INTRINSIC VERSUS EXTRINSIC FACTORS
ASSOCIATED WITH
EMBRYONIC NERVE-MUSCLE SPECIFICITY

By

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ABSTRACT

Previous analyses showed that following heterotopic transplantation of a thoracic neural tube segment into the region of the extirpated brachial neural tube in experimental (Thor-Br) chick embryos at embryonic day -2 (day -2E) the wings of hatched Thor-Br chicks are immotile. It was not known, however, if functional nerve-muscle interaction occurs during embryogenesis of Thor-Br embryos, although structural nerve-muscle connections do form in wing muscles during initial development of this experimental model. From day 8.5-9E onward, however, thoracic nerve-brachial muscle unions progressively uncouple in individual wing muscles.

The present study explored the nature of initial nerve-muscle contacts and employed a well documented functional parameter, wing motility, to monitor daily the development of functional nerve-muscle interactions in wings of individual Thor-Br embryos. Control embryos were either unoperated (UC) or received a homotopic brachial neural tube transplant (Br-Br). The results demonstrated that Thor-Br embryos exhibited normal frequencies of wing movement up to day -8E; but the frequency of wing movement became greatly reduced from that of controls following this initial developmental period. The loss of wing motility in Thor-Br embyos coincided temporally with the withdrawal of intramuscular axons from individual wing muscles reported previously. Thus, foreign thoracic nerves did establish initial functional contacts with wing muscles, however, these connections were subsequently deemed inappropriate and nerve-muscle unions progressively uncoupled.

To investigate factors responsible for the nerve-muscle uncoupling phenomenon observed in experimental (Thor-Br) embryos the development of heterotopically transplanted thoracic neural tubes was compared to that of neural tubes in control (Br-Br and UC) embryos. Parameters analysed include the pattern of

peripheral nerve outgrowth, neural tube histogenesis, the source of motor innervation to individual wing muscles and the pattern of motoneuron death, growth and differentiation. The results showed that while the pattern of nerve outgrowth was controlled by local environmental signals, developmental events within the neural tube were governed autonomously, independent of the periphery. Thus, heterotopically transplanted thoracic neural tubes developed according to their site of origin.

In addition to the heterotopic neural tube (Thor-Br) transplantation experiments a second series of surgically manipulated chick embryos (Thor-Br/som) was employed to determine if nerve-muscle incompatibility is limited to a thoracic nerve-brachial muscle combination. Experimental (Thor-Br/som) embryos were produced by transplanting thoracic somitic mesoderm into the site of extirpated brachial somites at day -2E. Daily wing motility analyses were performed to determine the extent of functional nerve-muscle interaction between *in situ* brachial nerves and wing muscles derived from heterotopically transplanted thoracic somitic mesoderm. The results demonstrated that functional nerve-muscle interaction did occur in wings of Thor-Br/som embryos and, in contrast to experimental (Thor-Br) embryos, wing motility was maintained in Thor-Br/som embryos throughout the developmental period analysed (day -6E through day -16E). Nerve-muscle uncoupling, therefore, did not occur in heterotopic somitic mesoderm transplantation experiments.

It was concluded that eventual nerve-muscle incompatibility observed following heterotopic neural tube (Thor-Br) transplantation is related to the rigidity of developmental processes within the spinal cord. Whereas heterotopically transplanted thoracic somitic mesoderm exhibited a high degree of plasticity and conformed to peripheral signals derived from the brachial environment, transplanted thoracic neural tubes were unable to respond to environmental signals and were eventually deemed inappropriate.

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To Connie, Steven and David

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LIST OF ABBREVIATIONS

day -6E:	embryonic day 6
St 29:	Hamburger and Hamilton (1951) stage 29 chick embryo.
Thor-Br:	Heterotopic thoracic neural tube transplant model.
Thor-Br/som:	Heterotopic thoracic somitic mesoderm transplant model.
DThor-NBr/som:	Dystrophic thoracic somitic mesoderm transplanted into the site of extirpated brachial somites in normal host.
NThor-NBr/som:	Normal thoracic somitic mesoderm transplanted into the site of extirpated brachial somites in normal hosts.
Br-Br:	Control embryos in which the brachial neural tube was removed and replaced with a donor brachial neural tube.
PBR:	Prebrachial neural tube removal embryos.
Thor-C:	control *in situ* thoracic neural tubes in UC embryos.
Br-C:	control brachial neural tubes in Br-Br and UC embryos.
UC:	unoperated control embryos.
52 h *in ovo*:	52 hours *in ovo*.
HBSS:	Hank's balanced salt solution.
CER:	chick embryo Ringer's solution.
M/10:	number of right wing movements in 10 minute observation periods.
M/min:	number of right wing movements per minute of activity.
LMC:	lateral motor column.
MMC:	medial motor column.
CT:	sympathetic preganglionic column of Terni.
HRP:	horseradish peroxidase.

INTRODUCTION

During embryonic development, structural and functional connnections are formed between motor neurites exiting the neural tube and developing muscle fibres. To understand fully nerve-muscle interaction, it is necessary to study the relationship between developing nerves and muscles during embryogenesis, i.e., prior to 4 days *in ovo* when the nerve and muscle components are physically separated and during the remainder of embryonic development when physical interaction exists. The present report is a detailed study of the development of nerve-muscle interactions in the chick embryo extending from St 29(day -6E) through St 42 (day -16E).

The chick embryo model, as opposed to mammalian models, is amenable to numerous experimental treatments and manipulations at early embryonic stages and experimental embryos are easily observed for extended developmental periods through openings in the egg shell. For these reasons, many studies have employed avian embryos to examine embryonic nerve-muscle development. The following review is, therefore, concerned primarily with investigations into avian neuromuscular development.

A. Motor Nerves Control Muscle Phenotypes After Hatching

Previous cross-reinnervation experiments demonstrated that after birth motor nerves exert profound control over muscle fibre-type differentiation in both mammalian (Buller, Eccles and Eccles, 1960; Bárány and Close, 1971; Douglas and Cosmos, 1974; Law, Cosmos, Butler and McComas, 1976) and avian (Koenig and

Fardeau, 1973; Cosmos, Butler, Allard and Mazliah, 1979; Mazliah, 1980) species. Two particular brachial muscles in the chicken, the anterior (ALD) and posterior (PLD) latissimus dorsi muscles, are especially well suited for cross-reinnervation studies because of their close proximity to each other and since each muscle expresses distinct phenotypic characteristics (Cosmos, Butler, Allard and Mazliah, 1979; Mazliah, 1980; Mazliah, Cosmos and Butler, 1987). When the PLD (a fast-twitch, glycolytic muscle) is denervated and immediately cross-reinnervated with the nerve of the ALD (a slow-tonic, oxidative muscle) in newly hatched chicks, the experimental PLD assumes structural, biochemical and physiological properties of the ALD (Cosmos, Butler, Allard and Mazliah, 1979; Mazliah, 1980; Mazliah, Cosmos and Butler, 1987).

The phenotypic alterations in cross-reinnervated PLD muscles are most obvious when one compares the muscle fibre-type profiles between unoperated and cross-reinnervated muscles using myosin ATPase histochemical reactivity; the activity of this enzyme is correlated with the speed of muscle contraction (Bárány, 1967). As is typical for fast-twitch muscles, unoperated PLD muscle fibres stain intensely for myosin ATPase activity after alkali preincubation only (alkali stable) and exhibit no reactivity following acid preincubation (acid labile) [Cosmos, Butler, Allard and Mazliah, 1979]. Conversely, fibres of the slow-tonic ALD muscle exhibit a strong myosin ATPase histochemical reaction after both alkali and acid preincubation (Asiedu and Shafiq, 1972).

Similar to unoperated ALD fibres, PLD muscle fibres successfully cross-reinnervated after hatching by the ALD nerve are characterized by dual alkali and acid stability, a muscle fibre phenotype not normally seen in the mature PLD (Koenig and Fardeau, 1973; Cosmos, Butler, Allard and Mazliah, 1979; Mazliah, Cosmos and Butler, 1987). Cross-reinnervation experiments performed in newly hatched chicks, therefore, indicate that motor nerves can dictate muscle fibre-type expression during *ex ovo* development. These experiments did not determine, however, if the ALD nerve actually induces *de novo* formation of this new muscle fibre phenotype in cross-reinnervated PLD muscles or, instead, de-represses an embryonic phenotype evident early in embryogenesis and repressed during subsequent development of this muscle. To investigate this question, Butler and Cosmos (1981a) first developed modified myosin ATPase and silver-cholinesterase histochemical reactions to examine muscle-nerve formation in chick embryos. Using these techniques, Butler and Cosmos (1981b) then performed a detailed study into the embryonic development of the latissimus dorsi primordium, extending from St 16 (51-56 h *in ovo*) through St 46 (day 20-21E). This study showed that at St 28 (day -5.5E), the earliest embryonic time that a distinct latissimus dorsi primordium is observed, the primordium is composed of myogenic cells which are alkali stable only. At St 29-30 (day 6-6.5E) the latissimus dorsi primordium divides into anterior (ALD) and posterior (PLD) parts and now myogenic cells within the ALD start to acquire dual acid and alkali stability. During incipient formation of the latissimus dorsi primordium, the PLD muscle expresses alkali stable myosin ATPase activity , similar to mature PLD muscles of adult chickens (Butler and Cosmos, 1981b). The differentiation of muscle fibres with dual alkali and acid stable myosin ATPase activity in PLD muscles cross-reinnervated by the ALD nerve, therefore, would represent *de novo* formation of this muscle fibre phenotype induced by the foreign ALD nerve.

An alternative explanation, however, based on the following observations must be considered. During the second half of embryogenesis, day -11E onward, a subpopulation of fibres appears within the PLD muscle which is both alkali and acid stable. These fibres, scattered throughout the PLD muscle, increase in number until a maximum of approximately 800-900 fibres (25% Of total) is noted between day-14E and day -16E (Toutant, Toutant, Renaud and LeDouarin, 1979; Butler and Cosmos, 1981a; Renaud, Gardahaut, Renaud and LeDouarin, 1983). Beyond this period, these fibres decrease in number; by 1 month after hatching only 78 fibres remain (Renaud, Gardahaut, Renaud and LeDouarin, 1983; Mazliah, Cosmos and Butler,1987). Thus, the possibility exists that the presence of ALD-type fibres within cross- reinnervated PLD muscles does not represent the *de novo* synthesis of these fibres due to the influence of the foreign ALD nerve but, instead, a preferential innervation of those ALD-type fibres which appear transiently during embryogenesis (Mazliah, Cosmos and Butler, 1987)

B. *In Ovo* Expression Of Muscle Fibre Types Is Independant Of Nerve Influences

The experiments of Butler and Cosmos (1981a,b) demonstrate that myosin ATPase activity is an excellent marker to monitor muscle differentiation in early chick embryos. In addition, these investigators demonstrated that nerves enter the primary premuscle masses of the wing bud coincident temporally with the onset of myosin ATPase activity (Butler and Cosmos, 1981b). It was not known, however, if nerves were necessary for the initial expression and differentiation of specific muscle fibre types. Subsequent analyses of developing brachial muscles rendered aneurogenic by surgical ablation of the brachial neural tube prior to peripheral nerve outgrowth, addressed this question directly (Butler, Brierley and Cosmos, 1980; Butler, Cosmos and Brierley, 1982a; Phillips and Bennett, 1984). These studies showed that aneurogenic and innervated (control) brachial muscles in chick embryos exhibit identical myosin ATPase profiles throughout the period analysed. Furthermore, it was reported that embryonic events associated with the early formation of individual brachial muscles, including migration of myogenic precursor cells to the forellimb bud and cleavage of the premuscle masses of the wing, occur on schedule in the absence of innervation (Butler, Cosmos and Brierley, 1982a). Similarly, Sohal and Sickles (1986) found that when an extra-ocular muscle, superior oblique, is made aneurogenic by destruction of the trochlear nucleus in early duck embryos, it too differentiates myosin ATPase profiles identical to innervated superior oblique muscles. Using the same duck model, Sohal and coworkers demonstrated that other myogenic processes, including formation of acetylcholine receptors (Creazzo and Sohal, 1983), differentiation of high molecular weight acetylcholinesterase and specialization of the sarcolemma to form postsynaptic folds and basal lamina (Sohal and Wrenn, 1984), also occur in aneurogenic superior oblique muscles. Together, these experiments show that a number of myogenic events during early *in ovo* formation and differentiation of skeletal muscle are controlled endogenously, independent of the nervous system (Butler, Cosmos and Brierley, 1982a; Phillips and Bennett, 1984; Sohal and Sickles, 1986).

Peripheral nerves, however, do influence the growth and survival of developing muscles *in ovo*. Aneurogenic muscles fail to grow normally and eventually degenerate and disappear in both duck (Sohal and Holt, 1980; Creazzo and Sohal, 1983; Sohal and Sickles, 1986) and chick embryos (Butler, Cosmos and Brierley, 1982a; Bloom, Butler, Brierley and Cosmos, 1985). Direct electrical stimulation, however, increases the growth and survival of aneurogenic brachial muscles, indicating that these myogenic processes rely on impulse-mediated activity (Bloom, Cosmos and Butler, 1983; Bloom, Butler, Brierley and Cosmos, 1985). Therefore, although the incipient differentiation of muscle fibre types occurs independent of nerves, the growth and survival of embryonic skeletal muscles is dependent on neuronal influences.

C. *In Ovo* Cross-Innervation Experiments

1. Foreign Nerves Do Not Affect Muscle Fibre-Type Differentiation *In Ovo*

To investigate further the respective roles of nerves and muscles during embryogenesis, surgically manipulated chick embryos were employed in Dr. Cosmos' laboratory in order to impose a foreign innervation on developing brachial muscles (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos,1986). In these cross-innervation experiments, the brachial neural tube was removed at St 13 (48-52 h *in ovo*), prior to peripheral nerve outgrowth, and replaced with a thoracic neural tube segment; thus, thoracic nerves derived from the heterotopic neural tube transplant were paired with brachial muscles (targets which thoracic nerves never innervate normally). Experimental embryos were analysed to determine if, similar to cross-reinnervation experiments performed after hatching, foreign motor nerves *in ovo* alter fibre-type expression of individual brachial muscles. Histochemical analyses of myosin ATPase activity, however, demonstrated that embryonic brachial muscles cross-innervated by foreign, and inappropriate, thoracic nerves exhibit appropriate fibre-types (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). Eventually, however, the union between brachial muscles and thoracic nerves is deemed inappropriate; the experimental muscles ultimately degenerate to the point where they resemble aneurogenic muscles (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). In contrast, brachial muscles cross-innervated by foreign but appropriate lumbosacral nerves survive throughout the embryonic period. Furthermore, these experimental muscles analysed during the last week of embryogenesis exhibit normal myosin ATPase patterns (Khaskiye, Toutant, Toutant, Renaud and LeDouarin, 1980; Laing and Lamb, 1983a). Recently, Vogel and Landmesser (1987) showed that chick embryo leg muscles innervated by foreign nerves following extensive rotations of the lumbosacral neural tube also exhibit normal myosin ATPase profiles, although a small percentage of the total number of muscles examined (14%) showed altered fibre types.

As reviewed recently by Sanes (1987), the pioneer research in Dr. Cosmos' laboratory (Butler and Cosmos, 1981a,b; Bloom, Butler, Brierley and Cosmos, 1985; Butler, Cauwenbergs and Cosmos, 1986), using differential myosin ATPase activity as a marker to study muscle fibre-type differentiation during both unaltered and experimentally altered development, has been amply confirmed by others who employed either the same parameter introduced by Cosmos or monoclonal antibodies for fast and slow muscle fibres. Thus, to date, experimental evidence supports the view that fibre-type differentiation occurs early *in ovo* and that this process proceeds independent of neuronal influences.

2. Foreign Nerves Induce Growth And Survival Of Embryonic Muscles

Although foreign nerves fail to influence muscle fibre differentiation *in ovo*, nerves derived from a thoracic neural tube transplanted to the site of the extirpated brachial neural tube exert a significant growth promoting affect on individual brachial muscles (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). Volumetric analyses of individual wing muscles demonstrated that, in contrast to aneurogenic wing muscles which exhibit limited growth (Butler, Cosmos and Brierley, 1982a; Bloom, Butler, Brierley and Cosmos, 1985), the growth of wing muscles innervated by foreign thoracic nerves is equivalent to that of control muscles during the first week of embryogenesis (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). In addition, the PLD is rescued by these nerves since PLD muscles innervated by foreign thoracic nerves survive well beyond the embryonic time when aneurogenic PLD muscles disappear (Butler, Cauwenbergs and Cosmos, 1986). In these experiments, therefore, thoracic nerves establish effective contacts with brachial muscles as evidenced by increased growth and survival of individual wing muscles innervated by nerves derived from the heterotopically transplanted thoracic neural tube.

During the second week of embryogenesis, however, initial nerve-muscle contacts in the brachial region of heterotopic neural tube transplant embryos uncouple and wing muscles degenerate progressively and are replaced by lipid (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). Although foreign thoracic nerves can initially support muscle growth and survival, permanent neuromuscular junctions are not observed in wing muscles of these heterotopic innervation experiments; thus, foreign thoracic nerves establish initial contacts with wing muscles, but these nerve-muscle unions are eventually deemed inappropriate and are severed.

D. Objectives And Rationale Of The Present Study

In the present study, analyses of the heterotopic innervation model used by Butler, Cosmos and Brierley (1982b) were performed to explore further the nature of initial nerve-muscle contacts which have been demonstrated to induce growth and survival of wing muscles in these experimental embryos. Specifically, a major aim of the present experiments was to investigate the expression of a functional interaction between brachial muscles and thoracic nerves derived from a transplanted thoracic neural tube. Furthermore, since compatibility at other levels is not expressed throughout embryogenesis, it was necessary to select a non-invasive parameter which was amenable to daily analyses of individual experimental embryos during an extended embryonic period, i.e., day -6E to day -16E. Wing motility, a well documented method to monitor functional nerve-muscle interaction (for review, Oppenhiem, 1982; Provine, 1986), satisfied this criterion.

1. Embryonic Motility

Embryonic movements were first examined by Preyer in the late 1800's; however, little effort was given to a thorough analysis of embryonic behavior until Hamburger and coworkers initiated the first systematic studies in the early 1960's. These investigators gave a detailed developmental account of chick embryonic behavior and also performed elegant surgical manipulations to determine its origin and significance (Hamburger and Balaban, 1963; Hamburger, Balaban, Oppenheim and Wenger, 1965; Hamburger and Oppenheim, 1967).

The first movements performed by chick embryos occur at day 3.5-4E and consist of so called S-wave contractions of the body axis (Hamburger and Balaban, 1963). These initial movements and all movements throughout embryogenesis are periodic, that is, they occur during periods of activity interrupted by quiescent periods of variable length. Limb movements are initiated on day -6E (Hamburger and Balaban, 1963; Hamburger and Oppenhiem, 1967), an event known to coincide temporally with the onset of polyneuronal bursts of activity within the spinal cord (Provine, 1972).

Hamburger and Balaban (1963) reported that reflex responses to sensory stimuli cannot be elicited until day -7.5E, and suggested that embryonic limb movements may be non-reflexogenic in nature. The spontaneous, non-reflexogenic nature of embryonic limb motility was subsequently proven by Hamburger, Wenger and Oppenheim (1966) who surgically isolated the lumbosacral neural tube at day -2E from all sensory, propriospinal (intersegmental) and supraspinal inputs and demonstrated high frequencies of leg motility even up to day -17E.

To investigate further the spontaneous origin of embryonic limb motility, Provine and coworkers initiated several pivotal studies on the neurophysiological basis of behavioral development (Provine, Sharma, Sandel and Hamburger, 1970). Specifically, by using sensitive recording electrodes placed at precisely defined loci within the lumbosacral spinal cord of chick embryos, Provine (1972) showed that, although electrical signals were recorded from most spinal cord regions, prominent electrical burst discharges were restricted to the ventral spinal cord. Ripley and Provine (1972) demonstrated that these polyneuronal bursts of electrical activity represent the neural correlate of embryonic limb motility, since bursting activity and limb motility are temporally coincident.

Provine (1980) demonstrated further that wing and leg motility phases occur concurrently in chick embryos. The electrophysiological studies of Provine and colleagues indicate that spontaneous polyneuronal bursts of electrical activity originate throughout the ventral spinal cord and rapidly propagate along its length (Provine, 1971; Provine and Rogers, 1977). Motor output from the brachial and lumbosacral spinal cord regions, therefore, occurs synchronously and elicits concurrent wing and leg motility (Stokes, 1976; Provine, 1980). Propagation of electrical activity through the developing spinal cord is thought to occur along propriospinal (intersegmental) connections, demonstrated as early as day -5E in chick embryos (Oppenheim, Chu-Wang and Foelix, 1975; Singer, Skoff and Price, 1978; Nornes, Hart and Carry, 1980). Synchronous motility of ipsilateral wings and legs in embryonic chicks, therefore, is an overt expression of the synchronized pattern of motor signals emanating from the spinal cord.

During embryogenesis of the chick, therefore, electrical signals can be recorded from the developing spinal cord from the earliest stage examined (day -5E) onward (Provine, 1972). Initially (day -5E) only unit activity is evident, indicating that at this time in development isolated neurons discharge singly. Polyneuronal burst discharges, which appear to be simple accelerations of unit activity, become evident by day 5.5-6E. Then, as development proceeds, clusters of burst discharges occur, first separated by 1-2 minutes of inactivity at day -6E (onset of limb motility) followed by a progressive increase in the duration of bursting activity; by day -13E, when peak frequencies of limb motility are observed (Hamburger, Balaban, Oppenheim and Wenger, 1965), interburst intervals, periods when bursting activity is not observed, disappear. From day -13E through day -15E, the long duration burst discharges become replaced by shorter, more frequent bursts which occur at irregular intervals. Provine (1972) suggested that altered electrical activity after day -13E may reflect either the decreased capacity of spinal neurons to discharge spontaneously or the influence of inhibitory circuits, possibly from supraspinal levels. It was later demonstrated that supraspinal input does not influence the frequency of limb movements until after day -13E (Oppenheim, 1975; Sohal, 1976; Provine and Rogers, 1977; Butler, Cauwenbergs and Cosmos, 1986). During late embryonic stages, however, coordinated movements necessary for hatching fail to occur in the absence of supraspinal control (Oppenheim, 1975).

These motility studies, therefore, eliminated the former stimulus-response theory of embryonic motility held by most early embryologists (for review, Provine, 1973; 1986; Oppenheim, 1982) and firmly established the spontaneous origin of limb motility within the developing spinal cord. The absence of wing motility in chick embryos from which the brachial neural tube was removed at day -2E demonstrated further the neurogenic origin of limb motility (Bloom, Butler, Brierley, and Cosmos, 1985). Limb motility, therefore, is an overt expression of the response of limb muscles to electrical signals generated autonomously by spinal motoneurons. Wing and leg movements observed from day -6E onward can, therefore, be used to monitor the development of functional interactions between limb muscles and spinal motorneurons.

For the present study, embryonic wing motility analyses were employed to determine if indeed functional interactions occur between wing muscles and foreign nerves derived from a heterotopically transplanted thoracic neural tube. If wing motility is evident in these heterotopic innervation experiments, it was reasoned that abnormal frequencies of wing movements might reflect the nerve-muscle uncoupling phenomenon reported previously (Butler, Cosmos and Brierley, 1982b).

2. Analyses Of Developmental Events Within The Spinal Cord

A second aim of the present study was to perform a detailed developmental analysis of the heterotopically transplanted thoracic neural tube in an effort to understand possible factors responsible for the eventual incompatibility observed between wing muscles and foreign thoracic nerves. Parameters examined include the pattern of peripheral nerve outgrowth and neural tube histogenesis, to determine the morphological properties of transplanted neural tubes, as well as retrograde horseradish peroxidase (HRP) motoneuron labelling to define precisely the origin of motor innervation of wing muscles supplied by foreign thoracic nerves. In addition, the pattern of motoneuron death, growth and differentiation, parameters never before analysed in thoracic neural tubes, were examined to investigate the effects of contact with an enlarged periphery (wing musculature) on thoracic motoneurons.

(a) Properties of the Thoracic Neural Tube: The developing spinal cord becomes regionalized very early in development, prior to day -2E (Wenger, 1951). Distinct morphological regions, however, are not recognized until day 4.5-5E (Levi-Montalcini, 1950). The thoracic neural tube characteristically develops a medial motor column (MMC) which is continuous with and similar in size to the MMC of both the brachial and lumbosacral neural tube regions (Wenger, 1951). The lateral motor column (LMC) of the thoracic spinal cord, however, is composed of small intermittent clusters of neurons located ventrolaterally, similar to the location of the LMC in limb innervating spinal regions where the LMC is large and prominent. Also, the thoracic neural tube region typically develops a discrete column of neurons located medially near the dorsolateral margin of the central canal, called the column of Terni (Levi-Montalcini, 1950; Oppenheim, Maderdrut and Wells, 1982). The column of Terni (CT), which in chick embryos is evident only in the thoracic and first two lumbar spinal segments (Oppenheim and Chu-Wang, 1983), is composed of sympathetic preganglionic neurons which innervate target postganglionic cells within the sympathetic chain of ganglia located bilaterally adjacent to the vertebral column.

The following is a brief summary of the developmental events which give rise to the LMC, MMC and CT as first described by Levi-Montalcini (1950). All three cell columns are comprised of cholinergic neurons (Oppenheim, Maderdrut and Wells, 1982) which originate early in development (day -3E) from a single morphologically uniform column of cells located ventrolaterally in the neural tube. Starting at day -4.5E, neurons destined to form the MMC and CT migrate medially from this primordial cell column, while cells forming the LMC remain in a ventrolateral position. Then, while MMC motoneuron migration stops in a ventromedial position, CT neurons separate from the MMC and migrate dorsally to reach their final location near the central canal by day -5.5E. It should be noted that the source of all neurons in the nervous system, including those early cells which form the uniform column of cells mentioned above, is the ependymal layer of the neural tube. It was demonstrated, however, that primitive neuroblasts of the ependymal layer only proliferate to produce presumptive motoneurons up to day -4E (Hamburger, 1948; Corliss and Robertson,, 1963; Hamburger, 1975; Hollyday and Hamburger, 1977) and once migration of motoneurons from the ependymal layer is initiated, proliferation ends.

Although motoneuron proliferation stops at day -4E, lateral migration of primordial motoneurons from the central proliferative region of the neural tube into the LMC continues to day -5.5E (Hamburger, 1975). Thus, during incipient formation of the LMC, MMC and CT primitive neurons migrate in opposite directions: MMC and CT neurons migrate medially away from the uniform, ventrolateral cell column and at the same time LMC motoneurons migrate laterally into this column.

Coincident temporally with these migratory events all three neuronal populations (LMC, MMC and CT) extend neurites into the periphery (Levi-Montalcini, 1950). LMC, MMC and CT fibres all exit the neural tube via the ventral roots, but, while LMC and MMC neurites join sensory fibres to form segmental spinal nerves, CT fibres immediately upon exiting the neural tube branch away from the ventral roots to form communicating rami which enter the sympathetic chain ganglia, where postganglionic target cells are located. Since the CT is restricted to the thoracic and upper lumbar spinal segments, communicating rami are observed only at these spinal levels and never occur in the brachial region (Levi-Montalcini, 1950; Oppenheim, Maderdrut and Wells, 1982). An abortive CT which forms early in embryogenesis and then disappears has been described, however, in the cervical region of chick embryos (Levi-Montalcini, 1950). This transient cell column, which follows the same developmental pattern as noted for the thoracic CT, extends neuronal processes into adjacent cervical sympathetic ganglia via temporary communicating rami. The cervical CT fibres then, for reasons that remain unknown, abruptly withdraw from the cervical sympathetic ganglia, communicating rami disappear and the cell column degenerates by day -5E (Levi-Montalcini, 1950). If, however, the cervical neural tube is transplanted into the thoracic neural tube region, the CT within the heterotopically transplanted neural tube no longer degenerates and stable structural contacts with thoracic sympathetic chain ganglia are established (Shieh, 1951). The observations of Shieh (1951) indicate that the cervical CT can substitute for thoracic CT neurons and, in doing so, establish contacts with suitable target cells within the sympathetic chain ganglia.

In the present study, the development of structural phenotypes within transplanted neural tubes, such as the LMC, MMC and CT, were investigated to determine if morphogenetic factors in transplanted thoracic neural tubes are related to the eventual uncoupling observed between wing muscles and foreign thoracic nerves derived from a heterotopic neural tube transplant.

(b) Source of Motor Innervation : In addition to structural analyses of transplanted neural tubes, the source of motor innervation to individual wing muscles innervated by foreign thoracic nerves was determined using HRP retrograde labelling of spinal motoneurons. Based on the well documented knowledge that individual limb muscles are innervated in a highly specific manner by motoneuron pools localized precisely within the spinal cord (for reviews, Landmesser, 1980; Grinnell and Herrera,

1981) it was hypothesized that abnormal nerve-muscle connectivity might account for the eventual incompatibility observed between wing muscles and foreign thoracic nerves.

Outgrowing motor neurites follow distinct, stereotyped pathways into the developing limb bud to establish initial nerve-muscle contacts (Lance-Jones and Landmesser, 1981a). Thus, individual neurites form specific connections with appropriate limb muscles [i.e., embryonic nerve-muscle connectivity is the same as that of adult animals (Landmesser, 1978a,b; Landmesser, 1980)]. Starting at the earliest embryonic time [St 27-28 (day 5-5.5E)] that nerve-muscle contacts can be demonstrated in leg muscles using *in vitro* stimulation of spinal nerves (Landmesser and Morris, 1975), discrete clusters of motoneurons, located in characteristic positions along the rostrocaudal and mediolateral axes of the LMC in the limb innervating regions of the spinal cord, are connected precisely with specific limb muscles (Landmesser, 1978a,b; Hollyday, 1980; 1983; Oppenheim, 1981a). Motoneurons located in a dorsomedial position within the LMC innervate limb muscles derived from the ventral premuscle mass (flexors), while ventrolateral motoneurons in the LMC innervate muscles which originated from the dorsal premuscle mass (extensors). Nerve-muscle specificity, therefore, is expressed throughout embryogenesis. Inappropriate nerve-muscle connections, however, also occur in normal development, but these are rare and are subsequently eliminated (Landmesser, 1978b; Laing, 1982; Hollyday, 1983; but see Pettigrew, Lindeman and Bennett, 1979).

Since the majority of axons establish appropriate connections, neither retraction of inappropriate nerve projections nor death of motoneurons having incorrect muscle connections is the primary mechanism for generation of nerve-muscle specificity. In fact, when naturally occurring motoneuron death (see summary of motoneuron development below) is experimentally reduced using chronic injections of d-tubocurarine, a neuromuscular blocking agent, peripheral motor nerve projections are unaltered from the normal pattern, indicating that natural motoneuron death during embryogenesis does not function to remove errors in motor nerve connectivity (Oppenheim, 1981a).

To date, the developmental mechanisms which control nerve-muscle specificity are not clearly defined, although evidence from surgically manipulated chick embryos indicates that motoneurons are specified for particular muscle targets prior to the onset of peripheral nerve outgrowth (Landmesser, 1980; 1984). Lance-Jones and Landmesser (1980a) demonstrated that following deletions of a small portion of the lumbosacral neural tube which normally innervates proximal leg muscles, peripheral motor nerves exiting the remaining portion of the neural tube grow past uninnervated proximal muscles and innervate only appropriate distal leg muscles. Remaining motor neurites, therefore, do not respond to environmental signals which normally direct growing axons to proximal leg muscles. Further experiments of Whitelaw and Hollyday (1983) and Tosney and Landmesser (1984) demonstrate that when hindlimb buds are partially deleted appropriate motor innervation develops in the remaining leg muscles. Motoneurons deprived of their normal muscle targets, due to limb deletion, selectively exhibit excessive degeneration during the period of natural motoneuron death (Hamburger, 1958; Oppenheim, Chu-Wang and Maderdrut, 1978). These experiments (Lance-Jones and Landmesser, 1980a; Whitelaw and Hollyday, 1983; Tosney and Landmesser, 1984) indicate that motoneurons which normally innervate particular leg muscles can recognize and respond to specific environmental cues which guide them to appropriate muscle targets.

