Embryonic wing and leg motoneurons have intrinsically different survival properties

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SUMMARY

Although spinal motoneurons in the chick embryo are born in a rostro-caudal gradient, the timing of their naturally occurring cell death varies in the opposite sense: brachial motoneurons (MNs) die later than lumbar MNs. We used in vitro methods to determine whether this difference results from factors intrinsic or extrinsic to the MNs. Embryonic MNs were purified from E5 lumbar and brachial spinal cord by a method that enriches for MNs with heightened trophic requirements; they were cultured in serum-free medium. In the absence of muscle extract, death of both populations was rapid; similar results are obtained in ovo after limb ablation. In the presence of muscle extracts, however, lumbar MNs always died more rapidly than brachial MNs, as in the normal embryo. We tested the ability of wing and leg bud extracts to provide trophic factors for MNs in culture. MNs from a given level responded equally well to wing or leg extracts. However, dose-response studies showed that near-maximal survival of brachial MNs was obtained with lower concentrations of limb extract than those required for optimal survival of lumbar MNs. These results suggest that the delayed apoptosis in the brachial MN population is not a result of differing peripheral trophic support, but is intrinsically programmed, at least partially through a higher sensitivity to trophic factors.

Key words: lumbar spinal cord, brachial spinal cord, apoptosis, motoneuron, chicken embryo

INTRODUCTION

Regions of the mature spinal cord situated at different rostro-caudal levels show clear differences in morphology and peripheral and central connectivity. The determination of these differences during embryonic development has been the object of many studies, mostly involving early transplantation of different segments of the spinal cord (Weiss, 1955). In the chick embryo, this specification has been found to be the result of influences both intrinsic and extrinsic to the spinal cord. For instance, many of the cytoarchitectonic characteristics of brachial, thoracic and lumbar spinal cord seem to be specified already by 2 days of incubation, as these characteristics are maintained during growth at ectopic sites after transplantation at this age (Wenger, 1951; Straznicky, 1963). The same is apparently true of the establishment of circuitry involved in stereotypic movements: embryos whose lumbosacral cord has been replaced by a brachial transplant subsequently show simultaneous leg movements that in several respects resemble wing flapping (Narayanan and Hamburger, 1971). In contrast, the formation and structure of peripheral nerves, and the innervation of the principal muscles, seem to occur correctly according to the level at which the transplant is placed, at least until later stages (Narayanan and Hamburger, 1971; O'Brien et al., 1990).

These results do not allow for identification of the cellular basis of the specified and plastic properties of early spinal cord. Another phenomenon potentially more open to detailed analysis is that of regional variation in naturally occurring motoneuron cell death. In the chick embryo, motoneurons (MNs) become postmitotic following a rostro-caudal gradient : their birth begins at stage 15 (embryonic day 2) in the brachial region and at stage 17 (embryonic day 2) in the lumbar region (Hamburger, 1975; Hollyday and Hamburger, 1977). Lineage studies show that the members of a given clone of MN precursors do not spread far along the rostro-caudal axis (Leber et al., 1990). By stage 23 (E4), virtually all MNs have been generated.

Variations in MN number along the rostro-caudal axis are initially set out by differential proliferation, giving rise to motor columns in brachial and lumbar segments that are larger than those of non-limb-innervating segments (Oppenheim et al., 1989). Subsequently, this pattern is accentuated and refined by apoptosis that occurs after motoneuron axons arrive in the limb around stage 27 (E5) (Landmesser and Morris, 1975). This cell death varies in both extent and timing according to the rostro-caudal position. In the lumbar

and thoracic regions, there is a steep fall in the number of living cells from stage 29 to 36 (E6 to E9), reaching a plateau at 60% of the cells originally present (Fig. 1). However, in the brachial region, only a slight fall is seen before stage 34 (day 8), and death continues until stage 44 (day 18), with only 50% survival (Hamburger, 1975; Oppenheim and Majors-Willard, 1978; Laing, 1982) (Fig. 1).

Transplantation of E2 thoracic spinal cord segments to the lumbar region leads to a significant increase in numbers of MNs surviving up to E12, probably as a result of the increased target size (O'Brien and Oppenheim, 1990). This experiment demonstrates that the cell death pattern characteristic of a given segment is not specified at E2, as already demonstrated by other experiments involving limb ablation or grafting (Laing, 1982; Hamburger, 1975; Hollyday and Hamburger, 1976; Oppenheim et al., 1978; Lanser et al., 1986) or treatment of embryos with crude extracts from embryonic hindlimbs (Oppenheim et al., 1988; Houenou et al., 1991). However, it has less bearing on the differences between brachial and lumbar segments, both of which are limb-innervating. We sought therefore to answer the following questions: is the delayed apoptosis in the brachial as compared to the lumbar motor column due to differences in trophic factor availability at the periphery, does it reflect different properties of the two spinal cord environments, or may it be due to cell-autonomous properties of these MN subpopulations? We thus compared the in vitro trophic requirements of purified MNs from the two regions and the ability of wing and leg extracts to promote MN survival. Our results argue for intrinsic differences between wing and leg MNs.

