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To cite this version:

Fabrice Chatonnet, Eduardo Domínguez Del Toro, Octavian Voiculescu, Patrick Charnay, Jean Champagnat. Different respiratory control systems are affected in homozygous and heterozygous kreisler mutant mice.. European Journal of Neuroscience, Wiley, 2002, 15, pp.684. <10.1046/j.1460-9568.2002.01909.x>. <hal-00083807>

HAL Id: hal-00083807
https://hal.archives-ouvertes.fr/hal-00083807
Submitted on 5 Jul 2006

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Different Respiratory Control Systems are affected in Homozygous And Heterozygous kreasler Mutant Mice.

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Running title: Respiratory phenotypes of kreasler mutants mice.

Key words: apneic breathing, neonate mice, kreasler, Krox20, rhombomeres.

Number of word in summary: 196, introduction: 476, whole paper: 7726.

Number of tables: 1, number of pages: 33.
Abstract

During embryonic development, restricted expression of regulatory genes *Krox20* and *kreisler* are involved in segmentation and antero-posterior patterning of the hindbrain neural tube. The analysis of transgenic mice in which specific rhombomeres (r) are eliminated points to an important role of segmentation in the generation of neuronal networks controlling vital rhythmic behaviours such as respiration. Thus, elimination of r3 and r5 in *Krox20*<sup>−/−</sup> mice suppresses a pontine anti-apneic system (Jacquin *et al.*, 1996). We now compare *Krox20*<sup>−/−</sup> to *kreisler* heterozygous (+/kr) and homozygous (kr/kr) mutant neonates. In +/kr mutant mice, we describe hyperactivity of the anti-apneic system: analysis of rhythm generation *in vitro* revealed a pontine modification in keeping with abnormal cell specifications previously reported in r3 (Manzanares *et al.*, 1999b). In kr/kr mice, elimination of r5 abolished all +/kr respiratory traits, suggesting that +/kr hyperactivity of the anti-apneic system is mediated through r5-derived territories. Furthermore, collateral chemosensory pathways that normally mediate delayed responses to hypoxia and hyperoxia were not functional in kr/kr mice. We conclude that the pontine anti-apneic system originates from r3r4. A different rhythm-promoting system originates in r5 and *kreisler* controls the development of anti-apneic and chemosensory signal transmission at this level.
Introduction

Hindbrain segmentation into rhombomeres (r) is a transient feature of early development (Lumsden & Keynes, 1989; reviewed by Lumsden & Krumlauf, 1996) followed by a dramatic reconfiguration of neuronal networks during foetal and neonatal brainstem maturation. Primordial rhythmic activities start in the hindbrain near the end of the segmentation process (Fortin et al., 1994a, 1995), and the activity of specific rhythm-promoting connections has been recently found to require a 2-segment repeated rhombomeric code (Fortin et al., 1999). Whether and how segmentation influences neuronal network properties remains an open issue. Our physiological approach to this problem is the analysis of the respiratory rhythmic neuronal network located in the pons and the medulla (reviewed by Bianchi et al., 1995).

Among the genes expressed in rhombomere-restricted patterns (reviewed by Schneider-Maunoury et al., 1998), Krox20, Hoxa1 and kreasler are implicated in the control of segmentation at distinct antero-posterior levels, since their inactivation leads to the elimination of rhombomeres r3 & r5, r4 & r5, and r5, respectively (Mark et al., 1993; Schneider-Maunoury et al., 1993; Cordes & Barsh, 1994; Manzanares et al., 1999b). Previous studies in Krox20-/- (Jacquin et al., 1996) and Hoxa1-/- (Dominguez del Toro et al., 2001) mice demonstrated that a normal embryonic expression of these genes in r3 and r5 or r4 and r5 respectively, is necessary for normal postnatal neuronal network function. Krox20-/- mutants exhibit a respiratory phenotype including low frequency and increased time spent in apnoeas, correlated with the absence of anti-apneic structures normally originating from r3 or r5. The present study seeks for a similar role for kreasler, in an attempt to elucidate the respective implication of r3 and r5 in respiratory controls.
The *kreisler* gene encodes a *mafB*-related transcription factor. It is expressed in r5, in r6 and in the roof plate along the hindbrain. It upregulates the expression of *Hoxa3* in both r5 and r6, and of *Hoxb3* in r5 (Manzanares *et al.*, 1997, 1999a). The *kreisler* mutants were generated by X-ray mutagenesis, the homozygous mutant animals (*kr/kr*) being hyperactive, a behavioural pattern characterised by head-tossing, running in circles, and deafness due to absence of a functional inner ear (Hertwig, 1942, 1944; Deol, 1964). These defects result from the early role of *kreisler* in r5 formation, since r5 is the only rhombomere that fails to form in *kr/kr* embryos. Later patterning in r6, including the caudal expression of *Phox2b* in a lateral cell column, is altered in *kr/kr* mice, even though the formation of r6 is not affected. Moreover, in both heterozygous (+/kr) and homozygous *kreisler* mutant embryos, patterning defects have also been observed in r3. *Hoxa3*, normally expressed posterior to r4, is upregulated and *ephrinB2* downregulated in r3 (Manzanares *et al.*, 1999b). We now show that this abnormal specification in r3 correlates at birth with alterations in the anti-apneic system and we identify specific defects resulting from the elimination of r5.

