

Requirement for downregulation of kreisler during late patterning of the hindbrain

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SUMMARY

Pattern formation in the hindbrain is governed by a segmentation process that provides the basis for the organisation of cranial motor nerves. A cascade of transcriptional activators, including the bZIP transcription factor encoded by the kreisler gene controls this segmentation process. In kreisler mutants, r5 fails to form and this correlates with abnormalities in the neuroanatomical organisation of the hindbrain. Studies of *Hox* gene regulation suggest that kreisler may regulate the identity as well as the formation of r5, but such a role cannot be detected in kreisler mutants since r5 is absent. To gain further insights into the function of kreisler we have generated transgenic mice in which kreisler is ectopically expressed in r3 and for an extended period in r5. In these transgenic mice, the *Fgf3*, *Krox20*, *Hoxa3* and *Hoxb3* genes have ectopic or prolonged expression domains

in r3, indicating that it acquires molecular characteristics of r5. Prolonged kreisler expression subsequently causes morphological alterations of r3/r5 that are due to an inhibition of neuronal differentiation and migration from the ventricular zone to form the mantle layer. We find that these alterations in r5 correlate with an arrest of facial branchiomotor neurone migration from r4 into the caudal hindbrain, which is possibly due to the deficiency in the mantle layer through which they normally migrate. We propose that the requirement for the downregulation of segmental kreisler expression prior to neuronal differentiation reflects the stage-specific roles of this gene and its targets.

Key words: kreisler, Hindbrain, Segmentation, Neuronal differentiation, Migration, Mouse

INTRODUCTION

During neurogenesis, a large variety of different neuronal cell types are generated in a defined spatial organisation that provides the basis for the functioning of the adult nervous system. Owing to its well-characterised structure and the identification of molecular markers and regulatory genes, the hindbrain has been used as a model system for studying molecular mechanisms that control pattern formation in the central nervous system. Crucial to patterning of this brain region is an early transient subdivision of the neural tube into repeated morphological units, termed rhombomeres (r). Rhombomeres act as compartments in which the movement of cells across rhombomere boundaries is restricted (Fraser et al., 1990). This restriction of cell movements enables rhombomeres to maintain a distinct identity conferred by regulatory genes with rhombomere-specific expression patterns (reviewed by Lumsden and Krumlauf, 1996). Hindbrain segmentation plays an important role in craniofacial

morphogenesis through the segmental specification and migration of neural crest cells to the branchial arches (Lumsden et al., 1991; Sechrist et al., 1993), and underlies the segment-specific differentiation of several neuronal cell types (Clarke and Lumsden, 1993; Gilland and Baker, 1993; Lumsden and Keynes, 1989). Each rhombomere contains a set of reticular neurones that first form in the even-numbered rhombomeres r2, r4 and r6, and later in the odd-numbered rhombomeres r3 and r5. In the chick, the branchiomotor nerves are organised with a two segment periodicity in which the nuclei of the trigeminal (Vth), facial (VIIth) and glossopharyngeal (IXth) nerves are generated in pairs of rhombomeres, r2/r3, r4/r5 and r6/r7, respectively. The axons of these nerves leave the neural tube at specific exit points located in the dorsal part of the even-numbered rhombomeres and project towards the branchial arches. Somatic motoneurons of the trochlear (IVth), abducens (VIth) and hypoglossal (XIIth) cranial nerves arise in specific rhombomeres, and their axons project through ventral exit points to innervate muscles derived

from paraxial and prechordal plate mesoderm. Thus, each rhombomere gives rise to a specific set of cranial motor nerves, reflecting the acquisition of a unique identity by each segment.

Analyses of hindbrain patterning have identified a cascade of transcriptional regulators that underlie segmentation and anteroposterior (AP) specification. The clustered Hox genes are key regulators of AP identity that are expressed in nested, overlapping segmental domains in the hindbrain (McGinnis and Krumlauf, 1992). Consistent with this role of Hox genes during hindbrain segmentation are the effects of retinoic acid treatment, which induces changes in the expression pattern of several Hox genes that correlate with altered identities of cranial motor nerves (Kessel, 1993; Marshall et al., 1992). Similarly, ectopic expression of *Hoxa1* or *Hoxb1* results in the transformation of r2 to an r4 identity (Alexandre et al., 1996; Bell et al., 1999; Zhang et al., 1994), and, conversely, loss of their expression transforms r4 to an r2 identity (Goddard et al., 1996; Rossel and Capecchi, 1999; Studer et al., 1996; Studer et al., 1998). Direct evidence for a role of Hox genes in the specification of neuronal phenotypes has been obtained from the inactivation of *Hoxb1*, which leads to defects in the migration of r4-specific facial (VIIth) and vestibuloacoustic (VIIIth) efferent neurones (Studer et al., 1996). Furthermore, *Hoxa2* is required for normal pathfinding of r2 and r3 motor axons (Gavalas et al., 1997), and *Hoxa2* and *Hoxb2* functionally synergize in controlling the development of ventral neuronal subtypes in r3 (Davenne et al., 1999).

Important information concerning the role of rhombomeres as neuronal specification units has been obtained from the analysis of mutant mice showing defects in the formation of hindbrain segments. The *Krox20* gene (*Egr2* – Mouse Genome Informatics) encodes a zinc-finger transcription factor expressed in r3/r5, with the onset of its expression occurring before segmentation becomes morphologically overt (Nieto et al., 1991; Wilkinson et al., 1989). The formation of odd numbered rhombomeres seems to be initiated correctly in *Krox20* null mutant mice, but they subsequently fail to form definitive r3 and r5 (Schneider-Maunoury et al., 1997; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). This loss coincides with effects on the migration of motor axons originating in neighbouring segments (Schneider-Maunoury et al., 1997). *Krox20* has also been shown to act as a direct transcriptional regulator of *Hoxa2* (Nonchev et al., 1996), *Hoxb2* (Sham et al., 1993) and *EphA4* (Theil et al., 1998), thus coupling formation of hindbrain segments to specification of their AP identity and to restrictions to cell mixing.

