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Vesicular glutamate transporter 2 is required for the respiratory and parasympathetic activation produced by optogenetic stimulation of catecholaminergic neurons in the rostral ventrolateral medulla of mice *in vivo*

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Abstract

Catecholaminergic neurons of the rostral ventrolateral medulla (RVLM-CA neurons; C1 neurons) contribute to the sympathetic, parasympathetic and neuroendocrine responses elicited by physical stressors such as hypotension, hypoxia, hypoglycemia, and infection. Most RVLM-CA neurons express vesicular glutamate transporter (VGLUT)2, and may use glutamate as a ionotropic transmitter, but the importance of this mode of transmission *in vivo* is uncertain. To address this question, we genetically deleted VGLUT2 from dopamine- β -hydroxylase-expressing neurons in mice [$D\beta H^{Cre/0}; VGLUT2^{flox/flox}$ mice (cKO mice)]. We compared the *in vivo* effects of selectively stimulating RVLM-CA neurons in cKO vs. control mice ($D\beta H^{Cre/0}$), using channelrhodopsin-2 (ChR2–mCherry) optogenetics. ChR2–mCherry was expressed by similar numbers of rostral ventrolateral medulla (RVLM) neurons in each strain (~400 neurons), with identical selectivity for catecholaminergic neurons (90–99% colocalisation with tyrosine hydroxylase). RVLM-CA neurons had similar morphology and axonal projections in $D\beta H^{Cre/0}$ and cKO mice. Under urethane anesthesia, photostimulation produced a similar pattern of activation of presumptive ChR2-positive RVLM-CA neurons in $D\beta H^{Cre/0}$ and cKO mice. Photostimulation in conscious mice produced frequency-dependent respiratory activation in $D\beta H^{Cre/0}$ mice but no effect in cKO mice. Similarly, photostimulation under urethane anesthesia strongly activated efferent vagal nerve activity in $D\beta H^{Cre/0}$ mice only. Vagal responses were unaffected by α_1 -adrenoreceptor blockade. In conclusion, two responses evoked by RVLM-CA neuron stimulation *in vivo* require the expression of VGLUT2 by these neurons, suggesting that the acute autonomic responses driven by RVLM-CA neurons are mediated by glutamate.

Keywords

C1 neuron; channelrhodopsin 2; conditional knockout; gene disruption in mice; glutamate

Introduction

Catecholaminergic neurons located in the rostral ventrolateral medulla (RVLM-CA neurons) are activated by a variety of stresses (pain, hypotension, hypoxia, hypoglycemia, and infection), and contribute to the autonomic and neuroendocrine responses elicited by these stimuli (Guyenet *et al.*, 2013). Most RVLM-CA neurons are C1 neurons, which, by definition, express all of the catalytic enzymes required for the production of adrenaline [tyrosine hydroxylase (TH), dopamine- β -hydroxylase (D β H), and phenylethanolamine *N*-methyl transferase (PNMT)] (Hokfelt *et al.*, 1974; Ross *et al.*, 1983; Phillips *et al.*, 2001). Despite C1 neurons having a catecholaminergic phenotype, evidence suggests that they release glutamate as a primary neurotransmitter in adult rodents. PNMT-containing terminals form asymmetric synaptic contacts, consistent with the ultrastructure of classic excitatory synapses (Milner *et al.*, 1987, 1988, 1989; Agassandian *et al.*, 2012; Depuy *et al.*, 2013). Glutamate uptake by synaptic vesicles is mediated by one of three vesicular glutamate transporters (VGLUTs): VGLUT1, VGLUT2, and VGLUT3 (Bellocchio *et al.*, 2000; Takamori *et al.*, 2000; Gras *et al.*, 2002; Fremeau *et al.*, 2004). Only VGLUT2 transcripts and protein are detectable in C1 neurons of rats and mice (Stornetta *et al.*, 2002a; Herzog *et al.*, 2004; Rosin *et al.*, 2006; Abbott *et al.*, 2012). Furthermore, optogenetic activation of the C1 axons elicits monosynaptic glutamatergic excitatory postsynaptic currents in para-sympathetic preganglionic neurons *in vitro* (Depuy *et al.*, 2013), which is consistent with previous *in vivo* pharmacological evidence (Morrison *et al.*, 1991; Morrison, 2003; Abbott *et al.*, 2012).

The evidence that C1 neurons use glutamate as a fast transmitter is substantial, but, for the most part, is indirect (histological and pharmacological) or has been obtained *in vitro*. Glutamate release by C1 neurons should rely on VGLUT2, but this has not been directly tested. The aims of the present study were to determine the contribution of VGLUT2-dependent glutamate release to RVLM-CA neuron signaling in intact mice, and to determine the significance of VGLUT2 in this process. To accomplish this aim, we crossed D β H^{Cre/0} mice, in which noradrenergic and adrenergic neurons express Cre-recombinase, with mice in which exon 2 of the VGLUT2 gene is flanked by LoxP sites (Tong *et al.*, 2007; Abbott *et al.*, 2013). In the resulting progeny [D β H^{Cre/0};VGLUT2^{flox/flox} mice (cKO mice)], VGLUT2 is largely eliminated from the terminals of RVLM-CA neurons (Depuy *et al.*, 2013). To determine the contribution of VGLUT2 and glutamate release to RVLM-CA neuron signaling *in vivo*, we compared the effects of stimulating these neurons on respiration and vagus nerve efferent activity in D β H^{Cre/0} and cKO mice by using a Cre-dependent channelrhodopsin-2 (ChR2)-based optogenetic method.

Materials and methods

Animals

Animal use was in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and was approved by the University of Virginia Animal Care and Use Committee. D β H^{Cre/0} mice were obtained from the Mutant Mouse Regional Resource Center at the University of California, Davis, CA, USA [Tg(D β H-cre)KH212Gsat/Mmcd; stock no. 032081-UCD]. D β H^{Cre/0} mice were maintained as hemizygous (Cre/0) on a C57BL/6J

background. Homozygous VGLUT2^(flox/flox) mice (JAX stock no. 012898; STOCK Slc17a6^{tm1Lowl/J}) (Tong *et al.*, 2007) were bred with DβH^{Cre/0} mice to generate DβH^{Cre/0};VGlut2^{flox/0} mice, and subsequently crossed with Vglut2^{flox/flox} mice for two generations to generate cKO mice, in which VGLUT2 is absent from any Cre-expressing neurons (Depuy *et al.*, 2013). A total of 11 DβH^{Cre/0} mice (five males; six females) and 10 cKO mice (five males; five females), aged between 10 and 22 weeks, were used for these experiments.

