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The vesicular glutamate transporter VGLUT3 contributes to protection against neonatal hypoxic stress

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Key points

- Hypoxic stress is an important cause of morbidity and mortality in neonates.
- We examined the role of VGLUT3, an atypical transporter of glutamate present in serotonergic neurons involved in breathing and heat production, in the response to hypoxia.
- The respiratory responses to chemical stimuli and the turnover of serotonin in the brainstem were impaired in newborn mice lacking VGLUT3.
- Under cold conditions, metabolic rate, body temperature, baseline breathing and the ventilatory response to hypoxia were disrupted.
- Thus, VGLUT3 expression is required for optimal response to hypoxic stress in neonates.

Abstract Neonates respond to hypoxia initially by increasing ventilation, and then by markedly decreasing both ventilation (hypoxic ventilatory decline) and oxygen consumption (hypoxic hypometabolism). This latter process, which vanishes with age, reflects a tight coupling between ventilatory and thermogenic responses to hypoxia. The neurological substrate of hypoxic hypometabolism is unclear, but it is known to be centrally mediated, with a strong involvement of the 5-hydroxytryptamine (5-HT, serotonin) system. To clarify this issue, we investigated the possible role of VGLUT3, the third subtype of vesicular glutamate transporter. VGLUT3 contributes to glutamate signalling by 5-HT neurons, facilitates 5-HT transmission and is expressed in strategic regions for respiratory and thermogenic control. We therefore assumed that VGLUT3 might significantly contribute to the response to hypoxia. To test this possibility, we analysed this response in newborn mice lacking VGLUT3 using anatomical, biochemical, electrophysiological and integrative physiology approaches. We found that the lack of VGLUT3 did not affect the histological organization of brainstem respiratory networks or respiratory activity under basal conditions. However, it impaired respiratory responses to 5-HT and anoxia, showing a marked alteration of central respiratory control. These impairments were associated with altered 5-HT turnover at the brainstem level. Furthermore, under cold conditions, the lack of VGLUT3 disrupted the metabolic rate, body temperature, baseline breathing and the ventilatory response

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to hypoxia. We conclude that VGLUT3 expression is dispensable under basal conditions but is required for optimal response to hypoxic stress in neonates.

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Abbreviations AI, apnoea index; AU, arbitrary unit; pFRG, parafacial respiratory group; 5-HIAA, 5-hydroxyindoleacetic acid; HVD, hypoxic ventilatory decline; IS, irregularity score; L-Trp, L-tryptophan; MAOA, monoamine oxidase A; PB, phrenic burst; RMg, raphe magnus; ROb, raphe obscurus; RPa, raphe pallidus; RPn, raphe pontis; RRG, respiratory rhythm generator; RTN, retrotrapezoid nucleus; VGLUT3, vesicular glutamate transporter type 3.

Introduction

Maintaining oxygen homeostasis through independent breathing is a critical ability throughout extrauterine life. Hypoxic stress, whether due to increased oxygen demands as in the cold, or to reduced oxygen delivery to tissues as in asphyxia, is a common cause of morbidity and mortality in neonates (Perrone *et al.* 2002). Neonates respond to hypoxia initially by increasing ventilation, and then by markedly decreasing ventilation (hypoxic ventilatory decline, HVD) and oxygen consumption (hypoxic hypometabolism), mainly through a centrally mediated inhibition of heat production (Saiki *et al.* 1994; Merazzi & Mortola, 1999; Bollen *et al.* 2009). This centrally mediated process is prominent in neonates but gradually vanishes with age (Massaro *et al.* 1995; Gautier, 1996; Mortola, 2004; Kamae *et al.* 2011). The neuronal substrate of hypoxic hypometabolism is unclear, but recent results support a strong involvement of the serotonin (5-hydroxytryptamine or 5-HT) system in the coupling of ventilatory and thermogenic responses to hypoxia (Alenina *et al.* 2009; Hodges *et al.* 2011; Osaka, 2011).

In this study, we examined the possible role of VGLUT3, the third subtype of vesicular glutamate transporter, in the ventilatory and thermoregulatory responses to neonatal hypoxic stress. Our interest in VGLUT3 originated in the intriguing observation that, contrary to VGLUT1 and VGLUT2, which are expressed by genuine glutamatergic neurons (Fremeau *et al.* 2004), VGLUT3 is present mostly in modulatory neurons (El Mestikawy *et al.* 2011). This is the case of cholinergic interneurons from the striatum, some GABAergic neurons from the cortex and the hippocampus, and most importantly with regards to oxygen homeostasis, in 5-HT neurons (El Mestikawy *et al.* 2011). In serotonergic neurons, VGLUT3 stimulates 5-HT transmission and provides the means to signal with glutamate (Amilhon *et al.* 2010; Varga *et al.* 2009). We anticipated that VGLUT3 may be important for the newborn's ability to generate the appropriate respiratory responses to hypoxic stress for two reasons. First, the two networks that compose the respiratory rhythm generator (RRG, Thoby-Brisson *et al.* 2009), the pre-Bötzinger complex (Smith *et al.* 1991) and the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG Onimaru & Homma, 2003), contain glutamatergic neurons and are both strongly modulated by 5-HT afferents. The RTN/pFRG, which is pivotal to central chemoreception (Guyenet *et al.* 2008), is densely innervated by VGLUT3 terminals from the raphe nuclei (Rosin *et al.* 2006). Second, thermoregulatory circuits contain VGLUT3 expressing cells (Morrison *et al.* 2008; Nakamura *et al.* 2005*a*; Schafer *et al.* 2002). In particular, VGLUT3 expressing cells in the raphe innervate the main neonatal thermogenic organ, the interscapular brown adipose tissue (Schafer *et al.* 2002; Nakamura *et al.* 2005*a*; Morrison *et al.* 2008).

To address the role of VGLUT3 in the response to hypoxic stress in neonates, we analysed mice lacking VGLUT3 (*Vglut3*$^{-/-}$) using anatomical, biochemical, electrophysiological and integrative physiology approaches. Previous studies in *Vglut3*$^{-/-}$ mice showed that VGLUT3 is a resistance factor against psychological stress as early as infancy, but its possible role against physiological stress has never been investigated (Amilhon *et al.* 2010). We found that while the lack of VGLUT3 did not affect the histological organization of brainstem respiratory networks or respiratory activity in normal conditions, it disrupted respiratory and thermogenic responses to cold and hypoxia, and in particular, HVD and hypoxic hypometabolism. These results indicate that VGLUT3 expression is required for optimal response to hypoxic stress in neonates.

Methods

Ethical approval

Experimental protocols were approved by local committees, in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU) for animal care, and were conducted in accordance with Canadian and French laws for animal care. The experiments also complied with the policies and regulations outlined by *The Journal of Physiology* (Drummond, 2009). All efforts were made

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to minimize animal suffering, especially by using fully non-invasive functional tests. Newborn mice used in *in vivo* experiments were killed by decapitation upon completion of physiological tests.

Animals

All experiments were performed in newborn ($n = 474$) wild-type, heterozygous and homozygous littermates obtained from crossing heterozygous *Vglut3*$^{+/-}$ mice (129/Sv × C57/BL6). *Vglut3*$^{+/-}$ parents (Gras *et al.* 2008) were housed at 24°C with a 12 h light–dark cycle and fed *ad libitum*.

