

Induction of a Parafacial Rhythm Generator by Rhombomere 3 in the Chick Embryo

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Observations of knock-out mice suggest that breathing at birth requires correct development of a specific hindbrain territory corresponding to rhombomeres (r) 3 and 4. Focusing on this territory, we examined the development of a neuronal rhythm generator in the chick embryo. We show that rhythmic activity in r4 is inducible after developmental stage 10 through interaction with r3. Although the nature of this interaction remains obscure, we find that the expression of *Krox20*, a segmentation gene responsible for specifying r3 and r5, is sufficient to endow other rhombomeres with the capacity to induce rhythmic activity in r4. Induction is robust, because it can be reproduced with r2 and r6 instead of r4 and with any hindbrain territory that normally expresses *Krox20* (r3, r5) or can be forced to do so (r1, r4). Interestingly, the interaction between r4 and r3/r5 that results in rhythm production can only take place through the anterior border of r4, revealing a heretofore unsuspected polarity in individual rhombomeres. The r4 rhythm generator appears to be homologous to a murine respiratory parafacial neuronal system developing in r4 under the control of *Krox20* and *Hoxa1*. These results identify a late role for *Krox20* at the onset of neurogenesis.

Key words: central pattern generator; transcription factor; *Krox20*; rhombencephalon; chick embryo; neurogenesis

Introduction

By developmental HH stage 10 (Hamburger and Hamilton, 1951) in chicks, the hindbrain region is subdivided along the anteroposterior (AP) axis into seven to eight rhombomeres (r) and starts neurogenesis (Lumsden and Keynes, 1989). Rhombomere-restricted gene expression patterns such as *Krox20* and *Hox* (for review, see Lumsden and Krumlauf, 1996) orchestrate rhombomere formation. How rhythmic neuronal circuits are generated in relation to rhombomeres is not known. The r3/r4 territory has been implicated in rhythmogenesis both by microsurgical rhombomere isolation experiments in chick embryos (Fortin et al., 1999) and by inactivating genes that are involved in specifying r3 or r4 development (e.g., *Krox20*, *Hoxa1*) (Jacquin et al., 1996; Helmbacher et al., 1998; Dominguez del Toro et al., 2001; Chatonnet et al., 2002; Borday et al., 2003). Furthermore, physiological studies of neonatal mice have identified rhythmic neurons of the parafacial respiratory group, located ventral to the

facial motor nucleus (deriving from r4), as an important component of primary respiratory rhythmogenesis (Mellen et al., 2003; Onimaru and Homma, 2003).

In both mouse and chick embryos, the intrinsic neuronal activity of the hindbrain starts with a low frequency (LF) (range, min⁻¹) bursting pattern. In chick, activity evolves during embryonic day 6 (E6) when each LF burst is followed by a progressive accumulation of cyclical bursts at high frequency (HF) (range, sec⁻¹). By E7–E9, network activities are dominated by these episodic activities generated at the facial level (Fortin et al., 1999). In rhombomere isolation experiments in chick, we found that the episodic rhythm in r4 emerges when r4 is left in contact with its anterior neighbor (r3) but does not emerge when r4 is left in contact with only its posterior neighbor, r5 (Fortin et al., 1999).

The aim of the present study was to investigate the relationship between r3 and r4, whereby a segmental program initiated in r3 instructs formation of a parafacial rhythmic network. Our approach has centered on grafting individual rhombomeres heterotopically, so as to produce artificial rhombomere pairs. *Krox20* is a candidate to initiate the r3 program as it confers odd identity to rhombomeres 3 and 5 (Giudicelli et al., 2001). We used *in ovo* electroporation to force *Krox20* expression in *Krox20*-negative rhombomeres and to investigate the previously observed polarity of interaction between r3 and r4 (Fortin et al., 1999) performed AP reversals of these rhombomere. Altogether, grafting, electroporation, and reversal procedures identify *Krox20*-expressing territories as sufficient for instructing episodic behavior in r4, regardless of the cellular environment (r3, r5, r1, r4) in which the gene is expressed. We also show that the interaction depends on an intrinsic AP polarity in r4.

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Materials and Methods

Animals and hindbrain preparations: the rhombomere isolation procedure. Fertilized commercial hens' eggs were incubated to HH9–HH10. Donor neural tubes for the rhombomere-grafting experiments were obtained after the hindbrain had been dissected out and treated with dispase (1 mg/ml in Leibovitz 15 medium) for 15 min. The neural tube, dissected free of adherent tissue using flame-sharpened tungsten needles, was transected at the level of rhombomere boundaries, making sure that the boundary and the immediately adjacent cells were excised; the AP orientation of the excised rhombomere was marked by leaving tags of dorsal ectoderm on one-half of the explant. All rhombomere explants included both left and right sides. For transplantation, stage-matched host eggs were windowed and the vitelline membrane reflected. A gap in the neural tube corresponding to the desired heterotopic site of insertion of the graft was created using tungsten needles. The graft was then moved into place, the right or left placement of the ectoderm being maintained or inverted in the host embryo, and the egg resealed. For experiments in which r1 was grafted in place of r3, HH9 donor embryos were used to ensure that r1 and r3 would be similar in size. After grafting, eggs were reincubated for ~2 hr before further isolation of segments and segment pairs. Isolations were made by excising both the immediately rostral and caudal pairs of rhombomeres as described previously (Fortin et al., 1999). The isolation procedure results in an "island" of rhombomere tissue with no connections either anteriorly or posteriorly with the remaining regions of neural tube. These islands round up at their cut surfaces to form ovoid vesicles. The eggs were then sealed again and incubated for either 24 hr (for whole-mount *in situ* hybridization; see below) or for 5–6 d until the embryos reached E7 (for electrophysiological recordings). Preparations consisting of either whole hindbrains or isolated rhombomere islands were then made by dissecting out the tissues and transferring them to a 2 ml recording chamber superfused at a rate of 2 ml/min with a physiological solution composed of the following (in mM): 120 NaCl, 8 KCl, 0.58 NaH₂PO₄, 1.15 MgCl₂, 1.26 CaCl₂, 21 NaHCO₃, 30 glucose, aerated with carbogen (5% CO₂, 95% O₂), pH 7.3, at 30°C (Fortin et al., 1999).