MATERIALS AND METHODS

Culture media and supplements

Poly-L-ornithine (30,000 M_r) and medium supplements were from Sigma. Laminin was prepared from the EHS sarcoma. Metrizamide was obtained from Serva. Neonatal muscle extract was a high-speed supernatant of denervated chicken muscle prepared in the presence of protease inhibitors as described (Henderson et al., 1983). Complete culture medium was L15 (Gibco-BRL), containing sodium bicarbonate (0.63 mg/ml), insulin (5 µg/ml), putrescine (0.1 mM), conalbumin (0.1 mg/ml), sodium selenite (30 nM), progesterone (20 nM), glucose (20 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Buffered L15 was L15 with the indicated concentration of sodium bicarbonate only.

Dissection of spinal cords

Brachial (segments 14-16/17, Laing, 1982) and lumbar (segments 23-29, Laing, 1982) regions of spinal cords were dissected from White Leghorn chicken embryos that had been incubated at 37.6°C for 5 days (stage 25-26). They were kept in Ca^{2+}, Mg^{2+}-free PBS at room temperature (RT) for a maximum of 1 hour before treatment with 0.05% trypsin in PBS for 15 minutes at 37°C. They were washed twice with buffered L15 and resuspended in buffered L15 supplemented with BSA (1 mg/ml) and DNase (60 µg/ml). After trituration by eight gentle passages through a Gilson blue cone, cells were counted with a haemocytometer to quantify cell yield and to verify absence of aggregates.

Centrifugation on metrizamide cushions

Cell suspensions were loaded on to 1.5 ml cushions of 6.8% (w/v) metrizamide in buffered L15 at RT in 10 ml Sterilin polystyrene tubes. They were centrifuged for 15 minutes at 400 g at RT, the band of cells at the metrizamide interface removed in 0.5 ml and resuspended in 5 ml of buffered L15. This suspension was layered onto a 1 ml cushion of BSA (4% w/v) in buffered L15 and centrifuged for 10 minutes at 300 g. After careful removal of the supernatant, the cell pellet was resuspended in complete culture medium and counted. These cells are referred to as 'large motoneurons'.

Purification of 'small motoneurons'

The pellet of cells that were dense enough to pass through the metrizamide cushion was panned to purify SC1-positive cells as follows : cells were resuspended in 10 ml buffered L15 and placed in a Petri dish that had been coated with SC1 antibody as described (Bloch-Gallego et al., 1991). After 1 hour at RT, the dish was washed rapidly eight times with PBS and adherent cells were detached by gentle agitation with 3 ml of SC1 hybridoma supernatant. They were diluted with 3 ml of buffered L15 and loaded onto a BSA cushion as for 'large MNs'. Floor-plate cells were co-purified by this procedure with 'small MNs'. However, they represented less than 5 % of this population, showed a distinctive flat-cell morphology and were not counted in the survival assay. The cells purified in these conditions are referred to as 'small motoneurons'.

Cell culture

Substrata were coated with poly-L-ornithine (1.5 µg/ml in water for 30 minutes at RT), allowed to dry and incubated with laminin (3 µg/ml in buffered L15) for 2 hours in the CO_2 incubator. Immediately before cell culture, the laminin was replaced with fresh culture medium. Cells were seeded at a density of 2000 cells in 2 ml per 35 mm well, 20 000 cells in 600 µl per 16 mm well or 100 cells in 10 µl per Terasaki well. 3 hours after seeding, initial cell attachment was quantified in 10 low-magnification microscopic fields (35 mm and 16 mm wells) or in 12 independent wells (Terasaki) using a phase-contrast microscope. At this stage, all
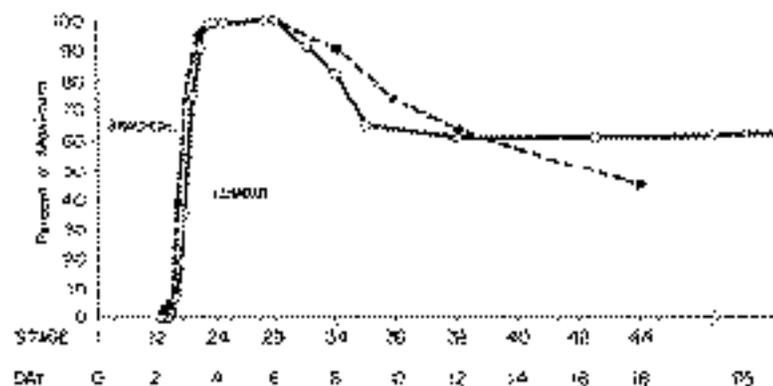

Fig. 1. Timing of MN birth and death in the lateral motor columns of the chick embryo. The number of MNs in the brachial (filled circles) and the lumbar (open circles) lateral columns is plotted as a function of embryonic stage and age. The data are from Hamburger (1975), Hollyday and Hamburger (1977) and Lanser and Fallon (1984). Reprinted with permission from Leber et al. (1990).

phase-bright cells were counted. On subsequent days, only those cells with neurites >2 cell diameters in length were taken into account when calculating survival.

