by restriction endonuclease digestion asindicated in Fig. 1, then cleaned with two phenol/chloroform/isoamyl alcohol extractions and two sodium acetate/ethanol precipitations. cRNA antisense probes were generated using T7 (GAP 43) or SP6 (α-tubulin) bacteriophage RNA polymerases as described by the manufacturer of the *in vitro* transcription kit (Promega, Madison WI) using α^{32}P-labelled CTP (40mCi/ml, 800Ci/mmol; Amersham, Oakville Ont.). Unincorporated nucleotides were removed by spin column chromatography using Sephadex G-50 (Sambrook et al, 1989).

Incorporation efficiency was crudely estimated by Geiger counter (Ludlum Measurements Inc.) measurement of the beta particle emissions from the purified probe compared with the emissions from the column containing the unincorporated nucleotides, and was usually 40-50%. Under the conditions of the reaction used, this routinely gave enough probe to hybridize with approximately $2x10^6$ cpm/ml of hybridization solution as determined by liquid scintillation counting of a 1μl aliquot of the radiolabelled probe using a Beckman LS 5000 TA liquid scintillation counter.

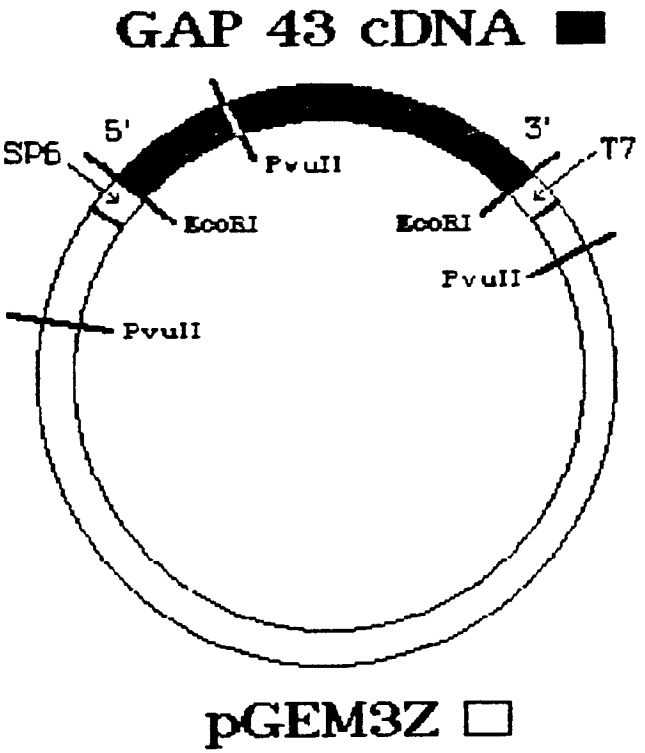

Figure 1: Construction of the GAP 43 cDNA. This diagram indicates the orientation of the chicken GAP 43 cDNA cloned into the EcoRI site of the plasmid vector pGEM 3Z (Baizer et al, 1990). After linearizing with PvuII (cuts at base 403 of the 1000 bp clone), *in vitro* transcription with T7 RNA polymerase and radiolabelled nucleotides produces an antisense cRNA probe. The total α-tubulin probe (Miller et al, 1987a,b) is in a similar vector, but opposite orientation, requiring linearization with Hind III and transcription with SP6 RNA polymerase.

Before hybridization, blots were rinsed for 15 min. in 2X SSPE plus 2% SDS with agitation to remove any remaining methylene blue stain. The blots were prehybridized in 5X PIPES buffer (25mM piperazine-N,N'-bis[2-ethane-sulfonic acid] pH 7.4, 750mM NaCl, 25mM EDTA) containing 50% formamide, 0.2% SDS, 200μg each of denatured salmon sperm DNA and baker's yeast tRNA (Boehringer Mannheim), 5X Denhardt's (1X Denhardt's is 0.02% Ficoll, 0.02% Polyvinylpyrrolidone, 0.02% Bovine Serum Albumin) for a minimum of 2 hours at 65°C. Hybridization was done in buffer with the same composition, with the addition of the labelled probe, overnight at 65°C. All blots were probed with both GAP 43 and total α-tubulin cRNAs sequentially. The first probe was stripped by incubating blots in 80% formamide at 80°C for 1 hour prior to re-probing with the second cRNA.

Following the hybridization, blots were rinsed for 15 min. in 2X SSC + 0.2% SDS at ambient temperature, then 0.5X SSC + 0.2% SDS at 55°C, then 0.1X SSC + 0.2% SDS at 65°C to remove any unbound and non-specifically bound probe. Blots were wrapped in saran wrap and apposed to Kodak XAR x-ray film at -70°C. The x-ray film was pre-flashed using a Vivitar camera flash unit and 17 Kimwipe tissues between the flash unit and the x-ray film at a distance of 170cm to generate a 0.15 absorbance unit change prior to exposing the film with the blots (Laskey and Mills, 1975; 1977).

The x-ray films were developed using a Kodak X-omat automatic processor, and the density of the bands in each lane quantified using a computerized scanning densitometer (Molecular Dynamics (MD) with MD Imagequant 3.0 software). The densitometer provided a plot of the peak densities of the bands in each lane, the area under each peak calculated by integration of the density plot. Each number generated in this fashion therefore represented a single sampling from a single animal except for animals younger

than E11 used for the developmental study (tissue pooled prior to RNA isolation as described above).

The initial blots run for this study included RNA size markers (Boehringer Mannheim, data not shown) to determine the sizes of the mRNA bands detected by the GAP 43 and total α-tubulin probes. The GAP 43 cDNA clone encodes chicken GAP 43, therefore the cRNA probe used here is 100% homologous to the GAP 43 mRNA. As the hybridizations were performed at high stringency (0.1X SSC + 0.2% SDS at 65°C final wash), the bands that were detected represent the authentic GAP 43 mRNAs. The banding pattern described here is also identical to that described by Baizer et al (1990) as being authentic GAP 43 mRNA.

The total α-tubulin cDNA used here encodes rat α-tubulin, but the coding sequences of the α-tubulin gene families across species are very well conserved (Cleveland et al, 1980; Cleveland and Sullivan, 1985; Pratt and Cleveland, 1988). The high stringency conditions employed in this study ensured that the appropriately sized band (1.8kb) detected with the total α-tubulin cRNA represented authentic chicken α-tubulin mRNA.

Data from the developmental blots were presented as integrated densitometric measurements from the GAP 43 and total α-tubulin cRNA probed blots.

The integrated densitometric measurements obtained from the GAP 43 probed blots of transected versus control animal RNA were subject to an additional control for variations in the amount of RNA loaded in each lane. The data from the total α-tubulin re-hybridizations did not demonstrate changes as a result of transection. The small variations in the total α-tubulin signal from lane to lane within a blot represented small variations in the amount of RNA loaded in each lane (see Figure 2). Therefore, data from the total α-tubulin re-hybridizations were used to transform the GAP 43 data to remove variations in

the detected GAP 43 mRNA levels resulting from unequal loading and transfer of lanes in a gel. An adjustment factor for each lane of the GAP 43 blots was generated by dividing the average total α-tubulin signal across all lanes of animals the same age by the total α-tubulin signal in that lane. The GAP 43 densitometric data from the corresponding lane was then multiplied by this ratio. The adjusted GAP 43 data was then expressed as a ratio of experimental to average age-matched control values. The log of the experimental/control GAP 43 expression ratio was tested by one-way analysis of variance to discriminate statistically significant ($p<0.05$) variations from zero.

RESULTS

RNA Isolation

The amount of RNA isolated from a given animal remained relatively constant in terms of μg/wet weight of hindbrain (approximately 0.5–1μg/mg). Transection of the spinal cord did not consistently alter this parameter. The amount of RNA isolated from animals older than E10 was sufficient to run five or more Northerns, but animals E10 and younger required pooling of tissue from two (E10) to ten (E4) animals to provide sufficient quantities of RNA.

RNA Sample Quantification, Equality of Lane Loading

Spectrophotometric determination of RNA sample concentrations was not accurate enough to ensure equal amounts of mRNA were loaded in each lane of a Northern blot, as it did not account for pipetting errors while diluting samples for the spectrophotometer or loading the gel, or the inequities in RNA transfer to Nytran. In an effort to control these sources of error, blots were stained after the transfer step with methylene blue to evaluate the quantity of RNA loaded in each lane of the blot. Methylene blue allowed the visualization of the major RNA species in the cytoplasmic extracts, the ribosomal RNA (rRNA) bands (Figure 2c). Gels containing lanes which appeared to be over- or underloaded based on the intensity of the methylene blue-stained rRNA bands were run again, with small corrections in the amount of RNA loaded in the lanes. This technique allowed a qualitative estimation of the gel loading and transfer efficiency for the developmental expression autoradiograms.