Plasmid constructs and electroporation. A *Krox20* expression plasmid, pCAB-*mKrox20*, was constructed by subcloning a *Clal/NotI* sequence containing the 1.4kb full-length sequence of *mKrox20* into the pCAB^{CMV}_{IRES} *mGFP6* plasmid. The *mKrox20* sequence was excised from the *pSlax-mKrox20* adaptor plasmid usually used for cloning into retroviral vectors. The internal ribosomal entry site (IRES) in pCAB^{CMV}_{IRES} *mGFP6* is not active, and therefore only *mKrox20* is transcribed in electroporated cells. The activity of this construct was tested by *in situ* hybridization. The activity of the CAB-*mGFP6* reporter plasmid was verified by green fluorescent protein (GFP) fluorescence ~6–8 hr after electroporation. An equimolar solution of pCAB-*mKrox20* and pCAB-*mGFP6* at concentrations of 0.5 µg/µl was pressure injected into the lumen of the neural tube through glass capillaries (tip diameter, ~10 µm) inserted through the roof plate at the 1–2 somite level. The DNA was diluted in PBS with 1.5% Fast Green to make the DNA solution visible. Electroporation was performed by delivering four to six square pulses (10 V and 50 msec) with a pulse-isolated stimulator (Digitimer, Hertfordshire, UK) through anodal and cathodal silver wire (diameter, 1 mm) electrodes placed ~1.5 mm apart on either side of the embryo at the r1/r2 or r4 level. Polarity of the current could be reversed or not so as to produce bilateral (data not shown) or unilateral penetration of the plasmids. Control embryos were electroporated with the GFP plasmid only. Electroporated donor embryos destined to grafting experiments were reincubated for 1–2 hr before being prepared (as above).

Electrophysiology. Nerve recording techniques have been reported previously (Fortin et al., 1995). The proximal root of a motor nerve was aspirated into a suction electrode connected to a high gain AC amplifier

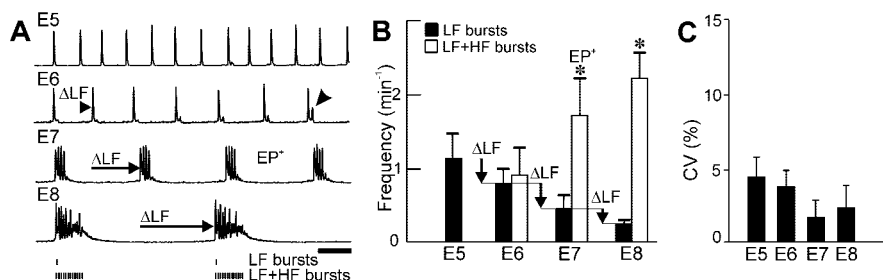


Figure 1. Appearance of episodic patterning of activities (EP⁺): transition from LF primordial activity. Central pattern generators for LF and episodes are identified by recording neuronal activities after isolation of the hindbrain *in vitro*. *A*, Integrated facial neurograms recorded at different developmental stages (E5–E8) show a progressive decrease (Δ LF; horizontal arrows) in the frequency of LF bursts initiated before E6 and maintained thereafter. *B*, The EP⁺ pattern is identified by the significant difference (*) between the frequency of LF bursts (black bars) and that of LF + HF bursts (empty bars). Note the exclusive presence of LF bursts at E5 and the emergence (A, arrowhead on the E6 trace) of HF bursts at E6. *C*, Regularity of rhythm generation, measured by the CV of inter-LF burst interval, is maintained during development. Scale bar, 1 min.

(Grass P511; Grass Instruments, Quincy, MA). Amplified neurograms filtered using a bandwidth (3 Hz to 3 kHz) were rectified and integrated through an analog integrator (Grass 7P3B; Grass Instruments) with a time constant of 50 msec. Upward deflections of the rectified, integrated neurogram corresponding to burst discharges of the nerves were acquired using the Acquis1 software package (developed by G. Sadocq, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France) and automatically detected using a threshold device.

Anatomy. *In situ* hybridization with digoxigenin (DIG)-labeled and fluorescein isothiocyanate (FITC)-labeled riboprobes for *mKrox20* and DIG-labeled riboprobes for *cHoxb1* was performed as described previously (Wilkinson, 1992). Embryos were harvested in Howard's Ringer or Tyrode's saline and fixed in 4% paraformaldehyde at 4°C until ready for processing. Endogenous *cKrox20* was detected by hybridizing the embryos with the *mKrox20* probe at 65°C. DIG-labeled probes were detected using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium as substrates (blue stain) and FITC-labeled probes using FastRed (red stain). Biocytin-labeled neurons were revealed using signal amplification with the ABC method (Vectastain Elite ABC kit PK6100; Vector Laboratories, Burlingame, CA) and standard diaminobenzidine procedure.

Assessment of the rhythm regularity. A modal distribution of inter-burst intervals shorter than 15 sec corresponding to bursts generated at high frequency (HF bursts) indicated an episodic patterning of activity. The episodic pattern (EP⁺) has been validated statistically (Mann–Whitney *U* test) by rejection of the null hypothesis that the medians for both frequency of LF bursts (longer than 15 sec) (Fig. 1A, LF burst marks under E8 neurogram) and that of the overall LF bursts and HF bursts production (Fig. 1A, LF+HF bursts marks under E8 neurogram) were equal ($p < 0.01$) (Fig. 1B, asterisks on histograms). In this way, EP⁺ and EP[−] patterns can be discriminated from pacemaker mechanisms controlling the regularity of activities otherwise quantified by measurement of the mean coefficient of variation (CV, SD/mean) of inter-LF burst intervals (Fig. 1C). For preparations having an activity exclusively composed of LF bursts, only the frequency of LF bursts (black bars) is presented on summary histograms. In the text and Table 1, data are expressed as mean \pm SD, and the figure error bars represent SD.

Results

EP⁺ identification

Electrophysiological recordings of the facial nerve in whole hindbrain preparations at E5–E8 (Fig. 1A) showed that the low-frequency generation of single-burst discharges (LF bursts) recorded at E5 begins to change during E6 when additional bursts are produced at high frequency (HF bursts) after LF bursts to form an episode of activity (LF+HF bursts). This episodic burst production at E6 is preceded by (E5–E6) and later associated with (E7–E8) a decrease of the frequency at which LF bursts are produced (Fig. 1A,B, Δ LF). The regularity of the episodic rhythm,

