Initiation of facial motoneurone migration is dependent on rhombomeres 5 and 6

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SUMMARY

In mammals, facial branchiomotor (FBM) neurones are born in ventral rhombomere (r) 4 and migrate through r5 to dorsal r6 where they form the facial motor nucleus. This pattern of migration gives rise to the distinctive appearance of the internal genu of the facial nerve, which is lacking in birds. To distinguish between extrinsic cues and intrinsic factors in the caudal migration of FBM neurones, this study takes advantage of the evolutionary migratory difference between mouse and chick in generating mouse-chick chimaeras in ovo. After the homotopic transplantation of mouse r5 and/or r6 into a chick embryo, chick ventral r4 neurones redirected their cell bodies towards the ectopic mouse source and followed a caudal migratory path, reminiscent of mouse FBM neurones. In a second series of grafting experiments, when mouse r4 was transplanted in place of chick r4, mouse r4 neurones were unable to migrate into chick r5, although mouse and chick cells were able to mix freely within r4. Thus, these data suggest that local environmental cues embedded in mouse r5 and r6 are directly involved in initiating caudal migration of FBM neurones. In addition, they demonstrate that chick FBM neurones are competent to recapitulate a migratory behaviour that has been lost during avian phylogeny.

Key words: Mouse-chick chimaera, Tissue transplantation, Motoneurone migration, Cell aggregation, Facial motoneurone markers

INTRODUCTION

Cell migration plays a crucial role in a wide variety of biological systems. In the developing central nervous system (CNS), most neurones are generated at different sites from those in which they permanently reside. After they have finished dividing, cells segregate from adjacent progenitors, extend a leading process and then move along specific pathways, a process known as neuronal migration (Rakic, 1990; Rakic, 1999). Interactions between the migrating neurones and the surfaces of neighbouring cells are crucial for the selection of migratory pathways (Pearlman et al., 1998). Neuronal migration differs from axonal pathfinding, because in the first case the cell body translocates, whereas in the latter case the cell body remains stationary while the axon projects towards its target. Although a huge number of descriptive studies have been reported, the cellular and molecular mechanisms that direct the intrinsic migratory predisposition of a single neurone and the migration of distinctive neuronal populations remain largely unknown. Recent data suggest that molecules involved in specification and migration are diverse and include transcription factors expressed in motoneurone subsets and cell surface receptors, which confer responsiveness to cues in the environment (reviewed in Hatten, 1999; Jurata et al., 2000).

The facial nerve of the vertebrate embryo represents an ideal system with which to explore neuronal migration. Facial motoneurones originate ventrally within a column on either side of the floor plate, occupying rhombomere (r) 4 and r5 of the segmented hindbrain (Lumsden and Keynes, 1989). Later in development, two subpopulations of facial motoneurones emerge, branchiomotor (FBM) neurones, which innervate the muscles of the second branchial arch, and visceral motoneurones (VMN), which innervate parasympathetic ganglia (Gilland and Baker, 1993). In most vertebrates, embryonic FBM neurones undergo a striking and complex neuronal migration while the hindbrain is still segmented. In the mouse, from E10 their cell bodies form a distinct cluster in the mantle layer and start migrating tangentially along the lateral margin of the floor plate, reaching first r5 and then r6. In rostral r6, these neurones begin a lateral and subsequently a radial migration towards the pial surface, where they form the facial motor nucleus at around E14 (Ashwell and Watson, 1983; Auclair et al., 1996; Studer et al., 1996; Garel et al., 2000). In almost all vertebrate species examined so far, FBM neurones exhibit this characteristic migration by giving rise to the internal genu of the facial nerve (Altman and Bayer, 1982; McKay et al., 1997). In shark, lizard or salamander, the location and organisation of facial motoneurones are similar to those in mammals (Barbas-Henry, 1982; Roth et al., 1988; Gilland and Baker, 1993), whereas in zebrafish they migrate into r6 and r7 (Chandrasekhar et al., 1997). In avian embryos,
however, FBM neurones translocate laterally and radially within r4, in a way similar to trigeminal motoneurones in r2, and therefore lack the characteristic genu (Lumsden and Keynes, 1989; Szekely and Matesz, 1993). A recent report has elegantly shown that a subpopulation of FBM neurones in chick embryos can migrate as far as r5, but as their position remains more lateral, their migration path does not form a genu (Jacob and Guthrie, 2000).

The identification of several mutations that affect the development of cranial motor nuclei in mouse has led to a recent renewal of interest in studying the cellular and molecular mechanisms involved in guiding facial motoneurones to their final location. In Hoxb1 loss-of-function mutants, FBM neurones do not exhibit their normal caudal migration but progress laterally within r4, behaving similarly to chick neurones (Goddard et al., 1996; Studer et al., 1996). FBM neurones are not fully differentiated in these mice, and lack expression of GATA and Phox2 family genes (Pata et al., 1999; Gaufo et al., 2000), consistent with the hypothesis that a defect in specifying FBM precursors would result in altered neuronal migration.