Tissue extracts

Whole limb buds were dissected from 6- to 8-day-old chicken embryos in PBS. Tissue (500 mg) was homogenized at 4°C in a Dounce homogenizer in 1 ml of PBS containing EDTA (2 mM), EGTA (2 mM), aprotinin (20 U/ml), pepstatin (1 μg/ml) and phenylmethane sulphonyl fluoride (1 mM). Homogenates were centrifuged at 25,000 revs/min for 2 hours and the clear supernatants collected. Protein concentration was immediately determined by the Bio-Rad protein mini-assay, and adjusted to 2.5 mg/ml before freezing aliquots at −20°C. A final protein concentration of 100 μg/ml thus corresponded to 4% (v/v) of PBS-protease inhibitor buffer. This buffer alone had no effect on motoneuron survival at concentrations up to 10%, whereas higher concentrations showed moderate toxicity (not shown).

Immunostaining of neuronal cultures

Cells were seeded on glass coverslips in 16 mm wells. After 1 day of culture, cells were incubated for 1 hour at 37°C with a 10-fold dilution of the SC1 hybridoma supernatant. After three gentle washes with L15 medium, they were fixed by layering under the culture medium a solution of 4% paraformaldehyde, 20% sucrose in 0.1 M phosphate buffer, pH 7.4 and incubating for 15 minutes at RT. After washing twice with PBS and once with PBS/BSA (2% w/v), the coverslips were incubated sequentially with biotinylated goat anti-mouse antibody (1:100 in PBS; Amersham) and streptavidin-fluorescein (1:100 in PBS; Gibco-BRL) before being mounted using Citifluor anti-fadant. Coverslips were counted using the Nomarski and fluorescence optics of a Nikon Microphot-FXA. Over 150 cells were counted for each condition and the results were expressed as the percentage of healthy neurons that were distinctly labelled by the antibody.

Assay for choline acetyltransferase (ChAT)

ChAT activity was assayed by a modification of the method of Fonnum (1975). Briefly, cell homogenates were prepared by trituration in 0.2% Triton X-100, 0.2 M NaCl and stored at −20°C. Small volumes (10 μl) of homogenate were incubated with an equal volume of assay cocktail containing [3H]acetyl coenzyme A for 20 minutes at 37°C. The reaction was stopped and toluene-soluble radioactivity was counted using a scintillation counter. Background samples in which cell homogenate was replaced by extraction buffer routinely gave less than 5% of maximal values; all measured activities were corrected by subtraction of background. More than 95% of the activity was inhibited by acetylcholinesterase (400 U/ml; Sigma).

RESULTS

Morphological and biochemical characterization of brachial and lumbar MNs

We sought first to determine whether the earlier death of lumbar motoneurons might simply reflect a greater degree of biochemical maturation, as gauged by the expression of two molecules characteristic of early motoneurons: choline acetyltransferase (Phelps et al., 1990) and the SC1 antigen (Tanaka and Obata, 1984). Motoneurons were enriched using an adaptation of the metrizamide gradient method (Schnaar and Schaffner, 1981; Dohrmann et al., 1987; El M'Hamdi and Henderson, unpublished data). Brachial and lumbar regions of spinal cords from 5-day chicken embryos were dissociated and loaded onto 6.8% metrizamide cushions (see Materials and Methods). After centrifugation, the cells remaining on top of the cushion in each case ('large cells') were counted. Yields of 9,500±1,000 (mean ± s.e.m.; *n*=12) cells per brachial spinal cord and 10,500±1,000 per lumbar region were obtained, representing 3.4% and 2.8% of loaded cells, respectively.