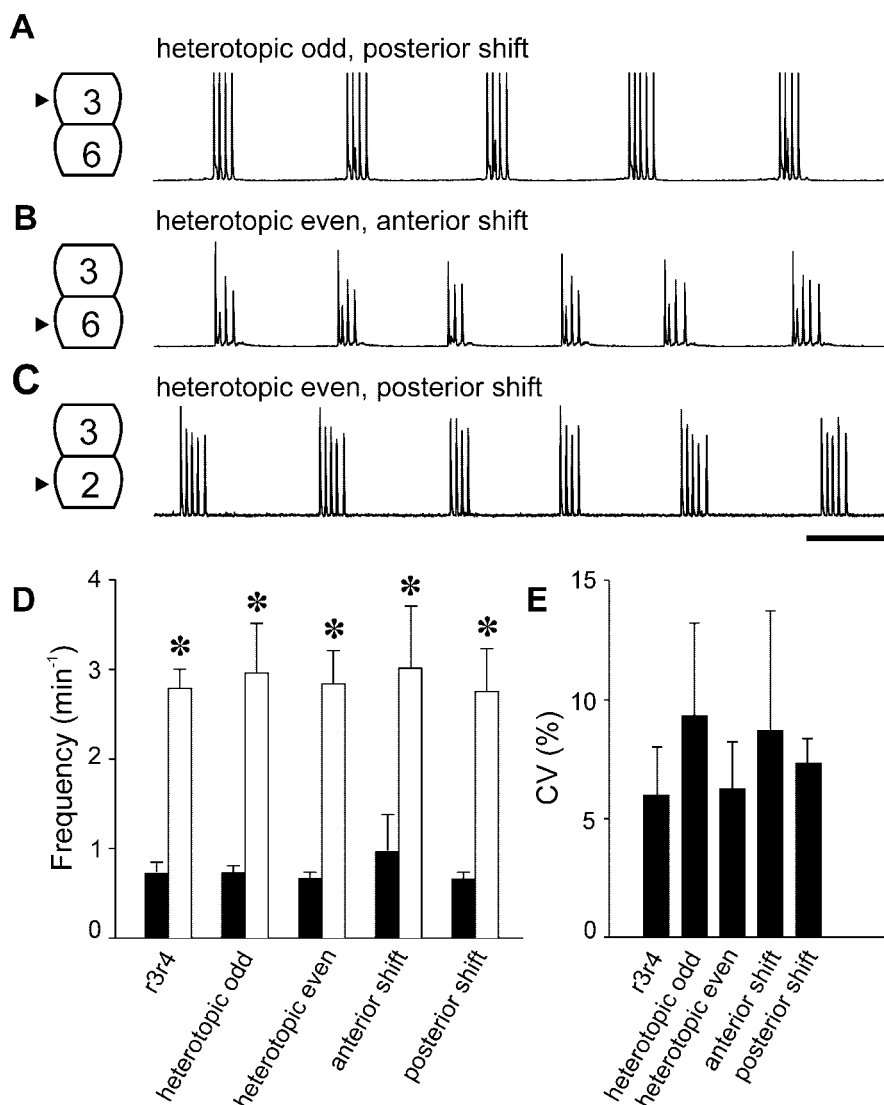


Figure 2. Anterior-odd rhombomeric code for the induction of EP⁺. Integrated glossopharyngeal (A, B) or trigeminal (C) neurograms obtained after heterotopic grafting and isolation. Transplantations of the odd r3 (in r5 position; A, arrowhead) and of the even r6 (in r4 position; B, arrowhead) or r2 (in r4 position; C, arrowhead) lead to r3r6 or r3r2 preparations showing an episodic patterning similar to that obtained from the normal configuration r3r4. D, E, Summary histograms of the frequencies of LF bursts and LF + HF bursts (D, filled and empty bars, respectively) and of CV (E) obtained after pooling preparations according to the heterotopic status of either odd (r3, r5) or even (r2, r4, r6) rhombomeres (detailed in Table 1) and the anterior or posterior status of the displacement along the neuroaxis (anterior shift and posterior shift) of rhombomeres, whatever their parity (detailed in Table 1). In all cases, an EP⁺ pattern comparable with that of the normal r3r4 pair resulted. Surgical isolation, shift, and grafting of rhombomeres do not interfere with the development of the EP⁺ in artificial rhombomere pairs in which the odd rhombomere is in contact with the anterior edge of the even rhombomere. Scale bar, 1 min.

estimated from the low values of CV of inter-episode time intervals (Fig. 1C, CV), remains high during this process. Thus, we could ascertain the ability for a preparation to produce episodes (EP⁺) from the significant difference ($p < 0.01$) (Fig. 1B, asterisk) between frequency values of LF and LF + HF burst discharges recorded at E7, a developmental stage at which the hindbrain has been found to be EP⁺ in 100% of cases (Table 1). In EP⁺ preparations comprising single odd rhombomeres or odd–even pairs of rhombomeres (e.g., r3r4) (Table 1) (Fortin et al., 1999), an episodic pattern was observed in 80% of cases at E6 (16 of 20) but in 0% at E5, demonstrating that the rhombomere isolation procedure does not interfere with the kinetics of activity maturation. In contrast, when recording from preparations comprising single even rhombomeres or even–odd pairs of rhombomeres (e.g.,

r4r5) (Table 1) (Fortin et al., 1999), the episodic activity does not appear at E6 and is absent at E7 (EP[−]). At E7, all EP[−] preparations retained an immature elevated frequency of LF bursts, lacking the characteristic Δ LF of EP⁺ segments (Table 1, compare values for r4r5 at E6 and E7). At later stages of development, EP[−] preparations tended to develop the episodic pattern. Indeed, the occurrence of EP⁺ noted in 10% (2 of 17) of the cases at E7 began to increase at E8 (37%; 3 of 8 cases) reaching significance (63%; 5 of 8 cases; χ^2 , $p < 0.01$) at E9. Thus, EP[−] preparations seem considerably delayed in the developmental program leading to episodic patterning, which is tightly linked to the development of LF.

A rhombomeric code enabling development of episodic bursts

The EP⁺ pattern could be recorded from the facial motor root (r4) in all cases where r5 was grafted in heterotopic position anterior to the even rhombomere (r5⁺r4) (Table 1). The EP⁺ pattern was also recorded in one case where r4 was grafted posterior to r5. Episodic activities were comparable with those obtained with normal r3r4 segments.

Although episodic rhythm generation at the facial level predominates in the intact hindbrain (Fortin et al., 1999), EP⁺ was also obtained from the glossopharyngeal motor root (r6) in normal r5r6 preparations (Table 1) or when r3 had been grafted into the r5 position (Fig. 2A) or r6 into the r4 position (Fig. 2B). EP⁺ was also produced by the trigeminal motor root (r2) when r5 was grafted anterior to r2 (data not shown) (Table 1) or r2 caudal to r3 (Fig. 2C). As for the facial nerve, the trigeminal was EP[−] in preparation where r1 was left in normal contact with the even rhombomere (r1r2) (Table 1). In contrast, an EP[−] identical to that obtained with r4 alone (Fig. 3A) was obtained by grafting r1 anterior to r4 (Fig. 3C) or by grafting r3 posterior to r4 ($p = 0.147$).