To identify mechanisms that are directly implicated in the caudal migration of FBM neurones, a second series of mutants that lack or have defects in the rhombomeres into which they migrate, have been extensively analysed. In kreasler (Mafs – Mouse Genome Informatics) and Kro20 (Egr2 – Mouse Genome Informatics) mouse mutants or in valentino (val) mutants in zebrafish, FBM neurones adopt either aberrant trajectories (Chandrasekhar et al., 1997) or a dorsal migration characteristic of r6 (McKay et al., 1997; Schneider-Maunoury et al., 1997; Manzanares et al., 1999; Garel et al., 2000). In contrast, Ebf1 mutant embryos have an apparently normal segmented hindbrain, however a subpopulation of FBM neurones express prematurely the cell-surface molecules TAG-1 and Cdh8, and migrate laterally within r5 (Garel et al., 2000). Although these data on mutant embryos tend to suggest a constant interaction between migrating cells and their environment, they do not directly address the role of the environment in initiating caudal migration. Are FBM neurones pre-programmed to follow a specific pathway or do they just migrate laterally within r4 if the conditions in the adjacent environment are not favourable? In addition, are FBM neurones able to respond to specific attractive and/or repulsive cues secreted by cells in the adjacent environment?

The experiments described here challenge the origin of the differences in FBM migratory behaviours in mouse and chick embryos. Because of conserved cellular and molecular strategies in mouse and chick early development, inter-specific transplants between mouse and chick have been used to study general developmental mechanisms (Itasaki et al., 1996; Fontaine-Perus et al., 1997; Fontaine-Perus, 2000). In replacing chick rhombomeres with mouse rhombomeres and generating in ovo mouse-chick chimaeas, this study provides evidence that the mouse environment, i.e. r5/6, plays an essential role in initiating the caudal migration of ventral r4 neurones. In the presence of either mouse r5 or r6, or both, chick FBM neurones redirect their cell bodies towards the ectopic mouse tissue and follow a caudal and lateral pathway that is characteristic of other vertebrate classes but absent in birds. These data indicate that in chick, FBM neurones are competent to reiterate an evolutionary conserved migratory pathway when exposed to appropriate cues. In addition, these results strongly suggest that different signalling cues located in r5/6 in chick and mouse are responsible for the species-specific migratory behaviours of FBM neurones.

**MATERIALS AND METHODS**

Experiments were performed using Rhode Island Red hens’ eggs, CD1 mice from Charles River and the Rosa26 transgenic mouse line (Zambrowicz et al., 1997). Mouse, chick and mouse-chick chimaeric embryos were staged according to the somite number, chick embryos were staged according to the incubation day (Hamburger and Hamilton, 1992) (HH) and mouse embryos were staged according to gestation period (Kaufman, 1995).

**In situ hybridisation, immunohistochemistry and retrograde labelling**

Embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Whole-mount single and double in situ hybridisation, immunohistochemistry and β-galactosidase staining were performed as described (Goddard et al., 1999). Probes and antibodies were for chick Isl1 (Varela-Echavarria et al., 1996) and mouse Isl1 (Tuschida et al., 1994); chick Phox2b (gift from J. F. Brunet) and mouse Phox2b (Pattyn et al., 1997); chick Hoxb1 (Bell et al., 1999) and mouse Hoxb1 (Murphy et al., 1989); kreisler (Cordes and Barsh, 1994), B2-repeat (Bollag et al., 1999) and anti-mouse Hoxb1 antibody (Goddard et al., 1996). At a hybridisation temperature of 70°C, mouse and chick orthologues did not cross-hybridise. For photography, hindbrains were dissected out, flattened and analysed by bright field and Nomarski microscopy. For retrograde labelling, embryos were pinned ventral side upwards and the surrounding mesenchymal tissue was dissected out to expose cranial facial nerve roots. The facial nerve and its branches were transected and rhodamine-dextran (Molecular Probes, Eugene, OR) was applied for retrograde axonal tracing as previously described (Jacob and Guthrie, 2000; Varela-Echavarria et al., 1996). After overnight fixation in 4% PFA, hindbrains were flat-mounted and viewed under a confocal microscope (BioRad, MRC-600).