Immunofluorescence

Six-day-old mice were anaesthetized deeply by an intraperitoneal injection of 1% sodium pentobarbital (60 mg (kg body weight)$^{-1}$), and perfused transcardially with 150 ml warm 0.9% saline followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected, conserving the pons, post-fixed by immersion in the same fixative, cryoprotected in phosphate-buffered saline (PBS) containing 10% sucrose and frozen in isopentane at −30°C. Ten-micrometre coronal sections were made with a cryostat at −20°C and thaw-mounted onto glass slides. Sections were hydrated with PBS and immersed in EDTA (10 mM). They were then washed with PBS containing gelatin (2 g l^{-1}) and 0.25% Triton X-100 (buffer B) and incubated with buffer B supplemented with VGLUT3 guinea pig polyclonal antiserum (1/10000; Gras *et al.* 2008), PHOX2B rat monoclonal antiserum (1/800; kindly provided by Dr Christo Goridis, Ecole Normale Supérieure, Paris), 5-HT mouse monoclonal antiserum (1/50; Chemicon), VMAT2 rabbit monoclonal antiserum (1/5000; Chemicon) and NK1R rabbit monoclonal antiserum (1/5000; Sigma-Aldrich). VGLUT3, PHOX2B and 5-HT primary antibodies were detected with anti-guinea pig, anti-rat or anti-mouse IgG coupled to Alexa Fluor 488, Alexa Fluor 544 and Alexa Fluor 544 (1/2000; Molecular Probes, USA), respectively. VMAT2 and NK1R primary antibodies were detected using anti-rabbit IgG coupled to Alexa Fluor 544 (1/2000; Molecular Probes, USA). Sections were observed under a conventional fluorescence microscope (Zeiss, Germany) at 10× and 40× magnification. The *Vglut3*$^{-/-}$ mice is a constitutive knockout obtained by targeting exon 2 of the *Vglut3* gene. This strategy results in a loss of this coding exon and a one base frame shift. Consequently, there is a complete loss of both mRNA and protein (Gras *et al.* 2008).

Electrophysiology

Two- to three-day-old mice were chilled over crushed ice for several minutes until they were immobile and failed to respond to a tail pinch (Phifer & Terry, 1986). The brainstem and the first segment of cervical spinal cord were isolated and placed ventral side upward in a recording chamber (volume: 2 ml) as described (Suzue, 1984; Voituron *et al.* 2010). This *en bloc* preparation is devoid of peripheral chemoreceptors including the carotid bodies, O_2 sensitive cells in the hypothalamus and peripheral thermoreceptors. Under basal conditions, the preparation was superfused at a rate of 4 ml min^{-1} at 27°C with artificial cerebrospinal fluid (aCSF; in mM: 129.0 NaCl, 3.35 KCl, 1.26 $CaCl_2$, 1.15 $MgCl_2$, 21.0 $NaHCO_3$, 0.58 NaH_2PO_4 and 30.00 glucose) saturated with O_2 and adjusted to pH 7.4 by bubbling with carbogen (95% O_2 and 5% CO_2). Recordings were performed as described (Voituron *et al.* 2010). Briefly, the electrical activity of a C4 ventral root was recorded using a suction electrode, filtered (100–3000 Hz), amplified (×5000), integrated (time constant 100 ms) and digitized through a Spike2 data analysis system (CED, Cambridge, UK; 5 kHz sampling frequency). Phrenic burst (PB) frequency (expressed in Hz) was defined as the frequency of spontaneous rhythmic C4 bursts. Integrated C4 activities were used to measure the duration of PBs (expressed in seconds) and the irregularity score (IS) of the PB cycle period. The IS was used to assess the variability of the respiratory cycle and was obtained by applying the formula: 100 × ABS $(P_n - P_{n-1})/P_{n-1}$, with P being the period of the nth respiratory cycle (Viemari *et al.* 2005). After dissection, the preparations were superfused for at least 30 min with normal aCSF. The phrenic responses to 5-HT, acidosis and anoxia were examined on different preparations. To examine the PB response to 5-HT, normal aCSF was replaced with aCSF containing 5-HT (25 μM) for 5 min. The PB response to acidosis was examined by replacing normal aCSF (pH 7.4) with modified aCSF containing a reduced level of $NaHCO_3$ (10.0 mM instead of 21 mM, pH 7.1; acidosis) (Voituron *et al.* 2010). The PB response to anoxia was examined by replacing normal aCSF by aCSF bubbled with 95% N_2 and 5% CO_2 for 5 min (anoxia). When subjecting preparations to modified aCSF, test-induced changes in PB frequency in a given preparation were expressed as a percentage of baseline levels determined during the 5 min period preceding the test.

Plethysmography

Breathing variables were measured noninvasively using a battery of four whole-body flow barometric plethysmographs, allowing the simultaneous measurement of breathing variables as previously described (Bollen *et al.* 2009). Each plethysmograph was composed of two 50 ml Plexiglas chambers, immersed in a thermoregulated water bath to maintain their temperature at given levels (26°C or 33°C). A 200 ml min^{-1} flow of dry

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air (Brooks airflow stabilizer, Urlo, Holland) was divided into two 100 ml min^{-1} flows through the chambers. The differential pressure between the chambers (GE Sensing transducer, Asnières, France, range: ±0.1 mbar) was converted into a digital signal at a sampling rate of 100 Hz, and processed (Labview, National Instruments, Austin, TX, USA).

The apparatus was calibrated before each session using a built-in pump incorporating a micro-syringe (Ito corporation, Fuji, Japan), which injected a sinusoidal air-flow with a maximal amplitude of 2 μl and a frequency of 6 Hz into the animal chamber (Ramanantsoa *et al.* 2006). We measured respiratory frequency (R_f, Hz), tidal volume (V_T, $\mu l\,g^{-1}$), minute ventilation ($\dot{V}_E$, $\mu l\,s^{-1}\,g^{-1}$), and the number of apnoeas, defined as respiratory pauses longer than twice the preceding breath (Simakajornboon *et al.* 2004). The limitations of the plethysmographic method in newborn mice have been discussed elsewhere (Lofaso *et al.* 2007; Mortola & Frappell, 1998). Briefly, the absolute values of V_T and $\dot{V}_E$ derived from the Drorbaugh and Fenn equation (Drorbaugh & Fenn, 1955; Epstein & Epstein, 1978) are purely indicative, whereas R_f and apnoea duration, as well as the relative change from baseline of V_T, R_f and $\dot{V}_E$, are reliable measurements. O_2 consumption ($\dot{V}_{O_2}$) was measured in six-day-old pups in cold condition as previously described (Bollen *et al.* 2009). Baseline levels of breathing variables were calculated over the 3 min of air breathing prior to exposure to hypoxia (10% O_2).

Biochemical analysis of the brainstem serotonergic system

The medulla was quickly removed after decapitation on postnatal day 2 and kept at −80°C until analysis (Viemari *et al.* 2005). The medullary content of 5-HT, its precursor L-tryptophan (L-Trp) and its main metabolite from the monoamine oxidase A (MAOA) degradation pathway, 5-hydroxyindoleacetic acid (5-HIAA), were measured with high pressure liquid chromatography (HPLC) and electrochemical detection (Waters System: pump P510, electrochemical detector EC2465; Atlantis column DC18; mobile phase: citric acid, 50 mM; orthophosphoric acid, 50 mM; sodium octane sulfonic acid, 0.112 mM; EDTA, 0.06 mM; methanol, 5%; NaCl, 2 mM; pH 2.95). Contents were expressed in nanograms per gram of medulla.

Statistics

Lack of VGLUT3 was expected to impair 5-HT metabolism, respiratory control *in vitro* and physiological responses to hypoxic stress *in vivo*. We used Student's unpaired two-tailed *t* test for genotype group comparisons (mutant *versus* controls). Respiratory variables and $\dot{V}_{O_2}$ were firstly analysed in baseline conditions and in response to chemical stimuli using analyses of variance and Student's unpaired two-tailed *t* test. Litter had no significant effect, either as a main between factor or in interaction with genotype, and will not be mentioned further. All quantification was performed by investigators blind to the genotype, which was determined after all tests were completed. Statistical analyses were conducted using Statview 5 (Abacus Concepts, Berkeley, CA, USA).

Results

As data from *Vglut3*$^{+/+}$ and *Vglut3*$^{+/-}$ newborn mice were indistinguishable regardless of the variable considered, these two groups were pooled and thereafter designated as control pups.

