Segmental specification of GABAergic inhibition during development of hindbrain neural networks.

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Odd-rhombomeric specifications control appearance of GABAergic inhibition in the postsegmental hindbrain.

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It is unclear whether and how behavioural patterns in vertebrates might be set genetically during development. Segmentation of the neural tube is a general strategy by which developmental genes control the antero-posterior (AP) organisation of brain regions. Segmentation of the hindbrain leads to the formation of successive bulges along the AP axis that are called rhombomeres (r1-r8)[1]. In keeping with neuronal network function being influenced by developmental genes, the end of segmentation of the chick hindbrain is timed with the development of a primordial rhythm generating neuronal network[2]. However, r's are transient features of early development later followed by a dramatic reconfiguration of neurons and synapses during postsegmental (and neonatal) stages of maturation so that the influence of segmentation on the function of hindbrain neuronal networks remains an open issue. We now show that segmentation influences a postsegmental developmental step by which a GABAergic rhythm generator is incorporated into the primordial network and increases rhythm frequency to near mature values. This process depends on specifications in r3 and r5 that control on a two segment repeat basis later maturation of GABAergic inhibition in the other parts of the hindbrain.

Rhythmic activities of the cranial nerves in the isolated hindbrain of the chick embryo, eventually linked with the onset of respiratory control (a branchial function), have revealed the existence of an active network of reticular interneurons associated with the efferent motor nuclei [3]. Activity in motor roots reflects the activity of bursting reticular neurons (BRNs) in which transient membrane depolarizations (the bursts) are followed by gating processes that prevent further burst elicitation during a precise window of time (ref. 2 and unpublished observations). From end-segmental stage HH24[4] to stage 28, bath application of a selective $GABA_A$ receptor antagonist, bicucullin (Figure 1A) demonstrates an excitatory control of the rhythm by endogenous GABAergic neurons. At these early stages, the network generates a « low frequency » (LF) pattern (Figure 1a), one order of magnitude slower than the rhythm produced by de-afferented postnatal brainstem preparations from permanently breathing vertebrates (Table I). A closer-to-mature activity with high frequency (HF) episodes is turned on at around stage 29 (Figure 1b) and increases in importance afterward. HF burst generation is episodic, like breathing in lower vertebrates (2 and refs. herein), shows robust interaction with the BRN network so that bursts are distributed in 3 statistically distinct populations according to the duration of adjacent inter-burst intervals (i_n, i_{n+1}, Figure 1b3). Maturation of GABAergic synapses acting on $GABA_A$ membrane receptors are responsible for HF generation. (i) Bath application of bicucullin (10µM) totally blocked the HF activity, LF bursts being unaffected (Figure 1b).

All the other synaptic blockers tested (including blockers of alpha2-nordadrenergic or mu/delta enkephalinergic inhibitions) were ineffective on HF generation. (ii) The HF activity correlates with the appearance of a new class of rhythmically inhibited reticular neurons (IRN, Figure 1c) forming about half of the active neuronal population (19/37cells) in the hindbrain network. IRNs exhibit GABAergic chloride dependent inhibitory postsynaptic potentials (IPSPs) elicited rhythmically (Figure 1c1) increasing the membrane conductance (Figure 1c2) and abolished by bicucullin (Figure 1c3). IPSPs are lacking in BRNs so that there is exclusive connection between inhibitory GABAergic neurons and IRNs. (iii) Postinhibitory rebound depolarizations in IRN (Figure 1c1, arrowheads) probably trigger the excitatory signal responsible for the generation of HF bursts. An artificial model of the network (coll. J.F. Vibert and coll., unpublished) including IRN capable of rebound excitation confirms that HF can result from switching GABAergic synapses from immature excitatory to mature inhibitory function, a process widely described in many postnatal (and some adult) brain systems [5,6,7].