Virus injection and fiber optic implantation

Adeno-associated virus (AAV2–DIO–EF1α–ChR2(H134R)–mCherry (AAV2–ChR2–mCherry) and AAV2–DIO–EF1α–ChR2(H134R)–eYFP (AAV2–ChR2–eYFP) (titer, 10¹² virus molecules per milliliter) were purchased from the University of North Carolina vector core [constructs courtesy of K. Deisseroth (Stanford)]. This vector features an enhanced version of the photosensitive cationic channel ChR2 [ChR2(H134R)] fused to a fluorescent reporter, mCherry or enhanced yellow fluorescent protein (eYFP), under the control of the promoter for elongation factor 1α (EF1α). The ChR2–mCherry sequence is flanked by double lox sites (LoxP and lox 2722).

Unilateral microinjections of AAV2–ChR2–mCherry into the left rostral ventrolateral medulla (RVLM) and placement of a fiber optic were performed under aseptic conditions in mice anesthetised with a mixture of ketamine (100 mg/kg) and dexmedetomidine (0.2 mg/kg; intraperitoneal), as previously described (Abbott *et al.*, 2013). Adequate anesthesia was judged by the absence of the corneal and hind-paw withdrawal reflex. Additional anesthetic was administered as necessary (20% of the original dose, intraperitoneal). Following injections of AAV2–ChR2–mCherry (~400 nL) into the left RVLM, an implantable 200-μm-diameter fiber optic was placed with its tip 400 μm above the injection site (~4.7 mm ventral to the dorsal surface of the brain), and the fiber optic was secured to the skull with cyanoacrylate adhesive. The implantable fiber optic consisted of a desheathed optical fiber (core, 200 μm; numerical aperture, 0.39; Thorlabs) glued into a zirconia ferrule (outside diameter, 1.25 mm; bore, 230 μm; Precision Fiber Products). A subset of mice (DβH^{Cre/0}, *N* = 3; cKO, *N* = 2) injected with AAV2–ChR2–mCherry were not implanted with a fiber optic, and were later used to determine the response of single presumed C1 neurons to photostimulation under anesthesia. A second subset of mice (*N* = 3) used for *in vitro* experiments were injected with AAV2–ChR2–eYFP. Mice received postoperative boluses of atipemazole (α₂-adrenergic antagonist, 2 mg/kg, subcutaneous), ampicillin (125 mg/kg, intraperitoneal), and ketoprofen (4 mg/kg, subcutaneous). Ampicillin and ketoprofen were read-ministered 24 h postoperatively. Mice were housed in the University of Virginia vivarium for >4 weeks after virus injection. During this time, mice gained weight normally and appeared to be unperturbed by the implanted fiber optic.

Single-unit recording and photostimulation of putative ChR2-expressing RVLM-CA neurons

Extracellular recordings of RVLM neurons were obtained in a subset of freely breathing anesthetised mice (DβH^{Cre/0} and cKO) with a modification of a method previously developed for rats (Abbott *et al.*, 2012). These mice were anesthetised with urethane (1.6

g/kg dissolved in H₂O, delivered intraperitoneally as a 20% w/v solution), and placed in the stereotaxic frame in a prone position. RVLM neurons located in the C1 region, i.e. immediately caudal to the facial motor nucleus and at a depth of 5–5.3 mm from the surface of the brain, were recorded by the use of glass pipettes introduced vertically in the brain. Light (8–9 mW) was delivered to the RVLM through a fiber optic inserted into the brain at a 15° angle from the vertical in the transverse plane, so that its tip was within 500 µm of the region explored during unit recordings. Faithful pulse-by-pulse neuronal activation up to 20 Hz was taken as evidence that the recorded cells were directly photostimulated and therefore probably RVLM-CA neurons.

Whole-cell recording and photostimulation of Chr2-expressing RVLM-CA neurons in slices

Slice preparation was performed as described in Depuy *et al.* (2013). Briefly, 6 weeks after AAV2 injection, three DβH^{Cre/0} mice were anesthetised with a mixture of ketamine (120 mg/kg) and xylazine (12 mg/kg) given intraperitoneally, and mice were decapitated when they had been rendered unresponsive to a firm toe pinch. The brainstem was sectioned with a vibrating microtome in the transverse plane in ice-cold *N*-methyl-D-glucamine-substituted artificial cerebrospinal fluid (ACSF) containing 92 mM *N*-methyl-D-glucamine, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 10 mM MgSO₄, 0.5 mM CaCl₂, 20 mM HEPES, 30 mM NaHCO₃, 25 mM glucose, 2 mM thiourea, 5 mM sodium ascorbate, and 3 mM sodium pyruvate (~300 mOsm/kg). After incubation for 10 min at 33 °C, brain slices were transferred to aerated physiological extracellular ACSF containing 119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM MgSO₄, 2 mM CaCl₂, 26 mM NaHCO₃, 12.5 mM glucose, 2 mM thiourea, 5 mM sodium ascorbate, and 3 mM sodium pyruvate, and maintained at room temperature (23 °C). All recordings were performed at room temperature in submerged slices continuously perfused with aerated (95% O₂, 5% CO₂) physiological ACSF. Glass pipettes (tip resistance, 2–6 MΩ) were filled with a solution containing 110 mM potassium gluconate, 20 mM potassium chloride, 10 mM HEPES, 10 mM tris-phosphocreatine, 3 mM ATP-Na, 0.3 mM GTP-Na, 1 mM EGTA, 2 mM MgCl₂, and 0.2% biocytin. The junction potential was 14.8 mV, and corrected membrane potentials are reported in the text and figures. Recordings were performed with a Multiclamp 700B amplifier and pCLAMP 10 software (Molecular Devices, Sunnyvale, CA, USA). Signals were low-pass filtered at 4 kHz and digitised at 10 kHz. Only cells with series resistance that remained below 25 MΩ were included in the analysis. Further analysis was performed with SPIKE2 v7.10 (CED, Cambridge, UK). Photostimulation of visually identified Chr2-eYFP-expressing neurons was performed with a 200-µm-diameter fiber optic coupled to a 473-nm DPSS laser (IkeCool Corporation, Anaheim, CA, USA), as previously described (Depuy *et al.*, 2013). The tip of the fiber optic was positioned 150 µm above and 250 µm lateral to the recorded neuron. The fiber optic intensely illuminated an ellipse of ~0.342 mm², and produced an estimated average irradiance of ~15 mW/mm². Delivery of light pulses (duration, 5 ms) was triggered by a digitiser (Digidata 1440A; Molecular Devices) controlled by episodic protocols run in pCLAMP 10 (Molecular Devices). The fiber optic was calibrated for 5-mW steady-state output prior to each experiment.

