Vav3 IS INVOLVED IN GABAergic AXON GUIDANCE EVENTS IMPORTANT FOR THE PROPER FUNCTION OF BRAINSTEM NEURONS CONTROLLING CARDIOVASCULAR, RESPIRATORY, AND RENAL PARAMETERS

by

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SUPPLEMENTARY INFORMATION*

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SUPPLEMENTARY TEXT

Arterial walls show normal signaling and mechanoelastic properties in Vav3-deficient mice

To explore whether the hypertension of $Vav3^{+/−}$ mice could derive from alterations in the reactivity and mechanoelastic features of their blood vessels, we first compared the contractility and dilatation properties of renal and mesenteric arteries from four month-old $Vav3^{+/−}$ and control animals in ex vivo perfusion experiments. No differences in the contractility of those vessels were detected upon stimulation with either KCl (Supplementary Fig. S1) or phenylephrine (Fig. S1). Likewise, normal vasodilatation responses were observed upon treatments with acetylcholine (Fig. S1) or isoproterenol (data not shown). However, both types of $Vav3^{+/−}$ arterial vessels did display higher constriction rates to Angiotensin II (AngII) (Figs. S1A,D and S2A,B) and, in agreement with this, we observed that primary cultures of $Vav3^{+/−}$ vascular smooth muscle cells also contracted more strongly (Fig. S2C) and proliferated better (Fig. S2D) than wild type cells when challenged with AngII. Further analyses indicated that this response was probably derived from the high levels of the AngII receptor type 1a (Agtr1a) present in $Vav3^{+/−}$ vascular smooth muscle cells (Sauzeau et al., 2006). Indeed, the higher proliferation of vascular smooth muscle cells to AngII was blocked by losartan, an Agtr1a antagonist (Fig. S2D). Moreover, we found that both the enhanced contractile responses of those cells to AngII (Fig. S2C) and their high $Agtr1a$ mRNA levels (Fig. S2E) were lost after several passages of cells in culture, indicating that these responses were epistatic to the hypertensive condition found in $Vav3^{+/−}$ animals. Consistent with this view, we did not see
any differential levels of the *Agtr1a* transcript in vascular smooth muscle cells obtained from one month-old animals (Sauzeau et al., 2006), an age in which *Vav3* null animals have not yet develop any cardiovascular remodelling (Sauzeau et al., 2006).

Finally, we measured the mechanoelastic properties of aorta rings obtained from wild type and *Vav3*-deficient mice. To avoid confounding influences from the vascular remodelling induced by the hypertensive state, these studies were conducted in one month-old animals. No detectable differences were observed in the elasticity of vessel rings regardless of the mouse genotype used (Fig. S3). Taken together, these results indicate that the hypertension and sympathetic nervous system (SNS) hyperactivity of *Vav3*−/− mice are not caused by intrinsic signalling or mechanical problems in resistance or conductance arterial vessels.

**The hyperventilation phenotype does not induce hypoxia, hypercapnia, acidosis or pulmonary hypertension in Vav3-mutant mice**

Given that both hypoxia and hypocapnia can trigger high blood pressure, we also investigated whether the hypertension of *Vav3*+/− mice could derive from defects in O₂/CO₂ exchange in their lungs due to the tachypneic state. However, we found no evidence of hypoxia, hypercapnia or acidosis in these mice (Table S1). A lack of hypoxia/hypercapnia in these animals was also indirectly confirmed by the fact that they had normal hematocrite values (Fig. S4A), erythropoietin plasma levels (Fig. S4B), and erythroblast numbers (data not shown). The absence of pulmonary artery remodelling (Fig. S4C) and right heart ventricle hypertrophy (Sauzeau et al., 2006), two common events triggered by hypoxia
conditions (Howell et al., 2004), was also consistent with the absence of hypoxia and/or hypercapnia states. These results confirmed that the hypertension of Vav3<sup>−/−</sup> mice was not due to defects in the O<sub>2</sub>/CO<sub>2</sub> exchange in their lungs as a consequence of their tachypnea.