Further evidence from surgically manipulated chick embryos show that both active and passive guidance of outgrowing axons to appropriate limb muscles occurs (Stirling and Summerbell, 1985). Limb bud transplantation studies in which limb primordia were dorsoventrally or anteroposteriorly reversed, indicate that in some cases invading axons make contact with appropriate muscle targets and in other operated embryos inappropriate nerve-muscle connections result (Stirling and Summerbell, 1980; 1983; 1985; Summerbell and Stirling, 1981; Ferguson, 1983; Laing, 1984). For example, in dorsoventrally reversed wings (Laing, 1984) and legs (Ferguson, 1983) appropriate neuromuscular connections develop, indicating that outgrowing neurites are able to actively seek out appropriate pathways and muscle targets. Others have reported, however, that appropriate nerve-muscle connectivity is evident only in anteroposteriorly reversed wings and that inappropriate connections are observed if wings are dorsoventrally reversed (Summerbell and Stirling, 1981; Stirling and Summerbell, 1985). These investigators (Stirling and Summerbell, 1985) suggest that the differences between their findings and those of others may be due to the exact location of the plane between host tissue and reversed donor tissue in each experiment. If the reversal plane is positioned distal to the nerve plexus of the limb, then invading axons first sort into specific nerve tracts within the plexus and then, as growth continues, they encounter the reversal plane and passively follow inappropriate nerve pathways, unable to correct for their abnormal position within the reversed limb. The inability of motor nerves to seek out appropriate targets under these experimental conditions may be due to the fact that individual axons are displaced a large distance from their normal position in the limb and are now unable to respond to local environmental cues (Landmesser, 1984; Stirling and Summerbell,, 1985). If, however, the reversal plane is proximal to the nerve plexus, then outgrowing axons encounter the reversed peripheral tissue before entering the plexus and now are able to compensate for their displaced position, sort into appropriate nerve tracts and establish connections with their normal muscle partners (Stirling and Summerbell, 1985).

Neural tube reversal experiments in which varying lengths of the lumbosacral neural tube were reversed craniocaudally show that motoneurons within spinal segments displaced a short distance from their normal position in the spinal cord are able to extend neurites into the developing leg and actively seek their appropriate muscle targets (Lance-Jones and Landmesser, 1980b; 1981b). On the other hand, if a large length of the lumbosacral neural tube is reversed craniocaudally, so that spinal segments are shifted a large distance from their normal position, motor neurites growing into the periphery are no longer able to correct for their abnormal position and consequently innervate inappropriate leg muscles (Lance-Jones and Landmesser, 1981b; Vogel and Landmesser, 1987). Under extreme experimental conditions, therefore, inappropriate motor innervation does occur and is related to the distance motor neurites are displaced from their normal position (Lance-Jones and Landmesser, 1981b; Stirling and Summerbell, 1985). Foreign motor nerves invading peripheral tissues distant from their normal environment, therefore, may be too far removed from and unable to respond to specific, localized cues which would normally guide them to appropriate muscle targets (Landmesser, 1984). Thus, inappropriate innervation occurs only after extreme experimental manipulations.

The present study sought to determine, using retrograde HRP labelling analyses, the source of motor innervation to wing muscles within a thoracic neural tube transplanted to the site of the extirpated brachial neural tube. In these heterotopic innervation experiments, nerves exiting the heterotopically transplanted thoracic neural tube enter a brachial environment far removed from their normal peripheral field. It was reasoned that abnormal localization of motoneuron pools supplying wing muscles may be a factor contributing to the inability of foreign thoracic nerves to maintain structural unions with wing muscles.

Once the origin of motor innervation to wing muscles in these heterotopic innervation experiments was defined, it was then possible to investigate the development of thoracic motoneurons known to be in contact with target (wing) muscles which they never innervate normally. Specifically, the developmental pattern of motoneuron death, growth and differentiation during the period extending from St 29 (day -6E) to St 42 (day -16E) was studied in heterotopically transplanted thoracic and appropriate control neural tubes. The analyses of motoneuron development were based on the reports of Hamburger (1934; 1939a,b) which demonstrated that factors derived from the periphery influence the number of motoneurons which develop in the limb innervating segments of chick embryo spinal cords.

(c) Pattern of Motoneuron Death, Growth and Differentiation: The control of motoneuron numbers during embryogenesis was examined further by Hamburger and coworkers who reported that following limb bud removal before peripheral nerves exit the neural tube, many fewer motoneurons develop in the neural tube region that normally supplies the missing limb (Hamburger and Keefe, 1944). Hamburger and Keefe (1944) suggested that the size of the peripheral target field may determine either the recruitment or differentiation of neurons from a population of undifferentiated, pluripotent stem cells to supply the limb, a theory which was subsequently disproven.

Up to the time when the report of Hamburger and Levi-Montalcini (1949) appeared, embryonic development was considered to be totally progressive in nature and the idea of regressive developmental mechanisms, such as cell death, had not occurred to embryologists of the day. Hamburger and Levi-Montalcini (1949) indicated that massive loss of dorsal root ganglion cells occurs during normal development. Subsequent re-examination of the limb bud removal experiments confirmed the presence of a period of massive motoneuron death during normal ontogeny and this cell loss was accelerated and increased greatly from normal in the absence of a limb (Hamburger, 1958; Chu-Wang and Oppenheim, 1978a). Detailed quantitative comparisons of the lumbosacral LMC following limb bud removal at day -3E demonstrated that by day -5E equivalent numbers of motoneurons develop on the operated and control sides (Hamburger, 1958, Chu-Wang and Oppenheim, 1978a). Thus, removal of the limb bud early in development did not influence either the initial production or migration of motoneurons during formation of the LMC. Between St 29 (day -6E) and St 35 (day -9E) a period of cell degeneration is observed in the lumbosacral spinal cord during which 40% of motoneurons present initially are lost through natural cell death on the control side and up to 90% of motoneurons die during the same period on the limb bud removal side (Hamburger, 1958; 1975; Chu-Wang and Oppenheim, 1978a,b). These observations identified the period of naturally occurring motoneuron death in the lumbosacral LMC during normal development and further indicated that the onset of induced (target deprived) motoneuron death coincides temporally with that of natural neurothanasia of these cells. Similarly, a period of natural motoneuron death extending from St 34 (day -8E) through St 40 (day -14E) has been identified in the brachial LMC of chick embryos (Oppenheim and Majors-Willard, 1978; Laing, 1982; Cauwenbergs, Cosmos and Butler, 1983; 1986).

Although much has been learned concerning the pattern of motoneuron death during embryogenesis, to date the ontogenetic mechanisms which control motoneuron death remain poorly understood and controversial (Hamburger and

Oppenheim, 1982; Oppenheim and Chu-Wang, 1983; Oppenheim, 1984). Hamburger (1958) first suggested that the temporal coincidence of induced and natural motoneuron death may indicate the presence of a trophic or maintenance factor(s), similar to nerve growth factor (NGF), which is derived from the periphery and acts to regulate motoneuron death during normal ontogeny. Tanaka and Landmesser (1986) indicated further that such a trophic factor is derived from developing myotubes and, therefore, motoneuron death is inversely related to the number of myotubes present in the periphery, although McLennan (1984) suggested that target size is not the primary factor governing motoneuron death.

Recent evidence indicates that survival of cultured chick embryo sensory neurons is supported by a neurotrophic factor(s) extracted from pectoralis muscles of day -18E chick embryos and that this neurotrophic activity was greatest during the period of naturally occurring death of these cells (Davies, Thoenen and Barde, 1986). Similarly, Hulst and Bennett (1986) reported that the survival of chick embryo motoneurons *in vitro* is promoted by media conditioned over dissociated embryonic skeletal muscle and that this neurotrophic effect is mediated by a trophic factor(s), similar to NGF, derived from the cultured muscle cells.

Other reports indicate that the regulation of naturally occurring motoneuron death is more complex than originally anticipated (for reviews, Oppenheim, 1981b; 1984). For example, initiation of motoneuron death during normal development may be related to the formation of permanent neuromuscular junctions (Hamburger, 1975). Also, synaptic activity at the myoneural junction functions to determine the degree of motoneuron death since chemical blockers of both acetylcholine release (botulinum toxin) and postsynaptic acetylcholine receptors (curare or α-bungarotoxin) reduce motoneuron death, so that in the absence of synaptic activity

93% of initial motoneuron numbers survive the normal cell death period (Pittman and Oppenheim, 1978; 1979).

Natural motoneuron death, on the other hand, is not related to an inability of motoneurons destined to die either to differentiate normally or to extend peripheral processes. Oppenheim, Chu-Wang and Maderdrut (1978) showed that after limb bud removal at day -3E all motoneurons on the operated side send out axonal processes which enter a neuroma (a densely packed tangle of nerve fibres) at the base of the removal site. Although no synapses developed in the neuroma, nearly all motoneurons were labelled retrogradely after HRP injection into the neuroma at day -5E. Oppenheim, Chu-Wang and Maderdrut (1978) also demonstrated that target deprived LMC motoneurons develop normal ultrastructural features and differentiate levels of cholinergic enzymes (choline acetyltransferase and acetylcholinesterase) similar to those of control motoneurons prior to the onset of motoneuron degeneration. Thus, these embryonic properties of motoneurons are controlled autonomously, independent of peripheral factors.

Available evidence, therefore, supports the competition hypothesis for control of naturally occurring motoneuron death, as proposed by Hamburger (1975). According to this hypothesis, large numbers of motoneurons send axonal processes into a peripheral target field which is restricted in availability of synaptic sites. Invading neurons consequently compete either for specific anatomically defined targets or for a limited supply of trophic substance or maintenance factor derived from target cells.

Based on the fact that peripheral factors influence natural motoneuron death during embryogenesis, the present investigation was directed toward elucidating the pattern of motoneuron death first in unoperated control thoracic neural tubes, since this was previously unknown, and then in heterotopically transplanted thoracic neural tubes. Thus, quantitative comparisons of motoneuron numbers were performed between experimental and control neural tubes from St 29 (day -6E) through St 42 (day -16E) to determine if structural contacts and interactions with an enlarged periphery (wing musculature) affect the pattern of motoneuron death in heterotopically transplanted thoracic neural tubes. These analyses also allowed comparisons of peripheral (wing) and central (spinal cord) embryonic events, first during the initial developmental period when structural nerve-muscle contacts are observed in heterotopically innervated wings and then following nerve-muscle uncoupling and muscle degeneration. It was reasoned that loss of previously compatible nerve-muscle unions observed in these heterotopic innervation experiments during the second week of embryogenesis (Butler, Cosmos and Brierley, 1982b) might severely affect the survival of foreign thoracic motoneurons.

3. Somitic Origin Of Skeletal Muscle

In addition to the heterotopic innervation experiments, a second series of surgically manipulated chick embryos was employed in the present study to determine if the nerve-muscle uncoupling phenomenon observed in heterotopic innervation experiments is unique to a thoracic nerve-brachial muscle combination. Whereas in the first experimental series the source of motor innervation to brachial muscles was replaced by a thoracic neural tube, in the second series of experiments the source of myogenic precursor cells was altered surgically. Specifically, the brachial somites, from which the myogenic component of wing muscles is derived normally (Jacob, Christ and Jacob, 1978), was removed and replaced by thoracic somitic mesoderm which normally gives rise to abdominal muscles (Christ, Jacob and Jacob, 1978a). As in the present heterotopic neural tube transplantation experiments, wing motility analyses were performed from St 29 (day -6E) through St 42 (day -16E) to determine the extent of functional nerve-muscle interaction following heterotopic somitic mesoderm transplantation.

During early somitogenesis two distinct regions are evident in the somites, the ventral sclerotome and dorsal dermatomyotome (Christ, Jacob and Jacob, 1977; 1978b). As development proceeds, the dermatomyotome subdivides further into outer dermatome and inner myotome layers, and it is from the myotomal layer that myogenic precursor cells of skeletal muscles originate (Christ, Jacob and Jacob, 1978b). In the brachial region of chick embryos presumptive myogenic cells start their migration from the myotomal region of individual somites at St 14 (48-52 h *in ovo*), shortly after segmentation of brachial somites is complete (Christ, Jacob and Jacob, 1977; Jacob, Christ and Jacob, 1978). Similarly, myogenic cells begin their migration into the hindlimb bud immediately following segmentation of the lumbosacral somitic mesoderm (Jacob, Christ and Jacob, 1979).

Several studies which utilized the distinctive nucleolar marker in chick/quail chimaeras developed by LeDouarin (1973) showed that individual skeletal muscles are derived from two separate mesodermal structure (Chevallier, Kieny and Mauger, 1977; 1978; Christ, Jacob and Jacob, 1977; 1978b; Chevallier, 1979). Only the myogenic component of skeletal muscles is derived from donor somitic mesoderm when somitic mesoderm of quail genotype is transplanted homotopically (sites of origin and implantation are the same) into the region of extirpated brachial (Christ, Jacob and Jacob, 1977; Chevallier, Kieny and Mauger, 1977), thoracic (Christ, Jacob and Jacob, 1978a; 1983; Chevallier, 1979) or lumbosacral (Chevallier, Kieny and Mauger, 1977) somites in chick hosts. Connective tissue elements within skeletal muscle, such as tendons, perimysium and endomysium, are derived from the somatopleure (lateral plate mesoderm) [Chevallier, 1979; Christ, Jacob and Jacob, 1983]. The somitic origin of muscle cells was demonstrated further by Chevallier, Kieny and Mauger (1978) and Lewis, Chevallier, Kieny and Wolpert (1981) who showed that skeletal muscles fail to develop in regions where the somitic mesoderm was removed surgically or by X-irradiation.

Chevallier and coworkers demonstrated further that the development of individual limb muscles is not dependent on the segmental origin of the somitic mesoderm giving rise to these muscles (Chevallier, Kieny and Mauger, 1977; Chevallier, 1979). Thus, the development of wing muscles following interspecific transplantation of either cervical, brachial, thoracic or lumbosacral somites into the site of extirpated brachial somites is unaltered from that of unoperated controls (Chevallier, Kieny and Mauger, 1977; Cauwenbergs, Butler and Cosmos, 1986b). Myogenic somitic mesoderm, therefore, is not regionalized at day -2E when the heterotopic somite transplantations were performed.

The aim of the experiments of Chevallier and colleagues was to use the structural marker in chick/quail chimaeras to determine the myogenic potentials of somitic mesoderm taken from various segmental levels. Consequently, experimental embryos were analyzed to day -12E only (Chevallier, Kieny and Mauger, 1977; Chevallier, 1979). For the present study, thoracic somitic mesoderm was transplanted heterotopically into the site of extirpated brachial somites and wing motility analyses were compared between operated and control embryos. The specific aim of the present investigation was to determine if in the intraspecific chimaeras used, functional interactions (wing motility) occur between *in situ* brachial motor nerves and wing muscles derived from the heterotopic somitic mesoderm graft. Moreover, motility analyses were carried out to St 42 (day -16E) to see if functional nerve-muscle interactions were maintained in experimental Thor-Br/som embryos or if, instead, nerve-muscle uncoupling occurred, as was the case for heterotopic neural tube (Thor-Br) transplant experiments.

E. Summary Of Aims And Objectives

The specific aim of the present study was to investigate the nature of initial nerve-muscle contacts observed previously in experimental chick embryo wing muscles cross-innervated by foreign thoracic nerves (Butler, Cosmos and Brierley, 1982b). Analyses were directed toward gaining a better understanding of possible factors intrinsic and extrinsic to the spinal cord responsible for the eventual nerve-muscle uncoupling reported previously (Butler, Cosmos and Brierley, 1982b), as summarized in the following list of objectives:

1. To utilize the well documented functional parameter, wing motility, to determine if initial nerve-muscle unions between wing muscles and foreign nerves derived from a heterotopically transplanted thoracic neural tube are functional.

2. To define the extent of functional nerve-muscle interaction by performing wing motility analyses in individual heterotopic innervation experiments throughout an extended embryonic period (day -6E through day -16E).

3. To investigate developmental events within the heterotopically transplanted thoracic neural tube including neural tube histogenesis, the origin of motor innervation (HRP analyses) and the pattern of motoneuron death, growth and differentiation during the embryonic period from St 29 (day -6E) to St 42 (day -16E).

4. To determine if the eventual nerve-muscle incompatibility observed in heterotopic thoracic neural tube transplant embryos is limited to a thoracic nerve-brachial muscle combination, wing motility analyses were performed in a second

experimental series in which, now, *in situ* brachial nerves innervate wing muscles which originate from heterotopically transplanted thoracic somitic mesoderm.

During the course of this thesis research, the following papers have been published:

Cauwenbergs, P., Cosmos, E. and Butler, J. (1983): Alterations of wing motility and motoneuron number following heterotopic neural tube transplantation in chick embryos. *Soc. Neurosci. Abstr. 9*:373.

Cauwenbergs, P., Cosmos, E. and Butler, J. (1986): Pattern of neuronal cell death within the medial motor column (MMC) of a thoracic neural tube transplanted to the brachial region (Thor-Br) of chick embryos. *Soc. Neurosci. Abstr. 12(2)*:871.

Butler, J., Cauwenbergs, P. and Cosmos, E. (1986): Fate of brachial muscles of the chick embryo innervated by inappropriate nerves: structural, functional and histochemical analyses. *J. Embryol. Exp. Morph. 95*:147-168.

Cauwenbergs, P., Butler, J. and Cosmos, E. (1986a): Impaired muscle-nerve interaction (motility) characterizes the brachial region of dystrophic embryos. *Exp. Neurol. 94*: 41-53.

Cauwenbergs, P., Butler, J. and Cosmos, E. (1986b): Intraspecific chick/chick chimaeras: dystrophic somitic mesoderm transplanted to a normal host forms muscles with a dystrophic phenotype. *Neurosci. Lett. 68*: 149-154.

Butler, J., Cosmos, E. and Cauwenbergs, P. (submitted): Positional signals: Evidence for a possible role on muscle fibre-type patterning of the embryonic avian limb. *Development* Under Review.

MATERIALS AND METHODS

A. Embryos

The majority of experiments in this study were performed on normal chick embryos from a local strain of White Leghorn chickens (Martindale Farms). In addition fertile eggs from a strain of chickens (S_1) afflicted with hereditary muscular dystrophy were obtained from the University of Connecticut (Storrs). The S_1 dystrophic strain was initially produced by outcrossing the Davis line 301 of dystrophic chickens (New Hampshire strain) to normal White Leghorn birds because of the latters' superior fertility and vigor (Chung, Morton and Peters, 1960). More recently the genetically related dystrophic (S_{10}) and normal (S_{11}) strains have become available from the University of Connecticut (Storrs). All four embryonic lines were used in the present study.

1. Preparation of Embryos

All eggs were incubated on their sides, without rotation in table top incubators at a temperature of 37.4° C and 56% relative humidity for approximately two days. This method of incubation rendered the embryos accessible for surgical manipulation, since, due to the relative densities of the embryo and yolk, the embryo floated to the top of the egg. Embryos which were to be used as thoracic tissue donors (neural tube or somitic mesoderm) were incubated four hours in advance of those to be used as hosts. After two days of incubation the embryos were exposed by the following technique: A small hole was made through the shell into the air space at the blunt end of the egg with fine scissors and another was made over the embryo. The shell overlying the embryo was then carefully lifted away from the adherent shell membrane and a small slit was made in this membrane, allowing the air space to shift and come to overlie the embryo. In this way the embryo dropped away from the shell allowing enlargement of the shell opening. At this point eggs designated as unoperated controls (UC) were fitted with glass coverslips (24 x 30 mm), sealed with soft dental wax and returned to the incubator. The glass window provided excellent visibility for subsequent motility analyses. Eggs to be used for transplantation experiments were sealed with cellophane tape and placed in the incubator until surgery. Host embryos were removed from the incubator approximately 15 minutes prior to surgery and left at room temperature. This procedure was observed to prevent damage to developing blood vessels during surgery since the heart rate slowed considerably at the cooler temperature. Occasionally host and donor eggs were kept at room temperature for variable lengths of time (up to two hours) in order to maintain the embryos at the desired stages for transplantation procedures. Although it has been shown that avian embryos may develop abnormally and exhibit growth retardation with prolonged (2-10 days) or continuous exposure to low incubation temperatures (Romanoff,1972), no deformities were observed following the short exposures employed in the present study. similar to the findings of Romanoff (1960),however, the formation of somites was slowed in embryos cooled for a brief period; thus, it was possible to extend slightly the period during which donor and host embryos were at the desired stages. All embryos were staged prior to surgery (and throughout this study) according to the morphological criteria established by Hamburger and Hamilton (1951).

B. Surgical Procedures

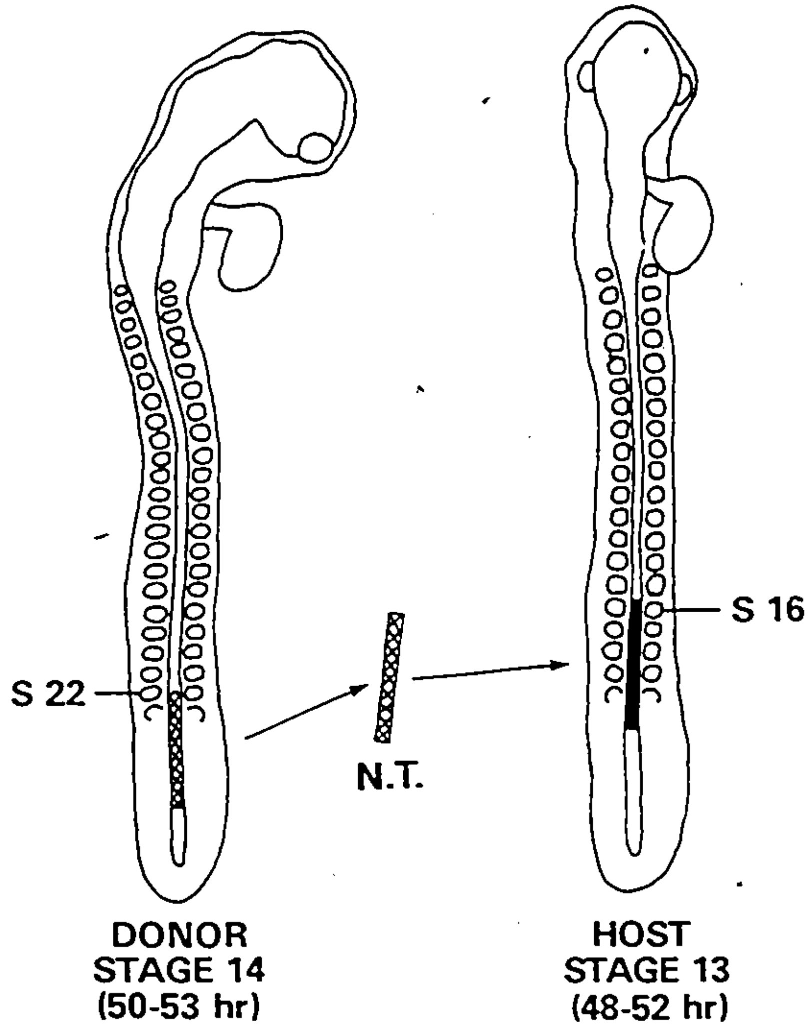
To investigate factors which influence nerve-muscle interaction during embryogenesis, two series of experimental surgical manipulations were employed: removal and heterotopic transplantation of either (1) neural tube segments or (2) somitic mesoderm. The surgical techniques employed were established and are used routinely in Dr. Cosmos' laboratory (Butler, Cosmos, and Brierley, 1982a; Butler, Cauwenbergs and Cosmos, 1986; Cauwenbergs, Butler and Cosmos, 1986b). For the neural tube transplantation series the brachial neural tube segment of the host embryo was removed and replaced by (1) the thoracic neural tube segment from a donor embryo (Thor-Br) or (2) by a donor brachial neural tube (Br-Br). The somitic mesoderm transplantation series of experiments (Thor-Br/som) were comprised of two groups of operated embryos. One group in which the brachial somites of the host embryo were extirpated and replaced by thoracic somitic mesoderm from a donor embryo of normal genotype (NThor-NBr/som) and a second group in which the host brachial somites were replaced by thoracic somitic mesoderm from a donor embryo of dystrophic genotype (DThor-NBr/som). All host embryos in the somitic mesoderm transplant series of experiments were of the local normal strain.

1. Neural Tube Transplantation

(a) Experimental Embryos: In the first series of experimental embryos heterotopic neural tube transplantation (Thor-Br) was achieved by removing the brachial neural tube from host embryos at stage (St) 13 (48-52 h *in ovo*) and inserting into the removal site a thoracic neural tube from a St 14 (50-53 h *in ovo*) donor embryo (Fig. 1). The brachial neural tube develops adjacent to somites 16 to 21 inclusive

Figure 1. Diagram to illustrate heterotopic neural tube transplantation, *i.e.*, replacement of host brachial neural tube segment by thoracic neural tube (*nt*) segment of donor embryo. S refers to somite number.

THORACIC → BRACHIAL NEURAL TUBE TRANSPLANT



(Butler, Cosmos and Brierley, 1982a), whereas the thoracic neural tube forms opposite somites 22 to 26 inclusive; thus, the number of formed somites and an estimate of unformed somites (caudally) was used to define the cranial and caudal limits of both the brachial neural tube removed from host embryos and thoracic (Thor-Br) or brachial (Br-Br) neural tube taken from donor embryos.

(i) Preparation of Donor Tissue: The donor thoracic neural tube was obtained as follows: The donor embryo was removed from the egg by cutting around the area vasculosa with small iridectomy scissors and placed in a small petri dish containing sterile Hank's balanced salt solution (HBSS; see Appendix Ia). The vitelline membrane was then teased away using forceps, the embryo was orientated dorsal side up and excess HBSS was suctioned away. The dish was then placed on a black background to facilitate visualization of the embryo with a dissecting microscope (X40) illuminated with a fibre optic light source. Next the donor embryo was staged and the cranial and caudal boundaries of the thoracic neural tube were delineated as described above (Fig. 1). The entire thoracic region of the donor embryo was removed by making two transverse cuts, one at the level of somite 22 and a more caudal cut between presumptive somites 26 and 27, with an electrophoretically etched tungsten needle. To maintain proper cranio-caudal and dorso-ventral orientation of the donor neural tube, the caudal incision was made at a slight angle. The transverse incisions were then joined laterally by longitudinal incisions. The excised tissue was then placed in a sterile 0.25% trypsin solution (Gibco Laboratories) (see Appendix Ib) for 10-15 minutes while the brachial neural tube segment was removed from the host embryo. After trypsinization the donor neural tube was carefully dissected free of extraneous tissues (ectoderm, endoderm, somitic mesoderm and notochord) and immersed for approximately 10 seconds in a trypsin inhibiting solution of 5% fetal bovine serum (Gibco

Laboratories) in complete HBSS (see Appendix Ic). The donor neural tube was then transferred to the host egg by pipette and positioned as described below.

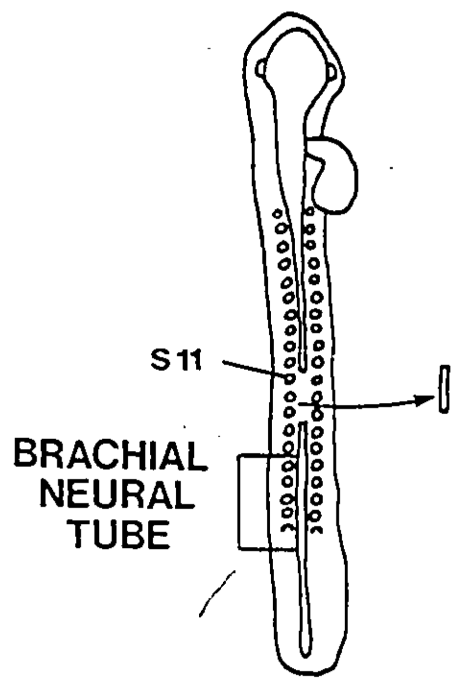
(ii) Preparation of Host Embryos: While the donor tissue was being trypsinized, the host embryo was prepared as follows: The host egg, which was opened previously as described above, was reopened by removing the cellophane seal. A small volume (approximately 0.2 ml) of sterile India ink (Pelikan #17, diluted 3:1 with HBSS) was injected between the embryo and the underlying yolk with a finely drawn glass needle (Butler, Cosmos and Brierley, 1982a) to allow proper visualization of the embryo. All surgical procedures were performed using a dissecting microscope at a magnification of 40X. To expose the brachial region of the host embryo, a drop of sterile HBSS was placed on the vitelline membrane and a small incision was made through this membrane. An additional drop of sterile HBSS was then added to raise the vitelline membrane and allow better access to the embryo beneath. To prevent the embryo from becoming dehydrated, additional drops of HBSS were added when necessary. Next, two longitudinal incisions were made on either side of the brachial neural tube, separating it from adjacent brachial somitic mesoderm (somites 16-21). Care was taken not to damage the somites or underlying endoderm. The neural tube was then transected at the cranial (between somites 15 and 16) and caudal (between presumptive somites 21 and 22) limits of the brachial region. Removal of the host brachial neural tube was done with fine forceps and any fragments were suctioned away with a finely drawn capillary pipette. After removal of the neural tube, the somites, notochord and endoderm were carefully inspected for injury resulting from the operation and injured areas were recorded.

Once the host embryo was prepared, the donor thoracic neural tube was removed from the trypsin solution, rinsed in trypsin inhibitor and placed into the host egg. The donor tube was then aligned with the host neural tube and in cases where it was longer than the removal site a small piece was trimmed off caudally. Then, with a finely etched needle the donor neural tube was gently manipulated into the host removal site, while maintaining proper cranio-caudal and dorso-ventral orientation. Once the donor neural tube was positioned correctly, excess fluid overlying the operated embryo was suctioned away to allow the donor thoracic neural tube to sink down into the removal site. Operated eggs were then fitted with glass coverslips, sealed with soft dental wax and returned to the incubator.

(b) Surgical Controls: In addition to the unoperated control (UC) embryos, surgically manipulated control embryos included the Br-Br group. For the Br-Br embryos the brachial neural tube was removed at St 13 (48-52 h *in ovo*) as described for experimental (Thor-Br) embryos and replaced by a brachial neural tube from a donor embryo at St 13 (48-52 h *in ovo*). The surgical techniques used in this procedure (Br-Br) were identical to those outlined for the Thor-Br experimental series.

A second series of surgically manipulated control embryos, namely the pre-brachial removal (PBR) group, was used specifically for wing motility analyses to determine if the loss of proper supraspinal or propriospinal (intersegmental) inputs would affect adversely either the qualitative or quantitative nature of wing motility. The PBR embryos were prepared by the surgical removal of a small portion of the pre-brachial (cervical) neural tube opposite somites 11-13 inclusive at St 13 (48-52 h *in ovo*) (Fig. 2). The surgical technique employed was the same as that described for removal of the brachial neural tube from host embryos in Thor-Br experiments; however, in PBR embryos, the extirpated neural tube segment was not replaced. Following surgery, the eggs were sealed with a glass

Figure 2. Diagram to illustrate removal of a prebrachial neural tube segment (PBR embryo) adjacent to somites 11 through 13. S indicates somite number.



PBR EMBRYO
STAGE 13
(48–52 hr)

coverslip and soft dental wax and placed in the incubator. Dissection of PBR embryos at the termination of the experiment revealed that in all cases the neural tube had not regenerated into the removal site and a large spinal gap persisted in the upper cervical region.

2. Somitic Mesoderm Transplantation

The second series of experiments consisted of surgically manipulated embryos in which thoracic somitic mesoderm was transplanted heterotopically into the site of the extirpated brachial somites (Thor-Br/som) which normally give rise to the wing muscles (Beresford, 1983). The surgical techniques used are described below.