Further clues to the relationship between rhombomere formation, segmental identity and the neuronal architecture of the hindbrain has come from the characterisation of the mouse mutant *kreisler* and its zebrafish equivalent *valentino*. *kreisler* mutant mice display circling and head tossing that are due to deafness and loss of vestibular function (Deol, 1964). These defects are correlated with abnormalities in the embryonic hindbrain, which is morphologically unsegmented posterior to the r3/r4 boundary (Deol, 1964) because of a loss of r5 (Frohman et al., 1993; Manzanares et al., 1999b; McKay et al., 1994), and are analogous to the phenotype of *valentino* mutants (Moens et al., 1998; Moens et al., 1996). This disruption to segmentation is caused by a loss of hindbrain expression of a member of the Maf subfamily of basic domain leucine zipper

transcription factors, *Krml1* and *valentino*, which are normally expressed in r5/r6 (Cordes and Barsh, 1994; Moens et al., 1996). Several cranial motor nerves show abnormalities in the *kreisler* mutant, including a loss of the abducens (VIth) and the general visceral component of the facial motor nerve (VIIth), both of which originate from r5 (McKay et al., 1997). In addition to segmentation of r5, *kreisler* directly regulates the rhombomere restricted expression of the *Hoxa3* and *Hoxb3* genes (Manzanares et al., 1999a; Manzanares et al., 1997) suggesting it controls regional identity through Hox genes. After its downregulation in r5/r6, *kreisler* (*Krml1*; *Mafb* – Mouse Genome Informatics) shows a second phase of expression in the hindbrain in the acoustic and several vestibular nuclei (Eichmann et al., 1997), raising the possibility of later functions of *kreisler* in the development of hindbrain nuclei. However, owing to the absence of r5 in *kreisler* mutants, it has not been possible to demonstrate a role of *kreisler* in establishing r5 identity.

To test these potential roles of *kreisler*, we generated transgenic mice in which there is a gain-of-function expression of the *kreisler* gene. We describe the effects of an ectopic and prolonged expression of *kreisler* in r3 and r5 on the development of these rhombomeres that indicate it has a role in specifying r5 identity. Unexpectedly, we find that correct downregulation of *kreisler* expression is required for the onset of neuronal differentiation and migration from the ventricular zone, and for establishment of an environment that allows migration of facial branchiomotor neurones into the caudal hindbrain.

MATERIALS AND METHODS

Plasmid construction and generation of transgenic mice

A 1.6 kb *NcoI/SacI* bearing the *EphA4* r3/r5 enhancer was inserted into the end filled *HindIII* site of pGZ40 (Yee and Rigby, 1993) giving rise to the plasmid pSekG40. The *kreisler*-coding region was amplified by PCR from the previously described *kreisler* cDNA (Cordes and Barsh, 1994) and subcloned into *EcoRI/BamHI* cut Bluescript KS. For ectopic expression, an *NdeI/NcoI* (partial digest) fragment was inserted into the *NcoI/NdeI* sites of pSekG40. After testing the integrity of the construct by sequencing on both strands a fragment for the generation of transgenic mice was released by *Sall/NotI* digest. Transgenic mice were generated by microinjection of fertilised eggs from crosses between F₁ hybrids (CBA×B10) as described previously (Theil et al., 1998) and were identified by polymerase chain reaction using extra-embryonic yolk sac or tail biopsies.

Transgenic mouse lines, β-galactosidase and alkaline phosphatase staining

For r2 labelling experiments, a transgenic line was used carrying a 1.1 kb fragment of *Hoxa2* linked to a human placental alkaline phosphatase reporter gene, and staining reactions were performed as described previously (Studer et al., 1996). The caudal migration of facial motoneurons was monitored using a *Hoxb1/lacZ* transgenic line (Marshall et al., 1992), and r3/r5 were detected by using a transgenic line carrying the *Hoxb2* r3/r5 enhancer driving *lacZ* expression (Sham et al., 1993). β-galactosidase staining reactions were performed as described (Theil et al., 1998).

In situ hybridisation

Whole-mount in situ hybridisation was performed as described (Wilkinson, 1992). The RNA probe for *kreisler* was derived from a

650 bp *KpnI/NcoI* fragment of the mouse kreisler cDNA (Cordes and Barsh, 1994). Probes for *Hoxb1*, *Hoxa3*, *Hoxb3* and *Krox20* were as described (Hunt et al., 1991; Wilkinson et al., 1989). For expression analysis on sections, mouse embryos were immediately frozen on dry ice upon dissection from uterine tissue and 16 μ m cryostat sections were prepared. In situ hybridisation was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993).

RESULTS

Ectopic expression of kreisler in the hindbrain using an *EphA4* r3/r5 enhancer

To alter the segmental patterns of expression of kreisler, we used a regulatory element of the *EphA4* gene, identified in a previous study, which mediates expression in r3 and r5 of the hindbrain (Theil et al., 1998). In transgenic mouse embryos, reporter gene activity is first detected at the three- to four-somite (s) stages in a narrow stripe of cells corresponding to presumptive r3, which then broadens, and by 7s in a row of presumptive r5 cells, which then broadens. By the 12s stage, low levels of expression also occur in r2. This pattern is stably maintained until at least embryonic day E12.5. To use this regulatory element for ectopic expression experiments, the kreisler cDNA was cloned into a vector containing this *EphA4* r3/r5 enhancer, a β -globin minimal promoter and an SV40 polyadenylation signal (Fig. 1A). After injection of the corresponding construct into fertilised mouse oocytes, three independent transgenic lines were established. Identical results were obtained from these lines, which we refer to as kreisler^{r3/r5}. These lines are heterozygous for the transgene and are viable, but homozygous adults could not be obtained by interbreeding, suggesting that a stronger defect occurs when the level of transgene expression is increased.

To confirm specific expression of the transgene in the hindbrain, we first performed an in situ hybridisation study using a kreisler probe. At E9.0, kreisler transcripts can be detected in wild-type embryos at approximately equal levels in r5/r6, in neural crest cells migrating towards the third branchial arch and in the roof of the neural tube (Fig. 1B) (Cordes and Barsh, 1994)). kreisler^{r3/r5} embryos, however, show an additional strong expression domain in r3 (Fig. 1C,D). The expression in r5 appears to be elevated compared with r6, indicating that the kreisler transgene is expressed at high levels in sites of *EphA4* enhancer activity. As expected from the time course of *EphA4* enhancer activity, transgenic kreisler expression persists in r3 to later stages, and is prolonged in r5 compared with wild-type embryos (not shown). Therefore, use of the *EphA4* r3/r5 enhancer results in the ectopic expression of kreisler in r3 and a higher level of expression in its normal r5 domain.

