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Mafa-dependent GABAergic activity promotes mouse neonatal apneas

Laure Lecoin1,2,6✉, Bowen Dempsey2,7, Alexandra Garancher1, Steeve Bourane3,4, Pierre-Louis Ruffault5, Marie-Pierre Morin-Surun2, Nathalie Rocques1, Martyn Goulding3, Alain Eychène1, Celio Pouponnot1✉, Gilles Fortin2,7✉ & Jean Champagnat2

While apneas are associated with multiple pathological and fatal conditions, the underlying molecular mechanisms remain elusive. We report that a mutated form of the transcription factor Mafa (Mafa4A) that prevents phosphorylation of the Mafa protein leads to an abnormally high incidence of breath holding apneas and death in newborn Mafa4A/4A mutant mice. This apneic breathing is phenocopied by restricting the mutation to central GABAergic inhibitory neurons and by activation of inhibitory Mafa neurons while reversed by inhibiting GABAergic transmission centrally. We find that Mafa activates the Gad2 promoter in vitro and that this activation is enhanced by the mutation that likely results in increased inhibitory drives onto target neurons. We also find that Mafa inhibitory neurons are absent from respiratory, sensory (primary and secondary) and pontine structures but are present in the vicinity of the hypoglossal motor nucleus including premotor neurons that innervate the geniohyoid muscle, to control upper airway patency. Altogether, our data reveal a role for Mafa phosphorylation in regulation of GABAergic drives and suggest a mechanism whereby reduced premotor drives to upper airway muscles may cause apneic breathing at birth.
The neural control of breathing is affected in many pathological conditions such as Congenital Central Hypoventilation Syndrome, Rett syndrome, and Multiple System Atrophy and presumably in Sudden Infant Death Syndrome (SIDS). Although the etiologies are varied and depend on the age, apneic events are typical traits of these syndromes. Furthermore, the biggest public health challenges that involve apnea, beyond the increasingly recognized contribution of central and obstructive apneas to "sleep disordered breathing" or "complex apnea", are neonatal apnea in the premature child. While the pathophysiological consequences of apnea are fairly well described, the underlying regulatory molecular mechanisms remain elusive.

Active interruption of the respiratory airflow is essential in a number of neonatal behaviors, for example to direct milk toward the alimentary tract, to protect airways from the inhalation of dust or irritant chemicals, or to increase subglottal pressure before uttering a sound. When their duration extends beyond the point at which blood oxygenhemoglobin desaturates, respiratory pauses can be life threatening. While central apneas refer to a failure of the central respiratory rhythm generator to maintain respiratory frequency, obstructive apneas, the most prevalent type of apneas, are characterized by a stop of the airflow despite persistent respiratory efforts, due to collapse of the upper airway (reviewed in Ref. [10]). Sleep-dependent reductions of pharyngeal muscles tonicity are known to increase the resistance of upper airways [11, 12]. Pharyngeal constrictor muscles have only a marginal effect on upper airway closure, but the main pharyngeal dilator muscles - the genioglossus and geniohyoid muscles - that pull forward the hyoid bone are crucial for the maintenance of upper airway patency [14, 15]. Benzodiazepines exacerbate oropharyngeal obstructions by depressing the motor drive in hypoglossal and recurrent laryngeal nerves that innervate upper airway muscles, incriminating overactive GABAAergic neuronal drives in apneic episodes [16, 17]. However, the endogenous mechanisms that may potentiate synaptic inhibition to cause apneic breathing remain unclear, as well as the inhibitory neuronal substrate.

Mafa is a member of the large MAF family of transcription factors (TFs) that also includes MafB, c-Maf, and Nrl [18, 19]. MAF factors belong to the larger b-ZIP TFs of the AP1 superfamily (like c-Fos or c-Jun), but harbor a specific DNA binding domain that participates in the recognition of the Maf Responsive Element (MARE). MAF activity is tightly controlled posttranslationally [20, 21]. More precisely, phosphorylation is a key regulatory mechanism, as shown by the Ser64Phe human mutation that impairs Mafa phosphorylation while causing familial diabetes mellitus and insulinomatisosis [22]. This mutation prevents Mafa phosphorylation on Ser65 by a priming kinase and the subsequent phosphorylation of Mafa by GSK3 kinase on 4 Ser/Thr residues. These residues (S61, T57, T53, S49) are highly conserved among MAF family members, and their phosphorylation mediates proteasome-dependent degradation of the protein through the proteasome [21, 22]. While Mafa is mainly known to control insulin gene transcription in β-cells of the pancreas [23, 24], it is also expressed in restricted neuronal populations of the peripheral nervous system and spinal cord [25–29] as well as in the brainstem (this study).

To understand the neurophysiological role of Mafa, we have generated conditional knock-in Mafa-4A mutant mice in which the 4 Ser/Thr residues are mutated into alanine. As a consequence, the mutant Mafa protein can no longer be phosphorylated by GSK3 nor be degraded by the proteasome, leading to intracellular accumulation and modification of its transactivation activity [21]. At birth, Mafa-4A mutants show an abnormally high incidences of apneas and died within 48h. Moreover, we find that Gad2 transcription is a mechanism target of Mafa dysregulation and propose that increased inhibitory premotor drive to upper airway muscles may be causal to the respiratory deficit.

**Results**

Mafa phosphorylation is essential for postnatal survival. To examine the physiological roles of Mafa, we have generated two cre-dependent Mafa knock-in mouse lines in which the Mafa coding sequence is either replaced by LacZ (Mafa<sup>lox<sub>LacZ</sub></sup>) or by a mutated Mafa<sup>4A</sup> allele (Mafa<sup>lox4A</sup>; Supplementary Fig. 1a–c) encoding a non-phosphorylatable form of the Mafa protein. Both lines were crossed to the PGK<sup>Cre</sup> line to obtain Mafa<sup>LacZ/LacZ</sup> knock-out and Mafa<sup>4A/4A</sup> knock-in mice, respectively. Mafa immunoreactivity in wildtype mice was lost in Mafa<sup>LacZ/LacZ</sup> mice and, conversely, was increased in Mafa<sup>4A/4A</sup> mutants, in keeping with the longer half-life of the non-phosphorylatable form of the Mafa-4A protein which can no longer be degraded by the proteasome (Supplementary Fig. 1d). As previously described by us and others [25, 26], Mafa<sup>LacZ/LacZ</sup> knock-out animals were viable, fertile and breathed normally. In contrast, no living Mafa<sup>4A/4A</sup> mutants were recovered at weaning stage from intercrosses of Mafa<sup>4A/4A</sup> mutants generated from two independent ES clones (Supplementary Table 1). At birth (postnatal day zero, P0), Mafa<sup>4A/4A</sup> mutant pups displayed normal weight, general morphology and were able to suckle and vocalize (Supplementary Table 2). They were however hypoactive and apart from seldom jerks or twitches, did not show postural nor chest wall movements suggesting exacerbated inspiratory or expiratory efforts. They developed cyanosis and died within 12h (Supplementary Fig. 1e, f). The viability of Mafa<sup>null</sup> pups in face of the rapid death of Mafa<sup>4A/4A</sup> mutant pups suggested that lethality may be correlated to gene dosage and the overall Mafa protein levels. In support of this, Mafa<sup>LacZ/+</sup> carrying one Mafa<sup>WT</sup> allele or Mafa<sup>LacZ/4A</sup> pups with a single Mafa<sup>4A</sup> mutant allele were fully viable, while heterozygous Mafa<sup>4A/+</sup> pups carrying one Mafa<sup>4A</sup> allele and one Mafa<sup>WT</sup> allele had a 60% survival rate at P21 (Supplementary Table 1).

Altogether, these data demonstrate that the Mafa<sup>4A</sup> allele is not itself lethal and that the abrogation of Mafa phosphorylation leading to an excess of Mafa protein in Mafa<sup>4A/4A</sup> mutants has a dramatic consequence on survival at birth.