**Material and methods**

*Genotype analysis*

The *kreisler* mutation was maintained in a 129 background in association with the *agouti* mutation. DNA was extracted from the tail of the mouse as described elsewhere (Lufkin *et al.*, 1991). The genotype at the *kreisler* locus was subsequently determined by a PCR assay using a set of oligonucleotide primers allowing amplification of the restriction site polymorphism associated with the *kreisler* mutation (Cordes & Barsh, 1994).
**Plethysmograph recordings**

Respiratory activity was measured using a modified barometric method previously employed in neonates (Fortin et al. 1994b). The plethysmograph chamber (20 ml) equipped with a temperature sensor (LN 35 Z) was connected to a reference chamber of the same volume. The pressure difference between the two chambers was measured with a differential pressure transducer (Validyne DP 103-14) connected to a sine wave carrier demodulator (Validyne, CD15). The spirogram was stored on a computer using a Labmaster interface at a sampling frequency of 1 kHz. Calibrations were recorded at the end of each recording session by injecting 2.5 - 5 µl of air in the chamber with a Hamilton syringe.

Neonates were removed individually from the litter and placed in the plethysmograph chamber kept hermetically closed and maintained at 31°C during the recording session (165 s). In each sample, we identified the periods of quiet breathing recorded in the absence of limb or body movements. **Duration of these limb, body and head movements were used to determine activity of the neonate mouse during recording.** During quiet breathing, a computer-assisted method was used to measure the durations of inspiration and expiration from which respiratory frequency (f) is derived and the tidal volume (VT, µl/g) from which minute volume (V(dot)=f*VT/1000, ml/g/min) is derived. Periods of apneic breathing are not included in frequency calculation. Comparisons between two sets of data were performed by paired Student’s t-tests. Naloxone (3.33 mg/kg in 50 µl saline was administered subcutaneously using an Hamilton syringe). During hyperoxic exposure (40 s) the chamber was flushed with a humidified 100% O₂ gas during recording, during hypoxic exposure (40 s) the chamber was flushed with a humidified gas mixture of 12% O₂ in N₂.
**Anatomical observation and immunochemistry**

Thirty days old mice (6 homozygous mutants, 6 heterozygous mutants and 4 wild-type animals) were anaesthetised and intracardially perfused for 15 min with 0.01M phosphate buffer (pH 7.4), followed by a mixture of 4% paraformaldehyde in 0.01M phosphate buffer (pH 7.4). The brain was removed and placed overnight in the same fixative. The brains were then rinsed in phosphate buffer containing 20% sucrose and stored at 4°C until used. Serial parasagittal 40 µm sections were cut on a freezing microtome. Sets of four adjacent sections were processed using cresyl violet and polyclonal antibodies to tyrosine hydroxylase and choline acetyltransferase (ChAT). Sections processed for tyrosine hydroxylase immunochemistry were incubated in the presence of Triton X-100 overnight at room temperature with the antibody (Boeringer, 1:1000 in PBS, pH 7.4) and for 2 hours with biotinylated anti-mouse serum (Amersham, 1:200, pH 7.4). Peroxidase was subsequently revealed in a staining mixture containing 0.05% 3,3’-diaminobenzidine hydrochloride (DAB, Sigma) and 0.03% H₂O₂ (1 hour at room temperature). Sections processed for ChAT immunochemistry were incubated overnight at room temperature with the antibody (Chemicon International Incorporated, 1:2000 in PBS), which was subsequently revealed using the Vectastain ABC kit (Vector).

**In vitro perfused brainstem**

The brainstem-spinal cord preparations were isolated from anaesthetised 1 to 3 days old mice as described previously (Suzue 1984, Reckling et al., 1996). To isolate the hindbrain-spinal cord without the cerebellum, the more rostral section was performed at the rostral part of the pons (ponto-mesencephalic preparation, PM). The caudal section was performed between the cervical and thoracic rootlets to preserve the integrity of the C6 (phrenic) level of the spinal cord.
The preparations were pinned down for electrophysiological recordings and transections with the ventral surface upwards in a 2 ml chamber and bathed in a ringer solution of the following composition (mM): KCl, 5.4; NaCl, 130; MgCl₂, 1; NaHCO₃, 26; D-glucose, 30; CaCl₂, 0.8 equilibrated with a 10% CO₂ in O₂ gas mixture at 26°C, pH 7.0 and perfused at a rate of 1 ml/min. Transections of the ventral (Borday et al., 1997) pontobulbar respiratory controls were performed using a razor blade driven in the recording chamber. A pontobulbar (PB) section was performed at the level of the inferior cerebellar arteries to isolate the medullar generator form its pontine afferences. After PB sections, rostral and caudal parts of brainstem were fixed in 4% paraformaldehyde in Phosphate Buffer. Coronal sections stained with cresyl violet and analysed under light microscope indicated the same level of PB sections, at the caudal end of the facial motor nucleus, in +/kr (n=5) and kr/kr (n=4) animals, as in control animals. In +/kr (n=2) and +/- (n=2) animals, a trigemino-facial (TF) section was performed immediately rostral to the exit point of the facial nerve to eliminate rostral pons deriving from r3 and r4.