Formation of hindbrain segments is not affected in kreisler^{r3/r5} embryos

To analyse whether the global segmentation of the hindbrain has been affected in kreisler^{r3/r5} embryos, the expression of several marker genes for even-numbered segments was examined. r4 appears normal, based on the segmental expression of *Hoxb1* in wild-type and

transgenic embryos (Fig. 2A,D). To assay the characteristics of r2 we crossed kreisler^{r3/r5} mice with a transgenic line carrying an alkaline phosphatase reporter gene under the control of a *Hoxa2* r2 enhancer (Studer et al., 1996). Reporter gene activity in r2 was identical in wild-type and kreisler^{r3/r5} embryos (Fig. 2B,E). Neuregulin is expressed in the dorsal part of all the even-numbered rhombomeres and in r1 (Meyer and Birchmeier, 1995), and this pattern of expression is also maintained in kreisler^{r3/r5} embryos (Fig. 2C,F). Taken together, these analyses indicate that the formation and number of rhombomeres remains undisturbed and that molecular markers of even-numbered rhombomeres are preserved in kreisler^{r3/r5} embryos.

Changes in r3 gene expression in kreisler^{r3/r5} embryos

To investigate the consequences of the ectopic expression of kreisler in r3 we analysed the expression patterns of several genes that are co-expressed with kreisler in r5 and/or r6, and are candidates for being downstream of this transcription factor.

The *Fgf3* gene is expressed in the hindbrain in a dynamic pattern in r5 and r6 between E8 and E9.5 (McKay et al., 1996; Wilkinson, 1990). Furthermore, *Fgf3* is required for the proper differentiation of the inner ear, as knockout mice often lack the endolymphatic sac and duct and show deformities in their subsequent growth (Mansour et al., 1993). Based on the spatial and temporal overlap in their hindbrain expression patterns, the similarities in the phenotype of kreisler and *Fgf3* mutant mice, and the loss of *Fgf3* expression in the hindbrain of kreisler mutant mice (McKay et al., 1996) it has been suggested that

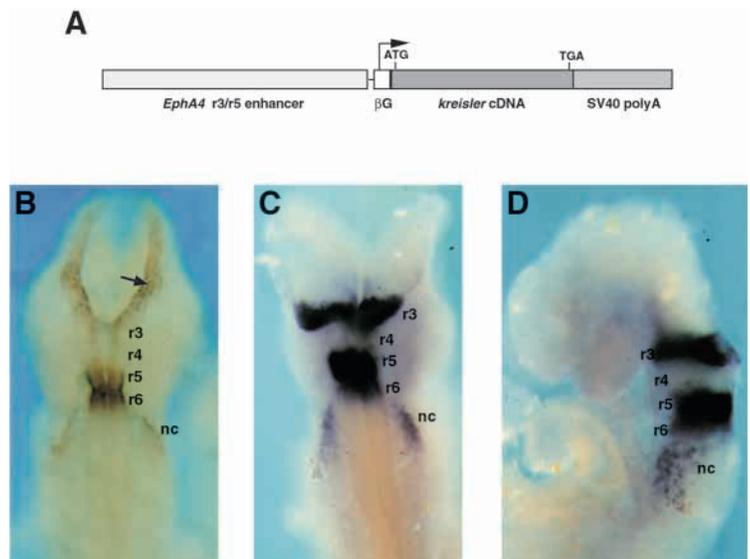


Fig. 1. Ectopic expression of kreisler in the hindbrain of transgenic mice. (A) Construct for the generation of transgenic mice. An *EphA4* r3/r5 enhancer in combination with a β -globin minimal promoter (β G) is used to drive expression of a kreisler mini gene in the hindbrain. (B-D) In situ hybridisation analysis of wild-type and transgenic embryos with a kreisler probe. (B) In wild-type E9.0 embryos, kreisler transcripts can be detected in r5/r6, in neural crest cells migrating to the third branchial arch and in the roof plate of the neural tube (arrow). (C) Dorsal view of an age-matched transgenic kreisler^{r3/r5} littermate showing additional kreisler expression in r3 and elevated expression levels in r5. (D) Side view of the same embryo shown in C revealing a flexure of the neural tube at the level of r3.

these genes are part of a genetic cascade. At E9, *Fgf3* is normally expressed in r5/r6 (Fig. 3A), whereas in *kreisler*^{r3/r5} embryos, patchy ventral expression is also induced in the position of r3 (Fig. 3D) and this ectopic r3 domain becomes stronger by E10 (Fig. 3E). Furthermore, in normal embryos, *Fgf3* expression becomes downregulated at approximately E9.5, first in r5 and later in r6 (Fig. 3B) (McKay et al., 1996). By contrast, at this stage, robust expression of *Fgf3* is still detected in r5/r6 of *kreisler*^{r3/r5} embryos (Fig. 3E). Hence, transgene-mediated expression of *kreisler* in r3/r5 leads to the ectopic induction of *Fgf3* in r3 and its prolonged expression in r5. This provides further evidence that *kreisler* lies genetically upstream of *Fgf3* and directly or indirectly regulates its expression.

The *Krox20* gene is expressed in r3/r5 and is required for the maintenance of these rhombomeres (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). In *kreisler* null mutants, there is a loss of the r5 expression domain of *Krox20* (Frohman et al., 1993; Manzanares et al., 1999b; McKay et al., 1994; Moens et al., 1996), but as r5 itself never forms it is not known whether *kreisler* regulates *Krox20*. In normal embryos, *Krox20* expression is downregulated in r3 such that by E10 *Krox20* transcripts can only be detected in r5 (Fig. 3C). By contrast, in *kreisler*^{r3/r5} embryos, the r3 stripe continues to be maintained at E10 (Fig. 3F). These data suggest that r3 now has an r5-like temporal pattern of *Krox20* gene expression.