In order to determine the degree of enrichment for MNs in each preparation, large cells were plated on coverslips coated with polyornithine/laminin, allowed to grow in culture for 20 hours in the presence of muscle extract and stained using the SC1 antibody (Tanaka and Obata, 1984), which recognizes only motoneurons and floor-plate in spinal cord at these ages (Fig. 2). All large cells that survived had clearly neuronal morphology, as reported by others (Schnaar
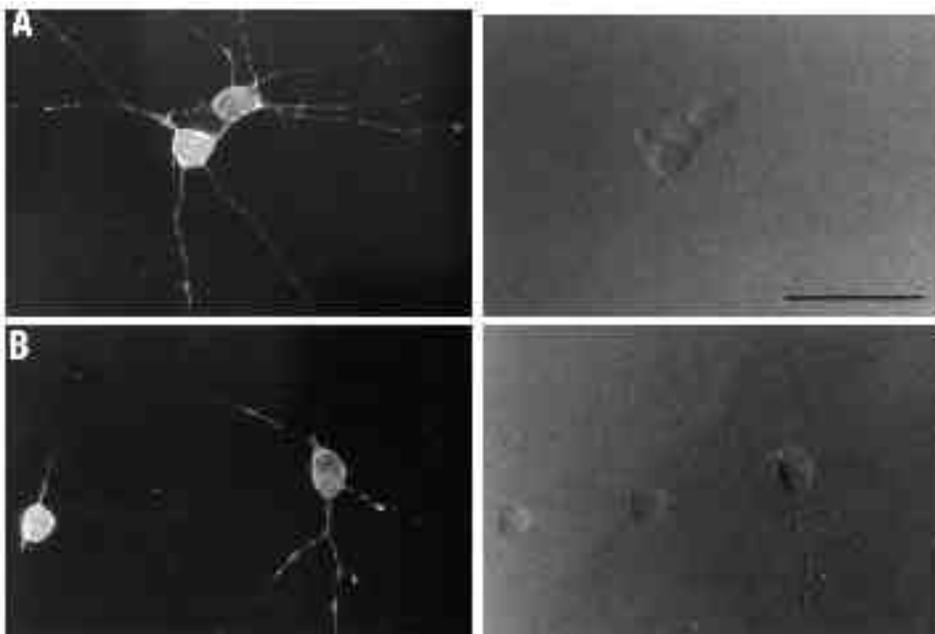

Fig. 2. Assessment of MN purity by immunofluorescent labelling with SC1 antibody of brachial (A) and lumbar (B) MNs. Purified MNs were cultured for 1 day on polyornithine-laminin in the presence of muscle extract and stained as described in Methods. Photomicrographs of the same cells are shown using fluorescence optics (panels on left) and Nomarski optics (panels on right). An SC1-negative cell is shown in each preparation. Scale bar, 50 μm.

Table 1. Variation of specific choline acetyltransferase activities of motoneuron preparations with rostrocaudal level

Preparation	'large cells' ChAT activity	SC1-positive cells (%)	Motoneuron ChAT activity
E5 brachial	62±13 (n=8)	77±2 (n=3)	81±17 (n=8)
E5 lumbar	38±7 (n=8)	82±2 (n=3)	47±9 (n=8)

Choline acetyltransferase (ChAT) activities are expressed in disints/minute/100 cells. The specific ChAT activity per motoneuron (disints/minute/100 cells) was calculated assuming that SC1-negative cells were ChAT-negative.

and Schaffner, 1981; Dohrmann et al., 1987); floor-plate cells were completely eliminated. At E5, this single-step enrichment gave preparations that contained 77±2% and 82±2% (n=3) SC1-positive cells in cultures of large cells from wing and leg segments, respectively.

The ChAT activity was measured for each preparation of large cells. This value was used, after correction for the fraction (ca. 20%) of non-motoneurons, to calculate an estimated ChAT activity per motoneuron in each region (Table 1). At E5, the ChAT activity for lumbar MNs was 57±7% (n=8) of that for brachial MNs. When the same experiment was performed at E6 (not shown), lumbar MNs contained 87±11% (n=4) of the specific ChAT activity of brachial MNs. In other experiments, we observed that the

previously described loss of SC1 immunoreactivity on MNs in tissue sections from E8 onwards (Tanaka and Obata, 1984) occurs first at brachial levels and only later at lumbar levels (not shown).

By these criteria, therefore, it appears that a rostro-caudal gradient of MN maturation is maintained throughout the cell death period. It is thus unlikely that the delayed death in the brachial with respect to the lumbar motor columns is a secondary result of the general developmental differences between them.

Differential survival of large MNs from brachial and lumbar regions

The behaviour in vitro of purified motoneurons from brachial and lumbar regions was compared. Large E5 MNs from both regions were seeded in Terasaki plates in serum-free complete medium, in the absence of muscle extract (Fig. 3A,C), and counted daily. MN death was rapid in these conditions: nearly all the cells had disappeared within 24 hours. Although lumbar MNs reproducibly died faster than did brachial MNs (Fig. 4A), the differences were small and concerned a population (10% of total cells) that could theoretically have been non-MNs, given the purity (80%) of the preparations.