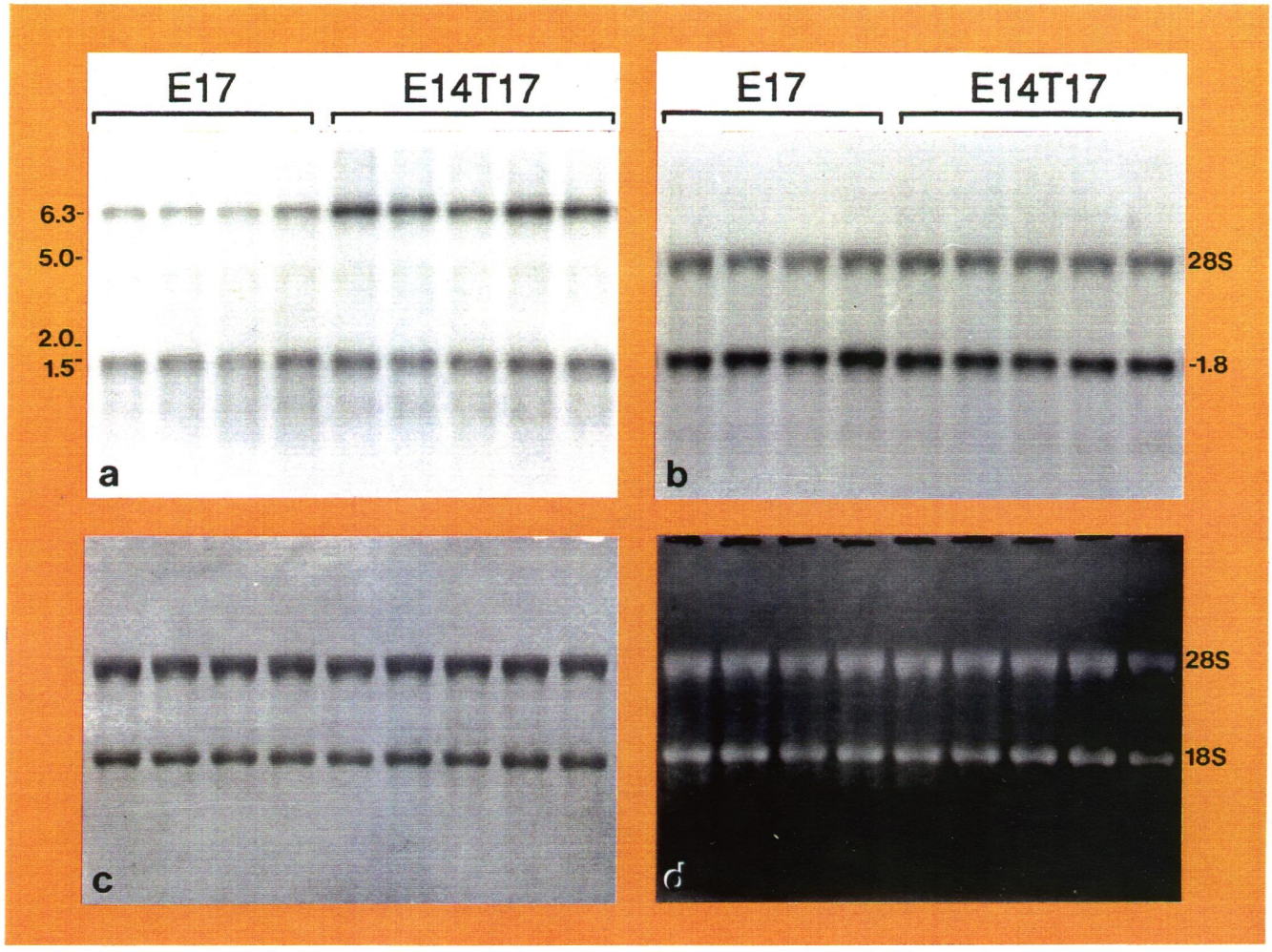

Figure 2: Quantification of RNA amounts loaded in lanes. (a) Northern blot of E17 control mRNA (first four lanes, E17) and E14 transected animals sacrificed on E17 (next five lanes, E14T17). The E14T17 animal group showed increased GAP 43 mRNA levels compared to the age-matched controls. (b) The same blot as in (a) stripped of the GAP 43 cRNA and re-probed with the total α-tubulin cRNA. Total α-tubulin levels were essentially equivalent in all lanes, providing a quantifiable assessment of the minor variations between lanes not detected by methylene blue staining (c) or ethidium bromide staining (d). Non-specific binding to 28S ribosomal RNA band is indicated in (b). Indicated sizes are in kilobases.

The results from the total α-tubulin probed transection blots (multiple lanes of RNA from animals the same age) equalized in this manner indicated that the variation of any one lane was not great. For example, the average densitometric value obtained from Figure 2b was 56.65 arbitrary units, and the greatest variation from that value in any one lane was less than 30%. This suggested that the discrimination of a tenfold change in mRNA levels over the embryonic development period of the chick could easily be accomplished using the methylene blue staining technique. Ideally, it was necessary to re-probe the developmental blots with a constitutively expressed mRNA probe, to provide a quantitative analysis of mRNA amounts in each lane. Such a probe was unavailable for studying the development of the chick CNS, therefore methylene blue staining was the best alternative.

Both probes used in this study have been well characterized (Baizer et al, 1990; Miller et al, 1987). The GAP 43 cRNA detected two mRNAs (6.3kb and 1.5kb) as previously described (Baizer et al, 1990). Although the total α-tubulin probe was derived from murine brain, the coding sequences of the α-tubulin gene families across species were very well conserved (Cleveland et al, 1980; Cleveland and Sullivan, 1985). This cDNA was capable of detecting the Tα1 tubulin isotype when cRNA probes were transcribed from the 3' untranslated region, however the cross-species variation in this region of the mRNA was too great between rat and chick to discriminate different α-tubulin isotypes in this fashion (data not shown). However, under highly stringent washing conditions (0.1X SSC + 0.2% SDS at 65°C), cRNA probes generated from the entire cDNA did recognize a single mRNA band of the correct size (1.8kb) for all α-tubulins expressed in the chick hindbrain.

GAP 43 and α-Tubulin Expression During Development

Another problem arose from the absence of probing the developmental blots for a constitutively expressed mRNA. Without such an internal standard, it was not appropriate to combine data from different blots for several reasons. There were numerous variables associated with the production of an autoradiogram including variations in amount of mRNA loaded per lane, the specific activity of the probe, final probe concentration, length of exposure time of the x-ray film, and determination of the baseline for densitometric analysis which all affected the actual densitometric values obtained from blot to blot. The inability to combine data from different developmental blots also precludes the use of statistical analysis with the developmental study data, rendering it descriptive at best. However, the raw densitometric values from all blots plotted on a single set of axes provided an indication of the trends which occurred during development of the chick embryo hindbrain.

Probing with the GAP 43 cRNA revealed a large (6.3kb) and a small (1.5kb) form of GAP 43 mRNA detected during the development of the hindbrain (Fig. 3a). The results from all developmental RNA blots shown in Figure 4 indicated the changes occurring in GAP 43 mRNA levels during development. GAP 43 mRNA levels followed a growth-associated pattern of expression. Early in development levels were low, then rose to a peak at E10-12, then fell to near-adult levels by E21 (Figure 4). For simplicity, the data from a representative blot (Figure 5) was chosen to illustrate these changes, as it also indicated the relative amounts of the large and small GAP 43 mRNAs. Levels of GAP 43 mRNA in the E4 embryo were almost below the level of detection of these techniques, but notably, the 1.5kb message was slightly more abundant than the 6.3kb message at this stage of development (Figure 3a, 5). As