Altogether, the graft configurations match an “anterior odd” rhombomeric code for EP⁺ patterning: all rhombomere pairs in which the odd rhombomere r3 or r5 (but not r1) is in contact with the anterior edge of the even rhombomere produce the EP⁺ pattern (Fig. 2D) with a low CV (Fig. 2E), regardless of which rhombomere had been displaced or whether it had been displaced anteriorly or posteriorly along the neuraxis (Fig. 2, Table 1).

Development of the episodic network is induced by *Krox20* electroporation in r4

To identify a possible role of *Krox20*, which is expressed in r3 and r5 but not r1, and which is known to specify aspects of r3 and r5 character (Giudicelli et al., 2001), we followed a gain-of-function

strategy. First, we wanted to see whether ectopic expression of *Krox20* in r4 would induce EP⁺ when the rhombomere is isolated from both r3 and r5. Normal isolated r4 is EP[−] (Fig. 3A), indicating that a prolonged odd–even interaction is required to develop an episodic network. However, when *Krox20* is ectopically expressed from HH10 in r4, the isolated rhombomere developed EP⁺ (Fig. 3B, Table 1). As demonstrated by Giudicelli et al. (2001), ectopic expression of *Krox20* in r4 initiates a program that attempts to convert cells to an r3 or r5 identity generating mixed cellular populations. Assuming that ectopic *Krox20* has the same outcome in our hands, it is likely that the production of an indeterminate mix of r3, r5, and r4 cells in the targeted territory confers properties sufficient to induce formation of an episodic generator.

Krox20-expressing r1 exerts an EP⁺-inducing influence on r4

To further explore the role of *Krox20*, we next combined the electroporation procedure with heterotopic grafting to form abnormal rhombomere pairs. We made r1r4 preparations (Fig. 4) because r1 does not normally express *Krox20* or any of the *Hox* genes expressed in posterior rhombomeres (Lumsden and Krumlauf, 1996). As shown above, the r1r4 preparations are EP[−] in all cases (Fig. 3C, Table 1). After electroporation of donor embryos with *Krox20* and *GFP* plasmids, we grafted r1 in place of host r3, followed by microsurgical isolation of the rhombomere pair. In these preparations, the r1 territory expressed both *Krox20* and *GFP* or *GFP* alone (Fig. 4) (data not shown). All *Krox20*-expressing r1r4 preparations (Fig. 3D) and none of those expressing *GFP* only developed EP⁺ (Table 1). Robustness of induction is shown by the ability to obtain the EP⁺ pattern even when restricting (2 of 5 cases) *Krox20* expression to one side of the donor r1 segment by unilateral electroporation (Fig. 4). Altogether, the results obtained by electroporation suggest that *Krox20* expression in r1 is sufficient to drive a specific AP interaction leading to episode generation in r4.

AP reversal of r4, but not r3, impairs development of the rhythm generator

To identify which of the two rhombomeres in the r3r4 EP⁺ preparation exerts directional control on the other, we inverted the AP polarity of each rhombomere independently. Inversion of r3 [Table 1, flipped (f) (r3)r4] did not change the EP⁺ outcome (Fig. 5E, compare a, b), showing that AP polarity of r3 is not critical. This is consistent with the view that r3 acts as a homogenous source of EP⁺-inducing activity. However, reversal of r4 resulted in dramatically altered development of the rhythm (Fig. 5C). First, an EP[−] pattern resulted [Table 1, r3f(r4)]. Second, the rhythm became irregular as indicated by an increased CV (from

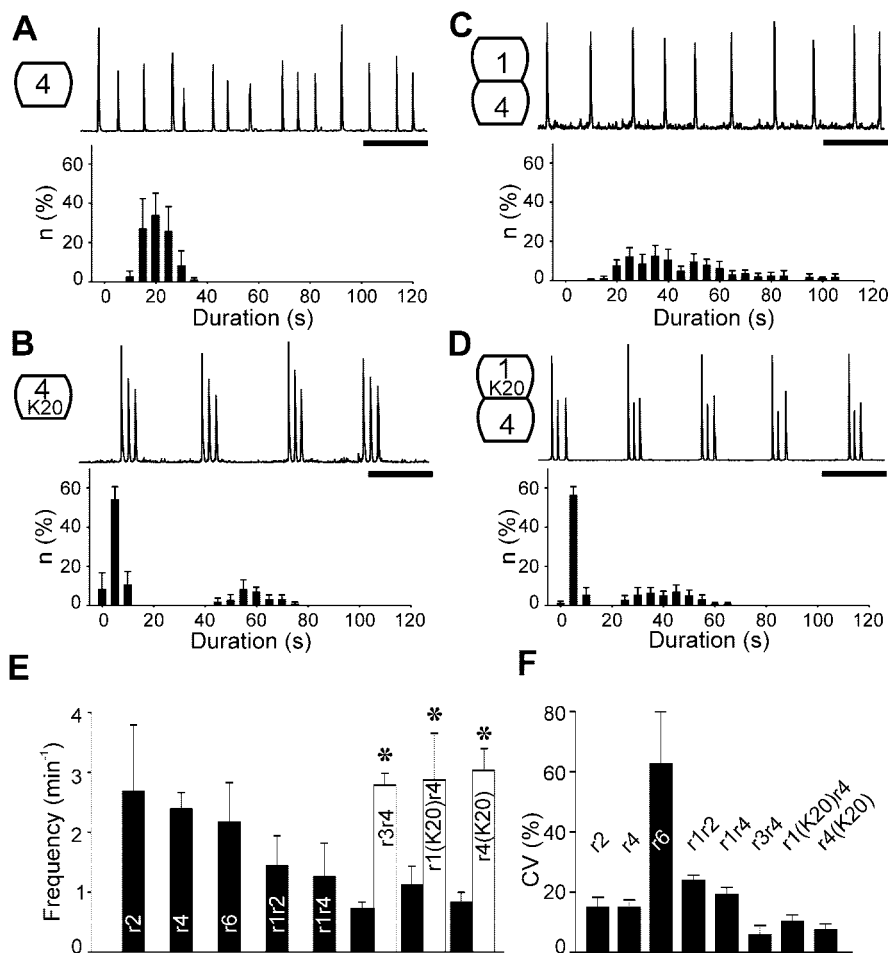


Figure 3. Electroporation of *Krox20* confers the EP⁺ pattern to the r4 and r1r4 EP[−] segments. Integrated neurograms (top) and corresponding compound distribution of interburst interval durations (bottom; n% ordinates; duration abscissae) recorded at E7 from the facial nerve root after development of the neuronal network deriving from EP[−] r4 (A) and r1r4 (C). An EP⁺ pattern of activity (B, D) results in 4 misexpressing *Krox20* (4K20 in B) and in r1r4 segments when r1 misexpresses *Krox20* in the pair (1K20 in D) resulting in a bimodal distribution of interburst interval. E, Summary histogram of the frequency of LF bursts and LF + HF bursts (E, filled and empty bars, respectively) showing that r2, r4, r6, r1r2 (normal configurations), and r1r4 are EP[−]. Electroporation of *Krox20* induces EP⁺ in r4 and r1r4 (asterisks; compare with the normal configuration, r3r4). F, CV is not significantly affected in these experiments and is particularly large for r6. Scale bar, 1 min.