Previously, we have shown that *kreisler* directly regulates *Hoxb3* in r5, and *Hoxa3* expression in r5 and r6 (Manzanares et al., 1999a; Manzanares et al., 1997), so we analysed the

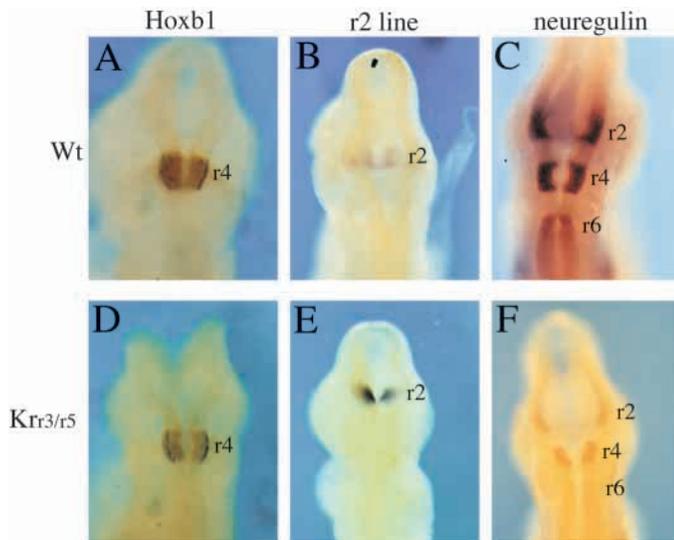


Fig. 2. Segmental expression in the hindbrain has not been affected in *kreisler*^{r3/r5} mice. Wild-type (A-C) and *kreisler*^{r3/r5} embryos (D-F) were analysed for the expression of several marker genes. Whole-mount in situ hybridisation for *Hoxb1* revealed its expression in r4 of both wild type (A) and *kreisler*^{r3/r5} embryos (D). Expression of an alkaline phosphatase reporter gene under the control of an r2 enhancer remains confined to r2 in wild-type (B) and transgenic embryos (E). Transcripts of the *neuregulin* gene can be detected in the dorsal part of the even-numbered rhombomeres and of r1 (C). This pattern of expression is maintained in *kreisler*^{r3/r5} mice (F); the weaker signal is due to the staining having been carried out for less time.

influence of ectopic *kreisler* on the expression of these genes. In *kreisler*^{r3/r5} embryos, expression of the endogenous *Hoxa3* and *Hoxb3* genes is induced in r3 (Fig. 3K,L), in addition to their normal domains of expression in r5 and r5/6 (Fig. 3G,H). To confirm these findings, transgenic mice expressing the *lacZ* reporter gene under the control of the *Hoxa3* (r5/r6) or the *Hoxb3* (r5) segmental regulatory regions (Manzanares et al., 1999a; Manzanares et al., 1997) (Fig. 3I,J) were mated to *kreisler*^{r3/r5} mice and double transgenic embryos assayed for

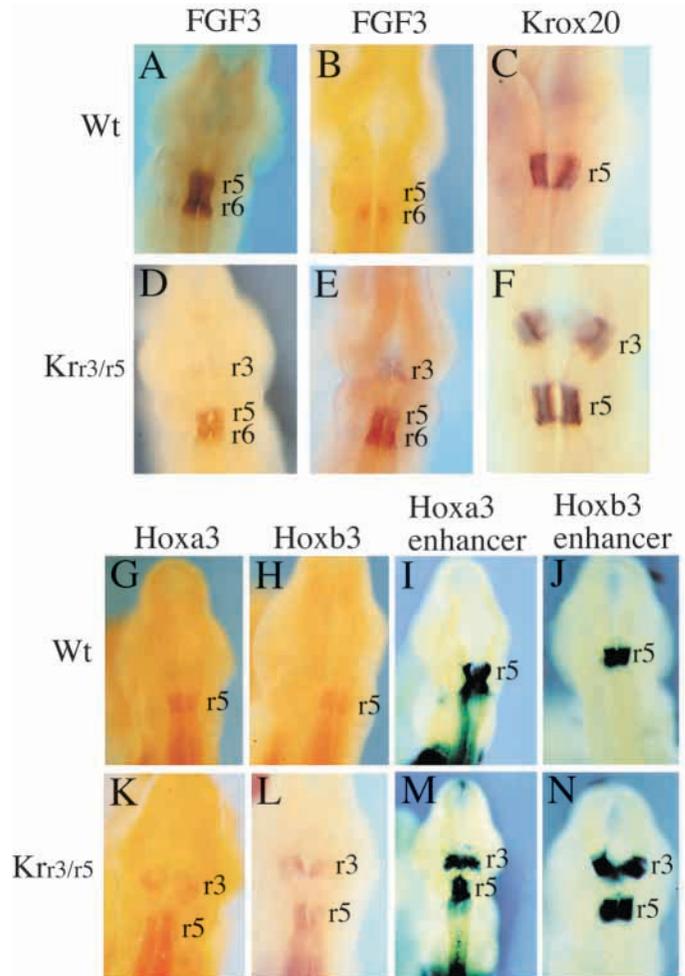


Fig. 3. Expression analysis of r5 specific marker genes. Dorsal view of wild type (A-C; G-J) and *kreisler*^{r3/r5} embryos (D-F; K-N). (A,D) 16s embryos hybridised with *Fgf3* antisense RNA showing weak ectopic activation in r3 of *kreisler*^{r3/r5} embryos (D). (B,E) *Fgf3* expression in E10.0 embryos. Ectopic transcripts can be detected in r3 and prolonged expression occurs in r5. (C,F) E10.0 embryos analysed for *Krox20* expression. By this time point *Krox20* expression is completely downregulated in r3 of wild-type embryos, but remains in *kreisler*^{r3/r5} embryos. (G,K) E9.25 embryos hybridised with *Hoxa3* antisense RNA. In wild-type mice, *Hoxa3* is expressed up to the r4/r5 boundary (G), *kreisler*^{r3/r5} embryos show an additional expression domain in r3 (K). (H,L) In situ hybridisation analysis using a *Hoxb3* probe. In *kreisler*^{r3/r5} mutants, *Hoxb3* is ectopically activated in r3. (I,M) Activity of a *lacZ* reporter gene under the control of a *Hoxa3* regulatory element is confined to r5 and r6 in wild-type embryos, whereas *kreisler*^{r3/r5} embryos show additional activation in r3. (J,N) Similarly, activity of a *Hoxb3* r5 enhancer is ectopically activated in r3 in *kreisler*^{r3/r5} embryos.

lacZ expression. Ectopic expression of *kreisler* results in the induction of β -galactosidase staining in r3 in embryos carrying each of these enhancers (Fig. 3M,N). Taken together, the ectopic induction of *Fgf3*, *Hoxa3* and *Hoxb3*, and the prolonged expression of *Krox20*, suggests that r3 adopts characteristics of r5 in *kreisler*^{r3/r5} mice and that *kreisler* plays a role in regulating segmental identity in addition to segmentation.