Lack of VGLUT3 does not alter the histological organization and function of respiratory areas under basal conditions

We first compared coronal sections of the medulla of *Vglut3*$^{-/-}$ and control pups. No differences were seen in the organization of the characteristic NK1R signal of the pre-Bötzinger complex (Fig. 1*A* and *B*), or the medial bundle of NK1R-expressing fibres or the raphe pallidus (RPa), raphe magnus (RMg) and raphe obscurus (ROb) areas (Fig. 1*A* and *B*). Finally, we observed very ventral PHOX2B-expressing neurons in the RTN/pFRG area of control and *Vglut3*$^{-/-}$ pups, without any noticeable difference between these groups (Fig. 1*A* and *B*).

We next examined whether the lack of VGLUT3 affected neonatal RRG function. We compared the activity produced by isolated RRGs from *Vglut3*$^{-/-}$ and control pups. Under basal conditions, phrenic bursts (PBs) had similar shapes, amplitudes, frequencies and irregularity scores in *Vglut3*$^{-/-}$ and control preparations (Fig. 1*C* and *D*, and Table 1 for statistical analyses).

As these *in vitro* results were obtained using completely deafferented medullary RRGs, we decided to examine breathing variables in intact, unrestrained pups using plethysmography under thermoneutral conditions (33°C, Fig. 1*F*). Statistical comparisons between *Vglut3*$^{-/-}$ and control pups are summarized in Table 2. *Vglut3*$^{-/-}$ and control pups had similar weights (Fig. 1*G*), temperatures (Fig. 1*H*) and breathing patterns under normoxia, regardless of the breathing variable considered (Fig. 1*I*–*L*).

Lack of VGLUT3 affects the 5-HT modulation of respiratory activity and 5-HT metabolism

VGLUT3 is expressed in 5-HT neurons from the dorsal and medial raphe (Amilhon *et al.* 2010). Because raphe

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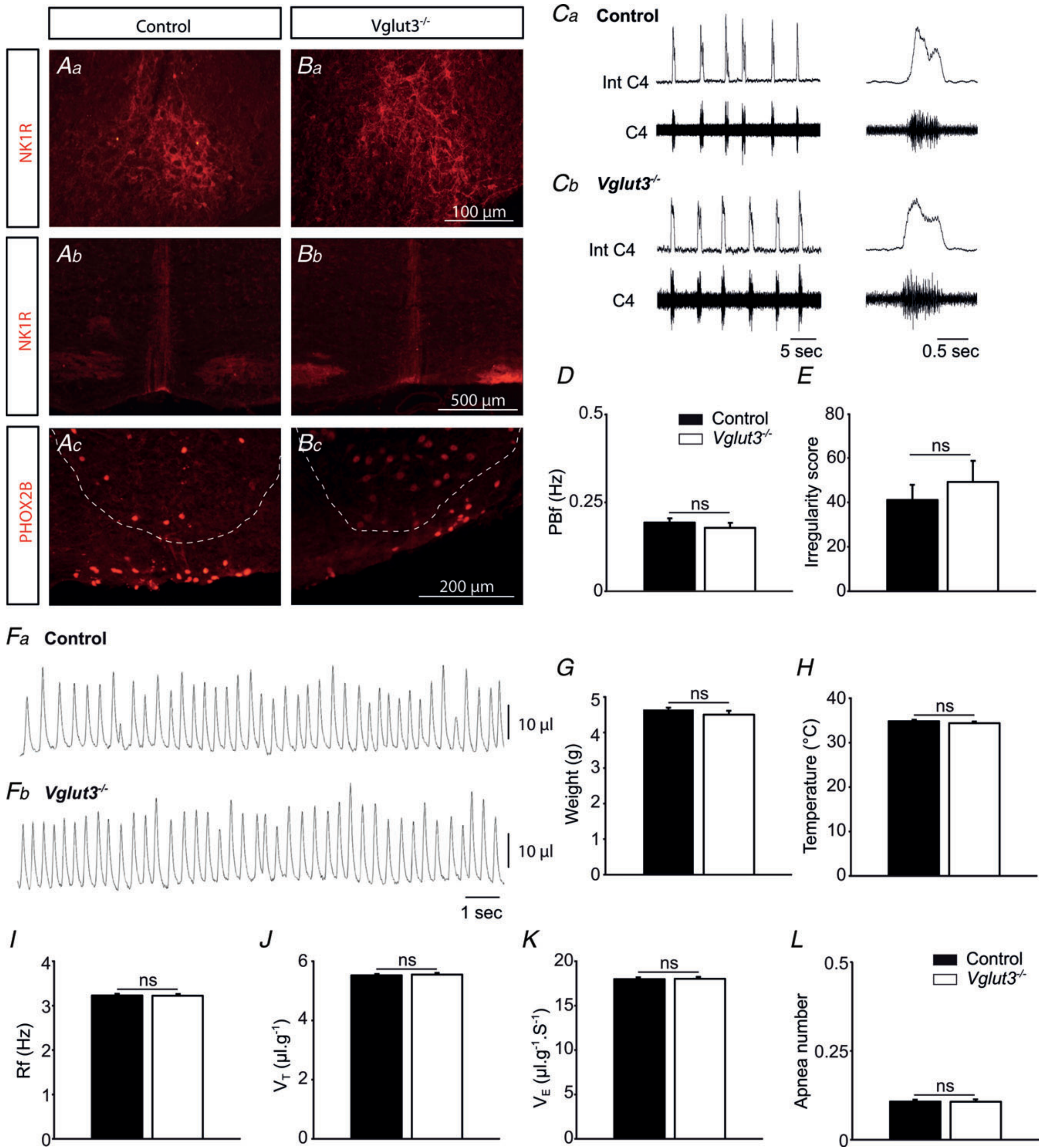

Figure 1. Lack of *Vglut3* does not alter the histological organization and function of respiration-related areas under basal conditions

See Supplemental Fig. 1 for schematic representation of the brainstem sections. *A* and *B*, immunolabelling showing that NK1R (red) in the pre-Bötzinger complex (*Aa* and *Ba*) and at the midline raphe level (*Ab* and *Bb*) and Phox2B (red) at the RTN/pFRG level (*Ac* and *Bc*) have similar expression patterns in control and *Vglut3*$^{-/-}$ pups. Anatomical limits of the facial nucleus have been superimposed on *Ac* and *Bc* section (dotted line, Lazarenko *et al.* 2009). *C*, examples of phrenic bursts (PBs) recorded from the C4 root of *in vitro* brainstem preparations from control (*a*) and *Vglut3*$^{-/-}$ (*b*) pups. Int C4: integrated C4 activity. *D* and *E*, neither phrenic burst frequency (PBf) (*D*) nor the irregularity score (*E*) was significantly affected by the *Vglut3* mutation under basal conditions (control $n = 64$; *Vglut3*$^{-/-}$ $n = 22$). *F*, examples of recordings of respiratory variables using plethysmography in one control (*a*) and one *Vglut3*$^{-/-}$ (*b*) newborn mouse. *G* and *H*, *Vglut3*$^{-/-}$ pups ($n = 53$) had normal weights (*G*) and body

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Table 1. RRG responses to chemical stimuli

	Control	*Vglut3*$^{-/-}$
Baseline	$n = 64$	$n = 22$
PB frequency (cycles min^{-1})	11.6 ± 0.6	10.7 ± 1.0
PB duration (s)	0.9 ± 0.1	0.9 ± 0.1
PB irregularity score (AU)	41.1 ± 6.8	49.2 ± 9.3
Response to 5-HT	$n = 14$	$n = 11$
PB frequency (%) at 1 min	130.9 ± 9.3††	118.5 ± 8.0†,*
Response to acidosis	$n = 12$	$n = 4$
PB frequency (%) at 5 min	132 ± 4†††	153 ± 13††,*
Response to anoxia	$n = 18$	$n = 8$
PB frequency (%)		
at 1 min	87.2 ± 6.3†††	94.7 ± 5.7
at 5 min	57.8 ± 5.5††††	97.4 ± 14.5***

PB: Phrenic burst; %: percentage of pre-stimulus value; AU: arbitrary units. *,**,***: significant difference between *Vglut3*$^{-/-}$ and control groups ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, Student's unpaired *t* test.). †,††,†††,††††: significant difference between post-stimulus and pre-stimulus levels ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively, Student's paired *t* test). Values are means ± SEM.