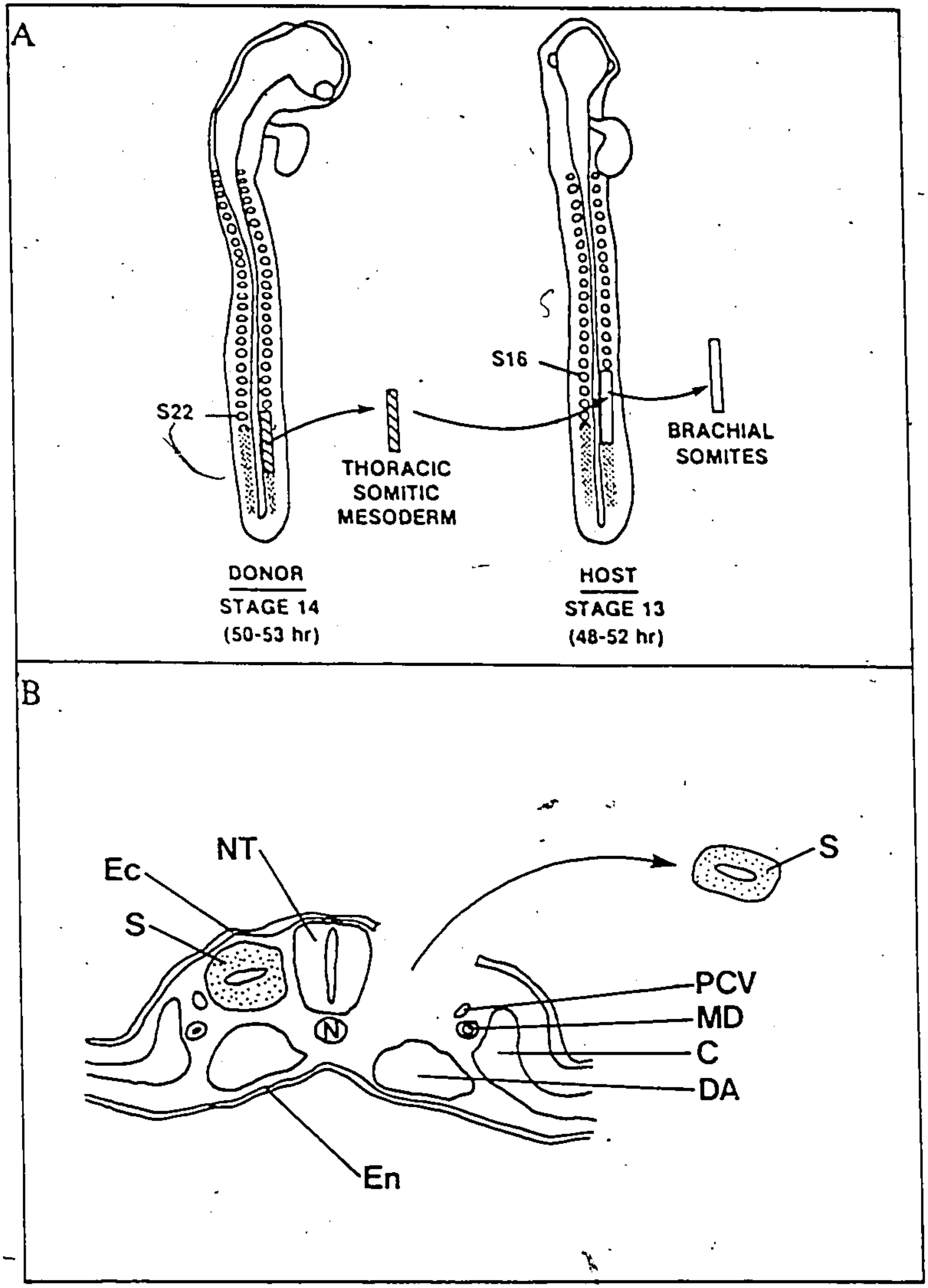
(i) Preparation of Donor Tissue: As in the neural tube transplantation experiments, donor embryos were removed from the egg at St 14 (50-53 h *in ovo*) placed in a petri dish containing sterile HBSS, and the thoracic region extending from somite 22 to presumptive somite 26 inclusive was excised. The excised tissue was then placed in a sterile solution of 0.25% trypsin/ Ca^{++} and Mg^{++} free HBSS for 10-15 minutes, while the brachial somites were removed from the host embryo. Following trypsinization the thoracic somitic mesoderm from the right side of the donor embryo was dissected free from surrounding tissues (ectoderm, endoderm, neural tube, mesonephric duct and lateral plate mesoderm), transferred by pipette to trypsin inhibitor (5% fetal bovine serum/ HBSS) for approximately 10 seconds, and then placed in the host egg.

(ii) Preparation of Host Embryos: Host embryos were prepared by extirpating the brachial somites (somite 16 to presumptive somite 21 inclusive) from the right side of the embryo at St 13 (48-52 h *in ovo*) (Fig. 3). Removal of host brachial somites was achieved by using an electrophoretically sharpened tungsten needle. After penetrating the vitelline membrane as described previously (Section B-1(a) of MATERIALS AND METHODS) a longitudinal incision through the surface ectoderm was made dorsal to the right lateral edge of the brachial neural tube. Transverse incisions through the ectodermal layer were then made to join this initial cut, first at the cranial end of the removal site (between somites 15 and 16) and then at the caudal end (between presumptive somites 21 and 22). The flap of ectoderm overlying the brachial somites was then folded laterally to expose the somites below. Two deeper longitudinal incisions were then made medial and lateral to the brachial somites, being careful not to damage either the posterior cardinal vein, underlying aorta or mesonephric duct (Fig. 3B) which is just forming at this stage (Patten, 1957). Transverse incisions were then made at the cranial and caudal limits of the removal site and the brachial somites were gently lifted away with fine forceps. After removal of the brachial somites, the operation site was suctioned with a finely drawn glass pipette to remove any possible fragments of somitic mesoderm left behind and the removal site was inspected for injuries to either the brachial neural tube or lateral plate mesoderm; damaged embryos were discarded.

Following removal of the host brachial somites, the donor thoracic somitic mesoderm was placed into the host egg and manipulated into the removal site using a fine wire probe. By cutting the caudal end of the donor tissue at a slight angle at the time of removal from the donor embryo, it was possible to maintain proper cranio-caudal and dorso-ventral orientation of the graft. Occasionally the donor tissue did not completely fill the host removal site. In these cases (20 of the 177 total), a short length (equal to approximately 1 somite) of unsegmented thoracic somitic mesoderm from the left side of the donor embryo was used to fill the space at the caudal end of the removal site. To do this, the somitic mesoderm

Figure 3. (A) Diagram to illustrate the heterotopic transplantation of thoracic somitic mesoderm from a donor embryo to replace the extirpated brachial somites of a host embryo. S refers to somite number and stippled area represents unsegmented somitic mesoderm.

(B) Diagram to illustrate a cross-section of a St 13 (48-52h *in ovo*) host embryo following removal of the brachial somites on the right side (arrow). PCV, posterior cardinal vein; MD, mesonephric duct; C, embryonic coelom; DA, dorsal aorta; En, endoderm; Ec, ectoderm; NT, neural tube; N, notochord; S, somite.


A
S22
THORACIC SOMITIC MESODERM
S16
BRACHIAL SOMITES
DONOR
STAGE 14
(50-53 hr)
HOST
STAGE 13
(48-52 hr)
B
NT
Ec
S
N
S
PCV
MD
C
DA
En

from the opposite side was rotated 180° to preserve proper medio-lateral orientation of the transplant. These embryos were not segregated from Thor-Br/som embryos which received donor tissue from only the right side since subsequent wing motility analyses indicated neither qualitative nor quantitative differences between these experimental groups. Following surgery, the eggs were sealed with a glass coverslip and soft dental wax and placed in the incubator.

C. Analyses

A number of analyses were used to investigate the development of functional nerve-muscle interaction in chick embryos. These included wing motility measurements in both experimental series (Thor-Br and Thor-Br/som) of embryos and analyses of the development of transplanted neural tubes in experimental (Thor-Br) and control (Br-Br and UC) embryos. These investigations were performed: (1) to determine the extent of functional nerve-muscle interaction (wing motility) in experimental (Thor-Br and Thor-Br/som) embryos and (2) to better understand ontogenetic events which occur within the developing spinal cord under experimental (Thor-Br) conditions.

1. Embryonic Motility

Embryonic limb motility originates within the developing spinal cord from spontaneous, polyneuronal bursts of electrical activity and is non-reflexogenic in nature (for review, Oppenheim, 1982). Limb motility is, therefore, an excellent, non-invasive parameter which can be used to investigate functional nerve-muscle interaction during embryogenesis.

To monitor the development of functional interaction between brachial muscles and their motor innervation, qualitative and quantitative analyses of wing motility were compared between experimental and control groups of embryos. First wing motility in experimental (Thor-Br, n=36) and control (Br-Br, n=33; PBR, n=33 and UC, n=74) embryos was analysed. Wing motility analyses were then also performed on experimental (Thor-Br/som, n=51) embryos. Of these (Thor-Br/som) operated embryos, 39 received donor thoracic somitic mesoderm of dystrophic genotype and 12 received donor thoracic somitic mesoderm of normal genotype. In addition to the UC control group, two strains of unoperated dystrophic (S_1, n=63 and S_{10}, n=20) as well as a group of unoperated S_{11} normal (n=15) embryos served as controls for the somite transplant experimental series.

As a further internal control for wing motility analyses in unoperated dystrophic and normal embryos, motility measurements were performed on the ipsilateral wing and leg of a separate series of dystrophic (S_1, n=14) and local White Leghorn (n=14) embryos at embryonic day -8 (day -8E) and day -12E. This analysis was done to determine if genotypically dystrophic leg muscles, which are less severely affected than wing muscles by the dystrophic process during *ex ovo* development(for review, Cosmos *et al.* 1979; 1980), exhibit embryonic motility patterns similar to dystrophic wings.

Motility measurements were carried out as follows: Daily counts of wing motility were performed from day -6E, when overt wing movements are initiated (Hamburger and Balaban, 1963), through day -16E. Since wing motility analyses were performed daily on individual experimental and control embryos, the data are expressed as the number of right wing movements/10 minute observation period (M/10) versus days *in ovo*. To ensure that the developmental stage of the embryos matched their chronological age (days *in ovo*), all embryos were staged daily using the morphological criterial of Hamburger and Hamilton (1951). Prior to day -13E, the embryos were staged accurately through the glass shell window at the time of motility measurements. Embryos at day -13E or older, however, could not be staged through the window due to their increased size. All embryos were staged upon removal from the egg at the termination of the experiment to ensure that their developmental stage continued to correlate with their chronological age.

(a) Total Frequency of Wing Movements: To quantify wing motility, the embryos were placed in a temperature (38° C) and humidity (56%) controlled viewing chamber and were allowed to equilibrate for 10-15 minutes. The embryos were illuminated with a fibre optic light source and were observed through a low power magnifying glass. Since chick embryos characteristically lie on their left side, the total number of right wing movements (total frequency) which occurred in a ten minute observation period (M/10) was recorded on a hand counter. All spontaneous right wing movements which occurred during the observation period were counted. Single counts were defined as any movement of the wing in relation to the body, including movements at the shoulder, elbow and carpus. In addition, if the wing was moving in one direction and then abruptly changed direction, this combination was counted as two separate movements. Simultaneous movements of more than one segment of the wing were treated as a single count. All passive movements of the wing which resulted from either contractions of the amnion or motility of other body parts were excluded.

(b) Absolute Frequency of Wing Movements: In addition to daily analyses of the total frequency of wing movements, the total time occupied by activity versus inactivity phases during a ten minute observation period was determined using a stop watch in experimental (Thor-Br) and control (Br-Br and UC) embryos. To accomplish this, an inactivity phase was operationally defined as any period greater than 5 seconds duration during which no wing or leg movements were observed. It is known that activity and inactivity phases for wing and leg motility, as well as the total frequency of wing and leg movements are highly correlated in unoperated normal chick embryos; i.e., wing and leg movements occur synchronously during normal development and the duration of activity/inactivity phases is determined by the synchronous outflow of electrical discharges from the developing spinal cord to both limbs (Provine, 1973; 1980). Similarly, in experimental (Thor-Br) embryos wing activity and inactivity phases were observed to be temporally coincident with those of the ipsilateral leg. This between limb synchronization of motility in Thor-Br embryos provided a useful means to identify periods of electrical activity and inactivity within the spinal cord as a whole. Although the frequency of wing motility in Thor-Br embryos was reduced at specific embryonic stages, the phasic nature of leg motility in Thor-Br embryos remained unchanged from that of controls and could, therefore, be used as a true indicator for the duration of spinal electrical activity and inactivity phases. Thus, the initiation and cessation of leg motility was used to designate the onset and termination of spinal electrical activity. During each activity phase only wing movements were counted. Using this technique the absolute frequency of wing movements was determined as follows: With a stopwatch the total inactivity period during a ten minute observation was measured and from this both the activity period (10 minutes minus the inactivity period) and the absolute frequency of wing motility (frequency of wing movements/minute of activity) were calculated. In this way it was possible to determine if alterations from the normal developmental pattern of wing motility observed in Thor-Br embryos resulted from a change in either the duration of spinal electrical activity or the absolute frequency of wing motility.

All motility data were stored in a computor and were analyzed for statistical significance using both the Mann-Whitney U-test and the Student's t-test at both the 95% and 99% confidence levels.

2. Development of Transplanted Neural Tubes

In addition to developmental events which occur in the periphery (wing motility)analyses of spinal cord ontogenesis were compared between experimental (Thor-Br)and control (Br-Br and UC) embryos. Experimental and control embryos were sacrificed between St 29 (day -6E) and St 42 (day -16E). The embryos were dissected free from extra-embryonic membranes and yolk sac, weighed, staged (Hamburger and Hamilton, 1951) and decapitated. After evisceration, embryos to be used for analyses of neural tube morphogenesis and motoneuron number were fixed in 2% glutaraldehyde/0.1M phosphate buffer (pH 7.2) for 4 hours. The tissues were then washed (5 minutes) in 0.1M phosphate buffer, processed for paraffin embedding and cut in cross-section. Prior to paraffin embedding, St 40 (day -14E) and St 42 (day -16E) embryos were decalcified in 12.5% EDTA (disodium salt) for two weeks in order to facilitate sectioning of calcified bony structures [ossification of the vertebral column and limb girdles is initiated at St 40 (day -14E)(Johnston and Comar,1955)]. The section thickness was selected according to the age of the embryo, thus, allowing for growth of the motoneurons which were to be enumerated: 8 μm for embryos younger than St 36 (day -10E), 10 μm for St 36 embryos and 12 μm for embryos older than St 36 (Hamburger, 1975). The sections were mounted on albumin coated slides and stained with 0.05% aqueous thionin (Chu-Wang and Oppenheim, 1978a).

An additional group of Thor-Br and UC embryos were terminated at St 29 (day -6E) and either frozen by immersion (30 seconds) in 2-methylbutane (J.T. Baker Chem. Co. Phillipsburg, N.J.) suspended in liquid nitrogen (-160 °C) for subsequent histochemical analysis using a silver-cholinesterase reaction (Butler and Cosmos, 1981a) or fixed in Bouin's fluid (4 hours) and embedded in paraffin. The paraffin embedded tissues were cut in cross-section and mounted on albumin coated slides and stained with the urea-silver nitrate method of Ungerwitter (1951) (see Appendix II). Both silver-stained preparations were used to determine the pattern of nerve fibre outgrowth from experimental (Thor-Br) and control (UC) embryos. In addition the peripheral nerve pattern within the brachial plexus was determined using reconstructions from serial thionin-stained cross-sections drawn with the aid of a camera lucida drawing tube (Mag = 100X).

(a) Morphogenesis of Transplanted Neural Tubes and Brachial Plexus: To assess the development of transplanted neural tubes, camera lucida drawings of thionin stained cross-sections (Mag = 100X) of the second spinal segment contributing to the brachial plexus (segment 14 in UC embryos) were compared between experimental (Thor-Br) and control (Br-Br and UC) embryos between St 29 (day -6E) and St 42 (day -16E) inclusive. Spinal segment 14 was chosen since it contains the most prominent lateral motor column and the greatest number of motoneurons amongst the brachial spinal segments in UC embryos. In addition, similar drawings of the second thoracic spinal segment (segment 18) in UC embryos were used to compare the development of an *in situ* thoracic neural tube with that of a heterotopically transplanted thoracic neural tube. This thoracic segment was chosen since in experimental (Thor-Br) embryos this segment corresponded to the second brachial segment (segment 14) of control (Br-Br and UC) embryos. On these drawings the presence of: (1) a lateral motor column (LMC); (2) a medial motor column (MMC); and (3) the sympathetic preganglionic column of Terni (CT) were indicated.

Also during the course of these morphometric analyses, it was observed that a few neural tube transplant (Thor-Br and Br-Br) embryos exhibited a small spinal gap at either the cranial or caudal end of the neural tube transplant, indicating that the grafted neural tube segment had not established morphological contact with the host neural tube in these regions. The number of experimental (Thor-Br) and control (Br-Br) embryos displaying such a gap was therefore recorded. This data was important since it was not certain that the absence of proper supraspinal or propriospinal (intersegmental) influences could affect adversely wing motility in experimental (Thor-Br) embryos. In addition, the pattern of nerve outgrowth from transplanted (Thor-Br and Br-Br) and unoperated (*in situ* brachial) neural tubes into the brachial plexus was analysed using serial reconstructions of the brachial plexus with the aid of a camera lucida drawing tube (Mag=100X). Although the brachial plexus formed bilaterally in all experimental (Thor-Br) and control (Br-Br and UC) embryos, the pattern of peripheral nerves was analysed on the right side only. The morphogenetic analyses of the neural tube and brachial plexus were performed on all experimental (Thor-Br) and control (Br-Br and UC) embryos used for analyses of the pattern of motoneuron death (see below).

(b) Localization of Motoneurons Innervating Wing Muscles of Thor-Br Embryos: Horseradish peroxidase (HRP) retrograde labelling analyses were used to identify the spinal origin of peripheral motor nerves innervating wing muscles of Thor-Br versus Br-Br and UC embryos. Two developmental ages were examined in this study: (1) St 34 (day -8E), a time when the total frequency of wing motility was equivalent in experimental (Thor-Br) and control (Br-Br, PBR and UC) embryos and (2) St 38 (day -12E), a time when greatly reduced frequencies of wing motility were observed in Thor-Br embryos. By employing the method of Oppenheim (1981), either the biceps brachii or the triceps brachii muscle was injected with HRP in individual experimental (Thor-Br) and control (Br-Br and UC) embryos. These muscles were chosen since extensive, detailed HRP analyses of their motor innervation in unoperated chick embryos were available in the literature (Oppenheim, 1981). For HRP injection, the glass window was removed from the egg and the embryo was viewed through a dissecting microscope (Mag = 6.3X). All surgical instruments were sterilized with 70% ethanol at the start of each experiment. To expose the embryo, a small incision through the chorio-allantoic and amniotic membranes was made avoiding major blood vessels of the extra-embryonic circulation. Then, using a fine sterile hair loop, the right wing was pulled through this artificial opening and secured to the egg shell with soft dental wax, thus exposing the upper segment of the wing. Occasionally a similar hair loop around the foot was required initially to move the embryo into a more favourable position for looping the wing. In these embryos, the foot loop was released after the wing had been secured. Periodic drops of sterile chick embryo Ringer's solution (CER) were used throughout the injection procedure to keep the exposed wing moist. Prior to HRP injection, a small incision was made through the skin overlying the muscle to be injected, resulting in a small amount of bleeding which always stopped within a few seconds. Next, using a finely drawn glass needle fixed to a 10 μl Hamilton syringe, a 0.5 μl volume of a 50% HRP/sterile CER was injected into the belly of the muscle. Leakage of HRP solution from the injection site occasionally occurred at the time of injection and was cleared away into the extra-embryonic fluid with a few drops of sterile CER. The hair loop was then removed and the wing was placed back into the amniotic sac. Immediately after HRP injection, the majority of embryos were treated with 50 μl of 0.1% hydrocortisone/CER to promote survival after injection, as suggested by Oppenheim (1985). The eggs were then refitted with a glass coverslip, sealed with soft dental wax and returned to the incubator. Injected embryos were inspected every 30 minutes and only those which survived beyond 3 hours post-injection were frozen by immersion (30 seconds) in 2-methylbutane (J.T. Baker Chem. Co., Phillipsburg, N.J.) suspended in liquid nitrogen (-160 °C) for subsequent histochemical analysis. The frozen tissues, mounted on cork blocks, were cut into 12 μm cross-sections using a cryostat set at -25 °C and serial sections were mounted on albumin coated slides. The histochemical method used to visualize the HRP reaction product in spinal motoneurons (see Appendix III) was similar to the technique described by Sickles and Oblak (1983) which used the chromogen 3,3',5,5' tetramethylbenzidine (Sigma Chem. Co., St. Louis, M.O.). All sections were counterstained with 1% neutral red/4mM sodium acetate buffer (pH 4.8).

Preliminary observations indicated that each HRP labelled cell was present in up to three consecutive serial sections; therefore, to prevent double counting of labelled cells, the total number of densely labelled cells was counted on camera lucida tracings of every fifth serial section. In this way, estimates of the total number of HRP labelled cells and their cranio-caudal distribution within the motor columns were compared between experimental (Thor-Br) and control (Br-Br and UC) embryos. Also, the medio-lateral and dorso-ventral position of HRP labelled cells within the motor columns was noted.

A total of 6 experimental (Thor-Br) and 11 control (Br-Br, n=5; UC, n=6) embryos was analyzed by the HRP technique at St 34 (day -8E). Due to the exceedingly high post-operative mortality of Thor-Br (77.5%) and Br-Br (72.9%) embryos and the additional mortality (56.3% and 64.3% respectively) following HRP injection, only one St 38 (day -12E) Thor-Br embryo survived the HRP injection procedure and was analyzed and compared to similarly treated UC (n=3) embryos of the same stage.

(c) Pattern of Motoneuron Death: To assess the pattern of motoneuron death in Thor-Br versus Br-Br and UC embryos, motoneuron counts were performed from St 29 (day -6E) to St 42 (day -16E). The total number of α-motoneurons/spinal segment in the lateral motor column (LMC) and medial motor column (MMC) of the brachial neural tube in control (Br-Br and UC) embryos was first counted. These values were then compared to the total number of motoneurons/spinal segment present in the MMC of both the heterotopically-transplanted thoracic neural tube of Thor-Br embryos and the *in situ* thoracic neural tube of UC embryos. To prevent double counting of single motoneurons, all counts were done on the right side of every tenth serial cross-section (thionin stained) of the neural tube (Hamburger, 1975) with the aid of camera lucida tracings (Mag = 400X). Since α-motoneurons are characterized by having large nuclei containing relatively little heterochromatin and prominent nucleoli, only motoneurons with nuclei containing one or more distinct nucleoli were counted (Hamburger, 1975). In addition, the total number of degenerating motoneurons was recorded. Degenerating cells were characterized by shrunken, pyknotic nuclei and condensed cytoplasm (Chu-Wang and Oppenheim, 1978a). Cell fragments were not counted since these could have originated from more than one degenerating cell.

The data are expressed as the total number of motoneurons (healthy or degenerating) per spinal segment. To determine these values, it was first necessary to define accurately the cranial and caudal boundaries of each spinal segment analysed. This was done as described by Hamburger (1958) by counting the number of serial cross-sections of the neural tube extending from the last ventral rootlette of one spinal segment (say segment 13) to the first ventral rootlette of the next (more caudal) segment (segment 14). Dividing this number of sections by 2 therefore gave the midpoint between the two spinal segments (13 and 14). This section (midpoint) was then designated as the boundary between segment 13 and 14. Thus, the borders between all spinal segments analysed were determined by this method. With this information it was then possible to determine the total number of healthy or degenerating motoneurons in each spinal segment analysed. The data were stored in a computor and compared statistically using the Student's t-test at both the 95% and 99% confidence levels.

(d) <u>Motoneuron Growth and Differentiation</u>: To determine if the growth of motoneurons in the transplanted neural tube of Thor-Br embryos was affected by their association and interaction with brachial muscles, measurements of the cross-sectional area of a random sample of motoneuron nuclei were compared between experimental (Thor-Br) and control (Br-Br and UC) embryos (Oppenheim and Chu-Wang, 1983). This analysis was performed on the same embryos used for motoneuron counts. Thus, all measurements were made on camera lucida tracings (Mag = 1000X) of thionin stained cross-sections. The population of motoneurons measured in each embryo was obtained by taking random samples throughout the appropriate length of the neural tube. To do this the cross-sectional area of 100 motoneuron nuclei distributed throughout the length of the brachial LMC in Br-Br and UC embryos was measured with the aid of an image analysis system (Carl Zeiss Videoplan). Mean values were compared to the means of similar measurements made on 50 motoneuron nuclei sampled from the MMC of either the transplanted thoracic (Thor-Br embryos) or *in situ* thoracic (UC embryos) neural tube.

To determine the state of motoneuron differentiation in experimental (Thor-Br) and control (Br-Br and UC) embryos, the number of nucleoli (3, 2 or 1) present in their nuclei was recorded on the same tracings used for measurements of nuclear growth rates. As motoneurons grow and differentiate, the number of nucleoli evident in their nuclei changes from 3 (immature state) to 2 (intermediate state) to 1 (mature state) (Hamburger, 1958). Therefore, the percentage of motoneuron nuclei containing 3, 2 or 1 nucleoli was used as an estimate of the state of motoneuron differentiation.

A total of 66 experimental (Thor-Br, n=25) and control (Br-Br, n=14; UC, n=27) embryos were used for analyses of neural tube morphogenesis, and motoneuron number, growth and differentiation.

RESULTS

A. Survival of Operated Embryos

To examine the respective roles of developing motor nerves and peripheral muscle targets during chick embryogenesis, two series of surgically manipulated embryos were employed in this study: 1. The brachial neural tube was extirpated and replaced by a thoracic neural tube segment (Thor-Br) and 2. The brachial somitic mesoderm was removed and replaced by a thoracic somitic mesoderm transplant (Thor-Br/som). Both series of operated embryos experienced exceedingly low survival rates post-operatively (TABLE 1). A total of 293 embryos underwent the Thor-Br transplantation procedure and 66 (22.5%) survived for analysis. In addition a total of 133 Br-Br and 99 PBR control operations were performed. Of these 36 (27.1%) Br-Br and 33 (33.3%) PBR embryos survived for analysis. Similarly, in the somitic mesoderm transplantation series of experiments a total of 143 DThor-NBr/som and 34 NThor-NBr/som operations were performed and only 39 (27.3%) and 12 (35.3%) operated embryos, respectively, survived for subsequent wing motility analysis (TABLE 1). Mortality of operated embryos was greatest during the 24 hour period immediately following surgery. After this initial peak in post-operative mortality many surgically manipulated embryos died at various times throughout the embryonic period analysed, even as late as St 42 (day - 16E). In many cases where the operated embryo survived for a period of time and then died abruptly, the dead embryos were developmentally malformed with either spina bifida or open thorax. In addition, a small number of operated embryos exhibiting abnormal morphology did not die as a result of the defect (see below).

TABLE 1

Post-Operative Survival

Experimental Manipulation	No. of Operations	No. Survived	Survival (%)
Thor-Br	293	66	22.5
Br-Br	133	36	27.1
PBR	99	33	33.3
DThor-NBr/som	143	39	27.3
NThor-NBr/som	34	12	35.3

B. Heterotopic (Thor-Br) Neural Tube Transplantation

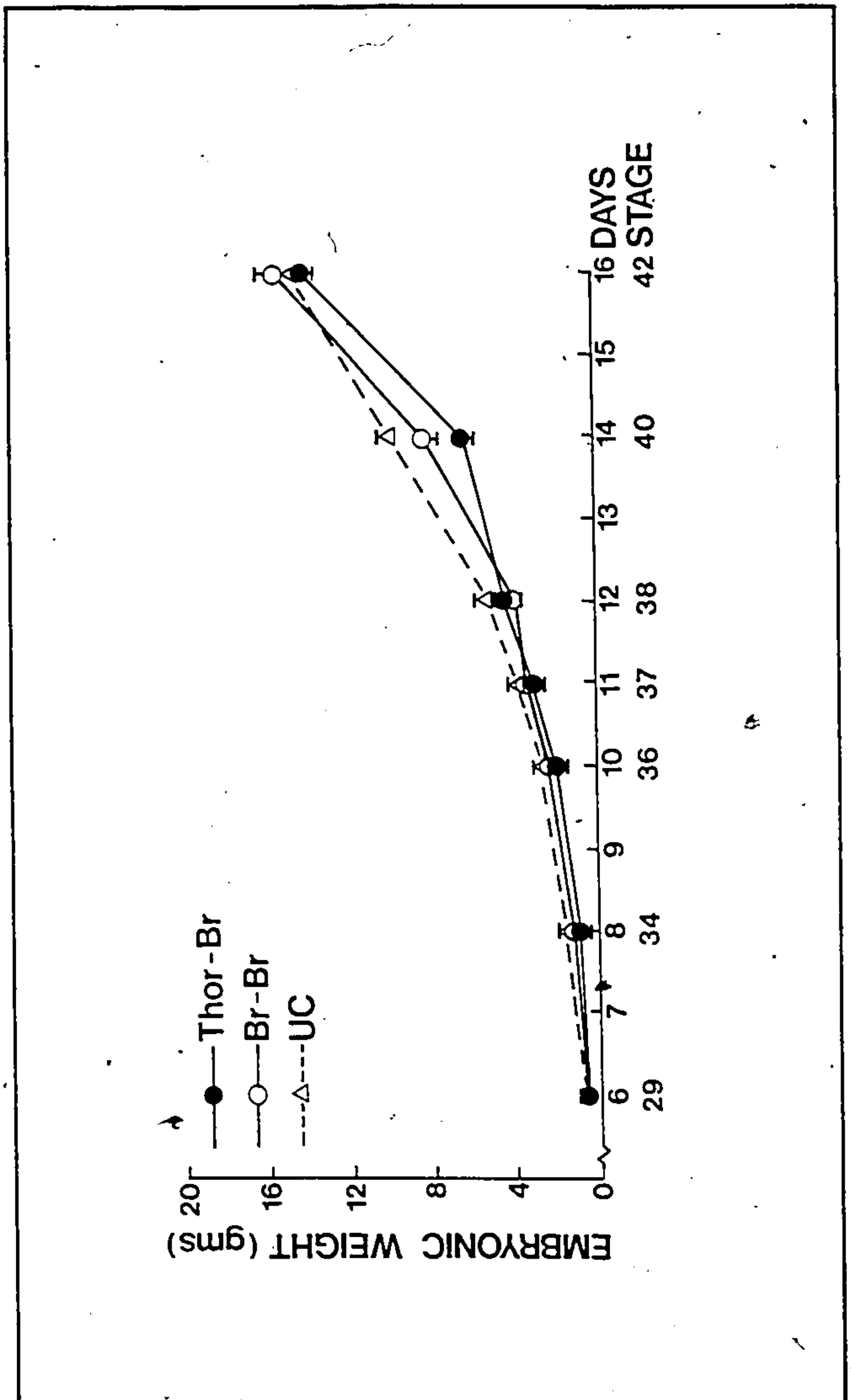
In the first series of experiments a thoracic neural tube of a donor embryo was transplanted to the region of the extirpated brachial neural tube of a host embryo to study the development of nerve-muscle interactions between ectopic thoracic motoneurons and *in situ* brachial muscles.

1. Morphology of Thor-Br Experimental Embryos

Examination of experimental (Thor-Br) and control (Br-Br) surgically manipulated embryos revealed that the majority of operated embryos exhibited normal morphology and their chronological age (days *in ovo*) matched the morphological staging criteria of Hamburger and Hamilton (1951). Thus, for the majority of experimental (Thor-Br) and control (Br-Br) operated embryos the surgery *per se* did not alter normal morphogenesis. A small number of Thor-Br embryos which survived, however, exhibited either a spina bifida (n=4 (6.1%)), open thorax (n=5 (7.6%)) or scoliosis; i.e., curvature of the vertebral column (n=2 (3.1%)) in the brachial region. Similarly a few surgical control (Br-Br) embryos developed either a spina bifida (n=1 (2.8%)), open thorax (n=4 (11.1%)) or scoliosis (n=1 (2.8%)) in the brachial region. The low percentage of operated embryos which exhibited either a spina bifida or open thorax were eliminated from this study. The three operated embryos which displayed a slight scoliosis, however, were retained in the study since this malformation did not affect wing motility adversely.