Ectopic *kreisler* causes changes in the morphology of hindbrain motor neurone populations

As hindbrain segmentation underlies the segment-specific differentiation of several neuronal cell types, we were interested in whether the ectopic and prolonged expression of *kreisler* resulted in changes in hindbrain neurones. We therefore performed an *in situ* hybridisation analysis using *Isl1* and *Ret* as molecular markers for motoneurone development. While this analysis revealed the normal organisation of many motoneurones, an unexpected difference became evident between wild-type and *kreisler*^{r3/r5} mice in the migration pattern of the medial group of facial branchiomotor (fbm) neurones. Fbm neurones originate in the basal plate of r4 and their cell bodies undergo a complex migration in the developing hindbrain. From E10.0, these neurones start migrating posteriorly adjacent to the ventral midline through r5 into r6 and r7 where they turn towards the dorsal aspect of the neural tube (Studer et al., 1996). At E11.5, this migration pattern of fbm neurones is evident in wild-type embryos using *Isl1* as a probe (Fig. 4A). By contrast, in *kreisler*^{r3/r5} mutant embryos, *Isl1* positive cells are not detected caudal to r4, and there are many more labelled cells adjacent to the ventral midline and dorsolateral in r4 (Fig. 4B,C). At E12.5 these *Isl1*-positive neurones are piled up to form a massive nucleus close to the ventral midline (Fig. 4D). *In situ* hybridisation to coronal and longitudinal sections revealed that this nucleus extends into the lumen of the fourth ventricle and expresses *Ret* as well as *Isl1* (Fig. 4E,F). This suggests that the ectopic or prolonged *kreisler* expression has changed the migration pattern of the r4 fbm neurones.

To confirm that this ectopic nucleus does derive from fbm neurones that fail to migrate caudally, we crossed *kreisler*^{r3/r5} mice with a mouse line transgenic for a *Hoxb1/lacZ* reporter gene, which marks r4 and its derivatives during their development (Marshall et al., 1992; Studer et al., 1996). Flat-mount preparations of the wild-type hindbrain at E12.5 revealed facial motoneurones that originated in r4 and migrated caudally along the ventral midline (Fig. 4G). By contrast, in *kreisler*^{r3/r5} embryos, these neurones did not migrate caudally from r4, and a β -galactosidase-positive ectopic motor nucleus is present (Fig. 4H,I).

We noticed that r4 appeared to be flanked

by abnormal clefts in the *kreisler*^{r3/r5} embryos (compare Fig. 4A and Fig. 4B,C). To examine this in more detail, we hybridised E11.5 embryos with probe for *Phox2b* (*Pmx2b* – Mouse Genome Informatics), which is expressed in fbm neurones and dorsolateral groups of neurones in a characteristic pattern in each rhombomere (Fig. 4J) (Pattyn et al., 1997). In agreement with the findings described above, *Phox2b*-positive fbm neurones fail to migrate caudal to r4 and many coalesce to form a ventrally located nucleus (Fig. 4K,L). Furthermore, r3 and r5 are greatly reduced in size and there is much weaker staining of dorsolateral *Phox2b*-expressing neurones in these rhombomeres compared with wild-type embryos. It is possible that the weaker *Phox2b* staining was due to a decrease in the number of neurones formed or to a normal generation but subsequent loss of neurones. To examine this, we analysed *Phox2b* and *Isl1* expression at early steps of neurogenesis at E10.5 (Fig. 5A–D). We found that at this stage, there was already a deficiency

