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The formation of axonal projections of the mesencephalic trigeminal neurones in chick embryos

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SUMMARY

Horseradish peroxidase–wheat germ agglutinin conjugate (HRP) was injected into masticatory and eye muscles of 6- to 15-day-old chick embryos and posthatched chicks to establish the timetable of axonal outgrowth and distribution of central and peripheral terminations of mesencephalic trigeminal neurones (MTN).

HRP-labelled MTN first appeared on the 10th day of incubation and by the 14th day most of MTN became labelled, indicating that axonic outgrowth to peripheral targets occurred between the 10th and 14th days of incubation. Peripheral targets included the pterygoideus lateralis and medialis, protractor quadratus, pseudotemporalis superficialis and profundus, and adductor mandibulae of jaw-closing muscles. HRP-filled central axonic processes of MTN were identified first on the 13th day of incubation and they terminated exclusively in the motor nucleus of the trigeminal nerve.

Between the 10th and 13th days of incubation, at the peak of naturally occurring cell death in the MTN pool, a consistently lower percentage of neurones could be labelled with HRP than in older embryos and in posthatched chicks. This finding suggests that many MTN die before establishing contact with peripheral targets.

INTRODUCTION

The neurones of the mesencephalic nucleus of the trigeminal nerve (MTN) represent one of the most distinct collections of cells in the central nervous system. There is good indirect evidence that at least the majority of MTN, if not all, derive from the cephalic neural crest and secondarily migrate into the roof of the developing mesencephalon (Narayanan & Narayanan, 1978; Piatt, 1945). Morphologically and functionally they are first order sensory neurones comparable to those found in the dorsal root ganglia of the spinal cord and the sensory ganglia of the cranial nerves. In amphibia MTN form the afferent limb of a proprioceptive reflex arc of masticatory and extraocular muscles (Gobrawi & Tarkhan, 1967; Rubinson, 1970; Hiscock & Straznicky, 1982) and in mammals these neurones are associated with masticatory and extraocular muscles and to an extent with facial muscles (Szentágothai, 1948; Jerge, 1963; Matesz, 1981). In contrast, avian MTN appear to have more restricted central and peripheral terminations (Alvarado-Mallart, Batini, Buisseret-Dalmas & Corvosier, 1975;

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Arends & Dubbeldam, 1982; Eden, Correira & Steinbuller, 1982). Although about 4500 MTN are initially generated in chick embryos, about 75 % of them are eliminated between the 10th and 13th days of incubation and by day 14 the residual 1150 MTN correspond more or less to the adult number (Rogers & Cowan, 1973).

Naturally occurring neurone death during development has been shown to be ubiquitous in neurone pools of the central and peripheral nervous system, and it may involve 15–85 % of the originally generated neurones (Clarke, 1981; Oppenheim, 1981*b*). Naturally occurring neurone loss can be exaggerated by removal of the target field (Hamburger & Levi-Montalcini, 1949) or reduced, if the target field is enlarged experimentally (Hollyday & Hamburger, 1976). Neuronal death in development has been thought to have a major function in matching up the size of neurone pool with the size of the available peripheral target since neurone loss occurs around the time when nerve fibres reach the target (Hamburger, 1961; Oppenheim, 1981*a*). Of the originally generated 50 000 neurones in the chick trigeminal sensory (Gasserian) ganglion, less than 30 000 survive after the 12th day of incubation (Straznicky & Rush, 1985). The bulk of neurone loss between the 7th and 10th days of incubation coincides well with the axonic outgrowth from the Gasserian ganglion and with the formation of cutaneous innervation of the head region (Noden, 1980). Similar correlation can be found between trigeminal motoneurone loss and motor fibre outgrowth to masticatory muscles in chick embryos (Arens & Straznicky, 1985).