Measurements of embryonic wet weights indicated that surgically manipulated (Thor-Br and Br-Br) embryos grew normally (Fig. 4). By St 42 (day -16E) the mean wet weights of Thor-Br, Br-Br and UC embryos were 14.3 ± 0.13, 15.7 ± 0.72 and 14.8 ± 0.91 gms, respectively. The slightly lower wet weight of the Thor-Br embryos

Figure 4. Comparative growth of experimental (Thor-Br) and control (Br-Br and UC) embryos from St 29 (day -6E) through St 42 (day -16E). Significant differences were not observed at any days tested. Values represent mean wet weight ± SEM of embryos minus yolk sac.


Thor-Br
Br-Br
UC
EMBRYONIC WEIGHT (gms)
0
4
8
12
16
20
6
7
8
9
10
11
12
13
14
15
16 DAYS
29
34
36
37
38
40
42 STAGE

at St 40 (day -14E) and St 42 (day -16E) was statistically significant at St 40 (day -14) only and may be due to the loss of muscle mass observed in the brachial region of Thor-Br experimental embryos during this embryonic period (Butler, Cauwenbergs and Cosmos, 1986).

A striking characteristic of Thor-Br experimental embryos appeared after St 37 (day -11E). Whereas the wings of Thor-Br embryos were held in a normal position prior to and including St 37 (day -11E), beyond this embryonic period the wings of all Thor-Br embryos exclusively were in a downward position. In surgical control (Br-Br and PBR) and unoperated embryos the wings were held close to the body in a flexed position throughout the developmental period analysed; however, in experimental (Thor-Br) embryos older than St 37 (day -11E) the wings were not flexed and hung between the lower limbs in a downward position. This abnormal wing position which was maintained even after hatching (Butler, Cauwenbergs, and Cosmos, 1986) was not due to joint ankyloses since the wing joints were freely moveable when manipulated with forceps.

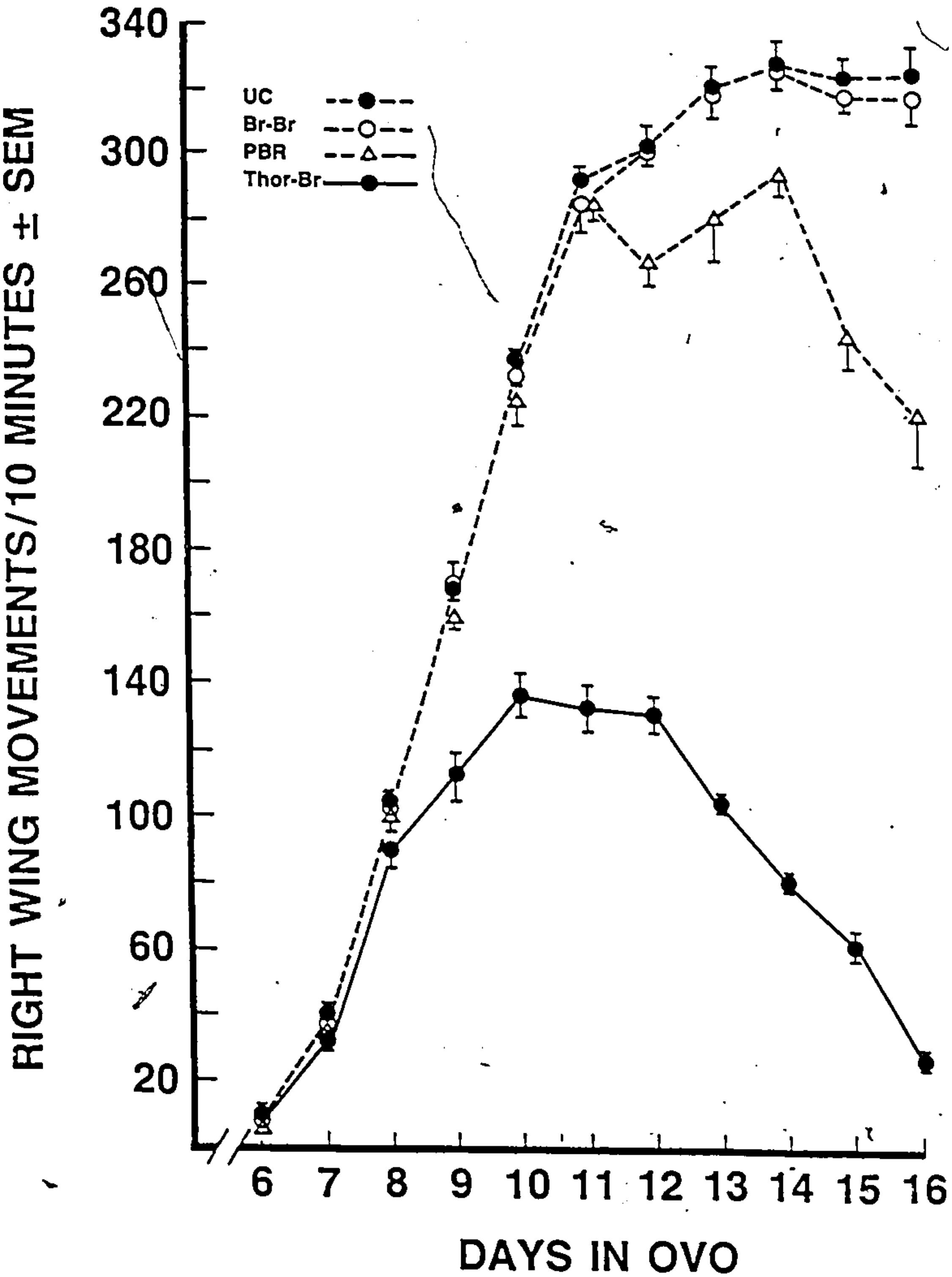
2. Wing Motility Analyses of Thor-Br Experimental Embryos

(a) Total Frequency of Wing Movements : To monitor the development of functional nerve-muscle interaction between ectopic thoracic motor nerves and *in situ* brachial muscles, the total number of right wing movements per ten minute observation period (M/10 = total frequency) was compared between experimental (Thor-Br) and control (Br-Br, PBR and UC) embryos from day -6E to day -16E, inclusive (Fig. 5). The total frequency of wing movements characteristic of UC embryos increased from 9 ± 0.8 M/10 on day -6E to a peak value of 328 ± 7.1 M/10 on day -14E; the latter frequency was maintained to day -16E. The total frequency of wing movements exhibited by Br-Br control embryos was statistically equivalent to that of UC embryos throughout the developmental period analysed, reaching a peak level of 326 ± 6.2 M/10 on day -14E. From day -6E through day -11E the total frequency of wing motility observed in PBR embryos also paralleled that of UC embryos. After this period, however, PBR embryos exhibited lower frequencies of wing movement; this reduction, however, was only statistically significant ($p < 0.05$) at day -15E and day -16E.

Conversely, the pattern of wing motility recorded for Thor-Br experimental embryos was strikingly different from that of control embryos (Fig. 5). Although the M/10 values of Thor-Br experimental embryos were equivalent to those of control (Br-Br, PBR and UC) embryos during the initial perod extending from day -6E through day -8E, the total frequency of wing motility for experimental (Thor-Br) embryos was significantly reduced ($p < 0.05$) from day -9E onward. Experimental (Thor-Br) embryos exhibited a peak M/10 value of only 135 ± 5.1 on day -10E and this level of activity was maintained up to day -12E. After day -12E the total frequency of wing movements observed in experimental (Thor-Br) embryos declined precipitously until by day -16E only 30 ± 2.6 M/10 were counted.

(b) Absolute Frequency of Wing Movements : To calculate the absolute frequency of wing movements (i.e.,the number of wing movements/minute of activity) for experimental (Thor-Br) and control (Br-Br and UC) embryos it was first necessary to determine the length of time each embryo spent moving (activity period) during a ten minute observation period. This was done by using a stopwatch as described in Section C-1B of MATERIALS AND METHODS. The activity periods observed for both experimental (Thor-Br) and control (Br-Br and UC) embryos increased from approximately 0.6 minutes on day -6E to a peak value of approximately 7.3 minutes on day

Figure 5. Mean frequency ± SEM of wing movements characteristic of experimental (Thor-Br) and control (Br-Br, PBR and UC) embryos from day -6E through day -16E. Wing motility in Thor-Br embryos was significantly reduced from control values from day -9E onward and declined rapidly after day -12E.

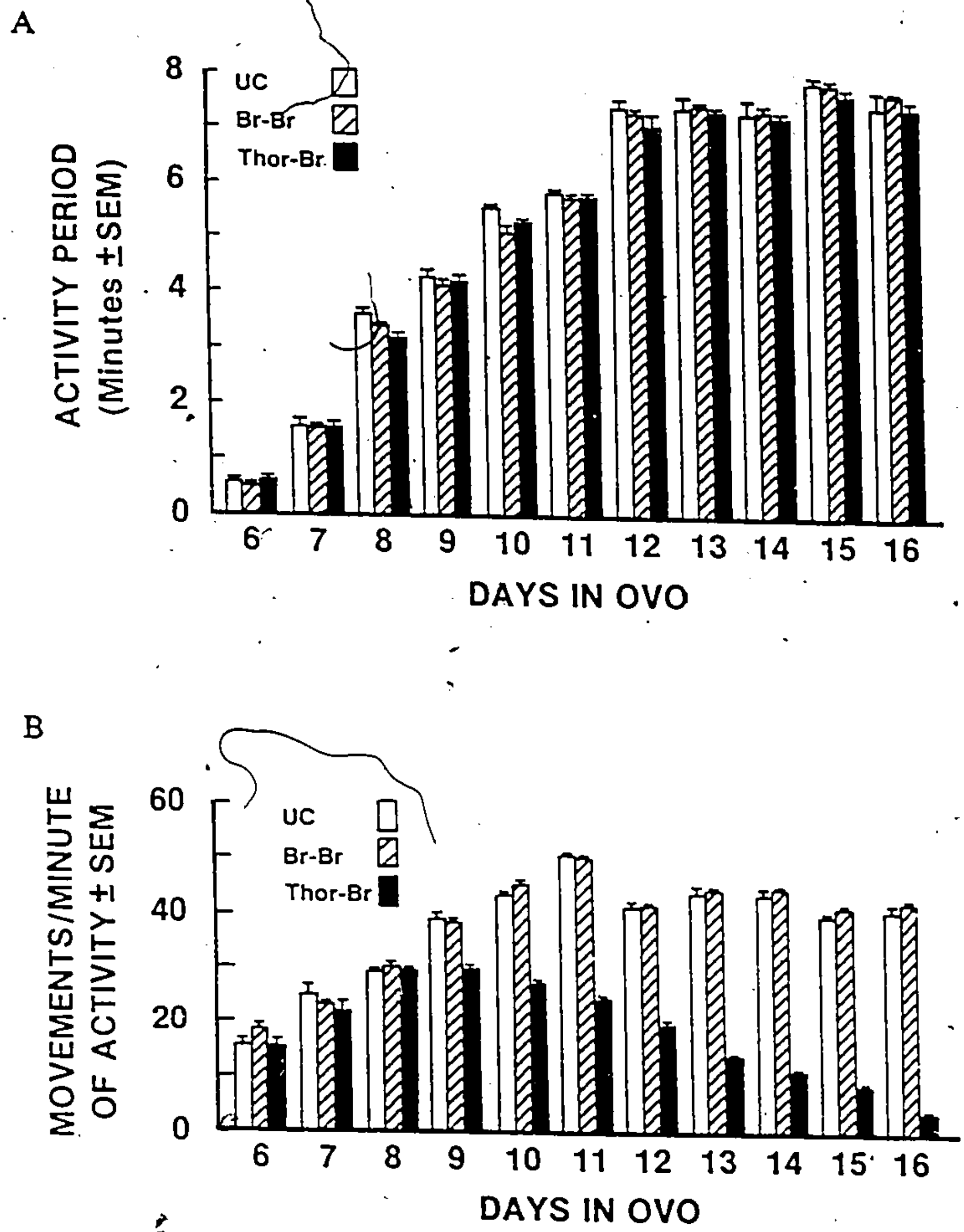

RIGHT WING MOVEMENTS/10 MINUTES ± SEM
340
300
260
220
180
140
100
60
20
UC
Br-Br
PBR
Thor-Br
6 7 8 9 10 11 12 13 14 15 16
DAYS IN OVO

-12E; this level was maintained through day -16E (Fig. 6A). No statistically significant differences were found between the activity periods of experimental (Thor-Br) embryos and those of control (Br-Br and UC) embryos at any days tested.

In contrast, striking differences were found between experimental and control embryos when the absolute frequencies of wing movement were calculated (Fig. 6B). During the initial developmental period extending from day -6E through day -8E experimental (Thor-Br) and control (Br-Br and UC) embryos showed statistically equivalent absolute frequency values increasing from approximately 16 movements/minute of activity (M/min) on day -6E to about 30 M/min on day -8E (Fig. 6B). During subsequent days of development, control (Br-Br and UC) embryos showed a continual increase in the absolute frequency values and reached a peak level of approximately 50 M/min on day -11E. By day -12E absolute frequency values for control embryos were reduced slightly from day -11E values to about 40 M/min and this level of activity was maintained to day -16E. Conversely, after day -8E experimental (Thor-Br) embryos exhibited a progressive daily reduction in the absolute frequency of wing movements which was statistically significant ($p < 0.05$) from day -9E onward. This developmental period (day -9E through day -16E, inclusive) coincided temporally with the period during development when the total frequency of wing movements was reduced in experimental (Thor-Br) embryos. Thus, the reduced M/10 (total frequency) values observed in experimental embryos after day -8E (Fig. 5) were correlated with a signifcant reduction in the absolute frequency (M/min) of wing motility (Fig. 6B), even though throughout the developmental period analysed no differences in the length of activity periods were observed between experimental (Thor-Br) and control (Br-Br and UC) embryos.

Figure 6. (A) Mean activity period ± SEM characteristic of experimental (Thor-Br) and control (Br-Br and UC) embryos from day -6E through day -16E. No significant differences were observed at any days analysed.

(B) Mean absolute frequency (M/min) ± SEM characteristic of experimental (Thor-Br) and control (Br-Br and UC) embryos from day -6E through day -16E. Absolute frequency values for Thor-Br embryos were significantly reduced from control values from day -9E onward ($p < 0.05$).


A
8
6
4
2
0
UC
Br-Br
Thor-Br
ACTIVITY PERIOD
(Minutes ± SEM)
6
7
8
9
10
11
12
13
14
15
16
DAYS IN OVO
B
60
40
20
0
UC
Br-Br
Thor-Br
MOVEMENTS/MINUTE
OF ACTIVITY ± SEM
6
7
8
9
10
11
12
13
14
15
16
DAYS IN OVO

(c) Qualitative Analyses of Wing Motility: Qualitative analyses of the type of wing movements observed in experimental (Thor-Br) and control (Br-Br, PBR and UC) embryos indicated a marked difference between experimental and control embryos. At the onset of wing motility on day -6E, initial wing movements in all embryos consisted of small flexions and extensions of the shoulder and elbow joints whereas movement at the carpal joint was not initiated until day -7E. Control (Br-Br, PBR and UC) embryos exhibited daily increases in the range of motion at all joints as development proceeded until by day -10E the maximum range of flexion, extension, abduction and adduction was observed in complex combinations of movements at all wing joints. Beyond day -10E, control embryos maintained this pattern of wing movement for the duration of the study. In contrast, wing motility of experimental (Thor-Br) embryos was characterized throughout the developmental period analyzed by a limited range of movement at all joints. Thus, while control embryos performed smooth movements through the full range of each wing joint, wing movements of experimental (Thor-Br) embryos were small and jerky even though passive manipulation of the wing with forceps showed that the full range of movement was possible at all wing joints. Although wing movements of experimental embryos were of limited range, the movements were distinct and easily recognized from day -6E to day -12E. After day -12E, however, wing movements became progressively weaker in Thor-Br embryos until by day -16E each movement was barely discernible. Therefore, while the total frequency of wing movements of experimental (Thor-Br) embryos was equivalent to that of control embryos from day -6E through day -8E and became reduced from control values subsequently, qualitative differences between the types of wing movements observed in experimental and control embryos were striking throughout the developmental period analysed.

3. Morphogenesis of the Transplanted Neural Tube and Brachial Plexus

Analyses of developmental events which occur in the periphery of experimental (Thor-Br) embryos, such as wing motility (innervation and growth of wing muscles were also examined, see Butler, Cauwenbergs and Cosmos, 1986), indicated that initially functional and structural contacts were established between foreign thoracic motoneurons and wing muscles. Following this initial embryonic period, however, these contacts were lost, as indicated by a progressive reduction in wing motility (Fig. 5), loss of intra-muscular nerve branches and muscle wasting (Butler, Cauwenbergs and Cosmos, 1986). In an effort to understand the uncoupling phenomenon, developmental events occurring centrally (i.e., within the transplanted neural tube of Thor-Br embryos *per se*) were examined. To provide a proper assessment of the development of the transplanted neural tube in its new environment, it was necessary to examine the 1) *in situ* thoracic neural tube; 2) transplanted thoracic neural tube; 3) *in situ* brachial neural tube; and 4) transplanted brachial neural tube. Five parameters were compared:

a) histogenesis of transplanted neural tubes

b) pattern of peripheral nerve outgrowth

c) localization of motoneurons innervating wing muscles using horseradish peroxidase (HRP) retrograde labelling

d) the pattern of motoneuron death

e) growth and differentiation of motoneurons

(a) <u>Histogenesis of Transplanted Neural Tubes</u>: Histogenetic analyses of transplanted neural tubes were performed to determine if transplanted thoracic neural tubes in Thor-Br embryos retained charateristics of *in situ* thoracic neural tubes or if they developed according to their new site. Examination of serial cross-sections (thionin stained) of transplanted neural tubes indicated that both heterotopic (Thor-Br) and homotopic (Br-Br) transplants were viable and formed a brachial plexus bilaterally. Further, analysis of neural tube histogenesis from St 29 (day -6E) through St 42 (day -16E) demonstrated that all transplanted neural tubes (Thor-Br and Br-Br) developed according to their site of origin. The surgical control (Br-Br) neural tube developed morphological characteristics typical of an *in situ* brachial neural tube. Both the unoperated brachial and homotopically transplanted (Br-Br) brachial neural tube developed a large lateral motor column (LMC), a medial motor column (MMC) similar in size to that of the thoracic neural tube region and absence of the sympathetic preganglionic column of Terni, as illustrated in Figure 7A-F. Similarly, *in situ* thoracic and transplanted thoracic (Thor-Br) neural tubes developed identical phenotypic characteristics. The LMC of both Thor-Br and *in situ* thoracic neural tubes was comprised of small, intermittent clusters of motoneurons located in the ventro-lateral region of the ventral horn of grey matter (Fig. 7G-L). The most prominent features of both Thor-Br and *in situ* thoracic neural tubes were a relatively large MMC and the presence of a column of Terni (CT) observed in all transplanted thoracic (Thor-Br) and *in situ* thoracic neural tubes. Thus, the transplanted thoracic neural tube in experimental (Thor-Br) embryos developed morphological characteristics which were distinctly different from those of the brachial neural tube in control (Br-Br and UC) embryos, and instead were typical of those of *in situ* thoracic neural tubes.

(b) Pattern of Peripheral Nerve Outgrowth: To assess the pattern of peripheral nerve outgrowth within the brachial plexus both silver-stained and thionin-stained cross-sections were employed. Analyses of serial reconstructions of the brachial plexus in experimental (Thor-Br) and control (Br-Br and UC) embryos revealed that the pattern

Figure 7. (A-F) Diagrams to illustrate the histogenesis of the brachial neural tube in both Br-Br and unoperated control embryos from St 29 (day -6E) (A) through St 42 (day -16E) (F). Both the homotopically transplanted (Br-Br) and *in situ* brachial neural tubes developed a large, lateral motor column (LMC) and a prominent medial motor column (MMC). Bar = 0.029 mm

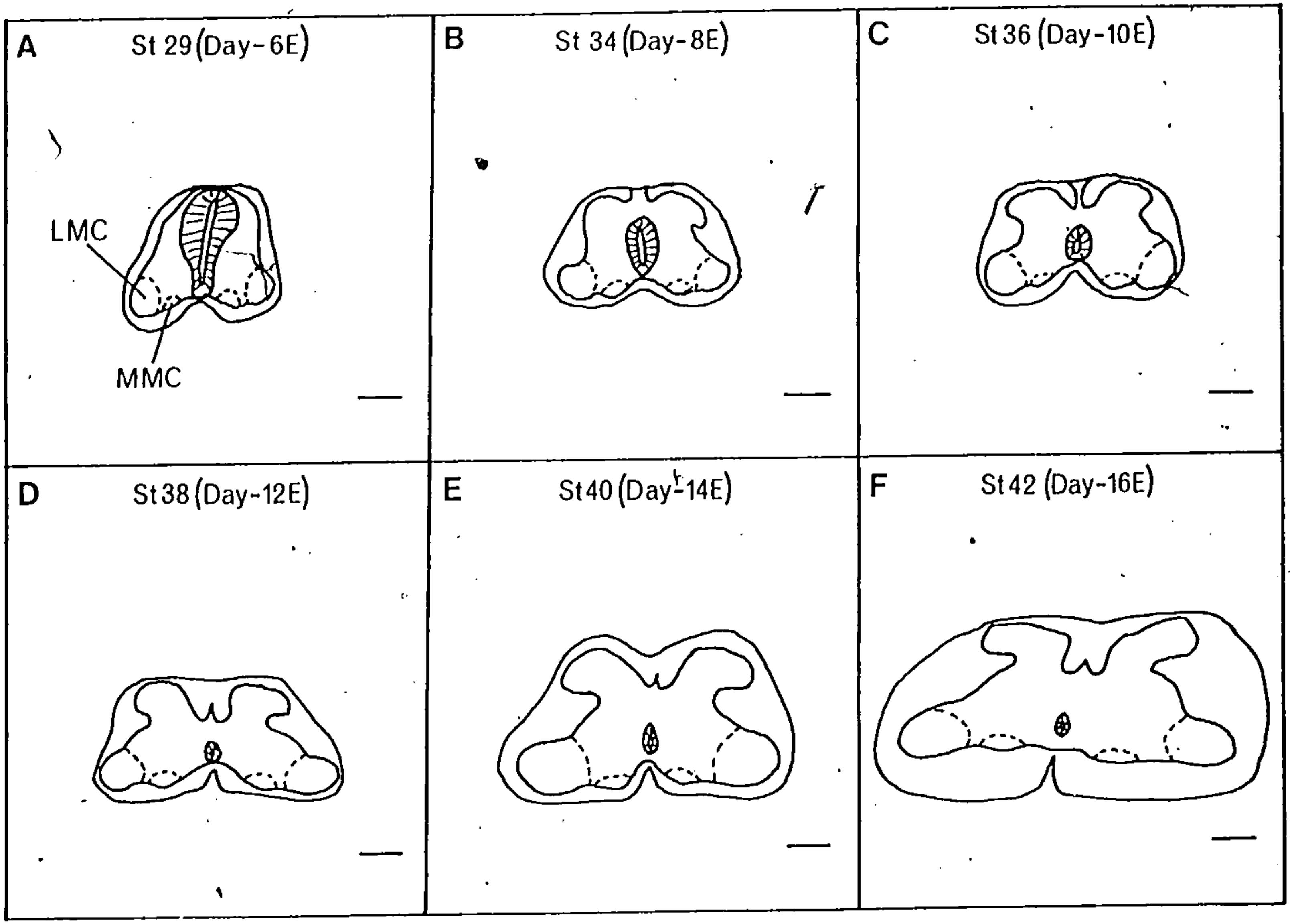
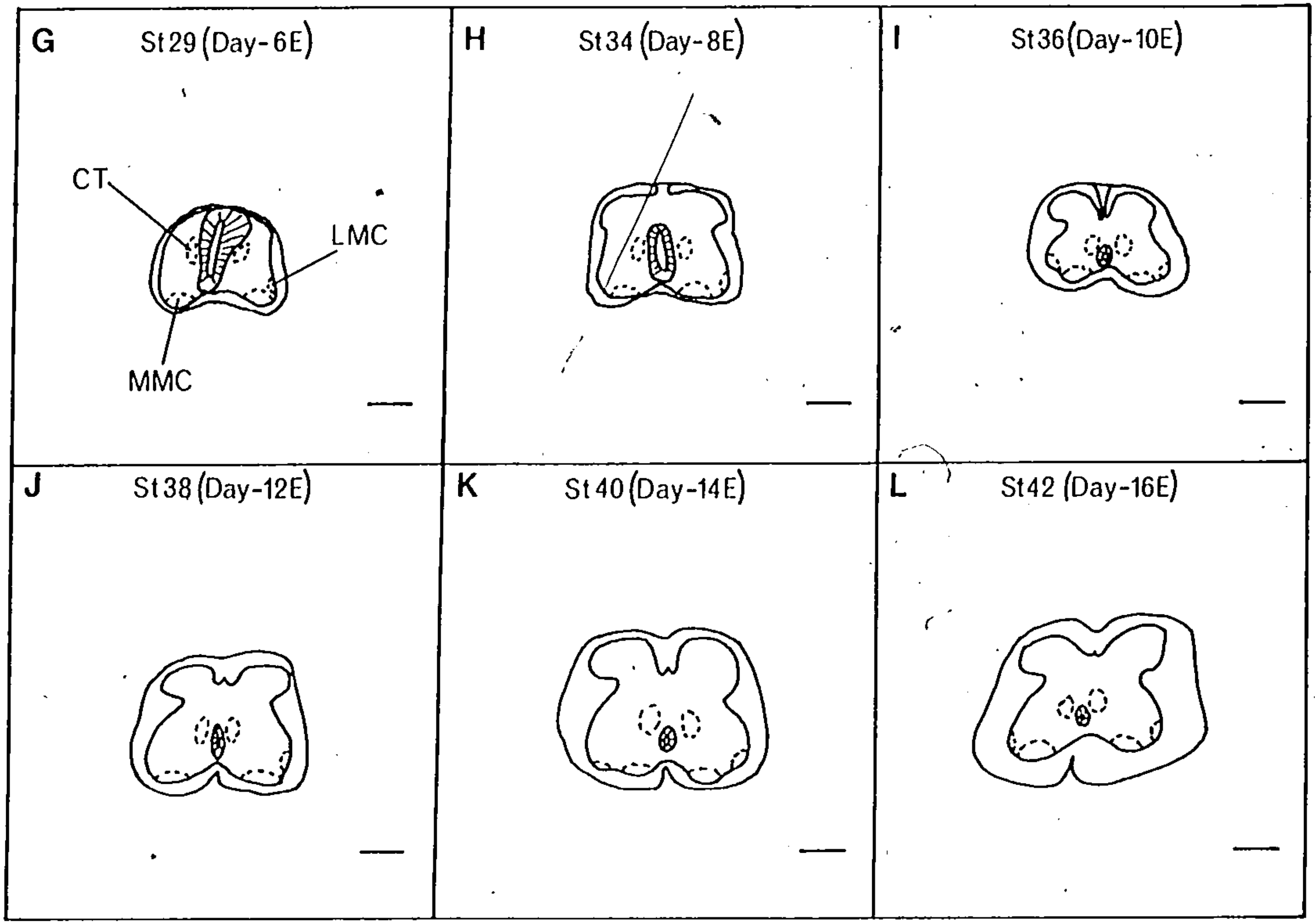

A
St 29 (Day-6E)
LMC
MMC
B
St 34 (Day-8E)
C
St 36 (Day-10E)
D
St 38 (Day-12E)
E
St 40 (Day-14E)
F
St 42 (Day-16E)

Figure 7. (G-L) Diagrams to illustrate the histogenesis of the heterotopically transplanted thoracic and *in situ* thoracic neural tubes in experimental Thor-Br and unoperated control embryos, respectively, from St 29 (day -6E) (G) through St 42 (day -16E) (L). Both the heterotopically transplanted and *in situ* thoracic neural tubes developed a small, intermittent lateral motor column (LMC) [present in only some cross-sections], a prominent medial motor column (MMC) and a sympathetic preganglionic column of Terni (CT). Bar = 0.029 mm.


G
St29 (Day-6E)
CT
LMC
MMC
H
St34 (Day-8E)
I
St36 (Day-10E)
J
St38 (Day-12E)
K
St40 (Day-14E)
L
St42 (Day-16E)

of nerve outgrowth from transplanted thoracic neural tubes was similar to that of neural tubes in control (Br-Br and UC) embryos, as described previously by others (Wenger, 1951; Straznicky, 1963; 1967). The brachial plexus in UC embryos was formed by peripheral nerve roots originating from spinal segments 12 through 17, inclusive (Table 2 and Fig. 8A) (see also; Roncalli, 1970). The contribution of these spinal segments to the brachial plexus, however, was variable as summarized in table 2. In 22 (81.5%) of 27 UC embryos examined only four nerve roots, originating from spinal segments 13 through 16, inclusive, gave rise to the brachial plexus. In the remaining 5 UC embryos analysed 5 spinal segments; either segments 12 to 16, inclusive, in 3 (11.1%) UC embryos or segments 13 to 17, inclusive, in 2 (7.4%) UC embryos, formed the brachial plexus. In none of the UC embryos analysed did all six spinal segments (12 through 17) contribute to the brachial plexus.

Similarly, the brachial plexus was formed by spinal segments 13 through 16, inclusive, in 11 (78.6%) of 14 surgical control (Br-Br) embryos examined. Also, the brachial plexus in 2 Br-Br embryos was derived from 5 spinal segments; segments 12 to 16, inclusive, in 1 (7.1%) Br-Br embryo and segments 13 to 17, inclusive, in 1 (7.1%) other Br-Br embryo (Table 2). In 1 (7.1%) additional Br-Br embryo the brachial plexus was formed by only 3 spinal segments (13 through 15, inclusive), however, in this operated embryo a spinal gap persisted at the caudal end of the grafted brachial neural tube and spinal nerve 16 was absent. Even though the spinal origin of the brachial plexus in this Br-Br embryo was deficient, the pattern of nerve roots contributing to the brachial plexus followed that typical of spinal nerves 13 through 15, inclusive, in unoperated embryos (see below). In all Br-Br embryos analysed the transplanted neural tube was continuous with the host neural tube at the cranial end of the graft.

TABLE 2

SEGMENTAL ORIGIN OF THE BRACHIAL PLEXUS

Experimental Manipulation	Number Analysed (n)	Spinal Segments 13 to 16 n (%)	Spinal Segments 12 to 16 n (%)	Spinal Segments 13 to 17 n (%)	Spinal Segments 14 to 16[1] n (%)	Spinal Segments 13 to 15[2] n (%)
Thor-Br	25	19 (76.0)	—	—	2 (8.0)	4 (16.0)
Br-Br	14	11 (78.6)	1 (7.1)	1 (7.1)	—	1 (7.1)
UC	27	22 (81.5)	3 (11.1)	2 (7.4)	—	—

1. Spinal nerve 13 was absent due to a spinal gap at the cranial end of the neural tube transplant.

2. Spinal nerve 16 was absent due to a spinal gap at the caudal end of the neural tube transplant.

In the majority of control (Br-Br and UC) embryos, therefore, the brachial plexus originated from four spinal segments (13 through 16, inclusive) and the morphology of the plexus was characterized as indicated in figure 8A. Typically ventral roots from spinal segments 13 and 14, as well as those from segments 15 and 16, joined to form two nerve trunks. In those embryos that were found to have 5 spinal segments which contributed to the brachial plexus (see above) a small ventral root from either segment 12 or segment 17 joined the plexus at the level of these two nerve trunks (Fig. 8A - dashed lines). The nerve trunks in all control (Br-Br and UC) embryos then coursed laterally for a short distance and merged to form a single combined nerve coursing towards the base of the wing and giving rise to two smaller nerve branches; 1) the axillary nerve which penetrated muscles of the shoulder region and 2) the pectoral nerve which innervated the pectoralis and supracoracoideus muscles. The brachial plexus in all embryos examined ended near the gleno-humeral joint in two large terminal branches; 1) the superior brachialis nerve which innervated the triceps brachii and extensor muscles of the forewing and 2) the inferior brachialis nerve which entered the biceps brachii and other flexor muscles of the wing.