In the presence of neonatal muscle extract (Fig. 3B,D), however, clear differences were observed. Large MNs from the brachial region survived consistently better than did their
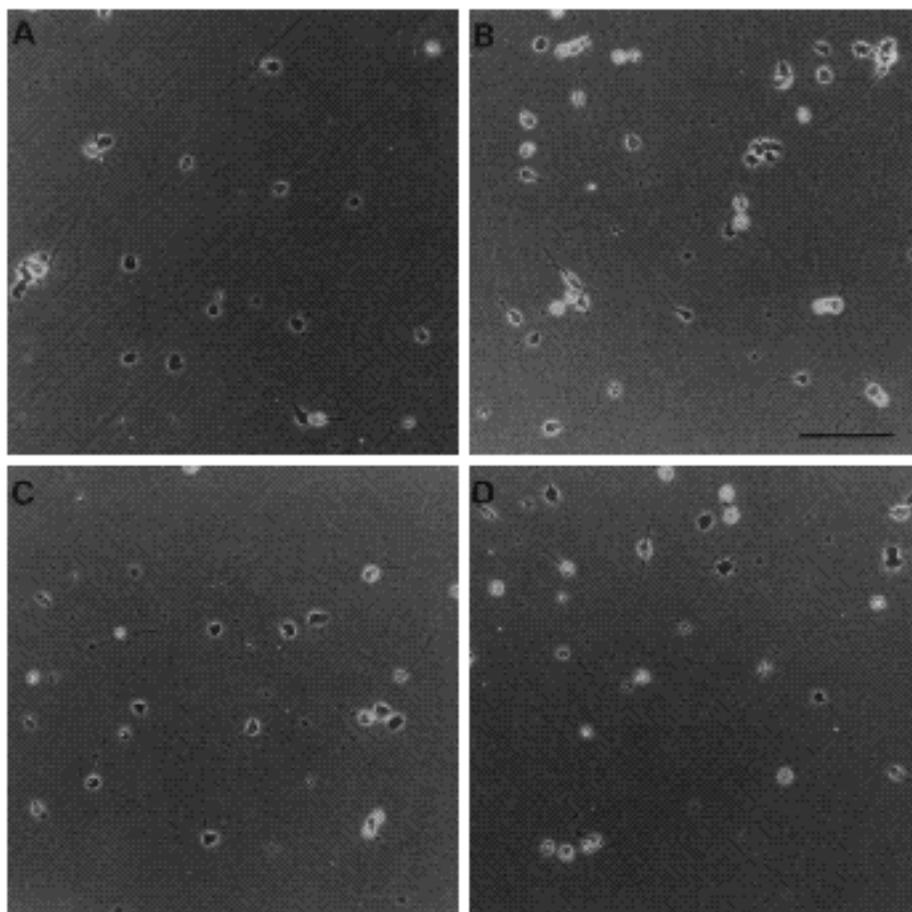

Fig. 3. Differential survival of 5-day embryonic brachial and lumbar MNs in culture. Phase-contrast micrographs are shown of brachial (A, B) and lumbar (C, D) large MNs cultured for 2 days on polyornithine-laminin in the absence (A, C) or the presence (B, D) of 10 µg/ml neonatal muscle extract. Scale bar, 100 µm.
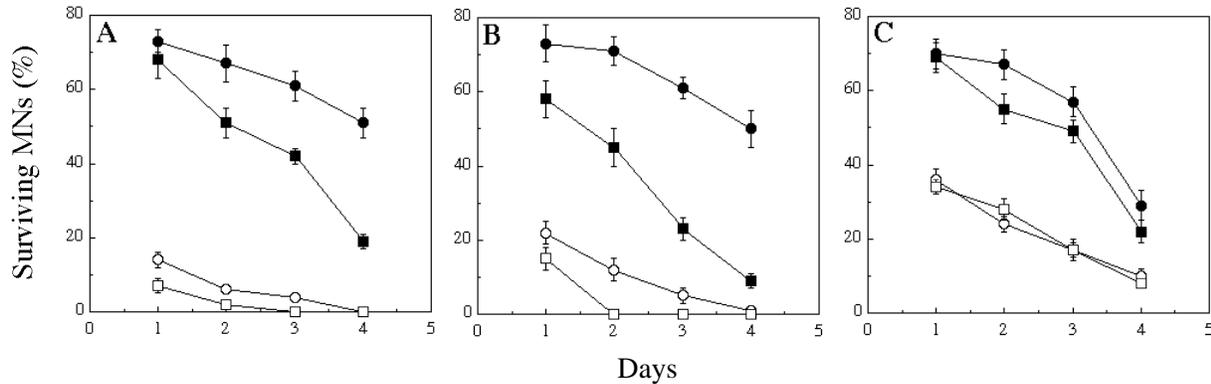
Fig. 4. Comparison of the survival of large (A,B) and small (C) MNs from brachial (circles) and lumbar (squares) regions in the presence (filled symbols) and absence (open symbols) of 10 µg/ml neonatal muscle extract. In B, lumbar MNs are 6 hours older than brachial MNs in the same experiment. The number of cells attached after 3 hours was taken as 100%. Indicated values are means ± s.e.m. of 12 Terasaki wells.

lumbar counterparts : in a typical experiment in Terasaki dishes, survival was still 50% on the 4th day, whereas lumbar MN survival was only 20% after the same period (Fig. 4A). A similar decrease of MN number was observed in 16 mm and 35 mm wells (not shown). As lumbar MNs are more immature than brachial at any given embryonic stage, we purified lumbar MNs from eggs that had been incubated 6 hours longer (Hollyday and Hamburger, 1977) than those used for brachial MN preparation. Even in these conditions, lumbar MNs survived significantly less well than brachial MNs (Fig. 4B). Longer delays gave similar results: when purified by metrizamide followed by panning, E6 lumbar MN survival was still lower than E5 or E6 brachial MNs (not shown).