Since neuronal death occurs about 4 days later in development in the MTN than the corresponding trigeminal motor and sensory neurone pools, it is of particular importance to establish the timetable of axonal outgrowth of MTN to their targets and to correlate it with the timetable of neurone death. In this work we investigated the peripheral and central termination patterns of MTN and the formation of these projections during development with the aid of horseradish peroxidase histochemistry (HRP). We also attempted to quantify the HRP preparations in order to gain further insight into the cause of the unusually high rate of naturally occurring cell death in this neurone pool.

METHODS

Experiments were carried out in White Leghorn chick embryos. Fertilized eggs, obtained from local poultry suppliers, were incubated at 37·7°C in a forced-draft incubator at 70 % relative humidity and rotated every 4 h throughout the period of incubation.

Injection procedure

Between the 6th and 15th days of incubation eggs were opened and the embryos exposed by tearing the chorioallantoic and amniotic membranes over the embryo avoiding damage to large vessels. Injection of 0·5 μl 1 % solution of horseradish peroxidase–wheat germ agglutinin conjugate (HRP), SIGMA, into the developing masticatory and eye muscles was made using a glass capillary tube of tip diameter approximately 50 μm. After the injection eggs were sealed with sterile tape and returned to the incubator. Each embryo had at least two or three injection sites in order to fill all nerve fibres in the developing muscles. Under 222-tribromoethanol (ALDRICH) anaesthesia 2- to 5-day-old posthatched chicks received either a single 1·0 μl HRP

injection into individual masticatory and eye muscles or a total of 3 μl HRP into all the jaw-closer muscles.

Histological processing

After 5 h postinjection survival in embryos or 20–24 h in posthatched chicks, animals were perfused transcardially first with 0·9 % saline and then with a 1·25 % glutaraldehyde and 1 % formaldehyde solution in phosphate buffer, at pH 7·2. The dissected brains were postfixed for 1–2 h or overnight in 2·5 % glutaraldehyde in phosphate buffer for embryos in which fixative perfusion was not successful. The brains were then embedded in gelatine–albumin and infiltrated with 30 % sucrose phosphate buffer overnight. The following day the brains were serially sectioned on a freezing microtome at 40 μm in the transverse plane. The free-floating serial sections were stained for peroxidase activity according to the tetramethyl benzidine method of Mesulam (1978). The reacted sections were flat mounted from rinsing solution onto subbed slides, counterstained with neutral red and safranin-O, air dried and coverslipped in Permount.

Morphometry

The number of HRP-labelled and unlabelled neurones were counted separately in the MTN and the trigeminal neurone pools and the percentage of HRP labelling established in each case. In 12- to 15-day-old embryos and in posthatched chicks the position of labelled MTN and the course of their HRP-filled axonic processes were drawn with the aid of a camera-lucida attachment. The course of the HRP-filled axons of MTN was reconstructed from the serial sections.

RESULTS

This report is based on successful histological preparations obtained from 21 embryos and 24 posthatched chicks. MTN are large pseudounipolar cells similar in appearance to the neurones of the dorsal root and cranial sensory ganglia. Because of their distinct size and shape they are easily distinguishable from other neurones and cells of the central nervous system. MTN form two continuous groups, the medial is located in the posterior and intertectal commissures, and the lateral in the anterolateral one third of the midbrain tectum in the stratum griseum periventriculare (Fig. 1A,B). HRP administration into extraocular muscles and depressor madibulae (jaw opener) in posthatched chicks resulted in labelling of oculomotor, trochlear, abducens and facial motoneurones only. In contrast, injections into pterygoideus medialis and lateralis, pseudotemporalis superficialis and profundus, and adductor mandibulae (jaw closers) brought about intense and always ipsilateral MTN and trigeminal motoneurone labelling (Table 1). The number of labelled MTN varied according to the size of the jaw-closing muscles and the amount of HRP successfully injected into the muscle. Each jaw-closing muscle had representation both in the medial and lateral groups. However, MTN supplying adductor mandibulae and pseudotemporalis profundus were found mostly in the rostral part of the lateral group. The other three small jaw-closing muscles were represented across the whole extent of the MTN pool.