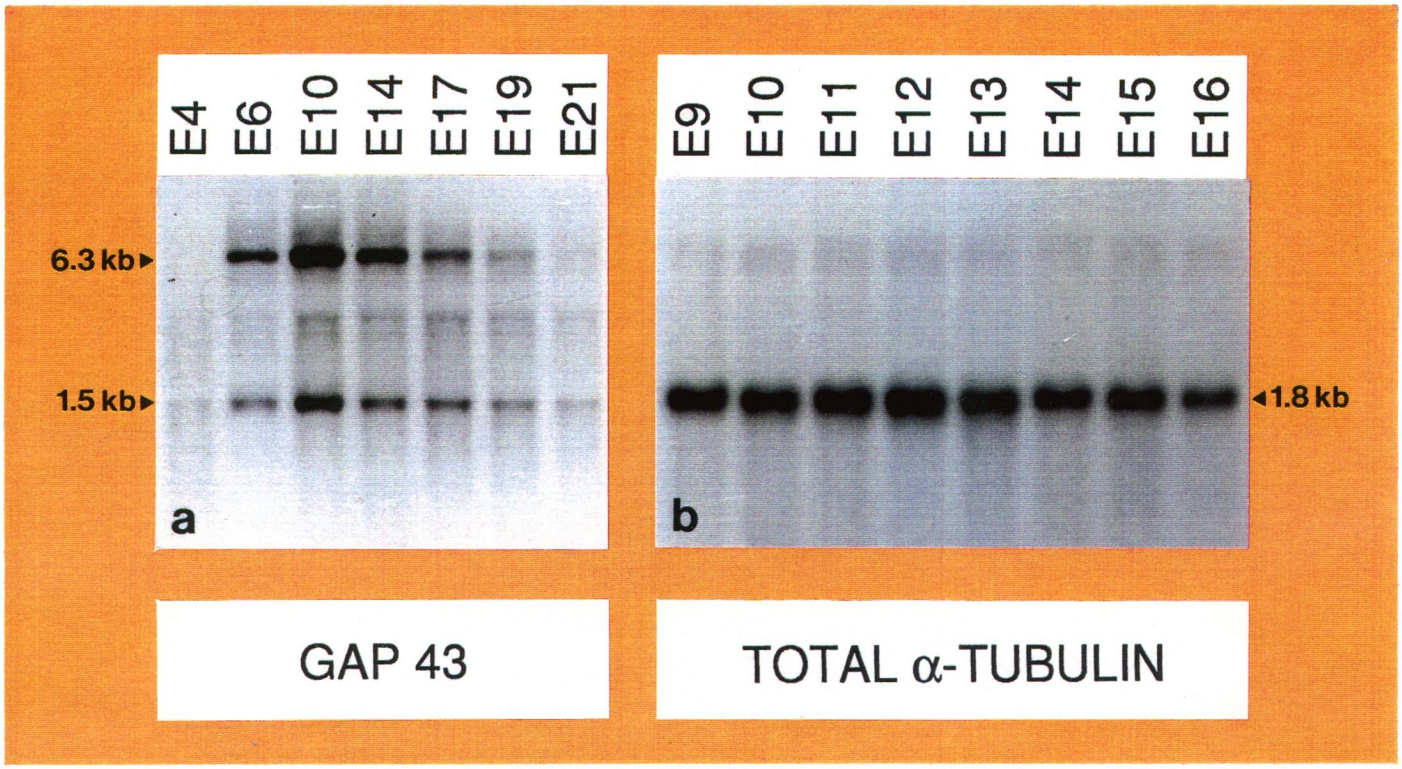

Figure 3: Typical Northern blots of GAP 43 and Total α-Tubulin mRNA levels during development. (a) GAP 43 mRNA levels were almost undetectable at E4, rose to a peak at E10-12, then declined to near-adult levels by E21. (b) Total α-tubulin mRNA levels followed a similar time-course, but the peak expression occured over a broader range of developmental ages. Compare E10-E14 expression levels for GAP 43 and total α-tubulin. Sizes of the transcripts were as indicated previously, developmental stage of embryos (days of incubation) indicated above each lane.

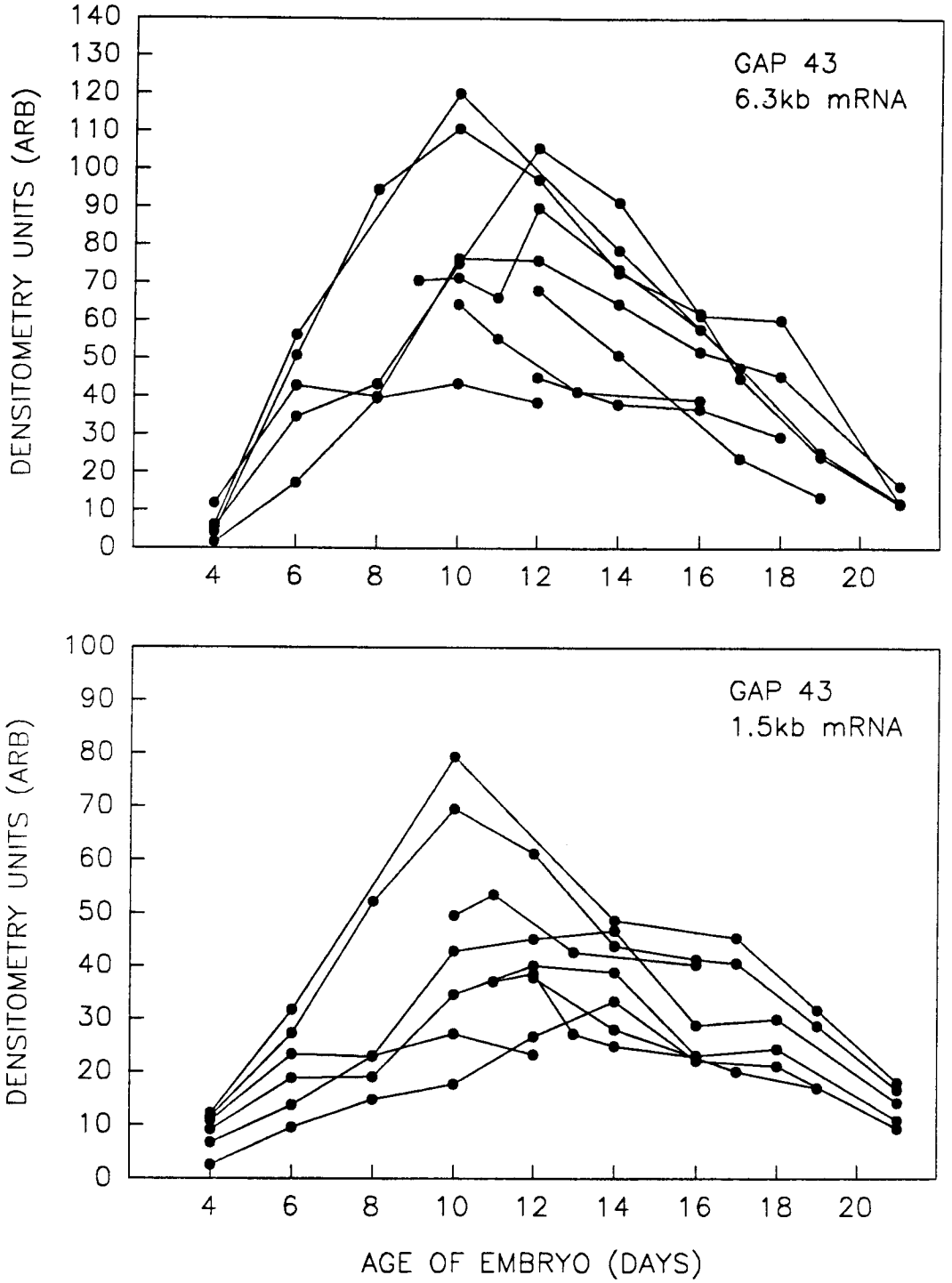

Figure 4: Densitometric analysis of GAP 43 mRNA levels during development. Each line in these plots represented an individual blot containing lanes of RNA isolated from animals of varying age, probed with the GAP 43 cRNA. These data demonstrated the growth-associated pattern of GAP 43 mRNA expression for the 6.3kb mRNA (a) and the 1.5kb mRNA (b) as development progressed.

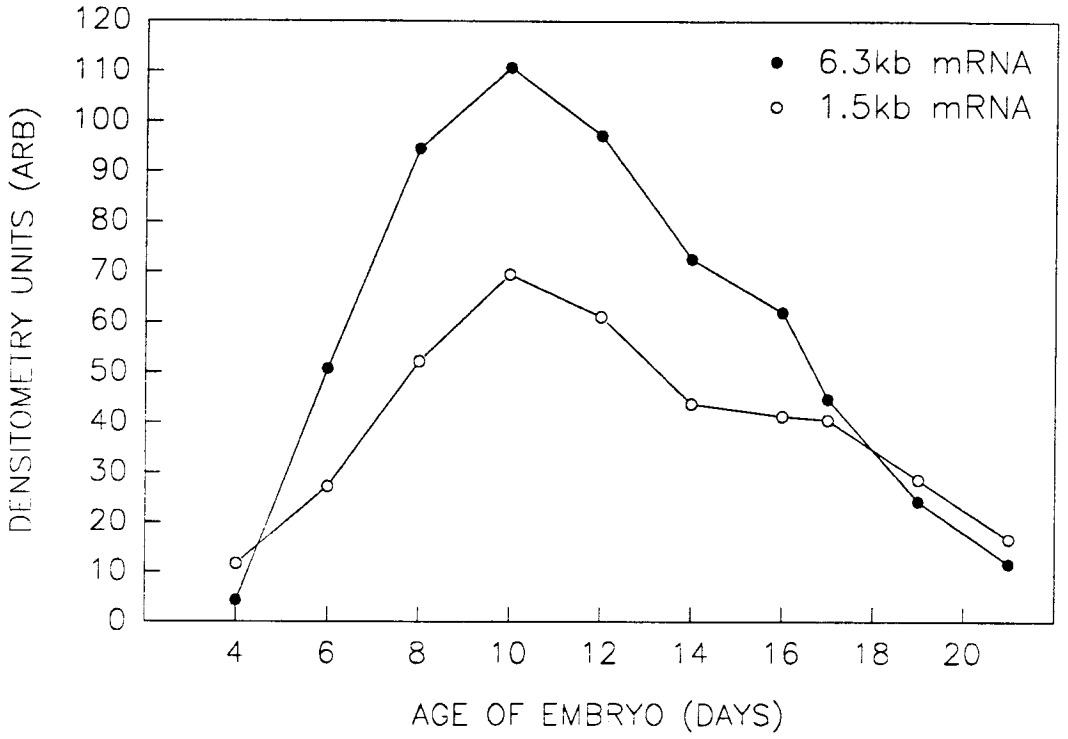

Figure 5: Typical developmental changes in GAP 43 mRNA levels from a selected blot. Selected GAP 43 mRNA developmental expression data which illustrated the changes in the relative amounts of 6.3kb and 1.5kb mRNA over the developmental period examined (E4-E21).