Mafa-4A mutation in the central nervous system causes neonatal apneic breathing. When the Mafa<sup>lox4A/lox4A</sup> line was crossed with a nestin Cre line (Fig. 1a), no more than 10% (3/34 expected) conditional nestin<sup>Cre</sup>/+; Mafa<sup>lox4A/lox4A</sup> mutants (hereafter designated Mafa<sup>4A/4A</sup>, survived up to weaning stage (Supplementary Table 3). Most Mafa<sup>4A/4A</sup> newborns, like Mafa<sup>4A/4A</sup> ones, were found hypoactive, developed cyanosis and died between P0 + 12h and P2 (Supplementary Fig. 2a) indicating that the Mafa apneic phenotype is CNS specific.

We then investigated the ventilation of Mafa<sup>4A/4A</sup> mice at birth. Non-invasive unrestrained whole-body plethysmography was performed on control (n = 29), and Mafa<sup>4A/4A</sup> (n = 23) mutant pups over the P0-P2 period. At birth, all littermates established within 1h a regular breathing pattern, which then rapidly deteriorated in mutants via an abnormal increase in the incidence of respiratory pauses (Fig. 1a). Twelve hours after birth (P0 + 12h), Mafa<sup>4A/4A</sup> mutant pups presented with an approximately halved minute volume ventilation (V<sub>MV</sub>) compared to control littermates, which was accounted for by an abnormally high incidence of apneic events that lowered the breathing frequency (f<sub>B</sub>) while tidal volumes (V<sub>T</sub>) were maintained normal (Supplementary Fig. 2b). To quantify the incidence of apneic events (i.e., respiratory cycles longer than normal), we statistically...
**Fig. 1** Impaired Mafa phosphorylation in the nervous system results in lethal breath holding apneas. 

*a* Top, genetic scheme to introduce the Mafa4A mutation in neurons. Bottom, typical plethysmographic recordings (inspiration: upward) of control (black traces) and Mafa\(^{4A/n4A}\) (red traces) littermates at P0 + 12 h showing repetitive and apneic breathing cycles (the superposed traces are adjacent segments of a continuous record). Apneas (gray triangle in controls) are more frequent in the mutant (red diamonds). 

*b* Poincaré plots of the duration of consecutive respiratory cycles \(n + 1\) (ordinates) as a function of \(n\) (abscissae) in a control (left) and a Mafa\(^{4A/4A}\) (right, 5 min recording samples). Repetitive short cycles (filled symbols) are clustered on the diagonal below the CORC (vertical and horizontal dotted lines), apneic cycles (empty symbols) give points away from the diagonal beyond the CORC. 

**Note that the Mafa\(^{4A/n4A}\) mutant shows an increased incidence of apneas.** 

c) Evolution of the apneic time fraction (left ordinates axis, bar plot) and mortality (right inverted ordinates axis, vertical arrows) in control (gray, \(n = 29\)) and Mafa\(^{4A/n4A}\) mutants (red, \(n = 21\)) pups. Each symbol represents measurement of a single pup (One-way ANOVA between the two genotypes \(P < 0.0001\), degree of freedom (df) = 1, \(F = 34.38\)). 

d) Percent relative change in the number of apneas (ordinates) compared to their controls for Mafa\(^{4A/n4A}\) (Statistical test between mutant and control littermates: n4A vs Cre\(^{+/+}\) littermates: \(n = 17\) for both genotypes, two-sided unpaired t-test \(P = 0.0004\), df = 32, \(t = 3.965\)) and Mafa\(^{LacZ/LacZ}\) (null \(n = 13\) vs wild type \(n = 10\) littermates, two-sided Mann-Whitney test \(P = 0.0041\), \(U = 20\)). 

e) Example traces of the two types of apneas in Mafa\(^{4A/n4A}\) mutant pups. At left, breath holding apneas characterized by post-inspiratory onset (black arrowheads) of limited expiratory flow prolonging lung inflation (plateau above dotted baseline) followed by the resuming of inspiratory efforts of the lung or occasionally an inspiration (i below the click trace, 4 of 5 clicks) de \(b\)eviating the lung or occasionally an inspiration (i below the click trace, 1 of 5 clicks,) further inflating the lung. 

**f** Joint plethysmographic (top) and click audio recordings (bottom trace) during an epoch of breath holding apneas. Clicks that follow a post-inspiratory (arrowheads) breath holding apnea are associated to small upward pressure shifts (downward arrows) that immediately precede onset of an expiration (e below the click trace, 4 of 5 clicks) deflating the lung or occasionally an inspiration (i below the click trace, 1 of 5 clicks,) further inflating the lung. 

**g** Quantification of the apneic time fraction for apnea types in control pups (Mafa\(^{fox4A/4A}\), \(n = 23\)) at P0 + 12 h (two-sided Mann-Whitney test \(P = 0.4100\), \(U = 226.5\)). 

**h** Quantification of the breath holding apneic time fraction in Mafa\(^{4A/n4A}\) (nCre/+, \(n = 10\) pups, red), Mafa null (\(n = 13\) pups, blue) and that of respective control (n+/+, \(n = 10\) pups, gray, two-sided unpaired t-test \(P = 0.0145\), df = 18, \(t = 2.704\)) and wild type (light gray, \(n = 9\) pups, two-sided Mann-Whitney test \(P = 0.0052\), \(U = 14\)) littermates. Plots represent mean ± sem.
analyzed respiratory cycles in each breathing record, and defined a "Cut-Off Respiratory Cycle” duration (CORC) beyond which respiratory cycles were considered apneic (see Methods). Respiratory cycles were subdivided into two groups, (i) repetitive short cycles corresponding to regular breathing (i.e., followed and preceded by a similar short cycle; "repetitive" in Fig. 1a), yielding a low value cluster on the diagonal of Poincaré plots (filled symbols, Fig. 1b) and (ii) isolated cycles longer than the CORC (i.e., preceded and followed by a short cycle; "apneic" in Fig. 1a) yielding the values distributed away from the diagonal of Poincaré plots (empty symbols, Fig. 1b). With this tool in hand, we analyzed the temporal emergence of apneas in mutant and control neonates by comparing the percentage of time spent in apnea (apneic time fraction) during periods of quiet breathing (Fig. 1c). In control mice, we observed a progressive increase in the apneic time fraction over the P0 + 2–12 h period after which the incidence of apneic events gradually decreased. By contrast, Mafan4A/n4A mutant neonates displayed exaggerated apneic breathing compared to controls that was first evident at P0 +4 h and which worsened in frequency (Supplementary Fig. 2c) and duration (Supplementary Fig. 2d) to culminate in an apneic time fraction twice that of control littermates at P0 + 12 h (Mafan4A/n4A, 29.3 ± 3.5%, n = 17 vs control: 12.3 ± 2.5%, n = 17, Fig. 1c). Whereas all Mafan4A/n4A mutant pups survived until P0 + 12 h, they began to die thereafter (Fig. 1c, red vertical arrows). Therefore, an apneic time fraction of less than 15% as observed in control pups, appears to be physiologically acceptable (at least for some hours), while its increase over 25%, as in the mutants, compromises survival. Intriguingly, in contrast to Mafan4A/n4A mutants, Mafa null mutants showed a reduced incidence of apneas when compared to controls, further confirming the pro-apneic action of Mafa and suggesting a gain-of-function mutation (Fig. 1d).

Plethysmographic traces of the breathing patterns of control and Mafan4A/n4A mutant pups displayed two types of apneic events. The first type was characterized by an abrupt post-inspiratory reduction of expiratory flow that prolonged lung inflation, suggesting a closure of the airways, and that we termed “breath holding apneas”, the second type consisted of a respiratory pause at the end of expiration (lung deflated) and was reminiscent of central apneas (Fig. 1e). In breath holding apneas, the slow deflation of the lung could be accompanied by a resuming of rhythmic inflation efforts that progressively, on a cycle-to-cycle basis, showed increased tidal volumes and likely resuming of rhythmic inflation efforts (Supplementary Fig. 3b) that immediately precede expiratory-like lung deflations terminating breath holds. Interestingly, clicks were virtually absent during both (non-vocal) eunpneic breathing (Supplementary Fig. 3c) and central-like apneas (Supplementary Fig. 3d, e). Although a direct measurement of airway patency was not feasible in our experimental conditions, these data strongly support the obstructive nature of breath holding apneas in mutant pups.

