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Induction of a Para-facial Rhythm Generator by Rhombomere 3 in the Chick Embryo

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SUMMARY

Observations of knockout mice suggest that breathing at birth requires correct development of a specific hindbrain territory corresponding to rhombomeres (r) 3 and 4. Focussing on this territory, we have 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, since 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 para-facial 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.

INTRODUCTION

By developmental stage HH10 in chicks (Hamburger and Hamilton, 1951), the hindbrain region is subdivided along the anteroposterior (AP) axis into 7-8 rhombomeres (r) and starts neurogenesis (Lumsden and Keynes, 1989). Rhombomere-restricted gene expression patterns, such as *Krox20* and *Hox* (reviewed in Lumsden and Krumlauf, 1996), orchestrate rhombomere formation. How rhythmic neuronal circuits are generated in relation to rhombomeres is not known. The r3r4 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 para-facial respiratory group, located ventral to the facial motor nucleus (deriving from r4), as an important component of primary respiratory rhythmogenesis (Onimaru and Homma, 2003; Mellen et al., 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 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 neighbour (r3) but does not emerge when r4 is left in contact with only its posterior neighbour, 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 para-facial rhythmic network. Our approach has centred 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 behaviour 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.

MATERIALS AND METHODS

Animals and hindbrain preparations: the rhombomere isolation procedure

Fertilized commercial hens' eggs were incubated to HH9-10. Donor neural tubes for the rhombomere grafting experiments were obtained after the hindbrain had been dissected out and treated with dispase (1mg/ml in L15 medium) for 15 minutes. 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-hand 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 re-incubated for approximately 2 hours prior to further isolation of segments and segment pairs. Isolations were made by excising both the immediately rostral and caudal pair of rhombomeres as previously described (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 (Fig. 4). The eggs were then sealed again and incubated for either 24 hours (for whole-mount *in situ* hybridisation, see below) or for 5-6 days, until the embryos had 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 2ml recording chamber superfused at a rate of 2ml/min with a physiological solution composed of (in mM) NaCl, 120; KCl, 8; NaH₂PO₄, 0.58; MgCl₂, 1.15; CaCl₂, 1.26; NaHCO₃, 21; glucose, 30 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, pCA β -*mKrox20*, was constructed by subcloning a *Clal/NotI* sequence containing the 1.4kb full length sequence of *mKrox20* into the pCA β ^{eCMV} IRES *mGFP6* plasmid. The *mKrox20* sequence was excised from the *pSlax-mKrox20* adaptor plasmid usually used for cloning into retroviral vectors. The IRES in pCA β ^{eCMV} IRES *mGFP6* is not active, therefore only *mKrox20* is transcribed in electroporated cells. The activity of this construct was tested by *in situ* hybridization. The activity of the CA β -*mGFP6* reporter plasmid was verified by GFP fluorescence approximately 6-8 hours after electroporation. An equimolar solution of pCA β -*mKrox20* and pCA β -*mGFP6* at concentrations of 0.5 μ g/ μ l was pressure injected into the lumen of the neural tube through glass capillaries (tip diameter ca. 10 μ m) 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 4 to 6 square pulses (10 V and 50 ms) with a pulse isolated stimulator (Intracel or Digitimer) through anodal and cathodal silver wire (diameter 1mm) electrodes placed about 1.5mm 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 bi-lateral (not shown) or uni-lateral (Fig. 4) penetration of the plasmids. Control embryos were electroporated with the GFP plasmid only. Electroporated donor embryos destined to grafting experiments were re-incubated for 1-2 hours 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 (Grass P511). Amplified neurograms filtered using a bandwidth (3Hz-3kHz) were rectified and integrated through an analog integrator (Grass 7P3B) with a time constant of 50ms. 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, Gif-sur-Yvette) and automatically detected using a threshold device.

Anatomy

In situ hybridization with digoxigenin- (DIG) and fluorescein isothiocyanate- (FITC) labelled 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% PFA 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 Labs, Burlingame, CA) and standard diaminobenzidine procedure.