In all cases, labelled axonic processes of MTN could be observed within the roof of the midbrain and the brainstem. HRP preparations revealed axon trajectories from the MTN pool to the brainstem trigeminal motoneurone pool extremely well

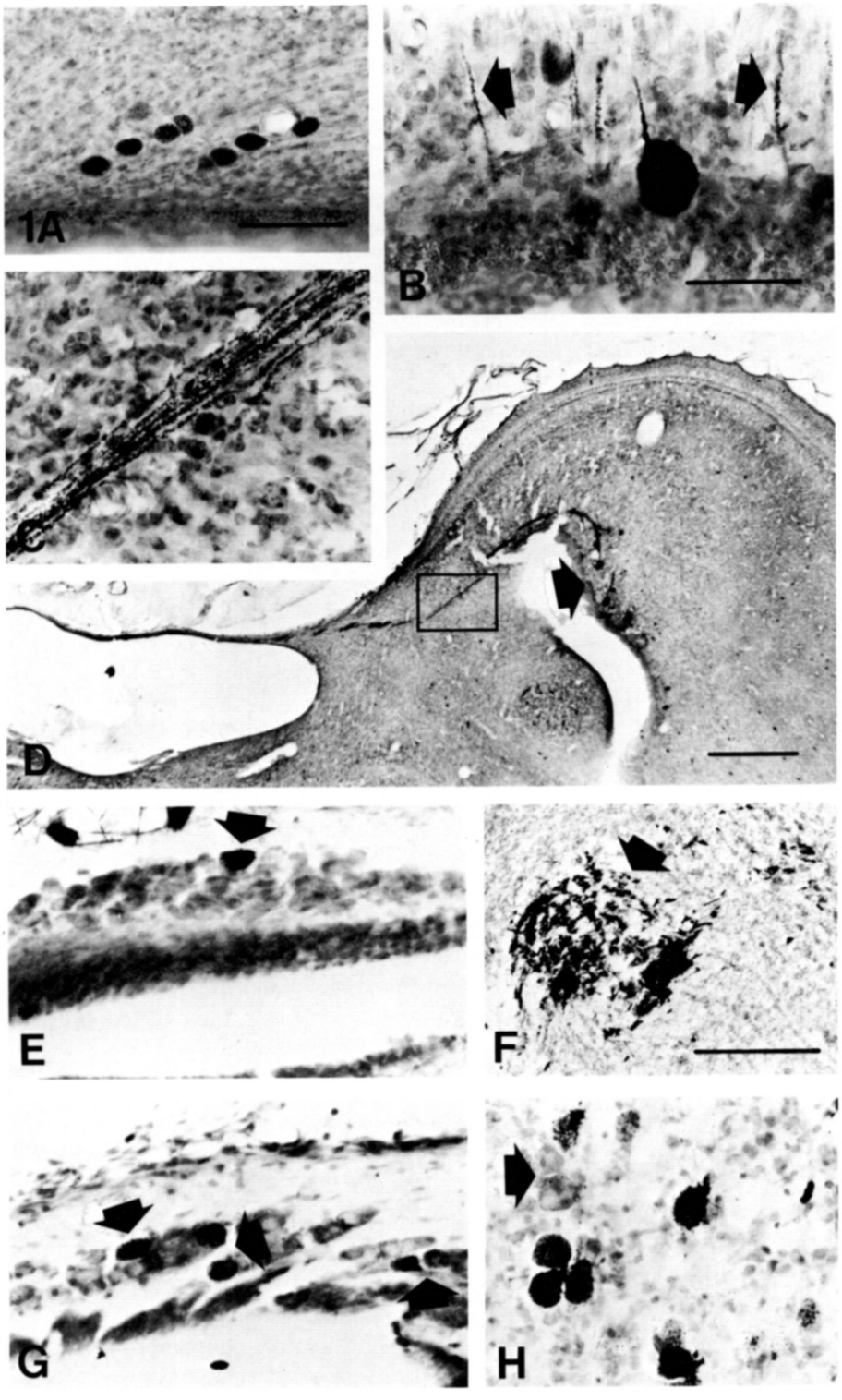
1A
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Table 1. *Retrograde labelling of MTN from various peripheral targets in posthatched chicks*

