Monosynaptic Glutamatergic Activation of Locus Coeruleus and Other Lower Brainstem Noradrenergic Neurons by the C1 Cells in Mice

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The C1 neurons, located in the rostral ventrolateral medulla (VLM), are activated by pain, hypotension, hypoglycemia, hypoxia, and infection, as well as by psychological stress. Prior work has highlighted the ability of these neurons to increase sympathetic tone, hence peripheral catecholamine release, probably via their direct excitatory projections to sympathetic preganglionic neurons. In this study, we use channelrhodopsin-2 (ChR2) optogenetics to test whether the C1 cells are also capable of broadly activating the brain's noradrenergic system. We selectively expressed ChR2(H134R) in rostral VLM catecholaminergic neurons by injecting Cre-dependent adeno-associated viral vectors into the brain of adult dopamine- β -hydroxylase (*DBH*)^{Cre/0} mice. Most ChR2-expressing VLM neurons (75%) were immunoreactive for phenylethanolamine *N*-methyl transferase, thus were C1 cells, and most of the ChR2-positive axonal varicosities were immunoreactive for vesicular glutamate transporter-2 (78%). We produced light microscopic evidence that the axons of rostral VLM (RVLM) catecholaminergic neurons contact locus coeruleus, A1, and A2 noradrenergic neurons, and ultrastructural evidence that these contacts represent asymmetric synapses. Using optogenetics in tissue slices, we show that RVLM catecholaminergic neurons activate the locus coeruleus as well as A1 and A2 noradrenergic neurons monosynaptically by releasing glutamate. In conclusion, activation of RVLM catecholaminergic neurons, predominantly C1 cells, by somatic or psychological stresses has the potential to increase the firing of both peripheral and central noradrenergic neurons.

Introduction

The locus coeruleus (LC) is the largest cluster of CNS noradrenergic neurons. The activity of this nucleus is state-dependent and facilitates arousal and attention by increasing neuronal excitability in the cortex and elsewhere (Aston-Jones and Cohen, 2005; Carter et al., 2010; Berridge et al., 2012). Noradrenaline released by LC neurons also directly activates astrocyte metabolism (Sorg and Magistretti, 1991; Hertz et al., 2010). The ponto-medullary region contains several other much less studied clusters of noradrenergic neurons (A1, A2, A5, A7) that primarily target subcortical regions involved in nutrient intake and cardiorespiratory and hormonal regulations (Dahlstrom and Fuxe, 1964; Byrum and Guyenet, 1987; Ritter et al., 1998; Appleyard et al., 2007; Fenik et al., 2008; Bruinstroop et al., 2012).

LC unit activity is activated by hypotension in several species (Elam et al., 1984; Morilak et al., 1987a; Curtis et al., 1993) including humans (Mitchell et al., 2009). LC neurons are also activated by hypoglycemia, hypoxia, hypercapnia, and bacterial

infection (Elam et al., 1981; Morilak et al., 1987b; Erickson and Millhorn, 1994; Haxhiu et al., 1996; Teppema et al., 1997; Yuan and Yang, 2002; Schiltz and Sawchenko, 2007). Several observations suggest that these physiological perturbations could activate the LC via the C1 cells, a group of neurons with a dual catecholaminergic/glutamatergic phenotype which resides in the rostral ventrolateral medulla (VLM; Guyenet et al., 2013). The C1 cells densely innervate the LC (Milner et al., 1989; Pieribone and Aston-Jones, 1991; Card et al., 2006; Guyenet, 2006; Abbott et al., 2013). C1 and LC neurons respond in a qualitatively similar manner to the above-mentioned stressors (Ritter et al., 1998; Guyenet, 2006; Moreira et al., 2006; Verberne and Sartor, 2010; Abbott et al., 2013). Optogenetic stimulation of the C1 cells activates the LC in anesthetized rats (Abbott et al., 2012). Finally, the C1 neurons express VGluT2 and produce glutamatergic EPSCs (Stornetta et al., 2002a; DePuy et al., 2013). Based on light microscopy evidence, the C1 cells may also target additional clusters of lower brainstem noradrenergic neurons, such as the A1 and A2 cell groups located respectively in the caudal VLM and the nucleus of the solitary tract (Card et al., 2006; Abbott et al., 2013). Finally, as is well known, the C1 cells drive sympathetic preganglionic neurons monosynaptically, plausibly by releasing glutamate, and therefore broadly regulate the release of noradrenaline in the periphery (Morrison et al., 1989; Jansen et al., 1995; Stornetta et al., 2002b; Guyenet, 2006; Marina et al., 2011).