Assessment of the rhythm regularity

A modal distribution of inter-burst intervals shorter than 15s corresponding to bursts generated at high frequency (HF bursts) indicated an episodic patterning of activity (see histograms in Fig. 3B,D; Fig. 6A,B; Fig. 7C,D). The episodic pattern (EP⁺) has been validated statistically (Mann-Whitney test) by rejection of the null hypothesis that the medians for both frequency of LF bursts (longer than 15s, LF burst marks under E8 neurogram on Fig. 1A) and that of the overall LF bursts and HF bursts production (LF+HF bursts marks under E8 neurogram on Fig. 1A) were equal ($p < 0.01$, stars on histograms in Fig. 1B). 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 (Fig. 1B, 3E, 7F). In the text and Table 1, data are expressed as mean \pm SD, on the figures error bars represent SD.

RESULTS

Episodic pattern (EP⁺) identification

Electrophysiological recordings of the facial nerve in whole hindbrain preparations at embryonic day 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 (Δ LF in Fig. 1A-B). The regularity of the episodic rhythm, estimated from the low values of coefficients of variation of inter-episode time intervals ("CV" in Fig. 1C), remains high during this process. Thus, we could ascertain the ability for a preparation to produce episodes (EP⁺) from the significant difference ($p < 0.01$, star in Fig. 1B) between frequency values of LF and LF+HF bursts discharges recorded at E7, a developmental stage at which the hindbrain has been found to be EP⁺ in 100% cases (see Table 1). In EP⁺ preparations comprising single odd rhombomeres or odd/even pairs of rhombomeres (e.g. r3r4, Table 1 and Fortin et al., 1999), an episodic pattern was observed in 80% of cases at E6 (16/20) 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 and 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 (compare values for r4r5 at E6 and E7 in Table 1). At later stages of development EP⁻ preparations tended to develop the episodic pattern. Indeed, the occurrence of EP⁺ noted in 10% (2/17) of the cases at E7, began to increase at E8 (37%, 3/8 cases) reaching significance (63%, 5/8 cases, Chi Square, $p < 0.01$) 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 to those obtained with normal r3r4 segments. 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 (Fig. 6D; $p = 0.147$).

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 (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).

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 and 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 if 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 a 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). Following 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 and 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 (2/5 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 of 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 (f(r3)r4 in Table 1) did not change the EP⁺ outcome (compare a and b in Fig. 6), 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. Reversal of r4, however, resulted in dramatically altered development of the rhythm (Fig. 6C): first, an EP⁻ pattern resulted (r3f(r4) in Table 1); second, the rhythm became irregular as indicated by an increased CV (from 0.05 to 0.23). This was not observed after reversal of the entire r3r4 (Fig. 5C, f(r3r4) in Table 1) that led to normal EP⁺.

Mechanisms responsible for the regularity of the rhythm would be distinct from those generating episodes because grafting r4 rostral to r3, with its correct AP orientation, produces a regular bursting pattern (CV=0.12) but no episodes (Fig. 6D, Table 1: r4*r3). Altogether, these experiments show that AP reversal of r4, but not of r3, alters the functional organization of the rhythmogenic neuronal network. This suggests that in the r3r4 pair, the rhythmogenic 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. 6 C,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 (Fig. 5D,7), a good model to investigate how EP⁺ potentialities in the other *Krox20* expressing rhombomere, r5, interact with r4 in the absence of r3 (compare a and d in Fig. 7). In the r4r5 preparation, we inverted the AP polarity of the r4 segment (Fig. 5E,7C) or of the entire r4r5 region (Fig. 5F,7B). 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. 7B; compare c to b in Fig. 7E). Finally, the EP⁺ was fully restored by grafting r5 anterior to r4 (Fig. 7D) or r4 posterior to r5 (not shown, Table 1). These experiments failed to reveal any EP⁺-repressing (Fig.

7C,E) or EP⁺-promoting (Fig. 7A, 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 (compare CV in a and b, c and d in Fig. 7F) confirming that distinct mechanisms control episode formation and the regularity of their recurrence.

DISCUSSION

Our results help to understand development of a para-facial rhythmogenic system, and allow us to propose a novel functional role for *Krox20*, in initiating the induction of the episodic generator during a restricted period of development, between HH10 (time of grafting) and HH15-20 (*Krox20* down-regulation). *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 neighbouring 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) have no obvious influence on the future pattern of rhythmic activities. The processes leading to episodes certainly begin after HH10 and probably cease with the down-regulation of *Krox20* expression in r3 at HH15-20 (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 HH15-20, 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 differentiation 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 HH 10**

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., 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, signalling initiated by *Krox20* expression can control the functional fate of differentiating neurons (Takasu et al., 2002). Signalling initiated by *Krox20* expression and leading to the formation of rhombomere boundaries may be important during development of neuronal networks including rhythm generators (Kullander et al., 2003).