Comparison of small motoneurons from brachial and lumbar regions

We recently showed (El M'Hamdi and Henderson, unpublished data) that the 5-day chicken spinal cord contains a population of 'small motoneurons' that are dense enough to pass through a 6.8% metrizamide cushion, and can be purified by panning using the SC1 antibody (see Materials and Methods). They seem by several criteria to be less mature than the 'large MNs' purified from the same embryos and, in particular, die more slowly in the absence of trophic support. They are present at all rostro-caudal levels; we therefore compared the properties of small MNs (>90% SC1-positive neurons) from lumbar and brachial levels (Fig. 4C). Only slight differences were observed between the two populations, either in the presence or the absence of neonatal muscle extract.

Effect of wing and leg extracts on survival of brachial and lumbar motoneurons.

Delayed death of brachial MNs could be due to more effective trophic support by the wing than by the leg from embryonic day 5 to 8. To test this hypothesis, we made extracts from wing and leg buds of 6-day embryos, because at this stage massive cell death has already begun in the lumbar region but has not started in the brachial

region. Embryonic extracts have already been shown to be active on MN survival in vivo (Oppenheim et al., 1988).

Initial experiments having shown that embryonic extracts did have trophic activity for MNs in vitro (Fig. 5),
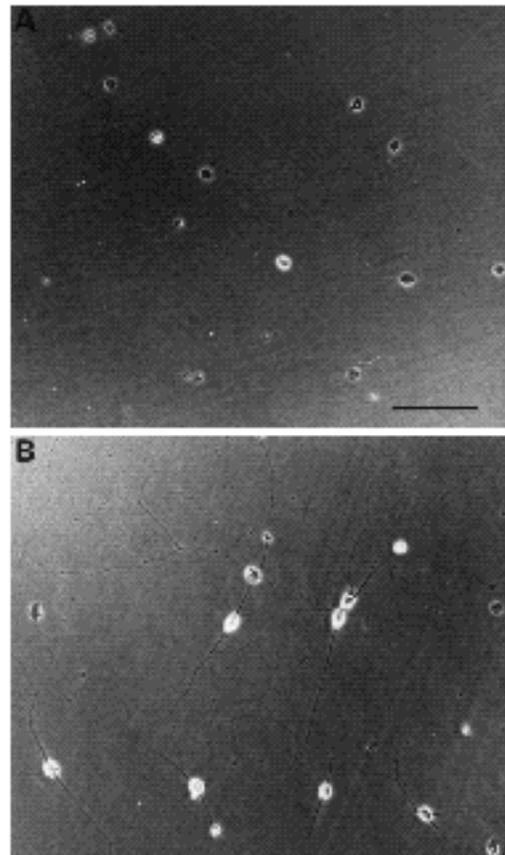

Fig. 5. Effect of embryonic leg extract on lumbar MN cultures. Phase-contrast micrographs are shown of MNs cultured for 2 days in the absence (A) or the presence (B) of 100 µg/ml E6 leg extract. Scale bar, 100 µm.
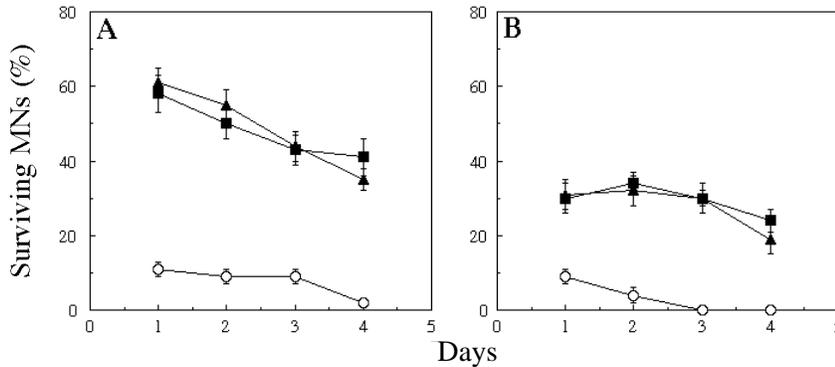
Fig. 6. Survival of large MNs from brachial (A) and lumbar (B) regions in the absence of extracts (open circles) or with E6 wing (filled square) or leg (filled triangle) extracts at 100 µg/ml.

brachial and lumbar large MNs were seeded in Terasaki wells with complete medium containing either no extract, or 100 µg/ml of E6 wing or leg extract. Survival of brachial MNs was supported by embryonic wing extract as efficiently as by leg extract (Fig. 6A), and the effects of the two extracts on lumbar MN survival were also indistinguishable (Fig. 6B). Lumbar MN survival was once again lower than that of brachial MNs in the presence of trophic supply.