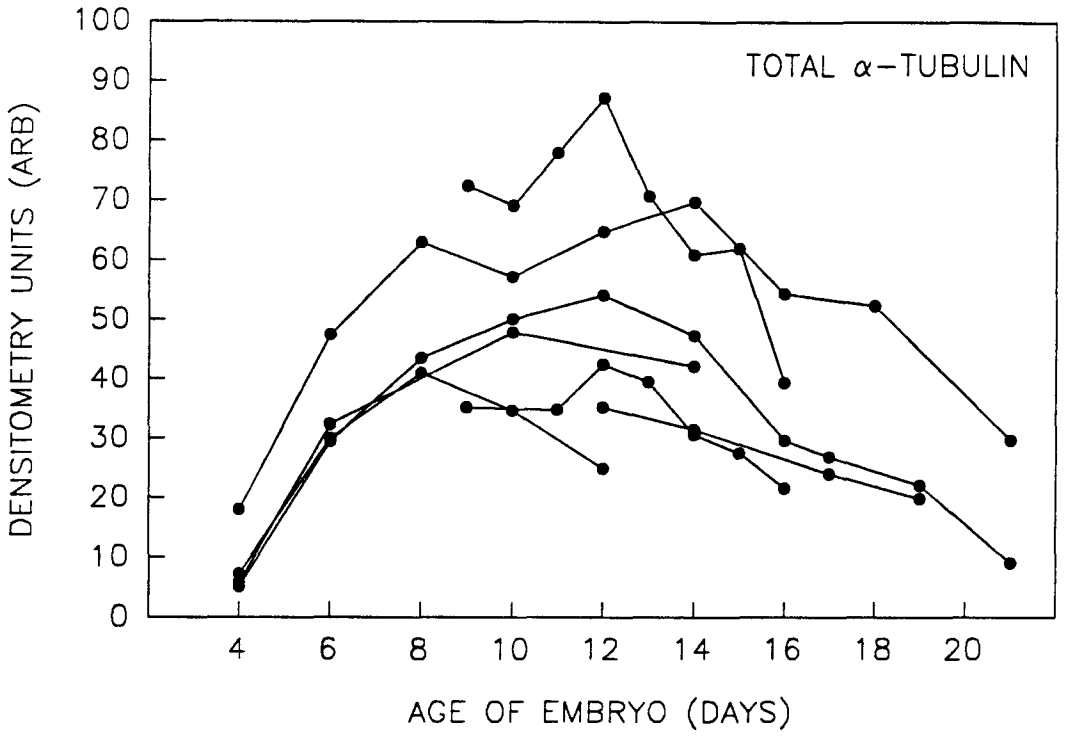

Figure 6: Densitometric analysis of total α-tubulin mRNA levels during development. Each line in this plot represented an individual blot containing lanes of RNA isolated from animals of varying age, probed with the total α-tubulin cRNA. This data demonstrated the growth-associated pattern of total α-tubulin mRNA expression as development progressed.

development progressed, the 6.3kb GAP 43 message level increased 20-30 fold to peak on E10-12, while the 1.5kb message increased 5-10 fold over a similar time course. Levels of both message then declined to near adult levels by E21; the 6.3kb species all but disappeared, and the 1.5kb species decreased to approximately one-fifth of the peak expression levels (Figure 3a, 5).

Probing with the total α-tubulin cRNA revealed a single mRNA band 1.8kb in length. The developmental time course of α-tubulin expression was similar to GAP 43, but the peak expression occurred over a broader time period (Figure 3b, 6). Levels were almost undetectable at E4, then rose approximately 10 fold by E8. There was a much smaller increase through to the peak expression at E12, then a gradual decline (approximately 5 fold) to near adult levels by E21 (Figure 6).

Effects of Spinal Cord Transection

Statistical Analysis

There was no detectable change in α-tubulin expression after any of the survival periods post-E11 or -E14 transection. For example, Figure 2a,b demonstrated the GAP 43 and total α-tubulin mRNA levels in nine animals aged E17, five of which received spinal cord transections on E14. The methylene blue- and ethidium bromide-stained blots (Figure 2c,d) demonstrated the near-equivalence of RNA amounts in each lane. The GAP 43 signal from the transected animals was greater than the control GAP 43 signal, yet the same blot re-probed with the total α-tubulin cRNA yielded only minor variation in the total α-tubulin mRNA levels across all the lanes (Figure 2a,b). The slight variations in the total α-tubulin signal did not correlate with either control or experimental

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	7	-.087	.023	.033	-.154	-.021*
2	7	-.046	.029	.033	-.112	.019
3	5	-.061	.027	.039	-.139	.016
4	5	-.094	.027	.039	-.172	-.015*
5	6	.082	.033	.035	.010	.153*
6	6	.059	.030	.035	-.012	.130
7	6	.006	.019	.035	-.065	.077
8	6	-.070	.018	.035	-.142	.0007
9	4	-.095	.023	.044	-.183	-.007*
10	4	-.151	.009	.044	-.238	-.063*
11	9	-.025	.025	.029	-.084	.032
12	9	-.078	.022	.029	-.136	-.020*
13	8	.437	.040	.031	.375	.499*
14	8	.193	.030	.031	.131	.255*
15	8	.402	.069	.031	.340	.463*
16	8	.283	.036	.031	.221	.345*
17	5	.477	.013	.039	.398	.555*
18	5	.086	.016	.039	.007	.164*
Total	116	.087	.008	.008	.071	.103

Table 2: **Analysis of Variance of Log-transformed GAP 43 expression levels** after transection. The ANOVA levels are coded as follows: 1, 2: E11T12 (E11 transect with E12 sacrifice) 6.3kb, 1.5kb mRNA respectively, as with all other groups; 3,4: E11T13; 5,6: E11T14; 7,8: E11T16; 9,10: E11T18; 11,12: E14T15; 13,14: E14T17; 15,16: E14T19; 17,18: E14T21. The count indicates the sample size for each group. Asterisks indicate statistical significance ($p<0.05$).

groups, but were indicative of minor variations in the amount of RNA in each lane which was undetectable with methylene blue or ethidium bromide staining. This allowed the use of the total α-tubulin data from transected animal blots as an internal control, and provided a quantitative assessment of the actual variation in RNA amounts in each lane. This allowed an adjustment to be made of the GAP 43 signal of a particular lane by multiplication with the ratio of the average total α-tubulin signal across the blot to the total α-tubulin signal for that lane. The adjusted GAP 43 values in the transected animal lanes were then expressed as a ratio to the average adjusted age-matched control value, log-transformed, and subjected to one-way analysis of variance (ANOVA). The results from the ANOVA are presented in Table 2, with 95% confidence intervals calculated for the (log-transformed) mean change over control from each group. If the 95% confidence interval included zero, the results were not statistically significant.

The adjusted GAP 43 expression levels after transection were also expressed in terms of percent change from control values (Figures 7c, 8c), then these changes were superimposed upon the typical normal developmental expression graphs provided in Figure 5 to illustrate the relative change with respect to the changing baseline which resulted from the normal developmental decline in GAP 43 mRNA levels over the one week survival period examined in this study (Figure 9).

Effects of Spinal Cord Transection in the Permissive Period on GAP 43 mRNA Levels

One day following an E11 transection, average GAP 43 levels dropped 17% for the higher size message, and 12% for the lower size message (Figure 7a,c), but the variability in this transected animal group prevented the difference from being statistically significant for the smaller mRNA species. Two DPT (Figure 7c) the larger GAP 43 message was closer to the control values (12.5% drop), but the smaller message dropped to 18% below control values (significance level $p<0.05$). Message levels 3 DPT (Figure 7a,c) were 22% above for the larger size ($p<0.05$) and 16% above for the smaller size (not statistically significant). By 5 DPT (Figure 7b,c), the larger size GAP 43 message had returned to control levels (2% difference) while the smaller size had dropped 14% below control values (not statistically signigficant). Message levels continued to drop below control values 7 DPT (Figure 7c), with the larger GAP 43 message 19% below control ($p<0.05$), and the smaller message 29% below control ($p<0.05$). These changes were very small and insignificant compared to the developmental levels of GAP 43 mRNA being expressed during this period. These results suggest there was an increase in GAP 43 expression 3 DPT, which returned to control levels 5 DPT but this was inconclusive (Figure 7c).