**Innervation routes of GABAergic neurons of the caudal ventrolateral medulla**

In addition to the ipsilateral and contralateral migration pathways described for the axons of CVLM GABAergic cells in the Main Text (Fig. 6A,B), we could detect in both Vav3<sup>−/−</sup> and control mice dextran amine-labeled axons descending bilaterally from the injection site in the CVLM to the nucleus of the solitary tract, the external cuneate and spinal cord. We also found bilateral ascending tracks to the lateral paragigantocellular nucleus, the parvocellular reticular nucleus and the dorsomedial hypothalamic nucleus. Retrograde, dextran amine-labeled somas were observed bilaterally in the nucleus of the solitary tract and in the CVLM contralateral to the injection site (data not shown). These migration routes are in accordance with previous studies about the connections of CVLM cells in other species (Van Bockstaele et al., 1989; Roder and Ciriello, 1992; Yu and Gordon, 1996; Hardy et al., 1998; de Sousa Buck et al., 2001; Cobos et al., 2003). Highest numbers of dextran amine-labeled neuropils were found in the RVLM, spinal cord and lateral paragigantocellular nucleus. Significant lower numbers were detected in the rest of nuclei (data not shown). With the exception of RVLM and CVLM sections, we have not investigated whether the dextran amine-labeled structures in other nuclei were GABAergic.
SUPPLEMENTARY MATERIALS AND METHODS

Reactivity of blood vessels. For ex vivo experiments, either renal arteries or mesenteric arterial beds were canulated and perfused at 1 ml/min with a Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 11 mM glucose) at 37 ºC and equilibrated with an atmosphere of 95% O₂ plus 5% CO₂. Catheters were connected to a pressure probe linked to a digital data recorder (MacLab/4e, AD Instruments). At the indicated times, arteries were perfused with 80 mM KCl, 1 µM phenylephrine (Sigma), 0.1 µM AngII (Sigma) and serial inputs (0.01, 0.1 and 1 µM) of acetylcholine (Sigma). In the case of renal arteries, we included an extra input of and 10 µM acetylcholine. These perfusions were always conducted in Krebs-Henseleit solution. Recordings were analyzed using the Chart v3.4 software (AD Instruments). For in vitro studies, aortic vascular smooth muscle cells from indicated culture passages were maintained under the microscope at 37°C and stimulated with either 40 mM KCl or 10 µM AngII. Cell contractility was recorded in real-time before and after the stimulation using a cool-SNAP camera (Princeton Instruments) and image data stored and analyzed using the Metamorph/Metaview software (Universal Imaging). Contraction rates were measured taking into account the length of the major cell axis before and after stimulation. At least 150 single cells were analyzed per experiment.

Aortic smooth muscle cell culture. Aortas were collected from mice, incubated in cold phosphate buffered saline solution and cleaned manually to remove fat and adherent connective tissue. The endothelium was then carefully removed by gently rubbing intimal
surfaces with the tip of a small forceps. Cleaned aortas were cut in ≈ 5x5 mm pieces and cultured in DMEM with 10% fetal calf serum supplemented with antibiotics at 37°C and 5% CO₂. The culture medium was changed every 48 h. After approximately two weeks, the vascular smooth muscle cells populating the plate were trypsinized and reseeded in fresh DMEM supplemented with 10% fetal calf serum and antibiotics. The reseeded smooth muscle cells were considered as passage 1. Further passages were done by trypsinization of confluent cultures of vascular smooth muscle cells.

**Determination of proliferation rates.** 5,000 aortic smooth muscle cells of the indicated passages were seeded in six-well plates in DMEM supplemented with 0.5% fetal calf serum and antibiotics. A day after, AngII (10 µM) with or without losartan (1 µM, DuPont) was added to the appropriate cultures and, after three days, cells were trypsinized and counted.