0.05 to 0.23). This was not observed after reversal of the entire r3r4 (see Fig. 7C) [Table 1, f(r3r4)] that led to normal EP⁺.

Mechanisms responsible for the regularity of the rhythm would be distinct from those generating episodes because grafting r3 caudal to r4 produces a regular bursting pattern (CV, 0.12) but no episodes (Fig. 5D; Table 1, r4r3*). Altogether, these experiments show that AP reversal of r4, but not r3, alters the functional organization of the rhythmic neuronal network. This suggests that in the r3r4 pair, the rhythmic network is located in r4, not r3, whereas r3, not r4, is the source of an inter-rhombomeric influence that is necessary for it to become EP⁺ and function appropriately.

AP reversal of r4 in r4r5 preparations produces EP⁺

Our findings with r3r4 preparations (Fig. 5C, D) indicate that reversal of r4 disrupts the rhombomeric code enabling development of episodic bursts. These results suggest that even if a “contact with the anterior odd rhombomere” position along the neuraxis is preserved, the EP⁺ phenotype also requires correct AP polarity of the even rhombomere. We further investigated this hypothesis, starting from the EP[−] pair r4r5 (Figs. 6, 7D), a good

model to investigate how EP⁺ potentialities in the other *Krox20*-expressing rhombomere, r5, interact with r4 in the absence of r3 (Fig. 6, compare a, d). In the r4r5 preparation, we inverted the AP polarity of the r4 segment (Figs. 6C, 7E) or of the entire r4r5 region (Figs. 6B, 7F). Rhombomere 4 reversal, bringing its anterior edge into contact with r5, produced EP⁺ activity, which was not detected when reversing the AP orientation of entire r4r5 pair (Fig. 6B, compare c and b in E). Finally, the EP⁺ was fully restored by grafting r5 anterior to r4 (Fig. 6D) or r4 posterior to r5 (data not shown) (Table 1). These experiments failed to induce any EP⁺-repressing (Fig. 6C,E) or EP⁺-promoting (Fig. 6A,E) effects of r5 in its normal (caudal) situation. However, r5 shows the same property as r3 to induce formation of EP⁺ activity provided it is in contact with the anterior border of r4. Regularity of the rhythm is altered in r4r5 preparations when rendered EP⁺ by reversal of the AP polarity of r4 or when preserving EP[−] after AP inversion of the entire r4r5 region (Fig. 6F, compare CV in a and b, c and d), confirming that distinct mechanisms control episode formation and the regularity of their recurrence.

Discussion

Our results help to understand development of a parafacial rhythmic system and allow us to propose a novel functional

Table 1. Characterization of EP⁺ and EP[−] preparations

Preparations	n	LF bursts	LF + HF bursts
EP[−]			
E5 hindbrain	7	1.15 ± 0.34	id.
E6 hindbrain	5	0.80 ± 0.20	0.92 ± 0.21, NS
r2	3	2.66 ± 1.12	id.
r4	4	2.38 ± 0.28	id.
r6	5	2.15 ± 0.66	id.
r2r3	3	1.11 ± 0.27	id.
r4r5 (at E6)	5	2.25 ± 1.01	id.
r4r5	9	2.20 ± 0.67	id.
r1r2	2	1.42 ± 0.49	id.
r1r4	7	1.26 ± 0.57	id.
r1(GFP)r4	3	1.10 ± 0.23	id.
f(r4r5)	9	1.21 ± 0.35	1.96 ± 1.03, NS
r3f(r4)	4	1.28 ± 0.20	1.64 ± 0.36, NS
r4r3*	2	2.02 ± 0.12	2.21 ± 0.27, NS
EP⁺			
E7 hindbrain	21	0.45 ± 0.21	1.75 ± 0.51
E8 hindbrain	8	0.24 ± 0.06	2.27 ± 0.35
r3r4	6	0.72 ± 0.08	2.78 ± 0.21
r5r6	4	0.79 ± 0.10	2.55 ± 0.24
r5*r4	3	1.10 ± 0.51	3.46 ± 0.33
Heterotopic odd	5	0.72 ± 0.14	2.96 ± 0.54
Heterotopic even	4	0.67 ± 0.12	2.84 ± 0.39
Anterior shift	5	0.96 ± 0.41	3.02 ± 0.69
Posterior shift	4	0.66 ± 0.12	2.75 ± 0.49
r4(K20)	4	0.84 ± 0.15	3.01 ± 0.38
r1(K20)r4	5	1.05 ± 0.32	2.85 ± 0.81
f(r3)r4	7	0.75 ± 0.13	2.95 ± 0.62
f(r3r4)	7	0.72 ± 0.14	3.11 ± 0.94
f(r4)r5	5	0.96 ± 0.62	3.13 ± 0.82

Summary table of the values of LF burst and LF + HF burst frequencies (minute^{−1}) measured on the different preparations at E7. Anteroposterior position of rhombomeres in rhombomere pairs is indicated from left to right (e.g., r4r5-r4 anterior to r5). An asterisk to the right of a rhombomere indicates that the rhombomere has been heterotopically grafted (e.g., r4r3*-r3 grafted in heterotopic r5 position). The f preceding rhombomeres in brackets indicates their flipped anteroposterior orientation. GFP or K20 in a bracket to the right of a rhombomere indicates that this rhombomere was targeted by electroporation of the sole GFP or the GFP and *Krox20* expression vectors, respectively. Heterotopic odd and even refer to compound measurements pooling, respectively, r3*r6 (n = 1), r5*r4 (n = 3), and r5*r2 (n = 1) and r3r6* (n = 1), r3r2* (n = 2), and r5r4* (n = 1) preparations. Anterior and posterior shift refer to compound measurements obtained pooling, respectively, r3r6* (n = 1), r5*r4 (n = 3), and r5*r2 (n = 1) and r3*r6 (n = 1), r3r2* (n = 2), and r5r4* (n = 1) preparations. id., Idem; NS, no significant difference between frequency values of LF bursts and LF + HF bursts.