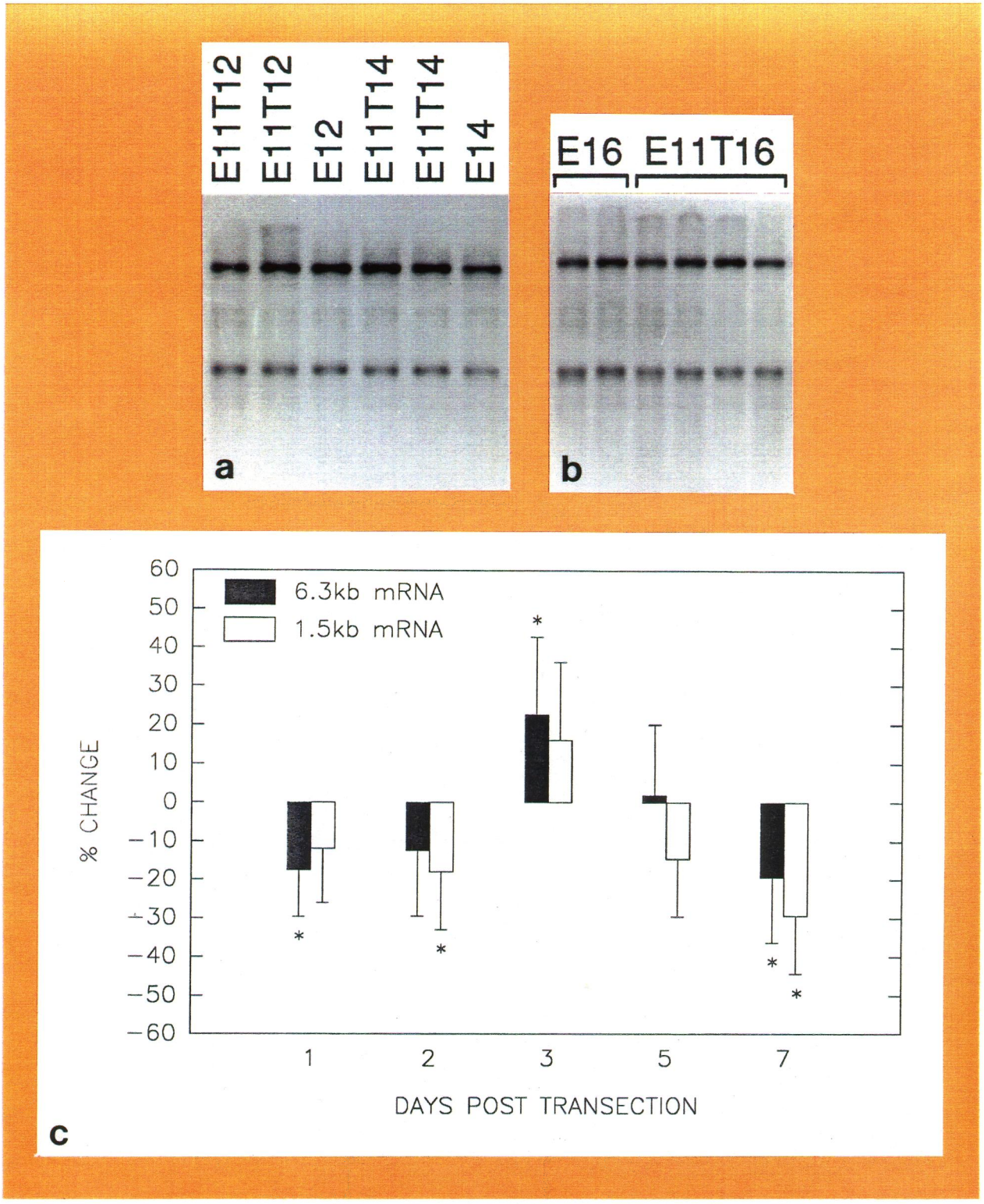

Figure 7: Changes in GAP 43 Expression After E11 Transection. (a) Representative Northern blot which showed GAP 43 expression one (E11T12) and three (E11T14) days post-E11 transection compared with age matched controls (E12 and E14 respectively). Note the near-equivalence of 1 DPT and E12 control GAP 43 mRNA levels, but 3 DPT animals showed an increase over E14 control mRNA levels. (b) GAP 43 mRNA expression 5 days post-E11 transection (E11T16) returned to control levels. (c) Plot of percent change in experimental animal GAP 43 mRNA levels (1,2,3,5 and 7 DPT) from age-matched control levels. (* indicates $p<0.05$, error bars calculated from ANOVA 95% confidence intervals)

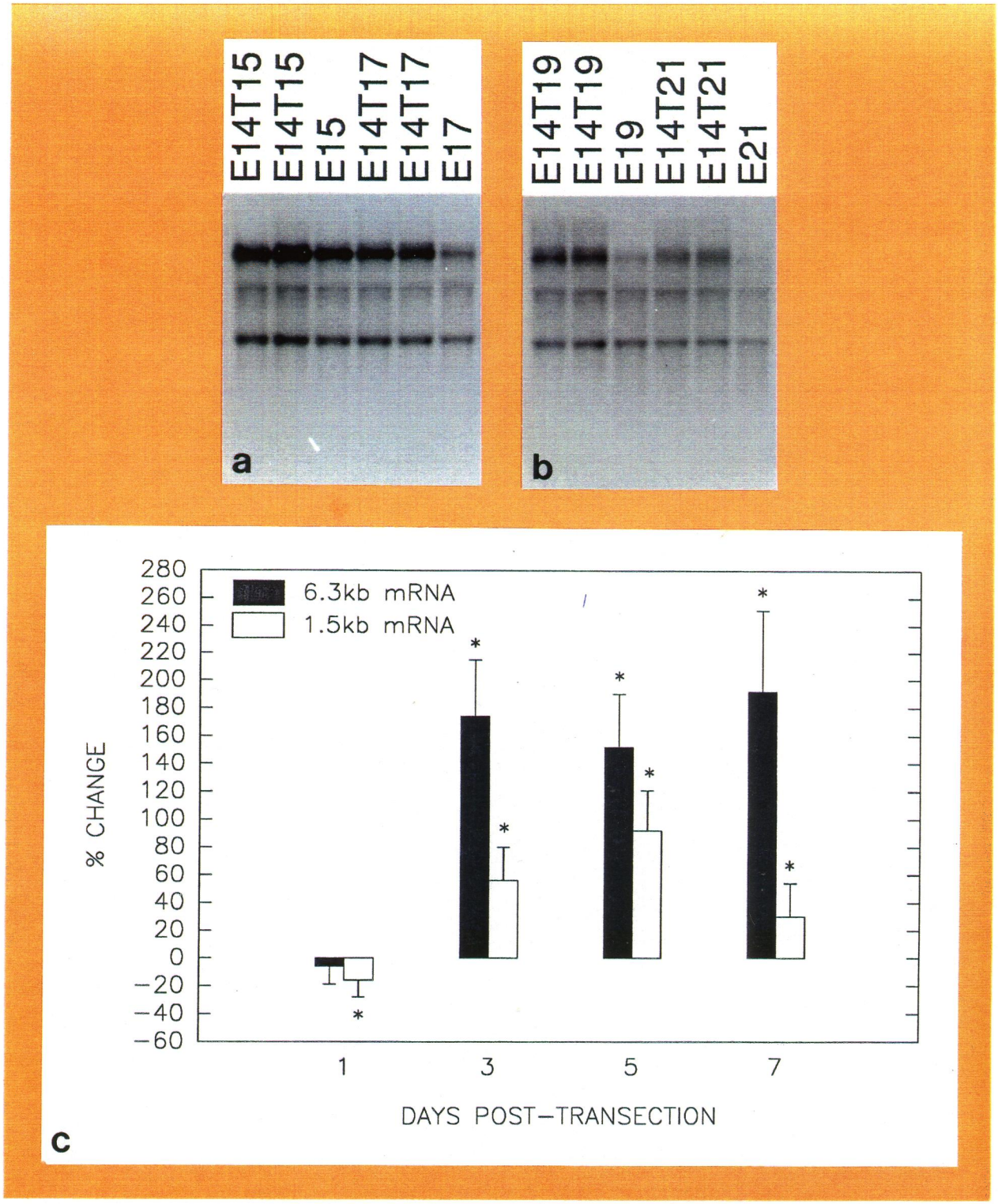

Figure 8: Change in GAP 43 Expression After E14 Transection. (a) Representative Northern blot which showed GAP 43 mRNA levels one (E14T15) and three DPT (E14T17) compared with age-matched controls (E15 and E17 respectively). GAP 43 mRNA levels were equivalent to control levels 1 DPT but increased above control amounts 3 DPT. (b) A similar Northern to (a) compared five DPT (E14T19) and seven DPT (E14T21) to age matched controls (E19 and E21 respectively). GAP 43 mRNA levels were still increased above control levels even 7 DPT. (c) Plot of percent change in experimental animal GAP 43 mRNA levels (1,3,5 and 7 DPT) from age-matched control levels. The data demonstrated a maintained re-expression of GAP 43 mRNA 3-7 DPT. (* indicated $p<0.05$, error bars calculated from ANOVA 95% confidence intervals)