Whole-body plethysmography and photostimulation in conscious mice

Photostimulation of the RVLM was performed as described previously (Abbott *et al.*, 2013). Prior to implantation, the light output of each implanted optical fiber was measured with a light meter (Thorlabs), and the laser setting was adjusted to deliver 8–9 mW. This setting was later used during experiments.

Breathing patterns were measured in conscious mice by the use of unrestrained whole-body plethysmography (EMKA Technologies), as described previously (Abbott *et al.*, 2013). The chamber was continuously flushed with dry room-temperature air delivered at 0.5 standardised liters per minute. Fluctuations in chamber pressure were amplified ($\times 500$) and acquired at 1 kHz with SPIKE 2 software (v7.06; CED). Respiratory frequency (f_R ; breaths/min) was calculated on the basis of the onset of inspiration during periods of behavioral quiescence. Mice were briefly anesthetised with isoflurane while the connection between the implanted fiber optic and the laser delivery system was established. Recordings were initiated at least 30 min after the mice had regained consciousness. To evaluate the effects of photostimulation on f_R , 10-s trains of light pulses (duration, 5 ms) were delivered at various frequencies (5–25 Hz) during behavioral quiescence. The reported changes in f_R reflect the average response of two or three trials at each stimulus frequency per mouse (inter-trial variability during 20-Hz trials: D β H^{Cre/0}, 7.6 ± 0.4 breaths/min; cKO, 3.5 ± 0.5 breaths/min; $N = 8$ each).

Recording of vagus nerve efferent activity in urethane-anesthetised mice

Recordings of the vagus nerve were performed under urethane anesthesia (1.6 g/kg dissolved in H₂O delivered intraperitoneally in a 20% w/v solution). Depth of anesthesia was assessed by absence of the corneal and hind-paw withdrawal reflex. Additional anesthetic was administered as necessary (10% of the original dose, intraperitoneal). Body temperature was maintained at 37.2 ± 0.5 °C with a servo-controlled temperature pad (TC-1000; CWE). Following induction of anesthesia, the fiber optic was connected to the laser, and mice were then placed in a stereotaxic frame in the supine position. A tracheostomy was performed, and mice were mechanically ventilated with pure oxygen (MiniVent type 845; Hugo-Sachs Elektronik). Fine Teflon-coated silver wires were placed subcutaneously in the chest area to record the electrocardiogram (ECG) and heart rate. To record vagus nerve efferent activity, ~15 mm of the vagus nerve was dissected free from the carotid arteries in the neck, and the distal end of the isolated nerve segment was crushed, amounting to a vagotomy. Following vagotomy, the central respiratory pattern became desynchronised from the ventilator, thereby establishing the loss of afferent vagus nerve activity. At this point, the rate and volume of mechanical ventilation were adjusted to eliminate spontaneous breathing (170–220 r.p.m. at 7–8 μ L/g), the adequacy of anesthesia was rechecked, and the paralyzing agent vecuronium was administered (0.1 mg/kg, intraperitoneal). After this point, adequate anesthesia was determined by the absence of changes in heart rate in response to a firm hind-paw pinch. The vagus nerve ipsilateral to the implanted fiber optic, and anterior to the section of the nerve crushed earlier, was placed on a bipolar platinum–iridium wire electrode (part no. 778000; A-M Systems) and embedded in silicone (Kwik-Sil, WPI). Physiological signals were filtered and amplified (ECG, 10–1000 Hz, $\times 1000$; vagus, 30–3000 Hz, $\times 10\,000$) (BMA-400; CWE) and acquired in SPIKE 2 software

(v7.06; CED). *Post hoc* high-pass filtering of vagus nerve recordings (high pass, 100 Hz; transition, 50 Hz) was used in cases showing ECG contamination. Three cases were excluded from analysis because of failed nerve recordings. The vagus nerve response to RVLM photostimulation was analysed by measuring the area under the curve (AUC) relative to the prestimulus AUC of waveform averages of rectified vagus nerve activity triggered from the first light pulse of 10–20 stimulation trials (1-s train of 5-ms pulses at 5, 10 and 20 Hz) delivered at 0.1 Hz. The resulting value was defined as the evoked vagus nerve activity during photostimulation [in arbitrary units (a.u.)]. To account for potential differences in the quality of the vagus nerve recording between mice, the evoked vagus nerve activity during photostimulation was normalised with respect to respiratory-related oscillations in vagus nerve activity [rectified and smoothed ($\tau = 0.01$)] during administration of CO₂ (6–7%) in response to the inspired gas. These oscillations reflect the periodic changes in para-sympathetic preganglionic neuron activity driven by the central respiratory pattern generator. Thus: normalised evoked vagus nerve activity during photostimulation (a.u.) = [(AUC vagus nerve activity during stimulation) – (AUC vagus nerve activity during the prestimulus period)]/(range of respiratory-related oscillations in vagus nerve activity during CO₂ administration).

For presentation purposes, the range of respiratory-related vagus nerve activity was assigned a scale of 0–100 a.u., and traces of nerve recordings and waveform averages were scaled accordingly. Note that 0 a.u. in vagus nerve recordings does not reflect absolute zero activity. Paired-pulse stimulation was evaluated in D β H^{Cre/0} mice by generating waveform averages of paired 50-ms laser pulses delivered at 0.2 Hz. Prazosin (1 mg/kg, intraperitoneal) was administered in D β H^{Cre/0} and cKO mice to evaluate the effects of central α_1 -adrenoreceptor blockade on the vagal response to photostimulation.