**Real-time PCR.** The expression levels of *Agtr1a* mRNA were quantified as described before (Sauzeau et al., 2006).

**Compliance determinations in aortas.** Four, 2 mm-long segments of the thoracic aorta from each mouse were dissected under a light microscope and mounted between two stain steel wires (diameter 40 µm) in a chamber wire myograph (MultiWire Myograph System, Danish Myo Technology) connected to the MacLab/4e digital data recorder. Before applying tensions, arteries were soaked in Krebs-Heinseleit solution at 37 °C in 5% CO₂ and equilibrated with frequent changes of Krebs-Heinseleit solution for 30 minutes. After
the equilibration step, the passive diameter-tension relationship was measured by increasing the inner diameter of aorta with the micrometer in serial steps of 10 µm. The stabilized-tension generated by aortas after each stretching step was collected and finally integrated using the Chart v3.4 software.

**Determination of breathing activities.** In the case of direct determinations of breathing activity, we anesthetized slightly the mice with 1 g urethane/kg body weight. In the case of stereotaxic experiments, we anesthetized the mice more deeply using 2 g urethane/kg body weight. After these preparatory steps, we attached forceps connected to a force transducer by a flexible wire to the mice at the diaphragm level and collected the respiratory amplitude and frequency using a digital data recorder (MacLab/4e, AD Instruments). Recorded data was finally integrated using the Chart v3.4 software (AD Instruments). In the case of hypercapnia experiments, we administered to anesthetized mice a CO₂-enriched air flux (6 ml/min) for 90 s. After this period, normal air was reapplied to mice.

**Long-term pharmacologic treatments.** Captopril (25 mg/l, Sigma) was added in the drinking water of one month-old mice for 3 months, as previously described (Sauzeau et al., 2006).

**Determination of blood parameters.** Arterial pressure of anesthetized mice was measured as described (Sauzeau et al., 2006). AngII and catecholamine plasma levels were measured as published (Sauzeau et al., 2006). Determinations of O₂, CO₂, pH, HCO₃⁻ and hematocrite
levels were done using a Synthesis25 apparatus (Instrumentation Laboratory). Erythropoietin levels in plasma were determined using a commercial ELISA kit (EPO ELISA, IBL).

**Other cardiovascular-related methods.** Protocols related to the characterization of the cardiovascular and renal phenotype present in Vav3−/− mice have been done exactly as described before (Sauzeau *et al.*, 2006; Sauzeau *et al.*, 2007).