Breath holding and central-like types of apnea contributed similarly (about 5%) to the apneic time fraction of control (Mafa+/lox/lox) pups at P0 +12 h (Fig. 1g). In Mafa+/lox/lox mutants at the same stage, the incidence of “breath holding apneas” was selectively increased about three-fold (n+/−/+, 8.3 ± 2.8%, n = 10 vs. nCre+/+, 22.6 ± 4.4%, n = 10). Conversely, in MafaLacZ/LacZ null mutants, breath holding apneas were more than five-fold reduced (Mafa−/−, 9.7 ± 1.9%, n = 9 vs MafaLacZ/LacZ, 1.7 ± 0.7%, n = 13, Fig. 1h). Altogether these data indicate that the incidence, specifically, of breath holding apneas is commensurate with the rates of Mafa protein in central neurons, pointing to an unexpected link between Mafa post-translational regulation and the control of airway patency at birth.

Mafa expression in the central nervous system includes inhibitory neurons in the caudal medulla. We looked for candidate neurons causal to the respiratory deficit by examining Mafa-expressing (Mafa+) neuronal populations using the Mafa−/−/lox/lox line. Mafa+ neurons were mostly found in sensory regions of the central and peripheral nervous system: the olfactory bulb, auditory networks of the pons, the spinal trigeminal nucleus, and, as previously shown, in dorsal root ganglia and in ventral and dorsal neurons of the spinal cord25–27,34,35 (Fig. 2a). None of these Mafa+ neuronal populations are known to participate in the control of breathing. Conversely, two regions with a prominent respiratory role, the preBötzing complex (Fig. 2b) that generates the respiratory rhythm (see Ref. 36 for a review) and the retrotrapezoid nucleus (Fig. 2c) that modulates it as a function of CO2 levels (see Ref. 37 for a review) were devoid of Mafa+ neurons. Accordingly, comparable rhythmic inspiratory-like activity was recorded from the fourth cervical spinal root in isolated brainstem preparations from Mafa+/lox/lox and control P0 littermates (Supplementary Fig. 4a, b), and Mafa+/lox/lox mutant pups had a preserved CO2 chemoreflex at birth (Supplementary Fig. 4c).

Taken together, these results show that the respiratory distress of Mafa+/lox/lox mutants is caused by alterations outside of the preBötC rhythm generator and of its attendant RTN modulator, in keeping with the notion that Mafa−/−/lox/lox mutants present with breath holding, rather than central-like, type apneas.

Interestingly, Mafa was most interestingly expressed in two discrete populations of the medulla reticular formation, one flanking the lateral and ventral margins of the hypoglossal motor nucleus (Mo12) here termed the peri-hypoglossal area (per12) including its accessory nucleus (accMo12, Fig. 2d, e, h), and another, more caudal population located close to the nucleus ambiguus (periAnnB, Fig. 2f, g, i). In situ hybridization indicated that Mafa+ neurons in both of these regions were predominantly inhibitory (83% S100A5+ (Glyt2+), 76% Gad1+, 72% Gad2+ compared to 7% Slc17a6+ (VGlut2+), Fig. 2g).

Taken together, these data suggest that the 4A mutation might enhance the incidence of breath holding apneas through an action on medullary inhibitory neurons.

The Mafa 4A mutation acts on GABAergic transmission. To examine the possibility that the respiratory deficit of Mafa+/lox/4A mutants may be related to inhibitory synaptic transmission, we injected newborn pups subcutaneously with pentylenetetrazol (PTZ), a GABA A receptor antagonist that crosses the blood brain barrier. Using a PTZ dose (40 μg/g) that does not induce seizures in neonates38, we compared the ventilation of PTZ treated Mafa+/lox/4A and control pups at the P0 + 12 h time, when the
incidence of apnea is maximal. After administration of PTZ, the regular breathing frequency of both control and mutant pups was found increased (Fig. 3a). In addition, PTZ injection, but not saline, reduced by more than 40% the apneic time fraction of Mafa4A/4A mutant pups but had no effect on that of controls (Fig. 3b). The PTZ-induced rescue of Mafa4A/4A mutants apneic breathing was transient (0.5–1 h) in line with the short half-life of PTZ49. None of the mutants (0/10) died within the one-hour period following PTZ injection, with 5/10 then dying in the next following two-hour period, when PTZ was no longer effective. Notably, PTZ reduced the apneic time fraction through a selective effect on apneas of the “breath holding” type (Fig. 3c) suggesting that these are caused by endogenously over-active inhibitory GABAergic circuits.

To further examine the role of inhibitory neurons in the 4A mutation-induced respiratory deficit and specifically in promoting breath holding apneas, we conditionally knocked in the 4A mutation in inhibitory neurons using a Slc32A1-tres-Cre line40 (VGATCre, Fig. 3d). VGAT is a vesicular inhibitory aminoacid transporter expressed in both GABAergic and glycinergic neurons. We crossed VGATCre;MafaVGAT4A with Mafaflox4A/4A mice and recorded the ventilation of VGATCre; Mafaflox4A/4A (MafaVGAT4A) mutant neonates. Fifty percent (6/11) of MafaVGAT4A mutant pups died before P2 compared to only 15% (2/13) death observed among control pups. Moreover, plethysmographic recordings indicated that MafaVGAT4A mutants showed an apneic time fraction double that of control littermates (Fig. 3e–g) accounted by a selective increase in the incidence of breath holding apneas (Fig. 3h). The reasons why MafaVGAT4A mutants show reduced morbidity compared to Mafafl4A/na4A mutant is presently unknown. These data show that restricting the 4A mutation to inhibitory neurons was sufficient to recapitulate the severe increase in the incidence of breath holding apneic events.

Mafa is expressed by premotor neurons controlling upper airway patency. We then refined the search for candidate Mafa inhibitory hindbrain neurons to examine whether breath holding apneas may result from (i) altered sensory control of breathing originating in trigeminal and vagally-derived sensory neurons that innervate the larynx and the lungs and mediate powerful protective reflexes to slow down or pause breathing41,42 and/or (ii) altered GABAergic transmission in dorsolateral pontine respiratory areas as the case in a mouse model of the Rett syndrome43. Using RNAscope, we report that the jugular and nodose vagal ganglia are largely devoid of Mafa+ neurons (Fig. 4a) and that the few present, as the case in the trigeminal sensory ganglia (Fig. 4b), co-expressed VGlt2 and are thus excitatory (Fig. 4c). Next, using the intersectional line VGATCre; Mafaflox4A/4A (MafaVGAT4A) to selectively label Mafa+ inhibitory neurons we found that they were also absent from the secondary sensory structures targeted by the above sensory afferents, namely the paratrigeminal nucleus (Pa5), the principal trigeminal nucleus (Pr5) and in the nucleus of the solitary tract (nTS). Furthermore, pontine dorsal respiratory areas comprising the Kölliker-Fuse and the parabrachial nucleus also lacked Mafa+ inhibitory neurons (Fig. 4d). These findings argue strongly that Mafa mutant apneic breathing is unlikely to be caused by alterations of respiratory sensory feedbacks or sensory integration at medullary of pontine levels.