Various dissected cranial nerves such as the V, VII, XII and C1 to C6 were recorded. In kr/kr animals, VI rootlet is missing, and ventral hypoplasia can be appreciated. Simultaneous recordings by pairs of any of those rootlets ipsilaterally or bilaterally were performed before and after ponto-bulbar sections. Rootlets were recorded using suction electrodes connected to Grass amplifiers. Data were stored on a PC computer using a Labmaster interface at a sampling frequency of 1 kHz. Smooth integration was performed from a full wave rectified signal with a 40 ms time constant.
Results

A total of 72 animals resulting from inter-crosses between kreasler mutants were analysed. 24 of these animals were kreasler homozygous mutants, 32 were heterozygous and 16 were wild-types, as determined by PCR genotyping. Analysis of the phenotype and genotype was performed independently in blind experiments and compared afterwards. Anatomical studies were performed on animals allowed to survive 30 days, while behaviour was analysed during the first week after birth. We have studied the link between the kreasler mutation and respiratory and motor deficits by measuring the respiratory frequency, VT (Table 1) and motor responses including activity, reactions to stress and the righting reflex (a normal reflex reaction of neonate mice, which turn back to prone position when placed in supine position).

Inactivation of a Single kreasler Allele Affects Respiratory Pattern at Birth While the Homozygous Mutation Eliminates Respiratory Traits of +/-kr Phenotype

The +/-kr mice showed a modified pattern of quiet breathing (Figure 1) with a higher than normal respiratory frequency (polypnea) resulting from shorter expirations and a lower tidal volume. This respiratory phenotype was highly penetrant in the mutant mice population when compared to wild-type animals (Figure 1C) and particularly significant during the first three days after birth (Figure 1B). During the first days after birth, wild-type mice exhibited irregular breathing including a significant number of apnoeas (i.e. expirations lasting more than 2 s). The total time spent in apnoeas was almost 1 order of magnitude less in +/-kr mutants than in wild-type littermates (Table 1). Comparison of kr/kr and +/-kr demonstrates a significant reversion of the +/-kr phenotype by the homozygous mutation (Table 1). In kr/kr mice, the in vivo
respiratory frequency was slightly less than normal (Figure 1B) and the duration of apnoeas was normal (Table 1).

Increased respiratory frequency can be related to stress, hyperthermia, hyperactivity or exaggerated chemosensitive responses. However, in the +/kr mice, no stress reactions nor hyperactivity were visible when observing the patterns of limb, neck or body movements in the plethysmographic chamber (Table 1). The buccal temperature (Table 1) and responses to hypoxic or hyperoxic stimuli (data not shown) were not significantly different from those of +/+ animals.

Therefore, inactivation of one of the two kreisler alleles resulted in rather selective modifications of the quiet and apneic breathing patterns, without interfering with survival. The homozygous mutation suppressed this phenotype.

**Pontine Origin of the Respiratory Phenotype in Heterozygous +/kr Mutants**

As previously done in Krox20−/− mice, central respiratory deficits were investigated using neonatal (P1-P2) hindbrain preparations, isolated and superfused in vitro, and from which a spontaneous, synchronised respiratory-like activity is recorded from the hypoglossal (12n, Figure 2A) or cervical spinal nerve roots. A ponto-mesencephalic section (PM, Figure 2A) delimited preparations including the pons and the medulla (PM frequency in Figure 2C). A section at the ponto-bulbar junction (PB, Figure 2A & 2B), caudal to the facial motor nucleus (see Methods), isolated the medulla which retains the rhythmic respiratory-like activity originating in the ventral bulbar respiratory group (PB activity in Figure 2C). To quantify the effect of the pons in +/kr and wild-type animals, we measured the changes in respiratory frequency before and after the ponto-bulbar transection for each type of animal (Δf=PB-PM, inset in Figure 2C). We found a pontine inhibition that was significantly less in +/kr mutants as compared to wild-type animals.
Δf=0.0788±0.0089 Hz in +/+, n=4, 0.0429±0.0089 Hz in +/kr, n=7, p<0.05). In +/kr mice, the PB frequency was normal (0.168±0.014 Hz, n=4) and the PM frequency was higher (0.133±0.018 Hz, n=7, p<0.01) than in wild-type littermates (0.090±0.009 Hz, n=4). Similar measurements led to contrasting observations in Krox20+/ mice, in which pontine inhibition was greater than normal (Jacquin et al., 1996).

In kr/kr mice, the pontine control (Δf=PB-PM, inset in Figure 2C) was normal, in keeping with the behavioural and in vivo analysis suggesting a reversal, rather than an exaggeration, of respiratory phenotypic traits observed in +/kr mice in vitro. Caudally to PB sections, the rhythmogenic function of the ventral respiratory group was preserved (Figure 2C), although the burst frequency was lower (0.106±0.018 Hz, n=4) than in wild-type or heterozygous mice (0.155±0.008 Hz, n=11, p<0.02). Thus, the kr/kr mutation seemed to alter the medullar rhythm generation and abolished all phenotypic traits modifying the control of resting, apneic and in vitro respiratory frequencies in +/kr animals.