Effects of Spinal Cord Transection in the Restrictive Period on GAP 43 mRNA Levels

One DPT, GAP 43 message levels were slightly below control levels (-6%, -16% upper and lower respectively), but only the smaller mRNA signal was significantly different ($p<0.05$; Figure 8a,c). Animals allowed 3 DPT recovery periods (Figure 8a,c) showed a large increase in GAP 43 message levels, 174% for the large size message ($p<0.05$) and 56% for the smaller message ($p<0.05$). This increase was maintained 5 DPT, with 152% increase in the larger message ($p<0.05$) and a 92% increase in the smaller message ($p<0.05$) over control values (Figure 8b,c). An increase in GAP 43 message levels was still evident 7 DPT (Figure 8b,c), with 192% increase in the larger message ($p<0.05$) and a 30% increase in the smaller message ($p<0.05$). Thus transection in the restrictive period for repair resulted in a maintained increase in GAP 43 mRNA levels (Figure 8).

An assessment of the absolute magnitude of the change relative to the decreasing baseline was obtained when the changes in GAP 43 expression after transection were related to the changing baseline of GAP 43 expression over the one week survival period. This figure was generated by applying the percent changes from baseline presented in Figures 7 and 8 to the values of GAP 43 expression indicated in the typical developmental plot illustrated in Figure 5. The magnitude of the observed increases 3 DPT was greater after E14 transection than after E11 transection, for both the 6.3kb (Figure 9a) and the 1.5kb (Figure 9b) mRNA. The magnitude of the peak change seen after E14 transection was similar to the peak changes seen over the course of normal development.

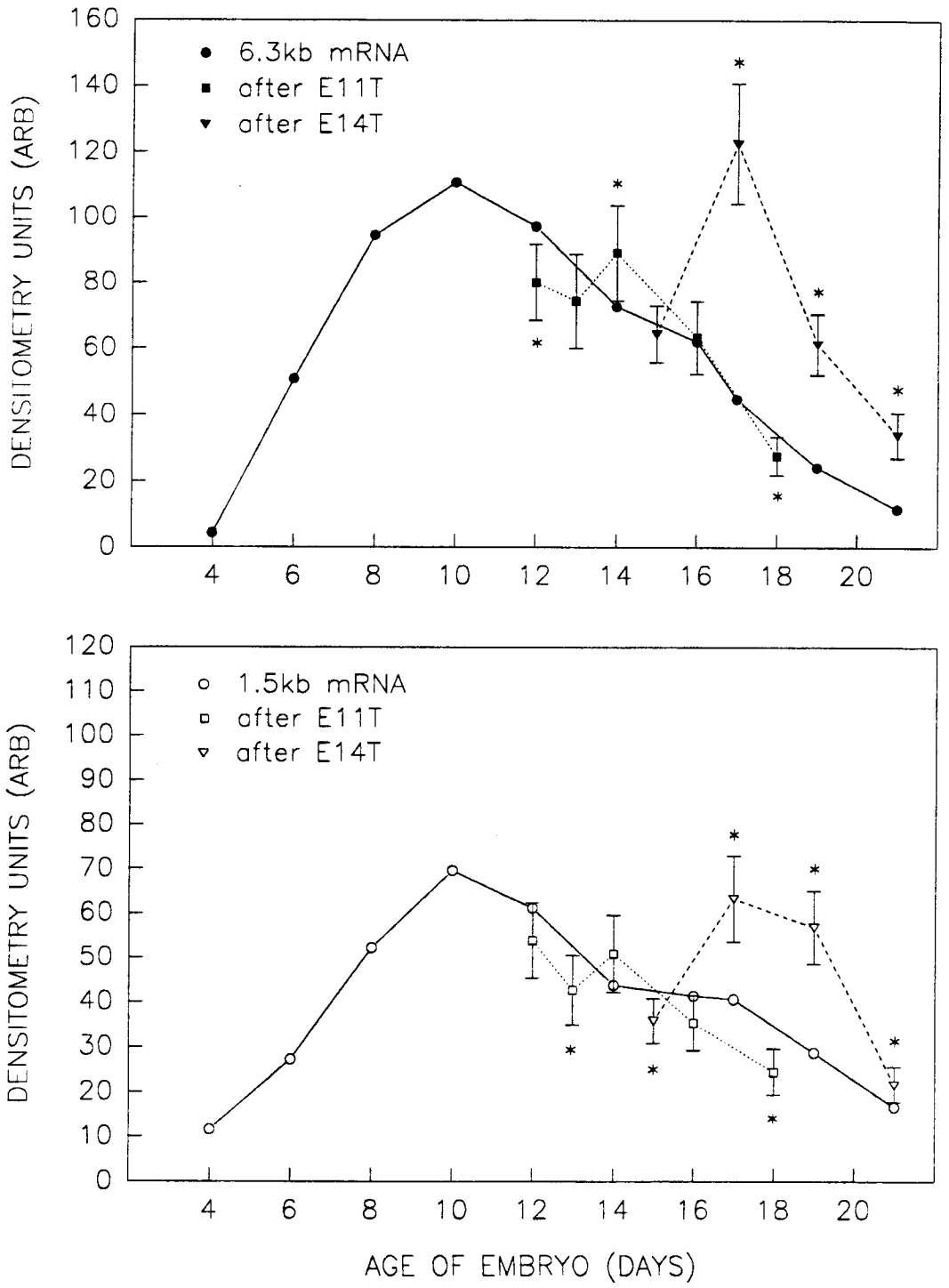

Figure 9: Changes in GAP 43 mRNA levels after transection superimposed upon typical developmental expression levels. This figure provides an indication of the magnitude of the changes in GAP 43 expression after transection relative to the decreasing baseline expression of the 6.3kb mRNA (a) and the 1.5kb mRNA (b). * indicates $p<0.05$, error bars calculated from the ANOVA 95% confidence intervals.

DISCUSSION

The results presented here illustrate a number of features of growth-associated protein expression in the developing hindbrain. Levels of expression of growth-associated mRNA peaked during periods of maximal process outgrowth from this brain region. After E11 transection, levels of GAP 43 and total a-tubulin mRNA expression did not increase above control levels despite regrowth of axons. After E14 spinal cord injury, however, GAP 43 (but not total α-tubulin) mRNA levels showed a second peak in expression (3 days after injury) which was maintained until at least 7 DPT. A comprehensive discussion of these results follows.

CRITIQUE OF THE METHODS

One of the most vital controls necessary with Northern blotting is to ensure that comparisons made between lanes on a blot account for differences in the relative amounts of mRNA in each lane. Some researchers have reported that quantification of the amount of ribosomal RNA in each lane, either by ethidium bromide staining of RNA in the gels (Bonini and Hofmann, 1991), or the membrane after transfer (Corea-Rotter et al, 1992) or hybridization with a 28S ribosomal RNA probe (de Leeuw et al, 1989) were accurate and reliable ways for assessing the equality of RNA loading across the lanes of a blot. These calculations assume that the relative amounts, and susceptibility to degradation, of ribosomal and messenger RNA remain constant across and within animal groups.

These assumptions must be questioned when studying a developing system. For example, Figure 2 demonstrated some variability in the total α-

tubulin signal which was not as obviously represented in either the methylene blue stained blot or the ethidium bromide stained gel. This variability did not correlate with the experimental treatment, but appeared to represent small differences in mRNA loading and/or individual variation in mRNA/rRNA ratio amongst animals. The margin of error which remained after equalization by methylene blue staining appeared to be on the order of $\pm$ thirty percent. Although this is almost an order of magnitude smaller than the changes seen in GAP 43 and total α-tubulin expression during normal development of the hindbrain, it is equal to or greater than the changes seen in expression following E11 spinal cord transection.

