Somatic motoneurone specification in the hindbrain: the influence of somite-derived signals, retinoic acid and Hoxa3

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SUMMARY

We have investigated the mechanisms involved in generating hindbrain motoneurone subtypes, focusing on somatic motoneurones, which are confined to the caudal hindbrain within rhombomeres 5-8. Following heterotopic transplantation of rhombomeres along the rostrocaudal axis at various developmental stages, we have found that the capacity of rhombomeres to generate somatic motoneurones is labile at the neural plate stage but becomes fixed just after neural tube closure, at stage 10-11. Grafting of somites or retinoic acid-loaded beads beneath the rostral hindbrain induced the formation of somatic motoneurones in rhombomere 4 only, and Hox genes normally expressed more caudally (Hoxa3, Hoxd4) were induced in this region. Targeted overexpression of Hoxa3 in the rostral hindbrain led to the generation of ectopic somatic motoneurones in ventral rhombomeres 1-4, and was accompanied by the repression of the dorsoventral patterning gene Irx3. Taken together, these observations suggest that the somites, retinoic acid and Hox genes play a role in patterning somatic motoneurones in vivo.

Key words: Somatic motoneurones, Rostrocaudal axis, Hindbrain, Retinoic acid, Hox genes, Somites, Quail, Chick

INTRODUCTION

During the development of the vertebrate nervous system, the fate adopted by each progenitor cell depends on its position along the rostrocaudal and dorsoventral axes of the neural tube, and on extrinsic signals and intrinsic gene expression. Some of these specification steps have been elucidated for motoneurones, which develop in the ventral neural tube and comprise a number of sub-populations. The early differentiation of motoneurones is known to depend on sonic hedgehog (Shh)-mediated signals which are transmitted from the notochord and the floor plate ventrodorsally across the neuroepithelium (reviewed by Tanabe and Jessell, 1996). Shh-mediated signalling leads to the expression of a key set of transcription factors within distinct dorsoventral progenitor domains, each of which generates a distinct class of neurones, including motoneurones (Briscoe et al., 1999; Briscoe et al., 2000; Novitch et al., 2001; Jessell, 2000).

Motoneurones later diversify into subpopulations that form discontinuous columns, occupying distinct domains along the rostrocaudal axis. In the cranial region, motoneurones are subdivided into branchiomotor (BM), visceral motor (VM) and somatic motor (SM) neurone subtypes, depending on their columnar organisation and synaptic target. These subpopulations are thought to originate within particular dorsoventral progenitor domains. For example, BM/VM neurones are born in a domain immediately dorsal to the floor plate, which expresses Nkx2.2, whereas SM neurones originate in a domain dorsal to this, which is Nkx2.2-negative but which expresses Pax6 at low levels and Olig2 (Briscoe et al., 1999; Briscoe et al., 2000; Novitch et al., 2001; Jessell, 2000).

Patterning of motoneurones along the rostrocaudal axis may depend on signals from the paraxial mesoderm because, in the trunk region, heterotopic transplantation of the paraxial mesoderm can alter some aspects of motoneurone phenotype (Ensini et al., 1998; Matise and Lance-Jones, 1996). Retinoic acid (RA), fibroblast growth factors (FGFs) and the TGFβ family member GDF11 have also been implicated in conferring rostrocaudal identity on spinal motoneurones (Liu et al., 2001; Sockanathan and Jessell, 1998). The actions of these, and possibly other mesoderm-derived molecules, appear to repattern the rostrocaudal expression domains of Hox genes, which are expressed in discrete rostrocaudal domains of the neural tube (Ensini et al., 1998; Liu et al., 2001), conferring upon Hox genes a central role in the interpretation of extrinsic signals in motoneurone patterning.

In the hindbrain, motoneurone patterning is linked to segmentation into rhombomeres, each of which contains a characteristic set of motoneurone subtypes with distinct axon pathways. BM, VM and SM neurone subtypes are differentially distributed with respect to the rostrocaudal axis: rhombomeres 2-4 contain only BM neurones (Lumsden and Keynes, 1989), whereas rhombomeres 5-8 contain BM, VM and SM neurones in various combinations. Segmentation occurs between stage 9 and stage 12 in the chick embryo, but...
a coarse rostrocaudal pattern is established even earlier, at neural plate stages, providing a substrate upon which later dorsoventral patterning acts (Lumsden and Krumlauf, 1996; Simon et al., 1995). Rhombomeres express different combinations of Hox genes, which play roles in segmentation, rhombomere patterning and neuronal differentiation (Lumsden and Krumlauf, 1996). In particular, some Hox genes, including Hoxb1, are expressed at early neural plate stages and it is plausible that they might act to establish rhombomeres as territories committed to generate particular repertoires of motoneurones. Recently, Hoxb1 and Hoxa2 have been shown to play a direct role in the specification of facial and trigeminal motoneurones, respectively (Gavalas et al., 1997; Bell et al., 1999; Jungbluth et al., 1999; Studer et al., 1996).

As in the spinal cord, patterns of Hox gene expression are responsive to environmental signals, including those from the mesoderm. Transposition of rhombomeres from the pre-otic region to the post-otic region at stage 10-12 resulted in respecification of the Hox ‘code’ and neuronal organisation (Grapin-Botton et al., 1995; Itasaki et al., 1996), whereas transpositions of rhombomeres between axial levels within the pre-otic region resulted in the maintenance of rhombomere identity and Hox gene expression (Grapin-Botton et al., 1997; Grapin-Botton et al., 1995; Guthrie et al., 1992; Itasaki et al., 1996; Kuratani and Eichele, 1993). The discrepancy seems to depend on differences in the paraxial mesoderm, which is unsegmented in the pre-otic region (Hacker and Guthrie, 1998), but forms somites caudal to the otic vesicle. Somite grafts adjacent to the neural tube in the pre-otic region were capable of respecifying Hox gene expression and some aspects of neuronal phenotype, effects that could be mimicked by RA (Gavalas and Krumlauf, 2000; Gould et al., 1998; Grapin-Botton et al., 1997; Grapin-Botton et al., 1995; Guthrie et al., 1992; Itasaki et al., 1996; Kuratani and Eichele, 1993). RA is known to be produced by the somites, making it a likely candidate for a caudalising signal in vivo (Maden et al., 1998; Swindell et al., 1999). An in vivo role of RA has been demonstrated in a series of studies on avian and mouse embryos, in which depletion or elimination of RA signalling leads to a failure in the development of rhombomeres 4-7 (Dupé and Lumsden, 2001; Gale et al., 1999; Maden, 1996; Niederreither et al., 2000).

However, in these studies, only selected aspects of rhombomere identity were examined and these did not include cranial motoneurone identities or axon trajectories. No systematic study has been carried out to elucidate the precise relationship between environmental signals, patterns of Hox gene expression and motoneurone specification. It is not clear at which timepoint rhombomeres become committed to generating particular motoneurone subtypes, nor is the source and nature of the inductive signals that control these processes known. In the present work we focus on these issues, and investigate in particular the mechanisms that govern somatic motoneurone generation in the hindbrain and confine it to caudal rhombomeres (5-8). We have performed transplantation experiments in early chick embryos, which indicate that the capacity of a rhombomere to generate SM neurones is labile at the neural plate stage but becomes fixed at stage 10-11, around the time of neural tube closure. Somites grafted rostrally were able to induce ectopic Hox gene expression (including that of Hoxa3) and SM neurones, in particular rhombomeres, in a restricted time period. RA-loaded beads grafted rostrally mimicked this effect, inducing SM neurones in the same region and within the same time window. We also tested a possible involvement of Hoxa3 in defining the territory that generates SM neurones, and found that ectopic expression of Hoxa3 in the rostral hindbrain induced SM neurones in rostral rhombomeres.