neurons have a profound influence on respiratory activity (Hilaire *et al.* 2010; Hodges & Richerson, 2010*a*), we examined the distribution of VGLUT3 in the rostral raphe nuclei, especially those involved in the control of breathing. We performed double immunolabelling for VGLUT3 and vesicular monoamine transporter 2 (VMAT2, Fig. 2*A*) on the one hand and for VGLUT3 and 5-HT on the other (Fig. 2*B* and *C*). As shown previously in adult mice (Amilhon *et al.* 2010), VGLUT3 colocalized poorly with VMAT2-positive somata and terminals in the dorsal raphe (DR), raphe pontis (RPn), RMg and RPa (Fig. 2*A*). Double-labelling for VGLUT3 and 5-HT confirmed the presence of VGLUT3-expressing neurons within the ROb and RMg (Fig. 2*C*). The colocalization of VGLUT3 and 5-HT signals in the somata of cells was observed in the ROb (Fig. 2*B*) and the RMg (Fig. 2*C*). No VGLUT3-expressing neurons were observed either in the pre-Bötzinger complex or in the RTN/pFRG areas of control pups, identified using anatomical landmarks and NK1R and PHOX2b expression (Rosin *et al.* 2006).

VGLUT3 and glutamate have been found to modify 5-HT vesicular filling by a mechanism named vesicular synergy (Amilhon *et al.* 2010). Thus, the lack of VGLUT3 may alter tissue concentrations of 5-HT and its metabolites in the brainstem. To address this issue, we measured endogenous levels of 5-HT, its precursor L-tryptophan (L-Trp) and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) using HPLC separation and electrochemical detection (Fig. 2*D*). The inverse (although non-significant) differences in 5-HT and 5-HIAA content between *Vglut3*$^{-/-}$ and control brainstems led to a significantly higher 5-HT/5-HIAA ratio in *Vglut3*$^{-/-}$ than in control brainstems (∼19%, see Fig. 2*D* for statistical analyses). This difference revealed an alteration in 5-HT turnover in *Vglut3*$^{-/-}$ pups, as previously reported in the hippocampus of adult *Vglut3*$^{-/-}$ mice (Amilhon *et al.* 2010).

Since alterations in 5-HT metabolism during development modify the formation of brain circuits (Deneris & Wyler, 2012), including the respiratory networks (Hilaire *et al.* 2010), we next examined whether the abnormal 5-HT/5-HIAA ratio in the brainstem of Vglut3$^{-/-}$ mice was associated with abnormal 5-HT modulation of respiratory activity. To this end, we applied exogenous 5-HT to the artificial cerebrospinal fluid (aCSF) of *Vglut3*$^{-/-}$ and control preparations for 5 min. Biphasic responses were observed in both preparations, but with quantitative differences (Fig. 2*E*, and Table 1 for statistical analyses). The initial component of this response corresponds to the medullary response and the late component to a spinal response with a tonic discharge of phrenic motoneurons (Bou-Flores *et al.* 2000). Although response latencies were not significantly different between groups, the response was shorter lived and of smaller amplitude in *Vglut3*$^{-/-}$ than in control preparations (see Fig. 2*F* for statistical analyses).

Lack of VGLUT3 disrupts respiratory control

We tested whether the lack of VGLUT3 affected the RRG responses to acidosis and anoxia, both of which are strongly modulated by 5-HT (Caubit *et al.* 2010). We measured the PB frequency response to central acidosis by replacing normal aCSF with acidified aCSF for 5 min (Fig 3*A*). This stimulus markedly stimulates PB frequency in normal preparations (Voituron *et al.* 2010). Acidosis increased PB frequency in both *Vglut3*$^{-/-}$ and control preparations (Fig. 3*A*). However, PB frequency reached

temperatures (*H*) compared to control pups ($n = 164$). *I–L*, breathing patterns were not affected by the *Vglut3* mutation under thermoneutral conditions, whatever the breathing variable considered: mean respiratory frequency (R_f) (*I*), tidal volume (V_T) (*J*), ventilation ($\dot{V}_E$) (*K*), or number of apnoeas per 30 s period (*L*). ns: non significant ($P > 0.05$); Student's unpaired *t* test. Values shown are means ± SEM. See Tables 1 and 2 for full statistical analyses.

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Table 2. Baseline breathing variables under thermoneutral (33°C) and cold conditions (26°C) in normoxia

	Thermoneutrality (33°C)		Cold (26°C)	
	Control $n = 164$	*Vglut3*$^{-/-}$ $n = 53$	Control $n = 200$	*Vglut3*$^{-/-}$ $n = 63$
Weight (g)	4.6 ± 0.9	4.5 ± 0.9	3.6 ± 0.8	3.4 ± 0.8
T (°C)	34.8 ± 0.1	34.4 ± 0.2	34.3 ± 1.1	34.1 ± 0.1
ΔT (°C)	0.5 ± 0.1	0.8 ± 0.2	–4.5 ± 0.1	–5.2 ± 0.1***
$\dot{V}_E$ (μl g^{-1} s^{-1})	16.0 ± 0.2	16.3 ± 0.4	18.1 ± 0.6	11.5 ± 0.6***
R_f (Hz)	3.23 ± 0.54	3.22 ± 0.57	2.83 ± 0.93	2.19 ± 0.61***
V_T (μl g^{-1})	5.52 ± 1.14	5.55 ± 1.11	6.27 ± 2.02	5.18 ± 1.92***
AI (apnoea min^{-1})	0.42 ± 1.2	0.42 ± 1.14	1.22 ± 2.84	3.68 ± 3.78***
			$n = 29$	$n = 7$
$\dot{V}_{O_2}$ (ml min^{-1} g^{-1})	—	—	0.084 ± 0.02	0.069 ± 0.02***

T (°C): body temperature before plethysmographic recordings (and before cold exposure in cold experiments); ΔT (°C): variation in body temperature after plethysmographic recordings (duration: 30 min). $\dot{V}_E$: ventilation; R_f: breathing frequency; V_T: tidal volume. AI: apnoea index, number of apnoeas per minute; $\dot{V}_{O_2}$: oxygen consumption. **,***: significant difference between *Vglut3*$^{-/-}$ and control pups ($P < 0.01$ and $P < 0.001$ respectively, Student's unpaired t test). Values are means ± SEM.

higher values in *Vglut3*$^{-/-}$ than in control preparations (Fig. 3*A*, and Table 1 for statistical analyses).

We then measured the RRG response to central anoxia by replacing normal aCSF with anoxic aCSF for 5 min (Fig. 3*B*). As expected (Hilaire *et al.* 2010), in control preparations, PB frequency was depressed throughout the 5 min of exposure to anoxia, and recovered slowly over 10–15 min after the resumption of normal aCSF (Fig. 3*Bb*). In contrast, in *Vglut3*$^{-/-}$ preparations, the anoxia-induced decrease in PB frequency was short lived, and PB frequency returned to control levels after 2–3 min despite the maintenance of central anoxia (Fig. 3*Bc*, and Table 1 for statistical analyses). However, resumption of normal aCSF produced an immediate decrease in frequency, which was greatly similar to that of control preparations. Taken together, these results suggest that the mechanisms controlling the depression of PB frequencies during anoxia and post-anoxia may be different, as previously proposed for HVD and post-HVD *in vivo* (Renolleau *et al.* 2001), and that only the depression of PB frequency during anoxia was affected by the lack of VGLUT3.