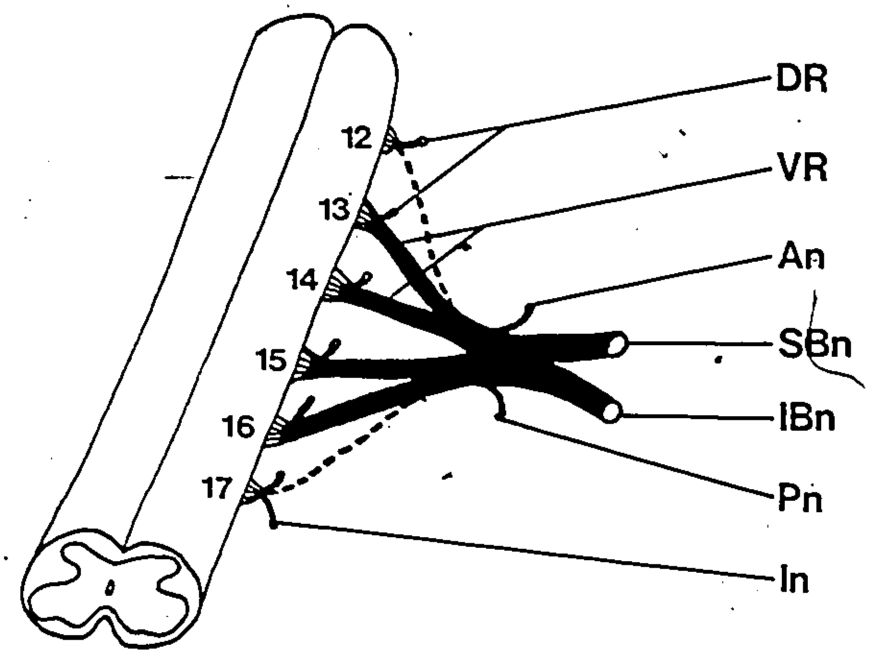
Serial reconstructions of the brachial plexus in experimental (Thor-Br) embryos produced similar results (Fig. 8B). Although the size of individual nerve roots contributing to the plexus was reduced from normal, the overall pattern of peripheral nerves within the plexus was similar to that of control (Br-Br and UC) embryos. The brachial plexus originated from spinal nerves 13 through 16, inclusive, in 19 (76.0%) of 25 Thor-Br embryos analysed and in no experimental embryos did the brachial plexus originate from 5 spinal segments (Table 2). Further, in 2 (8.0%) Thor-Br embryos in which a spinal gap was observed at the cranial end of the transplant and spinal nerve 13 was absent, the brachial plexus originated from only 3 spinal segments (14 through 16, inclusive) and these nerve roots followed the normal outgrowth

Figure 8. (A-B) Diagrams to illustrate the pattern of peripheral nerve outgrowth within the right brachial plexus of control (Br-Br and UC) (A) and experimental (Thor-Br) (B) embryos. Numbers represent spinal segments 12 through 17. DR, dorsal ramus to axial muscles; VR, ventral (Motor) root; An, axillary nerve; SBn, superior brachialis nerve; IBn, inferior brachialis nerve; Pn, pectoral nerve; In, first intercostal nerve.

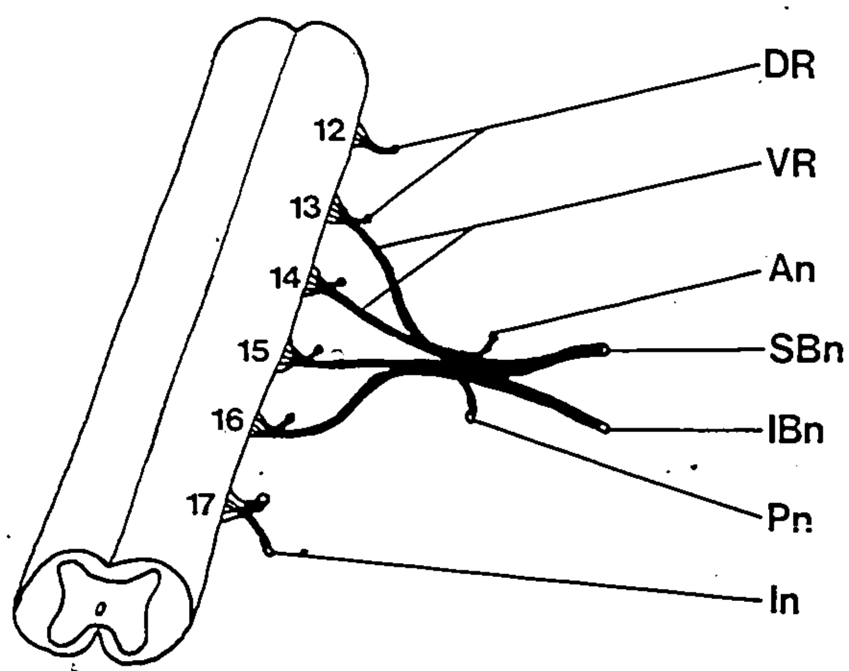
(A) The brachial plexus was formed by spinal nerves 13 through 16, inclusive, in 81.5% of control (Br-Br and UC) embryos. Spinal nerve 12 or 17 contributed to the brachial plexus in only 11.1% or 7.4%, respectively, of control embryos (dashed lines).

(B) The pattern of peripheral nerve outgrowth within the brachial plexus of experimental (Thor-Br) embryos was similar to that observed in control embryos. Nerves contributing to the plexus, however, were reduced in size and in no experimental embryos did spinal nerve 12 or 17 contribute to the plexus.

A


DR
VR
An
SBn
IBn
Pn
In
12
13
14
15
16
17

B


DR
VR
An
SBn
IBn
Pn
In
12
13
14
15
16
17

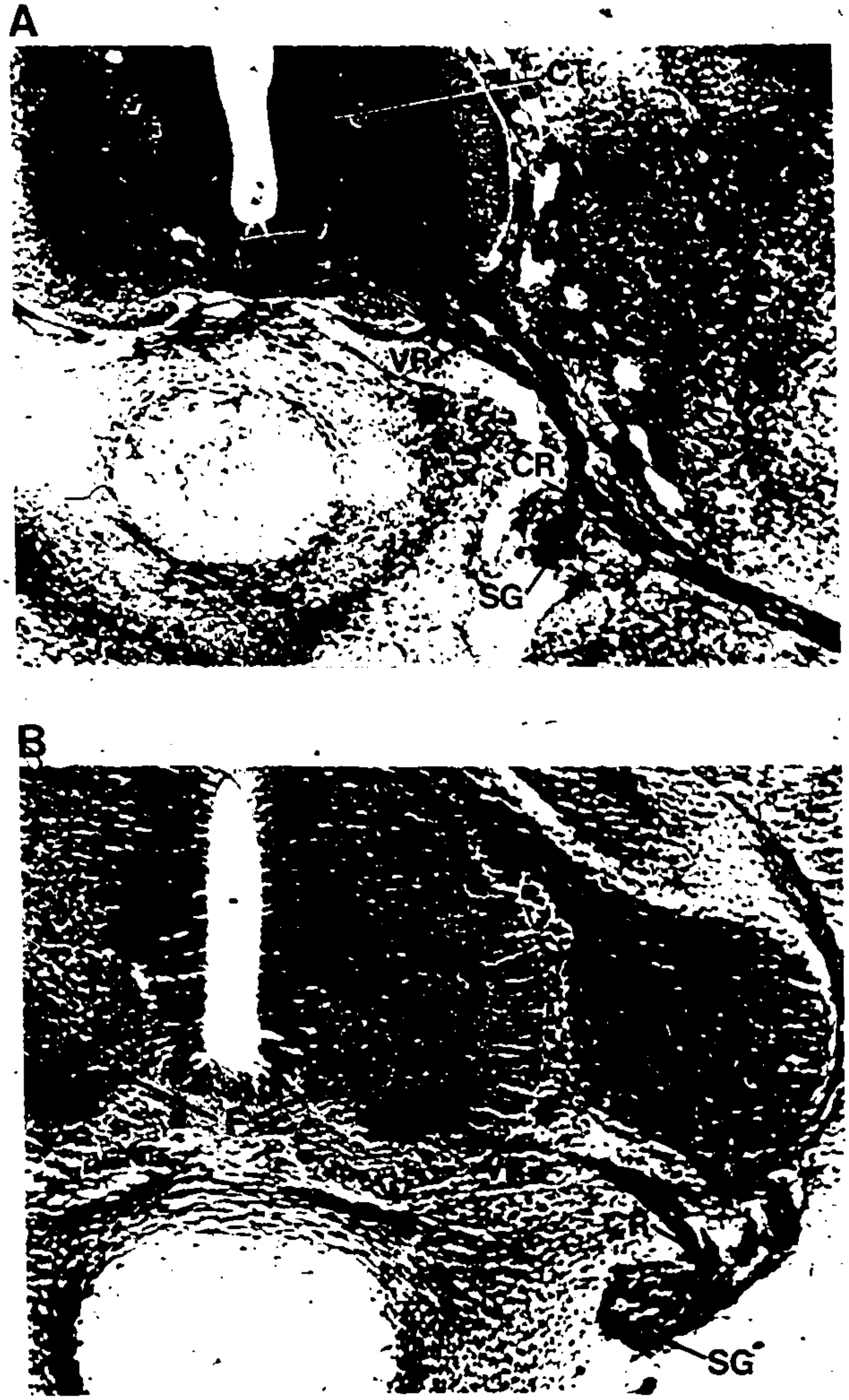
pattern observed for spinal nerves 14 to 16 in UC embryos. Also, in 4 (16.0%) Thor-Br embryos examined a spinal gap was found at the caudal end of the neural tube graft, which resulted in the absence of spinal nerve 16. The brachial plexus in the latter 4 experimental (Thor-Br) embryos was also derived from only 3 spinal segments (13 through 15, inclusive) and again the outgrowth pattern of the nerve roots was the same as that of nerve roots 13 to 15 in unoperated embryos (Fig. 8B).

Although the outgrowth pattern of peripheral nerves within the brachial plexus of Thor-Br embryos appeared morphologically normal a striking difference was observed between experimental (Thor-Br) and control (Br-Br and UC) embryos. Analyses of silver-stained serial cross-sections of the neural tube at St 29 (day -6E) revealed that sympathetic preganglionic nerve fibres originating in the CT were present in all transplanted (Thor-Br) and *in situ* thoracic neural tubes exclusively (Fig. 9 A-B) and these fibres formed ectopic communicating rami in the brachial region of Thor-Br embryos (Fig. 9A). Within the transplanted thoracic neural tube these nerve fibres were observed to course first ventrally from the CT, which was located near the midline of the neural tube adjacent to the spinal canal, as described previously for *in situ* thoracic neural tubes (Levi-Montalcini, 1950; Oppenheim, Maderdrut and Wells, 1982). The CT fibres then turned ventro-laterally to join motor fibres from the MMC and LMC and exited the neural tube via the ventral (motor) nerve roots. The sympathetic nerve fibres followed the ventral nerve roots for a short distance and then, in Thor-Br embryos, entered the sympathetic ganglia of the <u>brachial</u> region to form ectopic communicating rami (Fig. 9A). Sympathetic communicating rami are seen normally after day -3E in the thoracic region only (Levi-Montalcini, 1950). Similarly, in the present study communicating rami were observed in the thoracic region of UC embryos (Fig. 9B) and were not present in the brachial region of control (Br-Br and UC) embryos. Thus, sympathetic communicating rami connecting the ventral nerve roots

Figure 9.(A-B) Photomicrographs of cross-sections of the heterotopically transplanted thoracic (A), and *in situ* thoracic (B) neural tubes in experimental and control embryos at St 29 (day -6E). CT, sympathetic preganglionic column of Terni; VR, ventral root; SG, sympathetic ganglion; CR, communicating rami. Urea-silver nitrate stain. (160 X)

(A) Cross-section of the heterotopically transplanted thoracic neural tube in a Thor-Br embryo demonstrating the presence of the column of Terni (CT), sympathetic nerve fibres (F) and communicating rami (CR).

(B) Cross-section of the *in situ* thoracic neural tube in a UC embryo demonstrating the presence of the column of Terni (CT) and sympathetic nerve fibres (F) as well as communicating rami (CR).


A
CT
VR
CR
SG
B
SG

of the brachial plexus to the sympathetic chain ganglia were unique and characteristic in experimental (Thor-Br) embryos and their presence was the only deviation from the normal pattern of peripheral nerve outgrowth observed in the brachial plexus of control (Br-Br and UC) embryos.

(c) Localization of the Motor Innervation of Wing Muscles: To determine the precise origin of the motor innervation of specific wing muscles in Thor-Br embryos, horseradish peroxidase (HRP) retrograde labelling analyses were compared between experimental (Thor-Br) and control (Br-Br and UC) embryos at St 34 (day -8E) and St 38 (day -12E). Although the number of operated embryos analysed was small (Thor-Br, n=5; Br-Br, n=4) due to the high mortality rate following HRP injection, the HRP study yielded a striking result. Whereas HRP labelled cells were observed exclusively in the brachial LMC of control (Br-Br and UC, n=6) embryos following HRP injection into either the biceps brachii (Fig. 10 A,B) or triceps brachii (Fig. 11 A,B) muscle, the vast majority of labelled cells in all experimental (Thor-Br) embryos analysed were localized in the MMC of the transplanted thoracic neural tube (Fig. 10C and Fig. 11C). Only 2 labelled neurons were observed in the LMC of one transplanted thoracic neural tube following HRP injection into the triceps brachii at St 34 (day -8E).

Further analyses of the total number of HRP labelled cells revealed that following HRP injection into the biceps brachii muscle at St 34 (day -8E), 169 labelled cells and 131 labelled cells were counted in the brachial LMC of UC and Br-Br embryos, respectively,(Fig. 10A) and in these embryos labelled motoneurons were distributed predominantly in spinal segments 14 and 15 (Fig. 10B). Quantitative analyses of experimental (Thor-Br) embryos after HRP injection into the biceps brachii at St 34 (day -8E) indicated a total of 84 labelled neurons in the MMC of the transplanted thoracic neural tube (Fig. 10C) and again these cells were distributed predominantly in

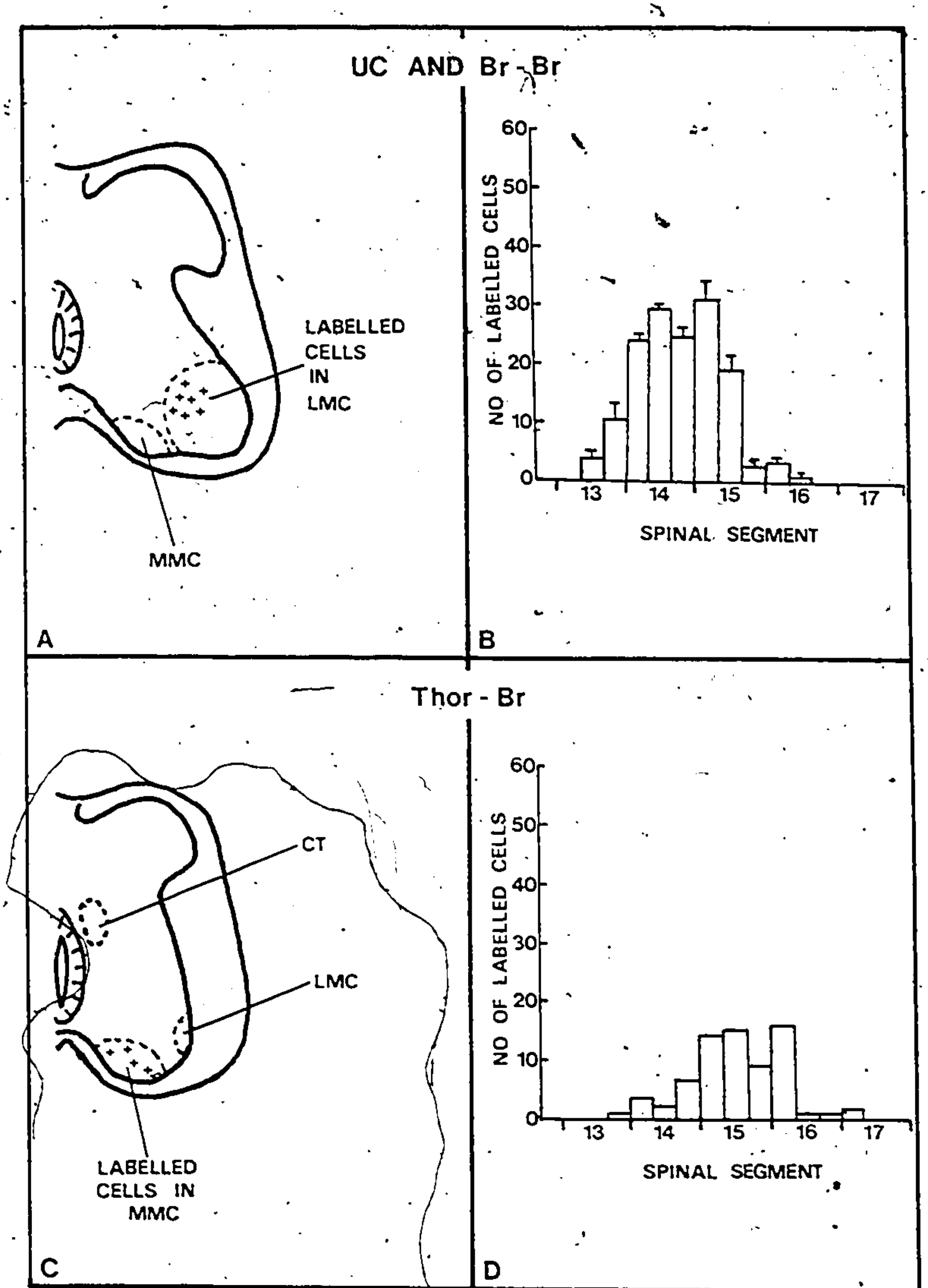
Figure 10. (A-D) Diagrams to illustrate the localization (A,C) and distribution (B,D) of HRP labelled motoneurons following HRP injection into the right biceps muscle in St 34 (day -8E) control (Br-Br and UC) and experimental (Thor-Br) embryos. LMC, lateral motor column; MMC, medial motor column; CT, column of Terni.

(A) Labelled cells were localized in a dorso-medial position within the LMC of control embryos.

(B) Motoneurons innervating the biceps muscle were distributed primarily in spinal segments 14 and 15 of control embryos. Values represent mean ± SEM.

(C) Labelled cells were positioned haphazardly within the MMC of heterotopically transplanted thoracic neural tubes.

(D) Similar to control embryos, motoneurons innervating the biceps brachii of experimental (Thor-Br) embryos were distributed primarily in spinal segments 14 and 15. Values represent mean ± SEM.


UC AND Br - Br
LABELLED CELLS IN LMC
MMC
A
60
50
40
30
20
10
0
NO OF LABELLED CELLS
13
14
15
16
17
SPINAL SEGMENT
B
Thor - Br
CT
LMC
LABELLED CELLS IN MMC
C
60
50
40
30
20
10
0
NO OF LABELLED CELLS
13
14
15
16
17
SPINAL SEGMENT
D

spinal segments 14 and 15 (Fig. 10D). HRP analyses following injection of the triceps brachii at St 34 (day -8E) showed that while a total of 130 (UC embryos) and 128 (Br-Br embryos) motoneurons contained the HRP label in control embryos, only 27 neurons were labelled in experimental (Thor-Br) embryos. In both experimental (Thor-Br) and control (Br-Br and UC) embryos, however, HRP labelled cells were distributed predominantly in spinal segments 15 and 16 (Fig. 11 B-D).

In addition the organization of HRP labelled neurons within the MMC of transplanted thoracic neural tubes was analysed and compared to that within the brachial LMC of control (Br-Br and UC) embryos. The analyses showed that unlike the orderly arrangement of HRP labelled cells observed in the brachial LMC of control embryos, experimental (Thor-Br) embryos were characterized by a disorderly organization of HRP labelled cells within the MMC of the transplanted thoracic neural tube. For example, similar to previous findings in unoperated chick embryos (Oppenheim, 1981), HRP labelled motoneurons in the brachial LMC of control (Br-Br and UC) embryos are localized in a dorso-medial position of the LMC after injection of the biceps brachii (Fig. 10 A) and in the ventro-lateral portion of the LMC following injection into the triceps brachii (Fig. 11A). In contrast, when HRP was injected into either the biceps brachii or the triceps brachii of Thor-Br embryos, labelled cells appeared randomly dispersed within the MMC of transplanted thoracic neural tubes (Fig. 10 C and Fig. 11 C). Motoneuron pools innervating particular wing muscles in experimental (Thor-Br) embryos, therefore, were not highly organized. Rather HRP labelled cells appeared to be positioned haphazardly within the MMC of transplanted thoracic neural tubes. This contrasted sharply with the organization of motoneuron pools observed within the brachial neural tube region of control (Br-Br and UC) embryos, in which motoneurons innervating individual wing muscles were localized into specific isolated regions of the brachial LMC.

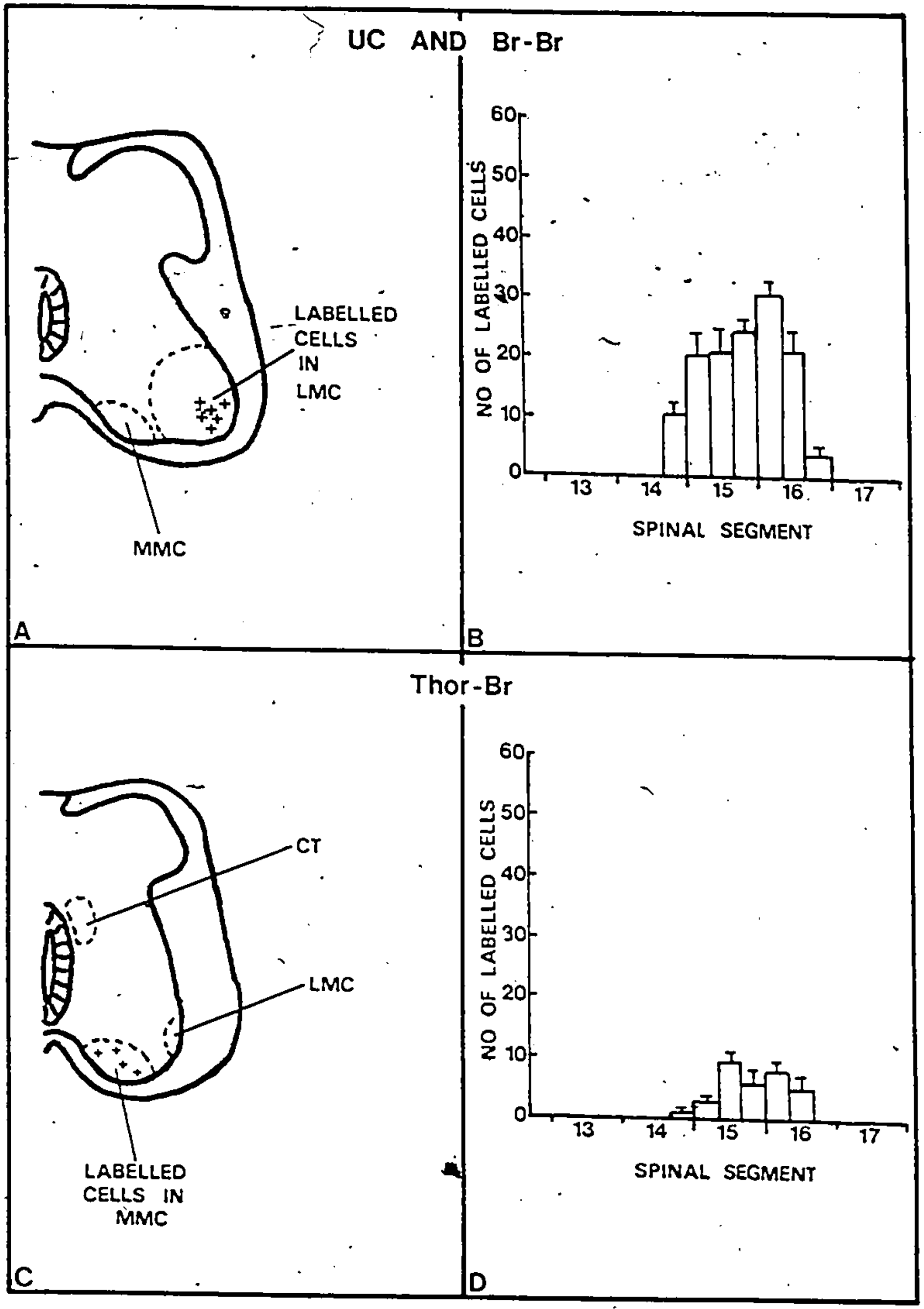
Figure 11. (A-D) Diagrams to illustrate the localization (A,C) and distribution (B,D) of HRP labelled motoneurons following HRP injection into the right triceps brachii muscle in St 34 (day -8E) control (Br-Br and UC) and experimental (Thor-Br) embryos. LMC, lateral motor column; MMC, medial motor column; CT, column of Terni.

(A) Labelled cells were localized in a ventro-lateral position within the LMC of control embryos.

(B) Motoneurons innervating the triceps brachii were distributed primarily in spinal segments 15 and 16 of control embryos. Values represent mean ± SEM.

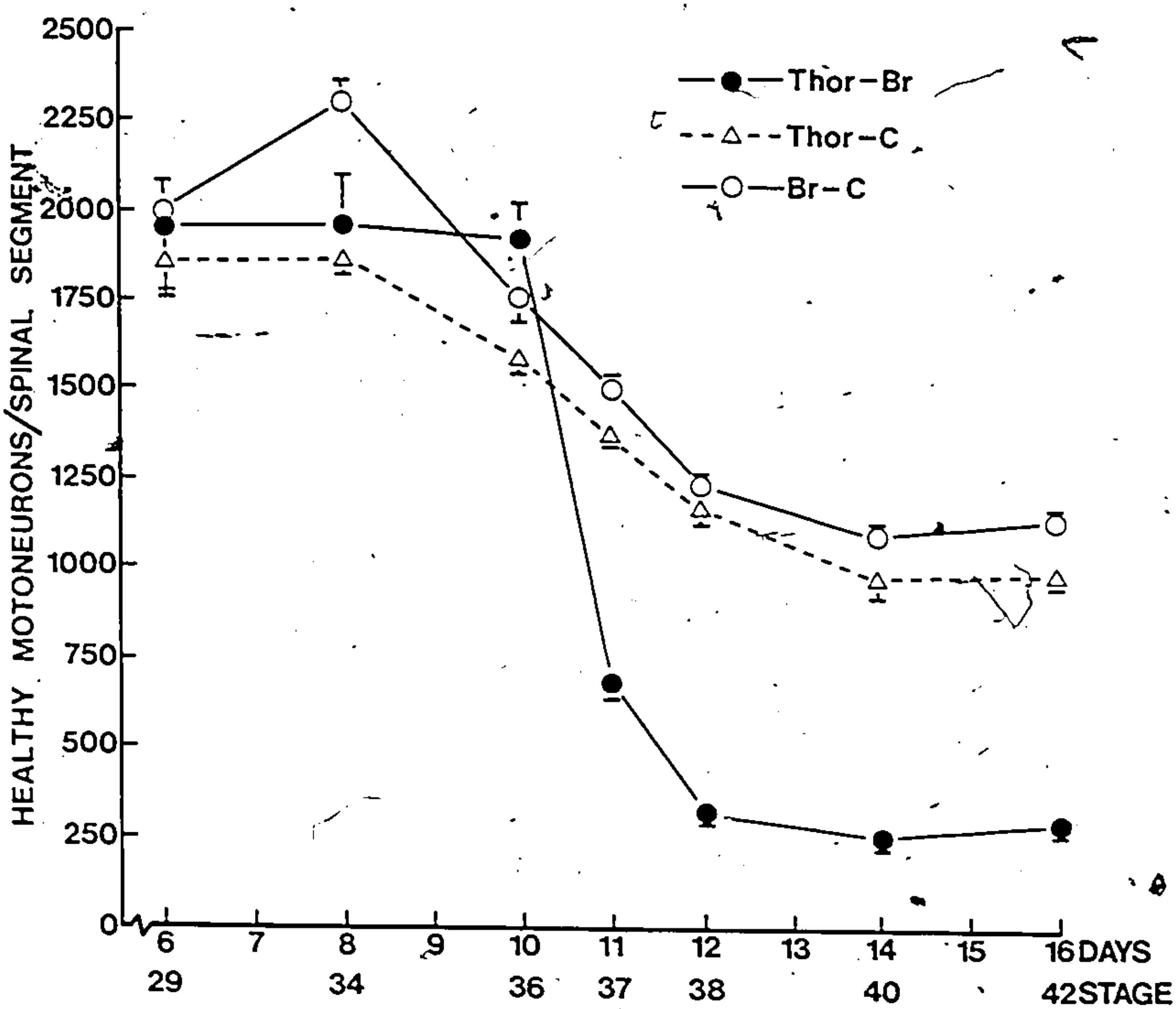
(C) Labelled cells were positioned haphazardly within the MMC of heterotopically transplanted thoracic neural tubes.

(D) Similar to control embryos, motoneurons innervating the triceps brachii of experimental (Thor-Br) embryos were distributed primarily in spinal segments 15 and 16. Values represent mean ± SEM.


UC AND Br-Br
LABELLED
CELLS
IN
LMC
MMC
A
60
50
40
30
20
10
0
NO OF LABELLED CELLS
13
14
15
16
17
SPINAL SEGMENT
B
Thor-Br
CT
LMC
LABELLED
CELLS IN
MMC
C
60
50
40
30
20
10
0
NO OF LABELLED CELLS
13
14
15
16
17
SPINAL SEGMENT
D

(d) The Pattern of Motoneuron Death in Transplanted Neural Tubes: The number of motoneurons/spinal segment in the MMC of transplanted thoracic (Thor-Br) and *in situ* thoracic (Thor-C) neural tubes were quantified and compared to similar analyses of the brachial MMC in ~~Br-C~~ (Br-Br and UC) embryos from St 29 (day -6E) through St 42 (day -16E) in order to assess the pattern of neurothanasia in a population of foreign thoracic motoneurons shown to be in contact structurally (HRP) and functionally (wing motility) with an enlarged peripheral target (wing musculature) (Butler, Cauwenbergs and Cosmos, 1986). Analyses of healthy (Fig. 12) and degenerating (Fig. 13) MMC motoneurons demonstrated a marked difference in the pattern of motoneuron death between neural tubes of experimental (Thor-Br) and control (Br-C and Thor-C) embryos. At St 29 (day -6E) neural tubes of Br-C and Thor-C embryos exhibited 1990 ± 76.8 and 1850 ± 96.0 healthy motoneurons/spinal segment, respectively. Initial numbers of MMC motoneurons in neural tubes of control (Br-C and Thor-C) embryos were maintained to St 34 (day -8E). After St 34 (day -8E) a period of natural motoneuron death extending to St 40 (day -14E) was observed with a cell loss of 43.4% in the brachial MMC of Br-C embryos and 47.9% within the *in situ* thoracic (Thor-C) MMC (Table 3). Final numbers of 1127 ± 38.4 and 963 ± 34.6 healthy motoneurons/spinal segment were maintained to St 42 (day -16E) in the MMC of control brachial (Br-C) and *in situ* thoracic (Thor-C) neural tubes, respectively. Similar to the MMC of control (Br-C and Thor-C) embryos, the initial number of motoneurons observed in transplanted thoracic neural tubes of Thor-Br embryos equalled 1900 ± 169.0/spinal segment (Fig. 12 and Table 3). In contrast to control embryos, however, the onset of neurothanasia in the MMC of grafted thoracic neural tubes (Thor-Br) was observed during the embryonic period following St 36 (day -10E). Between St 36 (day -10E) and St 38 (day -12 E) 83.8% of motoneurons present initially were lost in the MMC of heterotopically transplanted thoracic neural tubes (Fig. 12).

Figure 12. Mean number of healthy MMC motoneurons/spinal segment ± SEM observed in experimental (Thor-Br) and control (Br-C and Thor-C) embryos from St 29 (day -6E) through St 42 (day -16E). Initial numbers of healthy motoneurons in Thor-Br embryos were maintained to St 36 (day -10E); following this period 83.3% of MMC motoneurons had degenerated by St 38 (day -12E).