### MATERIALS AND METHODS

#### Transplantation experiments, somite and RA bead grafting

Stage 9-12 embryos were used in stage-matched quail-to-chick or bead grafting experiments. For all microsurgery, host eggs were windowed and embryos made visible by sub-blastodermal injection of India ink (1:20 dilution in Howard’s Ringer Solution). Surviving embryos were harvested at embryonic day 6 [E6: HH stage 26-29 (Hamburger and Hamilton, 1951)], when heads were removed and fixed in 3.5% paraformaldehyde (PFA) for 2 hours before in situ hybridisation or immunohistochemistry. Operations were performed as previously described (Guthrie et al., 1992). Somites for grafting were dissected from quail donors and grouped in a caudal population [caudal to and including somite 5 (s5)] and a rostral population (s1 to s3 only). For RA bead grafts, AG 1-X2 resin (Bio-Rad, 143-1255) was washed in phosphate buffered saline (PBS), incubated in the dark at room temperature for 1 hour in PBS containing all-trans-RA (Sigma, R2625; 1×10⁻⁴ M or 5×10⁻⁵ M) and finally washed again in PBS. Individual somites or beads were then grafted into isochronic chick hosts at various levels within the cranial paraxial mesoderm and beneath the neuroepithelium. Beads were washed in PBS prior to grafting.

#### Rhombomere fate mapping

Because rhombomere boundaries are not complete until stage 12 (Vaage, 1969), the territories corresponding with presumptive rhombomeres were mapped in both chick and quail embryos at stage 9. Small spots of Dil (Molecular Probes, Oregon, USA) were injected, using a micrometer graticule, at defined positions within the neural tube, relative to a caudal limit at the first somite and the rostral end of the neural tube. Embryos were incubated for 24 hours prior to analysis. Rhombomere 1 (r1) was mapped at 900 μm from the rostral end of the neural tube, and thereafter each rhombomere was approximately 100 μm long, with r7 located at level of the first somite. These measurements were used to ensure accurate dissection of rhombomeres for transplantation.

#### In ovo electroporation of chick embryos

In the electroporation experiments, we used full-length cDNA for tauGFP (green fluorescent protein), mouse Hoxa3 and human HOXB3 (gifts from Dr Andrea Brand, Dr Michael Hofmann and Dr Guy Sauvageau, respectively) under the control of a chicken β-actin promoter (pCA/link kindly provided by Dr Jon Gilthorpe). The tauGFP plasmid was co-electroporated with the mouse Hoxa3 or human HOXB3 plasmid to allow in vivo fluorescent screening of embryos. We found that a 1:3 ratio of tauGFP/Hoxa3 or HOXB3 gave the best co-localization of GFP and Hox gene expression. Plasmids were used at a combined final concentration of 2 μg/μl, whereas in control experiments the tauGFP plasmid alone was used at a final concentration of 0.5 μg/μl. Electroporation was performed as previously described (Itasaki et al., 1999). Briefly, eggs were windowed and embryos (stage 8-17) made visible. The vitelline membrane was opened and DNA solution was injected either into the neural tube (stage 9-17) or on top of the neural plate (stage 8). Two silver electrodes were then placed dorsal and ventral to the embryo at the rostrocaudal level desired, so that electroporation (10V, 5 pulses of 50 milliseconds) allowed the entry of DNA into the basal plate of the neural tube where motoneurone progenitors are located. Unilateral entry of DNA was obtained in most cases. Eggs were then sealed and
incubated for 48-96 hours before embryos were removed for fixation (at E4-E6).

In situ hybridisation

Whole-mount in situ hybridisation was performed essentially as published (Henrique et al., 1995), using Islet2 (Tsuchida et al., 1994), Hb9 (Tanabe et al., 1998), Hoxa3 (Saldivar et al., 1996), Hoxb3 (Rex and Scotting, 1994) and Hoxd4 (Grapin-Botton et al., 1995) chick-specific probes. A mouse Hoxa3 probe (full-length cDNA; GenBank Accession Number Y11717) was used to detect the expression of electroporated Hoxa3. Briefly, stage 28-30 embryos were fixed, dehydrated in methanol and stored at −20°C for a maximum of 2 weeks. Prior to pre-hybridisation and hybridisation at 70°C, embryos were treated with 10 μg/ml proteinase K for 20 minutes at room temperature. Digoxigenin (DIG)- or Fluorescein (Fluo)-labelled antisense RNA probes were synthesised according to the manufacturer’s instructions (Roche Molecular Biochemicals), and hybridised at 1 μg/ml. For double in situ hybridisation, probes were added simultaneously to the embryos, whereas antibody incubations (anti-DIG-AP and anti-Fluo-AP antibodies) and developing reactions were carried out sequentially. The NBT/BCIP reaction was always performed first, followed by a series of washes and a 30 minute incubation at 70°C to destroy residual alkaline phosphatase activity. Incubation with the anti-Fluo-AP antibody and Fast Red development followed. All reagents used for in situ procedures were from Roche Molecular Biochemicals, except for Fast Red (Sigma).

Immunostaining on whole mounts in rhombomere and somite grafting experiments

In grafting experiments, the correct graft position and integration was ensured by immunostaining with a monoclonal antibody to quail cells (QCPN). Following the in situ procedure, embryos were post-fixed, washed extensively in PBS containing 1% Triton X-100 (TX100), and left in blocking solution (PBS/10% heat-inactivated sheep serum (HSS)/1% TX100) for a minimum of 2 days prior to incubation with the primary antibody. After 2-3 days, embryos were washed extensively in PBS/10% HSS/1% TX100, incubated for further 2-3 days in secondary antibody, then finally washed overnight, dissected and mounted in DABCO/glycerol.

Immunohistochemistry on sections

Cryostat sections (10 μm) were immunostained by overnight incubation with primary antibodies in PBS/1% HSS/0.1% TX100. After several washes in PBS/0.02% TX100, sections were incubated for 2-3 hours at room temperature with fluorescent-conjugated secondary antibodies (FITC-, Cy3-, Alexa Fluor 568- or Cy5-labelled goat anti-mouse; goat anti-guinea pig or goat anti-rabbit antibodies), washed briefly and mounted in DABCO/glycerol prior to analysis. Vibratome sections (80 μm) were incubated with primary antibodies for 2 days in PBS/10% HSS/1% TX100. Sections were then washed extensively in PBS/1% TX100 and incubated overnight with secondary antibodies. After final washes, sections were mounted in DABCO/glycerol.

Primary antibodies used were polyclonal anti-neurofilament heavy chain (AB1991, Chemicon International), monoclonal anti-quail axons or quail cells (QN or QCNP, respectively; Developmental Studies Hybridoma Bank), monoclonals 4D5 (anti-Islet1/2), 4H9 (anti-Islet2), and polyclonals anti-Chx10 and anti-Lim3 (all kind gifts of Dr T. Jessell). We also used monoclonals anti-Nkx2.2, 81C10 (anti-Mnr2/Hb9) and anti-Pax6, and polyclonals anti-Irx3 and anti-Olig2 (also kind gifts of Dr T. Jessell). Finally, in misexpression experiments, a rabbit anti-GFP antibody (A-6455, Molecular Probes) was used to identify electroporated regions. All fluorescently-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, except for Alexa Fluor 568, which was from Molecular Probes.