Postsynaptic mechanisms, by e.g. the clustering of post-synaptic $GABA_A$ receptors [8], is required to account for the membrane conductance increase underlying GABAergic inhibition (Figure 1c3). Regulation of transmembrane gradient of chloride ions by voltage-dependent chloride channels (CLC) or energy-dependent chloride transporters [9,10,11] is also involved since GABAergic inhibition is associated with membrane hyperpolarization in virtually all IRN. We favour the idea that these two processes, postsynaptic differentiation and ionic gradient regulation, develop sequentially because membrane short-circuit is seen together with an immature Cl^- gradient in a small population of IRN (n=3, not shown). Central pattern generators (CPG's) are neuronal networks that are capable of generating a sequence of motor activity despite isolation[12]. Altogether, our observations show that inhibitory GABAergic synapses form CPG's for the HF activity (HF-CPG's) and that maturation of GABAergic transmission is responsible for postsegmental evolution of the hindbrain activity.

Recording activities, at postsegmental stages, from brain territories deriving from individual or pairs of rhombomeres isolated *in situ* at the time when they form, has allowed identification of presumptive LF- and HF-CPG's. To isolate these segments, immediate both rostral and caudal

pairs of adjacent rhombomeres were removed at stage HH10-12. Twenty-four hours later, the isolated segments showed normal expression patterns for *Krox-20*[13] in r3 (3/3) and r5 (3/3) and *Hoxb1*[14] in r4 (3/3) and similarly to controls sharp anteroposterior boundaries between expression domains when pairs of r3r4 (6/6) or r4r5 (5/5) rhombomeres were isolated (figure 2). At postsegmental stages, even rhombomeres presented typical motor roots and sensory ganglia appropriate to their axial levels. In the case of isolated r3's and r5's, regulatory anatomical reconfigurations allowed the formation of abnormal nerve roots, although proper *Krox-20* gene expression was verified (Fig.2a). We consider therefore that at least in part rhombomeric identities have been spared by the *in situ* isolation procedure and may therefore persist in the ventricular zone at late development stages[15] .

Recordings from single rhombomeres have revealed the segmental requirement for LF- and HF-CPG's. Consistent with the idea that rhombomeres are autonomous developmental units [1] comprising progenitors of the LF-CPG determined early, at the time of rhombomere formation, typical LF was obtained in all segments studied (r2 to r5) except for the most posterior segment r6 (Table 2). By contrast, consistent HF could only be recorded from isolated r3 (n=6) and r5 (n=11), but not from r2, r4 and r6 (Figure 3A, C): HF generation therefore depends on AP specifications in the hindbrain.

Recording from isolated segments containing pairs of anteroposterior adjoining odd/even or even/odd rhombomeres led to the observation that only those deriving from the former combinations yielded development of a normal HF-CPG (Figure 3B,D). Isolated r3r4 (7/7) or r5r6 (3/3) developed at stage 32, HF interburst intervals similar to controls at the same stage (Table 2) and similar sensitivity to GABA$_A$ antagonist bicucullin (10μM, not shown) or benzodiazepine modulation by zolpidem (5μM, not shown). (ii) By contrast neither isolated r2r3 (3/3) nor r4r5 (5/6) segments were able to generate typical HF. These results identify the specification of HF-CPG's on a two segment repeat basis within r3 and r5 and point towards the existence of rostral to caudal inter-rhombomeric influences for acquisition by even rhombomeres of HF-CPG's.

During later development, the trigeminal (r2r3 derived) and facial (r4r5 derived) segments acquire progressively the capacity to develop HF-CPG, as shown for example by isolating the facial segment by transsections of the hindbrain at stage 31 (Figure 4a). This facial segment contains all cell populations required to build up a HF activity and this CPG operates despite elimination of r3-derived structures. Furthermore, at this stage of development, isolated trigeminal segments do not yet produce HF activity (Figure 4b). It is therefore unlikely that HF in r4 results from cell lines that specified inside r3 to eventually connect r4-derived neurons. There is as well no evidence for a massive migration of pre- and postsynaptic neurons from r3 to r4, because the trigeminal segment exhibits fibers and terminals immunopositive to glutamic acid decarboxylase (GABA synthetizing enzyme, Figure 4c) and GABAergic inhibitions in IRN (Figure 4d). Therefore, in keeping with the HF-CPG being related to the maturation of GABAergic inhibitory synapses, it seems that r3 specifications affect cellular interactions in r3 and in r4, rather than the differentiation of neuronal types within r3.