The lack of difference between wing and leg extracts could have been due to the use of saturating concentrations of each. We therefore tested the survival-promoting activities for brachial MNs of both extracts at a range of concentrations (10 µg/ml to 500 µg/ml). Concentrations above 200 µg/ml showed a strong toxicity, which was not due to the PBS-protease inhibitor buffer. The trophic effect of both extracts was concentration-dependent, with the same slope, the maximum being approximately at 100 µg/ml (Fig. 7). Their specific activities were indistinguishable, whether extracts were prepared with or without protease inhibitors (not shown). Experiments performed using optimal concentrations of limb extracts from 7- and 8-day chicken embryos gave similar results (not shown).

Differential sensitivity to trophic factors between brachial and lumbar MNs

The difference in the rate of cell death between brachial and lumbar MNs may be due to a different sensitivity to trophic factors. We tested a range of concentration of embryonic wing extract on lumbar and brachial MN cultures (Fig. 8A). Brachial MN survival was close to its maximal value at a concentration (20 µg/ml) at which the response of lumbar MNs was still increasing. Similar results were obtained with embryonic leg extract (not shown) and with neonatal extract (Fig. 8B) tested on lumbar and brachial MNs prepared from embryos that differed by 6 hours in incubation time (see above). This tendency was confirmed by examining the ratio of brachial to lumbar MN survival. This ratio decreased with increasing extract concentration in 6 experiments out of 8, demonstrating that the dose-response curve of brachial MNs to any given extract is significantly displaced to the lower concentrations with respect to that of lumbar MNs. Increased sensitivity to limb-derived factors may therefore contribute to the enhanced survival of brachial MNs.

DISCUSSION

Our results using cultures of purified motoneurons from lumbar and brachial regions of the E5 chicken embryo strongly suggest that there are already at this stage intrinsic differences between the two populations insofar as their survival response to target extracts is concerned. In contrast, no significant differences could be detected between the trophic activities of leg and wing extracts on either MN population.

In the absence of muscle extract, both lumbar and brachial MNs from E5 spinal cord died rapidly (Fig. 4). This result is in agreement with those of Laing (1982) and Lanser et al. (1986), who showed that limb ablation resulted in MN cell death over the period E5-E10 at both lumbar and brachial levels, representing an acceleration of cell death among brachial MNs. The delayed death of brachial MNs in situ and in vitro is therefore not due to a lack of trophic dependence of these cells.

In order to reproduce more closely the situation in the unoperated embryo, in which MN axons at both levels form primitive contacts with the muscle before undergoing apoptosis (Prestige, 1976; Landmesser and Morris, 1975;
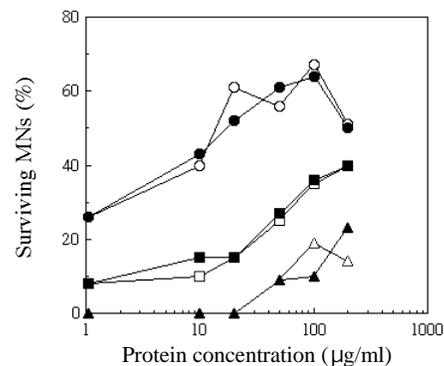

Fig. 7. Dose-response curves for the survival of brachial MNs in the presence of E6 wing (open symbols) or leg (filled symbols) extracts. Survival was counted after 1 day (circles), 2 days (squares) and 3 days (triangles) of culture. Error bars have been omitted for clarity; each point is the mean of 12 Terasaki wells. The lowest point on each curve represents the survival value in controls to which no extract was added.
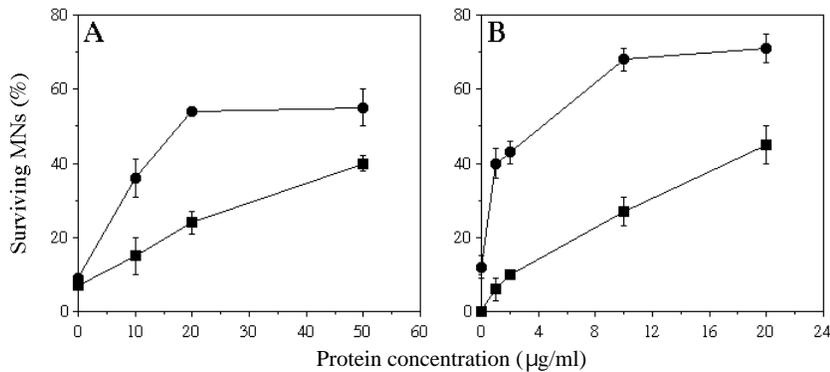
Fig 8. Dose-response curve for the survival of brachial (circles) and lumbar (squares) MNs in the presence of embryonic wing extract (A) or denervated neonatal muscle extract (B) at day 2. Lumbar MNs in B are 6 hours older than brachial MNs in the same experiment.