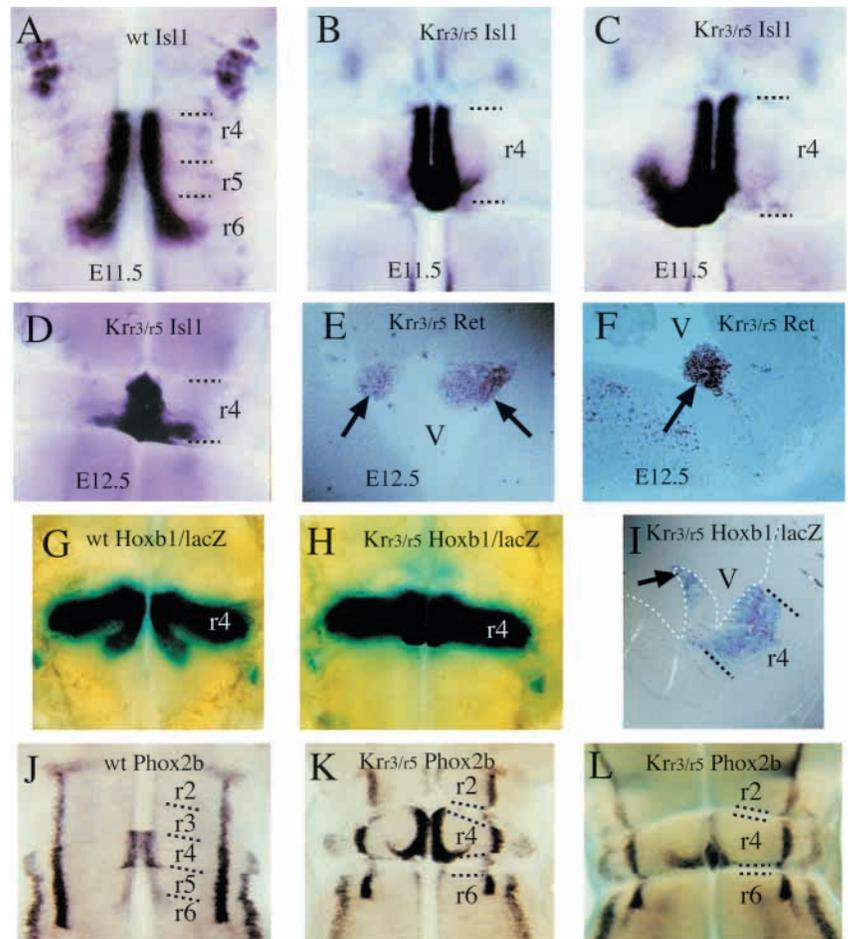


Fig. 4. Disruption of fbm neuronal migration in *kreisler*^{r3/r5} embryos. The expression pattern of neuronal markers and location of fbm neurones is shown in dorsal views and sections of embryos after *in situ* hybridisation or β -galactosidase staining of *lacZ* reporter line. (A–D) Dorsal views of *Isl1* expression in wild-type embryos at E11.5 (A) and *kreisler*^{r3/r5} embryos at E11.5 (B,C) and E12.5 (D). (E,F) *Ret* expression detected in coronal (E) and longitudinal (F) sections through hindbrain of E12.5 embryos. (G–I) *Hoxb1/lacZ* reporter gene expression in wild-type (G) and *kreisler*^{r3/r5} (H, dorsal view; I, sagittal longitudinal section) embryos at E12.5. (J–L): Dorsal views of *Phox2b* expression in wild-type (J) and *kreisler*^{r3/r5} (K,L) embryos at E11.5. The arrows in E,F,I point to the ectopic motor nucleus that protrudes into the hindbrain ventricle.

in the expression of these markers of specific neuronal cell types in the odd numbered rhombomeres.

Developmental defects in r3 and r5

Our analyses suggest that there are defects in the morphology of r3 and r5, and in the differentiation of specific neuronal populations. We therefore examined whether there is a general deficiency in neuronal differentiation and therefore in the formation of the mantle layer through which fbm neurones normally migrate. Immunodetection of β -tubulin, a marker of all differentiated neurones, revealed that whereas staining was almost uniform in the hindbrain of E12.5 wild-type embryos (Fig. 6A), in *kreisler*^{r3/r5} embryos there is an absence of staining at the clefts flanking r4 (Fig. 6B). This is seen most clearly in a lateral view that reveals that the clefts are due to a deficiency in the thickening of the neural tube, and that at this stage neuronal differentiation is absent in the neural tube adjacent to these clefts (Fig. 6D). In addition, staining for β -tubulin reveals that the fbm neurones at the ventral midline of r4 in *kreisler*^{r3/r5} embryos extend axons dorsally along the caudal boundary of r4 (Fig. 6B,C).

The thickening of the neural tube occurs because of the migration of neuronal precursors from the ventricular zone to form the deeper layers of differentiated cells. The simplest explanation for the defect in the thickening and differentiation was that prolonged expression of *kreisler*, and/or downstream targets, disrupted the normal migration and differentiation of neuronal precursors in r3 and r5. To examine whether the morphological defects correlated with r3 and r5, we crossed *kreisler*^{r3/r5} mice with transgenic mice carrying a *lacZ* reporter under the control of the *Hoxb2* r3/r5 enhancer that is directly regulated by *Krox20* (Sham et al., 1993). In transgenic wild-type E11.5 embryos, flat-mount preparations of β -

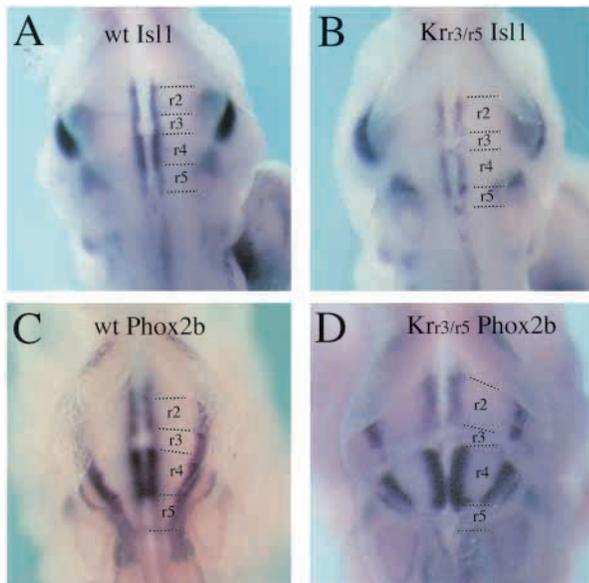


Fig. 5. Disruption of onset of *Isll* and *Phox2b* expression in neurones. (A,B) The expression of *Isll* was analysed in wild type (A) and *kreisler*^{r3/r5} (B) E10.5 embryos. (C,D) The expression of *Phox2b* was analysed in wild-type (C) and *kreisler*^{r3/r5} (D) E10.5 embryos. Expression of both markers was found to be reduced in r3 and r5 of *kreisler*^{r3/r5}, indicating that there is a decrease in the number of neurones expressing these markers that have initiated differentiation.