RESULTS

Motoneurone organisation and gene expression patterns in the chick hindbrain

Cranial motor nuclei are located at distinct positions along the rostrocaudal axis of the hindbrain, and may contain one or more of BM, VM and SM neuronal subtypes (Fig. 1A). Whereas BM and VM axons exit the neuroepithelium via large, single exit points, SM axons exit ventrally in small groups. Rhombomeres 2-4 (r2-4) contain only BM neurones and a small population of vestibulo-acoustic neurones (Lumsden and Keynes, 1989; Simon and Lumsden, 1993), whereas r5-8 contains BM, VM and SM neurones in various combinations. The trigeminal (V; r2 and r3) and cranial accessory (XI; r7 and r8) nuclei contain only BM neurones, whereas the facial (VII; r4 and r5), glossopharyngeal (IX; r6 and r7) and vagus (X; r7 and r8) nuclei contain BM and VM neurones. SM neurones are found in the oculomotor (III; midbrain), and trochlear (IV; r1), abducens (VI; r5 and 6) and hypoglossal (XII; r7 and 8) nuclei (Fig. 1A).

Whereas all cranial motoneurones express the Lim homeobox gene Islet1, SM neurones co-express Islet2 and/or Lim3 genes (Fig. 1A,B) (Varela-Echavarriá et al., 1996). Lim3 is expressed in the dividing progenitors of all SM neurones, but its expression is maintained only in subsets of post-mitotic hypoglossal and abducens SM neurones (Sharma et al., 1998; Varela-Echavarriá et al., 1996). Hypoglossal neurones express Islet1, Islet2 and Lim3, and at E6 a substantial subpopulation co-expresses Islet2 and Lim3. Neurones of the main abducens nucleus in r5 and r6 express Islet1 and Islet2, whereas those of the small accessory abducens nucleus in r5 migrate laterally, switch off Islet2, and thereafter express Islet1 and Lim3 (Varela-Echavarriá et al., 1996) (data not shown). For this reason, abducens and hypoglossal neurones can be distinguished based on the co-expression of Islet2/Lim3 by the latter, but not the former, population. The homeobox-containing gene Hb9 is expressed by post-mitotic SM neurones of both the abducens and hypoglossal nuclei (Fig. 1A,C) (Pfaff et al., 1996; Tanabe et al., 1998). The two other groups of SM neurones (oculomotor nucleus in the midbrain and trochlear nucleus in rostral r1) do not express Hb9 (Fig. 1C). In the chick, Hb9 and Islet2 are first expressed in hindbrain SM neurones at stages 16 and 22, respectively, whereas Lim3 expression is initiated in post-mitotic neurones at around stage 25 (Varela-Echavarriá et al., 1996). Thus, none of the markers are expressed at the time of grafting (stage 9-12), allowing us to test the commitment of rhombomeres to express these genes. Both in chick and mouse embryos, Hox3 paralogues have been reported to be expressed from the r4/5 boundary caudally (Grapin-Botton et al., 1995; Lumsden and Krumlauf, 1996; Rex and Scotting, 1994). However, although this was the expression pattern of Hoxa3 in the chick (Fig. 1D), we found that, at E6, Hoxb3 was also expressed in r4, with a rostral cutoff at the r3/4 boundary (Fig. 1A,E). Double in situ hybridisation for Hoxb3 and Islet2 confirmed this localisation, because the abducens neurones of r5 and r6 were completely contained within the Hoxb3-expressing territory (Fig. 1E). Hoxd4 was expressed from the r6/7 boundary caudally, in accordance with previous reports (Grapin-Botton et al., 1995), and double in situ hybridisation with Hoxd4 and Islet2 showed that hypoglossal neurones lay within the Hoxd4-expressing
domain, whereas abducens neurones lay rostral to this region (Fig. 1F). Thus Hoxa3, Hoxb3 and Hoxd4 were used as markers of more caudal rhombomere identity.

**In pre-otic rhombomere transplants, Lim gene expression and motoneurone identity is fixed from stage 10 onward**

To ascertain the timing of rhombomere commitment to generate SM neurones, r5 to r3 (r5/r3) quail to chick rhombomere transplants were performed at stage 10-12 (Fig. 2A), and operated hindbrains were assessed at stage 26-29 (Table 1A). Whole-mount in situ hybridisation on operated embryos showed that the transposed r5 maintained Islet2 expression in the ectopic r3 position (Fig. 2B), which suggests that, from stage 10 onwards, motoneurone progenitors within r5 are committed to express SM markers. Conversely, when r3 was transposed to the r5 position (r3/r5) at stage 10-12, the transposed r3 failed to express Islet2 (Fig. 2L, Table 1A), implying that r3 has maintained its rostrocaudal identity and failed to generate SM neurones. Thus, both r5/r3 and r3/r5 grafts after stage 10 showed rhombomere autonomy in gene expression patterns and SM neurone production.

To investigate the axon trajectory of the ectopic r5/r3 SM neurones, transverse sections of quail-chick chimeras were double-immunostained with various combinations of antibodies against neurofilament heavy chain (NF), Islet2 protein (Islet2), a quail-specific antigen (QCPN) or a quail axon-specific antigen (QN) (n=6; Fig. 2O-Q). In separate experiments, an antibody against Lim3 protein was used in combination with QCPN and QN antibodies (n=3; data not shown). In both sets of experiments, the ectopic r5 contained Islet2-positive and Lim3-positive quail neurones, which were closely associated with quail axons exiting the neuroepithelium ventrally (Fig. 2P,Q; data not shown). These axons formed an ectopic ventral exit point, which was absent on the unoperated side of the embryo because BM neurones in r3 form dorsal exit points only. The likely identities of these ventrally-projecting neurones are therefore SM abducens and accessory abducens neurones, which express Islet2 and Lim3, respectively. Axons from resident trigeminal motoneurones within r3 normally project dorsorostrally to exit the neuroepithelium in r2 (Fig. 1A). On the operated side at r2 level, quail axons extended via the trigeminal nerve dorsal exit point (data not shown), probably reflecting the pathways of facial motor axons from the transplanted r5, as has been shown previously in r5/3 grafts (Guthrie and Lumsden, 1991).

Do SM neurones within the r5 transplanted to r3 join the pathway of the endogenous abducens nerve? During normal development, abducens axons emerge from r5 and r6 as multiple rootlets, which fasciculate ventral to the brain, and extend rostrally to innervate the lateral rectus eye muscle, and the small pyramidalis and quadratus nictitans (P/Q) muscles (Wahl et al., 1994). In immunostained transverse sections of r5/r3 embryos, quail-derived axons exiting ventrally joined the host abducens nerve as it extended beneath the brain (Fig. 2Q),

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**Fig. 1.** Patterns of gene expression in rhombomeres and cranial motoneurones. (A) Diagram of a flat-mounted stage 28 chick embryo hindbrain and caudal half of the midbrain showing the different combinations of Lim homeobox genes expressed by each motor nucleus and the expression pattern of three Hox genes, Hoxa3, Hoxb3 and Hoxd4. Rhombomere boundaries are no longer visible at this stage but are shown for clarity. Motor nuclei: III, oculomotor; IV, trochlear; V, trigeminal; VI, abducens; VII, facial; IX, glossopharyngeal; X, vagus; XI, accessory; XII, hypoglossal [adapted, with permission, from Varela-Echavarria et al. (Varela-Echavarria et al., 1996)]. (B-F) Flat mounts of E6 chick midbrain and hindbrain preparations. Red lines indicate the midbrain-hindbrain boundary. Whole-mount in situ hybridisation for Islet2 (B), Hb9 (C), Hoxa3 (D), Hoxb3/Islet2 (E) and Hoxd4/Islet2 (F). mb, midbrain; hb, hindbrain; r, rhombomere; fp, floor plate; a, accessory; cva, contralateral vestibulocoustic. Scale bar: 500 μm.
Fig. 2. Rhombomere grafts in the pre-otic region. (A) Transplant of r3 at level of r5 (r3/r5) and of r5 at level of r3 (r5/r3). (B-N) Flat mount of E6 chick/quail chimera hindbrains in situ hybridised with probes for Islet2, Hb9, Hoxa3 or Hoxb3 as indicated. Labels in white bars above each panel indicate the type of graft and embryonic stage at which the graft was undertaken. Asterisk indicates grafted rhombomere.