**Expression studies.** Animals were perfused transcardially with 25 ml of fresh Ringer’s calcium-free buffer (145.45 mM NaCl, 3.35 mM KCl, 2.38 mM NaHCO₃, pH 6.9) at 37°C and then with 150 ml of freshly depolymerized 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. In the case of immunohistochemistry with anti-GABA antibodies, the fixative solution also contained 1.25% glutaraldehyde. Tissue slides were obtained either from paraffin-embedded blocks (5 µm of thickness) or with a freezing stage sliding HM430 microtome (Microm) (30 µm of thickness). In the case of paraffin-embedded tissue, sections were deparaffinized, incubated with the indicated primary antibodies, rinsed with phosphate buffered saline solution (PBS), incubated in a milk/PBS solution containing a goat anti-rabbit IgG coupled to horseradish peroxidase (GE Healthcare) for 1 h at room temperature, washed with PBS, and signals revealed with a 3’,3’ diaminobenzidine tetrahydrochloride solution containing hydrogen peroxide (Dako). In the case of immunofluorescence studies, we followed the above procedure but including Cy5- and Cy3-labeled secondary antibodies (Jackson ImmuneResearch). A home-made
rabbit polyclonal antibody recognizing Vav3 but not other family proteins has been described previously (Movilla and Bustelo, 1999; Quevedo et al., 2010). The rest of primary antibodies used in this study were obtained from either Millipore/Chemicon (tyrosine hydroxylase, v-GAT, GAD65 and GAD67) or Sigma (GABA_A receptor, GABA). The specificity of the primary antibodies to tyrosine hydroxylase, v-GAT, GAD65 and GAD67 was confirmed by western blot analysis of total cellular extracts from brain using, as negative control, lysates from vascular smooth muscle cells. All antibodies recognized proteins of the expected size without detectable cross-reactivity with other electrophoresed proteins (data not shown). In the case of experiments with anti-Vav3 antibodies, we used as negative control immunohistochemistries with tissue sections from Vav3^{−/−} mice. Quantification of fluorescence signals was done using the LSM Image Browser software (version 3.2.0.115, Zeiss). The optical density analysis was performed as described previously (Merchan et al., 2005). In the case of in situ hybridization studies, paraffin-embedded tissue sections were deparaffinized, treated with proteinase K (Dako), washed with PBS and soaked in 2x SSC. Digoxigenin-11-UTP-labelled cRNA sense and antisense Vav3 probes were generated from the pVS02 plasmid using the DIG RNA Labeling Kit (Roche Molecular Biochemicals). pSVS02 was generated by cloning a mouse Vav3 cDNA fragment (nucleotides 2181-2661) into the pSPT18 plasmid (Roche Molecular Biochemicals). After the probe hybridization for 6 h at room temperature, signals were detected using the DIG nucleic acid detection kit (Roche Molecular Biochemicals), according to the manufacturer’s protocol. To avoid bias in the analysis, the immunohistochemistry and in situ hybridization experiments were done and scored by
members of our research team lacking a priori information about the genotype of the mice under investigation. Furthermore, experiments were carried out simultaneously with tissues from control and mutant mice.
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**SUPPLEMENTARY TABLES**

**Table S1.** Analysis of blood parameters of 4 month-old mice

|                  | $Vav3^{+/+}$    | $Vav3^{-/-}$   |
|------------------|-----------------|----------------|
| pH               | 7.25 ± 0.01     | 7.24 ± 0.01    |
| $pO_2$ (mmHg)    | 125.25 ± 8.01   | 120.43 ± 7.65  |
| $pCO_2$ (mmHg)   | 44.75 ± 1.03    | 43.17 ± 0.84   |
| $HCO_3^-$ (mmol/l) | 19.80 ± 0.54  | 19.87 ± 0.53   |
LEGENDS TO SUPPLEMENTARY FIGURES

FIGURE S1. Response of resistance blood vessels to vasoconstrictor and vasodilator agents. (A) Representative real-time recordings of the response of mesenteric arteries from a wild type (WT, red, top panel) and a Vav3⁻/⁻ (blue, bottom panel) mice to the administration (arrows) of KCl, phenylephrine (Phe), several inputs of acetylcholine (ACh) and AngII. Scale bar, 10 min. (B,C) Variation of perfussion pressure (B) and percentage of vessel relaxation (C) induced by KCl (B), phenylephrine (B) and acetylcholine (C). Error bars represent the s.e.m. (n = 5-6). (D) Representative real-time recordings of the response of renal arteries from a WT (red, top panel) and a Vav3⁻/⁻ (blue, bottom panel) mice to the administration (arrows) of KCl, phenylephrine, several inputs of acetylcholine and AngII. Scale bar, 10 min. (E,F) Variation of perfussion pressure (E) and percentage of vessel relaxation (F) induced by KCl (E), phenylephrine (E) and acetylcholine (F) (n = 9-12). Error bars represent the s.e.m.