Competence of r4 to produce the episodic rhythm requires correct anterior-posterior polarity with respect to r3

Our observations on r4 suggest that differentiating neurons may be the target of signalling from r3. Boundaries between rhombomeres are characterized by enhanced extracellular space preferentially colonised by axons (Lumsden & 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 *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 while 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; Goddard et al., 1996; Studer et al., 1996; Barrow and Capecchi, 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., 2003) is also supported by the finding in *Krox20*^{-/-} transgenic mice that trigeminal motor axons are misrouted towards 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. Further studies using electrophysiology and selective markers will be necessary to investigate growth of axons and dendrites in the population of para-facial rhythmic neurons developing in r4.

Development of the para-facial 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 para-facial 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 para-facial rhythm in chicks exerts a prominent control over more posterior generators (Fortin et al., 1999). The chick para-facial generator could therefore retain caudal outputs comparable to those of the murine para-facial respiratory group (Onimaru and Homma, 2003) interacting with the pre-Bötzinger generator (Mellen et al., 2003) and spinal motor neurons (Jankzewski 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 para-facial rhythmic network in r4.

Regularity of the rhythm is a chick-specific, *Krox20*-independent phenotype that depends on the anterior-posterior 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; Pattyn et al., 2003; Dasen et al., 2003). Rhombomere 4-specific phenotypic choices are made at this developmental stage, including the generation of branchiomotor vs 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 para-facial 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 down-regulation 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 anterior-posterior *Hox* patterns (Brunet and Ghysen, 1999; Dominguez del Toro et al., 2001) as well as in the interaction of *Hox* genes with dorsal-ventral 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 have 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 para-facial circuit in the hindbrain reticular formation. By providing a link between regionalization of the neural tube and emergence of motor patterns, *Krox20* stands among the candidate molecules through which the genome may interact with behaviour during evolution.

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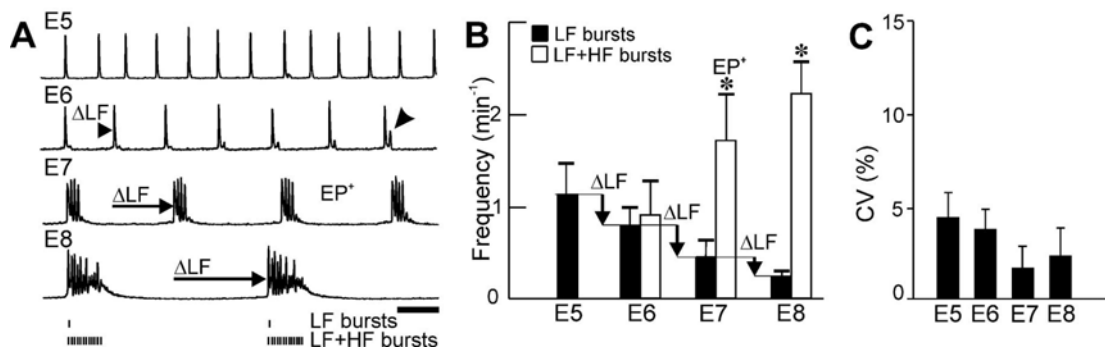
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Figures and legends

Figure 1. Appearance of episodic patterning of activities (EP^+): transition from low frequency (LF) primordial activity.