(G,N) Additional immunostaining of the embryos in F and M, respectively, with anti-quail cell antibodies (QCPN) to show position of the graft. (J,K) Higher magnification of H and I, respectively.

(O-S) Double-immunostaining (except S; single immunostaining) on sections of an E6 embryo that had received an r5 in r3 graft at stage 10; transverse cryostat sections through r3 (O-Q) or parasagittal vibratome sections (R,S). (S) Higher magnification of R. Antibodies as indicated: QCPN, anti-quail cells; NF, anti-neurofilament; QN, anti-quail axons; Islet2, anti-Islet2.

r, rhombomere; HH indicates Hamburger/Hamilton staging of embryo. White arrows indicate: (O-Q,S) axonal bundles from the graft joining the host abducens (Abd); (P) r5 Islet2-positive motoneuromes. Scale bar: 580 μm for B,E,F,I,L-N; 400 μm for C,D,J,K; 240 μm for O,Q; 120 μm for P; 500 μm for R; 350 μm for S.
whereas in parasagittal sections quail-derived axons fasciculated with the endogenous abducens and accessory abducens axons to extend towards the LR and P/Q muscles close to the orbit (Fig. 2R,S; n=4). It appears therefore that as early as stage 10, motoneurone progenitors in r5 are committed to express SM markers and to pursue an abducens pathway.

### In pre-otic transplantations, rhombomeres show plasticity at stage 9

To pinpoint the stage at which rhombomeres become committed to generate SM neurones, transplantation experiments were performed at stage 9 (Table 1A). Following r5/r3 transplants at this stage, the transposed rhombomere failed to express Islet2, even though QCPN immunostaining showed that the graft was correctly located and integrated (Fig. 2F,G). Thus, SM neurones were not generated in the transposed r5 at stage 9 and at stage 10, the transposed r5 maintained expression of Hoxa3 and Hoxb3 (Fig. 2D,E,H,K). Because in grafts at stage 9, the transplanted r5 did not express Islet2 (Fig. 2F,G), there is a discrepancy between Hox gene expression and cranial motoneuron identity at this stage. However, Hoxa3 and Hoxb3 expression in the transposed r5 was lower than in the host r5, at both stage 9 and stage 10.

### In pre-otic to post-otic rhombomere transplants, SM neurone production can be induced

Previous studies have shown that rostral rhombomeres transposed caudally (to post-otic levels) show caudalised Hox gene expression profiles due to mesoderm-derived signals (Grapin-Botton et al., 1997; Grapin-Botton et al., 1995). To further investigate SM neurone production in relation to other aspects of rhombomere phenotype, we grafted r3 or r4 into the post-otic region, cranial motoneuron identity at this stage. However, Hoxa3 and Hoxb3 expression in the transposed r5 was lower than in the host r5, at both stage 9 and stage 10.

### Table 1. Summary of neural tube grafting studies

#### A Pre-otic

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<td>4</td>
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</tbody>
</table>

n, number; T, total number; cs, control side; os, operated side; nd, not done.
Somatic motoneurone specification

Stage 11 compared with stage 9 (Table 1B). These data highlight a contrast in the behaviour of r3 and r4, as in transplants at stage 10 into r8p, only r4, and not r3, was able to produce SM neurones. In addition, in such r3 grafts there was a lack of correspondence between Hox gene expression and SM neurone production, as caudal Hox genes were induced in the absence of a concomitant repatterning of SM neurones appropriate for that Hox ‘code’.

In order to characterise further the putative SM neurones in r4 transplanted at stage 10, r4/r8p chimaeras were double-immuno-stained with various combinations of antibodies (n=7; Fig. 3K-O). Quail cells in the ectopic r4 expressed Islet2 protein, and quail axons exited ventrally from the neuroepithelium to fasciculate with chick hypoglossal axons (Fig. 3K,N,O). Some quail axons also exited the neuroepithelium dorsally, which suggests that some r4 motoneurones projected along vagus and cranial accessory axon pathways (data not shown). To confirm the hypoglossal identity of the newly induced SM neurones, the Lim homeobox ‘code’ of the transposed r4 was also investigated. Previous studies showed that r8 contains two distinct populations of motoneurones: vagus and cranial accessory neuronal somata are located laterally and express Islet1, whereas hypoglossal neurones lie more medially and express Islet1, Islet2 and Lim3 (Varela-Echavarría et al., 1996). We examined the Lim homeoprotein expression profiles of hypoglossal neurones in more detail at E6 in caudal r8, using antibodies against Islet1/2, Islet2 and Lim3 proteins. Because all motoneurones still express Islet1 at this developmental stage (Varela-Echavarría et al., 1996), we can conclude that hypoglossal neurones
express Islet1 and Islet2 or Lim3, with a subset co-expressing Islet2 and Lim3 (Fig. 3L-P). Each of these neuronal subpopulations occupied specific locations, with respect to the dorsoventral and mediolateral axes, that were distinct from those of abducens neurones at r5/6 level. For example, Islet2/Lim3-positive neurones were located medially and close to the ventral (pial) side of the neuroepithelium (Fig. 3O). Double-immunostaining on adjacent sections showed that the Lim homeobox code of the grafted r4/r8p mirrored perfectly that of the control r8 side (Fig. 3L-P), which shows that signals at r8p level could repattern r4 so that at least a proportion of motoneurones adopted a hypoglossal identity.

**Fig. 4.** Somite or RA-loaded bead grafts. (A) Somite and RA bead grafts at the level of r2/r3, r3/r4 and r6/r7. (B-J) Flat mounts of E6 chick hindbrain preparations after somite or RA bead grafts, in situ hybridised with probes for Islet2, Hb9, Hoxa3 or Hoxd4 as indicated. Labels in white bar above each panel indicate the type of graft and embryonic stage at which the graft was undertaken. (D) Double in situ hybridisation with probes for Islet2 and Hoxd4. Asterisks indicate the grafted side and double-headed arrows indicate ectopic gene expression. (K-M,O-T) Double-immunostaining on a sagittal vibratome section (M) or transverse cryosections (K,L,O-T) of E6 chick embryos that have received a somite (M,O-T) or an RA bead graft (K,L) adjacent to the r3/r4 (O-S) or r6/r7 (T) level at stage 10. Antibodies are as indicated, abbreviations as in Figs 1-3. (M) Rhombomere boundaries are shown by a white line whereas the white asterisk highlights r4 ectopic SM axons. (O) QCPN stains the mesoderm, identifying the grafted somite (as in M). P and S are higher magnifications of O and R, respectively, showing the grafted side only. (N) Diagram of a transverse section of a chick embryo at the level of r4 summarising the normal Lim code on the control side (right) in comparison with the one on the somite/RA bead-grafted side (left). Although Islet1-positive motoneurones are present on either side, only the grafted side contains Islet2-positive/Lim3-negative motoneurones, consistent with an abducens identity for the r4 ectopic SM neurones. Note that when the somite was grafted at the level of r6/r7 the grafted side contains Islet2/Lim3-positive motoneurones consistent with a hypoglossal identity for the r7 ectopic SM neurones (see also Fig. 3P). Scale bar: 720 μm for B-D; 480 μm for E-H; 190 μm for I,J; 60 μm for L,P,S; 100 μm for M; 120 μm for K,Q,R,T.
SM neurones can be induced by signals from the paraxial mesoderm