HEALTHY MOTONEURONS/SPINAL SEGMENT
Thor-Br
Thor-C
Br-C
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
6
7
8
9
10
11
12
13
14
15
16 DAYS
29
34
36
37
38
40
42 STAGE

Only 307 ± 36.5 healthy motoneurons/spinal segment were counted at St 38 (day -12E) and 278 ± 26.9 motoneurons/spinal segment survived to St 42 (day -16E) in the MMC of grafted thoracic neural tubes, indicating an overall cell loss of 85.4% (Table 3).

In addition to quantitative analyses of motoneuron numbers within the MMC of Thor-Br, Br-C and Thor-C embryos, similar analyses were performed on the brachial LMC of Br-C embryos in order to determine and compare the number of motoneurons shown (HRP) to innervate the wing muscles in both control (Br-C) and experimental (Thor-Br) embryos. The analysis showed that at St 29 (day -6E) 3754 ± 85.2 motoneurons/spinal segment were counted within the brachial LMC of Br-C embryos, and this number was maintained to St 34 (day -8E) (Table 3). Following this initial developmental time a period of natural motoneuron death extending from St 34 (day -8E) to St 40 (day -14E) was observed; by St 42 (day - 16E) 1157 ± 37.6 motoneurons/spinal segment were counted, indicating a cell loss of 69.2% in the brachial LMC of Br-C embryos. This final number, therefore, comprised the population of motoneurons which innervated the wing muscles normally. The MMC of transplanted thoracic neural tubes in St 29 (day -6E) Thor-Br embryos, on the other hand, contained only 50.6% of the number which innervated the wing muscles of control (Br-C) embryos prior to the period of motoneuron death (Table 3). Following the period of neurothanasia in St 42 (day -16E) Thor-Br embryos the final number of motoneurons available to innervate the wing musculature was only 24.0% of the normal number observed in the brachial LMC of control (Br-C) embryos. Thus, within the MMC of transplanted thoracic neural tubes in experimental (Thor-Br) embryos many fewer than the normal number of motoneurons are available to interact functionally with wing muscles.

TABLE 3

NUMBER OF MOTONEURONS/SPINAL SEGMENT BEFORE AND AFTER NEUROTHANASIA

Experimental Manipulation	Motor Column Analysed	Initial No./Segment[a]	% of Br-C LMC	Onset of Motoneuron Death	End of Motoneuron Death	Final No./Segment[b]	% of Br-C LMC	% Loss
Thor-Br	MMC	1900±169.0	50.6	St 36 (day -10E)	St 38 (day -12E)	278±36.5	24.0	85.4
Thor-C	MMC	1850±96.0	49.3	St 34 (day -8E)	St 40 (day -14E)	963±34.6	83.2	47.9
Br-C	MMC	1990±76.8	53.0	St 34 (day -8E)	St 40 (day -14E)	1127±38.4	97.4	43.4
Br-C	LMC	3754±85.2		St 34 (day -8E)	St 40 (day -14E)	1157±37.6		69.2

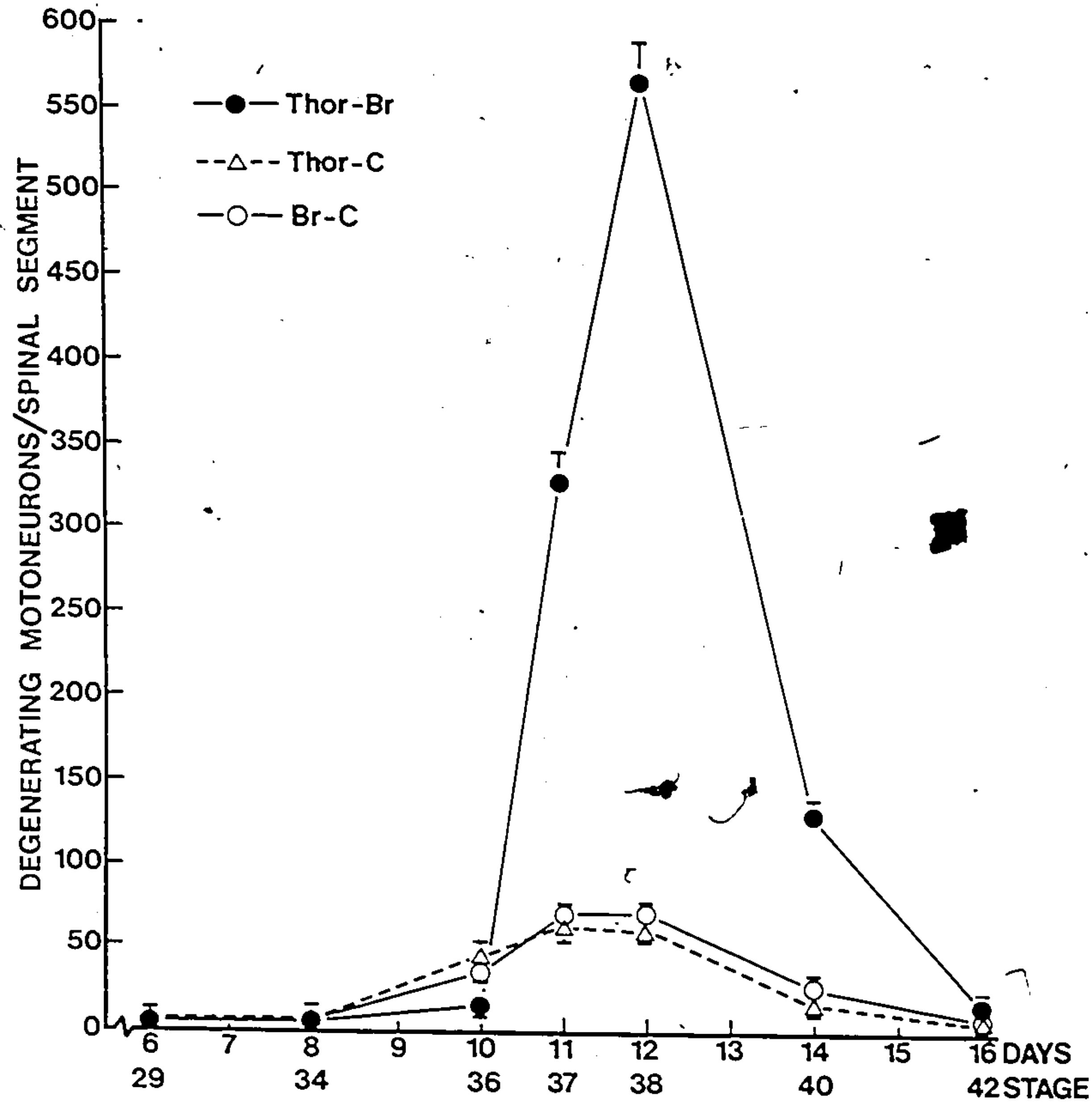
a. Values represent mean ± SEM at St 29 (day -6E)

b. Values represent mean ± SEM at St 42 (day -16E)

Further to analyses of healthy motoneuron numbers, quantification of the number of degenerating MMC motoneurons/spinal segment demonstrated a 9 fold increase in the peak number of degenerating cells within transplanted thoracic neural tubes of Thor-Br embryos when compared to brachial (Br-C) and *in situ* thoracic (Thor-C) neural tubes of control embryos (Fig. 13). Initially, few degenerating cells were observed in neural tubes of control embryos at St 29 (day -6E) and St 34 (day -8E). During subsequent days of development, however, the number of degenerating MMC motoneurons in neural tubes of control (Br-C and Thor-C) embryos increased , reaching peak values of 68.4 $\pm$ 5.45 (Br-C) and 66.0 $\pm$ 8.14 (Thor-C) degenerating neurons/spinal segment at St 37 (day -11E) (Fig. 13). This peak level was maintained to St 38 (day -12E); thereafter, few degenerating cells were observed in neural tubes of control embryos. In contrast to neural tubes of control (Br-C and Thor-C) embryos at St 36 (day -10E) in which 37.0 $\pm$ 6.3 and 46.2 $\pm$ 10.5 degenerating motoneurons/spinal segment, respectively, were observed (Fig. 13), low numbers of degenerating neurons were counted in transplanted thoracic (Thor-Br) neural tubes from St 29 (day -6E) (4.6 $\pm$ 4.2 degenerating cells/segment) through St 36 (day -10E) (16.9 $\pm$ 8.0 degenerating cells/segment). After St 36 (day -10E), however, the number of degenerating neurons in the MMC of transplanted thoracic (Thor-Br) neural tubes increased dramatically, reaching a peak value of 565.6 $\pm$ 21.7 degenerating cells/segment at St 38 (day -12E). The number of degenerating MMC motoneurons/spinal segment then dropped abruptly to 136.5 $\pm$ 11.3 at St 40 (day -14E) and 16.8 $\pm$ 8.8 at St 42 (day -16E) in transplanted thoracic (Thor-Br) neural tubes (Fig. 13).

Quantitative analyses of degenerating MMC motoneurons in neural tubes of both experimental (Thor-Br) and control (Br-C and Thor-C) embryos, therefore, demonstrated a close correlation between the number of degenerating cells and the pattern of healthy motoneuron numbers observed during the developmental period

Figure 13. Mean number of degenerating MMC motoneurons/spinal segment ± SEM observed in experimental (Thor-Br) and control (Br-C and Thor-C) embryos from St 29 (day -6E) through St 42 (day -16E). Few degenerating neurons were observed in the MMC of Thor-Br embryos during the initial embryonic period extending to St 36 (day -10E). Following St 36 (day -10E) a 9 fold increase in the number of degenerating cells was noted in Thor-Br embryos by St 38 (day -12E).



DEGENERATING MOTONEURONS/SPINAL SEGMENT
600
550
500
450
400
350
300
250
200
150
100
50
0
Thor-Br
Thor-C
Br-C
6 7 8 9 10 11 12 13 14 15 16 DAYS
29 34 36 37 38 40 42 STAGE

analysed. Thus, although the onset of MMC motoneuron death was delayed until after St 36 (day -10E) in neural tubes of Thor-Br embryos, neurothanasia was increased greatly during later development.

(e) Growth and Differentiation of MMC Motoneurons: Analyses of motoneuron growth and differentiation within the MMC of neural tubes in experimental (Thor-Br) and control (Br-C and Thor-C) embryos, as well as within the brachial LMC of neural tubes in Br-C embryos were performed from St 29 (day -6E) through St 42 (day -16E). The data indicated that heterotopic (Thor-Br) neural tube transplantation had no effect on the growth and differentiation of transplanted motoneurons.

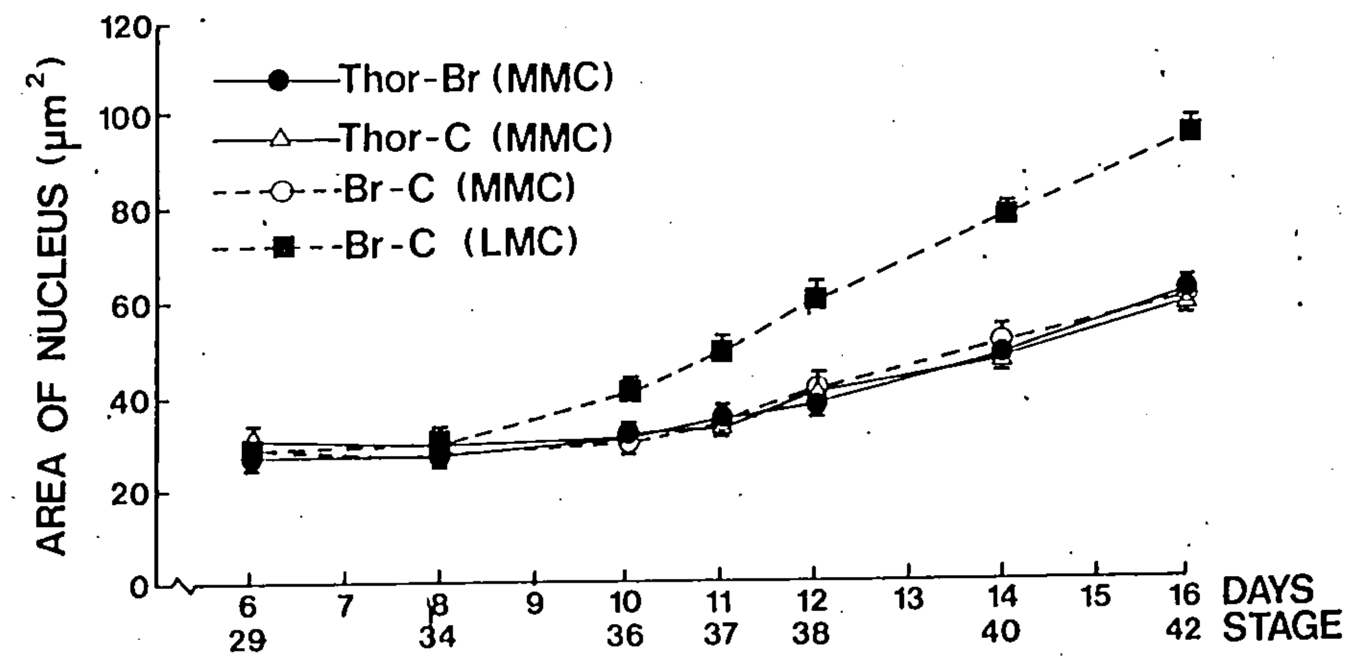
At St 29 (day -6E) motoneuron nuclei in all neuronal populations (LMC and MMC) analysed, exhibited mean cross-sectional areas of approximately 30 μm^2 (Fig. 14) and the nuclear size remained constant in all motoneuron populations through St 34 (day -8E). After St 34 (day -8E), however, LMC motoneuron nuclei grew significantly ($p < 0.05$) and continued to grow through St 42 (day -16E). MMC motoneuron muclei, on the other hand, did not grow significantly ($p < 0.05$) until St 38 (day -12E) and then showed continual growth during subsequent days of development through St 42 (day -16E). Consequently, as early as St 36 (day -10E) LMC motoneuron nuclei in brachial neural tubes of Br-C embryos were observed to have a mean cross-sectional area of $40.4 \pm 0.57\ \mu m^2$ and were clearly larger than MMC motoneuron nuclei in both transplanted thoracic neural tubes ($31.5 \pm 0.26\ \mu m^2$) and neural tubes of control (Br-C, $31.8 \pm 0.34\ \mu m^2$ and Thor-C, $31.3 \pm 0.25\ \mu m^2$) embryos. From St 36 (day -10E) onward the growth of brachial LMC motoneuron nuclei in neural tubes of Br-C embryos was greater than that of MMC motoneuron nuclei in neural tubes of both experimental (Thor-Br) and control (Br-C and Thor-C) embryos. By St 42 (day -16E) the mean cross-sectional area of brachial LMC motoneuron nuclei in neural tubes of Br-C embryos was

95.2 ± 0.26 μm^2 and that of MMC motoneuron nuclei in the neural tubes of Thor-Br, Br-C and Thor-C embryos was 61.9 ± 0.08 μm^2, 60.5 ± 0.40 μm^2 and 59.8 ± 0.46 μm^2, respectively. Interestingly, no differences were observed between the mean cross-sectional areas of MMC motoneuron nuclei in neural tubes of experimental (Thor-Br) and control (Br-C and Thor-C) embryos at any of the embryonic stages examined (Fig. 14).

Analyses of the growth of motoneuron nuclei, therefore, demonstrated a striking difference between the growth of MMC and LMC motoneurons. By St 42 (day -16E) brachial LMC motoneuron nuclei were 36.8% larger than all MMC nuclei examined. Furthermore, transplanted thoracic (Thor-Br) MMC motoneuron nuclei exhibited the same growth curve as *in situ* thoracic (Thor-C) motoneuron nuclei, indicating that the growth of motoneurons is independent of peripheral influences and is controlled autonomously.

Further, to assess the differentiation of healthy motoneurons from St 29 (day -6E) through St 42 (day -16E) the number of nucleoli were counted both in MMC motoneuron nuclei within neural tubes of experimental (Thor-Br) and control (Br-C and Thor-C) embryos and in LMC motoneuron nuclei within neural tubes of Br-C embryos. Since quantification of motoneuron nuclei within the LMC and MMC indicated that all motoneuron populations examined, irrespective of their location [LMC versus MMC or transplanted (Thor-Br and Br-Br) versus *in situ*], differentiated according to the same developmental schedule, the data obtained for experimental (Thor-Br) and control (Br-C and Thor-C) embryos were pooled and expressed as the mean percent of motoneurons which exhibited either 3,2 or 1 nucleoli (Fig. 15). In all cases LMC and MMC motoneurons remained undifferentiated (3 nucleoli) during early development up to St 34 (day -8E). The onset of motoneuron differentiation, as determined by

Figure 14. Growth of motoneuron nuclei in experimental (Thor-Br) and control (Br-C and Thor-C) embryos from St 29 (day -6E) through St 42 (day -16E). Values represent nuclear mean cross-sectional areas ± SEM. Brachial LMC motoneuron nuclei were significantly larger ($p < 0.05$) than MMC motoneuron nuclei from St 36 (day -10E) onward.


AREA OF NUCLEUS (μm²)
120
100
80
60
40
20
0
Thor-Br (MMC)
Thor-C (MMC)
Br-C (MMC)
Br-C (LMC)
6 7 8 9 10 11 12 13 14 15 16 DAYS
29 34 36 37 38 40 42 STAGE

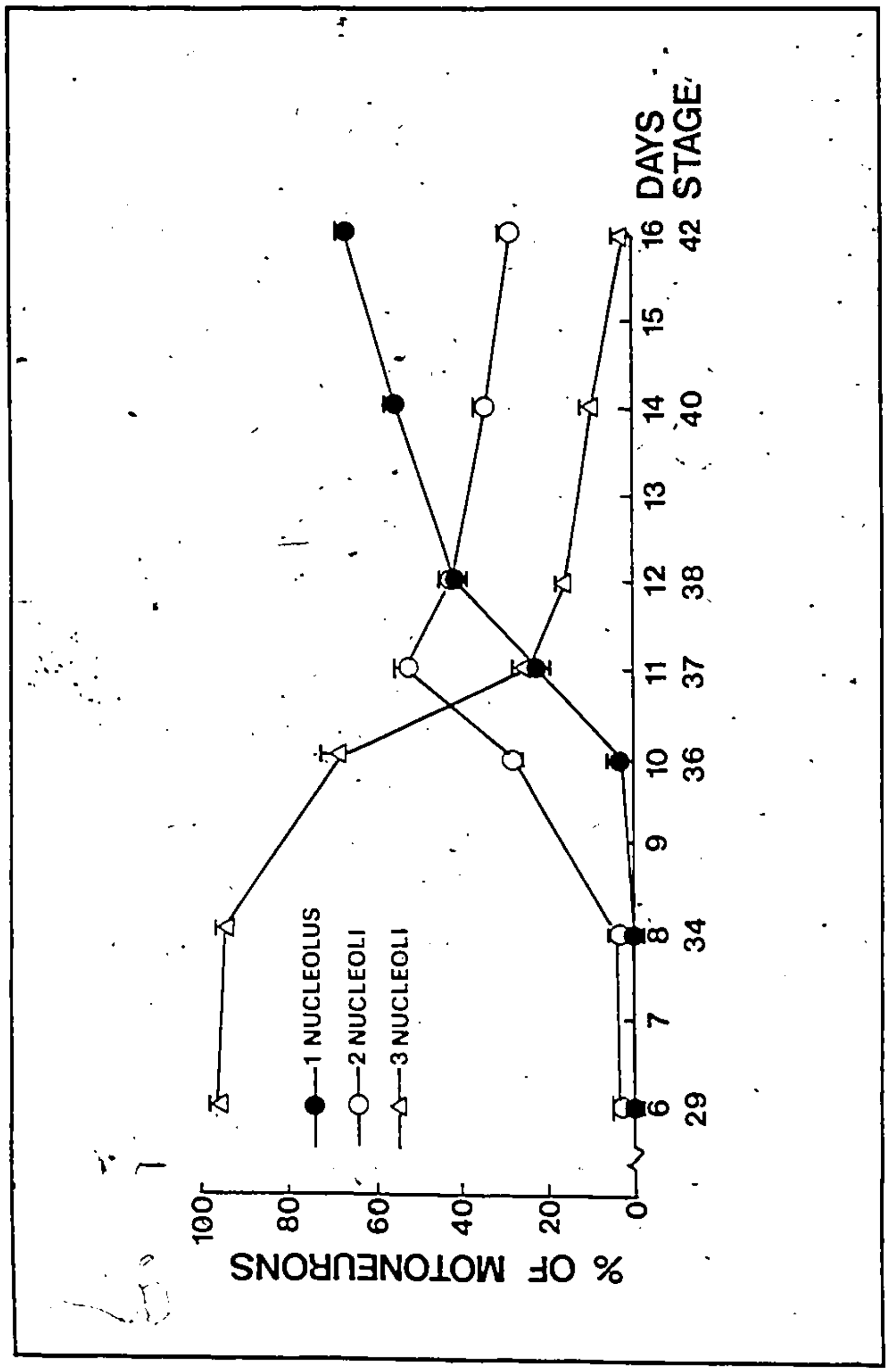
these criteria (see Section C - 2d of MATERIALS AND METHODS), was noted between St 34 (day -8E) and St 36 (day -10E). By St 36 (day -10E) 27.9 ± 0.87% of motoneuron nuclei contained 2 nucleoli and this increase in the number of differentiating cells occurred temporally coincident with a nearly equal drop in the percentage of undifferentiated (3 nucleoli) motoneurons. Only 3.8 ± 0.72% of motoneurons sampled were differentiated (1 nucleolus) at St 36 (day -10E). By St 37 (day -11E) approximately 50% of all motoneurons tested had started to differentiate (2 nucleoli) and now 22.1 ± 0.65% of motoneurons were differentiated (1 nucleolus). From St 37 (day -11E) onward the percentage of differentiated motoneurons continued to increase until by St 42 (day -16E) 67.2 ± 0.64% of the nuclei examined contained one large nucleolus. The increased percentage of differentiated nuclei during the later developmental stages examined was reflected by a gradual drop in the percentage of both differentiating (2 nucleoli) and undifferentiated (3 nucleoli) motoneuron nuclei. By St 42 (day -16E) only 3.45 ± 0.34% of nuclei examined contained 3 nucleoli and, therefore, had not yet started to differentiate.

Analyses of the differentiation of motoneuron nuclei indicate, therefore, that all motoneuron populations (LMC and MMC) examined differentiate according to the same developmental schedule.

C. Heterotopic (Thor-Br/som) Somitic Mesoderm Transplantation

The heterotopic neural tube transplant studies demonstrated that although foreign thoracic motor nerves initially interact functionally with wing muscles, such nerves are eventually deemed inappropriate (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). To investigate further the uncoupling phenomenon observed between inappropriate nerve-muscle partners, heterotopic

Figure 15. Differentiation of motoneuron nuclei as measured by the number of nucleoli present during the embryonic period extending from St 29 (day -6E) through St 42 (day -16E). No significant differences in the rate of differentiation were observed between any of the motoneuron populations tested when experimental (Thor-Br) and control (Br-C and Thor-C) embryos were compared, therefore, values were pooled and expressed as the mean percent of motoneurons ± SEM which contained either 1, 2 or 3 nucleoli.


% OF MOTONEURONS
100
80
60
40
20
0
1 NUCLEOLUS
2 NUCLEOLI
3 NUCLEOLI
6 7 8 9 10 11 12 13 14 15 16 DAYS
29 34 36 37 38 40 42 STAGE

somitic mesoderm transplantations (Thor-Br/som) were employed. Intraspecific chick/chick chimaeras were produced by transplanting thoracic somitic mesoderm from donor embryos of either dystrophic or normal genotype to the region of extirpated brachial somites in host embryos of normal genotype (see Section B-2 of MATERIALS AND METHODS). The aim of this series of experiments was to determine if *in situ* brachial motor nerves develop and maintain functional interactions with wing muscles derived from heterotopically transplanted (Thor-Br/som) thoracic somitic mesoderm.

1. Morphology of Thor-Br/som Experimental Embryos

Surgical (Thor-Br/som) manipulation of chick embryos at day -2E did not affect gross morphogenesis of experimental embryos during subsequent development. Examination of experimental (Thor-Br/som) embryos both through the shell window at the time of daily wing motility analyses and upon removal from the egg at the termination of the experiment revealed that surgically manipulated embryos adhered to the chronological staging criteria of Hamburger and Hamilton (1951) and that experimental embryos were morphologically similar to unoperated control embryos. Of a total of 51 operated embryos analysed, however, 3 (5.9%) exhibited a slight scoliosis of the brachial vertebral column, 9 (17.7%) were found to have shortened scapulae and 2 (3.9%) had developed a small curvature of the sternum. These experimental (Thor-Br/som) embryos were not eliminated from the study since re-examination of their motility records indicated that the slight deformities observed did not affect wing motility adversely.

2. Wing Motility Analyses of Thor-Br/som Experimental Embryos

To determine the extent of functional nerve-muscle interaction in experimental (Thor-Br/som) embryos at daily intervals from day -6E through day -15E the number of wing movements in 10 minute observation periods (M/10 = total frequency) were compared between two experimental groups: 1) DThor-NBr/som (dystrophic donors) and 2) NThor-NBr/som (normal donors).

Examination of limb motility in unoperated dystrophic and normal control groups of embryos demonstrated that abnormal frequencies (M/10) of limb motility specific to the brachial region characterized dystrophic embryos during a precise developmental period (see below). It was reasoned, therefore, that if this embryonic dystrophic phenotype of reduced wing motility, first reported by Cauwenbergs, Butler and Cosmos, 1986a, was also expressed in experimental (DThor-NBr/som) embryos, it could be used as an easily recognized, non-invasive marker for the viability of thoracic somitic mesoderm transplanted to replace extirpated brachial somites.

(a) Wing Motility of Unoperated Dystrophic Chick Embryos: To establish the developmental pattern of right wing movements in unoperated dystrophic (S_1) embryos, daily wing motility analyses were performed from day -6E through day -16E (as described in Section C-1a of MATERIALS AND METHODS) and compared to wing motility (M/10) values of unoperated normal white leghorn (WLH) embryos (UC in Fig. 5). Although no qualitative differences in wing movements were observed between dystrophic and normal embryos throughout the developmental period analysed, quantitative differences in the total frequency of wing movements (M/10) were noted during a specific embryonic period (Fig. 16 and Table 4). During the initial period extending from day -6E, when overt wing movements were first observed, through day -9E, no differences in M/10 values were observed between dystrophic (S_1) and normal groups. Both dystrophic and normal embryos exhibited daily increases in the frequency of wing movements, rising from 10 M/10 and 11 M/10 at day -6E to 168 M/10 and 172 M/10 at day -9E, respectively. M/10 values for dystrophic embryos were reduced significantly ($p < 0.05$) from those of normal embryos, however, from day -10E through day -15E. Dystrophic embryos characteristically did not exhibit the sharp increase in wing motility observed in normal embryos between day -10E and day -11E, resulting in M/10 values which were 25% reduced from normal values at day -11E (Fig. 16). The total frequency of right wing movements in dystrophic embryos increased on day -12E and reached a plateau by day -13E. By day -16E, M/10 values for dystrophic wings were still lower than normal values but the difference observed was not statistically significant at this time.

Additional wing motility analyses were performed on the genetically related S_{10} (dystrophic) and S_{11} (normal) lines of chick embryos to ensure that the difference in the total frequency of wing motility observed between S_1 (dystrophic) and the local white leghorn (WLH) embryos were not due to strain differences (Table 4). The data demonstrated that similar trends in the development of wing motility were evident when either the two dystrophic (S_1 and S_{10}) strains or the two normal (WLH and S_{11}) strains of embryos were compared. The total frequency of wing motility (M/10) was equivalent between the WLH and S_{11} normal embryonic lines throughout the period analysed. As in WLH normal embryos (UC in Fig. 5) motility values observed in S_{11} normal embryos increased from 9.7 M/10 on day -6E to a peak value of 324 M/10 on day -14E and this level of activity was maintained through day -16E (Table 4). Similarly, M/10 values of S_{10} dystrophic embryos were equivalent to those of S_1 dystrophic embryos, increasing from 9.5 M/10 on day -6E to 236.8 M/10 on day -10E. As in the S_1 dystrophic strain, S_{10} dystrophic embryos failed to show the sharp increase in the total frequency of wing motility observed in both WLH and S_{11} normal embryos

Figure 16. Comparative wing motility of normal (White Leghorn) and dystrophic (S_1') embryos from day -6E through day -16E. Statistically significant impaired motility characterized dystrophic wings from day -10E through day -15E.

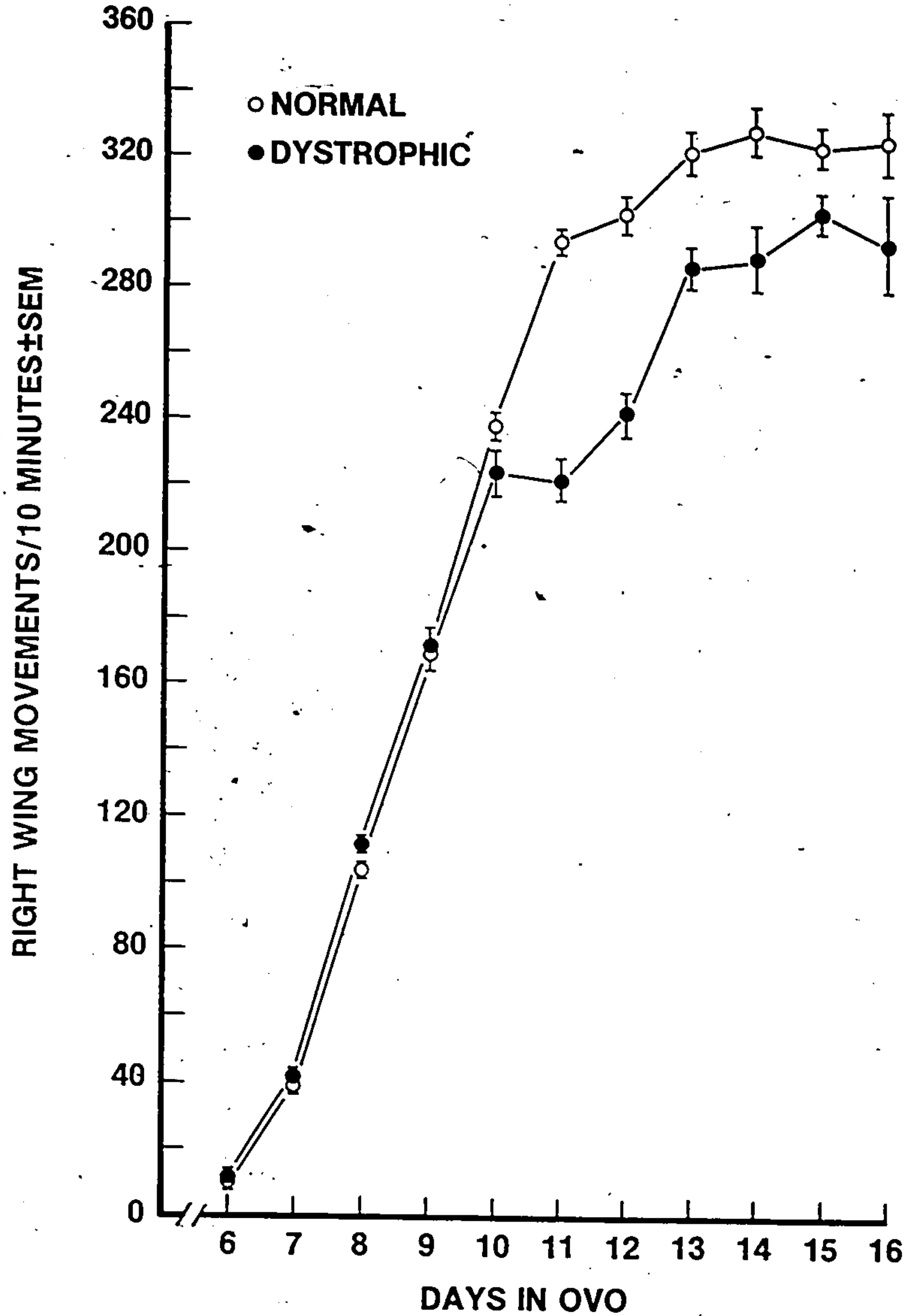

NORMAL
DYSTROPHIC
RIGHT WING MOVEMENTS/10 MINUTES±SEM
360
320
280
240
200
160
120
80
40
0
6
7
8
9
10
11
12
13
14
15
16
DAYS IN OVO

TABLE 4

Wing Motility of Normal and Dystrophic Chick Embryos

Age	N (WLH)[a]	D (S_1)[a]	N (S_{11})[a]	D (S_{10})[a]
6E	9.6 ± 0.8 (25)[b]	11.4 ± 1.1 (18)	9.7 ± 0.8 (10)	9.5 ± 0.8 (10)
7E	39.0 ± 2.1 (24)	39.9 ± 2.2 (21)	40.8 ± 2.4 (12)	39.7 ± 2.1 (11)
8E	104.3 ± 2.5 (28)	111.0 ± 3.1 (33)	106.6 ± 2.8 (10)	104.8 ± 2.9 (13)
9E	168.5 ± 3.5 (28)	171.8 ± 4.6 (29)	165.6 ± 2.3 (11)	165.5 ± 1.9 (13)
10E	238.3 ± 3.5 (26)	224.0 ± 6.8 (27)*	239.4 ± 2.0 (14)	236.8 ± 2.3 (13)
11E	294.0 ± 3.5 (25)	221.6 ± 6.5 (25)**	292.4 ± 2.7 (11)	228.1 ± 4.8 (15)**
12E	303.4 ± 5.6 (30)	242.5 ± 7.1 (26)**	303.7 ± 2.0 (9)	242.1 ± 5.1 (13)**
13E	321.8 ± 6.6 (22)	287.2 ± 5.5 (19)**	320.0 ± 2.4 (9)	281.3 ± 3.3 (9)**
14E	329.1 ± 7.1 (17)	290.4 ± 9.6 (16)**	324.4 ± 3.2 (7)	285.3 ± 6.0 (12)**
15E	324.4 ± 6.2 (17)	304.1 ± 6.0 (19)**	323.3 ± 4.8 (4)	314.1 ± 4.3 (11)
16E	325.7 ± 9.2 (18)	295.1 ± 14.9 (15)	316.5 ± 2.1 (2)	316.8 ± 3.8 (9)

[a] Motility (M/10) ± SE.
[b] Number in parentheses refers to number of embryos measured.
*,** Indicates dystrophic values significantly different from normal at the 0.95 and 0.99 confidence levels, respectively (Mann–Whitney U test).

between day -10E and day -11E. Total frequency values recorded for S_{10} dystrophic embryos were significantly reduced from normal M/10 values from day -11E (228 M/10) through day -14E (285 M/10). On day -15E and day -16E M/10 values were within the range of values observed in normal embryos. The only difference between S_1 and S_{10} dystrophic strains, therefore, was that S_{10} dystrophic embryos did not exhibit significantly lower motility values at either day -10E or day -15E, whereas M/10 values for S_1 dystrophic embryos were reduced significantly at these two embryonic days.