Next, we examined the breathing pattern of 6-day-old pups in air or hypoxia, in cold condition (Fig. 3*C*). Cold condition increases thermogenesis and oxygen demand, which, combined with hypoxia, result in strong hypoxic stress. Under normoxia, the ventilation ($\dot{V}_E$) of *Vglut3*$^{-/-}$ pups was significantly lower (about 37%) than in controls, mostly due to their decreased respiratory frequency, R_f (Fig. 3*D*, and Table 2 for statistical analyses). Hypoxia elicited a biphasic ventilatory response in both groups with an initial $\dot{V}_E$ increase reflecting the RRG response to carotid body chemoreceptor activation by peripheral hypoxia, and a subsequent HVD reflecting the RRG response to central hypoxia (Fig. 3*D*). The increase in $\dot{V}_E$ during the augmentation phase of the hypoxic response resulted from a combination of the rise in V_T and in R_f, as commonly reported (Bissonnette, 2000), although an R_f-based increase under similar conditions has also been reported (Bollen *et al.* 2009). Because of the delay in the wash-out of the hypoxic mixture (about 90 s), maximal ventilatory depression was reached after the switch to normoxia (Fig. 3*Da–c*). The depressive phase of $\dot{V}_E$ resulted from a decrease in V_T and R_f, as previously reported (Bissonnette, 2000; Bollen *et al.* 2009). When expressed as the percentage change relative to average pre-hypoxic baseline values, the initial $\dot{V}_E$ increase in response to hypoxia was not significantly different between groups, although the R_f increase in response to hypoxia was significantly higher in *Vglut3*$^{-/-}$ than in control pups (Fig. 3*E*). However, HVD, expressed as the percentage decrease from pre-hypoxic levels, was significantly attenuated in *Vglut3*$^{-/-}$ pups as compared to controls (Fig. 3*E*). Thus, both *in vivo* and *in vitro* data consistently showed that the lack of *VGLUT3* altered the inhibitory effect of hypoxia on respiratory activity.

Lack of VGLUT3 disrupts the thermogenic response to cold

The cold induced a greater decrease in body temperature in *Vglut3*$^{-/-}$ than in control pups (see statistics in Table 2). We assessed thermogenesis by measuring O_2 consumption

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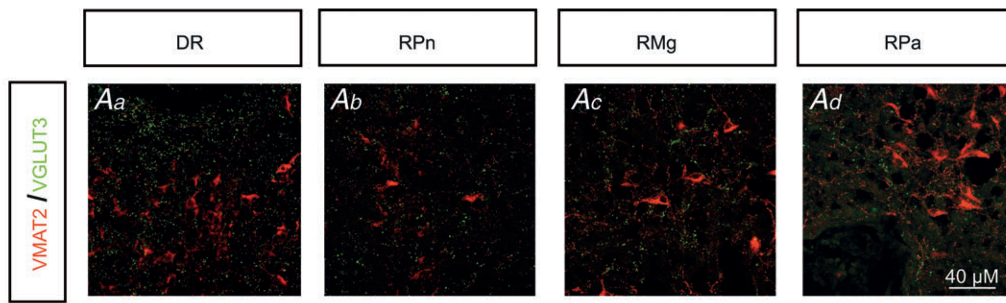

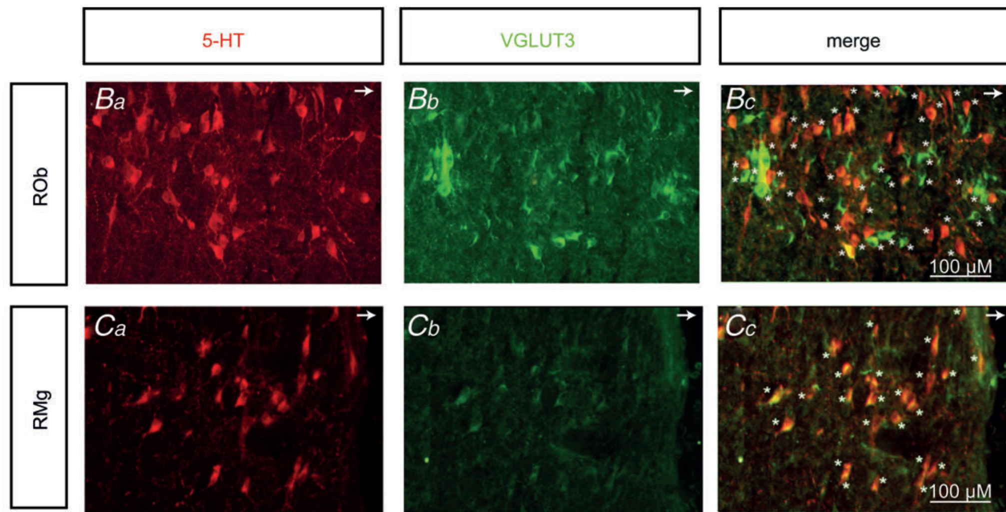

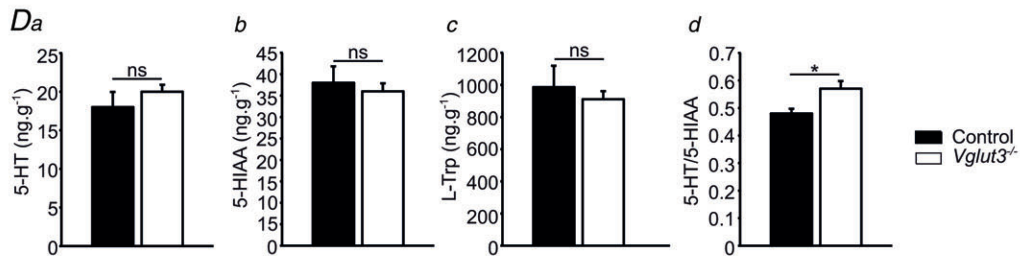

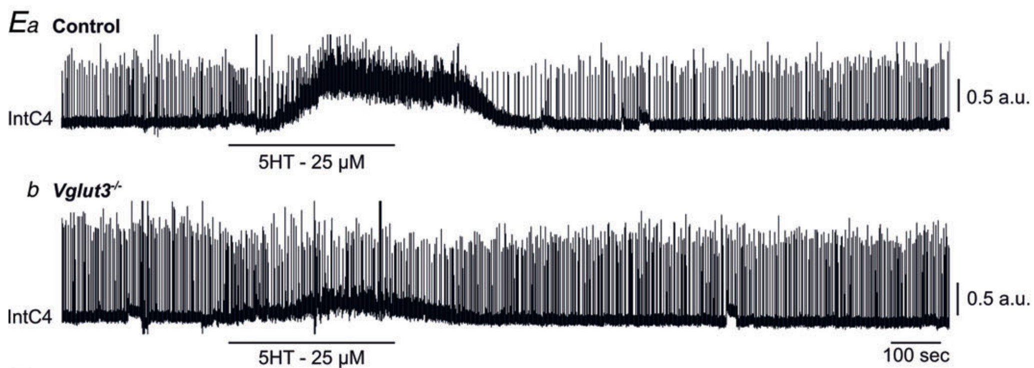

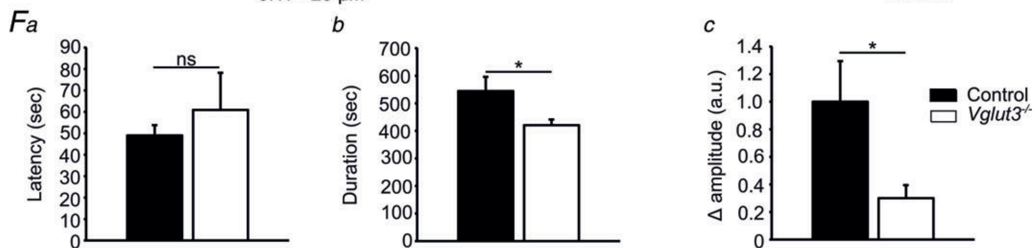

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($\dot{V}_{O_2}$) as a function of body weight during cold exposure. Both groups increased their $\dot{V}_{O_2}$ in an attempt to generate heat and maintain body temperature until a plateau was reached (Fig. 3*Dd*). However, *Vglut3*$^{-/-}$ pups exhibited a much slower rate of increase during the first 12–13 min of normoxia (Fig. 3*Dd*). Furthermore, the plateau level of $\dot{V}_{O_2}$ (steady state) was significantly lower in *Vglut3*$^{-/-}$ pups as compared to controls (Fig. 3*Dd*; see statistics in Table 2), revealing a weaker thermogenic response to cold. The difference in $\dot{V}_{O_2}$ between groups was small compared to the difference in $\dot{V}_E$ (18% *vs.* 36%, respectively), indicating that blunted thermogenesis in *Vglut3*$^{-/-}$ pups did not fully account for their lower $\dot{V}_E$ levels. Thus, the lack of VGLUT3 altered both the time constant and the steady state of the thermogenic response to cold, suggesting that the activation of brown adipose tissue was diminished in *Vglut3*$^{-/-}$ compared to control pups.