Because somites transplanted into the pre-otic region could repattern Hox genes and neuronal differentiation (Grapin-Botton et al., 1997; Itasaki et al., 1996), we tested a possible influence of the caudal paraxial mesoderm on somatic motoneurones. Quail somites were grafted into isochronic chick hosts (stage 9-11) at various levels within the cranial paraxial mesoderm and beneath the neuroepithelium (Fig. 4A; Table 2A), and induction of Islet2, Hb9, Hoxa3 and Hoxd4 was analysed at E6. For grafts of rostral somites (s1 to s3) beneath the rostral hindbrain (r2-4) none of the markers assayed were induced, which indicates that these somites are devoid of inductive capability at the stages tested (9-12), which is consistent with previous data (Itasaki et al., 1996). When caudal somites (s5 or more caudal) were grafted underlying r2 and r3 at stage 9-11, no Islet2 induction was observed, whereas Hoxd4 induction was observed in the alar plate of r2 and r3 (Table 2A; data not shown). Grafting of caudal somites underlying r3 and r4 at stage 11 led to a similar failure of SM neurone differentiation, and induction of Hoxd4 in the r3 and r4 alar plate only (Table 2A; data not shown). However, for transplants at stage 9-10, both Islet2 and Hb9 were induced in r4, although in some cases this was limited to the caudal region (Fig. 4B; data not shown). In these grafts, Hoxa3 and Hoxd4 expression was, in some cases, induced in the alar plate of r3 (data not shown) but was induced in r4 extending ventrally as far as the floor plate, corresponding with the region of SM neurone induction (Fig. 4C,D).

The phenotypes of the ectopic r4 SM neurones in somite-grafted embryos were analysed as described previously. In all cases (n=4), ectopic axons at r4 level exited the neuroepithelium ventrally as small rootlets and fasciculated with the endogenous abducens axons to extend towards the eye (Fig. 4M). To confirm an abducens phenotype of these neurones, transverse sections were double-immunostained to ascertain the Lim homeoprotein profile (Fig. 4O-S). As summarised diagrammatically (Fig. 4N), both the control and the somite-grafted side contained a lateral Islet1-positive motoneurone population belonging to the facial nucleus (blue), which was immunostained with pan-Islet1/2 antibodies but not anti Islet2 antibodies (Fig. 4N,O,R; data not shown). Only the somite-grafted side contained a medial Islet1/Islet2 positive, Lim3 negative motoneurone population (red), and a smaller Islet1/Lim3 positive population (yellow; Fig. 4N,P-S). On the basis of their Lim code, these two populations are likely to represent the abducens and accessory abducens neurones, respectively, with the abducens neurones positioned close to the ventricular side of the neuroepithelium and the accessory abducens neurones (Fig. 4O,P) positioned more laterally (Varela-Echavarria et al., 1996). No Islet1/Lim3-positive neurones were detected, which would have represented a hypoglossal phenotype (Fig. 4N,Q). Hence, at stage 9-10, somite-derived caudalising signals acting at the level of r4 repatterned r4 to produce SM neurones with abducens identity.

The apparent abducens identity of ectopic SM neurones following somite transplants presents a paradox, because the expression of Hoxa3 and Hoxd4 together (in embryos analysed 76 hours post-grafting; Table 2A) is consistent with a hypoglossal rather than an abducens phenotype. A possible reconciliation of these data lies in previous studies showing that following rostral somite transplants, caudal Hox genes take longer to be induced in rostral rhombomeres than more rostral Hox genes (Grapin-Botton et al., 1997; Itasaki et al., 1996). We therefore analysed Hox gene expression 24, 36 and 48 hours after somite grafting. After 24 hours, neither Hoxa3 nor Hoxd4 were expressed, whereas after 36 hours Hoxa3 but not Hoxd4 was expressed, and after 48 hours both Hoxa3 and Hoxd4 were expressed (Table 2B). These data demonstrate that there is indeed a timelag in Hoxd4 induction in response to somite-derived signals, so that although Hoxa3 is switched on at the equivalent of stage 15/16, Hoxd4 expression is not initiated until approximately stage 20. This may be consistent with the acquisition of an abducens phenotype by ectopic SM neurones in r4.

In order to further test the effects of somite grafts, we took

**Table 2. Summary of somite grafting studies**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Graft</th>
<th>Islet2 Induction</th>
<th>Hb9 Induction</th>
<th>Hoxa3 Induction</th>
<th>Hoxd4 Induction</th>
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<tbody>
<tr>
<td>9/10</td>
<td>s5 in r2r3</td>
<td>none</td>
<td>11</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>11</td>
<td>s5 in r3r4</td>
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<td>7</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
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<td>s5 in r3r4</td>
<td>r4</td>
<td>13</td>
<td>r4</td>
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<tr>
<td>10</td>
<td>s5 in r6r7</td>
<td>r7</td>
<td>4</td>
<td>r7</td>
<td>3</td>
</tr>
<tr>
<td>9/10</td>
<td>s1 in r3r4</td>
<td>none</td>
<td>5</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

s5, somite 5 caudally; s1, somite 1 to 3 only; n, number; T, total number; nd, not done; ap, alar plate.

NB in this table only gene expression in the grafted side has been presented.

**B Time course**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Graft</th>
<th>Hoxa3 24 hours</th>
<th>36 hours</th>
<th>48 hours</th>
<th>Hoxd4 24 hours</th>
<th>36 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
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<td>4/4*</td>
<td>3/3*</td>
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<td>0/5*</td>
<td>3/3*</td>
</tr>
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*Number of embryos ectopically expressing the marker/number of embryos analysed.
advantage of the fact that r7 does not express SM neurone markers (Fig. 1B,C). When caudal somites were grafted beneath r6/7 at stage 10-11 (Table 2A), Islet2 and Hb9 were induced in r7 at levels comparable to the rostral r8 expression, even at stage 11 (Fig. 4E,F). Thus, r7 was capable of responding to somite signals by generating SM neurones up until later stages of development than more rostral rhombomeres. We also tested the effects of somite transplants at r6/r7 level on Hoxa3 and Hoxd4 expression. When embryos incubated until E6 were subjected to in situ hybridisation for these genes but using shorter development times than usual, gradations in Hox gene expression between axial levels was apparent. For example, on the control sides of embryos (left hand side; Fig. 4G,H) Hoxa3 was expressed at a higher level in r6 than it was caudally, whereas for Hoxd4 the converse was true (see also Fig. 1D,F). However, for the operated, somite-grafted side, Hoxa3 and Hoxd4 expression were down- and upregulated, respectively, within the r6/7 region, reflecting a caudalisation of these rhombomeres (right hand side; Fig. 4G,H). Such a caudalisation was accompanied by the generation of Lim3/Islet1/2 neurones at medial positions, consistent with a hypoglossal identity adjacent to the transplanted somite (Fig. 4T; compare with Fig. 3M,P). In order to further confirm this hypoglossal identity, we sought to follow the axon pathway of induced SM neurones in r6/7, but were unable to trace nerve rootlets further than a short distance from the hindbrain (data not shown). However, the balance of evidence favours the idea that hypoglossal SM neurones were generated in r6/7 in response to somite grafts.

Taken together, these somite-grafting experiments indicate that there are rostrocaudal differences in the time-window during which cranial motoneurone progenitors are capable of responding to patterning signals from the somites. Rhombomere 7 is still capable of responding to these signals as late as stage 11, whereas r4 is refractory to these signals by stage 11, and r2 and r3 are not sensitive to the signal even as early as stage 9. These observations could imply either a complete inability of the rostral hindbrain to respond to somitic signals, or an earlier fate commission of rostral rhombomeres relative to more caudal levels. Finally, it is also possible that r2 and r3 require a higher level of inductive signal than a single grafted somite can supply.