By contrast, in keeping with the probable effect of the 4A mutation on: (i) upper airway motor control and (ii) the presence of inhibitory candidate neurons in the medulla reticular formation we then looked for evidence that the Mafa4A mutation targets neurons in the motor arm of respiratory control. We
that express only the rabies virus encoded mCherry) was located tracing to do this, we injected unilaterally, in motorizing an upper airway opener muscle by trans-synaptic viral accessory hypoglossal motor nucleus while the bulk of (i.e., that co-express both fluorophores) were found in the hyoid bone forward to increase airway patency (Fig. 4e) and at P3, a G-defective rabies virus variant encoding the fluorophore YFP in the geniohyoid muscle (i.e., that pulls the uorophore GFP labeled neurons in 4 inhibitory neurons (Fig. 4g, h) accounted for 10.0 ± 0.4% of all geniohyoid premotor neurons of the peri12 group of Mafa inhibitory neurons (Fig. 4f). There, we found that Mafa+ mCherry+ virally labeled geniohyoid premotor neurons (Fig. 4g, h) accounted for 10.0 ± 0.4% of all geniohyoid premotor neurons of the peri12 area (counted from 1123 mCherry+/GFP− labeled neurons in 4 pups). These data reveal the existence of a Mafa+ inhibitory premotor neuronal subset in a position to depress the motor drive of the geniohyoid muscles, thus facilitate the collapse of upper airways. We next sought the mechanism whereby Mafa expression could increase inhibitory neuronal activity.

Mafa-4A mutation does not alter neuronal development. As Mafa is expressed early in neural progenitors, we investigated the possibility that Mafa mutations may interfere with the development of inhibitory neurons. Focusing on neurons of the medulla which form an exclusive Mafa+ inhibitory population we have compared their number across the different Mafa mutant genotypes at P0 (Supplementary Fig. 5). Since the Mafa protein is not detectable by immunostainings in wild type animals, but only in 4A homozygous mutants where the protein is stabilized, we compared (n = 10 sections, from 2 animals/genotype) the number of Mafa+ neurons (immunostained for Mafa) in Mafa4A/4A and MafaLacZ/LacZ (immunostained for βgal). No significant differences in the number of Mafa+ neurons were detected between the different genotypes. These data indicate that Mafa affects neither the proliferation, nor the survival or the migration of inhibitory neurons in which it is expressed but, leave open the possibility that it may potentiate their synaptic efficacy.

Gad2 is a direct Mafa target gene. One way in which Mafa could regulate GABAergic transmission is by controlling GABA
Fig. 4 Absence of Mafa inhibitory neurons in sensory and pontine respiratory structures and presence of Mafa inhibitory premotor neurons controlling the geniohyoid muscle. a Left, transverse sections of P0 wild type pups showing the vagal jugular and nodose ganglia stained for DAPI (white) and by RNAscope using a probe against Mafa (red). Right, top, close-up views from the jugular ganglion probing Mafa (red), VGlut2 (green), Phox2b (blue) and the merge. Right, bottom, close-up views from the nodose ganglion. b Transverse sections of the trigeminal ganglion stained for DAPI (white) and Mafa (red). Right, close up views of the inset in (b) probing Mafa (red), VGlut2 (green) and Gad1&Gad2 (blue). Note the absence of Mafa expression in the nodose ganglion, expression of Mafa in the jugular (sparse) and trigeminal ganglia restricted to VGlut2-expressing excitatory neurons.

c Summary of RNAscope probing for co-expression of Mafa/VGlut2- and Mafa/Gad1&2-expression in vagal (5 sections, n = 3 ganglia) and trigeminal (7 sections, n = 3 ganglia) ganglia, nucleus tractus solitarius (nTS, 8 sections, n = 2 pups) and peri12 (8 sections, n = 2 pups). Plots represent mean ± sem.

d Left, transverse hemi-section of the brainstem at pontine level of a VGATCre/+; MafaFloxLacZ/+ P8 pup, showing absent Mafa+ inhibitory neurons (black) in the PB/KF, Pr5 areas but their presence in the SPO and DLL. Right, representative transverse hemi-section of the brainstem at medullary level showing the presence of Mafa+ inhibitory neurons in the peri12, peri-nAmb but their virtual absence in the Sp5, Pa5 and nTS (n = 2 pups).

e Monosynaptic tracing scheme showing unilateral injection of G-deleted Rabies viruses encoding mCherry and of a helper G- and YFP-encoding HSV virus into the geniohyoid muscle to transynaptically trace the position of premotor neurons.

f Representative transverse section (from n = 4 VGATcre/+; MafaFloxLacZ/+ pups) immunostained for βgal (white) in the caudal medulla showing mCherry+ virally labeled premotor neurons (red) and rare (at this axial level) seeding mCherry+/YFP+ geniohyoid motoneurons (yellow) in the accessory hypoglossal motor nucleus (arrowhead). g Close-up view of the inset in f showing that a fraction of mCherry+ premotor neurons indicated by arrows are Mafa+ (mCherry+/βgal+, white nuclear labeling). h 2D-reconstruction of the position of Mafa+ geniohyoid premotor neurons (red dots) and Mafa+ cells (gray dots) showing their location in the peri12 area. DLL dorsal nucleus of the lateral lemniscus, KF Kölliker-Fuse nucleus, PB parabrachial nuclei, Pa5 paratrigeminal nucleus, Pr5 principal sensory trigeminal nucleus, scp superior cerebellar peduncle, SPO superior paraolivary nucleus. Scale bars (µm): 100 (a, b left, c), 50 (a, b right), 1000 (d), 500 (f, h), 100 (inset).
Fig. 5 Gad2 is a direct target of the Mafa transcription factor. a Gad2 in situ hybridization (black) combined with anti-bgal staining (red) on P0 transverse sections of the caudal medulla of section of MafaLacZ/+ (left) and MafaLacZ/4A (right) pups. Note the reduced Gad2 signal surrounding bgal+ nuclei in the absence of Mafa. Data from three mice for each genotype. In DAOY medulloblastoma cell line (b) or primary culture of granule cell progenitors (c) that do not express Mafa (Ctl, left plots in b and c), transfection with either Mafa WT or Mafa 4A yields Mafa expression (left plots) that induces Gad2 transcription (middle plots). The right panel shows the fold induction of Gad2, compared to Mafa expression (n = 2 biological replicates each with n = 3 technical replicates). d The Gad2 promoter (878nt upstream of Gad2 transcription start, in between oligos used to clone Gad2 promoter in pGL3) contains MAF Responsive Elements (MARE, red boxes). e Luciferase assays in DAOY cells reporting Gad2 promoter (left) and control luciferase activity (right) when co-transfected with increasing doses of plasmids encoding Mafa WT or Mafa 4A. All assays were performed in triplicates. Two-way ANOVA was used to test that Mafa enhances Gad2 promoter activity in a dose-dependent manner (P < 0.0001, df = 3; F = 218.4) and that Mafa 4A induces endogenous Gad2 transcription more efficiently than Mafa WT (P < 0.0001, df = 1; F = 93.8). Plots represent mean ± sem. Scale bar (μm): 50 (a).