Histological procedures

Following completion of the *in vivo* experiments, mice were lethally anaesthetised with urethane, and transcardially perfused with 50 mL of heparinised saline followed by 100 mL of freshly prepared 2% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.4). Brains were extracted and post-fixed at 4 °C for 24–48 h in the same fixative. Coronal sections were cut at 30 μ m on a vibrating microtome, and stored in a cryoprotectant solution (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) at –20 °C.

Immunohistochemical procedures and microscopy were performed as previously described (Abbott *et al.*, 2013). The following antibodies were used: mCherry protein was detected with anti-DsRed (rabbit polyclonal, 1 : 500; Clontech #632496; Clontech Laboratories) followed by Cy3-tagged anti-rabbit IgG (1 : 200; Jackson ImmunoResearch Laboratories); TH was detected with a sheep polyclonal antibody (1 : 2000, Millipore #AB1542; EMD Millipore) followed by Alexafluor488-tagged donkey anti-sheep antibody (1 : 200; Jackson ImmunoResearch Laboratories); and eYFP was detected with a chicken polyclonal antibody (1 : 2000; Aves Labs, Tigard, OR, USA) followed by Alexafluor488-tagged donkey anti-chicken antibody (1 : 200, Jackson code 703-545-155; Jackson ImmunoResearch Laboratories).

The slices used for whole-cell recordings *in vitro* were rinsed, and incubated first in blocking solution with a Triton concentration of 0.5%, to enhance antibody penetration, and then in primary antibodies for TH and eYFP, as described above. eYFP was detected with a chicken polyclonal antibody (1 : 2000; Aves Labs) followed by Alexafluor488-tagged donkey anti-chicken antibody (1 : 200, Jackson code 703-545-155; Jackson ImmunoResearch Laboratories). Slices were then rinsed and incubated with secondary antibodies (as described above), and with NeutrAvidin-Dylight-649 (Thermo Fisher Scientific, Suwanee, GA, USA) to identify biocytin. Slices were finally rinsed and mounted, and coverslips were affixed.

All brain sections were examined under bright field and epifluorescence with a Zeiss AxioImager Z.1 microscope equipped with a computer-controlled stage, by use of NEUROLUCIDA software (v10; MBF Bioscience). Cell counts were performed in a 1:3 series aligned to the caudal extent of the facial motor nucleus, designated as -6.48 mm from bregma, after an atlas (Paxinos & Franklin, 2004). Images were obtained with a Zeiss MRC camera as TIFF files (1388×1040 pixels), and imported into CANVAS software (v10; ACD Systems) for the composition of figures. Output levels were adjusted to include all information-containing pixels. Balance and contrast were adjusted to reflect true rendering as much as possible. No other 'photo-retouching' was performed.

Statistical analyses

Microsoft Excel 2010 and GRAPHPAD PRISM 6 were used to collate and analyse data. The distribution of the data was tested for normality (D'Agostino and Pearson omnibus), and significant differences between samples were determined with one-way ANOVA (Kruskal–Wallis for non-Gaussian data) and two-way ANOVAS, and unpaired two-tailed *t*-tests (Mann–Whitney test for non-Gaussian data) with a significance threshold of $\alpha = 0.05$. Results are presented as mean $\pm$ standard error of the mean unless noted otherwise.

Results

ChR2-mCherry expression by RVLM-CA neurons in $D\beta H^{Cre/0}$ and cKO mice, and optical fiber placements

In $D\beta H^{Cre/0}$ mice ($N = 8$), 146 ± 19 ChR2–mCherry-positive neurons were identified in the RVLM (1:3 series; actual numbers of ChR2–mCherry-positive neurons were approximately three times larger). Most ChR2–mCherry-positive neurons were detectably catecholaminergic (i.e. expressed TH) ($95.4\% \pm 0.9\%$; range, 91.7–99.4%), accounting for $68.4\% \pm 2.3\%$ of the TH-positive neurons in the ipsilateral ventrolateral medulla in sections containing ChR2–mCherry-positive neurons (Fig. 1 A1, A2, and C1). In cKO mice ($N = 8$), 127 ± 16 ChR2–mCherry-positive neurons were identified in the RVLM, of which $94.9\% \pm 1.0\%$ (range, 90.4–99.3%) were TH-positive, accounting for $57.3\% \pm 5.0\%$ of the TH-positive neurons observed in sections containing ChR2–mCherry-positive neurons (Fig. 1 B1, B2, and C2). The RVLMs of $D\beta H^{Cre/0}$ and cKO mice contained a similar number of ChR2–mCherry-positive neurons ($t_{14} = 0.78$, $P = 0.45$), and a similar percentage of these neurons were TH-positive ($t_{14} = 1.38$, $P = 0.74$). Also, the rostrocaudal distribution of ChR2–mCherry-positive neurons in the RVLM was indistinguishable between $D\beta H^{Cre/0}$ and

cKO mice ($F_{16,214} = 0.23$ and $P = 0.99$ for the interaction between genotype and bregma level) (Fig. 1 C1 and C2). The absolute number of RVLM TH-positive neurons was equivalent between strains ($t_{14} = 2.61$, $P = 0.81$), as was the rostrocaudal distribution of TH-positive neurons in the RVLM ($F_{16,214} = 1.23$, $P = 0.24$). Finally, the proportion of RVLM TH-positive neurons expressing ChR2–mCherry was not different ($t_{14} = 4.59$, $P = 0.063$). Importantly, the distribution and density of the ChR2–mCherry-labeled terminals were similar in $D\beta H^{Cre/0}$ and cKO mice, indicating that the absence of VGLUT2 did not alter the gross morphology and projections of RVLM-CA neurons (Fig. 1 A3 and B3).

The tips of the optical fibers were typically found 300–500 μm above the ventral surface of the ventrolateral medulla, and had a similar distribution in $D\beta H^{Cre/0}$ and cKO mice (Fig. 1D).