To further locate pontine defects in +/kr mutants, we have sectioned between the exit point of the trigeminal and facial nerves (TF section, Figure 2A), thereby eliminating r3-derived and more rostral structures. A sequential PB section then eliminated r4- and r5-derived structures. In wild-type animals, the TF section was responsible for the increase in burst frequency (Figure 2D). Neither of the two sections were very effective in +/kr mice. We thereby exclude that in these animals, the inhibitory effect of the rostral pons (r3-derived) was compensated by an excitatory effect of more caudal (r5-derived) structures.
**Respiratory Phenotype in +/kr Mutants Does Not Result From the Elimination of Catecholaminergic or Enkephalinergic Neurons**

To demonstrate that the lack of pontine inhibition does not result from the elimination of certain neuronal populations, we have specifically investigated the two major systems that could possibly depress respiration at birth, the enkephalinergic system and the catecholaminergic A5 group. The group of noradrenergic neurons named A5, is a major pontine rhythm depressant system active *in vitro* in newborn rodents (Errchidi *et al.* 1991) and interacts with the enkephalinergic system (Romagnano *et al.*, 1991, Arvidsson *et al.*, 1995). Enkephalinergic neuron function was investigated pharmacologically *in vivo* by subcutaneous administration of the selective antagonist naloxone during quiet breathing. As noted previously (Jacquin *et al.*, 1996), naloxone has little effect in wild-type animals. In contrast, naloxone significantly stimulated respiratory frequency in +/kr mice, indicating that enkephalinergic inhibition might be hyperactive in these animals (Figure 3). This hypersensitivity to subcutaneous naloxone administration was reversed by homozygous mutation (Figure 3B).

Immunohistochemistry of tyrosine hydroxylase, an enzyme of the catecholamine synthesising pathway, was performed in 6 heterozygous mutants and 3 wild-type animals at P30 and showed that the neurons of the A5 group appeared normal (not shown). These observations demonstrate that major populations of respiratory-depressant pontine neurons are not eliminated in +/kr mutant.

**Pontine Neuronal Populations generated in Hoxa1^{-/-} mice are not seen in +/kr Mutants**

As in *Hoxa1^{-/-}* mutants (Domínguez del Toro *et al.*, 2001), an exaggerated pontine excitation might result from the formation of a novel population of r3-derived, *Krox20*
dependent, rhythm-promoting neurons. We have therefore investigated the anatomical correlates of this phenomenon previously identified in Hoxa1+/− mutants, namely: (i) a characteristic morphological abnormality of the anterior fourth ventricle, (ii) an increase in the antero-posterior length of the dorsal Pons and (iii) a compound reticular and motor supernumerary structure comprising radial stripes of reticular formation and ectopic trigeminal motoneurons, formed at the level of the wild-type parvocellular reticular nucleus, a dorsal pontine structure originating in r3. None of these phenotypic traits were observed in +/kr and kr/kr mutants. The parvocellular reticular nucleus, extending between the trigeminal motor nucleus and the facial nerve (length: 438±58.8 µm in +/+, n=4; 474±68.2 µm in kr/kr, n=4; 466±59 µm in +/kr n=4) and the morphology of the fourth ventricle were normal in kreasler mutants (Figure 4A and 4B).

**Delayed Responses to Hypoxia and Hyperoxia are Eliminated in kr/kr Mice**

Lethal deficits previously described in Krox20−/− mice were not seen in kr/kr mutants, indicating normal function of the anti-apneic system. The kr/kr mice were characterised by a lack of righting reflex and a respiratory depression resulting from a small V_T (Table 1). Respiratory depression was not life threatening because the mutation was lethal for only 3 kr/kr (out of 24); no wild-type or heterozygous animals died. The small V_T might be indicative of chemosensory abnormalities as suggested by previous observations on BDNF−/− mice lacking subpopulations of sensory neurons (Erickson et al. 1996). Chemosensory signals controlling respiration are conveyed by glossopharyngeal sensory neurons (Neubauer et al., 1990) that might be affected by the kr/kr mutation (Mc Kay et al., 1994). We have used hypoxic and hyperoxic tests at the age of P10 to investigate possible defects of the respiratory responses in kr/kr (Figure 5A) or +/kr mice (Data not shown). The responses were normal in +/kr animals. Significant changes in respiratory
minute volume were induced in all animals 30 s after the onset of these stimuli (Figure 5B). Hyperoxia which eliminates on-going chemosensory control, led to reduction of $V(\dot{\text{o}})$, whereas hypoxia increased $V(\dot{\text{o}})$ as a short term effect. Thus, chemoreceptors that normally detect the oxygen level and transmit this information to central structures (Gonzalez et al., 1994; Vizeck & Bonora, 1998) were operational. However, processing of this information within the central nervous system (90 s after the onset of stimuli, Figure 5B) was modified in kr/kr. Hyperoxia-induced reduction of $V(\dot{\text{o}})$ was sustained in +/+ mice, while it is no more effective after 90 s in kr/kr mutants. The secondary effect of hypoxia was a reduction of $V(\dot{\text{o}})$, which was not observed in kr/kr mice, suggesting the elimination of a previously described pontine control of hypoxic respiratory stimulation (Dillon et al., 1991). These results provide further evidence that the kr/kr mutation affects neuronal populations mediating the control of respiratory parameters in response of both hypoxia and hyperoxia, leaving intact more direct chemosensory controls of the rhythm generator.

**Anatomical Defects of the kr/kr Hindbrain, Consistent with the Elimination of r5, are not observed in +/kr animals**

In kr/kr mice, anatomical defects were all located caudal to the r4-derived descending facial nerve fasciculus (7n in Figure 6). All structures located rostral to the 7n were normal, in particular r3-derived structures affected by the Krox20$^{-/-}$ mutation (Jacquin et al., 1996). Caudal to the 7n, r5-derived structures were affected by both the kr/kr and Krox20$^{-/-}$ mutations. In particular, the abducens motor nucleus (6 in Figure 6) and the suprageniculate pontine nucleus (not shown) were eliminated, and the preganglionic facial nucleus (7pgg) and the superior olive (SO: largest diameter: 1197.5±78.5 μm in +/+, n=4, 537.5±114.4 μm in kr/kr, n=4, p<0.001)
were reduced. In extreme cases, we observed an unilateral ventral hypoplasia at the level of the facial exit point.