In both groups, hypoxia sharply depressed $\dot{V}_{O_2}$ (hypometabolic response to hypoxia, Fig. 3*Dd*), a centrally mediated defensive response to hypoxia in newborns (Mortola, 2004) that reflects the inhibition of thermogenesis (Mortola & Gautier, 1995). The $\dot{V}_{O_2}$ decrease relative to pre-hypoxic levels was smaller in *Vglut3*$^{-/-}$ than in control pups (see statistics in Fig. 3*Ed*), indicating a blunted hypometabolic response to hypoxia. Furthermore, unlike controls, *Vglut3*$^{-/-}$ pups failed to restore $\dot{V}_{O_2}$ to pre-hypoxic levels upon the return to normoxia (Fig. 3*Dd*). Thus, *Vglut3*$^{-/-}$ pups displayed a decreased ability to increase $\dot{V}_{O_2}$ and produce heat in response to cold, as well as a decreased ability to depress $\dot{V}_{O_2}$ in response to hypoxia.

Discussion

Whereas the lack of VGLUT3 did not significantly alter respiratory activity under basal conditions, it did so in response to chemical stimuli *in vitro*, revealing a dysfunction of the metabolic control of breathing. Furthermore, *in vivo*, the lack of VGLUT3 impaired respiratory and thermogenic responses to cold and hypoxia, and in particular, two main components of the defensive strategy to hypoxic stress: HVD and hypometabolism.

The histological analysis of brainstem respiration-related areas did not reveal any significant differences between *Vglut3*$^{-/-}$ and control mice, in line with functional results *in vitro* and *in vivo* under basal conditions. However, we cannot disregard the possibility that the lack of VGLUT3 led to subtle differences in the structure or the number of neurons in some populations under scrutiny.

Brainstem–spinal cord preparations produce periodic activity that corresponds to the respiratory rhythm generated in the brainstem of intact animals, although at a much lower rate (Suzue, 1984). This preparation is commonly regarded as a valuable tool for the analysis of central mechanisms of respiratory control (Johnson *et al.* 2012). However, these preparations present specific characteristics that may hamper extrapolation to *in vivo* conditions. In particular, under normal conditions, superficial medullary tissue layers, where respiratory rhythm is generated, are hyperoxic (Brockhaus *et al.* 1993; Okada *et al.* 1993*a*), whereas deep medullary layers are hypoxic (Okada *et al.* 1993*a*,*b*). Bath application of an anoxic mixture, as in the present study, may produce large and unpredictable gradients of oxygen levels in the preparation. However, we may preclude the possibility that anoxia caused notable damage to respiratory networks because the changes induced in PB frequency are reversible, as previously reported (Okada *et al.* 1998, 2007; Voituron *et al.* 2006, 2010, 2011).

As in most studies using brainstem–spinal cord preparations, we found that bath acidification led to an increase in the respiratory frequency rather than to an

Figure 2. Lack of Vglut3 impairs 5-HT metabolism in the brainstem, and the RRG response to 5-HT
See Supplemental Fig. 1 for schematic representation of the brainstem sections. *A*, immunolabelling showing that Vglut3 (green) colocalizes poorly with VMAT2 (red) in control mice at the level of the dorsal raphe (DR) (*a*), raphe pontis (RPn) (*b*), raphe magnus (RMg) (*c*) and raphe pallidus nuclei (Rpa) (*d*). *B* and *C*, immunodetection of 5-HT (red) and VGLUT3 (green) expression in coronal sections of control mice at the level of the raphe obscurus nucleus (ROb). Arrows in the upper right corner of panels *B* and *C* point toward the ventral medullary surface (*B*) and RMg (*C*). VGLUT3 was found in soma of cells at the ROb (*Bb*) and RMg (*Cc*) levels, and is co-expressed with 5-HT in cells of ROb and at RMg level (stars, yellow cells in *Bc* and *Cc*, respectively). *D*, 5-HT turnover in *Vglut3*$^{-/-}$ pups ($n = 12$) differs from that in controls ($n = 6$). Endogenous levels of 5-HT (*a*), its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) (*b*) and its precursor L-tryptophan (L-Trp) (*c*) in the brainstem of *Vglut3*$^{-/-}$ and control pups, measured by HPLC. The 5-HT/5-HIAA ratio was significantly higher in *Vglut3*$^{-/-}$ than in control pups (*d*). *E* and *F*, the spinal response to exogenous 5-HT is impaired in *Vglut3*$^{-/-}$ pups. *E*, examples of phrenic bursts recorded from the C4 root of *in vitro* brainstem preparations from control (*a*) and *Vglut3*$^{-/-}$ (*b*) pups, superfused with normal aCSF and aCSF containing 5-HT (25 μM, 5 min). Int C4: integrated C4 activity. *F*, tonic discharges in response to 5-HT appeared with the same latency in *Vglut3*$^{-/-}$ ($n = 11$) and control ($n = 14$) preparations (*a*) but were shorter (*b*) and of smaller amplitude (*c*) in *Vglut3*$^{-/-}$ preparations compared to control preparations (*$P < 0.05$; Student's unpaired *t* test, ns: non significant). a.u.: arbitrary units. Values given are means ± SEM. See Table 1 for full statistical analyses.

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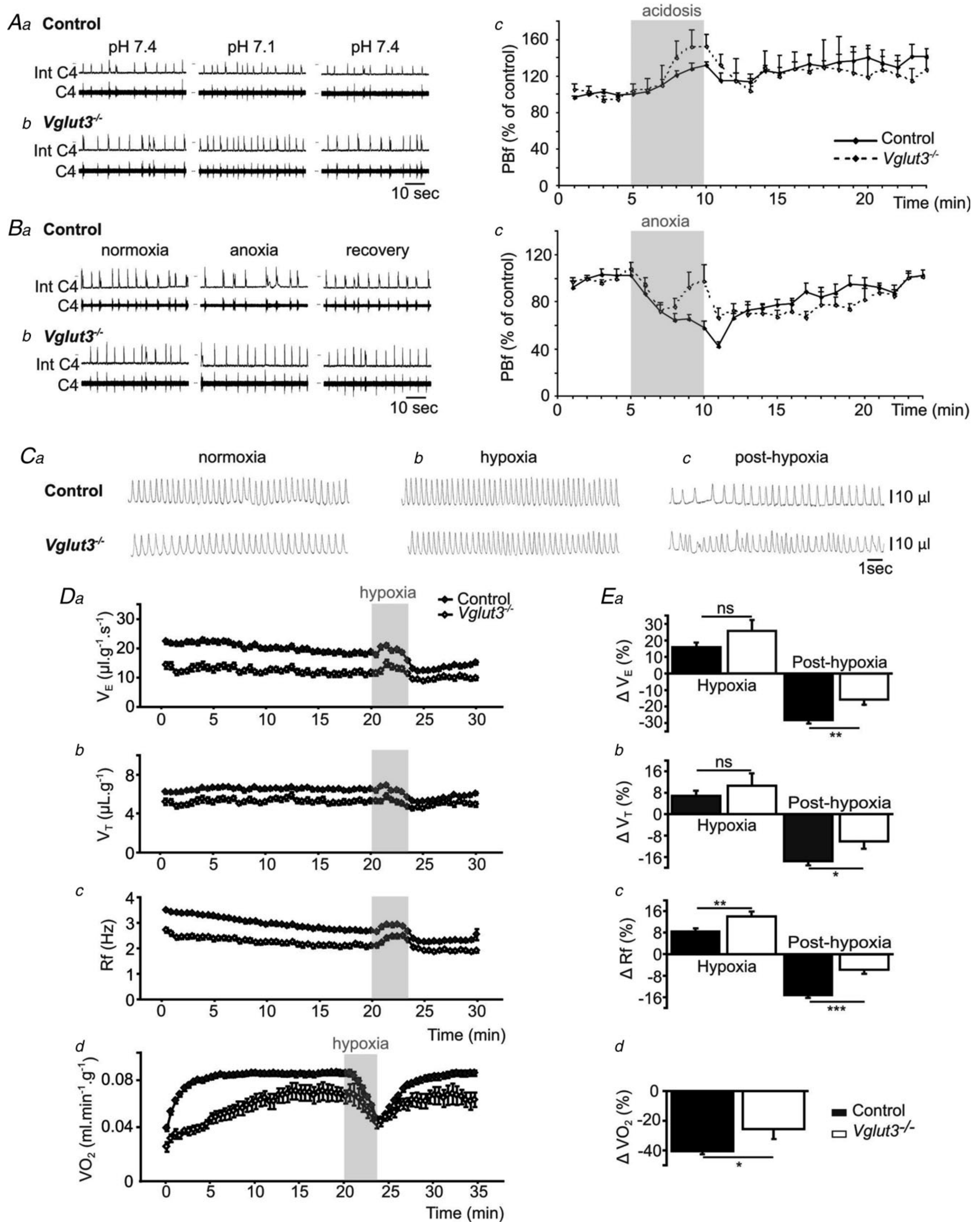