Photostimulation of RVLM-CA neurons in slices and *in vivo*

Optical stimulation of RVLM-CA neurons in brain slices—Whole-cell current clamp recordings of ChR2–eYFP-positive RVLM neurons were conducted in slices from adult $D\beta H^{Cre/0}$ mice to characterise the response of these neurons to photostimulation. ChR2–eYFP-positive neurons (nine neurons; three mice) were identified prior to patching by visualising eYFP fluorescence. These neurons were either silent or had a slow tonic discharge pattern (0.73 ± 0.32 Hz; range, 0–2.3 Hz, $N = 9$), and were exquisitely sensitive to photostimulation, each light pulse producing a strong depolarisation leading to a single action potential (Fig. 2A). Higher frequencies produced a tonic depolarisation without clear evidence of action potential generation (not illustrated). For this reason, we used a frequency of stimulation of <25 Hz in all our subsequent *in vivo* experiments. The amplitude of the stimulus-evoked spike gradually decreased during train stimulation at 10 Hz (Fig. 2A), and this was especially marked at 20 Hz (Fig. 2C). This tonic depolarisation is a characteristic of the particular ChR2 variant (H134R) used in this study (Boyden *et al.*, 2005; Lin *et al.*, 2009), and may be caused by fast sodium channel inactivation resulting from ChR2-dependent tonic depolarisation. All recorded neurons were filled with biocytin, and, after histological processing, every recovered biocytin-filled neuron ($N = 6$) contained eYFP and TH, demonstrating that they were ChR2-expressing RVLM-CA neurons (Fig. 2B).

Photostimulation of putative RVLM-CA neurons *in vivo*—These experiments were designed to compare the response of putative RVLM-CA neurons to photostimulation in $D\beta H^{Cre/0}$ and cKO mice. We recorded from neurons located immediately caudal to the facial motor nucleus in a region corresponding to the site of virus injection. We found four neurons in three $D\beta H^{Cre/0}$ mice and two neurons in two cKO mice in which neuronal activity could be entrained by stimulus trains up to 20 Hz (Fig. 2D and E). These neurons were spontaneously active and discharged tonically (range, 1.8–5.6 Hz), as opposed to the ON–OFF respiratory synchronous discharge that is typical of most non-catecholaminergic neurons in this region of the brain (Schreihofer & Guyenet, 1997). In both strains, the amplitude of the extracellular action potential decreased during photostimulation at frequencies >5 Hz. The decrease in spike amplitude was gradual, reaching steady state after 3–10 pulses, and reversible upon interruption of the stimulus (Fig. 2D and E). The same phenomenon has been previously described for ChR2-expressing C1 cells in rats (Abbott *et*

al., 2009). The decrease in extracellular spike amplitude is consistent with the reduced action potential amplitude observed *in vitro* (Fig. 2A and C). The silent period that followed high-frequency stimulation *in vivo* was presumably caused by hyperpolarisation, which was observable in the intracellular recordings in slices (Fig. 2C). Together, the evidence suggests that ChR2–mCherry-positive neurons are reliably entrained to stimulus frequencies up to 20 Hz, and that the stimulus–response relationship of putative ChR2–mCherry-positive RVLM-CA neurons is comparable in D β H^{Cre/0} and cKO mice.

In summary, the anatomical and electrophysiological evidence revealed no differences between D β H^{Cre/0} and cKO mice with regard to the morphology and distribution of RVLM-CA neurons, the level of expression of ChR2–mCherry by these neurons, and their response to light.

Respiratory effects produced by optogenetic stimulation of RVLM-CA neurons in D β H^{Cre/0} and cKO mice—During behavioral quiescence, D β H^{Cre/0} and cKO mice had similar f_R values (D β H^{Cre/0} vs. cKO, 131 ± 10 vs. 148 ± 3 breaths/min, $t_{12} = 1.7$, $P = 0.12$). Photostimulation increased f_R in D β H^{Cre/0} mice (Fig. 2A), as reported previously (Abbott *et al.*, 2013). The increase in f_R increased with light-pulse frequency up to a stimulus frequency of 15 Hz ($+54.1 \pm 11.6$ breaths/min, $N = 7$). In contrast, photostimulation had no effect on f_R in cKO mice ($+3.1 \pm 6.7$ breaths/min, $N = 7$) ($F_{4,48} = 6.62$ and $P = 0.0003$ for the interaction between stimulation frequency and genotype; Fig. 3A and B).

Vagal efferent activity elicited by optogenetic stimulation of RVLM-CA neurons in D β H^{Cre/0} and cKO mice—RVLM-CA neurons densely innervate the dorsal motor nucleus of the vagus, which contains parasympathetic preganglionic neurons (Fig. 1 A3 and B3) (Depuy *et al.*, 2013). Optogenetic experiments in slices maintained at room temperature have indicated that this connection is monosynaptic and primarily glutamatergic (Depuy *et al.*, 2013).

In urethane-anesthetised D β H^{Cre/0} mice, photostimulation of RVLM-CA neurons at 20 Hz activated vagus nerve efferents (Fig. 4 A1), with an onset latency of 24.1 ± 1.3 ms and latency to peak of 37.5 ± 1.7 ms from the start of the light-pulse train. Stimulation at 5 Hz and 10 Hz produced faithful short-latency responses that followed each light pulse (Fig. 4 B1). Unlike the sympathetic nerve response to photostimulation of RVLM-CA neurons (Abbott *et al.*, 2012), the vagus nerve response showed negligible paired-pulse depression when 50-ms pulses were delivered at intervals spanning 1000 ms, with a minimum interval of 250 ms ($F_{3,4} = 3.36$, $P = 0.14$; Fig. 4C). However, during 20-Hz stimulation, a modest accommodation in vagus nerve response was observed during the stimulus (1 s).

In contrast to the observations in D β H^{Cre/0} mice, photostimulation evoked no detectable vagal response in four of seven cKO mice tested (Fig. 4 A2 and B2), and a small discharge during 20-Hz stimulation in three of seven cKO mice (Fig. 4 D2). The discharge observed in responsive cKO mice had the same kinetics as the vagal response in D β H^{Cre/0} mice, but the amplitude of the response was much smaller when normalised to the fluctuations of vagal activity present during exposure of the mice to elevated CO₂ (Fig. 4D).