Chemogenic activation of Mafa+ inhibitory neurons triggers apneas. To confirm that the MafaLoA/A/+Mafa/4A/aapneic phenotype arises from increased activity of Mafa+ inhibitory neurons, we used an intersectional chemogenetic approach. We first verified that Mafa+ inhibitory neurons could be targeted by dual recombination with a Mafa/Flopo and a VGATCre reporter line (VGATCre/+, Mafa/Flopo/; RC::Fela/+), which specifically labels Mafa+/VGAT− (excitatory) neurons with GFP and Mafa+/VGAT+ (inhibitory) neurons with βgal (Supplementary Fig. 6a–d). Furthermore, we confirmed that Mafa+ neurons targeted in the caudal medulla were almost exclusively inhibitory. In addition to the cell groups detected by ISH for Mafa and MafaCre (excitatory) neurons with GFP, transient phase of Mafa expression in the rhombencephalon synthesis. We first confirmed qualitatively by ISH that inhibitory Mafa+ neurons in the medulla in Mafa null mutants, expressed lower amounts of Gad2 transcripts compared to heterozygous MafaLacZ/+ littermates (Fig. 5a). We next performed RT-qPCR to assess quantitatively the hypothesis that in MafaLoA/A mutants, the higher rates of Mafa would result in augmented Gad2 synthesis. To do this, plasmids encoding wildtype (WT) or Mafa-4A were transfected into a human neuronal cell line (medulloblastoma, DAOY) that do not express Mafa. As measured by RT-qPCR, Mafa significantly increased Gad2 expression in both cell lines (Fig. 5b) with Gad2 expression being four times higher in Mafa-4A expressing cells. These results were further confirmed on primary neuronal cells using mouse cerebellum primordia cultures (Fig. 5c). Inspection of the Gad2 proximal promoter region revealed the presence of at least 3 potential Maf Responsive Elements (MARE, red boxes). A Gad2 proximal promoter region containing these 3 potential MAREs was cloned into the pGL3 Luciferase reporter vector (Gad2-Luc) and a medulloblastoma cell line was transfected with either Mafa WT or Mafa 4A yields Mafa expression (>50 μm) in the absence of Mafa. Data from three mice for each genotype. In DAOY medulloblastoma cell line (b) or primary culture of granule cell progenitors (c) that do not express Mafa (Ctl, left plots in b and c), transfection with either Mafa WT or Mafa 4A yields Mafa expression (left plots) that induces Gad2 transcription (middle plots). The right panel shows the fold induction of Gad2, compared to Mafa expression (n = 2 biological replicates each with n = 3 technical replicates). d The Gad2 promoter (878nt upstream of Gad2 transcription start, in between oligos used to clone Gad2 promoter in pGL3) contains MAF Responsive Elements (MARE, red boxes). e Luciferase assays in DAOY cells reporting Gad2 promoter (left) and control luciferase activity (right) when co-transfected with increasing doses of plasmids encoding Mafa WT or Mafa 4A. All assays were performed in triplicates. Two-way ANOVA was used to test that Mafa enhances Gad2 promoter activity in a dose-dependent manner (P < 0.0001, df = 3; F = 218.4) and that Mafa 4A induces endogenous Gad2 transcription more efficiently than Mafa WT (P < 0.0001, df = 1; F = 93.8). Plots represent mean ± sem. Scale bar (μm): 50 (a).

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(Supplementary Fig. 6f, g). With this in mind, we used the dual Mafa/VGAT recombinogenic background to trigger expression of a conditional G-protein coupled receptor hM3Dq-mCherry fusion DREADD (Designer Receptor Exclusively Activated by a Designer Drug) in all these neurons (Fig. 6a, Supplementary Fig. 6h). The breathing behavior of 12 intersectional DREADD mutant neonates (P1–P2) was monitored before and after a single subcutaneous injection of the DREADD agonist clozapine-N-oxide (CNO, 5 mg/kg). Ten of twelve experimental pups displayed significantly increased apneas after CNO treatment (Fig. 6b, c). The incidence of apneas started to increase 10–20 min after CNO injection, reaching a plateau during which the pups presented on average with a 25% apneic time fraction. These experimental mice recovered normal breathing two hours after the CNO injection except for 2 that could not reverse the induced apneic breathing and died shortly after the end of recordings. No increase in apnea or lethality was observed following CNO injection in control littersmates (VGAT+/–; Mafaflpo/+;Fl:hM3Dq/+, Fig. 6d) and no effect of saline vehicle was observed on mutants (Fig. 6c). CNO treatment increased the incidence of breath holding apneas in keeping with preceding results following knock-in of the 4A mutation and PTZ pharmacological treatment. However, CNO treatment also augmented the incidence of central-like apneas (Fig. 6d) possibly through the spurious access to preBötC neurons revealed above in the lineage tracing analysis. We conclude that acute activation of Mafa– inhibitory neurons in vivo is able to trigger breath holding apneas thus mimicking the breath holding phenotype of Mafa+/−mice at birth.

Discussion

Apnea remains one of the major respiratory disorders spanning all ages from infants to adults. We found in mouse neonates a relationship between the stabilization of the transcription factor Mafa (via its 4A mutation) and an increased incidence of breath holding apneas incompatible with survival. We show that Gad2 is a target gene of Mafa; that the apneic phenotype can be rescued by blocking GABA_A receptor-mediated synaptic transmission and that apneas are promoted by chemogenetic activation of Mafa-expressing inhibitory neurons or by their selective expression of the 4A mutation. Our analysis of the ventilation of Mafa mutant neonates has revealed a spectacular link between Mafa and the incidence of apneas, particularly breath holding apneas. When compared to wild types, Mafa null mutants devoid of Mafa, and 4A mutants with higher than normal rates of Mafa, showed reduced and increased incidence of apneas, respectively, suggesting that Mafa transcription factor rates need to be regulated in order to limit the incidence of apnea immediately after birth at a physiologically tolerable level.

We have shown that increased Mafa protein levels in 4A mutant mice likely results in increased Gad2 transcription as indicated by our in vitro transcriptional analysis and the rate of apneas in vivo. GAD65, synthetized from the Gad2 gene, is a rate-limiting enzyme responsible for GABA synthesis, especially during intense synaptic activities. We propose that by mutating Mafa phosphorylation sites (4A mutation) and preventing Mafa phosphorylation by GSK3, GAD65 levels are increased. Moreover, a similar apneic phenotype is seen when the mutation is expressed either in all Mafa+ neurons or when it was restricted to the inhibitory Mafa+ cell fraction. This indicated a prominent mediation of the effect through inhibitory GABAergic rather than excitatory Mafa neurons. This was confirmed by recruiting the apneic phenotype by systemic injection of a GABA_A receptor antagonist. As increased presynaptic amount of cytosolic GABA, synthetized by GAD65, is known to potentiate GABA release, it is likely that increased transcription of Gad2 results in increased efficacy of GABAergic neurons harboring the 4A mutation. Indeed, Mafa phosphorylation by GSK3, through controlling cytosolic GABA level may impact synaptic vesicle filling and the size of the cycling vesicular pool, two interlocked processes through which augmented transmitter supply translates into augmented synaptic release. Finally, it has been shown that GSK3 is de-activated in an activity-dependent manner through intracellular calcium-dependent activation of PI3K and Akt signaling pathways. In Mafa+ inhibitory neurons, this activity-dependent inhibition of GSK3 activity would stabilize Mafa and ensure that GABAergic neurons harboring the 4A mutation is expressed either in all Mafa+ neurons or when it was restricted to the inhibitory Mafa+ cell fraction.
novel means to displace the excitatory/inhibitory balance of activity through interference with discrete inhibitory neuronal subsets in model circuits. For example, hypo-activity of Mafa4A mutants suggests an impairment of locomotor circuits54,55 that can now be addressed by intersectional genetic strategies, using the present Mafa1nk and Mafa1pp lines.

Mouse neonates presented with two distinct types of apneic events distinguishable by the lung inflation status and association to click sounds. About one half of apneic events in wild types were central-like apneas during which typically the lungs deflate normally, while the other half occurred while the lungs remain inflated, which is typical of "breath holds". Strikingly, Mafa1nk mutants and Mafa4A/VGAT4A/VGAT3A mutants showed an increase in the incidence of breath holding but not of central-like apneas, implying a central dysfunction other than in the rhythm generator which we confirmed was functional in vitro. The possibility that mutant breath holding apneas correspond to apneustic events (i.e., prolonged maintenance of inspiratory tone56) is unlikely. First, apneustic breathing requires deficient drives from both vagal afferents and posterior respiratory structures that would have disrupted the general pattern of breathing57,58. Second, breath holds developed after inspiration had ceased and were at times accompanied by tentative inspiratory efforts, two features incompatible with an ongoing apneustic "inspiratory cramp". Third, apneusis cannot be induced pharmacologically in mouse neonates59. Finally, breath holding apneas causing partial or complete retention of the inspired air in the lung were selectively accompanied by a production of a click time-locked to sudden deflations of the lung terminating the breath holds. This temporal organization is reminiscent of the re-opening of closed airways observed during vocal breathing albeit in the absence of the powerful expiratory effort compressing the lung and enabling call emission (this study and9). Although clicks have unknown origin, in these two breathing contexts they could strikingly stand as acoustic signatures of the re-opening of closed airways.

