The development of facial motoneurones in the mouse – neuronal death and the innervation of the facial muscles

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SUMMARY

The relationship between neuronal death and the formation of patterned connections was studied in the facial neuromuscular system of foetal, neonatal, and adult mice. The facial neuromuscular system was selected because two large, widely separated, facial muscles (the nasolabial and posterior auricular muscles) are innervated by clearly separated parts of the facial motor nucleus in the adult mouse. The number of motoneurones in the facial nucleus was counted in Nissl-stained sections at different stages of development. Over 6400 neurones were present in the facial nucleus at day 17 p.c. (post-coitum). After day 17 p.c. the number of neurones fell rapidly and only 2000 cells remain in the adult nucleus. This represents a loss of 68%, most of which occurs between days 18 and 20 p.c. Neurones with pyknotic nuclei are seen on day 17 p.c. and are most numerous on day 18 p.c. This leads us to believe that the fall in neurone numbers is due to cell death.

Indirect evidence provided by acetylcholinesterase histochemistry (time of earliest reaction in the facial muscles) and horseradish peroxide (HRP) tracing studies (time of earliest transport) indicate that facial motorneurone axons innervate the facial musculature before the period of cell death: diffuse acetylcholinesterase activity first appeared in the auricular muscles at day 15 p.c. and in the nasolabial muscles at day 17 p.c.; retrograde transport of HRP from the auricular and nasolabial muscles to the facial nucleus cannot be reliably demonstrated before day 17 p.c.

We assessed the topography of early facial neuromuscular innervation by making HRP injections into nasolabial and posterior auricular muscles of embryonic and neonatal mice. Injections of HRP at day 17 p.c. (the day before cell loss commenced) showed that the nasolabial muscle and posterior auricular muscles were innervated by the same subnuclei of the facial nucleus as in the adult – except that there was a small number (1–5%) of labelled cells located in parts of the nucleus not consistent with the adult innervation pattern. These data indicate that, except for a small number of neurones, topographically organized connections in the facial neuromuscular system are established before the period of cell death. We conclude that motoneurone cell death does not play a major role in the establishment of topographically organized connections in this system.

INTRODUCTION

The neuromuscular systems of vertebrates have been widely used as models for the study of the development of patterned neuronal connections (see reviews

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by Landmesser, 1980; Hollyday, 1981). The nerve-muscle connection is suitable for such studies because the developing nerve processes and their targets (the skeletal muscles) are accessible to experimental manipulation. Most studies on the development of neuromuscular connection patterns have been carried out on amphibian (e.g. Lamb, 1977, 1981; McGrath & Bennett, 1979) or avian species (e.g. Pettigrew, Lindeman & Bennett, 1979; Hollyday, Hamburger & Farris, 1977, Hollyday, 1981; Landmesser, 1978a,b) rather than mammals – probably because of the inherent difficulties in handling mammalian embryos. However studies on mammals have been carried out (e.g. Dennis & Harris, 1979; Lance-Jones, 1982) and the present report indicates that the study of development of some mammalian neuromuscular connections is quite feasible. This is important because most neuroanatomical data available today relates to mammalian rather than non-mammalian vertebrates.

A major question addressed by studies on the developing neuromuscular system has been the relationship between the formation of patterned (or specific) neuromuscular connections on the one hand and the death of a large proportion of the motoneuronal population on the other. Some workers consider that cell death serves to eliminate inappropriately connected motoneurones (Pettigrew et al. 1979; McGrath & Bennett, 1979) whereas others have argued that patterned connections are established before the time of motoneuronal death and therefore the major period of cell death is not causally related to the formation of patterned connections (Landmesser & Morris, 1975; Landmesser, 1978b; Lamb, 1976; Hollyday et al. 1977). The present study is a further attempt to clarify this problem. First of all we have aimed to establish the timing of neuronal death and the innervation of the muscles in the neuromuscular system of the mouse and then to examine the pattern (or lack of it) of early innervation of the facial muscles by the facial nucleus. In this way we hoped to determine whether cell death preceded or followed the time of innervation of the muscles. Although the suitability of the mouse facial neuromuscular system for such a study can be judged from the description of our results, we should point out here that the facial nucleus was selected for the following reasons: (i) it is well demarcated and easily recognisable; (ii) it is made up of a number of distinct subnuclei that innervate the facial muscles in a topographically ordered fashion (Ashwell, 1982); (iii) the two facial muscle groups selected for intensive study (nasolabialis and posterior auricular) occupy quite distinct and separate regions in the embryonic head and could therefore be separately injected with horseradish peroxidase (HRP) without the need to open the uterus widely. A further advantage of selection of the nasolabialis and posterior auricular muscle groups for the study of patterned connection formation is that the two motoneurone groups innervating these two muscles are widely separated in the adult facial nucleus (Ashwell, 1982).

The time of formation of neuromuscular connections was indirectly assessed by AChE histochemistry and by retrograde transport of HRP from the ingrowing
Mouse facial motoneurones

axons. The HRP technique can provide information on the arrival of nerve fibres in the periphery (Bunge, 1973; LaVail & LaVail, 1974). The appearance of cholinesterase activity in developing muscles has been correlated with the arrival of nerve fibres and the formation of neuromuscular connections (Atsumi, 1971, Mumenthaler & Engel, 1961).