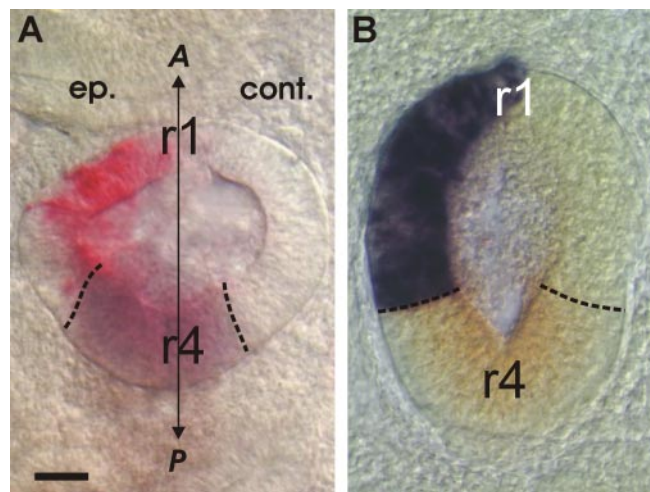


Figure 4. Whole-mount r1(K20)r4 islands 24 hr after electroporation grafting and isolation. *A*, *B*, Two examples of rhombomere islands (dorsal views) produced by heterotopic grafting in r3 position of r1 territories taken from donor embryos that had been submitted to unilateral electroporation of mixed GFP and *Krox20* expression plasmids at the r1 level, before engraftment and isolation of the r1r4 island. Note the overt healing at boundaries (*A* and *B*, dotted lines) separating r1 anterior and r4 posterior and the ovoid aspect taken by the isolated rhombomere pairs. Forced *Krox20* expression (red in *A*, dark blue in *B*) is visible on the electroporated side (ep.) on the left of the medial axis (*A*, vertical line) but not on the control side (cont.; right) of the r1 territory. *Krox20* expression is confined within r1 (*A*, *B*), and a sharp AP limit of expression is visible on the ep. side in *B*, whereas endogenous bilateral *Hoxb1* expression (blue in *A*, not probed in *B*) is restricted to r4. The activity produced by this rhombomeric configuration is illustrated in Figure 3D. *A*, Anterior; *P*, posterior. Scale bar, 50 μ m.

role for *Krox20* in initiating the induction of the episodic generator during a restricted period of development, between HH10 (time of grafting) and HH15–HH20 (*Krox20* downregulation). *Krox20* expression starts in r3 as early as at HH8, before the onset of hindbrain segmentation (Nieto et al., 1991; Irving et al., 1996; Giudicelli et al., 2001). However, we show that several hours later, at HH10, induction of the episodic activity is blocked in a variety of rhombomeric configurations but can be restored by *Krox20* electroporation. All even rhombomeres are EP[−], although by this stage, their neighboring rhombomeres already express *Krox20*. Hence, there is no early commitment to the production of the episodic pattern. Indeed, all artificial rhombomere pair combinations behave in strict accordance to a “anterior odd” code that either exists normally or can be produced by grafting. Thus, early AP positional value (Guthrie and Lumsden, 1992; Simon et al., 1995; Grapin-Botton et al., 1998; Dupé and Lumsden, 2001) has no obvious influence on the future pattern of rhythmic activities. The processes leading to episodes certainly begin after HH10 and probably cease with the downregulation of *Krox20* expression in r3 at HH15–HH20 (Wilkinson et al., 1989; Schneider-Maunoury et al., 1993, 1997).

Our results indicate the importance of heterochronic development of rhombomeres in the hindbrain by which gene expression, boundary formation, and neurogenesis do not take place at the same time in adjacent rhombomeres. Between HH10 and HH15–HH20, r3 and r5 display a marked delay compared with even rhombomeres in the timing of neuronal differentiation and axonal outgrowth (Lumsden and Keynes, 1989; Schneider-Maunoury et al., 1997; Giudicelli et al., 2001). We now show that with respect to the rhythm regularity and episodic patterning of activities, susceptibility to AP reversal at HH10 is restricted to r4. Therefore, heterochrony of neurogenic processes at the parafacial level of the neuraxis allows the simultaneous neuronal differentia-

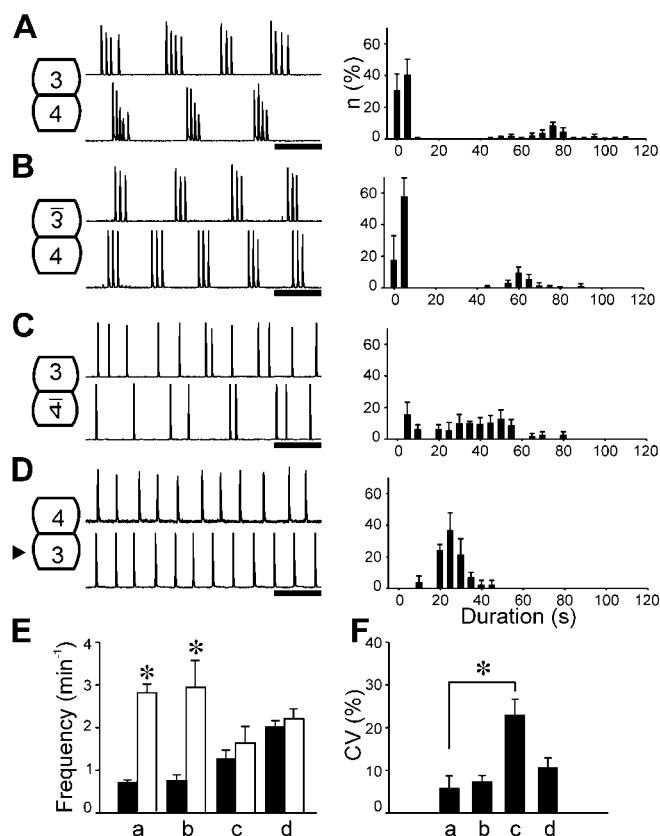


Figure 5. Anteroposterior orientation of r4, but not r3, is required for the development of a stable rhythm generator. *A–D*, Two examples (superposed traces) of integrated motor activity (left) recorded from distinct rhombomere pair preparations (left schematic) and compound histograms of the corresponding distribution of interburst interval duration (right) recorded from the facial nerve. *A, B*, Episodic patterning is present in r3r4 (normal configuration) and r3r4 after AP reversal of r3 (*B*), absent (*C, D*) in r3r4 after AP reversal of r4 (*C*), and in r4r3 after heterotopic transplantation of r3 (in r5 position; *D*, arrowhead). *E, F*, Summary histogram of the frequency of LF bursts and LF+HF bursts (*E*, filled and empty bars, respectively) and of CV (*F*) of the different preparations indicated by their lettering in the figure. Reversal of r4 eliminates regularity of the rhythm as indicated by significantly higher mean CV in *F* (compare *c, a*). Note also in *E* that modifying the relative position of r3 with respect to anteroposterior polarity of r4 abolishes (in *c* and *d*) the development of episodes (*a* and *b*). Scale bar, 1 min.

tion in r4 and *Krox20* expression in r3, a feature that seems important to the establishment of mature rhythmogenic properties.