More posterior defects, found in \textit{kr/kr} but not in \textit{Krox20} \textsc{+/−} mice, possibly involve r6-derived structures. These defects are located rostral to the dorsal vagal motor nucleus (9-10 in Figure 6), the ambiguus (Amb) subnuclei and the intermediate reticular nucleus (IRt), all identifiable by their intact population of choline acetyltransferase positive neurons. A dramatic reduction was found in the gigantocellular (Gi) and paragigantocellular reticular nuclei, whereas the more ventral facial nucleus (7Mo) was not significantly modified. As a result, the rostral (pars compacta, ca) Amb and IRt were shifted rostrally and dorsally (distance between caudalmost descending facial nerve fibers and rostralmost ambiguus neurons: 1215±31.3 µm in +/+, n=4 and 680±93.4 µm in \textit{kr/kr}, n=4, p<0.001). The facial nucleus itself was also shifted rostrally very close to the 7n (see Garel \textit{et al.}, 2000), indicating that hypoplasia affected the reticular domain extending between ascending and descending branches of the 7n (distance between anteriormost facial nerve fibers, and anteriormost limit of facial nucleus: d=432.5±35.9 µm in +/+, n=4, and d=215±47.3 µm in \textit{kr/kr}, n=4, p<0.001).

None of the anatomical defects described in \textit{kr/kr} mice were found in +/\textit{kr} mice therefore confirming that r5 and r6 were normal in heterozygous mutants. We observed normal distances between the 7n and the 7Mo (432.5±35.9 µm in +/+, n=4, and 420±59.4 µm in +/kr, n=4, NS), a normal sized SO (largest diameter: 1197.5±78.5 µm in +/+, n=4, 1267.5±99.8 µm in +/kr, n=4, NS) and normal AP lengths of the Gi (1215±31.1 µm in +/+, n=4, 1262±82.6 µm in +/kr, n=4, NS).
Discussion

We have analysed anatomical defects and the physiological phenotypes in newborn *kreisler* mutant mice. Our observations support the hypothesis that r5 derivatives are eliminated in *kr/kr* animals, and are consistent with previous reports showing that respiratory parameters are genetically controlled by early segmentation genes (Jacquin et al., 1996, Domínguez del Toro et al., 2001). In this context, observations in +/kr mice provide the first description of a segmentation-related respiratory phenotype that is not associated with rhombomere elimination. Our data suggest that genetic abnormalities in hindbrain development having no obvious consequences on survival and general hindbrain anatomy, might nevertheless result in abnormal respiratory parameters, a factor potentially contributing to sudden infant death, when combined with environmental factors.

*The Krox20-dependent Anti-apneic Neuronal System Originates from r3* Whereas *kr/kr Phenotypic Traits are Related to r5 and r6*

We conclude from the present study that the *Krox20<sup>-/-</sup>* and *Hoxa1<sup>-/-</sup>* respiratory phenotypes and lethality (Jacquin et al. 1996, Domínguez del Toro et al., 2001) result primarily from defects in r<sub>3</sub>/r<sub>4</sub>, since they are not reproduced in *kr/kr* mice in which r<sub>3</sub> and r<sub>4</sub> are present. In structures probably derived from r<sub>3</sub>/r<sub>4</sub>, many neurons (i) project to respiratory neuronal groups (Haxhiu et al., 1993; Dobbins & Feldman, 1994; Nunez-Abades et al., 1993), (ii) control the duration of inspiration in adult rodents (Jodkowski, et al., 1994), (iii) depress the respiratory rhythm frequency in rodent neonates *in vitro* (Errchidi et al., 1991, present results) and (iv) induce a periodic respiratory pattern in the foetal lamb (Dawes et al., 1983). As discussed previously (Jacquin et al. 1996), the *Krox20<sup>-/-</sup>* mutation spares rhythm-depressant
controls and eliminates the rhythm-promoting and the anti-apneic systems. We show that this r3/r4-derived system is not eliminated by the *kreisler* mutation.

Phenotypic particularities of *kr/kr* mice, not observed in */kr* animals, appear as a result of the abnormal development of different neuronal populations derived from r5 or r6. **Systems stimulating frequency are difficult to locate because pontine hypoplasia causes a rostral shift of bulbar structures relative to the facial nerve, so that PB transection removes more bulbar structures in *kr/kr* than in wild-type mice. Therefore, low frequency in *kr/kr* mice might result from the elimination of non vital rhythm-promoting reticular circuits or alternatively from the impairment of the medullar rhythmogenic circuits themselves.** The delayed chemosensory control of breathing seems also to require neurons originating in r5/r6. Among reticular structures that are reduced in *kr/kr* mice, the lateral paragigantocellular nucleus has been first suspected to control breathing by Von Euler (1986). This nucleus contains neurons that are connected to dorsal and ventral respiratory groups and respiratory premotoneurons (Andrezik et al., 1981; Ellenberger & Feldman, 1990, Nunez-Abades et al., 1993, Dobbins & Feldman, 1994). It also receives afferents from the primary relay nucleus of respiration-related sensory afferents, the nucleus tractus solitarius (Andrezik et al. 1981, Lovick, 1986). The *kr/kr* mutation may also affect the retro-trapezoid nucleus, a small group of neurons, located between the ventro-medial border of the facial nucleus and the ventral surface of the brain, controlling the delayed respiratory effects of CO2 (Nattie et al, 1990, 2000) and hypoxia (Bodineau et al. 2000). **Further experiments isolating in vitro the pontine CO2 sensitive structures would be needed to identify alterations of CO2 sensitivity in *kr/kr* mutant mice.**
**The Heterozygous kreira** Mutation Increases The Efficiency of Anti-apneic Neuronal Systems Originating from r3