**Short-term aggregation cultures**

Hindbrains from HH stage 14 chick embryos and E9.5 mouse embryos were processed for aggregation cultures as previously described (Wingate and Lumsden, 1996; Wizenmann and Lumsden, 1997). Mesenchyme-free hindbrains were subdivided into single rhombomeres and labelled with either CellTracker Green or CellTracker Red (Molecular Probes, Eugene, OR). Pooled rhombomeres were incubated in Ca2+-free medium (HBBS) and dissociated cell suspensions were obtained by gentle homogenisation. Cells from single rhombomeres were then mixed to produce different combinations (e.g. mouse r2-r3 or mouse-chick r5). Cell mixtures were allowed to aggregate for 12-24 hours at 37°C and the patterns of cell segregation or mixing in the resulting spherical cell aggregates were assessed under a fluorescence microscope equipped with dual wavelength optics. For detailed quantitative, qualitative and photographic analysis, aggregates were analysed as described previously (Wizenman and Lumsden, 1997).

**Microsurgery generation of chimaeras and explant cultures**

Fertilised hens’ eggs were incubated to HH stage 10 (33-38 hours of incubation at 39°C) and used as hosts. Mouse donor tissue (r2-r6) from E8.25 to E8.75 embryos (midday of the day of vaginal plug equalled E0.5) was dissected in L15 (Gibco) and transplanted homotopically into chick hosts. This specific mouse stage was chosen because of the appearance of a boundary between r4 and r5 and of clear pre- and
Migration of facial branchiomotor neurones in mouse-chick chimaeras

post-otic sulci as future boundaries between r2/3 and r4/5, respectively (Sakai, 1987; Ruberte et al., 1997). Because of the lack of boundaries between r5/r6 and r6/7, the adjacent somites were used as markers to ensure a reproducible posterior position of the neural grafts (Itasaki et al., 1996). In some experiments, the mouse tissue was first incubated in red CellTracker dye (CMTMR, Molecular Probes, OR) for 30 minutes before grafting. Unilateral grafts and ablations excised one side of the host neural tube along with the overlying ectoderm, leaving the floor plate untouched, except from Fig. 4D where the mouse floor plate was included in the graft. After grafting, embryos were incubated in ovo up to HH stage 23-24 before PFA fixation.

For explant assays, whole hindbrains (r2-r7) of E8.75 mouse embryos were embedded in Matrigel (Collaborative Biomedical Products) and allowed to congeal in a culture dish at 37ºC; the gel was subsequently overlaid with serum-free neurobasal medium (Gibco) containing B27 supplement (Gibco), L-glutamine and penicillin-streptomycin antibiotics (Gibco). Explant were immunostained with anti-mouse Hoxb1 after 2-3 days of incubation at 37ºC.

RESULTS

Comparative molecular and cellular analysis of facial motoneurones in mouse and chick

In mouse and chick embryos, the branchiomotor neuronal population (FBM in the figures) originated in r4, while the visceral motor subpopulation (VMN) arose in r5 (Lumsden and Keynes, 1989; Studer et al., 1996; McKay et al., 1997; Garel et al., 2000). Both populations projected into the periphery in the facial nerve from which they can be retrograde labelled with a fluorescent tracer dye (Fig. 1A,B). Whereas in the E11 mouse embryo FBM neurones migrated longitudinally along the ventral midline in r5 and r6 (arrow in Fig.1B), in HH stage 24 chick embryos, the majority of FBM neurones translocated their cell bodies laterally, similar to the VMN population in r5 (arrows in Fig. 1A). To understand whether the two species-specific neuronal paths were related to particular molecular properties, the expression pattern of markers labelling the differentiating and migrating facial populations in r4 and r5 was compared with the distribution of retrogradely labelled motoneurones. Although some of these markers were expressed in many other locations, Fig. 1 focuses exclusively on the regions of r4 and r5.

In chick, expression of Hoxb1 was exclusively restricted to r4 (Fig. 1C), whereas mouse Hoxb1 expression in mouse was also detected in r5 (arrow in Fig.1D) in a ventrolateral position that is characteristic of FBM neurones (inset in Fig. 1D; Studer et al., 1996; Garel et al., 2000). The early motoneurone marker Isl1 and the chick homeobox gene Phox2b were both expressed in differentiating and migrating neurones within r4 and r5, corresponding to FBM and VMN, respectively (Fig. 1E,G). By contrast, expression of the mouse orthologues, Isl1 and Phox2b, reproduced the rostral to caudal migratory pathway of FBM neurones in r5 and rostral r6 (arrows in Fig. 1F,H). Similar to chick, expression of mouse Phox2b was also