Pettigrew et al., 1978), we compared the survival of 'large' lumbar and brachial MNs in the presence of different embryonic and postnatal extracts (Figs 4, 6). These were purified by a metrizamide method that enriches for MNs with heightened trophic requirements. Whatever the extract used, lumbar MNs died more rapidly than did brachial MNs. In the experiment shown in Fig. 4A, a given degree of brachial MN survival is reached approximately 36-48 hours after the same value in the lumbar population. This delay is very similar to that observed in situ (Fig. 1); the intrinsic differences in the MN populations observed in vitro are thus themselves sufficient to explain the in vivo data.

One difference between the in vitro (Fig. 4) and the in vivo (Fig. 1) survival curves is that the former continue to drop below the minimum of 40% observed in ovo. One possibility is that, although MNs do not apparently require their central environment to express their regional differences, their prolonged survival may require, in addition to muscle extract, centrally derived factors that were absent from these cultures (Okado and Oppenheim, 1984 ; Dohrmann et al., 1987).

It was striking that the population of 'small MNs' purified from the metrizamide pellet by panning did not show such pronounced regional differences. Although the exact nature of this population is not clear (El M'Hamdi and Henderson, unpublished data), their relatively low level of biochemical maturation and trophic dependency make it possible that they represent a stage of motoneuron development between newly postmitotic MNs and mature MNs that are required to make a life-death decision. It may therefore be that they have yet to be regionally 'specified'. If this were the case, this level of determination would represent a relatively late stage in motoneuron differentiation (Ericson et al., 1992).

To test for a role of the periphery in regulating the timing of MN death at brachial and lumbar levels, we looked for differences between embryonic wing and leg extracts in promoting 'large' MN survival. Wing and leg extracts were not specific for the corresponding MNs populations, and wing extract was not better than leg extract in promoting survival of a given MN population. Furthermore, at embryonic days 7 and 8, there was not a relative drop of trophic activity in leg as compared to wing extracts. Our data do not therefore provide support for the hypothesis that the timing of MN death is peripherally regulated. However, the preparation of soluble extracts probably releases intracellular components that would not normally come into contact with the motoneuron, and it is possible that their activity

hides differences in the amounts of trophic activity that are secreted in wing and leg, respectively (Rassendren et al., 1992). Unfortunately, no data exist for the timing of motoneuron cell death in transplants of brachial spinal cord to lumbar levels, or vice versa. Such experiments might be expected to provide useful information on the role of the periphery in this phenomenon.

It has been suggested that MN death is regulated by the access that each MN has to peripherally derived factors rather than by the absolute amount of factor that is synthesized (Oppenheim, 1989; Houenou et al., 1991). One way in which a neuron may increase its access to such factors is by enhanced axonal branching and thus synaptogenesis. While it is likely that such mechanisms are involved in determining the long-term survival of individual MNs in situ (Landmesser et al., 1990), our results suggest that, at least as far as the wing-leg differences are concerned, they are not required for expression of the region-specific motoneuron cell-death clock. Not only did the 'large' lumbar and brachial MNs in our cultures have equal access to the source of trophic support (muscle or limb bud extract), but in addition their morphologies did not significantly differ.

Another way for the motoneuron to optimize its effective trophic support is to increase its affinity for neurotrophic factors. This may well be the case for brachial MNs, which survive much better at suboptimal concentrations of trophic extract than do lumbar MNs (Fig. 8). This could explain why, when competing for survival in normal conditions in vivo, brachial MN death occurs later and more slowly whereas it is fast when the target is removed. Molecular data on trophic factor receptor expression in lumbar and brachial MNs may provide further insight into this phenomenon. However, it is already clear that differential sensitivity to trophic factors may constitute one of the major mechanisms of rostro-caudal specification of survival properties. What might be the cause of the different properties of lumbar and brachial MNs? It is noteworthy that central deafferentation in situ (Okado and Oppenheim, 1984) seems to produce a deregulation in the timing of death of motoneurons (i.e. an increased loss of lumbar MNs which is prolonged beyond E10) without altering their gross peripheral connectivity (Laing and Lamb, 1985). It may therefore be that interactions with descending axons, which reach brachial levels several days earlier than lumbar levels (Glover and Eide, 1992), confer on limb motoneurons the cell-autonomous survival properties that we have demonstrated here.

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