Regardless of strain, administration of the α_1 -adrenoreceptor antagonist prazosin, at a concentration sufficient to block central α_1 -adrenoreceptors (Menkes *et al.*, 1981), did not alter the vagal activation caused by photostimulation ($D\beta H^{Cre/0}$ vs. responsive cKO, $+2.2\% \pm 14.0\%$ vs. $-0.4\% \pm 12.7\%$ change in vagal response after prazosin; $F_{1,9} = 0.21$ and $P = 0.66$ for the effect of prazosin) (Fig. 4D). Thus, the evoked vagal responses observed in $D\beta H^{Cre/0}$ and responsive cKO mice are unlikely to be generated by catecholamine release.

In summary, RVLM-CA neuron photostimulation produced much greater activation of the vagus nerve in $D\beta H^{Cre/0}$ mice than in cKO mice at all stimulation frequencies examined ($F_{2,22} = 13.11$ and $P = 0.0002$ for the interaction between stimulus frequency and genotype) (Fig. 4E).

Discussion

The present results show that VGLUT2 deletion from RVLM-CA neurons has no detectable impact on the number and morphology of these neurons, but almost completely eliminates two of the acute effects of activating these neurons *in vivo*. This work highlights the importance of VGLUT2 and, by inference, glutamate release in the physiological effects of RVLM-CA neuron stimulation in the intact mouse.

Evidence for deletion of VGLUT2 from RVLM-CA neurons

In an earlier study, we demonstrated that VGLUT2 immunoreactivity is almost completely absent from virally labeled RVLM-CA neuron terminals in the brains of cKO mice (Depuy *et al.*, 2013). In that study, only 8% of virally labeled axonal varicosities originating from RVLM-CA neurons contained detectable VGLUT2 immunoreactivity in cKO mice, as compared with 97% in $D\beta H^{Cre/0}$ mice (Depuy *et al.*, 2013). At the resolution of light microscopy, the appearance of VGLUT2 immunoreactivity in the terminals of RVLM-CA neurons in cKO mice may reflect superposition of virally labeled terminals and glutamatergic boutons of unknown origin. Other investigators have shown that VGLUT2 expression and function are effectively eliminated in Cre-expressing neurons when various Cre-driver mouse lines are crossed with the $VGLUT2^{flox/flox}$ mouse line used in the present study (Tong *et al.*, 2007; Liu *et al.*, 2010; Fortin *et al.*, 2012). On this basis, we conclude that VGLUT2 was effectively deleted from most but probably not all Cre-expressing noradrenergic and adrenergic neurons in our cKO mice.

cKO mice had no obvious abnormalities in the number, morphology and projection pattern of virally labeled RVLM-CA neurons, suggesting that VGLUT2-dependent vesicular glutamate release is not essential for the development of these neurons. This result is consistent with prior evidence that RVLM-CA neurons have normal embryological development in VGLUT2-null mutant mice (Wallen-Mackenzie *et al.*, 2006), and contrasts with evidence that VGLUT2 expression is important for the survival and the development of mesencephalic dopaminergic neurons after birth (Fortin *et al.*, 2012).

Photostimulation of RVLM-CA in slices and *in vivo*

The present whole-cell recordings have demonstrated that ChR2-expressing RVLM-CA neurons respond to light pulses in conventional manner. As in other neurons, the relatively

slow kinetics of the current elicited by ChR2(H134R) enable reliable neuronal activation only up to 20 Hz (Wang *et al.*, 2007; Lin *et al.*, 2009). At frequencies >10 Hz, fast sodium channel inactivation during tonic depolarisation may have caused a gradual reduction in action potential amplitude, and a reduction in the amplitude of extracellular spikes *in vivo*.

We did not obtain proof that the RVLM neurons that were light-activated on a pulse-by-pulse basis *in vivo* were ChR2–mCherry-positive catecholaminergic neurons. Conceivably, these cells could have been non-catecholaminergic neurons that were driven synaptically in D β H^{Cre/0} mice, but this is implausible in the case of cKO mice, in which the RVLM-CA neurons do not release glutamate.

Glutamate mediates the stimulatory effects of the C1 cells on respiration and the parasympathetic outflow *in vivo*

Up to 99% of the ChR2–mCherry-expressing neurons were demonstrably catecholaminergic in D β H^{Cre/0} and cKO mice. The RVLM contains catecholaminergic neurons primarily of the C1 phenotype (i.e. PNMT-expressing neurons), along with a small proportion of A1 neurons (i.e. noradrenergic neurons devoid of PNMT). Thus, given the location of ChR2–mCherry-expressing neurons in this study, most of these neurons are likely to be C1 rather than A1 neurons. A1 neurons, along with other noradrenergic neurons in the brainstem, do not commonly express transcripts for VGLUTs in rats (Stornetta *et al.*, 2002a), and are thought to use noradrenaline as a primary neurotransmitter. The fact that α_1 -adrenoreceptor blockade did not reduce the vagal response in D β H^{Cre/0} mice indicates that the release of noradrenaline from A1 (or C1) neurons or downstream targets was not essential for the observed responses. Accordingly, the robust and consistent respiratory and vagal stimulation produced by activation of RVLM-CA neurons in D β H^{Cre/0} mice was almost certainly caused by the activation of RVLM-CA neurons that normally express VGLUT2. Also, given that cKO mice were almost completely unresponsive to identical stimulation of a similar population of RVLM-CA neurons, the combined evidence indicates that VGLUT2, and thereby glutamate release, is necessary for physiological responses to C1 neuron stimulation.

The loss of VGLUT2 in catecholaminergic neurons that are activated during C1 stimulation may amplify the differences observed between D β H^{Cre/0} and cKO mice. VGLUT2 is expressed by the C2 and C3 adrenergic population in rats (Stornetta *et al.*, 2002a), but little is known about the function of these neurons. The circuit responsible for the respiratory stimulation elicited by photostimulation of RVLM-CA neurons is unknown. A direct connection of the C1 cells to the pre-Bötzinger complex is anatomically plausible, but many other routes are conceivable, given the complexity of the projection pattern of the RVLM-CA neurons (Card *et al.*, 2006; Abbott *et al.*, 2013). RVLM-CA neurons monosynaptically activate the preganglionic neurons of the dorsal vagal complex in mouse brain slices (Depuy *et al.*, 2013), which probably underlies the vagal efferent responses evoked by RVLM photostimulation *in vivo*, although indirect circuits may also contribute to this effect.