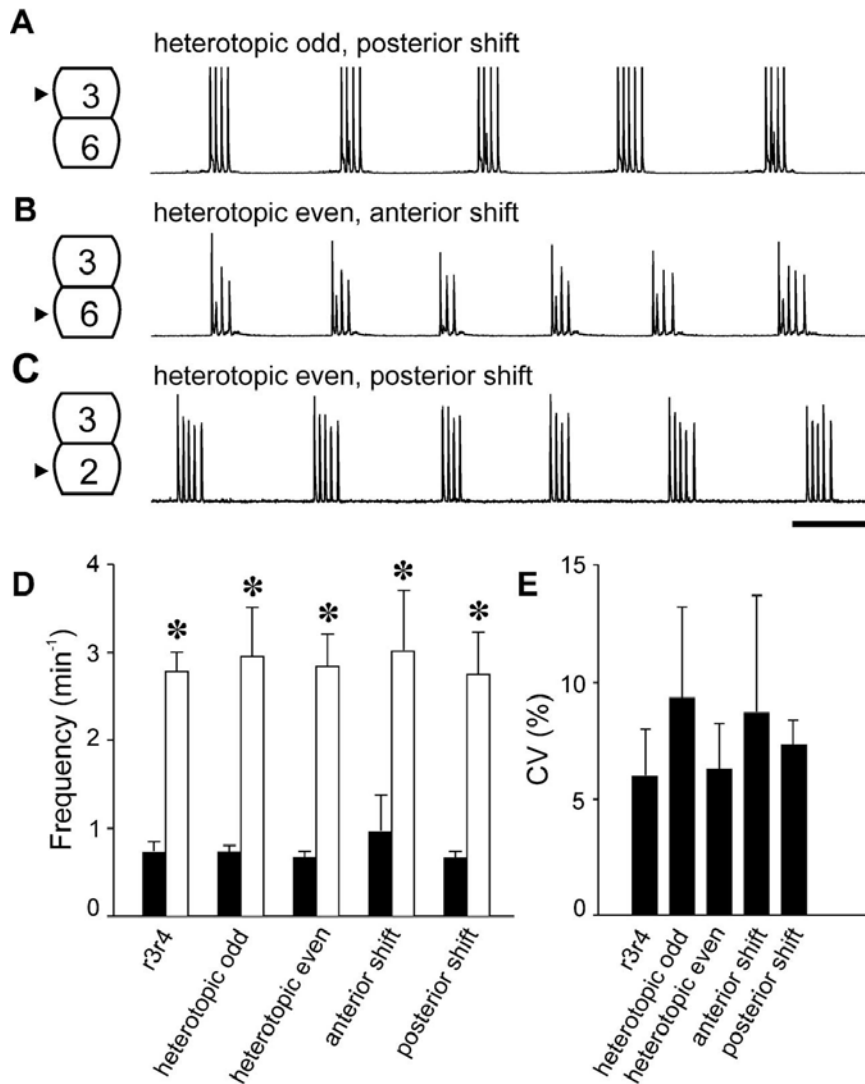
Figure 1 Coutinho et al.



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 (ΔLF , 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 (arrowhead on the E6 trace in A) of HF bursts at E6. C: Regularity of rhythm generation, measured by the coefficient of variation of inter-LF burst interval (CV), is maintained during development. Calibration bar: 1 min.

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.