In addition to a comparison of wing motility between the four strains of normal and dystrophic embryos a separate series of unoperated normal (WLH) and dystrophic (S_1) embryos was used to determine if aberrant motility observed in dystrophic strains was common to both the wings and legs of individual dystrophic embryos. Ipsilateral (right) wing and leg motilities were analysed in individual normal and dystrophic embryos at two specific embryonic times; day -8E, a period when wing motility was equivalent in normal and dystrophic embryos and day -12E, a period when wing motility was reduced significantly in dystrophic versus normal embryos (Table 5). Comparison of the data indicate that only M/10 values recorded for wings of dystrophic embryos at day -12E specifically, were significantly lower than values for normal wings. No statistical differences between normal and dystrophic embryos were observed in motility values recorded for hindlimbs at either day tested. Further, although ipsilateral wing and leg motility values for individual normal embryos were equivalent at both days, the total frequency of wing motility was significantly lower than that of ipsilateral legs in dystrophic embryos at day -12E.

Comparison of wing and leg motility analyses between normal and dystrophic embryos, therefore, demonstrate that impaired functional nerve-muscle interactions observed in dystrophic embryos during a precise embryonic period are specific to the brachial region.

(b) Wing Motility of Thor-Br/som Experimental Embryos: Daily recordings of the total frequency of wing motility (M/10) in experimental (Thor-Br/som) embryos demonstrated that similar to differences observed between unoperated dystrophic and normal embryos (Fig. 16) experimental DThor-NBr/som embryos exhibited M/10 values reduced significantly from those of NThor-NBr/som embryos during a specific embryonic period (Fig. 17) (Cauwenbergs, Butler and Cosmos, 1986b). As in unoperated embryos of either genotype (Table 5), M/10 values for both DThor-NBr/som and NThor-NBr/som operated groups were equivalent and increased daily from 10 M/10 and 9 M/10 at day -6E to 166 M/10 and 170 M/10, respectively, at day -9E. From day -10E through day -15E, however, the total frequency of wing movements in DThor-NBr/som embryos was significantly lower than that of NThor-NBr/som embryos. By day -11E and day -12E DThor-NBr/som embryos exhibited M/10 values that were reduced from NThor-NBr/som values by 21.6% and 19.3%, respectively. Comparison of Figures 16 and 17 demonstrates that wing motility in DThor-NBr/som and NThor-NBr/som embryos followed the same developmental pattern as exhibited by unoperated dystrophic and normal embryos, respectively.

Daily wing motility analyses, therefore, were an excellent functional marker and demonstrated the viability of the heterotopic thoracic somitic mesoderm transplant. The data indicate that transplanted thoracic somitic mesoderm gave rise to brachial muscles and these muscles made contact with *in situ* brachial motor nerves. The results also show that wing muscles derived from transplanted thoracic somitic mesoderm interact functionally with *in situ* brachial motor nerves and this functional interaction was maintained throughout the embryonic period analysed. This con-

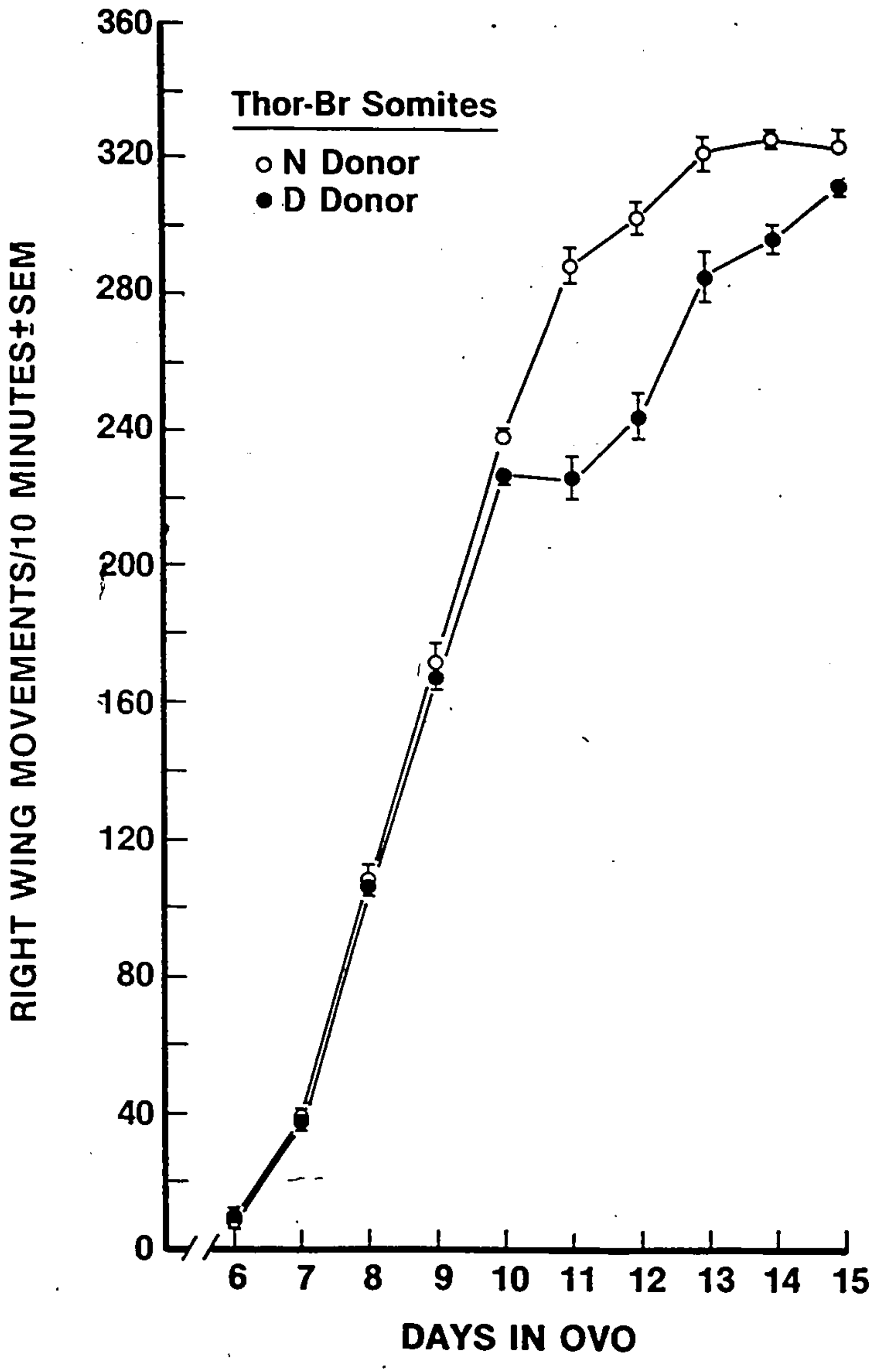
TABLE 5

Ipsilateral Wing and Leg Motility in Dystrophic Chicks

Genotype[a]	(N)	Age	Wing[b]	Leg[b]
N	(10)	8E	102.1 ± 2.8	102.3 ± 2.8
D	(14)	8E	111.1 ± 4.4	108.3 ± 2.6
N	(14)	12E	303.6 ± 5.4	301.5 ± 5.9
D	(12)	12E	233.6 ± 6.8*†	302.7 ± 6.0

[a] D and N refer to dystrophic (S_1) and normal (White Leghorn) embryos, respectively.
[b] Motility (M/10) ± SE.
*·† At day 12E only, the motility of the D wing differed significantly (0.95 confidence level, Mann–Whitney *U* test) from values for the N wing (*) and D leg (†).

Figure 17. Comparative motility analyses of wing muscles derived from NThor-NBr/som (normal donor) and DThor-NBr/som (dystrophic donor) experimental embryos from day -6E through day -15E. Statistically significant impaired motility characterized the wings of experimental (DThor-NBr/som) embryos from day -10E through day -15E.



Thor-Br Somites
N Donor
D Donor
RIGHT WING MOVEMENTS/10 MINUTES±SEM
360
320
280
240
200
160
120
80
40
0
6
7
8
9
10
11
12
13
14
15
DAYS IN OVO

trasts ~~sharply~~ with the results of the heterotopic neural tube transplant experiments, which showed that, although provisional functional contacts were established between foreign thoracic motor nerves and *in situ* brachial muscles early in embryogenesis, this functional interaction could not be maintained during later development (Butler, Cauwenbergs and Cosmos, 1986).

DISCUSSION

The purpose of the present study was to explore the nature of nerve-muscle interactions between brachial muscles and foreign nerves derived from a heterotopically transplanted thoracic neural tube in chick embryos. Developmental events in both the developing wing and spinal cord were investigated to define the extent of nerve-muscle interaction in Thor-Br embryos and to elucidate possible factors responsible for the eventual incompatibility observed between wing muscles and foreign thoracic nerves. The principle findings are summarized as follows:

1. The results show that initial nerve-muscle contacts in the wings of experimental (Thor-Br) embryos were functional since wing motility was observed in these embryos throughout the embryonic period analysed [St 29 (day -6E) to St 42 (day -16E)]. This finding indicates that impulse activity generated within the heterotopically transplanted thoracic neural tube reached wing muscles via foreign peripheral nerves; furthermore, frequencies of wing motility equal to those of control (Br-Br, PBR and UC) embryos were observed up to day -8E. From day -9E onward, however, frequencies of wing motility became progressively reduced in Thor-Br embryos until by day -16E few wing movements were noted. The decline in wing motility was correlated temporally with the onset of nerve-muscle uncoupling observed histochemically in specific wing muscles of experimental (Thor-Br) embryos (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986).

2. Histogenetic analyses revealed that transplanted thoracic neural tubes maintained the identity of *in situ* thoracic neural tubes. Thoracic neural tube transplants in Thor-Br embryos typically developed and maintained a CT and a small, intermittent LMC, morphological features characteristic of unoperated thoracic neural tubes. Histogenetic analyses revealed, therefore, that neural tube

development is controlled rigidly, since transplanted thoracic neural tubes were unable to conform to signals derived from the brachial environment.

3. Although morphogenesis of transplanted thoracic neural tubes was controlled autonomously, the pattern of peripheral nerve outgrowth in Thor-Br embryos was dependent on factors derived from the periphery.

Nerves derived from the transplanted thoracic cord, similar to *in situ* brachial nerves, formed a plexus in the brachial region, as reported previously (Wenger, 1951; Straznicky, 1967). The latter was essentially morphologically equivalent to the plexus formed in unoperated and Br-Br embryos.

4. HRP analyses of the origin of motor innervation showed that wing muscles in control (Br-Br and UC) embryos typically received innervation from motoneurons located in the LMC. Wing muscles of experimental (Thor-Br) embryos,however, were innervated by motoneurons localized in the MMC of the transplanted thoracic neural tube.

5. Quantitative analyses of MMC motoneuron numbers in transplanted neural tubes of Thor-Br embryos revealed that the pattern of motoneuron death in this neuronal population was dependent on peripheral events. During the initial embryonic period when intramuscular axons and functional nerve-muscle interaction was observed in wing muscles of Thor-Br embryos, initial numbers of MMC motoneurons were maintained in transplanted thoracic neural tubes. Coincident with the developmental time when motor nerves start to uncouple from individual wing muscles in Thor-Br embryos (Butler, Cauwenbergs and Cosmos, 1986), however, a rapid and near total degeneration of MMC motoneurons was observed in the transplanted thoracic neural tube. This finding indicated that during embryogenesis the survival of spinal motoneurons was dependent on the maintenance of sustained

interaction with target muscles in the periphery.

6. To determine if nerve-muscle uncoupling also occurs when *in situ* brachial nerves are made to innervate target muscles derived from heterotopically transplanted thoracic somitic mesoderm wing motility analyses were performed on a second series (Thor-Br/som) of operated embryos. Initial examination of unoperated embryos of normal and dystrophic genotype revealed that dystrophic chick embryos display reduced frequencies of wing motility during a specific phase of embryogenesis (Cauwenbergs, Butler and Cosmos,1986a). It was reasoned, therefore, that if muscles derived from thoracic somitic mesoderm taken from dystrophic donor embryos exhibit impaired motility during the same embryonic period then reduced frequencies of wing motility would serve as a useful marker for the viability of the mesodermal graft. The results showed that, indeed, wing motility in DThor-NBr/som (dystrophic donors) and NThor-NBr/som (normal donors) was equivalent to that of unoperated dystrophic and normal embryos, respectively (Cauwenbergs, Butler and Cosmos, 1986b). Thus, only embryos with wing muscles derived from somitic mesoderm of dystrophic genotype showed impaired nerve-muscle interaction. Wing motility, therefore, served as a suitable functional marker for the viability of transplanted somitic mesoderm.

7. Functional nerve-muscle interaction (motility) was maintained to day -16E (the oldest Thor-Br/som embryos analysed), a time well beyond the period (day -9E) when reduced frequencies of wing motility became evident in heterotopic neural tube transplant (Thor-Br) embryos. Sustained wing motility in experimental (Thor-Br/som) embryos indicates that, in contrast to Thor-Br embryos, uncoupling of functional nerve-muscle contacts was not expressed in wings of Thor-Br/som embryos. The nerve-muscle uncoupling phenomenon, therefore, was unique to a thoracic

nerve-brachial muscle combination.

A detailed discussion of these results is given below.

A. Extent Of Nerve-Muscle Interaction In Thor-Br Embryos

To monitor the extent of functional nerve-muscle interaction in individual experimental (Thor-Br) and control (Br-Br, PBR and UC) embryos , wing motility was quantified daily through an extended embryonic period from day -6E to day -16E. As reported previously for unoperated chick embryos (Hamburger and Balaban, 1963), the onset of wing motility was observed at day -6E in control (Br-Br, PBR and UC) embryos. Significantly, wing motility was also initiated at this stage in experimental (Thor-Br) embryos. The analyses showed that the total frequency of wing movements observed in Thor-Br embryos increased daily and equalled that of controls through day -8E. These findings indicated that the frequency of electrical activity emanating from the transplanted thoracic tube is normal since embryonic limb motility is correlated with polyneuronal bursts of electrical discharges generated spontaneously within the spinal cord (Ripley and Provine, 1972; Provine, 1973). Beyond this initial period, however, reduced frequencies of wing movement were observed in Thor-Br embryos and wing motility declined precipitously during the last week of development. At day -16E some wing movements were still observed indicating that a few functional nerve-muscle contacts persisted to this time.

Contrary to the suggestion of Mark (1980), the decline in wing motility observed in Thor-Br embryos was not due to a possible interruption of either supraspinal or propriospinal (intersegmental) input. Motility analyses of PBR embryos in which the brachial spinal cord was surgically isolated from supraspinal and propriospinal influences indicated that the frequency of wing movements in these operated embryos was significantly reduced from Br-Br and UC values only at days -15E and -16E, long after the time when wing motility became reduced in Thor-Br embryos. The present motility analyses, therefore, support the report of Provine and Rogers (1977) which demonstrated that frequencies of polyneuronal burst discharges (neural correlates of embryonic motility) are reduced from control values only from day -15E onward in chick embryo spinal cords following surgical removal of a portion of the cervical neural tube at day -2E. This late embryonic period (after day -15E) is characterized by the onset of coordinated limb movements and pre-hatching behavior in chick embryos, the latter motility patterns are controlled by supraspinal centres (Hamburger and Oppenheim, 1967; Sohal, 1976). Possible interruption of appropriate supraspinal or propriospinal inputs resulting from the hetero-topic neural tube transplantation procedure, therefore, does not account for the great reduction in wing motility observed in experimental (Thor-Br) embryos after day -8E.

Previous reports indicated that avian limbs innervated by thoracic nerves are either immotile or exhibit limited motility (Székely and Szentágothai, 1962; Straznicky, 1963; 1967). These investigations, however, were concerned primarily with coordinated limb movements during *ex ovo* development (for review, Mark, 1980). Székely and Szentágothai (1962) noted that when forelimb or hindlimb buds are transplanted to the midthoracic region of chick embryos at day -3E, limb movements are not observed in hatched experimental birds. Straznicky (1963), who noted that wings of hatched chicks are paralyzed when innervated by thoracic nerves derived from a heterotopically transplanted thoracic neural tube, concluded that "axons of thoracic motoneurons cannot establish effective myoneural junctions with wing musculature". Morris (1978) employed the same experimental model as Székely and Szentágothai (1962) and demonstrated using electrophysiological techniques that electrical stimulation of thoracic nerves innervating the transplanted limb elicits contractions of specific limb muscles; however, the study was limited to the embryonic period up to St 37 (day -11E) and involved small numbers of experimental embryos having limbs innervated exclusively by thoracic nerves. One study which used the same experimental (Thor-Br) model as the present study to achieve heterotopic innervation gave a detailed developmental account of histogenetic events in wing muscles innervated by foreign thoracic nerves (Straznicky, 1967). Although the chronology of degenerative events in embryonic wing muscles having thoracic innervation presented by Straznicky (1967) agrees with the more detailed analyses of Butler, Cauwenbergs and Cosmos (1986), Straznicky failed to compare the changes which occur within individual wing muscles. Furthermore, Straznicky (1967) was not concerned with the development of nerve-muscle function and indicated that muscle degeneration occurs in wings of Thor-Br embryos even though intramuscular motor axons are present. Previous findings (Butler, Cosmos and Brierley, 1982b; Cauwenbergs, Cosmos and Butler, 1983; Butler, Cauwenbergs and Cosmos, 1986) show that this is not the case. Indeed, degeneration of individual wing muscles in experimental (Thor-Br) embryos occurs as a consequence of progressive nerve-muscle uncoupling, withdrawal of intramuscular axons and loss of functional nerve-muscle interactions.

Wing motility in the present study served as a functional parameter reflecting developmental events recorded previously in the periphery (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). Experimental (Thor-Br) embryos exhibited reduced frequencies of wing movement coincident temporally with the onset of intramuscular axon withdrawal from individual wing muscles. The decline in wing motility reflects a progressive functional uncoupling of nerve-muscle contacts established initially by the foreign nerves and their targets, but, subsequently deemed inappropriate. Intramuscular nerve branches extending from the brachial plexus are observed within all brachial muscles of Thor-Br embryos from St 30 (day -6.5E) through St 35 (day 8-9E) (Butler, Cauwenbergs and Cosmos, 1986). After this initial embryonic period, however, intramuscular axons appear to withdraw from their muscle partners in wings of Thor-Br embryos (Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986). Although denervation does not occur synchronously in all experimental muscles, it does coincide with the time in normal development when permanent neuromuscular junctions are established within individual wing muscles (Atsumi, 1977; Adachi, 1983; Butler, Cauwenbergs and Cosmos,1986). To illustrate, the ALD muscle which forms endplates earlier than other brachial muscles exhibits withdrawal of innervation at St35 (day 8-9E) (Butler, Cauwenbergs and Cosmos,1986). In fact, mature endplates are never observed in wing muscles of experimental (Thor-Br) embryos. By St 42 (day -16E) only a limited number of intramuscular axons are observed in some wing muscles (triceps brachii, biceps brachii, scapulohumeralis posterior); other wing muscles in Thor-Br embryos at this stage either are replaced completely by lipid (ALD) or are absent (PLD) due to the denervation process (Butler, Cauwenbergs and Cosmos, 1986).

The nerve-muscle uncoupling phenomenon observed in Thor-Br embryos, however, is limited to appendicular brachial muscles (derived from the pre-muscle masses of the wing bud) and is not observed in brachial muscles of myotomal origin, such as the axial musculature (Butler, Cauwenbergs and Cosmos, 1986). Axial muscles are segmentally arranged along the entire length of the vertebral column and in the rat these muscles are a normal target for thoracic motor nerves (Smith and Hollyday, 1983). In experimental (Thor-Br) embryos, motor nerves growing out from the heterotopically transplanted thoracic neural tube encounter brachial axial muscles and establish stable endplates in these muscles. Two groups of nerve-muscle partnerships exhibiting distinct developmental patterns exist, therefore, in the brachial region of experimental (Thor-Br) embryos: 1. Foreign thoracic nerves associated with appendicular brachial muscles fail to establish permanent neuromuscular junctions and ultimately break initial connections; and, 2. Foreign thoracic nerves which couple with brachial muscles of myotomal origin form stable, compatible synapses.

It is known that axial and appendicular muscles differ phenotypically; whereas myotomally derived axial muscles exhibit similar myosin ATPase fibre-type profiles throughout the length of the vertebral column, the large locomotor muscles of the wing are characterized by diverse myosin ATPase profiles (Butler and Cosmos, 1981b; Butler, Cosmos and Brierley, 1982b; Butler, Cauwenbergs and Cosmos, 1986)). Thoracic nerves which are normally paired with axial muscles and never innervate wing muscles may not recognize appendicular muscles of the wing as suitable partners due to phenotypic fibre-type differences between these two muscle groups. Both the failure to develop mature neuromuscular synapses and the eventual uncoupling of nerve-muscle unions observed in appendicular brachial muscles of Thor-Br embryos may be due, therefore, to a mismatch between recognition factors specific to the nerve and/or muscle cells.

The degenerative processes observed in appendicular brachial muscles of experimental (Thor-Br) embryos are also evident in brachial muscles forced to develop entirely in the absence of nerves. Although embryonic brachial muscles made aneurogenic by surgical ablation of the brachial neural tube segment prior to peripheral nerve outgrowth form and express appropriate fibre-type profiles, they exhibit only limited growth and eventually fail to survive (Butler, Cosmos and Brierley, 1982a; Phillips and Bennett, 1984). It was demonstrated, subsequently, that these parameters (i.e., muscle growth and survival) are determined by neuronal influences associated with impulse-mediated activity (Bloom, Butler, Brierley and Cosmos, 1985). Functional nerve-muscle interaction is, therefore, necessary in order to maintain and promote embryonic muscle growth and survival.

Butler, Cauwenbergs and Cosmos (1986) reported that a heterotopically transplanted thoracic neural tube can substitute for the brachial neural tube and provides factors known to be associated with impulse activity (Bloom, Butler, Brierley and Cosmos, 1985) which support wing muscle growth and survival. The present results corroborate this conclusion since wing motility, an overt expression of the response of brachial muscles to electrical activity generated within the spinal cord, is observed in experimental (Thor-Br) embryos. Wing muscles innervated by foreign thoracic nerves, therefore, respond to electrical signals generated by the heterotopically transplanted thoracic neural tube and exhibit growth curves which parallel those of wing muscles in control (Br-Br and UC) embryos. This finding was most striking in individual wing muscles of Thor-Br embryos immediately before and then following the onset of nerve withdrawal from these muscles. Prior to St 35 (day 8-9E), the growth curves of specific wing muscles in Thor-Br embryos are similar to those of the same muscles in control (Br-Br and UC) embryos. From St 36 (day -10E) onward, however, when ALD muscles in Thor-Br embryos were totally denervated, the growth of these muscles is comparable to that of aneurogenic ALD muscles. Similarly, the PLD muscle in Thor-Br embryos becomes denervated by St 38 (day -12E). After this time this muscle rapidly degenerates and is absent by St 40 (day -14E) (Butler, Cauwenbergs and Cosmos, 1986), a fate also exhibited by aneurogenic PLD muscles (Butler, Cosmos and Brierley, 1982a). The survival of aneurogenic PLD muscles, however, is limited since these muscles disappear by St 32 (day -7.5E) in the absence of innervation. The presence of the PLD muscle in experimental (Thor-Br) embryos at St 38 (day -12E), well beyond the time when aneurogenic PLD muscles are absent, indicates that in addition to their growth promoting effect foreign thoracic nerves rescue the PLD. The observations that wing motility as well as increased growth and survival of individual brachial muscles occur in Thor-Br embryos indicate that nerves derived from a heterotopically transplanted thoracic neural tube interact functionally with wing muscles, albeit only for an initial embryonic period.

B. Properties Of Heterotopically Transplanted Thoracic Neural Tubes

In an effort to understand factors responsible for the nerve-muscle uncoupling phenomenon observed in experimental (Thor-Br) embryos, morphogenesis of transplanted and unoperated neural tubes was compared in experimental (Thor-Br) and control (Br-Br and UC) embryos during the period extending from St 29 (day -6E) to St 42 (day -16E). The results showed that both heterotopic and homotopic neural tube transplants in Thor-Br and Br-Br embryos, respectively, develop morphological properties in keeping with their site of origin. The heterotopically transplanted thoracic neural tube in Thor-Br embryos maintained the identity of a thoracic neural tube, thus indicating that neural tube development is determined prior to the time that the present operations were performed [St 13 (48-52 h *in ovo*)] and that it is controlled autonomously, as described previously (Wenger, 1951).

A prominent feature of heterotopically transplanted thoracic neural tubes, as opposed to brachial neural tubes, was the absence of a large LMC; a large LMC characterized the brachial neural tube of control (Br-Br and UC) embryos. The small, intermittent LMC of heterotopic thoracic neural tube transplants was identical to that of *in situ* thoracic neural tubes, thus indicating that the enlarged periphery (brachial musculature) did not influence formation of the LMC, even though the LMC normally innervates wing muscles

An outstanding characteristic of heterotopically transplanted thoracic neural tubes was the presence, throughout the embryonic period analysed, of the CT. The present study is the first to show that in all experimental (Thor-Br) embryos analysed fibres from the CT enter the brachial sympathetic chain ganglia via ectopic communicating rami. Although the CT and communicating rami are structures never normally seen in the brachial region of chick embryos (Levi-Montalcini, 1950; Oppenheim, Maderdrut and Wells, 1982; present observations), their sustained presence in the brachial region of Thor-Br embryos indicates that CT cells in heterotopically transplanted thoracic neural tubes were able to seek out appropriate targets within the sympathetic chain ganglia and establish contacts which are maintained to day -16E, when the present study was terminated. The results also emphasize the autonomous nature of neural tube development and demonstrate that the pattern of CT fibre outgrowth is determined by factors intrinsic to the thoracic neural tube.

Unlike the pattern of CT fibre outgrowth in the brachial region of Thor-Br embryos, however, the outgrowth pattern of motor nerves exiting the thoracic neural tube transplant was controlled by the periphery, as reported by Wenger (1951). Although thoracic nerves never form a brachial plexus normally, the brachial plexus formed by thoracic nerves growing from the heterotopic neural tube graft in Thor-Br embryos was essentially morphologically normal.

A large body of evidence from both neural tube transplant (Wenger, 1951; Straznicky, 1963; 1967; Butler, Cauwenbergs and Cosmos, 1986; present observations) and limb bud transplant (Hamburger, 1939; Morris, 1978; Hollyday, 1981; Swanson and Lewis, 1982; Straznicky, 1983; Summerbell and Stirling, 1982; Stirling and Summerbell, 1985) studies of chick embryos indicates that the pattern of nerve outgrowth during early morphogenesis is determined exclusively by environmental cues derived from the periphery and is unrelated to the segmental origin of the spinal nerves. Stirling and Summerbell (1977) were the first to show that following removal of a portion of the wing bud before peripheral nerve outgrowth, the nerve branching pattern within the remaining portion of the wing was normal. Similarly, experimental manipulations of the lumbosacral neural tube or hindlimb bud, demonstrate the ability of the peripheral signals to guide growing axons along stereotyped pathways into the developing leg (Lance-Jones and Landmesser, 1980a,b; 1981b; Ferguson, 1983). Whitelaw and Hollyday (1983a,b) found similar results using orthograde HRP labelling of motor nerves growing into partially deleted or duplicated chick hindlimbs and concluded that limb associated growth cues act to guide groups of motor axons into different regions of the limb. It was suggested by Tosney and Landmesser (1984) that since growing crural and sciatic motor nerves develop correct and specific branches within the lumbosacral plexus, even in the absence of all target tissues distal to the pelvic girdle, cues governing the formation of these branch points are independent of target tissues and may be derived from the local mesenchyme. On the other hand, target muscles have a specific, short range influence on motor nerve outgrowth since individual intramuscular nerve branches fail to develop in limbs devoid of target muscles (Lewis, Chevallier, Kieny and Wolpert, 1981; Tosney and Landmesser, 1984).

The present study also showed that the pattern of motor nerve outgrowth from a heterotopically transplanted thoracic neural tube into the brachial environment is controlled by the periphery. Motor nerves in the brachial plexi of experimental (Thor-Br) embryos grew into the forelimb region, followed pathways dictated by the brachial environment and established initial structural and functional contacts with wing muscles (Butler, Cauwenbergs and Cosmos, 1986; present observations). Conversely, the pattern of CT fibre outgrowth in the brachial region of the Thor-Br embryos was independent of peripheral influences and appeared to be controlled by factors endogenous to the heterotopic neural tube transplant. Although Wenger (1951) studied the morphogenesis of the spinal cord and peripheral nerves in chick embryos following heterotopic (Thor-Br) thoracic neural tube transplantation, the present study is the first to examine in detail the development of the CT in the brachial region of experimental (Thor-Br) embryos. The results showed that CT fibres and motor fibres both derived from the heterotopically transplanted thoracic neural tube differed in two respects: 1. The pattern of motor fibre outgrowth was governed by the periphery whereas that of CT fibres was endogenously controlled; and, 2. Motor fibres did not maintain initial contacts with target wing muscles while CT fibres were observed to enter and maintain structural contact with sympathetic chain ganglia throughout the embryonic period analysed.