A minority of cKO mice retained a weak vagal response that resembled the response observed in D β H^{Cre/0} mice. The fast kinetics of the residual vagal response in cKO mice are inconsistent with the actions of a slow-acting metabotropic transmitter, be it a catecholamine

or peptide. As already mentioned, noradrenaline release from stimulated C1 or A1 neurons is unlikely to explain the vagal response in cKO mice, as it was insensitive to α_1 -adrenoreceptor blockade. Other possible explanations for the small residual effect seen in a fraction of the cKO mice are the release of an unidentified transmitter from RVLM-CA, and the upregulation of either VGLUT1 or VGLUT3. However, in this case, a greater proportion of cKO mice should have had an appreciable response to photostimulation.

Failed or incomplete deletion of the VGLUT2 gene in a minority of RVLM-CA neurons sufficiently explains the small vagal response found in a minority of cKO mice. By experimental design, both the expression of ChR2-mCherry and the deletion of the VGLUT2 gene were contingent on the presence of genomic Cre driven from the D β H promoter. For this reason, we predicted that RVLM-CA neurons expressing Cre-dependent ChR2-mCherry would be neurons that express sufficiently high levels of Cre to also eliminate VGLUT2 in the cKO mouse, theoretically bypassing issues arising from variegated expression of Cre in the D β H^{Cre/0} mouse line. However, the efficacy of Cre recombination of LoxP sites within the VGLUT2 gene may be influenced by the chromosomal location of the LoxP sites and the distance between these sites (Schmidt-Suppran & Rajewsky, 2007; Liu *et al.*, 2013). Given this, differences in the recombination efficiency of the virally delivered floxed transgene vs. the floxed VGLUT2 gene may result in the expression of ChR2-mCherry in a subset of RVLM-CA neurons that retain functional VGLUT2 expression in the cKO mouse. This possibility accounts for the reduced amplitude of the vagal response in cKO mice, as well as the similarities in the kinetics of the vagal response in both strains. It would also account for the small fraction of virally labeled RVLM-CA terminals that appear to be VGLUT2-immunoreactive in cKO mice (Depuy *et al.*, 2013).

Altogether, the evidence supports the conclusion that VGLUT2 and glutamate release are required for the effects of RVLM-CA neuron stimulation *in vivo*. This interpretation is consistent with the presence of VGLUT2 mRNA and protein in C1 neurons in rodents (Stornetta *et al.*, 2002b, 2005; Herzog *et al.*, 2004; Abbott *et al.*, 2013) and the fact that optogenetic activation of RVLM-CA terminals in slices produces fast excitatory postsynaptic currents that are blocked by ionotropic glutamate antagonists (Depuy *et al.*, 2013).

Do C1 neurons release catecholamines?

In mesencephalic dopaminergic neurons, VGLUT2 may serve to facilitate the vesicular packaging of catecholamine (Hnasko *et al.*, 2010). The same mechanism could potentially exist in C1 neurons, which co-express vesicular monoamine transporter 2 and VGLUT2 (Sevigny *et al.*, 2008; Depuy *et al.*, 2013). If vesicular packaging of catecholamine depends on VGLUT2 in C1 neurons, the ability of these neurons to release catecholamine could be severely curtailed in cKO mice, which could explain the absence of detectable catecholamine release during RVLM-CA neuron stimulation in cKO mice. However, this interpretation requires the responses elicited in D β H^{Cre/0} mice to have a prominent catecholaminergic component, which does not appear to be true [see Depuy *et al.* (2013), Abbott *et al.* (2013), and the present results]. We did not explore the effect of β -

adrenoreceptor blockade on the vagal response in the present study, because the excitatory effect of catecholamines on dorsal vagal motoneurons have been reported to be entirely mediated by α_1 -adrenoreceptors (Martinez-Pena y Valenzuela *et al.*, 2004). Furthermore, we have previously shown that β -adrenoreceptor blockade does not attenuate the respiratory response to RVLM-CA neuron stimulation (Abbott *et al.*, 2013). Together, the lack of effect of α_1 -adrenoreceptors indicates that catecholamine release makes, at most, a very minor contribution to the acute respiratory and parasympathetic effects produced by RVLM-CA neuron stimulation *in vivo*. Clearly, this observation does not eliminate the possibility that catecholamines are released from C1 neurons in other conditions.

An uninvestigated possibility is that catecholamines released by C1 neurons have effects that are delayed. Indeed, catecholamines often produce long-term potentiation or even metaplasticity that develops over a much longer time period than the acute responses that we have examined (Neverova *et al.*, 2007; Inoue *et al.*, 2013). A similar argument can be made for any one of the neuropeptides known to be expressed by C1 neurons [for a review, see Guyenet *et al.* (2013)]. Another possibility is that the catecholamines released by C1 neurons activate the surrounding glia, and have only minor effects on synaptic transmission (O'Donnell *et al.*, 2012).

In conclusion, we have shown that genetic deletion of VGLUT2 from RVLM-CA neurons abolishes the acute respiratory and parasympathetic activation produced by selectively stimulating these neurons in mice. The collective evidence indicates that C1 neurons regulate the autonomic nervous system through the synaptic release of glutamate, which is consistent with their role in mediating rapid counter-regulatory responses to physical stresses.

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Abbreviations

AAV	adeno-associated virus
AAV2-ChR2-eYFP	AAV2-DIO-EF1 α -ChR2 (H134R)-eYFP
AAV2-ChR2-mCherry	AAV2-DIO-EF1 α -ChR2(H134R)-hmCherry
ACSF	artificial cerebrospinal fluid
a.u.	arbitrary units
AUC	area under the curve
ChR2	channelrhodopsin-2
cKO	D β H ^{Cre/0} ;VGLUT2 ^{flox/flox}
DβH	dopamine- β -hydroxylase

ECG	electrocardiogram
EF1α	elongation factor 1 α
eYFP	enhanced yellow fluorescent protein
f_R	respiratory frequency
PNMT	phenylethanolamine <i>N</i> -methyltransferase
RVLM	rostral ventrolateral medulla
RVLM-CA	neurons catecholaminergic neurons of the rostral ventrolateral medulla
TH	tyrosine hydroxylase
VGLUT	vesicular glutamate transporter

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Fig. 1.