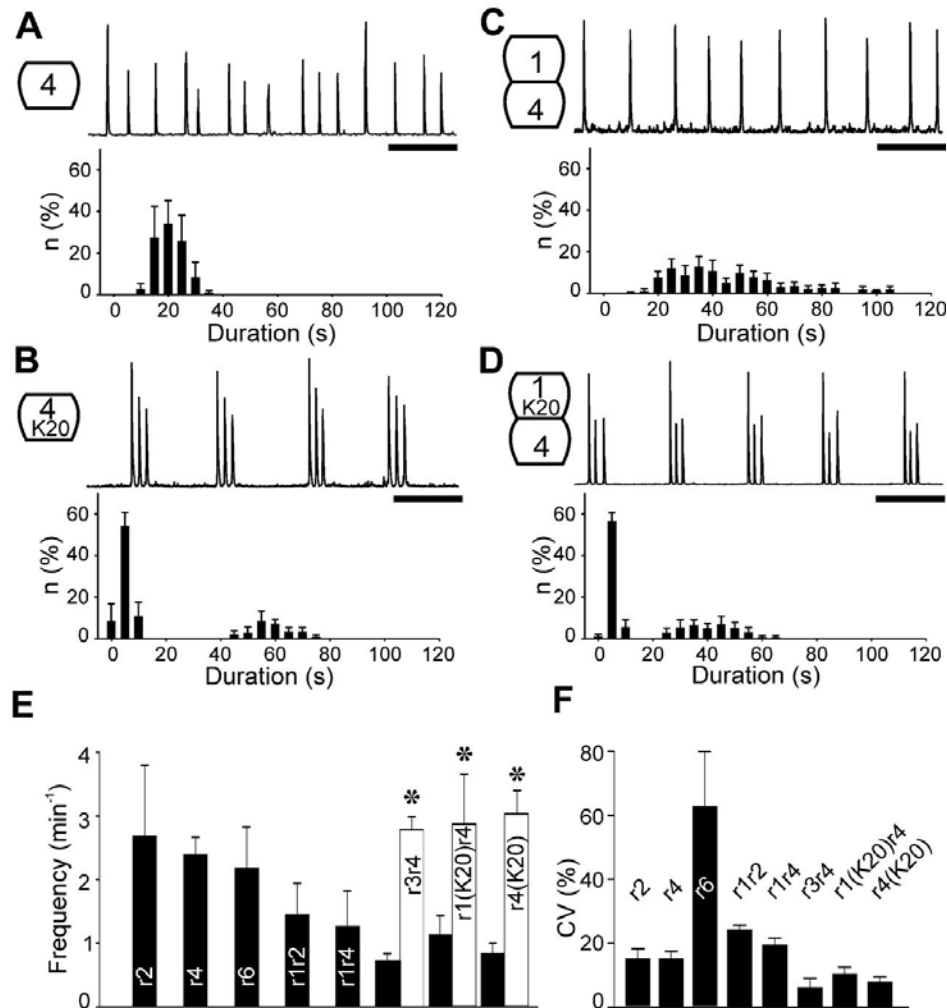
Figure 2 Coutinho *et al.*



Transplantations of the odd r3 (in r5 position, arrowhead in A) and of the even r6 (in r4 position, arrowhead in B) or r2 (in r4 position, arrowhead in C) lead to, r3r6 or r3r2 preparations showing an episodic patterning similar to that obtained from the normal configuration r3r4 (shown in Fig. 6A). D,E: Summary histograms of the frequencies of LF bursts and LF+HF bursts (filled and empty bars respectively in D) 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 to 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. Calibration bar: 1 minute.

Figure 3. *Electroporation of Krox20 confers the EP⁺ pattern to the r4 and r1r4 EP⁻ segments.*

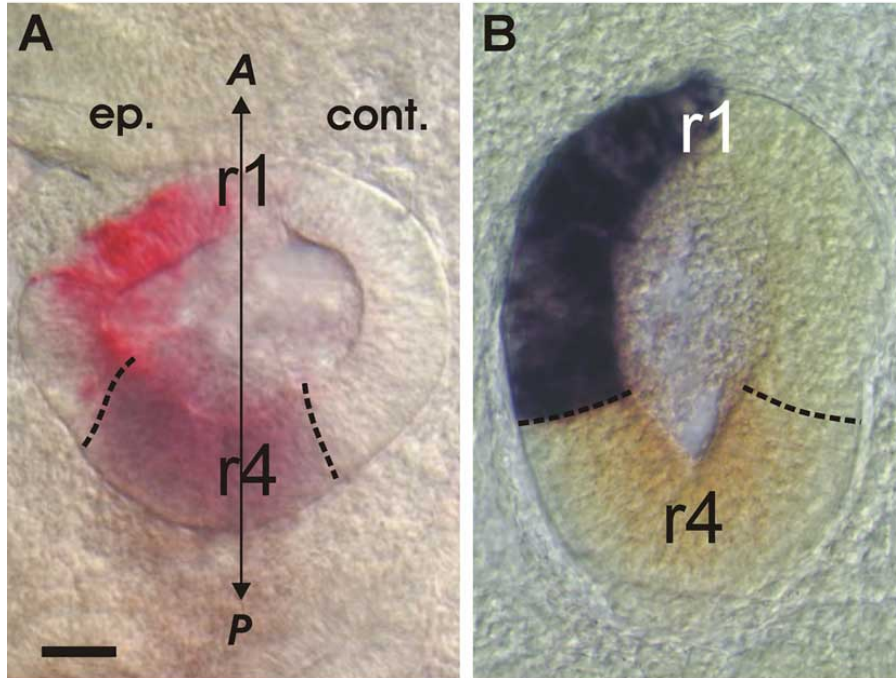
Figure 3 Coutinho et al.



Integrated neurograms (top) and corresponding compound distribution of inter-burst 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 r4 mis-expressing *Krox20* (4K20 in B) and in r1r4 segments when r1 mis-expresses *Krox20* in the pair (1K20 in D) resulting in a bi-modal distribution of interburst interval. E: summary histogram of the frequency of LF bursts and LF+HF bursts (filled and empty bars respectively in E) showing that r2, r4, r6, r1r2 (normal configurations) and r1r4 are EP⁻. Electroporation of *Krox20* induces EP⁺ in r4 and r1r4 (stars, compare to the normal configuration, r3r4). F: CV is not significantly affected in these experiments and is particularly large for r6. Calibration bar: 1 minute.