Krox20 in r3 exerts an EP⁺-inductive role after HH10

It is unlikely that *Krox20* effects the induction of EP⁺ in r4 by the generation of some specific subtype of r3 neuron with posteriorly directed processes, because AP inversion of r3 does not affect the induction of EP⁺. Indeed, other studies show that *Krox20* inhibits (rather than stimulates) neurogenesis and neuronal differentiation (Giudicelli et al., 2001).

In contrast, because r3, r5, and *Krox20*-misexpressing rhombomeres were equally effective, *Krox20* induction of the episodic activity most probably depends on its role in specifying r3/r5 identity and their ensuing properties. Hence, non-cell-autonomous mechanisms such as those extending the *Krox20* expression domain at the expense of r2 in the normal hindbrain, or r4 after *Krox20* electroporation (Giudicelli et al., 2001), are possibly involved. Rhombomere boundaries are potentially important for the action of inductive signals on developing neurons in an adjacent rhombomere. Once expressed, *Krox20* controls the expression of several gene products contributing to the establishment of the r3/r4 boundary (Theil et al.,

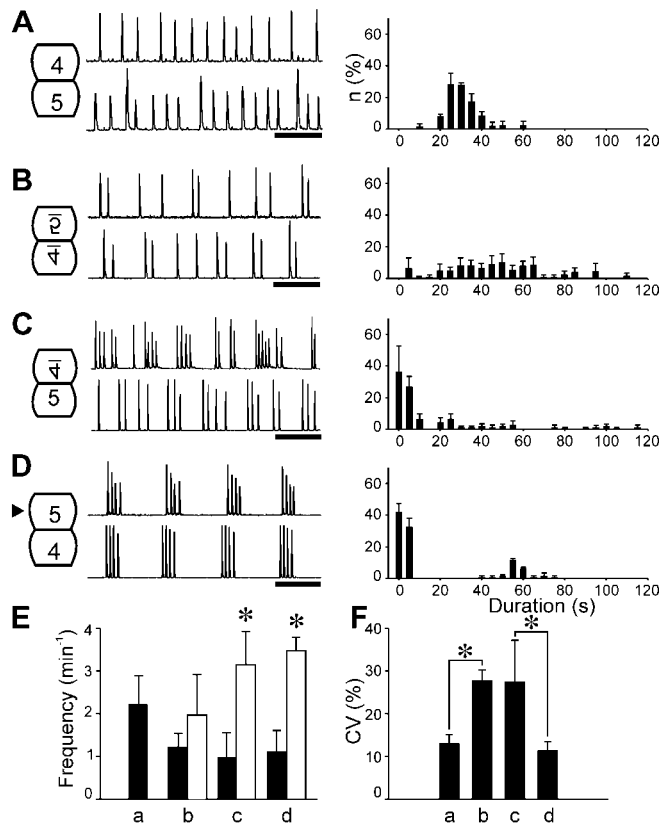


Figure 6. Anteroposterior reversal of r4 produces the development of an episodic generator in r4r5. *A–D*, Two examples (superposed traces) of integrated motor activity (left) recorded from distinct rhombomere pair preparations (left schematic) and compound histograms of the corresponding distribution of interburst interval duration (right) recorded from the facial nerve. *A, B*, Episodic patterning is absent in r4r5 (*A*, normal configuration) and after reversal of the entire r4r5 pair (*B*) and is produced (*C, D*) in r4r5 after AP reversal of r4 alone (*C*) and in r5r4 after heterotopic transplantation of r5 (in r3 position; *D*, arrowhead). *E, F*, Summary histogram of the frequency of LF bursts and LF+HF bursts (*E*, filled and empty bars, respectively) and of CV (*F*) of the different preparations. The EP⁺ pattern is produced (*E*, asterisks) by modifying the relative position of r5 with respect to the anteroposterior orientation of r4 (in *c* and *d* but not in *a* and *b*). Reversal of r4 (*B, C*) also eliminates regularity of the rhythm as indicated by significantly higher mean CVs (*F*, asterisks). Scale bar, 1 min.

1998; Xu et al., 1999; Mechta-Grigoriou et al., 2000). A target of *Krox20*, *EphA4*, encodes a cell surface molecule contributing to cell sorting during boundary formation (Wizenmann and Lumsden, 1997; Xu et al., 1999). Expression of these genes is highly dynamic and might contribute to inter-rhombomeric regulation well after HH10. Thus, signaling initiated by *Krox20* expression can control the functional fate of differentiating neurons (Takasu et al., 2002). Signaling initiated by *Krox20* expression and leading to the formation of rhombomere boundaries may be important during development of neuronal networks, including rhythm generators (Kulander et al., 2003).

Competence of r4 to produce the episodic rhythm requires correct anteroposterior polarity with respect to r3

Our observations on r4 suggest that differentiating neurons may be the target of signaling from r3. Boundaries between rhombomeres are characterized by enhanced extracellular space preferentially colonized by axons (Lumsden and Keynes, 1989). Thus, one of the potential roles of boundaries is to direct axonal growth within rhombomeres. Interestingly, *Krox20* electroporation experiments in chick (Giudicelli et al., 2001) have shown that extending axons of r4 neurons avoid the territories expressing