FIGURE S2. Vascular smooth muscle cells from four month-old Vav3⁻/⁻ mice show enhanced responses to AngII. (A,B) Level of vasoconstriction of mesenteric (A) and renal (B) arteries of mice of the indicated genotypes to AngII (n = 5-10). Error bars represent the s.e.m. **, P < 0.01 compared to wild type control. (C) Contractile response to AngII and KCl of primary vascular smooth muscle cells of the indicated genotypes after the second (left panel) and fifth (right) passage in culture (n = 3, each performed in duplicate). Error bars represent the s.e.m. **, P < 0.01 compared to wild type control. (D) Proliferation rates of cultures of primary vascular smooth muscle cells (second passage) cultured in 0.5% fetal
calf serum in the presence or absence of AngII with or without losartan ($n = 3$, each performed in triplicate). Error bars represent the s.e.m. $\ast\ast, P < 0.01$ compared to wild type control. (E) Expression $Agtr1a$ mRNA levels in primary vascular smooth muscle cells of the indicated genotypes and passages ($n = 3$). au, arbitrary units. Error bars represent the s.e.m. $\ast\ast, P < 0.01$ compared to wild type control.

**FIGURE S3.** Normal mechanoelastic properties of arterial vessels from $Vav3^{-/-}$ mice. Aorta rings from mice of the indicated genotypes were subjected to compliance determinations as indicated in Supplementary Methods.

**FIGURE S4.** Evaluation of pulmonary circulation and erythrocyte status in $Vav3$-deficient mice. (A,B) Hematocrit (A) and erythropoietin (B) levels of mice of indicated genotypes ($n = 5-6$). (C) Hematoxylin-eosin stained sections of lungs from mice of the indicated genotypes (top). Walls of pulmonary arteries are indicated by arrows ($n \geq 10$). Scale bar, 25 $\mu$m.

**FIGURE S5.** An example of the response of brainstem areas of wild type mice to L-glutamate microinjections. (A,D,G) Schematic representation of the brainstem at the indicated interaural coordinates (top). Amb, nucleus ambiguus; CC, central canal; LPGi, lateral paragigantocellular nucleus; LRt, lateral reticular nucleus; PY, pyramidal tract; Sp5I, spinal trigeminal nucleus interpolar. CVLMs and RVLMs are shaded on gray. D, dorsal; V, ventral. (B,E,H) Representative real-time recordings of blood pressure variations in a WT
mouse upon bilateral microinjections of glutamate (G) in the indicated interaural areas. The time of injection is indicated by arrows. Brackets (labeled 1 and 2) shown at the bottom of charts indicate the time intervals for the breathing recordings shown in panels C,F and I. Scale bar, 2 min. (C,F,I) Example of representative real-time recordings of the evolution of the respiratory frequency of a control mouse at the times indicated in panels B, E and H, respectively. Scale bar, 3 s.

**FIGURE S6.** *Vav3* null mice do not have reduced numbers of GABAergic cells in the CVLM. Coronal sections of the brainstem at the level of the CVLM and derived from mice of the indicated genotypes were subjected to immunohistochemistry with anti-GABA antibodies. After staining, GABA-positive cells were counted. au, arbitrary units. Error bars represent the s.e.m.

**FIGURE S7.** Incorporation of the dextran amine tracer in GABAergic cells of the CVLM. Coronal brainstem sections obtained from iontophoresed animals at the CVLM level were stained with anti-GAD65 plus anti-GAD67 antibodies, incubated with Cy2-labeled streptavidin and a Cy3-labeled secondary antibody, and subjected to confocal immunofluorescence microscopy. The Cy2- and Cy3-derived signals are shown in green and red, respectively. Areas of co-localization are shown on yellow. Scale bar, 10 µm.
SUPPLEMENTARY FIGURES
Supplementary Figure S3

Sauzeau et al. (2010)
Supplementary Figure S4

A

![Bar graph A](image)

B

![Bar graph B](image)

C

![Images of tissue samples](image)
Supplementary Figure S6

Cell number (au)

Genotype

Vav3 ^+/+  Vav3 ^-/-
Supplementary Figure S7

CVLM

Vav3^{+/+}  Vav3^{-/-}

Tracer  GAD65/67  Merge  Tracer  GAD65/67  Merge