Post-segmental setting up of the HF-CPG appears to be the earliest known neuronal function to be impaired by abnormal rhombomeric relationships with r3 or r5. This has certainly influence on later stages of development. In mice, inactivation of *Krox-20* controlling development of r3 and r5 has been shown to dramatically impair the rhythmogenic function of the hindbrain at birth, causing for example life threatening apneic (LF) breathing and abnormal pontobulbar synaptic relationships[16]. Within the segmented hindbrain, domains of gene expression, lineage restriction and cell surface properties coincide and provide cells with positional information values with a 2-segment repeat pattern along the AP axis [1]. Membrane surface properties lead to cell-sorting processes that have been demonstrated by monitoring the aggregation of cells from odd- and/or even-numbered

rhombomeres in suspension cultures [17]. Cell-to-cell contact is also known to trigger interaction between appropriate neurons that eventually lead to functional synapses [18,19]. Pattern of gene expression that are responsible for the establishment of a 2-segment repeated organisation appear therefore essential for cell-to-cell recognition and synapse formation required for the generation of adapted physiological rhythm by the hindbrain.

Methods

Animals and hindbrain preparations

Hindbrains of chick embryos staged according to ref. 21, surgically isolated as previously described [3] were transfered into a 2 ml recording chamber superfused with a physiological solution composed of in mM : NaCl, 120 ; KCl, 8 ; NaH_2PO_4, 0.58 ; $MgCl_2$, 1.15 ; $CaCl_2$, 1.26 ; $NaHCO_3$, 21 ; Glucose, 30 ; aerated with carbogen (5% CO_2, 95% O_2) ; pH = 7.3 at 30°C at a rate of 2ml/min. Pharmacological applications of bicucullin methiodide (Tocris, final concentration 10µM) or zolpidem (Sigma, 5µM) were bath applied for 10 minutes through the physiological solution.

Isolation of neural segments

Isolation of rhombomeric territories was performed at stage 10-11 (10-16 somites). Microsurgery was performed, after embryos were made visible by sub-blastodermal injection of india ink, through a small opening of the vitelline membrane using tungsten needles, flame-sharpened from 150µm diameter pure tungsten wire,. A variety of isolated rhombomeric segments were obtained by excision of both the immediately rostral and caudal pairs of adjacent rhombomeres. In the case of an isolated r4 bilateral transverse cuts were made along the boundaries between r1 and r2 (r1/r2) ; r3 /r4 ; r4/r5 and r6/r7. The r2r3 and r5r6 segments were dissociated from the surrounding mesoderm and excised together with their underlying notochord. Operated eggs were sealed with tape and returned to the incubator for approximately 6 days until they had reached stage 31-32. All operated embryos at at the time of recording showed discontinuous neural tube composed of three independent vesicle enwrapped with pial mesenchyme. Cut ends of the neural tube had ealed up and evolved into round shapes so that the dorsal aspect of isolated segments depending on their one or two rhombomere constitution looked either spherical or cylindical. Dorsoventral polarity seemed preserved in isolated segments as indicated by adequate location of motor nerve exit points and the dorsal presence of a ventricular like structure. When dissecting at stage 32 the neural tube together with the pial mesenchyme and often the surrounding mesodermic derivatives were dissected out and transferred into the recording chamber so as estimate isolation failures due to the presence of axonal bundles interconnections. Such connections were found in 5 out of 61 cases when activities originally synchronized in between isolated terrritories could be desynchronized by transverse sectionning in between the segments, these embryos were excluded from analysis. Isolation of trigeminal and facial levels at postsegmental stages were performed manually using razor blades on the isolated hindbrain under a dissecting microscope.