The present observations suggest that in mutants, stabilization of Mafa in inhibitory premotor neurons controlling upper airway patency might favor obstructions. We show that this may apply to the geniohyoid muscle known to increase airway patency. In addition, we identify breath holds as major targets of Mafa-related controls. Although the obstructive nature of breath holds would require further investigation, it is strongly supported by the ability to maintain lung inflation, the resuming of rhythmic inflation efforts and the final elicitation of a click, otherwise known to correspond to the opening of airway cavities from a closed state, a concept that has phonological relevance, in association to USV emission in mice (this study and9) and as a regular part of the consonant systems of many human languages60. This issue is important clinically, because example cases of airflow limitation during exhalation have been described in children following sighs61,62 and breath holds pushing air against closed airways are a common feature of abnormal breathing in Rett syndrome (RTT) patients63,64. In Mafa4A/VGAT3A mutant pups, the inspirations that precede breath holds were not augmented. In a mouse model of RTT, post-inspiratory breath holds where air is pushed against closed airways are thought to result from insufficient43,65 GABAergic drives to the KF that would entail prolongation and augmentation of post-inspiratory upper airway adductive, and abdominal muscle expiratory, drives43,66. In Mafa4A/VGAT3A mutant pups, the onset of the obstructive event would take place after inspiration has ceased in agreement with an impairment of the post-inspiratory coordination of pharyngeal muscles contractions albeit in the absence of noticeable joint expiratory contractions. Furthermore, Mafa4A inhibitory neuronal targets are lacking in primary and secondary sensory vagal and trigeminal neurons (including the nTS, the putative source of inhibitory drive to the KF thought to be depressed in RTT43). Breath holding apnea akin to Rett syndrome and breath holds associated to the Mafa4A mutation, although sharing post-inspiratory airflow closure, appear to arise from orthogonal contexts regarding (i) GABAergic drives respectively deficient (Rett) and augmented (Mafa4A) and (ii) the association to pronounced expiratory efforts, respectively present (Rett) and absent (Mafa4A) and thus should not be confused. As the Mafa4A mutation likely spares respiratory reflexes and the excitability of pontine respiratory circuits the deficit may rather owe to enhanced inhibitory premotor drive onto upper airway opener muscles favoring their collapse.

How increased inhibitory drives from Mafa+ neurons come to set the condition and the location (e.g., palate, tongue base, lateral walls, epiglottis) of airway collapse is not known. Mafa+ inhibitory neurons are resident in the peri12 and peri-nAmb areas. In the peri12, a fraction of them are premotor neurons to the geniohyoid, a muscle whose activity during resting breathing remains controversial67,68 but that activates during inspiratory-resistive breathing and during a way occlusion possibly through active eccentric contraction to regulate the position of the hyoid bone and increase upper airway size and stability59. Airway obstruction is unlikely caused by defective contraction of the sole geniohyoid muscle, further tracing experiments are required to check whether the same geniohyoid (through axonal collateralization69) or other Mafa+ inhibitory, premotor neurons lie upstream hypoglossal motoneurons innervating the genioglossus muscle, the other main pharyngeal opener muscle (reviewed in Ref.15), that also locate for part in the peri12 area71,72. Although the hypoglossal nucleus is often described as inspiratory, hypoglossal motoneurons were additionally categorized as pre-inspiratory, inspiratory/post-inspiratory and even expiratory discharging patterns while these patterns have been shown to evolve in context dependent manners73–75. This makes it possible that depressed hypoglossal motor drives impact upper airway patency outside of inspirations, notably and timely, during post-inspiration. Indeed, reduction of the hypoglossal motor drive during post-inspiration, a time when it should normally be reflexively increased by laryngeal inflation-related sensory drives75 (likely spared by the 4A mutation), could precipitate airway collapse and cause limited expiratory flow.

Nothing is presently known of the Mafa+ inhibitory neurons in the peri-nAmb area, the possibility that they might be premotor to pharyngeal constrictor motoneurons that reside in the nucleus Ambigus will need to be investigated. Paradoxically, when the airway volume is relatively small, inhibition of pharyngeal constrictor muscle tone further enhances pharyngeal wall flaccidity, and favors pharyngeal collapse14. The normal vocalizing behavior of Mafa mutants indicates preservation of adductive movements at laryngeal level. Thus, if obstructive, breath holding apneas of Mafa mutants likely originate in the pharynx mostly composed of soft tissues, thus most susceptible to collapse76. Such a “valve-like” breathing behavior affecting exhalation but not inhalation has been proposed to result from palatal prolapse in Obstructive Sleep Apnea (OSA) patients77,78.

We here identified Mafa as a marker for a set of inhibitory neurons of the reticular formation with probable impact on upper airway patency at birth. Interestingly, we found that 60% of Mafa+ neurons in the peri-hypoglossal reticular formation but not those of the peri-nAmb that flank the nucleus ambiguus, are V1 type neurons with a history of expression of the homeobox gene Engrailed1 (Supplementary Fig. 6i, k). The spatial proximity with somatic Mo12 motoneurons and the triple V1, Mafa+ and inhibitory nature of these cells are evocative of spinal Renshaw cells25 involved in recurrent inhibition of somatic spinal motoneurons79,80. Altogether, Mafa phosphorylation by GSK3,
by modulating GABAergic inhibitory transmission, may impact the resistance of upper airways through controlling the output gain of motor drives at both premotor and, via recurrent inhibitions, motor levels. To get further insights on the role of Mafa + inhibitory neurons in breathing circuits, it will be important, capitalizing on the present molecular signatures, to identify their presynaptic partners in pontine and medullary sites thought to generate post-inspiratory drives and in sleep related areas given the prevalence of obstructive sleep apneas.

The present study points to the possibility that abnormal Mafa gene function may contribute to human respiratory pathologies. It is noteworthy that Toruner et al. identified duplication and translocation of chromosomal 8q24.3 region encompassing the Mafa locus in a few cases of SIDS. An increase in Mafa protein level comparable to the one we describe, triggered by lack of phosphorylation, could occur through increased synthesis in case of gene duplication or other genomic alterations. SIDS has many etiologies, and upper-airway obstruction may be one of them. Pharyngeal collapse occurs in OSA without fatal issue, in 3–7% of studies and 1–4% of children. Lower and posterior position of the hyoid bone is widely recognized as a major trait in OSA etiologies, and upper-airway obstruction may be one of them. The present study suggests that the heritability of this trait has been demonstrated and the heritability of this trait has been demonstrated. Backward position of the hyoid bone can result either from intrinsic craniofacial features or from hypotonic supra-hyoid muscles as suggested here. Potential involvement of Mafa in OSA was also suspected in a human genetic study on whole genome scan for OSA susceptibility loci; instead, the highest linkage with the apnea/hypopnea index was observed in the 8q24 region encompassing the Mafa locus. In addition, numerous epidemiologic studies point to comorbidity between OSA and diabetes mellitus for which the role of Mafa is well-established (reviewed in Ref. ). Mafa dysregulation may be the link between these two pathologies.

Methods

Housing. Animals were group-housed with free access to food and water in controlled temperature conditions (room temperature 21–22°C, humidity 40–50%), and exposed to a conventional 12-h light/dark cycle. All experimental procedures and the handling of mice were done in accordance with the European Community Directive 86/609/EEC and following the recommendations of the French National Ethics Committee for Science and Health report on “Ethical Principles for Animal Experimentation” – CEEA n°59 Paris Centre et Sud – under agreement N° 2015071710462096. All efforts were made to reduce animal suffering and minimize the number of animals.