Comparing +/kr with Krox20−/− mice shows that the same respiratory parameters are affected in both mutants. **Thus, the +/kr respiratory phenotype was a mirror image of that previously found lethal in Krox20−/− mice, in which the respiratory frequency was low, the tidal volume was large and the time spent in apnoeas dramatically increased compared to wild-type animals. Similarly, present observations in vitro, showing that the pontine inhibition is significantly less than normal in +/kr mutants contrasts with observations in Krox20−/− mice, in which pontine inhibition was greater than normal as compared to wild-type animals. Altogether, results are therefore consistent with the hypothesis that the r3-derived part of the anti-apneic system is hyperactive in the +/kr mutants and probably contributes to the increased respiratory frequency. Furthermore, comparisons between +/kr and kr/kr mice, show that the +/kr mutation does not functionally modify r5-derived structures. These results may be in keeping with observations in +/kr embryos indicating that the pattern of gene expression is altered in r3 (Manzanares et al., 1999b). Alternatively, since the kreira mutation affects rather large genomic domains (Cordes & Barsh, 1994), other yet undetermined genes operating in r3/r4 might be involved.**

The expression of Hoxa3 is normally caudal to the r4/r5 limit and upregulated by kreira in r5 and r6 (Manzanares et al. 1997). In +/kr mutants, Hoxa3 has been found to be induced in r3, raising the possibility that there may be a partial change in r3 identity (Manzanares et al. 1999b), but no deficits were found in r4. We have recently investigated the functional consequences of a similar mis-specification. In Hoxa1−/− mutant mice, in which parts of r3 and r4 acquire an r2-like identity, the mutation induces formation of ectopic motor subnuclei and supernumerary neuronal
circuits increasing respiratory frequency after birth (Domínguez del Toro et al., 2001).
Generation of these new neuronal populations changes the antero-posterior length of the pons and
the morphology of the anterior end of the fourth ventricle. Present results show that production of
supplementary reticular neurons is unlikely to occur in +/kr mice. Lack of any obvious anatomical phenotype in the pons of +/kr mice suggests that the development of efferent connections of r3-derived neurons has been changed without significant modification in the size of neuronal populations.

Hyperactivity of The Anti-apneic Neuronal System in +/kr Mice Requires kreasler
dependent Neurons

Additional experiments, performed at embryonic stages, are required to investigate developing efferent connections of r3-derived neurons. Nevertheless, comparing +/kr with kr/kr mutants provides information on how these connections are modified. The behavioral analysis of kr/kr mice revealed a reversal, rather than an exaggeration, of respiratory phenotypic traits observed in +/kr mice, despite the mis-specifications of r3 persist in kr/kr mutants (Manzanares et al. 1999b). In vivo, the respiratory frequency was slightly less than normal and the duration of apneas was normal. Hypersensitivity to subcutaneous naloxone administration was also reversed. In vitro, studies of rhythm generation indicated that the pontine control was normal. Thus, the kr/kr mutation abolished all phenotypic traits modifying the control of resting, apneic and in vitro respiratory frequencies in +/kr animals. It seems therefore that hyperactivity of the anti-apneic neuronal system in +/kr mutants requires intact relay structures originating from r5/r6. Indeed, rhythm promoting ponto-bulbar pathways (Borday et al. 1997), deriving probably from a lateral embryonic axonal tract (Lumsden et al.
1994), cross r5 before reaching the rhythm generator. Hence, hyperactivity in +/kr mice may result from the development of additional axonal arborizations or increased synaptic efficacy in r5/r6-derived territories. After elimination of r5 in kr/kr mice direct functional connections are preserved with the major synaptic target, i.e. the rhythm generator. Therefore, the +/kr mutation may generate, in r5/r6-derived territories, a novel collateral relay of the anti-apneic neuronal system. Although abnormal, this relay persists and functions after birth.

The question whether this +/kr phenotype results exclusively from abnormalities in r3, remains open. Within r3, the mechanisms by which the mutation may influence cell specification remains poorly understood, because kreisler expression in this rhombomere, at the level of the dorsal roof, remains unaffected in the mutants (Cordes & Barsh, 1994). Furthermore, assuming an exclusive r3 origin of the phenotype, only Krox20-dependent neuronal systems would be affected. Our observations on the enkephalinergic control of the respiratory frequency show that, in fact, some neuronal populations that are not eliminated by the Krox20⁻/⁻ mutation (Jacquin et al. 1996), can also be hyperactive in +/kr mutants and presumably modified in kr/kr mutants, suggesting widespread abnormalities associated with the kreisler gene regulatory role, despite its restricted expression in r5 and r6. We hypothesise a contribution of r5/r6 kreisler-expressing cells in the establishment and/or maintenance of their presynaptic connectivity patterns. The same mechanism may also operate in wild-type animals, in which kreisler-dependent neurons receive collateral inputs from the chemosensory pathway. We suggest that the molecular signalling initiated by kreisler expression in r5/r6 may act presynaptically, to control the development of functional collateral relays, according to the identity of presynaptic neurons. In wild-type animals, this mechanism would prevent collateralization of pontine (anti-apneic) or
widely distributed (enkephalinergic) neuronal systems and promote collateralization of medullar (chemosensory) systems.