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Fig. 1. Mouse and chick facial branchiomotor neurones undergo different migratory pathways during development. (A,C,E,G) Ventral views of flat-mounted HH stage 23 to 24 chick hindbrains and (B,D,F,H) E11.0 to E11.5 mouse hindbrains. All the panels show the region of rhombomeres (r) 4 and 5 with the floor plate (fp) to the right (basal) and the r4 exit points to the left (alar). (A,B) Retrograde rhodamine-dextran labelling of facial branchiomotor (FBM) and visceral motor neurones (VMN) in chick and mouse embryos. The inset in D shows a transverse section of mouse Hoxb1 expression at the level of r4/r5. Note expression in the mantle layer lateral to the floor plate corresponding to migrating FBM neurones. (E) Chick Isl1 is expressed in chick in ventral r4 and r5, and in migrating FBM neurones within r4. (F) In mouse, a large stream of mouse Isl1-positive cells is present in ventral r4, r5 and rostral r6 (arrow in F). (G) Chick Phox2b is expressed at high levels in FBM and VMN neurones migrating laterally within r4 and r5, respectively. (H) In mouse, mouse Phox2b is expressed in ventral r4 and in the caudally migrating FBM population (arrow in H). G and H have an additional lateral Phox2b expression, which corresponds to the intermediate neural column expanding from r2 to r6.
detected in the migrating VMN population in r5, although at lower levels (Fig. 1H). Thus, the analysis of molecular markers for the various sub-populations of the facial nerve in mouse and chick shows that FBM neuronal properties are conserved at a cellular and molecular level, as r4 markers in both species correlate with the position of FBM neurones. Therefore, these markers can be used as molecular tools to identify FBM neurones in both species.

Mouse and chick cells have similar aggregation properties in r5 and r6

To ensure that the species-specific pattern of migration did not reflect differences in cell surface properties, short-term chimaeric aggregation cultures were made from mouse and chick hindbrains. In chick, cells from even-numbered rhombomeres sort out from cells of odd-numbered rhombomeres, whereas cells from either even- or odd-numbered rhombomeres mix freely, suggesting a difference in cell adhesive properties between adjacent rhombomeres (Wizenmann and Lumsden, 1997). To first assess whether rhombomere-specific segregation was conserved in mouse, cells derived from r2 to r6 of E9.5 mouse hindbrains were mixed in several combinations. The cell aggregates that subsequently formed were scored as ‘segregated’ or ‘mixed’ as previously reported (Wingate and Lumsden, 1996; Wizenmann and Lumsden, 1997). Fig. 2A,B shows micrographs of aggregates derived from mouse even-numbered rhombomeres (red) cultured for 24 hours with cells of odd-numbered rhombomeres (green). A segregation ratio of 68% for cell mixtures of r2 with r3 (number of aggregates, n=322) and 71% for r4 with r5 (n=356) suggested a difference in adhesive properties between odd and even mouse rhombomeres (Fig. 2C). The percentage of segregation was however slightly lower than those observed in chick (Wizenmann and Lumsden, 1997), which might reflect species-specific differences. By contrast, aggregates from two even-numbered rhombomeres (i.e., r4:r6) mixed well together with only 22% of segregation (Fig. 2C; n=340 and data not shown). Then, to assess the degree of segregation of r5 or r6 between mouse and chick, cells from E9.5 mouse r5 or r6 (in red) were mixed with those from HH stage14 chick r5 or r6 (in green; n=362 and 330, respectively). In both cases, mouse and chick cells mixed evenly (Fig. 2D,E) and had aggregation ratios of 81% and 83%, respectively (Fig. 2F), suggesting that mouse and chick r5 or r6 cells have similar adhesive properties. Similar results were obtained with mouse and chick r4 cells (data not shown).

These interspecies aggregates show that there are no differences in adhesive properties between mouse E9.5 and chick HH stage 14 for r5 and r6. This suggests that early differences in cell-surface properties might not be responsible for the differential migratory behaviour of FBM neurones. Moreover, the ability of free mixing between mouse and chick cells raises confidence that in subsequent mouse-chick chimaera transplantation experiments, species-specific cell properties would not be a significant factor in determining cell migration.

Mouse-chick chimaeras

It was first assessed whether mouse rhombomeres would effectively integrate into the chick hindbrain and maintain their rhombomeric identity. To this end, r5/6 from E8.5 mouse embryos, derived from a Rosa-26 LacZ transgenic line were transplanted homotopically into HH stage 10 chick hosts (Fig. 3A), and chimaeras were incubated for a further 36 to 70 hours (see also Table 1). To appraise the morphology and identity of the mouse grafts, embryos were stained for β-galactosidase activity or hybridised with kreasler, a mouse-specific probe for r5:r6 (Fig. 3B-D).
Migration of facial branchiomotor neurones in mouse-chick chimaeras

At HH stage 19, chimaeric hindbrains displayed the mouse tissue in the expected position and with the desired rhombomeric identity (Fig. 3B,C). Some chimaeric embryos generated a new boundary at the posterior position of the mouse graft (arrowhead in Fig. 3B), which was helpful in localising the mouse tissue in the chick environment (see also inset in Fig. 4F). At HH stage 24, the anteroposterior length of the mouse r5/6 graft was equivalent to a single rhombomere width when compared with the unoperated side (Fig. 3D). This suggests either a difference in growth rate between the two species or high regenerative capacity of the chick tissue through compensatory proliferation and migration (Diaz and Glover, 1996). Nevertheless, lacZ expression at the r4/5 boundary was sharp, suggesting regeneration of a normal boundary between chick r4 and mouse r5 (arrow in Fig. 3D). These experiments demonstrate that mouse r5/6 can maintain their rhombomeric identity when homotopically grafted into chick embryos of similar embryological stages and that the chick r4/mouse r5 boundary is well preserved.