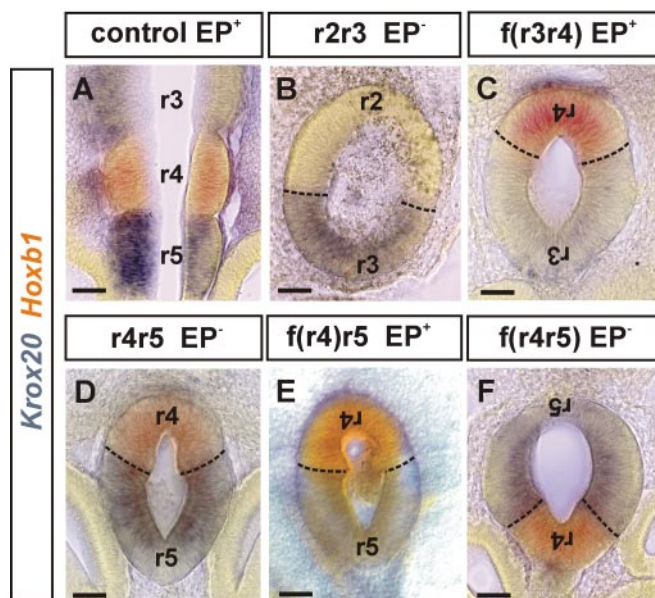


Figure 7. Horizontal sections of preparations, including the anteroposterior reversal of r4, 24 hr after surgical manipulation. *In situ* hybridization for *Krox20* (blue) and *Hoxb1* (red) in a control hindbrain (A) and in isolated rhombomere island preparations (B–F). Normal configurations, r2r3 (B) and r4r5 (D); modified configurations, AP inverted (f) pair of segment r3r4 (C) and r4r5 (F) and flipped r4 within the r4r5 pair (E). In all cases, *Hoxb1* expression is preserved in r4 and *Krox20* expression in r3 and r5, although episodic patterning is present (EP⁺ in A, C, E) or absent (EP⁻ in B, D, F). Scale bar, 50 μ m.

Krox20 or the patches of ectopic *Krox20* expression. Branchial motor neurons are inclined to extend their axons rostrally from odd rhombomeres (e.g., r3) through their anterior (e.g., r2/r3) boundary, whereas crossing their posterior boundaries (e.g., r3/r4) is avoided. There is a considerable amount of data demonstrating anomalies of this organization of motor neurons when segmental patterns of gene expression are modified (Marshall et al., 1992; Barrow and Capocchi, 1996; Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 1997). The importance of *Krox20* for the axonal navigation in r4 in addition to electroporation studies in chick (Giudicelli et al., 2001) is also supported by the finding in *Krox20*^{-/-} transgenic mice that trigeminal motor axons are misrouted toward the facial exit point in r4 (Schneider-Maunoury et al., 1997). We propose that a similar mechanism may link *Krox20* expression in r3 to the development of competent reticular neurons from which the episodic generator derives. It is also possible that yet unidentified AP gradients of molecules may produce polarization of r4 during neurogenesis and neuronal migration, such that competent progenitors are specified only at the anterior border of r4. However, there is as yet no firm evidence either for or against the existence of internal segment polarity within rhombomeres. Additional studies using electrophysiology and selective markers will be necessary to investigate growth of axons and dendrites in the population of parafacial rhythmic neurons developing in r4.

Development of the parafacial rhythm generator may be conserved in mice and chicks

The present study provides indications at molecular and regional levels that support the homology of chick and mouse parafacial generators. Although episodic breathing is a rather uncommon or pathological pattern in mammals, it is conserved at a specific embryonic stage in the mouse and in the mature control of pulmonary ventilation in amphibians and has been considered a

“physiological phylotypic stage” of hindbrain development in tetrapods (Abadie et al., 2000). The parafacial rhythm in chicks exerts a prominent control over more posterior generators (Fortin et al., 1999). The chick parafacial generator could therefore retain caudal outputs comparable with those of the murine parafacial respiratory group (Onimaru and Homma, 2003) interacting with the pre-Bötzinger generator (Mellen et al., 2003) and spinal motor neurons (Jankowski et al., 2002). In keeping with the vital respiratory role of r4 (Dominguez del Toro et al., 2001) and *Krox20* (Chatonnet et al., 2002) in mice, we now demonstrate that a segmental program initiated in r3 under the control of *Krox20* instructs formation of a parafacial rhythmic network in r4.

Regularity of the rhythm is a chick-specific, *Krox20*-independent phenotype that depends on the anteroposterior orientation of r4

Because AP reversal of r4 impairs the regularity of the rhythm, AP polarity cues within r4 are required after HH10 for the proper development of LF activities. Because regularity of the rhythm was observed in all preparations comprising odd and even rhombomere pairs, whether or not the odd contacts the anterior border of r4, *Krox20* is dispensable for regularity of the LF rhythm. Mechanisms whereby r6, unlike other isolated even rhombomeres, generates an irregular LF rhythm are presently not known. The high regularity of embryonic rhythms is a chick-specific character, contrasting with the irregular LF primordial rhythm of mice (Abadie et al., 2000). It seems that several other chick-specific, *Krox20*-dispensable processes start in r4 at the beginning of neuronal migration around HH12. *Hox* genes interact in r4 with neural specification programs to establish unique cell identities within each rhombomere (Davenne et al., 1999; Gaufo et al., 2000; Dasen et al., 2003; Pattyn et al., 2003). Rhombomere 4-specific phenotypic choices are made at this developmental stage, including the generation of branchiomotor versus serotonergic neurons (Pattyn et al., 2003) and the caudal migration of facial branchiomotor neurons through r5 and r6 (lacking in chicks) (Lumsden and Keynes, 1989; Pata et al., 1999; Garel et al., 2000; Gaufo et al., 2000; Studer, 2001). All of these processes are *Krox20* dispensable, although they depend on cell autonomous expression of *Hoxb1* and *Phox2b* (Pattyn et al., 2000), persisting after HH20 (Gaufo et al., 2000; Pattyn et al., 2003). The regularity of parafacial rhythmogenic properties thus suggest that AP reversal alters the organization of the generator as a result of the abnormal AP polarity in r4 during periods of neurogenesis and neuronal migration outlasting the downregulation of *Krox20* expression in r3.

Segmentation is a robust developmental strategy resulting from the regionalized expression of a limited number of genes (Von Dassow et al., 2000). Local changes in anteroposterior *Hox* patterns (Brunet and Ghysen, 1999; Dominguez del Toro et al., 2001) as well as in the interaction of *Hox* genes with dorsoventral establishment of neuronal diversity (Simon et al., 1995; Gaufo et al., 2003; Pattyn et al., 2003) may offer novel opportunities for the evolution of distinct subsets of neurons and the emergence of novel functions. We have now identified a robust developmental specification mechanism that is instructive for the maturation of the activity pattern of hindbrain neuronal circuits. We also provided insight into the possible adaptive role of odd rhombomeres. Operating upstream of *Hox* genes during the formation of r3, *Krox20* initiates development of a specific parafacial circuit in the hindbrain reticular formation. By providing a link between regionalization of the neural tube and emergence of motor pat-

terns, Krox20 stands among the candidate molecules through which the genome may interact with behavior during evolution.

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