Figure 3. Lack of Vglut3 disrupts respiratory control and thermogenesis in the cold
Aa and *b*, examples of changes in central respiratory drive induced by acidosis in Control (*a*) and *Vglut3*$^{-/-}$ pups (*b*) *in vitro*. Int C4: integrated C4 activity (C4). *Ac*, the effect of acidosis (shaded) on mean phrenic burst frequency

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increase in the amplitude of phrenic nerve bursting (Ballanyi *et al.* 1999). This is in contrast with previous data in newborn mice showing that hypercapnia primarily causes an increase in V_T, with a less prominent increase in respiratory frequency (Ramanantsoa *et al.* 2011). It is generally agreed that the attenuated *in vitro* effects of acidification on the amplitude of phrenic nerve bursting might be related to the removal of afferent sensory inputs in brainstem–spinal cord preparations (Ballanyi *et al.* 1999). Thus, while brainstem–spinal cord preparations are suitable for the analysis of the effects of acidification on the central respiratory network, direct extrapolation to *in vivo* conditions must be done cautiously. In fact, *in vivo* and *in vitro* approaches are complementary in this respect.

The increase in the 5-HT/5-HIAA ratio in the brainstem of *Vglut3*$^{-/-}$ pups is in line with previous observations in the hippocampus of adult *Vglut3*$^{-/-}$ mice (Amilhon *et al.* 2010). This increase may reflect decreased turnover of 5-HT in *Vglut3*$^{-/-}$ pups (El Mestikawy *et al.* 2011). The link between respiratory control and 5-HT metabolism in mice has been previously established by comparing two strains of inbred mice (C57BL/6J *vs.* FVB/N) that display differences in 5-HT metabolism in the medulla and in respiratory control (Menuet *et al.* 2011). In newborn mice, large differences in L-Trp and 5-HIAA content were observed between the two strains, leading to a 3-fold higher 5-HT/5-HIAA ratio in FVB/N mice. These differences in 5-HT metabolism were associated with greater respiratory irregularity in C57BL/6J mice, both *in vitro* and *in vivo* (Menuet *et al.* 2011). In the present study, L-Trp and 5-HIAA content was not significantly different between Vglut3$^{-/-}$ and control brainstems, and the 5-HT/5-HIAA ratio was 19% higher in *Vglut3*$^{-/-}$ mice when compared to controls. This relatively small difference in 5-HT metabolism was not associated with differences in respiratory activity under basal conditions, but might have been sufficient to impair this activity under highly demanding hypoxic conditions.

Both the initial (medullary) and the late (spinal) responses to 5-HT were reduced in *Vglut3*$^{-/-}$ preparations. The reduced response to 5-HT in *Vglut3*$^{-/-}$ preparations is consistent with its role in potentiating 5-HT vesicular accumulation and the exocytotic secretion of 5-HT. However, the present results do not indicate whether these abnormal responses reflect changes in 5-HT storage and release, or developmental changes in 5-HT receptor expression, although previous results do not indicate any major modification of 5-HT receptors (Amilhon *et al.* 2010). Previous studies have shown that 5-HT acts at the medullary level and that its effects are due to its specificity for the rostro-ventral respiratory column and not due to a diffuse action on all medullary respiratory centres (see Hilaire *et al.* (2010) and Hodges & Richerson (2010*b*) for recent reviews on the respiratory effects of 5-HT). Therefore, the putative impairment of 5-HT signalling due to Vglut3 inactivation may account for the abnormal response to 5-HT. However, we cannot fully discount the influence of 5-HT induced depolarization of larger cell populations in the brainstem (e.g. the A5 area of the caudal pons; Di Pasquale *et al.* 1992) and spinal cord, and the subsequent release of a large variety of inhibitory and excitatory neurotransmitters, on a part of the response of *Vglut3*$^{-/-}$ preparations in the present study. With these limitations in mind, considering that 5-HT is pivotal to respiratory function (Hilaire *et al.* 2010) and thermoregulation (Morrison *et al.* 2008), the present results support the view that the abnormal respiratory and thermogenic responses to cold in *Vglut3*$^{-/-}$ pups may be caused by abnormal 5-HT metabolism. Abnormal 5-HT signalling may also account for the abnormal RRG response to acidosis, which is closely dependent on 5-HT (Voituron *et al.* 2010) and mediated by pH-sensitive RTN/pFRG neurons (Guyenet *et al.* 2008), which receive dense 5-HT inputs from VGLUT3-positive terminals (Nakamura *et al.* 2005*b*; Stornetta *et al.* 2005; Rosin *et al.* 2006).

The fact that the lack of VGLUT3 increased the response of isolated RRG to acidosis is intriguing. It suggests that glutamate release by 5-HT neurons might inhibit breathing through its action on specific populations of

(PBf) was significantly increased in *Vglut3*$^{-/-}$ pups ($n = 4$) when compared to control pups ($n = 12$, $P < 0.05$; Student's unpaired *t* test). *Ba* and *b*, examples of changes in central respiratory drive induced by anoxia in control (*a*) and *Vglut3*$^{-/-}$ pups (*b*) *in vitro*. *Bc*, the effect of anoxia (shaded) was significantly increased in *Vglut3*$^{-/-}$ pups ($n = 8$) when compared to controls ($n = 18$, $P < 0.001$). *C*, examples of plethysmographic recordings in 6-day-old *Vglut3*$^{-/-}$ and control pups during normoxia (*a*), hypoxia (10% O_2) (*b*) and post-hypoxia (*Cc*). *Da–c*, breathing variables: ventilation ($\dot{V}_E$), tidal volume (V_T) and breathing frequency (R_f). *Vglut3*$^{-/-}$pups ($n = 63$) displayed decreased ventilation during both normoxia and hypoxia, compared to control pups ($n = 200$). Both groups displayed a biphasic response to hypoxia (shaded), with the initial increase in $\dot{V}_E$ being followed by a marked decrease (under the control of V_T and R_f). *Ea–c*, percentage change in breathing variables in response to hypoxia, relative to pre-hypoxic levels (hyperpnoeic response and hypoxic decline); HVD was significantly lower in *Vglut3*$^{-/-}$ pups. *Dd*, oxygen consumption ($\dot{V}_{O_2}$) at 26°C under normoxia and in response to hypoxia (shaded) in 6-day-old *Vglut3*$^{-/-}$ ($n = 7$) and control pups ($n = 29$). The smaller $\dot{V}_{O_2}$ in *Vglut3*$^{-/-}$ pups ($P < 0.001$) reflected their impaired thermogenesis. *Ed*, percentage change in $\dot{V}_{O_2}$ relative to pre-hypoxic levels was smaller in *Vglut3*$^{-/-}$ pups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, Student's unpaired *t* test. Values shown are group means ± SEM. See Tables 1 and 2 for full statistical analyses.