The pattern of neuromuscular connection formation was assessed by retrograde transport of HRP after injection into the nasolabialis or posterior auricular facial muscles.

MATERIALS AND METHODS

Over 160 foetal, neonatal and adult mice of the BALB/e strain were used for this study. Timed pregnancies were obtained by placing females with a male overnight and checking for vaginal plugs at 08:30 h the next morning. The day of appearance of the plug was taken as day 1 postcoitum (p.c.). Most pregnancies which were allowed to proceed to term resulted in birth on day 21 p.c.

Neonatal animals were aged by counting the day of the morning of the first appearance of the pups as day 1 postnatum (p.n.).

Material for neuronal counts

The brains of foetal (14–20 days p.c.) and neonatal (1, 5, 10, 16 days p.n.) mice were fixed in 10 % formalin, sectioned in a cryostat or on a freezing microtome, and stained with haematoxylin and eosin, neutral red or cresyl violet. Every third section was examined at x500 and the motoneurone nuclei counted (all counts were performed by K.W.S.A. to ensure that any observer error was consistent). Correction was made for double counting according to the methods of Abercrombie (1946). The modal nuclear size for the correction factor for each age was obtained from a sample consisting of 50 nuclei in the middle third of the rostrocaudal extent of the facial nucleus.

HRP experiments

Injections of HRP solution were made through the uterine wall into the facial musculature of more than 50 foetuses of age 15, 16, 17 or 18 days p.c. and also into 33 neonates aged between 1 and 5 days p.n.

The foetus was injected with HRP solution (50 % w/v) in either the nasolabial or posterior auricular region (Fig. 1A) using a 0.5 μl syringe.

After a survival of 6–16 h, the foetus was removed from the uterus, decapitated and the brain fixed in paraformaldehyde (1 %) and glutaraldehyde (2.5 %) fixative. Neonates were fixed after 8–24 h survival. Most of the nasolabial and posterior auricular injection areas were retained for histological examination, although in some cases the dissection of the brain from the skull had so damaged the posterior auricular tissue that further study of the area was
not possible. The tissues were sectioned on a freezing microtome at 15 or 50 \( \mu m \) and 50 \( \mu m \) for the portions of head containing the injection site. The sections of brain were then reacted for the demonstration of HRP by the Mesulam (1978) technique. Sections of head were reacted either by the TMB technique or the pyrocatechol/p-phenylene-diamine technique of Hanke, Yates, Metz & Rustioni (1977).

*Early musculotopic organization tested with HRP*

Foetuses injected in the nasolabial musculature where the HRP had diffused beyond the target confines were rejected. Due to the difficulty of obtaining posterior auricular injection sites, the only means available to minimize HRP spread was by using only the smallest volumes which still gave labelling of motoneurones.

The position of labelled motoneurones within the facial nucleus was mapped with the aid of a projection device. In many foetal brain sections the density of labelling made it difficult or impossible to see the outlines of individual motoneurones. Because of this the labelling was designated according to the following semiquantitative system using grades:--grade 1 -- labelling of more than 90% of the motoneurones in the region; grade 2 -- labelling of 50--90% of the motoneurones; grade 3 -- labelling of less than 50% of motoneurones in the relevant region of the nucleus. Combined with this second series of grades denoting the type of labelling of the individual motoneurones: a -- heavy, granular, and filling more than 50% of soma area; b -- medium, granular and filling 10--50% of soma area; c -- sparse granules in the soma. In the regions where labelled cells were very sparse, individual cells were drawn.

*Acetylcholinesterase histochemistry*

Two foetuses for each age of 14, 15, 16, 17, 18 and 20 days p.c. were decapitated and the heads placed in Lillie’s formal calcium overnight at 4°C. Nasal vibrissal pads from adults were cut off the deeply anaesthetized animal with a scalpel and fixed as for the foetal heads. The tissue was then sectioned on a freezing microtome at 25 \( \mu m \) or 50 \( \mu m \).

Free-floating sections were then incubated for demonstration of acetylcholinesterase by the copper ferrocyanide method of Karnovsky & Roots (1964). Eserine and iso-OMPA controls were examined in some cases.

**RESULTS**

*Nuclear morphology and neuronal counts*

The facial nucleus was distinguishable in some animals at 14 days p.c. but the margins of the nucleus are not clearly defined until 15 days p.c. Facial motoneurones at 14 days p.c. appear as rounded or slightly triangular cells (soma
diameter 8–10 μm) with centrally placed oval nuclei approximately 4 μm in diameter. The cells increase in size and by 17 days p.c. some, but not all, show prominent multipolarity. At this age the cytoplasm still stains faintly as do the one or two nucleoli visible within the oval nucleus. The number of dendritic stalks and the density of cytoplasmic staining increase up to the first day p.n., at

![Diagram](image)

**Fig. 1.** This diagram shows the pattern of distribution of labelled motoneurones in the facial nucleus of the adult mouse following injections of HRP in the nasolabial and posterior auricular musculature. Above is a sketch of an adult mouse head showing the injections sites referred to in the present study. Below are two drawings of a middle coronal section through the facial nucleus showing the distribution of labelling obtained in each type of experiment. The darker shading indicates that 50–100% of motoneurones in that region were labelled and the lighter shading indicates that less than 50% of the motoneurons in that region were labelled. The seven subnuclei of the facial nucleus are indicated (Lat = lateral, DL = dorsolateral, DI = dorsal intermediate, VI = ventral intermediate, DM = dorsomedial, VM = ventromedial, DA = dorsal accessory).
which age the motoneurones are still small but otherwise appear to be mature. The only changes visible from the first day p.n. to adult are increases in soma and nuclear size (to 20 µm and 8 µm respectively) and an increasing prominence of the nucleolus.