Figure 4. Whole-mount *r1(K20)r4* islands 24 hours after electroporation grafting and isolation.

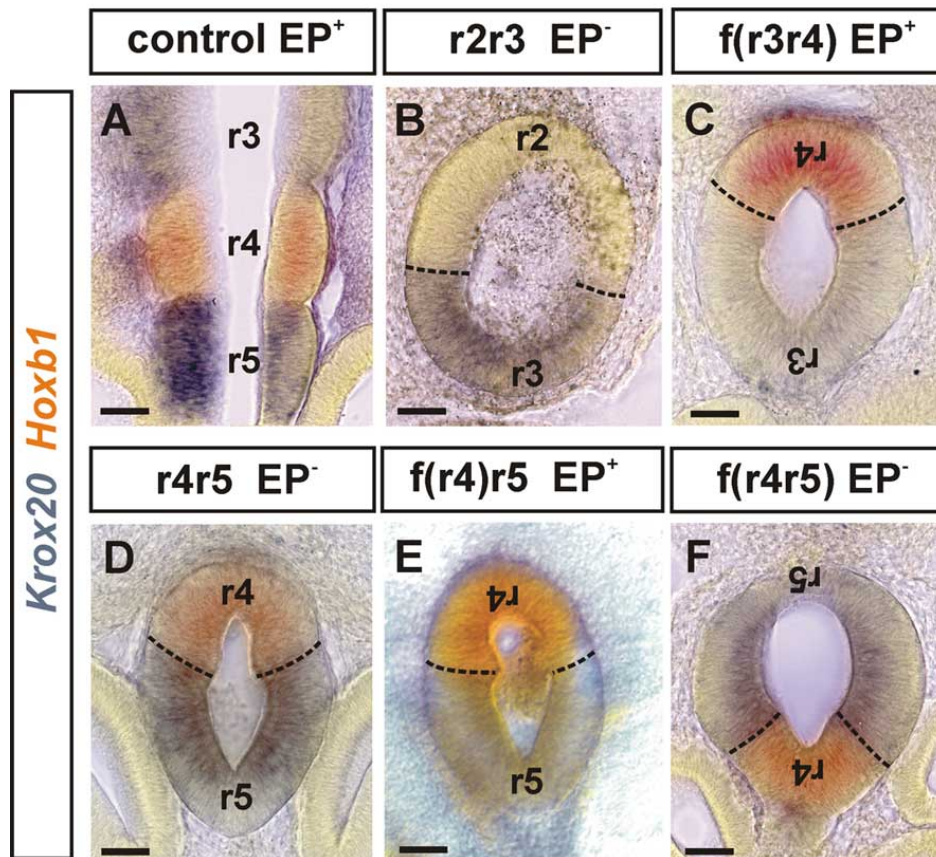
Figure 4 *Coutinho et al.*



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, prior to engraftment and isolation of the *r1r4* island. Note the overt healing at boundaries (dotted lines in A and B) 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 (vertical line in A) but not on the control side (cont. on the right) of the *r1* territory. *Krox20* expression, is confined within *r1* (A, B), a sharp AP limit of expression is visible on the ep. side in B while 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 Fig. 3D. A, anterior; P, posterior; calibration bar: 50µm.

Figure 5. Horizontal sections of preparations including the anterior-posterior reversal of r4, 24 hours after surgical manipulation.