Electrophysiology

Nerve recording techniques have been reported previously [3] briefly, the proximal root of a motor nerve was aspirated into a succion electrode connected to an high gain AC amplifier (Grass P511). Amplified neurograms filtered using a bandwidth (3Hz-3kHz) allowing monitoring of multifiber activity were rectified and integrated through an analog integrator (Grass 7P3B) with a time constant of 50ms. Blind whole-cell recordings were obtained using pipettes (7-10MΩ) pulled from borosilicate glass and filled with a recording solution containing in mM : K-gluconate, 130 ; $CaCl_2$, 1 ; $MgCl_2$, 1 ; BAPTA, 10 ; HEPES ½ Na, 10 ; Mg-ATP, 2 ; pH 7.4 at 30°C.

Rhythmic phenotype Identification

Upward deflections of the rectified integrated neurogram corresponding to burst discharges of the nerves were acquired by Acquis1 software package (developed by G. Sadocq) and automatically detected using a threshold device. Earliest activity of cranial nerves in the isolated hindbrain preparation appearing on preparations arising from stage HH 24 consist of spontaneous single burst discharges recurring at a low frequency (LF) of 1 every 30-40s. During the following 48 hours or up to stage 28-29 the maturation of activity consist of a steady state increase of LF interburst intervals (iLF). When iLF reaches a threshold for of $70.2 \pm 6.2s$ (measured on 14 embryos) single burst discharges are replaced by episodes of activity comprising high frequency (HF) cyclical burst discharges. The maturation progresses by further addition of extra HF burst discharges among episodes at a rate of 1 every 93.0 ± 34.4 min. We consider a neurogram to be produced by a LF rhythm central pattern generator (LF-CPG) if all interburst intervals recorded on the in vitro isolated hindbrain preparation are larger than 30s and if the coefficient of variation of the period (standart deviation/mean) is smaller than 0.30. For activity to be produced by the HF rhythm central pattern generator a population of short interburst interval less than 10s must have a size corresponding to n-1 times that of the iLF population where n is the number of burst discharges composing episodes.

Anatomy

Immunohistochemistry : Stage HH31 hindbrains were dissected and fixed overnight in 3.5% formaldehyde in PBS. Embryos were washed extensively in PBS and blocked overnight in 0.1% hydrogen peroxide in PBS. Embryos were incubated 24 hours with a primary monoclonal antibody anti-GAD (Chemicon) and then were exposed to a peroxidase-conjugated secondary antibody overnight and then developed using 0.5mg/ml diaminobenzidine (sigma) with 0.03% hydrogen peroxide. After washing in PBS, slices were mounted in DePeX and observed using bright field optics.

In situ Hybridization,.......STEFAN « ON »

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Legends of Figures

Figure 1 *Appearance of inhibitory GABAergic synaptic transmission is responsible for high frequency rhythm in the postsegmental chick embryo.* Integrated activity of a motor root (facial, 7n) and whole cell recording of inhibited reticular neurons (IRN in c) showing the effect of bicucullin (bic), a selective antagonist of endogenous transmission via GABA-A membrane receptors. **a** : At late segmental stages (HH27, a) the hindbrain generates a low frequency (LF) discharge of single burst (a2) forming a single population in 2D-histograms of adjacent inter-burst intervals i_n, and i_{n+1}, (a3, AIBIH) ; bic (n=3 summarized in AIBI, a3) reveals an excitatory function of GABAergic neurons. **b** : At postsegmental stages (HH31, b), extrabursts are generated at HF (b2) forming 2 additional populations in AIBIH (b3) ; bic (n=3 in b3) shows that HF generation is GABAergic. c : HF results from rhythmic burst-related GABAergic neurons generating inhibition followed by rebound excitation (arrowheads in c1). Inhibition results from membrane hyperpolarization, increase of membrane conductance (c2) and is abolished by bic (c3). IRN are only found when HF is generated ; only bursting neurons (not shown) are found previously. Note that bic transforms inhibition into depolarization (c3), suggesting that this IRN derives from a previously differentiated bursting neuron by maturation of GABAergic inhibitions.