In the adult mouse the nucleus is divided into seven subnuclei, six of which lie within the main nucleus, while the seventh, the dorsal accessory, lies slightly dorsal to the rostral parts of the nucleus (Fig. 1) (Ashwell, 1982). It was found difficult to distinguish motoneurones of the dorsal accessory nucleus from the surrounding reticular formation neurones at ages younger than day 1 p.n. For this reason, the counts of all motoneurones present in the nucleus at various ages do not include dorsal accessory motoneurones for foetal ages. This is unlikely to greatly affect the results as dorsal accessory motoneurones comprise only about 1% of the total number of facial motoneurones in ages for which it is possible to distinguish the dorsal accessory nucleus. Fig. 2 shows a low-power view of the nucleus on day 18 p.c.

The results of counts of the total number of motoneurones in the facial nucleus are summarized in Table 1 and Fig. 3. The number of neurones in the facial

Fig. 2. The developing mouse facial nucleus at 18 days p.c. The facial nucleus is a prominent feature in the ventral brainstem in this cresyl-violet-stained section. In this section the facial nucleus appears as a dark cluster of large cells almost surrounded by a pale-staining border (arrowed). The subnuclear organization cannot be discerned with certainty but there are some indications of subdivision, particularly in the lateral part of the nucleus (compare with the diagram in Fig. 1). The midline of the brainstem is represented by a white vertical strip near the right of the photograph. The calibration bar (representing 100 µm) is close to the ventral surface of the brainstem.
Table 1. The number of motoneurones in the developing mouse facial nucleus

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>n (no. animals)</th>
<th>N (no. motoneurones mean of Lt. &amp; Rt. Nuclei)</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 p.c.</td>
<td>2</td>
<td>5352</td>
<td>5524</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5695</td>
<td></td>
</tr>
<tr>
<td>16 p.c.</td>
<td>3</td>
<td>6384</td>
<td>6135</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5883</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6140</td>
<td></td>
</tr>
<tr>
<td>17 p.c.</td>
<td>3</td>
<td>5866</td>
<td>6430</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7103</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6323</td>
<td></td>
</tr>
<tr>
<td>18 p.c.</td>
<td>3</td>
<td>4218</td>
<td>4319</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4170</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4569</td>
<td></td>
</tr>
<tr>
<td>19 p.c.</td>
<td>4</td>
<td>3299</td>
<td>3164</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3346</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3107</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2902</td>
<td></td>
</tr>
<tr>
<td>20 p.c.</td>
<td>4</td>
<td>2647</td>
<td>3130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3431</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3314</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3128</td>
<td></td>
</tr>
<tr>
<td>1 p.n.</td>
<td>6</td>
<td>2290</td>
<td>2983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2583</td>
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<td>3368</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3303</td>
<td></td>
</tr>
<tr>
<td>5 p.n.</td>
<td>4</td>
<td>2051</td>
<td>2244</td>
</tr>
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<td></td>
<td></td>
<td>2254</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>2378</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2293</td>
<td></td>
</tr>
<tr>
<td>10 p.n.</td>
<td>2</td>
<td>2049</td>
<td>2115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2182</td>
<td></td>
</tr>
<tr>
<td>16 p.n.</td>
<td>1</td>
<td>2070</td>
<td>–</td>
</tr>
<tr>
<td>Adult</td>
<td>1</td>
<td>2030</td>
<td>–</td>
</tr>
</tbody>
</table>

nucleus for each animal was taken as the mean of the neuronal counts for the left and right nuclei.

Several points should be noted from these figures. Firstly, the number of motoneurones peaks on 17 day p.c. at a mean value of 6430. Secondly, neuronal loss has begun by the middle of the 18th day p.c. and reaches a peak rate at this age. The rate of decrease of neuronal numbers drops rapidly over the next two days and remains at a low rate into early postnatal life. Thirdly, the number of motoneurones lost (4400) represents a loss of 68% of the total number of motoneurones present on day 17 p.c., most of this occurring between 18 and 20 days p.c.
Neuronal counts for each subnucleus were made where the boundaries between subnuclei could be distinguished (animals 17 days p.c. and older). The time of rapid decline of neuronal numbers was the same in the medial (DM and VM in Fig. 1B), intermediate (DI and VI) and lateral (DL and Lat.) regions of the nucleus. This suggests that neuronal loss occurs simultaneously throughout the nucleus.

The pattern of distribution of pyknotic nuclei across the nucleus also suggests that neuronal death occurs simultaneously across the nucleus. These pyknotic nuclei appear in cresyl-violet-stained tissue as clumps of strongly chromophilic material with little or no surrounding cytoplasmic staining. The earlier stages of degeneration as described by Hughes (1961) for the developing amphibian ventral horn could not be discerned in this material. Pyknotic nuclei appear on 17 days p.c. and become very prominent on 18 days p.c. when they appear to be evenly distributed across the nucleus. They become less prevalent on the following 2 days and have virtually all disappeared by the first day p.n.