Distribution of ChR2–mCherry-positive neurons in the RVLM, and placement of fiber optics in DβH^{Cre/0} and cKO mice. (A1) Low-magnification image of ChR2–mCherry expression in a coronal section of the RVLM from a DβH^{Cre/0} mouse (approximately –6.64 mm from bregma). Scale bar: 400 μm. (A2) Higher magnification of the region outlined in A1, showing immunofluorescence for ChR2–mCherry (in red) and TH (in green). Colocalisation of ChR2–mCherry and TH is indicated by orange. Scale bar: 100 μm. (A3) ChR2–mCherry-immunoreactive axons within the dorsal medulla of a DβH^{Cre/0} mouse (approximately –7.48 mm from bregma). Scale bar: 200 μm. (B1–B3) Descriptions and scale bars are the same as in A1–A3, except that sections are from a cKO mouse. (C1 and C2) Rostrocaudal distribution of neurons expressing ChR2–mCherry, TH or both in the ipsilateral RVLM of DβH^{Cre/0} (C1, *N* = 8) and cKO (C2, *N* = 8) mice. (D) Locations of the tips of implanted fiber optics in DβH^{Cre/0} and cKO mice. 10N, dorsal motor nucleus of the vagus; 12N, hypoglossal motor nucleus; Amb, compact formation of the nucleus ambiguus; AP, area postrema; cc, central canal; IO, inferior olive; py, pyramidal tract; sol, solitary tract; VMS, ventral medullary surface.

Fig. 2. Characteristics of the response of ChR2-expressing neurons to light stimulation in DβH^{Cre/0} and cKO mice. (A) Current-clamp recording of a ChR2-eYFP-positive RVLM neuron in a slice (DβH^{Cre/0} mouse) during 20-Hz, 10-Hz and 5-Hz stimulation (10 × 5-ms pulses). Each light pulse is followed by a short-latency depolarisation and an action potential. Asterisks indicate spontaneous action potentials. (B) Example of biocytin-filled RVLM-CA neurons that were photo-responsive (arrowheads). All three neurons expressed ChR2-eYFP and TH. Asterisks indicate ChR2-eYFP-positive, TH-positive neurons not labeled with biotin.

Inserts correspond to the middle cell in the main panel (black-rimmed arrowhead), and show immunofluorescence for each marker separately. (C) Response of a ChR2–eFYP-positive RVLM-CA neuron to a 1-s stimulus train (20 Hz with 5-ms pulses), illustrating the reduction in action potential amplitude after the first light-induced spike and the hyperpolarisation that follows the stimulus train. (D) Extracellular recording of a single putative ChR2–mCherry-positive RVLM-CA neuron during photostimulation (10 Hz and 20 Hz with 5-ms pulses) in an anaesthetised $D\beta H^{Cre/0}$ mouse. Expanded traces (below) show the pulse-by-pulse activation typical of ChR2-expressing neurons, and the gradual reduction in spike amplitude during stimulation and the silent period following the stimulus train. (E) Identical experiment in a cKO mouse.

Fig. 3. Effect of stimulating RVLM-CA neurons on f_R in DβH^{Cre/0} and cKO mice. (A) Original plethysmography traces (inspiration upward) of a DβH^{Cre/0} mouse (upper trace) and a cKO mouse (lower trace) during photostimulation (5-ms pulses at 20 Hz, 10-s train). (B) Relationship between photostimulus frequency and increase in f_R in DβH^{Cre/0} ($N = 7$) and cKO ($N = 7$) mice. *** $P < 0.001$ for DβH^{Cre/0} vs. cKO (Bonferroni *post hoc* test following repeated measures ANOVA).

Fig. 4.

Effect of stimulating RVLM-CA neurons on efferent vagus nerve activity in $D\beta H^{Cre/0}$ and cKO mice. (A1 and A2) Left panel: integrated (upper trace) and raw (lower trace) recording of multi-unit vagus nerve activity in urethane-anesthetised $D\beta H^{Cre/0}$ (A1) and cKO (B2) mice, showing activation by photostimulation (5-ms pulses at 20 Hz, 1-s train). Right panel: respiratory-related oscillations in vagus nerve activity caused by hypercapnia in the same mouse (7% CO₂). The amplitude of oscillations in vagus activity under conditions of elevated inspired CO₂ (i.e. peak to trough) was assigned a value of 100 a.u. to normalise

recordings between experiments. Integrated vagus nerve activity is rectified and smoothed with a 0.03-s time constant. (B1 and B2) Normalised waveform averages of the vagal response to photostimulation (5-ms pulses, 15–20 trials per trace, averaging triggered from the onset of the 1-s stimulus train) in a $D\beta H^{Cre/0}$ mouse (B1) and a cKO mouse (B2). (C) Waveform average of the vagal response to paired-pulse stimulation (50-ms pulses, identified by S1 and S2) in a $D\beta H^{Cre/0}$ mouse; note that the magnitude of the second burst was similar regardless of the delay of the second pulse of light down to 250 ms. (D) Example of the of the vagal response to photostimulation (5-ms pulses at 20 Hz) in a $D\beta H^{Cre/0}$ mouse (D1) and a cKO (D2) mouse before and after α_1 -adrenoreceptor blockade (prazosin, 1 mg/kg). Waveforms were generated by averaging 10–15 trials per trace. Note that the cKO mouse used in the example had the largest response of all cKO mice tested. (E) Group data of the normalised vagus nerve activity during photostimulation (see Materials and methods) in $D\beta H^{Cre/0}$ ($N = 6$) and cKO ($N = 7$) mice. $*P < 0.05$ and $***P < 0.001$ for $D\beta H^{Cre/0}$ vs. cKO by the Bonferroni *post hoc* test.