Mouse rhombomeres 5 and/or 6 induce ectopic migration of ventral r4 motoneurones

To assess whether the adjacent environment is implicated in driving differentiated facial motoneurones out of r4, chick r5 and/or r6 were homotopically replaced in ovo with mouse r5 and/or r6. The position of the mouse graft was identified either by hybridising chimaeras with a mouse-specific probe or by labelling the mouse tissue with a fluorescent tracer before grafting, or by morphological criteria, i.e. presence of prominent boundaries around the graft and different morphology of cells.

In a first series of experiments, mouse r5/6 were grafted in place of chick r5/6 and chimaeric embryos were hybridised with chick Isl1 probe (Fig. 4B,C; see Table 1). At HH stage 20, before facial motoneurones started to migrate laterally within r4, chick Isl1-positive cells of chimaeric embryos accumulated ventrally in the most anterior portion of mouse r5/6 (arrow in Fig. 4B). These ectopic cells were either induced de novo by the mouse tissue or were just starting to migrate caudally towards the mouse graft. To follow the fate of this ectopic population, chimaeric embryos were incubated up to HH stage 24, when r4 motoneurones normally migrate laterally. In these embryos, a major stream of chick Isl1-positive cells invaded the grafted mouse tissue (circumscribed by red dots), whereas a minor proportion of motoneurones maintained their lateral pathway (asterisk in Fig. 4C).

Table 1. Transplantation and ablation experiments

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<th>Type of transplantation</th>
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<th>Number of chimaeras*</th>
<th>Type of analysis</th>
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<th>r4 expansion</th>
<th>Migration of chick cells</th>
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*Includes only those embryos that have incorporated the mouse graft.

1Includes embryos from HH stage 19 to stage 24.

2Includes only basal r4.
Moreover, levels of chick *Isl1* expression were increased in the migrating neurones suggesting a higher rate of cell proliferation at the operated side compared with the control side. To ensure that this chick-specific motoneurone population was effectively originating from r4, chimaeric embryos were hybridised with the r4-specific marker chick *Hoxb1*. Fig. 4D shows that chick *Hoxb1*-positive cells extended caudally from ventral r4 along the floor plate terminating with a fan-shape pattern, reminiscent of mouse FBM neurones. Thus, these data indicate that the initiation of caudal migration of ventral r4 motoneurones is dependent on cues present in its juxtaposed environment.

To test whether mouse r5 was sufficient to induce a migration of chick ventral r4 cells, chick r5 was replaced with mouse r5 and chimaeric embryos were hybridised with the chick *Hoxb1* probe. HH stage 24 chimaeric embryos showed a caudal extension of chick *Hoxb1* expression followed by a thin stream of cells invading mouse r5 (surrounded by red spots in Fig.4F). The inset in Fig. 4F is in a different focal plane and shows the prominent borders encompassing the mouse graft. Moreover, chick *Hoxb1* expression in r4 was unusually expanded at the operated side when compared with the unoperated side (Fig. 4F), possibly owing to an overgrowth of r4 tissue. These data show that mouse r5 is sufficient to attract chick *Hoxb1*-positive cells.

Finally, to assess whether mouse r6 would also be involved in initiating caudal migration of r4 motoneurones, mouse r6 was first labelled with a red CellTracker dye and then grafted in place of chick r6 (Fig. 4G). Surprisingly, the ventral domain of the operated r4 side was dramatically expanded compared with the control side and a thick stream of chick *Hoxb1* expression ran through r5 towards the direction of the fluorescent graft (Fig. 4H). Therefore, although mouse r6 was not in direct contact with chick ventral r4, cues released from the mouse tissue were sufficient to diffuse through the host territory and attract chick *Hoxb1*-positive cells, suggesting the involvement of long-range attractive cues in the migration of r4 motoneurones.