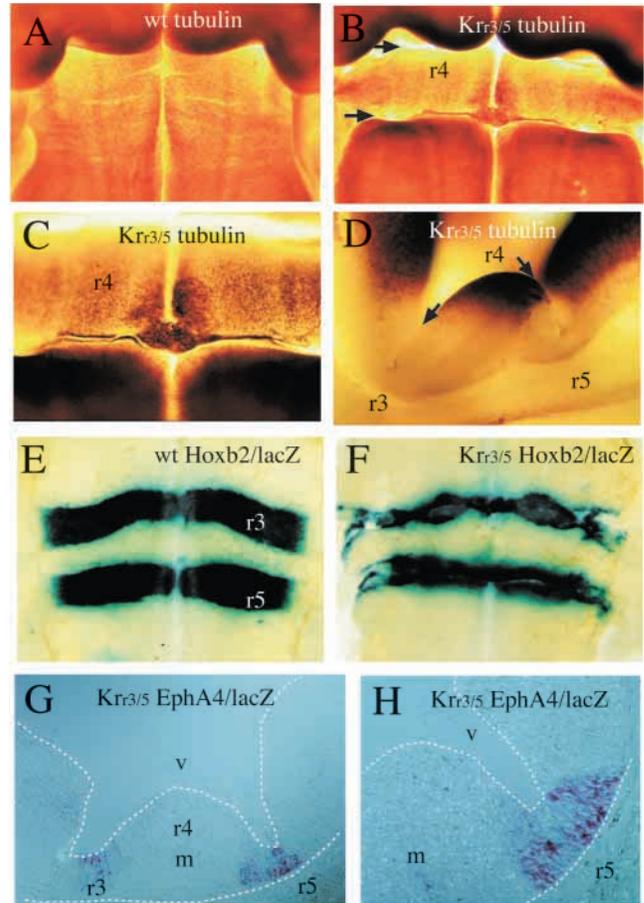


Fig. 6. Disruption of neuronal differentiation in *kreisler*^{r3/r5} embryos. (A-D) The differentiation of neurones revealed by immunodetection of β -tubulin is shown in dorsal views (A-C) and a lateral view (D) of E12.5 wild type (A) and *kreisler*^{r3/r5} (B-D) embryos. (C) A higher magnification view of the ectopic nucleus seen in B; axons are seen extending dorsolaterally along the cleft at the r4/r5 boundary. (E-H) β -galactosidase staining of r3/r5 *lacZ* reporter line shown in dorsal view at E11.5 (E,F) and parasagittal longitudinal section at E12.5 (G,H). v, ventricle; m, mantle region of neural tube. Arrows in B,D indicate the clefts that form at r3 and r5 in *kreisler*^{r3/r5} embryos.

galactosidase-stained hindbrains showed a smooth and uniform shape of r3 and r5 (Fig. 6E). By contrast, the overall morphology of r3 and r5 are clearly abnormal and clefts are present at the location of r3 and r5 in *kreisler*^{r3/r5} embryos (Fig. 6F). By E12.5, r3/r5 reporter gene expression has become restricted to narrow domains that precisely correlate with the regions of the neural epithelium that have failed to thicken (Fig. 6G,H).

DISCUSSION

Molecular and morphological changes in the hindbrain

In *kreisler*^{r3/r5} mice, formation of the normal number of hindbrain segments remains undisturbed, as indicated by the expression patterns of the transgene and of marker genes. The lack of a change in the total complement of segments can be

explained by the late and r3/r5 specificity of the *EphA4* enhancer used to direct ectopic kreisler expression. The ectopic expression of kreisler in r3 leads to changes at a molecular level consistent with an acquisition of r5 characteristics. The *Hoxa3*, *Hoxb3*, *Fgf3* and *Krox20* genes, which are co-expressed with kreisler in r5/r6 are either ectopically induced or show a prolonged expression in r3. However, the induction of *Fgf3* is relatively weak, suggesting either a requirement of additional kreisler-independent factors that are not present in r3 and/or a partial transformation of r3 to an r5 like identity.

Previous studies have shown that alterations in Hox gene expression patterns lead to transformations of rhombomere identity and to altered identities of cranial motor nerves (Alexandre et al., 1996; Bell et al., 1999; Goddard et al., 1996; Marshall et al., 1992; Rossel and Capecchi, 1999; Studer et al., 1998; Studer et al., 1996; Zhang et al., 1994). By analogy, the ectopic expression of *Hoxa3* and *Hoxb3* in r3 should result in changes in neuronal identity that are consistent with an r3 to r5 transformation, but because of the neuronal differentiation defect in r3, we were not able to analyse this. However, transient *EphA4* enhancer/kreisler transgenic embryos, some of which have lower levels of ectopic kreisler, display less severe defects in neuronal differentiation (data not shown). In these embryos, axons of r3 trigeminal motoneurons, which would normally leave the neural tube via an exit point located in r2, are now misrouted to an exit point in r4, consistent with conversion to an r5 facial motoneurone identity.

The finding that there is prolonged expression of *Krox20* in r3 because of ectopic kreisler expression suggests that *Krox20* maybe a direct or indirect target of kreisler. This is consistent with the deficiency in r5 in kreisler/*valentino* mutants (Frohman et al., 1993; Manzanares et al., 1999b; McKay et al., 1994; Moens et al., 1998; Moens et al., 1996), which could be due to a failure to upregulate expression of *Krox20* and other genes in presumptive r5 territory. As a consequence, a positive feedback loop between kreisler and *Krox20* may be established in kreisler^{r3/r5} mice, in which ectopic kreisler upregulates *Krox20*, which in turn upregulates the kreisler transgene under the control of the *Krox20*-dependent *EphA4* enhancer.

Abnormal facial branchiomotor neurone migration

Our analysis of cranial motor nuclei organisation in the hindbrain revealed an unexpected defect in the migration of fbm neurones. In wild-type embryos, the fbm nuclei migrate from r4 to finally settle in r6/r7 territory, whereas in kreisler^{r3/r5} embryos, fbm neurones pile up at the caudal boundary of r4 and form a massive nucleus, which erupts into the lumen of the fourth ventricle.

Recently, it has been shown that the migration of fbm neurones requires an interaction between migrating nuclei and the environment they encounter (Garel et al., 2000). Disruption of fbm neuronal migration could therefore indicate a differentiation defect where fbm neurones in r4 may have lost the ability to respond to environmental cues. Our molecular characterisation of these neurones and r4 markers suggests that r4 patterning is normal and argues against this possibility. The activity of the *EphA4* enhancer, which is used to drive kreisler gene expression in the hindbrain is tightly restricted to r3/r5, and fbm neurones have a normal pattern of expression of specification and differentiation markers, including *Isl1*, *Ret*, *Hoxb1* and *Phox2b*. Furthermore, kreisler^{r3/r5} mice are viable

and do not show behavioural abnormalities, suggesting that the facial motor nucleus is functional, despite its abnormal location in the lumen. Hence, the disruption of fbm migration appears not to arise from an intrinsic defect in their differentiation.

Alternatively, transgene expression may have influenced the environment of r5 through which fbm neurones have to migrate, such that it is not able to support their migration. Indeed, although the initial formation of r3/r5 is not affected by the transgene, subsequently there is a deficiency in r3 and r5 in the migration of neuronal precursors from the ventricular zone and subsequent differentiation. As fbm neurones migrate through the mantle layer adjacent to the ventricular zone (Gaufo et al., 2000), the deficiency of the mantle zone in r5 of kreisler^{r3/r5} mice could lead to lack of an appropriate substratum for fbm neuronal migration. We therefore favour the idea that alterations in the r5 environment, which are due to the extended expression of kreisler, underlie the disruption of fbm neuronal migration.