Neuronal size was measured (using oil immersion at ×1250) in three foetuses at 17 days p.c. – the age immediately prior to the onset of neuronal death. In each animal over 200 cells were selected for measurement on the basis of distinct
Mouse facial motoneurones

Fig. 4. Soma-size histograms for neurones of the three major divisions (lateral, medial, intermediate) in each of three day-17 p.c. mouse foetuses (No. 1, No. 2, No. 3). Note that the size distribution in the nasolabial division in two of the three foetuses shows a weak suggestion of bimodality but this is not evident in other histograms. (LAT = lateral; MED = medial; INT = intermediate)

nuclei and prominent nucleoli, and their position in the middle third of the rostrocaudal extent of the nucleus. The cells were grouped according to whether they came from the lateral (DL and Lat. subnuclei), intermedite (DI and VI subnuclei) or medial (DM and VM) division of the nucleus. The histograms plotted from these results as shown in Fig. 4. The histograms for the lateral portions of the nucleus (DL and Lat. subnuclei) show a suggestion of bimodality in the cell-size distributions of two out of three animals but other distributions appeared to be unimodal. We found no obvious indication of selective survival
of neurones of either small or large size and did not further test the possibility of an early bimodal distribution. In addition, if there were indeed two types of neurones with the smaller neurones being those susceptible to degeneration (such as described by Sohal, 1976) then the bimodality might be expected to be also present in the intermediate and medial divisions of the nucleus.

The timing of neuromuscular contacts – acetylcholinesterase histochemistry

On the 16th day p.c. most activity in the nasolabial region appears to be confined to nerve fibres which in some sections are traceable back to the semilunar ganglion. These nerve fibres appear to be sensory neurones, possibly supplying the vibrissal follicles. On the 17th day p.c. strong activity is visible in the developing muscle tissue as it traverses the region and loops around the vibrissal follicles. The development of AChE activity in the musculature inserting into the developing auricle is slower; activity is first present on day 15 p.c. and increases steadily until day 17 p.c.

Examination of the other facial musculature suggests that there is a temporospatial gradient of the development of AChE activity from the auricular to the nasal regions such that musculature closer to the stylomastoid foramen develops AChE activity before muscles situated more peripherally. For example, the orbicularis oculi muscle develops AChE activity after the auricular musculature and before the nasolabial musculature.

The distribution of activity within the muscle fibre appeared to be diffuse at the early stages, but concentrations are present at the myotendinous junctions and on the middle third of the fibre (a position likely to be occupied by the developing myoneural junction). Not until several days later were distinct endplates visible. For example, in the nasolabial musculature activity is visible at 17 days p.c. but endplates are not clearly distinguishable until 20 days p.c.

The timing of neuromuscular contacts – HRP experiments

The results of HRP injections into nasolabial and posterior auricular musculature are summarized in Table 2.

No labelling was present in the brains of 15 or 16 day p.c. foetuses after 8 h survival time. Since it is known that rapid retrograde axoplasmic transport is considerably slower in the early developmental stages than in the adult (Hendrickson & Cowan, 1971, Clarke & Cowan, 1976), foetuses in later experiments were allowed to survive for periods of 12 and 16 h. The only result of this was that faint labelling was found following one nasolabial injection with 12 h survival time. All other injections failed to yield labelled motoneurones in the nucleus. Labelled cells in a 17 day p.c. foetus are shown in Fig. 5.

Relatively large injections were made into each of the two muscle groups at 17 days p.c. and the number of labelled motoneurones was assessed in serial 15 μm sections through the facial nucleus. It was found that within the portion
Table 2. The results of HRP experiments

<table>
<thead>
<tr>
<th>Age (days PC)</th>
<th>Survival time (hours)</th>
<th>Injection sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nasolabial</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>0/2</td>
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<tr>
<td>15+</td>
<td>16</td>
<td>–</td>
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<td>16</td>
<td>6</td>
<td>0/2</td>
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<td></td>
<td>12</td>
<td>1/1</td>
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<td>17</td>
<td>8</td>
<td>7/7</td>
</tr>
<tr>
<td>18</td>
<td>6</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5/6</td>
</tr>
</tbody>
</table>

Total injected animals – 55
Total animals with labelling – 29

Values entered under the two injection sites indicate the success of HRP injection under those conditions. Denominator indicates the number of animals injected. Numerator indicates the number of animals showing labelled neurones.

of the nucleus in which that muscle is represented in the adult (Ashwell, 1982) the percentage of all cells labelled was over 90%.

The topography of developing neuromuscular connections – HRP experiments

a) Foetal animals

In animals in which the distribution of HRP labelled cells was mapped (day 17 p.c. to day 5 p.n.), it was found that the pattern of labelling within the various subnuclei of the facial nucleus proper was substantially similar in all coronal sections examined, regardless of the position of the section in the rostrocaudal extent of the nucleus. For this reason a picture of only one coronal section is used to summarize the results in each case presented.