Ideally, a constitutively expressed mRNA should also be probed and measured to allow a more quantitative analysis of the changes which occurred during development. A constitutively expressed mRNA is defined as demonstrating constant levels of expression in spite of experimental manipulation. Unfortunately, a neuron-specific, constitutively expressed mRNA for which a chicken cDNA is available has not as yet been found, if one indeed exists. The next best alternative was to rely on the methylene blue staining for an approximation of equality of loading across the lanes. Although this precluded a quantitative analysis, making the results descriptive at best, it did reveal the trends which occurred during development.

For the transection study, all data collected from experimental animals were carefully compared to data collected from age-matched control animals. Minor variation, however, arising from intrinsic variation in the animals or as a result of variation in the RNA isolation and Northern blotting procedures cannot be completely discounted with this approach. Methylene blue staining was used initially to approximate the equality of loading from lane to lane, however a more quantitative analysis was possible due to the inability to detect any

significant changes with the total α-tubulin cRNA probe (see Figure 2). There was always minor variation in the α-tubulin signal, but this variation did not correlate with the experimental treatment, and is probably accounted for by individual variation between animals and potential inequities in gel loading and transfer. Although it was initially hypothesized that total α-tubulin mRNA levels would change with transection, there are several possible explanations for why no changes were observed. It is most likely that the techniques employed are not sensitive enough to detect the changes in a-tubulin expression which should be occurring in the small subpopulation of cells in the hindbrain injured by the transection. The high levels of α-tubulin being expressed in the majority of cells in the hindbrain which were not injured probably diluted the signal from the injured neurons beyond the limits of detection. Alternatively, the levels of α-tubulin expression were already high enough that no further increases were necessary or possible to accomodate the regenerative process. What ever the case, in effect, the total α-tubulin mRNA levels acted as a constitutively expressed message, allowing a quantitative evaluation of the differences in the amount of RNA loaded in each lane which the methylene blue staining was unable to discriminate.

Following all attempts to normalize data and remove variability due to extraneous factors, the GAP 43 mRNA levels, when compared between animals within any one group on a blot (either within transected or control groups of the same age), still showed some minor variability. This undoubtedly reflects the many manipulations of the samples between the tissue isolation and the final densitometric measurement which introduces variation which is too difficult to eliminate. Hence, despite this effort, an accurate quantification of small changes in mRNA levels could not be attained with this technique.

Another major drawback of Northern blot analyses is that results can only be discriminated at the tissue level, with one datum obtained per animal (or pooled tissue sample). The data provides no clues to the cellular events underlying the observed changes. This is a critical point to consider when results are interpreted from a heterogenous collection of cells such as the hindbrain. The *in situ* hybridization experiments now underway should address these concerns. Northern blotting, however, did provide useful information concerning temporal changes in mRNA expression.

DEVELOPMENTAL EXPRESSION OF GROWTH-ASSOCIATED mRNA

It was interesting that the GAP 43 probe recognized two mRNAs during development. The 1.5kb mRNA was described as the authentic GAP 43 message from which the protein is transcribed, and the function of the larger species remains unknown (Baizer et al, 1990). The expression of multiple mRNAs for a single protein is not isolated to GAP 43 expression in the chick, but has also been reported from studies of nicotinic acetycholine receptor mRNA developmental expression in Drosophila CNS (Hermans-Borgmeyer et al, 1989). The large mRNA form found in that system was shown to contain intronic sequences using intron-specific probes on Northern blots. It is unknown, however, if a similar manifestation occurs with GAP 43 expression.

The results shown in Figures 3, 4, 5 and 6 support the first hypothesis, and demonstrated that the expression of GAP 43 and total α-tubulin during development of the chick hindbrain follows the classical growth-associated protein pattern of expression. That is, they are developmentally regulated, with very low levels of mRNA expression early in development, which rise to a peak correlating with the time period of axonal outgrowth and synaptogenesis and

then fall to near adult levels by hatching. These results are similar to the description of GAP 43 (Baizer et al, 1990) and total α-tubulin mRNA expression (Bhattacharya and Sarkar, 1991) in whole chick brain and the reported developmental expression of the 3D5 antigen, identified as chicken GAP 43 protein (Allsopp and Moss, 1989). To date there have been no published accounts of GAP 43 or total α-tubulin mRNA or protein expression in discrete brain regions of the chick.

The measured expression of total α-tubulin mRNA in this study provided an average of all the α-tubulin production across the hindbrain, as all cell types in this tissue produced α-tubulin mRNA(s) and the cRNA probe used to detect α-tubulin mRNA was not specific for any particular isotype. However, the observed peak expression matched the time frame when maximum axonal outgrowth, and hence tubulin requirement, occurred. The expression of tubulin mRNA has been found to be under strict auto-regulatory control (Cleveland, 1988; Cleveland, 1989) meaning there was only as much tubulin message around as was needed to provide tubulin protein for cellular needs. Maximal axon outgrowth from the hindbrain occurred between E8 and E13, when α-tubulin mRNA levels were very high. The peak expression was extended somewhat due to the needs of other cell types such as oligodendrocytes, known to begin myelination around E13 (Bensted et al, 1957; El-Eishi, 1967; State et al, 1977; Costa et al, 1981; Macklin and Weill, 1985). As well, there was a large increase in the size of the embryos from E13 to E18. Although the brainstem-spinal projections were not seeking new target sites during that period, total α-tubulin expression would be required at a somewhat elevated level to supply the microtubules necessary for axon extension to accommodate the growth of the animal. These factors resulted in the broad peak expression observed for the total α-tubulin mRNA.

The GAP 43 peak expression was more defined than the α-tubulin peak, and occurred towards the end of the maximal axonal outgrowth phase of hindbrain development. If GAP 43 was required for actual growth of axons as α-tubulin was, the peak expression should have occurred earlier and covered a broader range of developmental ages, as it did for α-tubulin expression (compare Figures 4 and 6). This suggested that the protein was not needed so much for axonal outgrowth *per se*, but events occurring nearer the end of the axon growth phase such as target recognition or target evaluation and/or synaptogenesis. This is purely speculative but an attractive hypothesis considering the reported growth cone/presynaptic functional correlations of GAP 43 in the developing and adult animal (Skene, 1989). A role for GAP 43 in signal transduction mechanisms has been postulated from the calmodulin binding properties, IP3 regulatory functions, correlations with phosphorylation and LTP induction, and interactions with G_o as described in the introduction (Nelson and Routtenberg, 1985; Lovinger et al, 1985; Skene, 1990; Strittmatter et al, 1990; De Graan et al, 1990). During development (or regeneration) then, GAP 43 may play a role in the process of evaluating potential targets by modulation of second messenger system activity during the course of axon extension. The results of this study correlate the peak of GAP 43 expression with the time when these postulated functions would be occurring. In this respect, mRNA levels did not peak until after axon outgrowth was well on its way, and levels dropped after E12 when all targets had been contacted.

GROWTH-ASSOCIATED mRNA EXPRESSION AFTER TRANSECTION

Total α-Tubulin

The second and third hypotheses, that mRNA levels for total α-tubulin would transiently increase after E11 transection and not change after E14 transection, were not supported by the data obtained by Northern Blotting. In all blots of experimental animals probed with the total α-tubulin cRNA, there was no consistent change in mRNA levels compared to age-matched controls. There was always minor variation in the α-tubulin signal which did not correlate with the experimental treatment, but can be accounted for by individual variation between animals and potential inequities in gel loading and transfer. Although there is a precedent in the literature for changes in α-tubulin levels after injury to CNS or PNS (Pearson et al, 1988; Miller et al, 1989; Miller and Geddes, 1990; Tetzlaff et al, 1991), there are a number of key differences between those reports and this study.

The work quoted above was done using the extremely sensitive technique of *in situ* hybridization with a neuron-specific α-tubulin isotype probe combined with retrograde tracing. This allowed a very precise cellular discrimination of the changes which occurred after injury only in those neurons that were injured. Northern blotting as performed in this study only discriminated changes at the tissue level, and essentially provided an assessment of the average expression across the tissue under study. Unfortunately the total α-tubulin cDNA, capable of discriminating the neuron-specific Tα1 tubulin isotype in the rat, failed to recognize a chicken homologue (data not shown). Therefore, the total α-tubulin cRNA as employed here was detecting all isotypes of α-tubulin being produced by all cells of the hindbrain. Previous studies of α-tubulin expression in chicken

identified 4 isotypes which were expressed in brain (Pratt and Cleveland, 1988). Cα1, cα3 and cα8 were expressed ubiquitously at low levels, although high levels of cα1 and cα8 were found in brain. Cα5 was only found in brain,testis and thymus (Pratt and Cleveland, 1988). It remains unknown which of these isotypes, if any, are expressed exclusively in neurons.