### Retinoic acid beads mimic the caudalising action of the somites

It has been proposed that retinoic acid (RA) mediates a part of the ability of the paraxial mesoderm to caudalise the rostrocaudal axis (Gavalas and Krumlauf, 2000; Gould et al., 1998). In the early chick embryo, the somitic mesoderm generates RA, with younger, more caudal somites generating higher levels of RA than older ones (Berggren et al., 1999; Maden et al., 1998; Swindell et al., 1999). As our somite grafting experiments showed that only caudal somites could induce SM neurones, this implies that RA is a candidate in SM neurone induction.

When beads treated with RA [10^{-4} M] were grafted in chick hosts at various axial levels within the cranial paraxial mesoderm and beneath the neuroepithelium (Fig. 4A; Table 3), Islet2 and Hb9 were induced exclusively in r4, and only in grafts at stage 9-10 at r3/4 level (Fig. 4I; Table 3). No induction was observed in grafts done at stage 11 or when beads were implanted at more rostral positions (data not shown; Table 3). When a higher concentration of RA was used (5×10^{-4} M), SM neurone induction was still exclusive to r4, although some Hb9 induction was observed in the r4 contralateral to the bead implantation (Fig. 4J). We also analysed Hox gene expression following RA bead implantation. No induction of Hoxa3 or Hoxd4 was observed in transplants performed at stage 11. However, for transplants of beads treated with RA [10^{-4} M] at stage 9/10, five out of five cases showed Hoxa3 induction, whereas three out of seven cases showed Hoxd4 expression (Table 3). This lower frequency of Hoxd4 expression was observed even when 5×10^{-4} M RA was used to soak the beads, in which case one out of three embryos showed induction (Table 3). The Lim homeobox gene expression of the ectopic r4 SM neurones was assessed in E6 embryos grafted at stage 10 (RA 10^{-4} M, n=3; RA 5×10^{-4} M, n=2), and Islet2 positive/Lim3 negative motoneurones were found on the grafted side (Fig. 4K,L). Hence, ectopic RA-induced SM neurones have an abducens phenotype, as in somite grafting experiments. The invariant induction of Hoxa3 and lower frequency of induction of Hoxd4 are broadly consistent with the abducens phenotype of the induced neurones.

The parallels between the results from somite grafting and RA bead grafting studies (compare Tables 2 and 3) support the idea that retinoic acid mediates a part of the caudalising ability of the paraxial mesoderm. Rhombomeres rostral to r4 did not respond to high concentrations of RA, even as early as stage 9, suggesting that the reason for the insensitivity of these rhombomeres to somite grafts in unlikely to be caused by an insufficiency of signal. Instead, such insensitivity might be explained by a suppressive effect of adjacent tissues, consistent

<table>
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<tr>
<th>Stage</th>
<th>Gift</th>
<th>Induction</th>
<th></th>
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</tr>
<tr>
<td></td>
<td></td>
<td>Islet2</td>
<td>Hb9</td>
<td>Hoxa3</td>
<td>Hoxd4</td>
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</tr>
<tr>
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<td>RA1  in r2/3</td>
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<td>5</td>
<td>none</td>
<td>2</td>
<td>nd</td>
</tr>
<tr>
<td>11</td>
<td>RA1  in r3/4</td>
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<td>4</td>
<td>none</td>
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</tr>
<tr>
<td>9/10</td>
<td>RA1  in r3/4</td>
<td>r4</td>
<td>2</td>
<td>r4</td>
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<td>r4*</td>
<td>5</td>
<td>nd</td>
<td>r4</td>
</tr>
</tbody>
</table>

*Some induction was observed also in the contralateral r4.
†Number of embryos ectopically expressing Hoxd4 in r4/number of embryos analysed.
RA1, 1×10^{-4} M; RA2, 5×10^{-4} M.
with the observation that r3 can acquire a caudal identity when transplanted caudally, away from the influence of such signals.

**Misexpression of Hoxa3 is sufficient to induce ectopic SM neurones in the rostral hindbrain**

Although most of our data indicate a correlation between rostrocaudal motoneurone identity and Hox gene expression, there were examples to the contrary. For example, in rostral to caudal grafts (r3/r8a), r3 expressed Hox genes typical of r8 identity, but failed to produce SM neurones. In order to test directly the role of Hox genes in specifying rostrocaudal identity of motoneurones, in ovo electroporation was used to misexpress the full-length Hoxa3 cDNA in the rostral hindbrain of stage 8-16 embryos (r1-r4 region). Hoxa3 is expressed from the rhombomere 4/5 boundary caudally (Fig. 1A,D) and is therefore a good candidate to impose an abducens phenotype on r5 and r6 motoneurones (Capecchi, 1997). To allow identification of the ectopic region of Hoxa3 expression in the chick hindbrain, a mouse Hoxa3 cDNA was used (Fig. 5A,C), and to facilitate the screening of electroporated embryos, a tauGFP plasmid was co-electroporated (Fig. 5B,C). The results of electroporation of the two constructs were compared with control electroporations of the tauGFP plasmid alone. GFP expression was observed in embryos 3 hours after electroporation, as previously reported (Momose et al., 1999). After 48 hours (equivalent of E4), embryos showing unilateral GFP expression in the rostral hindbrain were processed by whole-mount in situ hybridisation for Hb9. Ectopic Hb9 motoneurones were found only in those embryos that had received both tauGFP and Hoxa3 plasmids (n=21; Fig. 5D-G), but never in the control group (n=5; data not shown). Interestingly, each of rhombomeres 1 to 4 was found to produce Hb9-positive motoneurones in some cases, in contrast to the inability of r1-3 to generate SM neurones in somite or RA bead grafting experiments. In all embryos examined (n=5), some GFP-positive axons left the neuroepithelium from ectopic ventral exit points in the GFP/Hoxa3 positive region, consistent with the induction of SM neurones (data not shown). To assess Lim gene expression patterns of these ectopic SM neurones, embryos were grown up to E5, and GFP fluorescence...
was combined with double-immunostaining for anti-neurofilament antibodies and anti-Lim antibodies on serial sections (Fig. 6A-D). These experiments showed that a similar number of motoneurones were present in both the control and electroporated sides (Fig. 6A,B), but ectopic Islet2-positive motoneurones were present only on the GFP-positive electroporated side (Fig. 6A,C,D). The majority of Islet2-positive motoneurones were Lim3 negative, Which is indicative of an abducens phenotype, but occasional Islet2/Lim3-positive neurones were detected (Fig. 6C,D). Because in these experiments, embryos were fixed earlier (E5) than in other experiments (E6), this may reflect the fact that accessory abducens neurones (destined to be Islet1/Lim3) are in the process of migrating laterally and have yet to downregulate Islet2. Hence, misexpression of Hoxa3 at stage 8-16 is sufficient to induce SM neurones in the rostral hindbrain. However, the number of motoneurones within the Hoxa3 misexpression domain was relatively small compared with those at axial levels that normally generate SM neurones. Electroporation of Hoxa3 at stage 17 or later failed to induce Hb9 expression (Fig. 5H,I), showing that the window of competence to generate SM neurones extended only up to stage 16. As the domain of expression of Hoxb3 also coincides with the region that generates SM neurones, we also tested whether misexpression of Hoxb3 in r1-4 could induce ectopic SM neurones. However, co-electroporation of a human HOXB3

Figure 6. Dorsoventral patterning following Hoxa3 misexpression.
(A-T) Immunostaining on transverse cryostat sections of embryos electroporated with mouse Hoxa3/tauGFP at stage 10.
(A-D,E-H) Sections of two different embryos grown up to stage 27 prior to fixing.
(I-P) Sections through another embryo that was fixed at stage 18.
(Q-T) Sections through a second embryo fixed at stage 18. D,F,H,J-K,M-N are higher magnifications of C,E,G,I,L, respectively, showing only the electroporated side.
Antibodies are as indicated.
(A,E,F,J,M,Q,S) GFP natural fluorescence.
K,N,R,T are the same sections as J,M,Q,S, respectively, but without the GFP signal. White asterisks (A,B,C,G,I,L,O) indicate the electroporated side.
White arrows (D) indicate Islet2/Lim3-positive motoneurones. Asterisks (O,T) represent regions of repression of Pax6 and of induction of Olig2 expression, respectively.
(P) r8, (S,T) r1; all other axial levels shown are r3/4.
Scale bar: 120 μm for A,C,Q,T; 60 μm for D,E,G,I,L,O-P; 30 μm for F,H,J,K-M,N.
plasmid and a tauGFP plasmid at the same stages as for Hoxa3 (stage 8-16) resulted in a few ectopic Hb9-positive cells in the rostral hindbrain in only two out of seventeen cases (data not shown), and in the majority of cases no SM neurone induction was observed (Fig. 5J-K). Thus the effect of Hoxb3 in SM neurone induction appears to be weak or absent compared with that of Hoxa3.