The earliest age at which consistent uptake and retrograde transport was observed was 17 days p.c.. This is the day immediately prior to the onset of neuronal death. Examples of the pattern of distribution of labelled cells within the facial nucleus following nasolabial and posterior auricular injections at this age are shown in Fig. 6. The pattern is essentially the same as that seen in the adult (Ashwell 1982) (Fig. 1). The nasolabial injection shown in Fig. 6 was confined to the region of the pad supporting the vibrissal follicles and their attached musculature. The labelled motoneurones in the facial nucleus are predominantly in the lateral and dorsolateral subnuclei – sites where one would expect to find labelled cells after such an injection in the adult mouse (Ashwell, 1982). The results of one posterior auricular injection shown in Fig. 6 demonstrate labelled motoneurones in the ventromedial, dorsomedial and dorsal accessory subnuclei.
Fig. 5. (A) An example of a small HRP injection site in the nasolabial region as seen in a horizontal section through the snout of a 17-day p.c. mouse embryo. TMB reaction technique. Bar represents 1 mm. (B) Labelling in the facial nucleus resulting from an injection of a large volume of HRP in the nasolabial region of a 17-day p.c. foetus (a different animal from that shown in (A)). Bar represents 100 μm. (C) Higher power view of the same section as shown in (B). Bar represents 50 μm.
The ventromedial and dorsomediaal subnuclei respectively supply the posterior and anterior auricular muscles in the adult (and hence labelling here would be predicted by an auricular injection) but the dorsal accessory subnucleus is
labelled after injection of the posterior belly of the digastric (and probably stapedius and stylohyoid as well) in adult rodents (Ashwell, 1982; Watson, Sakai & Armstrong, 1982). We consider that the presence of labelled cells in the dorsal accessory subnucleus is probably due to the penetration of the needle into the posterior belly of the digastric muscle and/or the stylohyoid, since the depth of needle penetration could not be adequately controlled in these small animals. Apart from this, there is a close correspondence between the foetal pattern of labelling and that seen in the adult following injection in the same site.

Despite the marked similarities between the pattern of distribution in the 17 days p.c. animals and the adult, some labelled cells were observed in parts of the nucleus some considerable distance from the main mass of labelled motoneurones and separated from them by many unlabelled cells. Examples are shown in Fig. 6. These labelled cells are separated from the main group of labelled cells by such a distance that it appears unlikely that they are due to a diffusion of HRP not detected by examination of the head section. In the case of the posterior auricular injection the presence of these 'misplaced' labelled cells might be due to uptake of HRP across the perineurium of axons which are destined for the nasolabial musculature but which pass through the region of HRP at the posterior auricular injection site. However, this suggestion cannot explain the misplaced labelled cells resulting from nasolabial injections. These misplaced cells probably represent truly inappropriate connections, so that in the example shown, neurones in the dorsomedial subnucleus (which in the adult contains neurones supplying the anterior and posterior auricular musculature) are in this 17 days p.c. foetus making contact with the nasolabial musculature. It is important to note, however, that these aberrant connections are very few – comprising only a very small proportion (probably 1–5%) of the motoneurones connecting with the injected musculature.

The results for 18 days p.c. foetuses are comparable to those at 17 days p.c.. As Fig. 12 shows, the pattern of labelling is again similar to that seen for the adult, but the 'misplaced' motoneurones previously seen are still evident.

There was no indication in our experiments that contralateral connections between facial nucleus and muscles existed at any stage.

Fig. 7 and 8 show photographs of the pattern of labelling in these two foetal ages.

b) Neonatal animals

We have shown above that neuronal loss death is still occurring (at a reduced rate) in the period following birth and is not complete until 10 days p.a. – in fact it was found that 30% of the motoneurones present at birth will die soon after birth (Fig. 3). For this reason it is notable that the pattern of distribution of labelling in postnatal animals are very similar to those seen in the adult following injections in the relevant sites. Examples of the results obtained in 1 day p.n. animals are shown in Fig. 6. The most notable feature is that misplaced labelled cells seen in the foetal animals were not seen in neonatal animals.
Fig. 7. The pattern of distribution of HRP-labelled motoneurones in the facial nuclei of two representative foetuses at age 17 days p.c. (A) following a nasolabial injection; (B) following a posterior auricular injection. Note the presence of misplaced labelled cells (arrowed) in the dorsomedial subnucleus in case (A). In the case of the posterior auricular injection (B) labelled cells were confined to the medial parts of the dorsomedial and ventromedial subnuclei. Subnuclei indicated as in Fig. 1.
Fig. 8. The distribution of HRP labelled cells in the facial nuclei of two 18 days p.c. foetuses following a small volume injection into the nasolabial region (A) Labelled neurones are confined to the dorsolateral and the lateral parts of the lateral subnuclei. (B) Following a posterior auricular injection labelled neurons are confined to the medial subnuclei. Subnuclei indicated as in Fig. 1.
DISCUSSION

In this study, we have tried to relate the time of naturally occurring cell death amongst facial motoneurones to the time when the definitive pattern of innervation of the facial muscles is established – the term ‘innervation’ being used in its broadest sense (see below).

Limitations of the HRP technique

Before considering the significance of the results some comments on the limitations of the HRP technique for determining motor projection patterns must be made.