Mouse genetics. To generate the Mafafllox allele, Mafa-4A cDNA was cloned by PCR and introduced into the targeting vector using SalI – NruI cloning sites (after the 2 kb 3′ genomic region) to enhance homologous recombination. The temporal pattern of breathing in each recorded sample was characterized as regular or apneic from Poincaré plot (Fig.1b). A regular breathing pattern is an homogeneous characteristic of the entire apneic population. Thus, we defined the apnea/hypopnea index as the number of apneas is the number of cycles longer than CORC. In all recording sessions induced by touching their lip with a foam tip and body movements (measured as percentage of time over 5 min observation periods) were examined. Vocalizations were recorded in five minutes continuous audio recording periods (see below).

Phlethysmographic and audio recordings, detection and analysis of respiratory cycles, apnea definition. Breathing volumes were measured in unaesthetized, unrestrained animals by whole-body barometric plethysmography as described previously. Three hundred second continuous records were acquired at defined time points after birth (P0 = 1 h after birth, between 1 and 2 h after birth (P0 + 2 h), 3–5 h after birth (P0 + 4 h), 7–9 h (P0 + 8 h) after birth, 11–13 hours after birth and P1, P2 days after birth). The phlethysmographic transducer (Ti/g/min) was connected to a reference chamber of equal volume and the inter-chamber pressure difference was measured using a differential pressure transducer (Validyne DP-103-14) connected to a demodulator (Validyne CD15). The phlethysmographic signals recorded in the absence of limb or body movements were sampled at 1 kHz, stored on a computer and analyzed using the Elphy2 software (developed by G Sadoc at CNRS). The duration of inspirations (T1) and expirations (T2) were measured to calculate breath frequency Fb (Fb = 1/Ti + Te; breath/min); tidal volume, VT (microL); and ventilation, VE (VT × Fb) (microL/min). Calibrations were performed by injecting 2.5 µL of air in the chamber with a Hamilton syringe. The CO2 chemosensor was tested by measuring ventilatory changes induced by a two minute exposure to 8% CO2, 5% O2, 4% N2.

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P0 + 12 b) hours after birth and at P1, P2 days after birth). The phlethysmographic signal was recorded in the absence of limb or body movements and was sampled at 1 kHz, stored on a computer and analyzed using the Elphy2 software (developed by G Sadoc at CNRS). The duration of inspirations (T1) and expirations (T2) were measured to calculate breath frequency Fb (Fb = 1/Ti + Te; breath/min); tidal volume, VT (microL); and ventilation, VE (VT × Fb) (microL/min). Calibrations were performed by injecting 2.5 µL of air in the chamber with a Hamilton syringe. The CO2 chemosensor was tested by measuring ventilatory changes induced by a two minute exposure to 8% CO2, 5% O2, 4% N2.

The temporal pattern of breathing in each recorded sample was characterized as regular or apneic from Poincaré plot (Fig. 1b). A regular breathing pattern was a mix of repetitive cycles (regular breathing cycles followed and preceded by cycles of about the same duration) and of long isolated cycles (apneic cycles followed and preceded by short cycles). Apneic cycles were defined as being longer than a “cut-off respiratory cycles” (CORC) duration, measured in Poincaré plots from the longest repetitive cycle (CORC) located at the origin of the correlation (forming an ovoid cloud close to the origin) and the population of isolated apneic cycles (spreading along the x and y axes away from the diagonal, Fig. 1b).

Alternatively, we defined apneic respiratory cycles as cycles longer than a threshold duration (ThD). Using ThD’s classically used to define apneas (e.g., ThD = 3 s or longer in mice), we found that the total duration of apneic cycles (apnea time fraction, ATF) is proportional to log(ThD). Robustness of this relationship (whatever the ThD is), suggests that the constant ATF/log(ThD) ratio is an homogeneous characteristic of the entire apneic population. Thus, we extrapolated ThD toward shorter cycles (e.g., ThD < 3 s), until a limit beyond which AT/log(ThD) differed from the typical apneic value. This limit measures the CORC; ATF for ThD = CORC gives the total time spent in apnea; the number of apneas is the number of cycles longer than CORC. In all recording samples, identical values were obtained using our alternative definition of apneas (i.e., isolated events), indicating that both methods depict the same population of apneas. Figure 1 provides a characteristic example.

Breath holding apneic events in plethysmographic recordings were characterized by (i) a post-inspiratory airway closure (filled arrowheads in Fig. 1c, i) causing partial

Fig.1).
complete or unrestricted inspiration in the lung (plateau above the basal end-expiratory volume) either followed by (i) waning and resuming of rhythmic inspiratory efforts with airflow decreasing to near zero (large inspiration events, empty arrowheads, Fig. 1c) or (ii) the presence at the end of the breath holds of a brief (<1 ms) audio-electrophysiological event named “click” (Figs. 1f, 3e, 6b and Supplementary Fig. 3b). Click emissions were found temporally associated to inspiratory on-switch and terminalization of lung compression enabling USVs during vocal breathing sequences (Supplementary Fig. 3a). Note that the analysis of plethysmographic recordings during vocalizations (Supplementary Fig. 3a), must prominently take into account lung compression, thus the relationship between pressure, flow rate and resistance of upper airways (Boyle law) in addition to the vaporization of water during inspiration (ideal gas law) itself prominent during resting breathing. Similarly, during breathing holding apneic breathing clicks were time-locked both to inspiratory on-switch, and the termination of breath holding apneas (Fig. 1f and Supplementary Fig. 3b). Whether clicks associated to inspiratory on-switch also denote an end-inspiratory obstructive context remains to be investigated. Audio recordings were obtained with an UltraSoundGate condenser microphone capsule CM16 (sensitive to frequencies from 20 Hz to 180 kHz) and Avisoft Recorder software (sample rate, 250 kHz; format, 16 bit) from Avisoft Bioacoustics. To synchronize the acquisitions of plethysmographic and audio signals, a “send trigger” command is written into the Ethly program. This generates a 10 ms trigger pulse sent to both an analog input of the automated data collection system acquiring the plethysmographic signal and the Avisoft UltraSound Gate system acquiring the audio signal. Although the synchronization of the two signals can be obtained within 1 ms accuracy, the clocks of the two systems have a ±50 ppm rating, which can result in deviations of ±10 ms/min of recording. To take this drift into account, we gently knocked on the plethysmographic chamber with a metal rod before the end of the recording session to create synchronous signals from a pressure change and sound that were used after acquisition to adjust the timing for both signals. Repeated tests demonstrated that this procedure ensured that the latencies between the plethysmographic and the audio signals were <2 ms in a 10-min recording. Ethly configuration and program files are freely available at: http://yerelast.github.io.

A total of 1070 clicks were counted in joint audio-plethysmographic recordings (5 min duration) from WT animals (n = 6) and found preferentially associated to vocal breathing (81.3 ± 4.6%) and breath holding apneic breathing (15.0 ± 3.7%) while very scarce during eupneic and central apneic breathing (2.2 ± 0.7% and 1.1 ± 0.5%, respectively, Supplementary Fig. 3e). Examination of the occurrence of clicks in relation to respiratory contexts were performed on 5 s samples during vocalizing events (n = 70 samples from 4 wild type pups), eupnea (n = 40 samples from 3 wild type pups) and breath holding events (n = 15 samples from 5 Mafafl/fl mutants pups). This analysis was not performed during central apneic events for lack of clicks. In these samples, plethysmographic traces were aligned and centered on the inspiratory-on switch (n = 47 events) and end lung emptying (n = 34 events) for vocal breathing (1 s time window, Supplementary Fig. 3a), on small amplitude upward pressure shifts (n = 25 events) for breath holds (2 s time window, Supplementary Fig. 3b) and aligned on the inspiratory-on-switch and centered at approximately a mid-respiratory cycle time (n = 40 events) for eupneic breathing (1 s time window, Supplementary Fig. 3c). The about 50% value of the mode of the distribution (central 20 ms mode bin) for vocal breathing is due to the temporal excitation allowing click detections timed to the preceding or the following events. Central-like apneic events were characterized by a respiratory pause following a normal breath while the lung was deflated. Using these criteria breath holding apneas were validated manually and independently by two investigators, when the nature of the apneic cycle was found ambiguous (<1% of apneic cycles) the cycle was ruled as a central-like apneic cycle.