C. Source Of Motor Innervation In Thor-Br Embryos

Retrograde HRP labelling analyses of motoneurons were used in the present study to determine if the source of motor innervation to wing muscles was a factor that might be responsible for the eventual loss of initial nerve-muscle contacts in appendicular brachial muscles of Thor-Br embryos. Although the report by Wenger (1951) and the present observations showed that the structural organization of heterotopically transplanted thoracic neural tubes is identical to that of *in situ* thoracic neural tubes, Wenger did not investigate the precise localization of motoneurons supplying wing muscles in Thor-Br embryos. The results of the present HRP study demonstrate that the innervation of two specific wing muscles, biceps brachii and triceps brachii, was derived from the MMC in experimental (Thor-Br) embryos. The motor innervation to individual wing muscles, therefore, was highly atypical. Evidence from HRP labelling analyses indicates that during normal development appendicular muscles are innervated exclusively by LMC motoneurons (Landmesser, 1978; Hollyday, 1980; Oppenheim, 1981a; Lance-Jones and Landmesser, 1981b; Straznicky and Tay, 1983; present observations). Furthermore, Smith and Hollyday (1983) showed that in adult rats thoracic MMC motoneurons supply motor innervation to the axial musculature of the thoracic region, as well as some intercostal and abdominal muscles. Thoracic MMC motoneurons, therefore, characteristically never innervate large appendicular muscles.

The present study demonstrates that inappropriate connections were established between thoracic MMC motoneurons and wing muscles in experimental (Thor-Br) embryos. Localization of HRP labelled motoneurons within the MMC of heterotopically transplanted thoracic neural tubes was found consistently in Thor-Br embryos at St 34 (day -8E) and in one St 38 (day -12E) Thor-Br embryo, which survived HRP injection; in the latter embryo the number of HRP labelled cells was greatly reduced from that observed in St 34 (day -8E) Thor-Br embryos. The reduced number of HRP labelled cells noted at St 38 (day -12E) is due to two events observed in Thor-Br embryos: 1. A great loss of motoneurons through neuronal cell death was evident in experimental (Thor-Br) embryos between St 36 (day -10E) and St 38 (day -12E) (Cauwenbergs, Cosmos and Butler, 1986); and, 2. Uncoupling of nerve-muscle contacts and withdrawal of intramuscular axons occurred after St 35 (day 8-9E) in individual wing muscles of Thor-Br embryos (Butler, Cauwenbergs and Cosmos, 1986).

Previous studies showed that in chick embryos the biceps brachii is innervated predominantly by spinal nerves 14 and 15, whereas, the motor innervation to the triceps brachii originates predominantly from spinal segments 15 and 16 (Oppenheim, 1981a; Straznicky and Tay, 1983). The present HRP analyses of control (Br-Br and UC) embryos corroborates these findings and indicates further that the segmental origin of motor nerves supplying these two muscles in experimental (Thor-Br) embryos was unaltered from that of controls. This finding suggests that the craniocaudal extent of motoneurons pools supplying specific wing muscles was determined by the periphery and may be dependent on the pattern of peripheral nerve outgrowth.

In concurrence with previous reports (Oppenheim, 1981a; Straznicky and Tay, 1983), the present study demonstrates a precise topographic organization of motoneuron pools within the LMC of the brachial neural tube following HRP injection into either the biceps brachii or triceps brachii of control (Br-Br and UC) embryos. The biceps brachii muscle was innervated by motoneurons localized in a dorsomedial position within the brachial LMC, while, motoneurons located ventrolaterally within the brachial LMC innervated the triceps brachii muscle. Conversely, HRP labelled motoneurons in experimental (Thor-Br) embryos were randomly distributed within the MMC of the transplanted thoracic neural tube (i.e., HRP labelled cells were not localized to precise medial or lateral positions within the MMC) after injections into either the biceps brachii or triceps brachii. Thus, the highly ordered topographic distribution of motoneuron pools evident in control (Br-Br and UC) embryos was not observed in experimental (Thor-Br) embryos.

The random distribution of motoneurons innervating individual wing muscles in Thor-Br embryos is a logical explanation for the abnormal qualitative nature of wing motility observed in these experimental embryos. Jerky movements of limited range characterized Thor-Br embryos, exclusively, throughout the developmental period analysed. The results of the present HRP study indicate that since motoneuron pools in heterotopically transplanted thoracic neural tubes were comprised of randomly dispersed motoneurons within the MMC, motor activity generated in the foreign thoracic neural tube transplant probably reached individual wing muscles in a haphazard fashion and, therefore, gave rise to the altered qualitative nature of wing motility observed in Thor-Br embryos. To determine this conclusively, however, electromyographic recordings from individual wing muscles in experimental (Thor-Br) embryos would be necessary.

D. Motoneuron Development

The HRP analyses of the present study provides evidence that motoneurons within the heterotopically transplanted thoracic neural tube established structural contacts with wing muscles. These motoneurons, however, were localized in the MMC of the transplanted thoracic neural tube, a population of motoneurons that never normally contacts or interacts with locomotor muscles of the wing. Further analyses were performed, therefore, to determine if the pattern of motoneuron death, growth and differentiation within the MMC of transplanted thoracic neural tubes was related to the eventual nerve-muscle incompatibilty observed in wings of experimental (Thor-Br) embryos.

1. Pattern Of Motoneuron Death

Analyses of the number of healthy and degenerating MMC motoneurons in transplanted thoracic neural tubes of Thor-Br embryos from St 29 (day -6E) through St 42 (day -16E) demonstrated that a rapid and nearly complete loss of MMC motoneurons occurred coincident temporally with the period of intramuscular axon withdrawal from appendicular brachial muscles (Cauwenbergs, Cosmos and Butler, 1986). The small number of healthy motoneurons (15% of initial numbers) that were observed at St 42 (day -16E) represent cells that are either still in contact with specific wing muscles or have formed stable synapses with myotomally derived axial muscles of the brachial region. Up to St 35 (day 8-9E) intramuscular nerve branches derived from the heterotopically transplanted thoracic neural tube are present in all wing muscles. Furthermore, these initial nerve-muscle contacts were observed to promote wing motility as well as muscle growth and survival (Butler, Cauwenbergs and Cosmos, 1986). During this initial embryonic period of sustained, effective contact with wing muscles, healthy MMC motoneurons were maintained and few degenerating motoneurons were observed in the MMC of transplanted thoracic neural tubes. Subsequently, however, when structural and functional nerve-muscle contacts uncouple in individual wing muscles of Thor-Br embryos, approximately 85% of the initial number of MMC motoneurons degenerated. Thus, the present study indicates that the maintenance of structural and functional interactions with target muscles was ultimately necessary for the survival of these cells. Furthermore, analyses of Thor-Br embryos and the phenomenon of motoneuron death are analogous to previous studies whereby limb bud removal was performed; the studies of Hamburger (1958) and Oppenheim, Chu-Wang and Maderdrut (1978) demonstrated that the intrinsic capacity of motoneurons to survive initially in the absence of peripheral influences is limited. Following surgical ablation of the hindlimb bud at day -3E initial numbers of lumbosacral LMC motoneurons were maintained to day -6E, the embryonic time when natural motoneuron death is initiated normally in the hindlimb region; however, a rapid and near total degeneration of LMC motoneurons occurs on the limb bud removal side during subsequent development (Hamburger, 1958; 1975; Oppenheim, Chu-Wang and Maderdrut, 1978). The recent studies of Lanser and Fallon (1984) and Lanser, Carrington and Fallon (1986) on limbless mutant chick embryos also demonstrate the dependence of brachial LMC motoneurons on peripheral influences. Although normal numbers of motoneurons are present initially, only 10% of initial LMC motoneuron numbers survives the embryonic period in mutant embryos (Lanser and Fallon, 1984). Implantation of a genetically normal wing bud into limbless mutant embryos at day -3E, however, results in a rescue of more than 3.5 times as many LMC motoneurons on the implanted wing side compared to the unoperated wingless side (Lanser, Carrington and Fallon, 1986). These studies indicate that LMC motoneurons become dependent for their survival on factors derived from target muscles coincident with the developmental time when naturally-occurring motoneuron death is normally initiated. The nature of these peripheral factors, however, remains poorly understood (Oppenheim, 1981b) although available evidence indicates that the onset of naturally-occurring motoneuron death during normal chick embryogenesis coincides temporally with the formation of permanent neuromuscular junctions in limb muscles (Hamburger, 1975; Oppenheim and Chu-Wang, 1978; Hamburger and Oppenheim, 1982).

Pittman and Oppenheim (1978) were the first to show that neuromuscular activity may also act to regulate motoneuron death in chick embryos. By applying either pre- or post-synaptic blocking agents before the period of natural motoneuron death begins, they and others demonstrated a near total elimination of natural motoneuron death; 93% of motoneurons survived the normal cell death period (normal survival = 60%) in the absence of nerve-muscle electrical activity (Pittman and Oppenheim, 1978; 1979; Laing and Prestige, 1978; Oppenheim, 1984). In contrast, electrical stimulation of chick embryo limb muscles and nerves during the period of natural motoneuron death results in a 20% increase in motoneuron loss (Oppenheim and Nunez, 1982). These researchers suggest that interactions between acetylcholine, released from presynaptic terminals, and postsynaptic acetylcholine receptors may initiate events which lead to motoneuron death during normal ontogeny (Pittman and Oppenheim, 1979; Oppenheim and Nunez, 1982).

The results of the present study showed that during an initial embryonic period, up to St36 (day -10E), MMC motoneurons in transplanted thoracic neural tubes develop independently of the periphery. During subsequent development, however, survival of MMC motoneurons in transplanted thoracic neural tubes of Thor-Br embryos is dependent on factors derived from target muscles. Rapid and massive MMC motoneuron death occurred in transplanted thoracic neural tubes coincident temporally with the loss of peripheral interactions with wing muscles in Thor-Br embryos. This finding concurs with previous reports which indicates that in normal development peripheral nerve-target interactions operate to regulate the pattern of neuronal cell death in the developing spinal cord (Oppenheim, 1981b; 1985; Oppenheim and Chu-Wang, 1983; Cowan, Fawcett, O'Leary and Stanfield, 1984). The influence of the periphery, however, counteracts the process of motoneuron death since in the absence of the periphery (limb bud removal experiments) neurothanasia is initiated on schedule but is greatly accelerated (Hamburger, 1958; Oppenheim, Chu-Wang and Maderdrut, 1978). The present study demonstrated that coincident with the loss of peripheral contacts with target muscles in the wings of Thor-Br embryos thoracic motoneurons also die at an acceleratedrate.

The results of the present study also agree with the widely accepted competition hypothesis for motoneuron death which states that during normal ontogeny as neuromuscular synapses mature, motoneurons which fail to establish stable endplates start to degenerate (Oppenheim and Chu-Wang, 1983). Since mature neuromuscular junctions were not observed in wing muscles of Thor-Br embryos, MMC motoneurons contacting these muscles in experimental embryos eventually die when initial structural and functional nerve-muscle connections are withdrawn.

Interestingly, initial numbers of MMC motoneurons in heterotopically transplanted thoracic neural tubes were maintained beyond the embryonic time when natural motoneuron death occurred in *in situ* thoracic neural tubes. The period of natural motoneuron death in the thoracic MMC of unoperated embryos occurred between St 34 (day -8E) and St 40 (day -14E), coincident with the period in development when motoneuron death occurs in the brachial LMC (Oppenheim, Majors-Willard, 1978; Laing, 1982; Cauwenbergs, Cosmos and Butler, 1983). The onset of MMC motoneuron death in transplanted thoracic neural tubes of Thor-Br embryos, however, was observed to occur at St 36 (day -10E) and an accelerated rate of motoneuron death continued through St 38 (day -12E) (Cauwenbergs, Cosmos and Butler, 1986). The period of cell death of foreign thoracic MMC motoneurons innervating wing muscles of experimental (Thor-Br) embryos, therefore, was atypical and coincided with the developmental period when in these embryos intramuscular nerve branches withdraw from individual wing muscles (Butler, Cauwenbergs and Cosmos, 1986). Thus, during the initial period up to St 36 (day -10E) when intramuscular axons and functional nerve-muscle contacts were observed in the wings of Thor-Br embryos, nerve-muscle interactions supported the survival of foreign thoracic MMC motoneurons. These nerve-muscle unions, however, were subsequently deemed inappropriate and progressive uncoupling of previously compatible nerve-muscle contacts ensued. The present study is the first to demonstrate that accelerated and nearly complete death of foreign thoracic MMC motoneurons shown to innervate appendicular brachial muscles occurred coincident with the embryonic time when structural and functional nerve-muscle contacts are withdrawn in wings of Thor-Br embryos. This finding indicates that the survival of both foreign thoracic motoneurons and *in situ* brachial muscles is ultimately dependent on sustained interactions between them. For reasons that remain unclear, however, permanent neuromuscular junctions fail to form between wing muscles and nerves derived from a heterotopically transplanted thoracic neural tube, even though this developmental event is necessary for the continued survival of both partners.

2. Motoneuron Growth And Differentiation

Although analyses of motoneuron numbers indicated that initial structural and functional interactions between foreign thoracic MMC motoneurons and wing muscles promote the survival of these neurons, such interactions did not influence either growth or differentiation of MMC motoneurons. Regardless of the nature of the nerve-muscle partnerships (i.e., foreign thoracic MMC motoneurons coupled to wing muscles, or *in situ* thoracic MMC motoneurons coupled to their normal muscle partners, or *in situ* brachial MMC motoneurons coupled to brachial axial muscles), the pattern of MMC motoneuron growth and differentiation was the same. The growth and differentiation of foreign thoracic MMC motoneurons in heterotopically transplanted neural tubes, therefore, was unaltered from that of other MMC motoneuron populations coupled to their normal *in situ* muscle partners. This finding demonstrates that these developmental events (motoneuron growth and differentiation) are autonomously controlled and proceed on schedule irrespective of peripheral target influences. The present study, therefore, supports the previous report of Oppenheim, Chu-Wang and Maderdrut (1978) which showed that motoneurons deprived of their normal muscle targets due to early limb bud removal express, initially, intrinsic capacities for growth and differentiation equal to those of motoneurons coupled to their normal muscle targets.

When the growth of MMC and LMC motoneuron nuclei was compared between experimental (Thor-Br) and control (Br-Br and UC) embryos in the present study, brachial LMC motoneuron nuclei of Br-Br and UC embryos were significantly larger than MMC motoneuron nuclei of both experimental (Thor-Br) and control (Br-Br and UC) embryos from St 36 (day -10E) onward. This indicates that LMC motoneurons which normally innervate the large locomotor muscles of the wing have a much greater capacity for growth than MMC motoneurons. The present study showed, therefore, that although foreign thoracic MMC motoneurons interact functionally with appendicular brachial muscles in Thor-Br embryos, this interaction does not alter the growth pattern of these neurons. Thus, wing muscles in Thor-Br embryos were innervated by MMC motoneurons which were markedly smaller than brachial LMC motoneurons which normally innervate these muscles. The size difference observed between MMC and LMC motoneurons indicates that these two motoneuron populations are phenotypically different. Phenotypic differences between foreign thoracic MMC motoneurons shown to contact wing muscles in Thor-Br embryos (HRP analyses) and brachial LMC motoneurons coupled normally to appendicular brachial muscles may be responsible for the inability of thoracic MMC motoneurons to maintain structural and functional contacts with wing muscles.

It is also possible that an insufficient numbers of motoneurons in transplanted thoracic neural tubes may have resulted in the failure of these motoneurons to support the large target field of the wings, as projected by Morris (1978) in her supernumerary limb model. The present analyses showed that the initial number of MMC motoneurons in heterotopically transplanted thoracic neural tubes was only 51% of the initial number of brachial LMC motoneurons which innervated the wing muscles of control (Br-Br and UC) embryos. It is still unclear, however, why the few motoneurons which were observed in transplanted thoracic neural tubes and which made initial effective contacts with individual wing muscles could not support at least some wing muscle targets. The present investigation indicates that the eventual incompatibility between appendicular brachial muscles and foreign nerves derived from a heterotopically transplanted neural tube may stem from factors related to the source of motor innervation *per se* in experimental (Thor-Br) embryos. It is hypothesized that the autonomous nature of neural tube morphogenesis as well as intrinsic phenotypic differences between specific motoneuron populations (MMC and LMC) may be primary factors responsible for the nerve-muscle uncoupling observed in wings of Thor-Br embryos.

E. Source Of Target Wing Muscles

To test this hypothesis further a second series of experimental (Thor-Br/som) embryos was utilized in the present study. Thus, in Thor-Br/som embryos the brachial somites which give rise normally to the myogenic components of brachial muscles (Jacob, Christ and Jacob, 1978), were removed and replaced with thoracic somitic mesoderm which characteristically never forms wing muscles (Christ, Jacob and Jacob, 1978a). The objective of the present Thor-Br/som experiments was to determine if nerve-muscle uncoupling, as observed in heterotopic (Thor-Br) neural tube transplant experiments, is expressed when the mesodermal source of myogenic precursor cells of wing muscles is altered experimentally. Specifically, daily wing motility analyses were performed in Thor-Br/som embryos from St 29 (day -6E) to St 42 (day -16E) to define the extent of functional nerve-muscle interaction when *in situ* brachial nerves are paired with wing muscles derived from heterotopically transplanted thoracic somitic mesoderm.

Previous studies showed that somitic mesoderm transplanted from various segmental levels (including thoracic levels) of quail donors into the site of extirpated brachial somites of chick hosts is viable and forms morphologically normal wing muscles of quail genotype (Chevallier, Kieny and Mauger, 1977; Chevallier, 1979). These studies demonstrated that somitic mesoderm taken from a large range of craniocaudal levels at day -2E is highly plastic and that the pattern of muscle development is controlled by peripheral factors derived from the local environment. Chevallier and coworkers did not indicate, however, if functional nerve-muscle contacts are established in experimental wings of heterotopic somitic mesoderm transplant embryos. Furthermore, the studies of Chevallier, Kieny and Mauger (1977) and Chevallier (1979) were terminated at day -12E and, therefore, were not carried out beyond the time in development when in the present study loss of functional nerve-muscle interaction (wing motility) was observed in wings of heterotopc neural tube transplant (Thor-Br) embryos (Cauwenbergs, Cosmos and Butler, 1983; Butler, Cauwenbergs and Cosmos, 1986).

Instead of the interspecific chick/quail experimental model used in previous investigations (Chevallier, Kieny and Mauger, 1977; Chevallier, 1979), the present functional analyses were performed on an intraspecific chick/chick model; thus, experimental (Thor-Br/som) somitic mesoderm transplant embryos were analogous to experimental (Thor-Br) neural tube transplant embryos used in the first series of experiments in this study. Since previous analyses demonstrated that chick embryos of dystrophic genotype exhibit impaired wing motility during a specific phase of embryogenesis extending from day -10E to day -15E (Cauwenbergs, Butler and Cosmos, 1986a) and the myogenic component of appendicular muscles originates from somitic mesoderm (Chevallier, Kieny and Mauger, 1977; 1978), donor embryos of dystrophic lineage were utilized to determine if impaired wing motility could be used as a functional marker to monitor the viability of transplanted somitic mesoderm. The results show that experimental (DThor-NBr/som) embryos having wing muscles derived from heterotopically transplanted thoracic somitic mesoderm of dystrophic genotype exhibited impaired wing motility from day -10E through day -15E, similar to unoperated dystrophic embryos (Cauwenbergs, Butler and Cosmos, 1986b; present observations). This finding demonstrates that the functional parameter, wing motility, was a suitable marker for the viability of heterotopic thoracic somitic mesoderm transplants. These experiments with intraspecific chick/chick chimaeras are in agreement with the observations of Chevallier (1979) using interspecific chick/quail chimaeras which indicate that brachial muscles in heterotopic thoracic somitic mesoderm transplant embryos are derived from the thoracic somitic mesoderm graft.

The present study is the first to show that functional nerve-muscle contacts are established in wings of Thor-Br/som embryos since wing motility was observed throughout the period analysed; moreover, this functional nerve-muscle interaction was maintained since frequencies of wing motility observed in DThor-NBr/som (dystrophic donors) and NThor-NBr/som (normal donors) embryos were equivalent to those of unoperated dystrophic and normal embryos, respectively, at all days tested (Cauwenbergs, Butler and Cosmos, 1986b). Wing motility in experimental (Thor-Br/som) embryos, therefore, was maintained well beyond the time in development (day -9E) when functional interactions between foreign thoracic nerves and brachial muscles in Thor-Br embryos began to falter.

The present study demonstrates further the high degree of plasticity expressed by somitic mesoderm; thus, thoracic somitic mesoderm transplanted to the site of extirpated brachial somites form wing muscles known to be phenotypically different from muscles of the thoracic region (Butler and Cosmos, 1987b). Furthermore, the results indicate that factors which determine the compatibility of nerve-muscle partners in heterotopic innervation experiments may reside in the degree of plasticity expressed by donor tissues. For example, in Thor-Br/som experiments donor thoracic somitic mesoderm was highly plastic and functional nerve-muscle interaction was maintained whereas in Thor-Br experiments donor thoracic neural tubes were extremely rigid and developed according to their site of origin. The developmental pattern expressed by transplanted spinal cords, therefore, was unaltered by peripheral influences. Conversely, transplanted somitic mesoderm assumes the form and function of the site of transplant, indicating that the developmental pattern expressed by grafted somitic mesoderm was governed by local environmental cues. The ability of *in situ* brachial nerves to maintain contacts with wing muscles derived from heterotopically transplanted thoracic somitic mesoderm in Thor-Br/som embryos, therefore, may be due to the intrinsic capacity of muscles of thoracic origin to conform to peripheral signals and alter their phenotypic characteristics (Butler and Cosmos, 1987b).

Previous cross-reinnervation studies performed *ex ovo* also demonstrated that foreign nerves maintain contact with a new muscle target only when the target muscle assumes the characteristics of the original muscle partner of the transplanted nerve (Cosmos, Butler, Allard and Mazliah, 1979; Mazliah, 1980; Mazliah, Cosmos and Butler, 1987). During *ex ovo* development, therefore, nerves have the ability to alter the phenotypic characteristics of their muscle partners and permanent neuromuscular junctions are established and maintained in cross-reinnervation experiments. *In ovo*, however, foreign nerves do not exhibit a controlling influence over muscle fibre-type expression. Consequently, target muscles do not alter their phenotypes in experimental (Thor-Br) embryos, and initial structural contacts (intramuscular axons) are not maintained.

Recently Butler and Cosmos (1987a) demonstrated that the formation of permanent neuromusclar junctions is a critical developmental event which determines the fate of both nerve and muscle partners. They showed that mutant wing muscles of dystrophic chick embryos respond atypically to heterotopic thoracic innervation. Following heterotopic (Thor-Br) neural tube transplantation, as performed in the present study, permanent neuromuscular junctions do form in wing muscles of dystrophic embryos, wing motility is maintained and individual wing muscles exhibit increased growth throughout the period analysed. The response of brachial muscles in dystrophic embryos to nerves derived from a heterotopically transplanted thoracic neural tube is diametrically opposed to that of wing muscles in embryos of normal genotype used in the present study. Permanent neuromuscular junctions do not form between foreign thoracic nerves and appendicular brachial muscles of normal genotype; instead, initially effective nerve-muscle contacts are severed and both nerve and muscle partners degenerate (Butler, Cauwenbergs and Cosmos, 1986). The results of Butler and Cosmos (1987a) show that brachial muscles of dystrophic genotype express an inability to recognize foreign thoracic nerves as being inappropriate; whereas, in the present study brachial muscles of normal genotype in Thor-Br embryos do exhibit this ability. Thus, appendicular brachial muscles in experimental (Thor-Br) embryos reject foreign thoracic nerves and initial nerve-muscle contacts are broken.

F. Summary And Conclusions

To investigate the nature of nerve-muscle contacts in wing muscles innervated heterotopically, two series of experiments were performed in the present study. First, in Thor-Br embryos a thoracic neural tube was transplanted heterotopically into the site of the extirpated brachial neural tube. The second experimental series (Thor-Br/som) consisted of operated chick embryos in which the brachial somites were removed surgically and replaced by a heterotopic thoracic somitic mesoderm graft. Surgically manipulated embryos in both experimental series were allowed to develop for an extended embryonic period [up to St 42 (day -16E)] and analysed to determine the extent of nerve-muscle interaction. The results indicate that, contrary to previous suggestions, structural and functional nerve-muscle contacts are established in the wings of experimental (Thor-Br) embryos during early embryogenesis up to St 35 (day 8-9E). Wing motility recordings, analyses of peripheral nerve outgrowth and HRP retrograde labelling analyses all support this conclusion. At a time in development when mature neuromuscular junctions start to form normally, however, these nerve-muscle unions in Thor-Br embryos are gradually broken. Permanent neuromuscular synapses fail to form in the wing musculature of Thor-Br embryos even though the survival of both target muscles and their motor nerve supply is dependent on this developmental event. The nerve-muscle uncoupling process is exemplified by reduced frequencies of wing motility after day -8E, followed by a rapid decline in wing motility after day -12E. The reduction and eventual loss of functional nerve-muscle interaction after day -8E resulted in degeneration of both wing muscles and their motor innervation. The onset of these degenerative changes occurred coincident with the time in development when initial withdrawal of intramuscular axons and reduced frequencies of wing motility were observed. MMC motoneuron death in heterotopically transplanted thoracic neural tubes was not initiated until after St 36 (day -10E). This delayed onset of motoneuron death in transplanted thoracic neural tubes of Thor-Br embryos demonstrates that structural and functional nerve-muscle interactions in the wings of these embryos do support the survival of MMC motoneurons.

Several lines of evidence derived from analyses of both Thor-Br and Thor-Br/som experimental groups indicated that the eventual incompatibility observed between wing muscles and foreign thoracic motor nerves may be due to the autonomous nature of neural tube development and the consequent failure of thoracic motor nerves and wing muscles to accept or recognize each other as suitable partners:

1. Histogenetic development of transplanted neural tubes is controlled autonomously since heterotopically transplanted thoracic neural tubes developed structural phenotypes characteristic of *in situ* thoracic neural tubes.

2.The present HRP study is the first to show that the source of motor innervation to wing muscles in Thor-Br embryos is the MMC of the transplanted thoracic neural tube. Furthermore, in contrast to the highly ordered topographic organization of LMC motoneurons which normally innervate wing muscles, motoneuron pools supplying specific wing muscles in experimental (Thor-Br) embryos were randomly dispersed within the MMC of the transplanted thoracic neural tube.

3. Growth of MMC motoneurons in experimental (Thor-Br) embryos was distinctly different from that of LMC motoneurons in control (Br-Br and UC) embryos. It is hypothesized, therefore, that MMC motoneurons may be unable to maintain connections with large locomotor muscles of the wings due to their small size.

4. Stable neuromuscular junctions do form and are maintained between foreign thoracic motor nerves derived from the heterotopically transplanted thoracic neural tube and myotomally derived axial muscles of the brachial region. This finding indicates that there may be an inherent mismatch between nerve-muscle recognition factors, a mismatch which is specific to thoracic nerves and appendicular

brachial muscles and which renders foreign thoracic nerves inappropriate to appendicular brachial muscles.

In addition to analyses of the developing neural tube in Thor-Br embryos, further evidence was obtained from experimental (Thor-Br/som) embryos in which a peripheral structure (somitic mesoderm) was manipulated. Wing motility observations in Thor-Br/som embryos demonstrated that myogenic stem cells derived from heterotopically transplanted thoracic somitic mesoderm do conform to signals from the brachial environment and form brachial muscles which exhibit phenotypes characteristic of unoperated brachial muscles. The response of developing wing muscles in experimental (Thor-Br/som) embryos to environmental cues renders these experimental muscles compatible with *in situ* brachial motor nerves. This is in direct contrast to the observations made with the experimental (Thor-Br) embryos which do not respond to signals from the brachial environment and are ultimately deemed inappropriate. Thus, the incompatibility expressed between foreign thoracic nerves and appendicular brachial muscles is related to the rigid nature of neural tube development.

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Appendix I

(a) Hank's Balanced Salt Solution (HBSS): Sterile HBSS was prepared from a commercially prepared stock solution (GIBCO Laboratories) using the following procedure.

1. Approximately 1100 ml of super-distilled water was placed into a 2000 ml flask, sealed with autoclave tape and aluminum foil and then autoclaved.

2. Into a previously sterilized 1000 ml volumetric flask approximately 800 ml of sterile water was poured followed by 100 ml sterile GIBCO HBSS stock (without $NaHCO_3$).

3. Now, 3.5 ml of sterile $NaHCO_3$ was added and the volume brought to 1000 ml with sterile water.

4. Approximately 30 ml aliquots were poured into previously sterilized 100 ml glass bottles, sealed and stored at room temperature.

Note: All procedures were performed in a sterile tissue culture hood.

(b) Sterile 0.25% trypsin solution : The following steps were used to prepare the trypsin solution:

1. 250 mg of enzyme (trypsin) was dissolved in 100 ml of sterile Ca^{++} and Mg^{++} free HBSS (GIBCO Laboratories) and the pH brought to 7.2.

2. This solution was then filter sterilized into a sterile 100 ml glass bottle.

3. Approximately 5 ml aliquots were then poured into sterile 10 ml glass bottles, sealed and stored at room temperature.

(c) Sterile trypsin inhibiting solution : Trypsin inhibitor was prepared as follows.

1. Into 100 ml sterile complete HBSS (GIBCO Laboratories) 5 ml of sterile fetal bovine serum was added and the pH brought to 7.2.

2. This solution was then filter sterilized into a sterile 100 ml glass bottle.

3. Approximately 5 ml aliquots were then poured into sterile 10 ml glass bottles, sealed and stored at room temperature.

Appendix II

The urea silver nitrate method of Ungerwitter (1951) was used to visualize nerve fibres in Bouin's fixed tissues.

1. Paraffin embedded tissues were cut into 8 μm cross-sections and mounted on albumin coated slides.

2. Serial sections were then routinely deparaffinized in xylene and hydrated in alcohol and water.

3. Staining:

   (i) Place slides in silver solution (for preparation see below) for 3 hours at 50° C.

(ii) 3X distilled water rinses.

(iii) Place slides in reducer solution (see below) for 3-5 minutes at room temperature (shake for first 2 minutes).

(iv) 4X distilled water rinses.

(v) Repeat steps (i) through (iv) as many times as necessary (usually 2-3 times). Note: each repeat in step (i) should only be 10-15 minutes in duration.

4. Dehydrate and coverslip with permount.

Preparation of Silver Solution : Into 200 ml double distilled water dissolve: 3 gms $AgNO_3$, 40 gms Urea and 6 drops mercuric cyanide in picric acid.

Preparation of Reducer Solution : To prepare the reducer solution the following ingredients were added to 200 ml double distilled water in the order indicated: 20 gms anhydrous sodium sulfite, 4 gms hydroquinone, 40 gms urea (just before use).

Appendix III

HRP histochemistry was performed on serial cryostat sections cut at 12 μm thickness according to the method of Sickles and Oblak (1983).

1. Mount frozen sections on albumen coated slides.

2. Fix in 5% glutaraldehyde/0.1M phosphate buffer (pH 7.4) at room temperature for 15 minutes.

3. Rinse in 10% sucrose/0.1M phosphate buffer (pH 7.4)
   - (i) 10 dips
   - (ii) 2X 15 minute rinse

4. Presoak in 50 ml solution composed of solution A and solution B (see below for preparation) for 25 minutes at room temperature while kept in the dark.

5. Incubate in 50 ml solution composed of solution A and solution B + 0.5 ml of 0.3% hydrogen peroxide for 25 minutes at room temperature while kept in the dark.

NOTE: use fresh solutions for steps 4 and 5 for every set of slides.

6. Rinse in 0.01M sodium acetate buffer (pH 3.3) at room temperature (25 dips X5).

7. Counterstain with 1% Neutral Red/ 0.004M sodium acetate buffer (pH 4.8) for 4 minutes at room temperature.

8. Rinse quickly in distilled water (2X 7 seconds).

9. Dehydrate quickly in 95% ethanol (5-6 dips) and then in absolute ethanol (2X 5-10 seconds).

10. Dip in xylene until solution flows evenly over slides.

11. Clear in xylene (2X 5 minutes) and mount coverslips with permount.

Preparation of solution A : To prepare solution A add 5 ml of 0.2 M acetate buffer (pH 3.3) and 75 mg. sodium nitroferricyanide to 92.5 ml of super-distilled water. This solution should be straw coloured and stored in the dark.

Preparation of solution B : To prepare solution B add 5 mg 3,3',5,5' tetramethylbenzidine (TMB) to 2.5 ml absolute ethanol (Must be heated to 40° C in order to dissolve). The presoak solution must be made up (solution A + solution B) immediately before use.