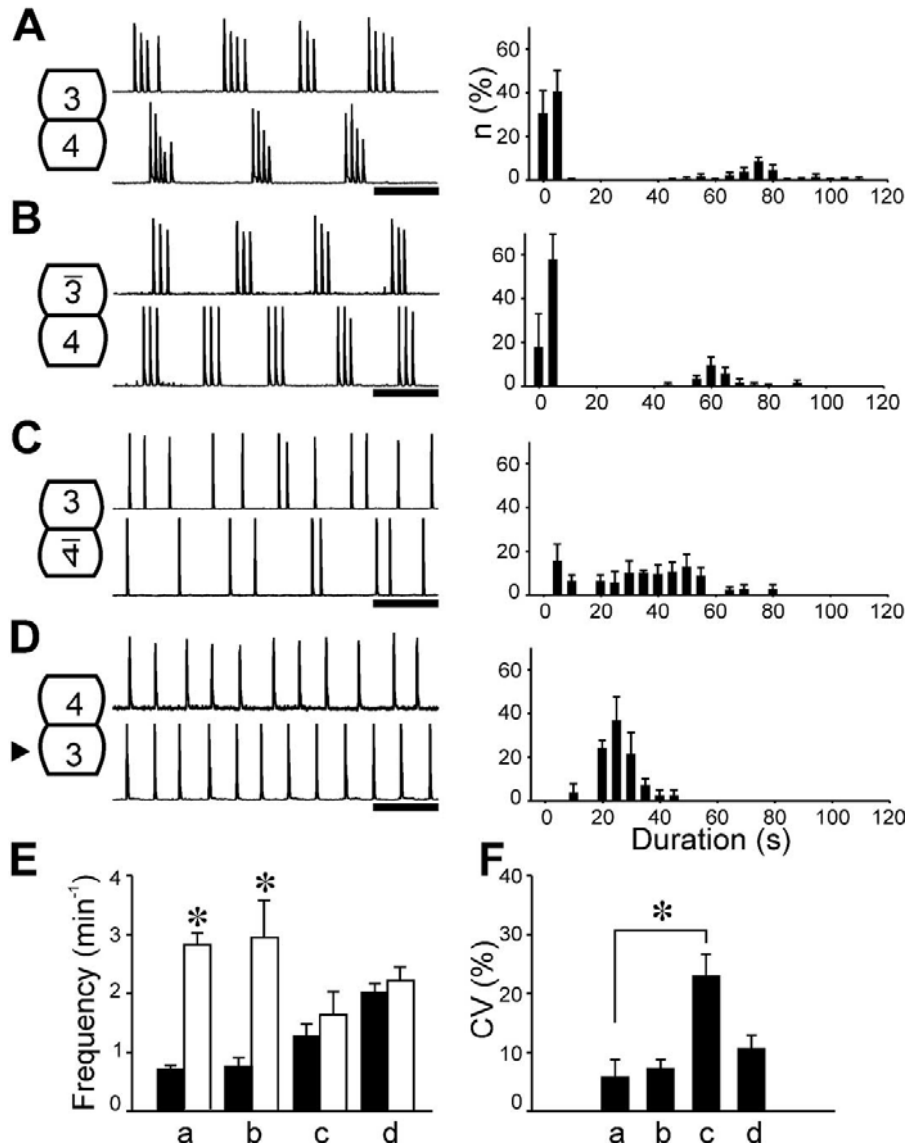
Figure 5 *Coutinho et al.*



In situ hybridisation for *Krox20* (blue) and *Hoxb1* (red) in a control hindbrain (A) and in isolated rhombomere islands preparations (B-F). Normal configurations: r2r3 (B) and r4r5 (D); modified configurations: AP inverted (flipped, 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). Calibration bar: 50μm.

Figure 6. Anterior-posterior orientation of r4, but not r3, is required for the development of a stable rhythm generator.