**Conclusion**

Our results provide additional evidence that hindbrain segmentation during early embryonic development controls the functional organisation of neuronal networks after birth. Developmental processes initiated in r3 generate neuronal systems that are crucial to prevent sudden death during the restricted period of time extending during the first postnatal days in mice. In contrast, despite massive hypoplasia of the paragigantocellular reticular nucleus in kr/kr mutants, no evidence was found for a vital role of neuronal networks originating from r5; some of these neurons exert a positive control over respiratory frequency that relays pontine or chemosensory information controlling respiratory frequency.

These observations have important clinical implications for neonatal physiopathology. Observations in mice models point out at least three distinct syndromes indicative of abnormal embryonic development of hindbrain circuits. They might be detectable in human by scoring simple reflex reactions such as (i) suction for Krox20⁻/⁻-like syndromes (Jacquin et al. 1996), (ii) righting for kr/kr-like syndromes and (iii) chemosensory responses that are affected in human Ondine Course syndrome (Erickson et al., 1996). Importantly, abnormalities of the suction-deglutition-respiration have been ascribed to different syndromes involving precise domains of the segmented hindbrain (Abadie et al., 1999). Because transcription factors orchestrating hindbrain segmentation are organized in a regulatory network, a single mutation may have many consequences in this network. Therefore, prediction of neuronal deficits in patients is not straightforward.
A punctual mutation may not necessarily eliminate function of neurons. Thus, previous and present work shows that genetic abnormalities of hindbrain segmentation are as able to suppress (kr/kr, Krox20−/−) or enhance (+/kr, Hoxa1−/−) the function of respiration controlling neuronal system.

**Acknowledgements**

This work was supported by a H.F.S.P. research grant 101/97. We wish to thank G. Fortin and S. Jungbluth for valuable discussions and comments on the manuscript. We thank M. Sahakian F.-H., Mss Fourcaudeau, Morales, Daoud & Callens for very useful technical assistance. We thanks Dr Barsh for kind gift of kreisler oligonucleotides. Work in J.C.’s laboratory was supported by ACI BDPI#57, Centre National de la Recherche Scientifique, Fondation pour la Recherche Médicale (FRM). E.D.T. was supported by European Community (BIO4-CT975-096) and FRM (EP001227/1) training grants. Work in P.C.’s laboratory was supported by Institut National de la Santé Et de la Recherche Médicale.
Abbreviations

+/+: wild-type mouse

+/kr: Heterozygous kreasler mutant

kr/kr: Homozygous kreasler mutant

ChAT: Choline acetyltransferase

NLX: Naloxone

PB: Ponto-bulbar preparation

PM: Ponto-mesencephalic preparation

TF: trigemino-facial section

V(dot): minute volume

V_T: tidal volume
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**TABLE 1.** Comparison of *kreisler* homozygous and heterozygous mutant and wild-type animals (+/+) during the first day (P0).

Respiration (f: frequency; $V_T$: tidal volume; $V(\text{dot})$: minute volume; apneas) was measured during quiet breathing. Durations of apnoeas and movements during plethysmographic recordings are expressed in % of the total time of recording (165s). Righting reflex measures time spent by the mouse for moving from supine to prone position. Data at P0 include 24 (-/-) and 32 (+/-) and 16 (+/+) animals from *kreisler* litters, 109 +/+ are added for respiratory parameters. Means ± SEM; ***: p<.001; **: p<.01; *: p< .05; NS: not significant.

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<th>Measure</th>
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<td>$f$ (min$^{-1}$)</td>
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<td>***</td>
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<td>$V_T$ (µl/g)</td>
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<td>9.7±0.5</td>
<td>***</td>
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<td>$V(\text{dot})$ (ml/g)</td>
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<td>NS</td>
<td>1.61±0.09</td>
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<td>0.75±0.07</td>
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<td>apnoeas (%t)</td>
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<td>*</td>
<td>0.6±0.31</td>
<td>***</td>
<td>9±2.1</td>
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<td>buccal T° (°C)</td>
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<tr>
<td>righting (s)</td>
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<td>12.5±1.6</td>
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<td>26.9±7.3</td>
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<td>Movement (%t)</td>
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**FIG. 1.** Respiratory frequency is elevated in +/kr mutant mice during the first days after birth. (A) Representative samples of whole-body plethysmographic recording of +/+, +/kr and kr/kr littermates during the first postnatal week (P0-P6); scale bars: abscissa: 2 s, ordinate: 50 µl. Inspiration is upwards. (B) Evolution of the respiratory frequency in +/kr (open diamonds), kr/kr (open triangles) and wild-type (closed circles) littermates during the first postnatal week. The most significant differences between +/+ and +/kr are found at P0 / P1 (+/+, n=16 from P0 to P4 and 7 at P5 and P6; +/kr, n=32 from P0 to P2 and 26 from P3 to P6; kr/kr, n=24 from P0 to P2 and 18 from P3 to P6): stars indicate significance: ***:p<0.001, **: p<0.01, *:p<0.5. (C) Individual values of tidal volume (ordinate) and respiratory frequency (abscissa) in +/kr (open diamonds, n=32), Krox20−/− (open circles, n=12, data from Jacquin et al., 1996) and wild-type mice (crosses: +/kr and kr/kr littermates, n=16; closed circles: animals added to provide an estimate of population variability, n=109) during the first postnatal day. The tidal volume is smaller in +/kr mutants and their respiratory frequency is abnormally high.