In the present study, we test the hypothesis that the C1 neurons also broadly regulate noradrenaline release within the CNS.

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We focus on three clusters of noradrenergic neurons: LC, A1 and A2 neurons. The existence of monosynaptic connections between the C1 cells and their noradrenergic targets is examined at the ultrastructural level and synaptic transmission between C1 and their noradrenergic neuronal targets is studied using channelrhodopsin-2 (ChR2) optogenetics in slices.

Materials and Methods

Animals. Animals were used in accordance with guidelines approved by the University of Virginia Animal Care and Use Committee. *Tg(Dbh-cre)/KH212Gsat/Mmcd* mice, stock no. 032081-UCD (*DBH-Cre*) were obtained from the Mutant Mouse Regional Resource Center at the University of California, Davis, CA and maintained as hemizygous (*DBH^{Cre/0}*) on a C57BL/6J background. *B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J}* mice (*ROSA-tdTomato*) were obtained from The Jackson Laboratory and crossed with the *DBH^{Cre/0}* mice to produce mice (*DBH^{Cre/0};ROSA-tdTomato*) in which noradrenergic neurons could be visualized in live slices.

Viral vectors and microinjections. The Cre-recombinase-dependent viral vectors DIO-eF1 α -hChR2(H134R)-mCherry adeno-associated virus (AAV) and DIO-eF1 α -ChR2(H134R)-eYFP AAV (both serotype 2; Atasoy et al., 2008) were obtained from the University of North Carolina Vector core (Chapel Hill, NC) at a titer of 10^{12} viral particles per milliliters. Twenty-eight, 6- to 13-week-old mice (median 9; 14 males, 14 females) were anesthetized with a mixture of ketamine (100 mg/kg) and dexmedetomidine (0.2 mg/kg) given intraperitoneally. After reaching an adequate plane of anesthesia (unresponsive to paw pinch and no corneal reflex) animals were placed into a modified stereotaxic device (Kopf) on a thermostatically controlled heating pad and received three injections of undiluted virus (240–360 nl total volume) into the left rostral VLM (RVLM) under electrophysiological guidance as described previously (Abbott et al., 2013).

Electrophysiology. Four to 12 weeks after AAV2 injection, the mice were anesthetized with a mixture of ketamine (120 mg/kg) and xylazine (12 mg/kg) given intraperitoneally, and after becoming completely anesthetized (unresponsive to hindpaw pinch) were decapitated. The brainstem was sectioned with a vibrating microtome in the transverse plane in ice-cold, *N*-Methyl-D-glucamine (NMDG)-substituted artificial CSF (ACSF) containing the following (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 0.5 CaCl₂, 20 HEPES, 30 NaHCO₃, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate (~300 mOsm/kg). After 10 min at 33°C, 200- to 300- μ m-thick slices were transferred to aerated physiological extracellular ACSF containing the following (in mM): 119 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, 12.5 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, and maintained at room temperature (23°C). All recordings were performed at room temperature in submerged slices continuously perfused with aerated physiological ACSF. Glass pipettes (2–6 M Ω tip resistance) were filled with a solution containing the following (in mM): 140 K-gluconate, 10 HEPES, 10 tris-phosphocreatine, 3 ATP-Na, 0.3 GTP-Na, 1 EGTA, 2 MgCl₂, pH 7.3. EPSCs were recorded at V-hold of –60 mV (–74 mV after junction potential correction). In selected experiments, K-gluconate was replaced with cesium-methanesulfonate (140 mM) and recordings were made at Vhold of –70 mV and +9 mV (respectively, –80 and 0 mV after junction potential correction). The calculated E[Cl[–]] was –88 mV. Biocytin-filled electrodes (0.2%) were used to label the recorded cells. Recordings were performed using a Multiclamp 700B amplifier and pClamp 10 software (Molecular Devices). Signals were low-pass filtered at 4 kHz and digitized at 10 kHz. Only cells with series resistance that remained <25 M Ω were included in the analysis. Further analysis (event-triggered averages, event-triggered histograms, EPSC detection and counting, curve fitting of PSC decay and measurement of decay time-constant) was done using Spike2 version 7.10 software (CED). Photostimulation of ChR2-expressing neurons, axons and nerve terminals was done with a 200- μ m-diameter optical fiber coupled to a 473 nm DPSS laser (IkeCool; 1 ms pulses, 5mW steady-state output) as previously described (Depuy et al., 2013). The optical fiber, held at 40 degrees from the horizontal, was positioned such that the tip was 150 μ m above and 250 μ m lateral to each recorded neuron. This setup intensely illuminated an ellipse of ~0.342