Muscle injected	No. of animals	Labelling of MTN
Pterygoideus medialis	2	++
Pterygoideus lateralis	3	+
Pseudotemporalis superficialis	5	++
Pseudotemporalis profundus	3	++
Adductor mandibulae	2	+++
Depressor mandibulae	2	–
Medial rectus	2	–
Superior oblique	2	–

\+ very scarce labelling (1–5 %)
++ sporadic labelling (5–15 %)
+++ widespread labelling (about 50 %)
– no HRP labelled neurones present

in three posthatched chicks with multiple injections into all the jaw-closer muscles (Fig. 1C,D). No other central termination of MTN was observed. Reconstructions from histological preparations show the axons of MTN converging onto the medial border of the tectum from where they pass the isthmic nucleus medially and dorsally travelling underneath the floor of the fourth ventricle towards the root of the trigeminal nerve (Fig. 2). Before exiting from the brainstem, collateral branches were given off from the axons of MTN to their central termination in the motor nucleus of the trigeminal nerve.

The results of the present study on embryos are summarized in Table 2. Following HRP injection into masticatory and extraocular muscles between the 6th and 9th days of incubation only motoneurones in the oculomotor, trochlear, trigeminal and facial motor nuclei became labelled. Counts of the trigeminal motoneurone pool showed that 55–100 % of cells were filled retrogradely with HRP (Fig. 1F). In contrast, MTN could first be filled in 9½-day-old embryos with only very few neurones (about 5–7 %) taking up HRP (Fig. 1E). In this and at later developmental stages MTN could be filled exclusively from jaw-closer muscles. Between the 10th–13th days the percentage of labelled MTN compared

Fig. 1. Photographs of HRP-filled MTN and trigeminal motoneurones following HRP administration into jaw-closing muscles. Transverse sections of the tectum in 2-day-old chicks showing MTN in the stratum griseum periventriculare (A,B) and axon trajectories of MTN (C,D). Arrows in (B) point at HRP-filled axons, whose corresponding neurones are not in focus. Boxed area in (D) is shown in (C). Note the HRP-filled axons coursing towards the intertectal commissure. (E,F) are HRP preparations from a 10-day-old chick embryo. Arrow in (E) points at an HRP-filled MTN, surrounded by a large number of unlabelled MTN. Two blood cells in the upper left corner are also HRP-positive. Arrow in (F) points at the motor nucleus of the trigeminal nerve, where most of the neurones are filled with HRP. Arrows in (G) point at HRP-filled MTN in a 13-day-old embryo. (H) HRP-filled and unfilled (arrow) MTN in the tectum of a 15-day-old embryo. Bars in (A), 100 μm; (B,C,E,G,H), 50 μm; (D), 250 μm and (F), 200 μm.