Dorsoventral patterning is altered following Hoxa-3 misexpression in the rostral hindbrain

The induction of SM neurones in the rostral hindbrain by Hoxa3 electroporation suggests that dorsoventral patterning has been altered in this region. In the hindbrain, BM/VM neurones originate from a domain of the neural tube that lies directly adjacent to the floor plate, the p3 domain, which is Nkx2.2-positive (Briscoe et al., 1999). In the spinal cord and caudal hindbrain, SM neurones originate from a domain dorsal to this, the pMN domain, which is Olig2-positive, Nkx2.2-negative and which expresses Pax6 at low levels (Briscoe et al., 1999; Novitch et al., 2001; Ericson et al., 1997). Lying dorsal to the pMN domain is the p2 domain, which is Olig2 and Nkx2.2-negative, and which expresses Pax6 at high levels and in addition expresses Irx3 (Briscoe et al., 2000). The p2 domain gives rise to interneurones that express the marker Chx10. However, the disposition of these domains within the rostral hindbrain has not been extensively investigated as it has been in the spinal cord. We found that in rhombomeres 2-4 of the rostral hindbrain at stage 18, the domains of Nkx2.2 (p3) and Irx3 (p2) expression abutted each other directly, without an intervening pMN domain (Fig. 6L) (C. William and T. Jessell, personal communication; J. Ericson, personal communication). It might also be expected that a domain of high Pax6 expression (p2) would directly abut the Nkx2.2 domain (p3) in the rostral hindbrain, rather than include an intervening domain of low Pax6 expression (pMN) as has been reported (e.g. Ericson et al., 1997). However, we were unable to detect any gradations in Pax6 expression in the hindbrain, at least at its ventral limit (Fig. 6R). However, this may have reflected a technical limitation in the sensitivity of our immunohistochemistry.

In Hoxa3 electroporated embryos, we performed double immunostaining on transverse sections showing GFP expression, to visualise the domains of expression of various dorsoventral markers of progenitor domains or cell fate. In particular, we focused on patterning changes in regions in which ectopic SM neurones were present, as shown by immunopositivity using an antibody against the SM markers Mn2/Hb9 (and which we shall refer to as Hb9-positive cells). In embryos analysed at stage 27, Hb9-positive induced SM neurones were located ventrally within the hindbrain, and were in some cases interpersed with or ventral to Chx10-positive interneurones (Fig. 6E-H). As the location of these cells would be consistent with the conversion of the p2 domain into an ectopic pMN domain, we analysed whether at earlier stages (e.g. stage 18) Olig2 had been induced in the GFP/Hb9-expressing domain. At this stage, ectopic Hb9-positive cells were observed laterally as well as ventrally, which suggests that some more laterally-located cells may die, leading to the ventral localisation of Hb9-positive cells observed at stage 27 (Fig. 6L-K). Only a few of the most ventral of these Hb9-positive cells were Islet1/2-positive, which is consistent with this idea (Fig. 6K; data not shown). However, no ectopic Olig2 was detected at r2-4 levels (Fig. 6O), despite the presence in the same embryo of an Olig2-positive domain at rhombomere levels 5-8 where resident Hb9-positive motoneurones were also detected (Fig. 6P). However, Olig2 expression was induced coincident with ectopic Hb9-positive cells in caudal r1 in response to Hoxa3 overexpression (Fig. 6S,T), perhaps reflecting an enhanced tendency for r1 to produce SM neurones (the SM trochlear nucleus lies in rostral r1 although it is Hb9-negative; see Fig. 1).

The p2 marker Irx3 was repressed in GFP-expressing regions within r2-4 (Fig. 6L-N,S,T). Irx3 and Olig2 have been shown to be mutually repressive (Novitch et al., 2001), and yet these data suggest that Hoxa3 is capable of repressing Irx3 and inducing SM neurones without induction of Olig2. The localisation and level of Nkx2.2 expression failed to change following Hoxa3 overexpression (Fig. 6L), whereas Pax6 expression was repressed to a modest extent, and particularly in dorsal regions (Fig. 6Q,R). Taken together, the downregulation of both Irx3 and Pax6 would be consistent with the induction of an ectopic pMN domain that generates ectopic SM neurones, although this domain apparently lacks Olig2 expression.

DISCUSSION

Our first major conclusion is that the capacity of a rhombomere to produce SM neurones is susceptible to alteration by rostrocaudal cues, but becomes fixed at stage 10-11 at around the stage of neural tube closure. Second, grafting of somites or RA beads in the rostral hindbrain induces SM neurones, which suggests that RA might be a component of somite-derived signals that pattern SM neurones in vivo. Third, SM neurone induction by somite grafts or by RA is concomitant with ectopic Hox gene expression, such as Hoxa3. Fourth, misexpression of Hoxa3 in the rostral hindbrain alters dorsoventral patterning and induces ectopic SM neurones, which implies that Hox genes expressed in r5-8 might be involved in patterning SM neurones.

Cranial motoneurone identity is plastic prior to neural tube closure

Our data indicate that cranial motoneurone commitment to a particular rostrocaudal identity must occur in a very restricted time period at stage 10-11 and coincident with the time of neural tube closure. These results are broadly comparable to those for the spinal cord, in which motoneurone progenitor identity is labile at stage 11 but becomes fixed just a few hours later, at around stage 12 (Ensini et al., 1998; Lance-Jones and Landmesser, 1980; Matise and Lance-Jones, 1996). In the hindbrain the first post-mitotic motoneurones are detected using Sc1 and Islet1 markers at late stage 13 (Ericson et al., 1992; Guthrie and Lumsden, 1992; Varela-Echavarria et al., 1996). As the acquisition of a generic motoneurone fate remains sensitive to local Shh signalling until late in the final cell cycle of motoneurone progenitors (Ericson et al., 1996), rhombomere commitment to generate a particular repertoire of motoneurones occurs relatively early in the process of motoneurone fate specification.
Rostrocaudal patterning of cranial motoneurones by the paraxial mesoderm and RA

Our results from transplantation experiments are consistent with the existence of a mesoderm-derived caudalising activity that is capable of eliciting somatic motoneurone differentiation from at least a subset of progenitors. The timing and modality of action of such a caudalising activity differs along the rostrocaudal axis, with caudal rhombomeres being more susceptible than rostral ones. Prior to stage 10, both r3 and r4 were competent to express Hb9/Islet2 and produce SM neurones in response to somite signals when transplanted into r8 caudal position. However, by stage 10 only r4, and not r3, could generate SM neurones following transposition to r8, whereas at stage 11, r7 was still capable of responding to caudal signals. This suggests that the competence to respond to somite signals by SM neurone differentiation decreases with time, and is switched off more rapidly in r3 than in r4 and more caudally. Moreover, there appears to be a gradient of signals along the rostrocaudal axis, because for the same rhombomere (r4) transplanted at the same stage (10/11), Islet2 was induced in grafts made into the caudal but not the rostral part of rhombomere 8.