Effect of delayed neuronal differentiation on hindbrain morphology

The failure of the mantle layer to expand in r3 and r5 in kreisler^{r3/r5} embryos, whereas r2/r4/r6 thicken, leads to a buckling of the ventricular zone of r3 and r5. Consequently, although in dorsal views r3 and r5 appear shorter along the AP axis, this does not reflect a decrease in the size of the r3/r5 ventricular zone. However, a failure of cells to exit and differentiate might predict that there would be a major expansion of the r3/r5 ventricular zone, whereas this is not seen. One possible explanation is that there is size regulation of the ventricular zone of segments, as observed in some mutants in which hindbrain segmentation is affected. For example, r3 and r5 fail to be maintained and are likely to switch to r2/r4/r6 identity in *Krox20* mutants (Schneider-Maunoury et al., 1997; Schneider-Maunoury et al., 1993), but these even-numbered segments acquire a normal size. This is not accompanied by a detectable change in cell death, raising the possibility that small changes in cell proliferation rates could account for size regulation. We have not detected changes in the amount of apoptosis in kreisler^{r3/r5} embryos (T. T., L. A.-M. and D. G. W., unpublished), and thus favour a similar explanation for the absence of an expanded r3 and r5 ventricular zone.

Relationship between persistent kreisler expression and neuronal differentiation

The simplest explanation for the disruption of r3 and r5 differentiation in kreisler^{r3/r5} mice is that neuronal precursors must downregulate kreisler and/or its target genes in order to initiate migration and differentiation, and that this downregulation has been hindered through the prolonged activity of the *EphA4* r3/r5 enhancer and/or establishment of a positive feedback loop between kreisler and *Krox20* gene expression. Several models that are not mutually exclusive can explain such a requirement to downregulate kreisler and/or *Krox20* gene expression.

First, as r3 and r5 are specified by *Krox20* (Giudicelli et al., 2001; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993) and normally have delayed neuronal differentiation compared with even-numbered rhombomeres (Lumsden and Keynes, 1989), *Krox20* may directly or indirectly regulate the

timing of differentiation. Indeed, misexpression of *Krox20* in even-numbered rhombomeres leads to a severe delay and impairment of neurogenesis (Giudicelli et al., 2001). However, it is possible that in these latter experiments, the delay in differentiation is because forced expression of *Krox20* from a constitutive enhancer prevents its downregulation in neuronal precursors rather than only being due to a respecification of cells in the ventricular zone to r3/r5 identity.

A second model is that the regional expression of kreisler, *Krox20* and/or Hox genes is involved in maintaining segmentally regulated cell proliferation in the neural epithelium, and thus their downregulation is required in cells that cease proliferation in order to become postmitotic neuronal precursors. Indeed, in *valentino* zebrafish mutants, there is a failure to expand r5 from a small population of *Krox20*-expressing cells, and the hindbrain is one segment shorter (Moens et al., 1996), consistent with a role in cell proliferation in r5. It is unlikely that *Krox20* is required for this expansion, as presumptive r5 territory is initially normally sized in *Krox20* mutants (Schneider-Maunoury et al., 1993). It is intriguing that *Phox2b* is required to generate the correct number of postmitotic precursors, and that forced expression of *Phox2b* in the ventricular zone causes premature cell cycle exit and differentiation (Dubreuil et al., 2000). This raises the possibility that the decreased number of *Phox2b*-positive neurones in r3 and r5 in kreisler^{r3/r5} embryos reflects a repression of *Phox2b* by kreisler or its targets. Such a regulatory relationship would ensure that in wild-type embryos, a switch occurs from maintenance of proliferation in the ventricular zone (by expression of kreisler and absence of *Phox2b*) to the differentiation and cell cycle exit of cells (downregulation of kreisler, enabling later upregulation of *Phox2b*). An analogous situation is suggested by studies of *Pax6* gene function in the developing telencephalon. *Pax6* is normally expressed in cortical progenitor cells and expression becomes downregulated with the onset of neurogenesis. Loss of *Pax6* function results in premature differentiation of neurones with early differentiation markers now present in the ventricular zone (Caric et al., 1997).

A third possibility is that downregulation of kreisler and *Krox20* expression or their Hox targets is required because the later expression of these genes in specific subsets of hindbrain neurones (Eichmann et al., 1997; Wilkinson et al., 1989) reflects later roles in aspects of neuronal specification. For example, the patterns of Hox expression are dynamic and vary along the DV axis with the birth of major classes of neurones (Graham et al., 1991). Furthermore, analysis of Hox mutants has also shown that there is abnormal patterning, differentiation or migration of subsets of neurones, indicating that Hox genes have important later roles in regulating neuronal development (Davenne et al., 1999; Tiret et al., 1998). A failure to downregulate kreisler, *Krox20* and/or their Hox gene targets in cells within the ventricular zone that initiate differentiation would thus lead to mis-specification of neurones. We propose that this is prevented by ensuring that neuronal differentiation does not proceed until correct downregulation of these genes has occurred. Segmental expression of kreisler and *Krox20* is transient and occurs in the ventricular zone only during early stages of neurogenesis, whereas segmental Hox expression is maintained throughout later stages. This pattern of expression reflects early roles of

Kreisler and *Krox20* in establishing hindbrain segments and regulation of early enhancers of Hox genes, whereas segmental Hox gene expression is subsequently maintained (Wingate and Lumsden, 1996) under later-acting enhancers. This persistent expression of Hox genes is required for the AP specification of cells in the ventricular zone, but needs to be downregulated in most neurones as they initiate differentiation. The downregulation of kreisler and *Krox20* expression is therefore unlikely to regulate the timing of differentiation directly, rather it may be a pre-requisite for switching Hox gene regulation to the later enhancers that are correctly downregulated during the onset of differentiation.

Whereas there is much knowledge of transcription factors and gene regulatory sequences that initiate and maintain segmental gene expression in the hindbrain (Trainor et al., 2000), less is known regarding the downregulation of genes. Two proteins, Nab1 and Nab2, bind to and repress the function of the transactivation domain of *Krox20* protein, and have been shown to be downstream targets of *Krox20* in r3 and r5 (Mechta-Grigoriou et al., 2000). These proteins are thus involved in a negative feedback loop that limits *Krox20* expression. It would seem likely that correct downregulation of kreisler would also require active mechanisms, and it will therefore be interesting to determine whether targets of kreisler act in a negative feedback loop.

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