Thus, it is possible that, as only a small subset of cells in the hindbrain were neurons projecting to the spinal cord, the up-regulation of α-tubulin mRNA which was predicted to have occurred in those neurons suffering transections would not be enough to be discerned above the large background of α-tubulin production from the majority of cells in the hindbrain. A second possibility was that the normal developmental α-tubulin mRNA production in the hindbrain was already high enough and no further increase was necessary or possible to accomodate the regenerative processes. The latter scenario was only tenable at the earlier stages of repair after transection when α-tubulin levels were developmentally high, and can not explain why at E21, when the α-tubulin signal was near adult levels in control animals, there were still no changes seen in, for example, the E14 transected, seven day survival group. Resolving this question awaits the completion of the *in situ* hybridization experiments now under way.

GAP 43

The second hypothesis, that GAP 43 mRNA levels would transiently increase after E11 transection was not supported by the data from the Northern blotting experiments. Data from the E11 transection group did not provide conclusive evidence for changes in GAP 43 expression during successful

regeneration. Any changes seen were small and difficult to detect at the tissue level using Northern blotting relative to the amount of mRNA present from the normal developmental expression.

Initially, a small decrease in GAP 43 mRNA levels was observed after both E11 and E14 transection, more so after E11 transection (see Figure 9). This may reflect the increased sensitivity to injury described for the embryonic CNS (Brodal, 1983). Previous experiments have not evaluated the extent of cell death in chick embryos as a result of spinal cord transection at different developmental stages. Perhaps the larger initial decrease in GAP 43 mRNA seen after E11 transection was a result of increased sensitivity to injury at that earlier age. The smaller initial decrease in GAP 43 seen for the E14 transection group suggested that more neurons survived the transection procedure, and resulted in a more robust expression of GAP 43 mRNA similar to that seen during the normal course of development.

Although there was a precedent in the literature for increased GAP 43 mRNA levels after neuronal injury when regeneration was successful (Skene and Willard, 1981a,b; Skene, 1984; Kalil, 1988; Tetzlaff et al, 1991), it is possible that the techniques employed in the present study are just not sensitive enough to detect a change in the minority of neurons which received transections above the high background levels of expression in the majority of hindbrain neurons. The normal developmental expression levels from E12 to E14 were still high (but decreasing) and increases in expression in the subpopulation of neurons receiving transections may simply have been masked. By E16 and E18, when the normal GAP 43 developmental mRNA levels were lower, still no change was detectable after E11 transection. The results from previous anatomical and physiological experiments indicate that regeneration is complete by this time (E16-E18, Hasan et al, 1991, 1992) thus GAP 43 mRNA levels in the transected

neurons had likely decreased as well. Another possibility was that GAP 43 mRNA levels were already high enough at the time of transection (E11) that no further increase was necessary or possible to accommodate the regeneration. This observation did not rule out the possibility of subsequent regeneration contributing to the repair process, however, data from double retrograde tracing experiments (Hasan et al, 1992) refute this possibility.

This was in marked contrast to the mRNA expression levels after a restrictive period transection, which rose dramatically by three days post-transection and remained elevated compared to control animals throughout the period examined in this study. This supports the third hypothesis as it applies to GAP 43. Recent reports in the literature suggested that GAP 43 mRNA levels increased after neuronal injury whether or not regeneration was successful (Doster et al, 1991; Tetzlaff et al, 1991) but the time course of the response differed between the two paradigms. When regeneration was successful, the re-expression of GAP 43 correlated with the re-growth of the injured axons, and was down-regulated at the time of target contact and formation of appropriate synapses (Tetzlaff et al, 1991). When regeneration failed, the re-expression of GAP 43 remained elevated long after the time it should have taken to grow to and re-connect with appropriate targets (Doster et al, 1991; Tetzlaff et al, 1991). The results from this study correlated well with these recent observations. Thus the rise in GAP 43 mRNA levels observed here may indicate a futile return to an earlier developmental growth "mode" with a prolonged re-instatement of the 6.3kb/1.5kb band density ratio reminiscent of the peak expression seen at E10. Perhaps it was the failure to re-connect with appropriate targets which did not allow the down-regulation of GAP 43 mRNA.

Recent work by the Benowitz group has shed some light upon the cellular mechanisms responsible for regulation of GAP 43 mRNA levels. They used

Northwestern blotting to identify GAP 43 mRNA binding protein(s) found in developing neocortex and differentiating PC12 cells which appeared to confer message stability (Irwin et al, 1991). These were small (17kDa) cytosolic protein(s) which bound to defined sites in the 3' untranslated region of the GAP 43 mRNA. Inhibition of protein synthesis in PC12 cells did not block the increased stability of the GAP 43 message during induction of neurite outgrowth (Cansino et al, 1991), which indicated that the protein(s) were present but activated only when increased GAP 43 message was required. The chicken GAP 43 cDNA sequence incorporates two segments within the 3' untranslated region which were highly conserved between avian and mammalian species (Baizer et al, 1990). One segment (nucleotides 934-959) was shown to produce a stem-loop structure which may alter message stability (Reeves et al, 1986), the second (nucleotides 992-1028) contains an AUUUA motif which has been shown to affect mRNA stability (Cleveland and Yen, 1989). It was likely that a similar mechanism occurred in our system, that the developmental expression and up-regulation seen after transection was due to increased message stability rather than an increased transcription rate.

CONCLUSIONS AND FUTURE EXPERIMENTS

The data supported the first hypothesis, indicating that GAP 43 and α-tubulin mRNA expression in the hindbrain of the embryonic chick was developmentally regulated and correlated with periods of axon outgrowth and synaptogenesis. Consistent changes in the hindbrain expression of α-tubulin after injury to the spinal cord were not measurable using Northern blotting. This was likely due to the insufficient sensitivity of the technique combined with the small proportion of neurons in the hindbrain which were injured by the transection. The second and third hypotheses concerning a transient re-expression of total α-tubulin after E11 spinal cord injury and a maintained re-expression after E14 injury were not supported by the data. Total α-tubulin levels after transection provided an internal control for measuring changes in GAP 43 expression. The data did not support the second hypothesis concerning a transient re-expression of GAP 43 after E11 spinal cord transection, as levels of GAP 43 mRNA in the hindbrain did not detectably change after permissive period spinal cord transection. In contrast, transection during the restrictive period resulted in a maintained increase in GAP 43 mRNA levels, which supported the third hypothesis concerning GAP 43. These results suggested that the embryonic CNS retained the ability to appropriately re-express a part of the genetic growth program after injury on E14 as indicated by the appropriate increase in GAP 43 mRNA levels when there was no physiological or anatomical evidence for successful regeneration.

To further characterize the neuronal response to injury in this system, several experiments are required. A broader range of GAPs will be studied at the cellular level using combined retrograde tracing and *in situ* hybridization histochemistry. This will allow a greater resolution of the response to injury of

identified brainstem-spinal projection neurons. Collaborations are underway with several groups supplying novel and identified GAP cDNAs for testing in this system.

It is unknown if the observed failure of regeneration seen after E13 is due to the absence of some facilitatory influence such as neurotrophic factors or the presence of some inhibitory factor such as myelin components. Growth factor administration has been shown to enhance the regenerative ability of responsive neurons within the CNS (Hefti, 1986; Williams et al, 1986 Kromer, 1987; Gage et al, 1988; Hagg et al, 1989), and our system can be used to rapidly screen the *in vivo* consequences of putative CNS neuronal trophic factors such as the neurotrophins, fibroblast growth factors, etc. With the added sensitivity of using *in situ* hybridization, very subtle effects of a variety of factors can be measured.

Coincidentally, the process of myelination begins around E13 in the chick embryo (Bensted et al, 1957; Costa et al, 1981; El-Eishi, 1967; Macklin and Weill, 1985; State et al, 1977). Evidence is accumulating for an inhibitory role played by myelin contributing to the failure of CNS regeneration. CNS myelin (Savio and Schwab, 1989) and oligodendrocytes (Schwab and Caroni, 1988) are poor substrata for neurite outgrowth *in vitro*. Recently, several proteins have been isolated from CNS myelin which contributed to its inhibitory properties (Caroni and Schwab, 1988). An elegant study by Schnell and Schwab (1990) demonstrated that immunochemical blockade of one of these proteins *in vivo* resulted in improved anatomical regrowth of severed adult rat corticospinal axons.

Recent efforts by a graduate student in our lab, Hans Keirstead, have demonstrated that the onset of myelination in the chick embryo can be delayed well into the restrictive period for repair. Injection of antibodies to galactocerebroside combined with serum complement proteins resulted in the

delay of the onset of myelination until E17. Preliminary experiments suggest that the absence of myelin enhanced the ability of the spinal cord to repair damage done on E15 (Keirstead et al, 1991). It may prove interesting to examine the molecular response to injury in the dysmyelinated spinal cord injured animals.

These experiments have opened the door to a careful molecular examination of the neuronal response to injury in a system well-suited for this type of study. Expanding the scope of GAPs in the repertoire and measuring the response at the cellular level with *in situ* hybridization should provide a sensitive system for examining the neuronal response to injury and the effectiveness of experimental intervention.

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