**No migration is induced when r5/6 is ablated, replaced with chick r5/6 or with mouse r2/3**

To ensure that the ectopic stream of expression of motoneurone markers in chimaeric embryos was not due to an artefact of the grafting procedures or to an attraction by any mouse tissue, a series of control experiments, shown in Fig. 5, was performed. First, chick r5/6 were unilaterally excised at HH stage 10 (Fig. 5A) and embryos were hybridised with chick *Hoxb1* at HH stage 24. It has been shown that ablating r4 will lead either to partial or total regeneration of the ablated region without affecting any axonal trajectory or neuronal pattern of migration of efferent neurones (Diaz and Glover, 1996). Fig. 5B shows an example of a very low rate of regeneration after r5/6 ablation. In this case, although the whole r4 domain of chick *Hoxb1* expression was abnormally enlarged along the anteroposterior axis, no ectopic stream of chick *Hoxb1*-positive cells was detected caudal to r4 (see also Table 1). When chick r5/6 were orthotopically replaced with chick r5/6 of another embryo, chick *Hoxb1* expression showed no obvious changes although some embryos had a slightly enlarged r4 domain at the operated side (Fig. 5D). Finally, to exclude the possibility of an artefact involving attraction of chick neurones by any mouse hindbrain tissue, mouse r2/3 were grafted in place of chick r5/r6 (Fig. 5E) and chimaeric embryos were incubated up to HH stage 24. In all the cases examined (Table 1), no
ectopic chick Hoxb1 expression was detected in the mouse tissue (Fig. 5F). In most cases chick Hoxb1 domain was enlarged similarly to Fig. 5B,D.

Thus, the presence of an ectopic stream of chick motoneurone cells in mouse neural grafts is specifically due to the presence of mouse r5/6.

Change of FBM trajectory after transplanting mouse r5/6 into chick r5/6

To investigate the neuronal identity of the ectopic chick neurones in chimaeric embryos and to verify whether they originated from r4, the rhodamine-dextran tracer was applied to retrogradely label the facial nerve after nerve transection. In normal chick embryos, when the dye was applied close to the brain, both FBM neurones and VMN were labelled in r4 and r5, respectively (Fig. 6B; Simon and Lumsden, 1993). Selective application of dextran at the hyoid branch labelled FBM neurones in r4, whose cell bodies migrated away from the floor plate (Fig. 6C). A few cells migrated into r5 in a lateral position, consistent with previous studies (arrowhead in Fig. 6C; Jacob and Guthrie, 2000); however, no contingent of cells was detected close to the midline. After grafting mouse r5/6 in place of chick r5/6, chimaeric embryos were incubated up to HH stage 24 and dextran was subsequently applied to the hyoid branch of the operated and unoperated sides. In five out of eight chimaeric embryos (Table 1) a stream of retrogradely filled cell bodies, whose neuronal leading processes were oriented caudolaterally, could be identified in ventral r5/6 (see arrow in Fig. 6D). On the grafted side, the majority of non-migrating FBM neurones remained in ventral r4 next to the floor plate compared with the control side, where most of FBM neurones had initiated lateral migration (Fig. 6C and data not shown). Thus, when mouse r5/6 are juxtaposed to chick r4, a subpopulation of ventral r4 neurones, which can be specifically retrogradely labelled from the hyoid branch, follow a caudolateral pathway characteristic of mouse FBM neurones. Together

Fig. 5. Attraction of chick r4 ventral motoneurones is specific to mouse r5/6. Diagrams of r5/6 chick ablation (A), orthotopic r5/6 graft (C) and heterotopic mouse-chick graft (E). (B,D,F) Dorsal views of flat-mounted HH stage 24 hindbrains after chick Hoxb1 in situ hybridisation. (B) The most severe case in which very little regeneration of the surrounding tissue has occurred and chick Hoxb1 expression is dramatically enlarged. (D) No obvious changes in chick Hoxb1 expression resulted after cr5/6 orthotopic grafts. (F) By replacing cr5/6 with mr2/3, chimaeric embryos showed a slightly enlarged chick Hoxb1 domain, but no extension of ventral expression.

Fig. 6. Chick FBM neurones reproduce a caudal and lateral migratory pathway characteristic of mouse FBM cells in mouse-chick chimaeras. (A) Schematic of ventral aspect of facial nerve and their projections into the periphery (adapted from Jacob and Guthrie, 2000). The red and green lines indicate the branchiomotor (FBM) axonal projections towards the hyoid nerve and the visceromotor (VMN) axonal projections towards the palatine nerve, respectively. (B-D) Ventral views of flat-mounted HH stage 23-24 hindbrains after retrograde labelling of facial motoneurones. Labelling of FBM and VMN subpopulations after rhodamine-dextran fills of the facial nerve (B) and of the hyoid nerve (C). An arrowhead in C marks the presence of a few neurones in r5, as previously reported (Jacob and Guthrie, 2000). Labelling of FBM neurones after rhodamine-dextran fills of the hyoid nerve in a chimaeric embryo after chick r5/6 were replaced with mouse r5/6 (D). Only a small subpopulation of FBM neurones in r4 migrates laterally (arrowheads in D), whereas the majority of FBM neurones remain either close to the ventral midline or migrate caudally. The arrow in D shows how within the caudal migration cells are oriented caudolaterally. Ba1, first branchial arch; Ba2, second branchial arch; ct, chorda tympani; ep, r4 exit point; fp, floor plate; h, hyoid nerve; Mx, maxilla; P, palatine nerve.
with the molecular data described above, these results indicate that the ectopic cells are indeed chick FBM neurones that have been attracted by mouse r5/6.