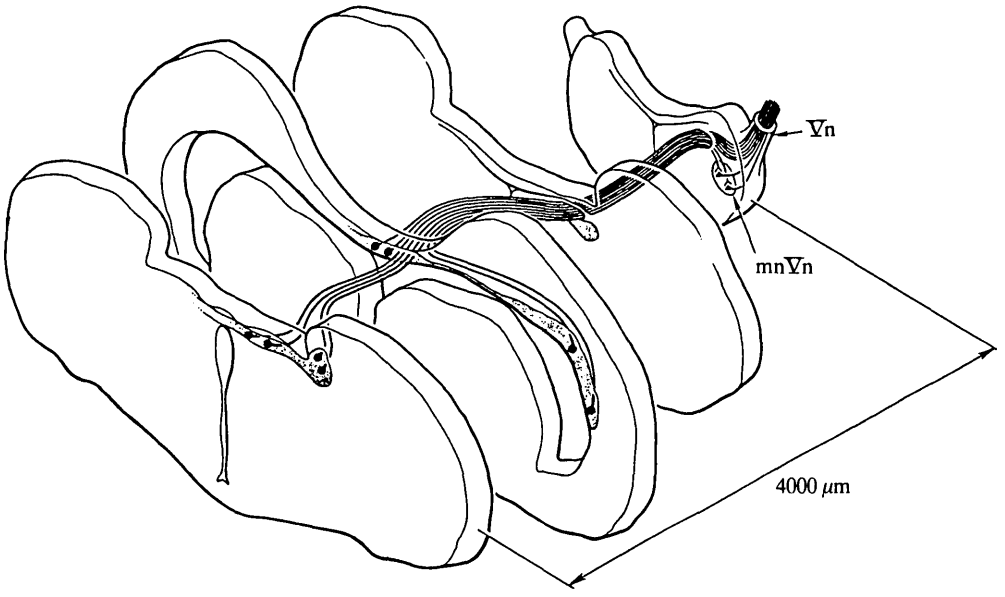

Fig. 2. Drawing of a three-dimensional reconstruction of the axonic trajectories of MTN from serial sections through the midbrain of a posthatched animal. Shaded area marks the position of the medial and lateral groups of MTN. Note that the two groups become discontinuous caudalwards in the midbrain. Filled dots and lines indicate the position of representative MTN and their axonal trajectories. The distance between the rostralmost occurring MTN in the midbrain and the root of the trigeminal nerve (Vn) is given. mnVn, motor nucleus of the trigeminal nerve.

to unlabelled MTN increased from 5 % to 57 %. Most of the labelled MTN were located in the intertectal commissure and in the stratum griseum periventriculare (Fig. 1H). At any time, only very few labelled neurones were observed in the posterior commissure in spite of the large number of MTN present (Fig. 1E,G). Regularly a much higher percentage of trigeminal motoneurones were filled with HRP than MTN in the same embryo. Because of the 40 μm thickness of the serial sections the number of HRP-filled or unfilled neurones were underestimated. However, the number of MTN and trigeminal motoneurones for various developmental stages have been previously established (Rogers & Cowan, 1973; Straznicky & Rush, 1985; Arens & Straznicky, 1985). Therefore, from the observed percentage label of MTN and their established total numbers, the total numbers of HRP-filled neurones can be estimated (Table 2). These figures clearly indicate that the total number of HRP-labelled MTN remained relatively constant, in the range of 800–1000 from the 11th day of incubation to past hatching. However, due to the striking decrease of the absolute number of MTN from 3100 down to 1100 over the same period, the percentage of labelled MTN continuously increased up to 95 % in posthatched animals. Axon trajectories of MTN in embryos were seen from the 10th day of incubation and their course was very similar to those shown in posthatched chicks. However, HRP-filled axon collaterals to the motoneurone pool appeared only from the 13th day of incubation.

Table 2. *The percentage of labelled cells in the MTN and trigeminal motoneurone pools following HRP injection into masticatory muscles in embryos and posthatched chicks*

Age at injection in days	MTN %	Trigeminal motoneurones %
6	—*	70
6½	—	100
8	—	55
8	—	83 (3450)†
8½	—	100
9	— (4500)‡	84
9½	5	100
9½	—	71
9½	7	100
10	19	88
10	12	100
10	30 (3550)	90
11	15	47
11	32 (3100)	75
12	37	100
12	38 (2000)	85
13	57 (1350)	100 (1700)
14	65	70
15	61	85
15	77	92
15	65 (1150)	81
PH§	87	90
PH	95 (1100)	100

* No HRP-labelled MTN were found.
† After Arens & Straznicky (1985).
‡ After Rogers & Cowan (1973); Straznicky & Rush (1985).
§ Posthatched chicks.