**FIG. 2.** Pontine respiratory depression *in vitro* is weaker than normal in +/kr mice. (A) Schematic drawing showing the levels at which transverse sections of the brainstem were performed (PM: ponto-mesencephalic, TF: trigemino-facial, PB: ponto-bulbar). The most caudal segment (PB) contains the rhythm generator and spontaneous rhythmic neural activity is recorded from the hypoglossal (12n) nerve root (arrow). TF and PB sections eliminates the rhythm-depressant effect of the caudal pontine reticular formation, rostral to the facial nerve (7n). Calibration bar : 500 µm. (B) Cresyl violet staining of a coronal section made immediately
caudal to PB section in a +/kr mutant mouse. Facial motor nucleus (7Mo) and vestibular nuclei (Ve) are visible. Calibration bar: 500 µm. (C) Mean burst frequency before (PM) and after (PB) PB transection in wild-type (left, n=4), +/kr (middle, n=7) and kr/kr (right, n=6) mice. Inset: Mean effectiveness of pontine depression in +/+ (n=4), +/kr (n=7) and kr/kr (n=6) littermate mice. Ordinates: difference in frequency before and after PB transection (f(PB)-f(PM) in Hz).

(D) Evolution of the frequency (ordinate) during the time of an experiment (in abscissa, scale bar 15 min.) including subsequent transections at the TF and PB levels (arrowheads); the +/+ (top) and +/kr (bottom) preparations behave the same except in control (PM) situation: functional differences between the two genotypes are therefore located rostral to TF. Dashed lines indicate respiratory frequency recorded in PM preparation.

**Fig. 3. Enkephalinergic control of respiration in +/kr and kr/kr mice in vivo.** (A) Whole-body plethysmographic recordings and (B) mean respiratory frequency (cycles/min) before (Control) and after (NLX) blockade of the depressant enkephalinergic control of respiration by subcutaneous administration of naloxone in vivo at P2. This treatment has no effect on respiratory frequency in wild-type animals (n=20) nor in kr/kr animals (n=6) and further increases frequency in +/kr mice (n=9, p<0.001), stars indicate significance: ***:p<0.001, **: p<0.01. Scale bars in A: abscissa: 2 s, ordinate: 25 µl.

**Fig. 4. None of the Hoxa1⁻/⁻ anatomical defects are found in +/kr mutant mice.** (A) Sagittal section of brainstem at trigeminal level stained by ChAT immunohistochemistry. Rostral is left and dorsal is up. Left side is wild-type animal, right is +/kr animal. Double arrow indicates the width of parvocellular reticular formation, measured between trigeminal motor nucleus (5Mo)
and facial nerve (7n). The parvocellular reticular formation does not increase in width, and does not contain ectopic trigeminal motoneurons in +/kr mice. (B) medial sagittal section showing the anterior part (V4a) of the fourth ventricle (V4). +/kr mice show normal, non invaginated V4a. A1, B1: wild-type, A2, B2: +/kr mice. Scale bars: 100 µm.

**FIG. 5. Respiratory responses to hyperoxia and hypoxia in kr/kr and wild-type mice at the end of the first postnatal week.** (A) Direct plethysmographic recordings at P10 before (Control) and 30 s after the onset of hypoxic or hyperoxic challenges, traces are calibrated for Vₜ, scale bar: 2 s. (B) Trace plot of average minute volume (V(dot), in % of control values) measured before and 30 s and 90 s after the onset of hyperoxia (B1) or hypoxia (B2), in +/+ (n=20, black dots) and in kr/kr (n=11, open triangles), stars indicate significance: *:p<0.5. In kr/kr mutants, the initial response is preserved (30 s) demonstrating function of chemoreceptive mechanisms, while later processing is abolished (90 s).
**FIG. 6. Anatomical defects in the hindbrain of kr/kr mice.** Sagittal sections of the brainstem of wild-type (+/+, left) and kr/kr (right) littermates showing ChAT immunoreactive neurons; top: location in the brainstem; bottom: structures (in gray) reduced in size by the mutation: the gigantocellular nucleus (Gi), the superior olive (SO), the preganglionic facial nucleus (7pgg); note the absence of the abducens nucleus (6) in kr/kr mice. The mutation does not affect the size of the trigeminal nucleus (5Mo), caudal pontine nucleus (PnC), facial nucleus (7Mo) and nerve (7n), intermediate reticular nucleus (IRt) and of the different subnuclei of the ambiguus nucleus, (Amb, ca: pars compacta, sca: pars semi-compacta, ra: pars retro-ambigualis). LC: locus coeruleus, Nts: nucleus tractus solitarius, Ve: vestibular nuclei, 9-10: dorsal glossopharyngeal and vagal nuclei; 12: hypoglossal nucleus. Scale bar: 200 µm. Arrows indicate rostral (r) and dorsal (d).