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respiratory neurons (Monnier *et al.* 2003). Furthermore, we cannot disregard the possibility that this effect is caused by non-5HT neurons with a potential impact on respiratory activity, as detailed below, or by compensatory mechanisms occurring during perinatal development.

Both our *in vitro* and *in vivo* results argue for the involvement of *Vglut3* in HVD. *In vitro*, the decrease in PB frequency in response to anoxia in control preparations confirmed the results of previous studies using similar preparations (Ballanyi *et al.* 1999), as well as transverse slice preparations (Ramirez *et al.* 1997). *In vivo*, hypoxia depressed ventilation, as consistently observed in newborn mammals (Teppema & Dahan, 2010). Thus, both *in vitro* and *in vivo* experiments confirmed that hypoxia decreased respiratory frequency, and showed that the lack of VGLUT3 disrupted this response. Since the RRG in *in vitro* preparations is isolated and lacks excitatory input from peripheral carotid body chemoreceptors, the abnormal RRG response of *Vglut3*$^{-/-}$ pups to anoxia can be ascribed to the inhibitory drive from hypoxia-sensing neurons of the ventral medulla (Voituron *et al.* 2006). In keeping with our *in vitro* data, HVD *in vivo* was blunted in *Vglut3*$^{-/-}$ pups, leading to greater $\dot{V}_E$ and R_f. Previous studies have shown that blocking 5-HT2 receptors in adult mice suppresses HVD (Kanamaru & Homma, 2009). Taken together, these results suggest that the lack of VGLUT3 disrupts a common mechanism accounting for both *in vitro* and *in vivo* findings. However, the decrease in PB frequency *in vitro* (frequently referred to as 'short-term depression' under these experimental conditions; Dick & Coles, 2000; Powell *et al.* 1998) and the decrease in ventilation *in vivo* had markedly different time courses, which may at least partially reflect different mechanisms. Therefore, caution must be used in attempting to integrate both results within a unifying framework, as previously noted (Bissonnette, 2000).

The weaker thermogenic response of *Vglut3*$^{-/-}$ pups to cold indicates that the range of phenotypic defects extends beyond respiratory control disorders. This defect is probably related to the fact that a brainstem network that includes raphe neurons that express VGLUT3 through postganglionic sympathetic neurons controls brown adipose cells (Schafer *et al.* 2002; Nakamura *et al.* 2005*a*; Morrison *et al.* 2008). Furthermore, the hypometabolic response to hypoxia (i.e. the decrease in thermogenesis and $\dot{V}_{O_2}$ consistently observed in newborn mammals during hypoxia; Mortola, 2004) was blunted in *Vglut3*$^{-/-}$ pups. The mechanisms underlying hypoxic hypometabolism are complex and, to date, elusive, but they may involve a decrease in central thermosensitivity at the level of the hypothalamic medial preoptic area (Tattersall & Milsom, 2009), a target for VGLUT3-expressing non-5-HT neurons of the midbrain raphe nuclei (Hioki *et al.* 2010). The drop in arterial P_{CO_2} that normally accompanies hypoxic hypometabolism during and after hypoxia may not have been present in *Vglut3*$^{-/-}$, thus counteracting HVD and accounting for the present ventilatory data.

The implication of 5-HT signalling defects in the *Vglut3*$^{-/-}$ phenotype is supported by the similarities between *Vglut3*$^{-/-}$ pups and those lacking central 5-HT neurons (*Lmx1b*$^{f/f/p}$ pups), which display decreased ventilation and body temperatures when exposed to cold (Hodges *et al.* 2009). Furthermore, newborn mice lacking 60–70% of their 5-HT neurons (*Pet-1*$^{-/-}$) display reduced thermogenic and ventilatory responses to cold (Cummings *et al.* 2011*b*) and reduced ability to survive episodic severe hypoxia (Cummings *et al.* 2011*a*). The critical role of 5-HT neurons in respiratory and thermoregulatory functions was recently confirmed in conscious mice using the inducible and reversible suppression of neuronal excitability, which attenuated the ventilatory response to CO_2 and the thermogenic response to cold (Ray *et al.* 2011).

While the role of VGLUT3 in 5-HT neuronal activity may account for the present results, further studies are necessary to substantiate this interpretation, and other mechanisms may also be involved. Firstly, in addition to 5-HT neurons, the raphe contains a population of VGLUT3-positive cells that are not serotonergic (Commons, 2009; Jackson *et al.* 2009). Both subclasses of VGLUT3-positive neurons from the raphe could exert modulatory effects on 5-HT transmission and contribute to the response to hypoxic stress in *Vglut3*$^{-/-}$ mice. Also, VGLUT3 expression is prominent in nitroxidergic neurons and glial processes of the dorsolateral, medial and commissural subnuclei of the nucleus tractus solitarii (NTS; Lin, 2009), all regions that receive afferents from carotid body chemoreceptors (Finley & Katz, 1992). The lack of VGLUT3 may also affect the response to hypoxic stress through its effects on these structures. Furthermore, as previously noted, VGLUT3 is expressed in various neuronal populations including cholinergic interneurons of the dorsal and ventral striatum, and subpopulations of cortical and hippocampal basket cells (Fremeau *et al.* 2002). VGLUT3 expression has also been reported in non-neuronal cells such as insulin secreting β-cells of the pancreas (Gammelsaeter *et al.* 2011), chromaffin cells of the adrenal medulla (Olivan *et al.* 2011), and in the liver (Gras *et al.* 2002). Some of these populations might be indirectly implicated in some aspects of breathing control and metabolism. Previous analyses have shown that adult mice lacking VGLUT3 have a number of diverse disorders such as pain, deafness and non-convulsive seizures (Ruel *et al.* 2008; Seal *et al.* 2008, 2009), suggesting that *Vglut3* may interfere with a large variety of neuromodulatory and behavioural phenotypes beyond the 5-HT system (El Mestikawy *et al.* 2011).

In conclusion, the present results identify VGLUT3 as a protective factor against hypoxic stress in newborns, thus extending its known role in the defence against

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psychological stress to autonomic functions. The use of temporal and spatial conditional mutants in future studies should improve our understanding of the molecular mechanisms underlying these defensive functions.

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Author contributions

S.E.M., G.H. and J.G. designed the experiments; S.M., N.V., A.S., E.V., L.M., N.R., B.A., O.P., E.L., B.M., S.M., N.V., A.S., S.E.M., G.H. and J.G. collected and analysed data; S.M., N.V., S.E.M., G.H. and J.G. analysed data and wrote the manuscript. All authors approved the final version for publication.

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Translational perspective

Hypoxia–ischaemia is a major cause of acute brain injury in neonates. Hypoxic stress may be aggravated by cold, especially in preterm infants (Miller *et al.* 2011). While keeping in mind the limitations of extrapolating data from mice to humans, the present results in 6-day-old mice might shed some light on the response to hypoxic stress in preterm human infants of 32–35 weeks of gestational age (Hagberg *et al.* 2002). These results suggest that the lack of VGLUT3 compromises the ability of preterms to produce two components of the defence response to hypoxia: hypoxic hypometabolism and hypoxic ventilatory decline. The present results consolidate the concept that major components of the response to hypoxic stress (ventilatory, thermoregulatory, behavioural) in newborns are tightly coupled at the central level, and that the newborn's inability to produce this integrated response may originate in its central organization rather that in its components. The involvement of Vglut3 in the response to hypoxic stress in newborns suggests that Vglut3 could be a potential genetic marker of vulnerability to hypoxic damage. However, further investigations are required to better understand the role of Vglut3 in this process and to identify it as a potential pharmacological target against hypoxic stress in neonates.

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