In caudal rhombomere transpositions, caudalising signals are likely to come from the somites, an idea supported by the results of somite grafting experiments. Somites transplanted beneath the neural tube at r2-4 levels induced SM neurone differentiation in r4, as well as a caudalisation of Hox gene expression. Perhaps surprisingly, somite grafts did not induce SM neurones in r3, even at a stage (stage 9) at which transplantation of r3 tissue to caudal r8 (adjacent to s5) would have resulted in SM neurone differentiation. This may suggest the existence of factors capable of repressing the caudalising activity, localised in rostral rhombomeres and/or the rostral (pre-otic) paraxial mesoderm. We found that r4 also manifested a differential response to caudal transplantation or juxtaposition of a somite, as in the former case hypoglossal neurones were induced, whereas in the latter case abducens neurones formed. The explanation for this finding appeared to be that Hox3 expression was induced in r4 more rapidly than Hoxd4, which is consistent with previous studies (Grapin-Botton et al., 1997; Itasaki et al., 1996). Expression of Hoxa3 in the absence of Hoxd4 is consistent with an abducens motoneurone phenotype, and by the time Hoxd4 was switched on, motoneurone fates and axon pathways might already have been established.

We have found that the rostral implantation of RA beads mimicked the action of the somite, inducing SM neurones in r4, but not r3, and with an identical stage dependence. Indeed, it has been proposed that RA mediates a part of the caudalising ability of the paraxial mesoderm in patterning the rostrocaudal axis (Berggren et al., 1999; Gavalas and Krumlauf, 2000; Gould et al., 1998; Maden et al., 1998; Swindell et al., 1999), and RA is produced by the somites (Maden et al., 1998). Our finding that only somites caudal to s5 were capable of inducing SM neurones is also consistent with the idea that RA is involved, because RA is generated at a high level in younger somites (Maden et al., 1998; Swindell et al., 1999) and the ability of the somites to repattern Hox gene expression is lost in a rostral to caudal wave (Itasaki et al., 1996). It is thus possible that RA influences SM neurone patterning through a rostral (low) to caudal (high) gradient in the somitic mesoderm and possibly the hindbrain (for a review, see Maden, 1999).

Application of RA beads reliably induced Hoxa3 but only induced Hoxd4 in a proportion of cases, in contrast to the invariant induction of Hoxd4 in somite grafts. This observation may be consistent with studies showing that somitic signals capable of inducing Hoxd4 are not entirely accounted for by RA; a higher molecular weight factor is involved (Gould et al., 1998). In view of the lack of RA-mediated induction of SM neurones in r3, higher doses of RA were applied: this did not induce ectopic SM neurones in r3, but instead induced SM neurones on both sides of r4. These results also tend to favour the idea that r3 or its surrounding mesoderm contains an inhibitory factor that modulates the action of RA, preventing SM neurone differentiation. Indeed, our recent results from in vitro experiments have shown that the ability of RA to induce SM neurones in the rostral hindbrain is modulated by the RA-degradative enzyme Cyp26, and that Cyp26 expression in the neuroepithelium is upregulated by factors derived from the mesoderm and endoderm (Guidato et al., 2003).

Hox genes as determinants of rostrocaudal motoneurone identity

Taken together, the results of our grafting experiments showed that rostrocaudal motoneurone identity and Hox gene expression profile are intimately linked. Nevertheless there were examples to the contrary. For example, r3 grafts in r8a expressed Hoxa3, Hoxb3 and Hoxd4 but failed to produce hypoglossal SM neurones, which are normally characteristic of neuroepithelium with that Hox ‘code’. In addition, somite grafts induced expression of all three Hox genes in r4, but the neurones produced were abducens rather than hypoglossal. Therefore in grafts of r3 to r8a, by the time Hox gene expression is initiated, the time-window for motoneurone specification is already over, whereas for somite grafts, Hoxa3 has an earlier onset of expression than Hoxd4, leading to an abducens phenotype. Another example of the mismatch between Hox expression and motoneurone fate concerns stage 9 r5 grafts to the r3 position; these grafts expressed Hoxa3 and Hoxb3 but did not generate SM abducens neurones. In this case, Hox expression was at a lower level than at more caudal axial levels and so levels of Hox protein may not have been high enough to maintain segment identity (Greer et al., 2000).

In a more direct test of the role of Hox genes, we misexpressed Hoxa3 rostral to its normal expression limit and obtained SM neurone induction in r1-4, which indicates that driving a high level of Hoxa3 expression could overcome any inherent inhibition of SM neurone production in the rostral hindbrain. Induction of SM neurones by Hoxa3 could be accomplished in electroporations performed up to stage 15/16. This data is broadly consistent with that from somite grafting experiments in which induction of Hoxa3 in r4 level occurred approximately 36 hours after grafting, when embryos were approximately stage 15/16, and this was sufficient to induce differentiation of SM neurones with abducens phenotype. Hoxa3 was found to repress Irx3 and Pax6, and Hb9-expressing SM neurones were found adjacent to, or interspersed with, Chx10-positive cells, which are derived from the Irx3-expressing domain. This suggests that ectopic SM neurones are induced at the expense of presumptive Chx10-positive interneurons. However, no induction of Olig2 was seen in r2-4 of the hindbrain, but it was detected in r1. As Olig2...
has been shown to play an important role in SM neurone generation, this implies that Hoxa3 can function in parallel with, or downstream of, Olig2 in hindbrain SM neurone differentiation, at least at r2-4 axial levels.

In Hoxa3 electroporated embryos, SM neurones were present in relatively large numbers in embryos analysed at stage 18, but were present in small numbers at stage 27, when they were restricted to a ventral column on either side of the floor plate and had a phenotype more consistent with abducens identity. These data are thus consistent with Hox3 paralogues being involved with SM specification, and the abducens phenotype in particular. However, the maintenance of these motoneurons following initial induction seems to occur only for more ventrally located cells, and thus may depend on other local factors. In both mouse and chick there are three Hox3 paralogues, Hoxa3, Hoxb3 and Hoxd3, which, based on a number of studies, are expressed to a rostral limit at the r4/5 boundary (Capecchi, 1997; Lumsden and Krumlauf, 1996). Although little is known about the early expression pattern of Hoxd3 in the chick hindbrain, Hoxa3 and Hoxb3 are expressed to this rostral limit in the early neural plate (Lumsden and Krumlauf, 1996; Rex and Scadding, 1994). Hence the early expression domains of Hoxa3 and Hoxb3 genes coincide with the territory of SM neurone differentiation, making them good candidates for patterning SM neurones. It therefore remains to be determined which Hox genes, and in which combinations, give rise to SM neurones of particular phenotypes. The analysis of various Hox-null mutant mice implicates Hox genes in conferring rostrocaudal identity upon motoneurones (Gavalas et al., 1997; Gavalas et al., 1998; Goddard et al., 1996; Lumsden and Krumlauf, 1996; Studer et al., 1996). In Hox3 mutants, there are defects in the formation of the ganglion of the IX cranial nerve (glossopharyngeal), which may relate to aberrant neural crest migration, and in addition glossopharyngeal (BM/VM) motoneurones in r6 project incorrectly, possibly as a result of the ganglionic defect (Watari et al., 2001). To our knowledge no defects in the abducens or hypoglossal SM neurone populations have been reported in Hox3 paralogue mutants, although neural crest and skeletal patterning are affected (Chisaka and Capecchi, 1991; Condie and Capecchi, 1993). However, because mice mutant for both Hoxa3 and Hoxd3 show defects not found in either of the single mutants (Condie and Capecchi, 1993), SM neuronal patterning might require further analysis in these or other double or triple Hox3 mutants.

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