DISCUSSION

The present study confirms that the peripheral termination of MTN in embryos and posthatched chicks includes only the ipsilateral jaw-closing muscles. Neither jaw-opening muscles, nor extraocular muscles receive proprioceptive sensory innervation from the MTN. Proprioceptive neurones supplying these muscles have been shown to be located in the Gasserian ganglion of the trigeminal nerve (Eden *et al.* 1982). Our observations are also in agreement with the results of recent findings in other avian species that the central axonic processes of MTN terminate in the motor nucleus of the trigeminal nerve forming direct monosynaptic contact with the motoneurones (Arends & Dubbeldam, 1982; Berkhoudt, Klein & Zeigler, 1982).

The main finding of the present study indicates a rather late outgrowth of MTN axons to jaw-closing muscles, in contrast to the much earlier arrival of motor fibres. This observation is interesting because MTN are generated between the 2nd and 5th days of incubation (Rogers & Cowan, 1973), similar to the large dorsal

root ganglion cells (Hamburger & Levi-Montalcini, 1949) and then the outgrowth of their axonic processess is delayed by about 5 days. Of the 4500 MTN originally generated only 1100 survive to maturity (Rogers & Cowan, 1973; Straznicky & Rush, 1985). Although the whole complement of MTN is still present on the 10th day of incubation, not more than 5–7 % of them could be filled retrogradely with HRP. A very large percentage of trigeminal motoneurones were labelled in the same embryos indicative of sufficient amount of HRP present for uptake in the injected muscles. In older embryos and in posthatched chicks an increasingly larger percentage of MTN were labelled keeping the number of HRP-filled MTN at a fairly constant level of 800–1000. These observations suggest the existence of two neurone populations within the MTN pool. One population appears to establish connections with their target muscles during the time of naturally occurring neurone death. The timing of the axonic outgrowth of these surviving MTN coincides well with the reported timetable of avian muscle spindle development (Tello, 1922). The other larger population of MTN, present in decreasing numbers from the 10th day of incubation, could be filled neither from the natural target nor from extraocular or jaw-opener muscles.

Relevant previous observations and the results of the present study do not readily lend themselves to a satisfactory explanation for the exceptionally high rate of MTN loss during development. There are several possibilities for further consideration. (i) Many of the axons of MTN fail to reach their natural targets and consequently such neurones die. In view of the results of the present study this is unlikely. Although all the muscles of the anterior aspect of the head region (masticatory and extraocular muscles) were tested, these results did not indicate the presence of transient erroneous projections of MTN during development besides the natural target of the jaw-closer muscles. Previous reports on motor fibre outgrowth to limb muscles (Landmesser, 1978) or fibre outgrowth from the isthmo-optic nucleus to the retina (Clarke & Cowan, 1976; O'Leary, Gerfen & Cowan, 1983) in chick embryos have indeed revealed a high degree of target selectivity. (ii) Competition for the target has been recognized as an important factor in determining the survival of ventral horn motoneurones and dorsal root ganglion cells. Neurones which fail to establish appropriate connections with the target are eliminated (Oppenheim, 1981*b*). If competition within the target for synaptic terminal space were to apply to MTN one would expect to find very large numbers of MTN labelled at the beginning of naturally occurring cell death and progressively less as more neurones die. The results of the present study do not show this. (iii) Another alternative explanation is that a large proportion of MTN may not develop axonic processes before the onset of neurone death. Such neurones, of course, cannot be labelled retrogradely with HRP. This possibility is supported by observations on the developing trochlear motoneurones in duck embryos, where two morphologically distinct neurone types, large and small, have been identified (Sohal, 1976). The small neurones do not appear to differentiate or

to form axonic processes, hence they die during the period of naturally occurring cell death.

Since target competition or correction of grossly erroneous axonic projections do not readily explain the exceptionally high rate of neuronal death in the MTN pool, we are inclined to support a previous suggestion that some neurones which perish during development may provide an evolutionary reserve (Clarke, 1981). In contrast to birds, in mammals MTN supply all masticatory and extraocular muscles (Jerge, 1963; Matesz, 1981) with a greater density of proprioceptive innervation, hence utilizing a greater proportion of originally generated MTN.

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