Firstly, there is the well-recognized problem of the extent of HRP diffusion away from the injection site (Pettigrew et al. 1979). This study has used two of the more sensitive methods of demonstrating HRP activity (the Mesulam, 1978 TMB and the Hanker et al. 1977 pyrocatechol/p-phenylenediamine techniques) for determining the extent of spread of HRP at the injection site. Thus, the maps of HRP spread at the injection sites in this study include regions of lower HRP concentration than would have been possible to demonstrate using diaminobenzidine as the chromogen. In any event, regardless of the chromogen used it has been shown that the source of HRP retrograde transport is usually restricted to the densely stained region of HRP injection sites (see Jones & Hartmann, 1978 for review). The problem of HRP diffusion at the injection site is more serious for those studies claiming to demonstrate initially widespread (non-selective) neuromuscular connections, e.g. Pettigrew et al (1979). In studies such as the present one (where the initial projection pattern is almost identical with that found in the adult) the difficulty in interpretation is minimized, because an undetected diffusion of HRP away from the target area can readily imitate diffuse or inappropriate connections, but cannot imitate a specific projection pattern.

In this study the earliest time of uptake and retrograde transport of HRP from the facial region was one of two criteria used to give an approximate indication of the time of formation of early neuromuscular contacts. Such an assumption can be criticised because it has been shown that all invading neurones early in development may transport HRP from an injection site (Clarke & Cowan, 1976). In addition, Kikuchi and Ashmore (1976) did not find definite synaptic contacts in an ultrastructural study of early neuronal ingrowth into muscles. Nonetheless, it seems that these early contacts are electrophysiologically functional (Landmesser & Morris, 1975; Landmesser, 1978b). In addition, the earliest time of HRP retrograde transport in the present study coincided with the first appearance of AChE activity in the nasolabial facial muscles. At a comparable time in the development of the chick limb bud, there is a rise in choline acetyltransferase activity (Giacobini, 1972; Giacobini et al. 1973) which may be a more reliable indicator of the onset of neuromuscular function.
Because it is difficult to prove with the above methods that synaptic contacts exist at some particular stage of development, we use the terms 'innervation' and contact only in the general sense that the nerves involved have invaded the territory of a particular muscle. Silver-staining techniques and electronmicroscopy could be employed to resolve this question.

**General observations on development of the facial nucleus**

Some general comments on the timing of events in the developing facial neuromuscular system are worthwhile making. The sequence of events is summarized in Table 3.

Neuroblasts leave the mitotic cycle on the 10 and 11th days p.c. (Taber-Pierce, 1973) and migrate to the ventral surface of the brainstem before the neuronal soma have reached their final resting place (15 or 16 days p.c.). The appearance of AChE activity in posterior auricular musculature on the 15th day p.c. suggests that axons have reached these muscles two days before neuronal numbers on the facial nucleus reach a peak. Axon growth, then, is probably not a simple matter of an axon growing away from some point on the axon (probably at the future internal genu of the facial nerve). One direction is towards the neuronal soma,

Table 3. *A summary of the sequence of events in the developing facial neuromuscular system. Observations and events surmised from these observations against age*

<table>
<thead>
<tr>
<th>Observations</th>
<th>Age (days)</th>
<th>Surmised events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facial motoneurones leave mitotic cycle (Taber-Pierce, 1973)</td>
<td>10,11 p.c.</td>
<td>Motoneurones migrate to ventral brainstem, axon outgrowth proceed</td>
</tr>
<tr>
<td></td>
<td>13 p.c.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 p.c.</td>
<td>Motor axons arrive in vicinity of auricular musculature</td>
</tr>
<tr>
<td>AChE activity appears in auricular musculature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motoneurone numbers peak, AChE activity appears in nasolabial musculature, HRP retrograde transport first observed from both sites</td>
<td>17 p.c.</td>
<td>Motor axons arrive in the vicinity of nasolabial musculature</td>
</tr>
<tr>
<td></td>
<td>19 p.c.</td>
<td>Neuronal degeneration and death</td>
</tr>
<tr>
<td>Ears open (Crispens, 1975)</td>
<td>1 p.n.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 p.n.</td>
<td></td>
</tr>
<tr>
<td>Whisking behaviour begins Eyes open (Crispens, 1975)</td>
<td>10 p.n.</td>
<td>Neuronal death complete</td>
</tr>
</tbody>
</table>
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and results from neuronal soma migration, the other is towards the periphery and results from axonal elongation.

Development of the facial neuromuscular connections would appear to be delayed relative to the neuromuscular connections to the trunk and limb musculature. This is suggested by observations in rat (quoted in East, 1931) and human (Humphrey, 1964) foetuses demonstrating that reflexes and spontaneous movement involving the facial musculature appear later than those involving the trunk and limb musculature.

Neuronal death in the facial nucleus occurs predominantly from 17 days p.c. to 20 days p.c. However, a considerable amount of neuronal death is still occurring while some motoneurones innervating buccinator are probably involved functionally in the vitally important suckling behaviour. Obviously the loss of 30% of the neurones present 1 day p.n. over the next 10 days does not interfere with the performance of suckling behaviour. (A similar instance of neuronal death occurring late into postnatal life has been observed by Zilles & Wingert (1973) who observed that neuronal death in the mouse oculomotor nucleus in not complete until 20 days after birth). In the case of the rest of the facial nucleus motoneurones, however, they are probably not required to perform important functions until the opening of the ears and eyes on the 3rd day p.n. and 13th day p.n. respectively (Crispens, 1975) and the development of whisking behaviour, which becomes noticeable on day 10 p.n. (personal observations).