Mouse r4 neurones do not migrate into a chick environment

The above results indicate that extrinsic cues present in the murine environment are involved in the choice of FBM neuronal migratory pathways. However, they cannot exclude the possibility that mouse FBM neurones are also intrinsically programmed to initiate caudal migration. Therefore, to assess whether mouse FBM neurones were able to migrate into a chick environment, chick r4 was replaced with mouse r4. In order to identify any migrating cells originating from the graft, chimaeric embryos were stained with anti-mouse Hoxb1 antibody, which does not crossreact in chick embryos (Bell et al., 1999), and labels r4 and migrating FBM neurones (Goddard et al., 1996). In a first series of transplants, E8.75 mouse r3/4 were grafted in place of chick r4 (Table 1; Fig. 7A), whereas in a second series of experiments only basal r4, in which facial motoneurones are born, was grafted in place of basal r4 of HH stage 10 chick hosts (Fig. 7C). As an internal control for mouse tissue, E8.75 mouse hindbrains were cultured in vitro as explant cultures for the same length of time as chimaeric embryos, i.e. 2-3 days post-surgery. While the mouse explants showed a normal caudal expression of Hoxb1 in ventral r5 (inset in Fig. 7D), no Hoxb1-positive cells caudal to the graft were observed in chimaeric embryos (Fig. 7B,D). Although only basal r4 was grafted in Fig. 7D, mouse Hoxb1-positive cells were able to mix and spread along the mediolateral axis of r4, indicating that there was free mixing between mouse and chick r4 cells, as previously suggested by a short-term aggregation assay (Fig. 2). These data indicate that mouse r4 neurones are unable to initiate caudal migration when juxtaposed to chick r5/6. Thus, the chick environment might be inhibitory to mouse r4 cells or the competence of mouse r4 neurones to migrate caudally is dependent on the environment.

DISCUSSION

In this study, the role of non cell-autonomous mechanisms in the regulation of the migratory pathway of facial branchiomotor neurones has been explored using a mouse-chick transplantation approach. By exchanging chick r5 and/or r6 with mouse r5 and/or r6, chick FBM neurones are able to re-route their leading processes towards the ectopic mouse tissue and follow a caudal migratory pathway typical of mouse FBM neurones. Moreover, the presence of ectopic mouse r4 precursors in a chick environment is not sufficient to induce caudal migration of mouse cells. Therefore, these data show for the first time that differences in the environment can account for changes in the migratory behaviour of the two species.

Intrinsic determination and extrinsic cues in the migration of FBM neurones

In mouse and chick r4, the first contingent to initiate migration is the vestibulo-acoustic (VA) efferent system, whose cell bodies move either ipsilaterally towards the r4 exit point or contralaterally across the floor plate (Fritzsch et al., 1993; Simon and Lumsden, 1993; Pata et al., 1999). Subsequently, FBM neurones follow a lateral pathway within r4 in chick, or a caudal and then lateral pathway within r6 in mouse.

As for other CNS systems, the particular combinations of transcription factors are crucial determinants of neuronal identity and specification in r4 (Pattyn et al., 2000). Hox genes are known to set the positional value of individual rhombomeres, and thereby control their identity and phenotypic specialisation (reviewed by Lumsden and Krumlauf, 1996). In the absence of Hoxb1 the differentiation of multiple neuronal subtypes in r4 is affected (Gaufo et al., 2000). Nevertheless, motoneurones do differentiate, express Isl1 and project their axons into the periphery, although they do not have a ‘facial’ identity and undergo a lateral migration within r4 (Studer et al., 1996). Moreover, ectopic expression of mouse Hoxb1 in chick r2 induces ectopic contralateral migration of VA neurones (Bell et al., 1999), and conversely absence of Hoxb1 in mouse abolishes migration of VA neurones (Studer et al., 1996), confirming that Hoxb1 is a determining factor in regulating r4 migration. However, little is known about how Hoxb1 controls neuronal migration. This might depend either on intrinsic properties of FBM cells or on extrinsic signals present in the adjacent environment, or on both. In the absence of Hoxb1, mouse FBM neurones might...
lack specific receptors, be unable to recognise particular cues in the environment and undergo a ‘default’ lateral path, in a similar way to other cranial nerves in the hindbrain. The present study, together with the conclusions of Garel et al. (Garel et al., 2000), support this hypothesis and show that the adjacent environment, e.g. r5 and r6, is instructive for the initiation and selection of their local migratory pathway, when FBM neurones are fully specified.