Figure 2. In situ hybridization ..... Stefan « ON »

Figure 3. *Odd rhombomeric specification of HF activities.* In A and B, the top set of three superposed traces are examples of neurograms recorded at stage 32 from segments deriving from different combinations of isolated single segments in A, from left to right r2, r3, r4, r5, r6 or isolated pairs of segment B, from left to right r2r3, r3r4,r4r5 and r5r6 performed at stage 10-11. Corresponding compound AIBIH presenting all the cases (r2, n=3 ; r3, n=6; r4, n=6 ; r5, n=11; r6, n=6 ; r2r3, n=3; r3r4, n=7; r4r5, n=5 ; r5r6, n=3) are presented in vertical register below the neurograms. C and D are histograms showing in ordinates the ratio: number of HF interburst intervals / number of LF intervals quantifying HF-CPG function within isolated segments composed of single rhombomeres C or pair of rhombomeres D.

Figure 4 *Analysis of the postsegmental HH31 neural network in r3-r4 derived structure : the onset of HF involves a local generator at the facial level and cell-to-cell interactions with the trigeminal level.* **a** : Transversal sections at HH31 abolish HF generation in the trigeminal (5n) but not in the facial root. The facial HF activity (quantified as the ratio : number of HF interburst intervals / number of long intervals ; **a3**) is also decreased at the facial level by rostral influences. **b** : Horizontal section of the right side of the hindbrain showing the fiber plexus Immunoreactive to GAD (glutamate decarboxylase synthetizing GABA) in r2-r3. **c** : drawing of the same area as in b, showing that GABAergic inhibitions in IRN (white circles) are recorded (see Fig. 1c) as soon as stage HH31 in the r2-r3-dervived area showing GAD immunoreactivity.

Table I.- Average embryonic LF-CPG interburst interval is one order of magnitude higher than that of embryonic HF-CPG or postnatal respiratory-CPG. Inter-respiratory burst intervals were monitored in chickens on postnatal day 6 to 8 (P6-P8) after vagotomy as chemosensory (e.g. vagal) afferents control respiratory frequency in the chicken[20]; embryonic hindbrains of mice (unpublished, see ref. 2) and brainstem of P0-P4 mice are recorded after isolation in vitro[16].

	Embryonic LF	Embryonic HF	Postnatal
chick	75.9 ± 18.8 s	4.1 ± 1.3 s	13.1 ± 6.9 s
	(HH24-27)	(HH28-32)	(P6-P8)
	(30)	(62)	(13)
mouse	69.5 ± 11.3 s	———	5.8 ± 1.9 s
	(E14)		(P0-P4)
	(3)		(15)

Table 2.- LF and HF activities at stages (29-31) in rhombomeric segments isolated *in situ* at stages (10-11). The table indicates the number of recorded preparation of each class, the mean coefficients of variation of corresponding LF activities and the mean value of HF interburst intervals. Note that r2, r4 and r6 do not produce HF activity and that in addition r6 fails to produce LF activity (CV>0.3).

isolated r's	n	CV (iLF)	iHF (s)
r2	3	0.15 ± 0.17	-
r2r3	3	0.06 ± 0.03	-
r3	6	0.06 ± 0.05	5.3 ± 0.9
r3r4	7	0.07 ± 0.04	4.1 ± 0.7
r4	6	0.26 ± 0.09	-
r4r5	5	0.09 ± 0.02	-
r5	11	0.11 ± 0.05	4.6 ± 0.8
r5r6	3	0.11 ± 0.03	4.0 ± 0.1
r6	7	(0.50 ± 0.24)	-
control	35	0.04 ± 0.02	5.5 ± 0.6