Cell counts and neuronal death

Neuronal death during ontogeny is a well-documented phenomenon (see the extensive reviews of Cowan, 1973 and Oppenheim, 1981) and the observations that it occurs in this neuromuscular system is not surprising. However, some points about the time of onset and completion of neuronal death are worth considering in detail.

Before turning to a consideration of neuronal death in the facial nucleus, some difficulties concerning the relationship about falling cell counts and neuronal death must be examined. Rogers and Cowan (1973) point out that the observed fall in cell numbers may actually be due to the misidentification of immature glial cells at the earliest stages so that our counts at days 15 and 16 p.c. could be inflated. In addition, we cannot be certain that some cell loss was not due to emigration of neurones, although we found no evidence of such a process. On the other hand we did observe small numbers of motoneurones with pyknotic nuclei during the period of natural cell death; the fact that only a few such degenerating neurones were seen in any one section is consistent with the findings of other investigators (e.g. Hughes, 1961). From another point of view it could be argued that the present study probably underestimates the degree of neuronal death, since in the younger foetuses some small cells were excluded from the count because their small soma size, small dark nuclei and thin faintly staining cytoplasm made them difficult to distinguish from immature glial cells.
Subsequent examination of cells labelled after HRP experiments showed that many of the small cells ignored in cell counts contained deposits of reaction product – suggesting that they were in fact motoneurones.

The pattern of early neuromuscular projections

A popular hypothesis has been that of random outgrowth of motoneurone axons; it is supposed that such axons would connect randomly with muscle groups but that developmental cell death would later delete incorrect connections (Hughes, 1965). The random pattern would thus be converted to a specific connection pattern (see reviews by Landmesser, 1980; Hollyday, 1981 and Oppenheim, 1981). The work of Landmesser (Landmesser & Morris, 1975; Landmesser, 1978a,b) on the developing chick hindlimb argues strongly against such an hypothesis because it was shown by electrophysiological and HRP retrograde tracing that almost all motoneurones had made appropriate connections prior to the period of motoneuronal death. The present study shows that the same situation pertains in mammals; facial motoneurones connected to appropriate facial muscles prior to onset of neuronal death in the facial nucleus. In both the mouse facial system and the chick hindlimb system (Landmesser, 1978b), a very small number of inappropriately connected neurones were present before the period of motoneurone death but it seems clear that in both cases ‘the process of cell death does not create a specific pattern out of an initially diffuse one’ (Landmesser, 1980).

Lamb (1977) has shown that some motoneurones make early inappropriate connections in the hindlimb of Xenopus and that these cells die. However the number of cells in this category is small and the great majority of motoneurones undergoing natural cell death do so after having made appropriate neuromuscular connections (Lamb, 1976).

It is possible that the present study and that of Landmesser (1978a,b) may have missed minor, or local, errors. For example some neurones within the lateral region of the mouse facial nucleus may have connected to an inappropriate part of the nasolabialis muscle yet the detection of such an error would be beyond the resolution of the HRP retrograde tracing technique used in the present study. In addition, very early connectional errors (at the time when motoneurone axons first invade the target area) could have occurred and have been connected before the time when specific connections were clearly demonstrated (17 days in this study; stage 27 (5 days) in the chick). Although Lamb (1976) has demonstrated initial connection errors such as these in developing Xenopus hindlimb, an HRP anterograde tracing study (Lance-Jones & Landmesser, 1980b) suggests that in the chick axons initially go directly to their appropriate region in the base of the hindlimb bud.

The work of Landmesser (1978, 1980) and the present study support the contention that motoneurones of the chick spinal cord and mouse facial nucleus possess some physical or chemical characteristics which identify them before
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they make contact with muscles and these characteristics are somehow matched to local physical or chemical features in the muscle field to be innervated.

On the other hand, evidence for the existence of an early, more random connection pattern prior to the major period of cell death has been provided by studies on the development of chick forelimb (Pettigrew et al. 1979) and axolotl forelimb (McGrath & Bennett, 1979). It is difficult to reconcile these results with those of Landmesser’s group or with the present study. Pettigrew et al (1979) found that the initial (stage 26) projection of axons to the biceps brachialis muscle of the chick was from motoneurones whose somas lie within spinal segments from 12 to 17. By stage 38 only the 14th and 15th spinal segments projected to those muscle, suggesting that neuronal death or some other process had removed those motoneurones in other spinal segments which made inappropriate connections with the biceps muscle. However, a problem of interpretation is raised by the statement by these authors that the volumes of HRP injected into the biceps muscle were in the range of 0.05 to 2 μl. They state that when sections of wing bud were reacted to demonstrate the extent of spread of HRP, animals which showed the HRP activity had spread more than 100 μm from the injection site were rejected from their study. This seems inconsistent with the fact that 0.05 μl is equivalent to the volume of a cube over 350 μm on a side. This seems to indicate that either there was considerable leakage from the injection site or there was a great deal of undetected spread of HRP throughout the wing bud. At another point in their report, the authors note when the experiments were repeated using smaller volume injections (0.02 μl), the results were similar; namely that HRP reaction product could still be observed in motoneurones in all spinal segments from 12–17 of stage-26 to -28 animals. This volume (0.02 μl) is equivalent to the volume of a cube 270 μm on a side, so that even with the smaller injection series, it seems unlikely that the injected volume was confined to a target of the dimensions claimed. If widespread diffusion had occurred, an apparently diffuse pattern of innervation could be explained, since in the younger animals the wing but is small and diffusion of HRP is more likely to imitate a diffuse or inappropriate projection pattern. Nonetheless, electrophysiological data in the same report support the anatomical data that indicate that the biceps receives innervation from many different segments in the early stages of development (but see Oppenheim, 1981).