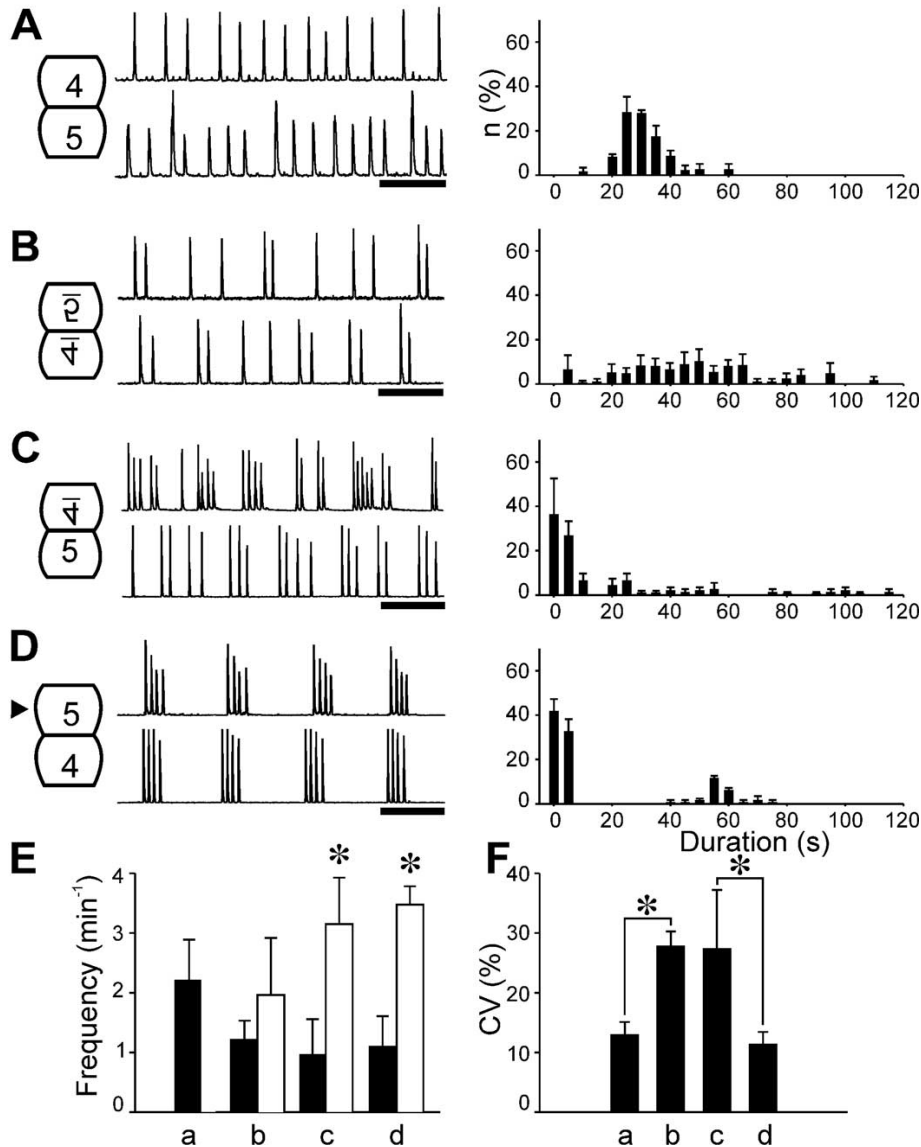
Figure 6 Coutinho et al.



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 inter-burst interval duration (right) recorded from the facial nerve. A,B: Episodic patterning is present in r3r4 (normal configuration) and in r3r4 after AP reversal of r3 (B) and absent (C, D) in r3r4 after AP reversal of r4 (C) and in r4r3 after heterotopic transplantation of r3 (in r5 position, arrowhead in D). E, F: Summary histogram of the frequency of LF bursts and LF+HF bursts (filled and empty bars respectively in E) 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 (compared c to a). Note also in E, that modifying the relative position of r3 with respect to anterior-posterior polarity of r4 abolishes (in c and d) the development of episodes (a and b). Calibration bar: 1 minute.

Figure 7. Anterior-posterior reversal of *r4* produces the development of an episodic generator in *r4r5*.

Figure 7 Coutinho et al.



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 inter-burst 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, arrowhead in D). E, F: Summary histogram of the frequency of LF bursts and LF+HF bursts (filled and empty bars respectively in E) and of CV (F) of the different preparations. The EP⁺ pattern is produced (stars in E) by modifying the relative position of *r5* with respect to the anterior-posterior orientation of *r4* (in c, d but not in a, b). Reversal of *r4* (in B, C) also eliminates regularity of the rhythm as indicated by significantly higher mean CV's (stars in F). Calibration bar: 1 minute.

Table 1. Characterization of EP⁺ and EP⁻ preparations

Summary table of the values of the frequencies (min^{-1}) of LF-burst and of LF+HF-bursts measured on the different preparations at E7.

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

Anterior-posterior 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 this rhombomere has been heterotopically grafted e.g. r4r3* – r3 grafted in heterotopic r5 position. f preceding rhombomeres in brackets indicates their flipped anterior-posterior orientation. GFP or K20 in a bracket to the right of a rhombomere indicates that this rhombomere was targeted by electroporation of respectively the sole *GFP* or the *GFP* and *Krox20* expression vectors. Heterotopic odd and even refer to compound measurements pooling respectively, r3*r6, n=1; r5*r4, n=3; r5*r2, n=1; and r3r6*, n=1; r3r2*, n=2; r5r4*, n=1, preparations. Anterior and posterior shift refer to compound measurements obtained pooling respectively, r3r6*, n=1; r5*r4, n=3; r5*r2, n=1; and r3*r6, n=1; r3r2*, n=2; r5r4*, n=1, preparations. id.: idem; ns: no significant difference between frequency values of LF-bursts and of LF+HF-bursts.