Pentylene tetrozole (PTZ) injections and chemogenetic activation of Mafa inhibitory neurons. We performed PTZ dose response analysis (2, 10, 20, 40 and 80 µg/animal) in mouse neonates to determine the maximal PTZ dose (40 µg/kg) that did not induce an epileptic crisis. A single subcutaneous injection of 0.02 ml (40 µg) PTZ was performed at P0 + 12 h. Plethysmographic signals were acquired just before and 30 min after treatment in control and Mafafl/fl mutants. A third group of Mafafl/fl mutants was injected with isotonic NaCl following events. Central-like apneic events were characterized by a respiratory pause following a normal breath while the lung was deflated. Using these criteria breath holding apneas were validated manually and independently by two investigators, when the nature of the apneic cycle was found ambiguous (<1% of apneic cycles) the cycle was ruled as a central-like apneic cycle.

Viral Tracking experiments and histology. Briefly, mouse pups between ages P2-4 were anesthetized by hypothermia and two microinjections of a viral solution were made. Following surgery, mice received a helper virus, HSV-hCMV-YPB (titer –3 e + 8) was pressure injected unilaterally into the geniohyoid muscle via a glass micro-pipette. Five days post-injection, the pups were transcardially perfused with cold PBS and 4% PFA and the brains collected for histological processing. Serial sections (30 µm thick, every 2nd section) were imaged using a 10X objective (Nikon) or a 20X objective (Olympus) at 256 X 256 X 256 pixel resolution. Images were analyzed using Image J and Photoshop.

Cell culture conditions and granule cell progenitors' purification. Medullo-blastoma cell lines DAOY and ONY-76 were cultured in MEM and RPMI 1640 medium (GIBCO), respectively, supplemented with 10% fetal bovine serum (GIBCO), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). 0.125 µg/ml fungizone (Invitrogen). DAOY Medium was supplemented with 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. Granule cell progenitors (GCP) were purified from mouse cerebella dissected at P7. Following dissociation (30 min, 37°C) in trypsin/DNase solution at 37°C and triturating in Dounce (with either a Leica TCS SP8 confocal microscope or using a slide scanner (Hamamatsu NanoZoomer S210). Mafa expressing cells in Mafafl/fl embryos were visualized using a BiGalan staining procedure. Briefly, dissected brainstem at different embryonic stages were fixed 2 h in 1% formaldehyde-0.2% glutaraldehyde in washing solution (2 mM MgCl2, 10 mM sodium citrate, 0.5% NP-40, 5 mM D-glucose buffer saline) and stained for 2–4 h in a solution of 1 mg/ml X-Gal (5 mM potassium ferricyanide, K4[Fe(CN)6]; 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40, in PBS).

The following antibodies were used for immunostainings: rabbit anti-Mafa (Bethyl IHC-00352; dilution 1:200 in PBS 0.05% Tween 20) followed either with a secondary goat anti-rabbit Cy3 antibody (Invitrogen, A10520). Since Mafa protein was barely detectable in wild type animals at P0 we also used a double amplification system: first anti-avidin/avidin (BioRad) coupled biotinylated secondary antibody and then goat anti-biotin antibody (Jackson). The Tyramide Signal Amplification (TSA Plus, Perkin Elmer, coupled Cy3).

In situ hybridization was performed on 14 µm thick frozen sections. cDNA clones encoding mouse Gad1 (IMGP9819102), mouse Gad2 (IMAG9980154122), mouse Glyt2 (IRC50914K88Q) were purchased from Sigma or provided by L. Gage and M. Garaschuk. Multiplex single molecule Fluorescent In Situ Hybridization (smFISH) was performed on 14 µm thick cryosections using a RNA probe (Advanced Cell Diagnostic) following manufacturer instructions. The following probes were used Mafa (C1), Phox2b (C2) and Vglut2 (C3) and revealed using the respective Tyramide substrate (Cy3, Opal520 and C5). In one set of experiment, the Phox2b probe was replace by a mix Gad1/Gad2 probe design in C2 channel. All probes were obtained from Advanced Cell Diagnostic company. Section were counter labeled using DAPI and slide mounted using ProLong Diamond antifade medium. Images were acquired using a confocal Zeiss LSM700, processed using Image J and Photoshop.

Fluorescent in situ hybridization. Hindbrain, vagal and trigeminal ganglia were obtained from P0 mice pups (C57B6 background) fixed in 4% paraformaldehyde. Multiplex single molecule Fluorescent In Situ Hybridization (smFISH) was performed on 14 µm thick cryosections using a RNA probe (Advanced Cell Diagnostic) following manufacturer instructions. The following probes were used Mafa (C1), Phox2b (C2) and Vglut2 (C3) and revealed using the respective Tyramide substrate (Cy3, Opal520 and C5). In one set of experiment, the Phox2b probe was replaced by a mix Gad1/Gad2 probe design in C2 channel. All probes were obtained from Advanced Cell Diagnostic company. Section were counter labeled using DAPI and slide mounted using ProLong Diamond antifade medium. Images were acquired using a confocal Zeiss LSM700, processed using Image J and Photoshop.

Articulation
1 h plating. GCPs were infected, washed and cultured (37 °C, 5% CO₂) in the medium for 48 h when their RNA was extracted.

**Real time RT-qPCR, constructs, transfection and retroviral production.** Total RNAs were extracted using RNeasy Plus mini kit (Qiagen) and reversely transcribed using Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen). Quantitative real-time PCR assays (see primer list on Supplementary Table 6) were conducted using SYBR Green real-time PCR Master Mix and real-time PCR amplification equipment (Applied Biosystem). The RT-qPCR analysis was normalized using TBP expression.

The pcDNA3-Mafa or Mafa-4A plasmids were obtained by inserting the Mafa or Mafa-4A coding region into the BamH1 and EcoRI restriction sites of the pcDNA3 vector (Invitrogen). Medullolobastoma cells DAOY and ONS-76 were transfected using Effectene reagent (Qiagen). Transfected cells were selected with neomycin. The retroviral constructs pMiGR-Mafa or Mafa-4A were obtained by inserting the Mafa or Mafa-4A coding region into pMiGR. Retroviruses were produced in 293T cells, by co-transfecting retroviral pMiGR-derived vectors and the packaging plasmids pCAG-4 and pMD.gag-pol, using lipofectamine 2000 (Invitrogen). Retroviral particles were harvested 48–72 h after transfection.

**Statistical analysis.** All data are reported as mean ± sem. Normal distributions of data points were tested using D’Agostino & Pearson or Shapiro–Wilk tests. One- and Two-way ANOVA was used to compare groups of data, unpaired and paired Student’s t-test were used to compare two Gaussian distributions of continuous variables, non-parametric tests (Mann–Whitney or Wilcoxon) were used otherwise. Chi square (χ²) test was used for discrete variables. All graphs and statistical analyses were generated using GraphPad Prism software. *p < 0.05, **p < 0.01, ***p < 0.001.

**Statistics and reproducibility.** For all experiments, the number of experiments is indicated in the legends of the relevant figure. Histological analyses and tracing experiments were reproduced a minimum of three times.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The data that support the findings of this study can be found in the Source Data provided with the paper. Microcopy data are available from the corresponding authors upon reasonable request.

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Competing interests
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Additional information
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Correspondence and requests for materials should be addressed to . Present address: Institut de Biologie de l’Ecole Normale Supérieure IBENS, École Normale Supérieure, CNRS, INSERM, PSL Université Paris, 75005 Paris, FranceLaure Lecoin, Celio Pouponnot or Gilles Fortin.

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