The results of an investigation by Dennis & Harris (1979) of the development of segmental innervation of rat intercostal muscle, indicated that there was a 40 % error rate in the innervation of these muscles at the earliest age examined (16 days gestation). However, the ages at which these errors are removed (18th and 19th days of gestation) are considerably later than the probable period of neuronal death. Harris-Flanagan (1969) noted that spinal motoneurones in the mouse underwent degeneration from the 11th to the 14th days of gestation. The time of spinal motoneurone death is involved in the correction of the errors in this case. It seems possible that the ‘errors’ removed in this situation coincide
with the period of normal reduction of polyneuronal innervation (see Landmesser, 1980 and Grinnell & Herrera, 1981).

In the present study, the close correspondence between the musculotopic organization of the adult and the 17 and 18 days p.c. foetuses (the earliest ages showing HRP retrograde transport) suggests that axons do not grow out at random but are directed (by factors unknown) to the portion of the facial musculature appropriate for the neuronal soma's position in the facial nucleus. It also indicates that neuronal death is not involved in the large scale correction of grossly inappropriate connections. However, neuronal death may function to remove those few (1–5 %) motoneurones which have made grossly inappropriate connections, or those motoneurones whose axons have made slight errors, the detection of which is beyond the limits of resolution of this study.

At the present time there appears to be little hope of resolving the widely disparate results of the above studies. Perhaps the variety of results obtained simply reflects a large variety of processes involved in the development of neuromuscular projection patterns in the animals and systems studied (Oppenheim, 1981).

Other possible mechanisms for formation of specific neuromuscular connections

If the early projection patterns are indeed appropriate, it still remains to explain how such a pattern comes about. It has been suggested (Landmesser & Morris, 1975) that topographic organization may arise as a result of temporal patterns of outgrowth of axons. In the case of the present system, this would involve those motoneurones lying within the medial subnuclei maturing and sending out their axons to the periphery before those motoneurones in the lateral subnuclei. These first axons would contact the nearest muscle groups (the auricular musculature), and later arriving axons would be forced to terminate on muscles situated further nasally.

Such a pattern is certainly suggested by the observed sequence of maturation and innervation of facial muscles. Gasser (1967a,b) noted that musculature close to the stylomastoid foramen (stapedius, posterior digastric and stylohyoid muscles) received innervation prior to more peripheral musculature. Furthermore, gradients in the time of origin of neurones across various nuclei (Clark, Rogers & Cowan, 1976; Sohal & Holt, 1977) provide the basic mechanism by which timed outgrowth might be achieved. However, Landmesser & Morris (1975) commented that a temporal patterning of peripheral connections appears unlikely for several reasons. They point out that polyneuronal innervation of muscle fibres is common in developing neuromuscular systems (Bennett & Pettigrew, 1974), and all muscle fibres in a given muscle do not mature simultaneously (Kelly & Zacks, 1968), so that axons arriving late at a muscle that was already innervated need not be forced to move on to a more peripheral muscle with no innervation. In addition, Lance-Jones & Landmesser (1980a) have shown that the axons of late-maturing cells arrive at the base of the limb bud at the same time as those from cells that mature earlier.
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Alternatively, the growing axons may be directed to the appropriate musculature regardless of the time of maturation and axon outgrowth of motoneurones. This might be achieved by chemotaxis, by a positional information co-ordinate system which directs growth, or some other unknown mechanism. However, irrespective of the mechanism some form of specification of motoneurones and target mesenchyme would be required. In the case of the motoneurones this could be achieved using a gradient of time of cell origin across the nucleus (Sohal & Holt, 1977) as already mentioned.

At the present time it is not possible to decide whether the precise pattern of projection in the developing mouse facial neuromuscular system is due to temporospatial gradients of maturation and axon outgrowth, or to guidance mechanisms dependent on precise positional information available to axons and target tissue. However, the present results suggest that neuronal death does not play a major role in the formation of the projection pattern in this system.

Since the completion of this study an account of motoneurone death in mouse spinal cord has been published (Lance-Jones, 1982). Her estimate of the extent of motoneurone death in the spinal cord (67 %) is remarkably similar to the level we found in mouse facial nucleus (68 %). Lance-Jones found that many spinal motoneurones which die had previously sent axons into the limb – a finding strongly supported by our observations that facial motoneurones which died had sent axons into the face.

Lance-Jones demonstrated that motoneurone death in the spinal cord extends from day 14 to 19 p. c. (actually reported as 13–18 days p. c. but changed to accord with the definition of day 1 used here – see Materials and Methods), whereas in the facial nucleus the motoneurone death began on day 17 and continued into the first post-natal week. This delay corresponds with other observations indicating that the facial neuromuscular system develops at a later stage than spinal counterparts (East, 1931; Humphrey, 1964).

REFERENCES


(Accepted 15 April 1983)