Why do mouse and chick FBM neurones behave so differently? One hypothesis is that the mouse environment has one or more attractive cues missing in chick; alternatively, the chick environment might be repulsive to murine FBM neurones. Data in this study show that the adjacent environment is instructive for initiating caudal migration and suggest that long-range cues originating from mouse r6 can attract chick Hoxb1-positive cells towards the mouse tissue (Fig. 4H). Therefore, the chick hindbrain might have lost, during evolution, the chemotraction originating from the caudal hindbrain, but have maintained expression of their receptor(s) on FBM neurones. Thus, mouse and chick FBM neurones could share the same combination of receptors; however, cues in the adjacent environment are of different nature in the two species.

Little is known about the distribution of guidance molecules and their receptors in the hindbrain. In zebrafish, cyclops mutants, which have a deletion of the floor plate at the ventral midline, show abnormal crossing of facial neurones, suggesting a role for floor plate-derived repulsive cues in the normal migration of these neurones (Chandrasekhar et al., 1997). Signals released by the floor plate, and in particular netrin 1, have also been characterised in the migration and projections of inferior olivary neurones (Bloch-Gallego et al., 1999). Facial motor axons in chick are repelled by netrin 1 and Sema3a expressed in the floor plate (Varela-Echavarria et al., 1997); however, it is not known whether the same guidance molecules involved in directing axons to their targets are also involved in instructing caudally migrating FBM neurones. It is plausible that facial motor axons and motoneurones respond to similar signals by expressing different receptors, as already suggested in the olfactory system (Wu et al., 1999).

Several studies have shown that chemotraction and promotion of growth are intimately linked in the guidance of growing neurones (Ebens et al., 1996; Bloch-Gallego et al., 1999; Caton et al., 2000). In this study, mouse r5/6 adjacent to chick r4 in mouse-chick chimeras induce an increase of chick Isl1 expression in ventral r4 and in the migrating population (Fig. 4C), which indicates an increased cell proliferation of r4 motoneurones. Thus, the mouse tissue might secrete specific factor(s) involved in both growth and migration of facial motoneurones. This study might therefore be a starting point for the identification of novel chemotactic cues involved in FBM caudal migration.

The role of r5 and r6 in initiating caudal migration of FBM neurones

Caudally migrating FBM neurones reach first r5 and then r6 where they initiate a lateral and radial migration. Based on a series of mouse mutants (kreisler, Krox20), it has been shown that in the absence of an r5 territory, FBM neurones migrate laterally and express a repertoire of markers characteristic of r6 (Manzanares et al., 1999; Garel et al., 2000). Although these data show that r6-specific cues are necessary in controlling the lateral migration of fully differentiated FBM neurones, they do not address the issue of initiating caudal migration. The present report demonstrates directly that both r5 and r6 can initiate caudal migration of FBM neurones (Fig. 4). In the presence of only mouse r5, chick ventral Hoxb1-positive cells migrate into r5 (Fig.4F), and by replacing chick r6 with mouse r6, a large stream of Hoxb1-positive cells migrate through the host r5 territory until they reach the rostral portion of mouse r6 (Fig. 4H). Thus, both r5 and r6 independently can attract and initiate FBM migration, which would explain why in the absence of r5, FBM neurones in kreisler and Krox20 mutants are still capable of exiting r4 and initiate neuronal migration. However, in most mouse-chick chimaeras the ectopic stream of r4 chick cells do not follow a solid paramedial course in r5 before turning laterally, as in the mouse embryo. Although these data are not sufficient to explain such a peculiar behaviour, different hypotheses can be postulated: (1) specific receptors expressed by chick FBM neurones can only respond to cues involved in lateral migration; (2) the mouse r5/6 graft included in the chick hindbrain is reduced to one rhombomere width at the stage when migration can occur, which could contract the caudal trajectory proportionally (see also Fig. 3D); and (3) the chick floor plate next to the mouse tissue could be involved in repulsing ectopic chick neurones. Further investigations are required to discriminate between these hypotheses.

In summary, the data obtained from mouse mutants and mouse-chick chimaeras demonstrate that to initiate caudal migration, FBM neurones need to be fully specified (i.e. express a receptor) and the environment need to be instructive (i.e. express the right ligand). If one of the two conditions is not fulfilled, such as in the case of Hoxb1 mutant mice where the neurones are incorrectly specified, or in the presence of an unsuitable environment, as in the normal chick embryo, then FBM neurones will not exit r4 and instead follow a lateral migratory pathway.

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