mm², which produced an estimated average irradiance of ~14mW/mm² comparable to that used by others previously (Grossman et al., 2013). Delivery of optical pulses was triggered by a digitizer (Digidata 1440A, Molecular Devices) controlled by episodic protocols run in pClamp 10 (Molecular Devices). The output of the laser/fiber was calibrated for 5 mW steady-state output before each experiment. The following drugs were used: tetrodotoxin (TTX; Fisher Scientific, final concentration 1 μ M); 4-aminopyridine (4-AP; Sigma-Aldrich; 100–200 μ M); kynurenic acid (Sigma-Aldrich; 1 mM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Bioscience; 10 μ M), D-(–)-2-Amino-5-phosphonopentanoic acid (AP-5, Sigma-Aldrich; 50 μ M).

Slices from electrophysiological recording experiments containing biocytin-filled cells were fixed in 4% paraformaldehyde (PFA) from 2 to 5 d. Sections were rinsed and incubated first in blocking solution using a Triton concentration of 0.5% to enhance antibody penetration in thicker tissue and then in primary antibodies (described below) to detect tyrosine hydroxylase (TH) and dsRed or EYFP. Slices were then rinsed and incubated with appropriate secondary antibodies as well as with NeutrAvidin-Dylight-649 (Fisher Scientific), rinsed, mounted and covered as described below.

Fluorescent light microscopy for distribution of PNMT and to confirm tdTomato expression in catecholaminergic neurons. Lower brainstem sections from 3 *DBH^{Cre/0}* mice injected 5 weeks prior with DIO-eF1 α -eYFP AAV2 were examined to determine which proportion of the transduced neurons expressed both TH and phenylethanolamine-*N*-methyl transferase (PNMT), i.e., were C1 cells. These mice were anesthetized with an overdose of pentobarbital and perfused transcardially with 10 ml heparinized saline followed by 80 ml of 2% PFA. The brains were postfixed in 2% PFA for 2–5 d. Brainstem sections from three *DBH^{Cre/0};ROSA26-tdTomato* mice were also examined histologically to test whether the red fluorophore, tdTomato, was confined to catecholaminergic neurons. Thirty-micrometer-thick transverse sections were cut with a vibrating microtome and collected into a cryoprotectant solution and stored at –20°C before further processing. All histological procedures were done with free-floating sections. For fluorescent immunohistochemistry, sections were rinsed, blocked with 10% normal horse serum in 100 mM Tris-Saline containing 0.1% Triton X-100 and incubated in primary antibodies diluted in this blocking solution for 16–18 h at 4°C as follows: sheep anti-TH (Millipore; 1:1000), rabbit anti-dsRed (Clontech Laboratories; 1:500) or chicken anti-GFP (AVES; 1:1000) and/or rabbit anti-PNMT (generously provided by Dr. Martha Bohn, Northwestern University Medical School, Chicago, IL; 1:3000; Bohn et al., 1987). Sections were then rinsed and incubated for 45–60 min in appropriate secondary antibodies as follows: DyLight 649 anti-sheep IgG, Cy3 anti-rabbit IgG, AlexaFluor 488 anti-chicken IgY all raised in donkey and at 1:200 (Jackson ImmunoResearch) then rinsed, mounted on gelatinized glass slides, dehydrated through a graded series of alcohols, and covered with DPX.

Light microscopy for imaging VGluT2 in terminals from C1 neurons and to examine close appositions from C1 neurons onto A1, A2, and A6 (LC) neurons. For fluorescent imaging of close appositions as well as for determination of VGluT2 immunoreactivity in terminals of C1 or A1 neurons, a one in three series of 30- μ m-thick coronal sections were processed as described above. A guinea pig anti-VGluT2 antibody (Millipore, AB2251; 1:2000) was substituted for the PNMT antibody. The secondary antibody for the VGluT2 primary was a Cy3-tagged anti-guinea pig IgG (Jackson ImmunoResearch; 1:200) used exactly as the other secondaries described above.

For bright-field imaging of close appositions of terminals from ChR2-mCherry labeled C1 neurons onto TH cells in A1, A2, and A6 (LC), sections from 3 *DBH^{Cre/0}* mice previously injected with DIO-eF1 α -mCherry as described above were incubated in blocking solution as described, rinsed, and incubated in 1% hydrogen peroxide, then rinsed and incubated in rabbit anti-dsRed (1:500; recognizes mCherry) in 0.5% “TNB” (proprietary blocking reagent, PerkinElmer) prepared in 100 mM Tris-saline for 16–18 h at 4°C. Sections were then rinsed and incubated in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch; 1:1000) for 45–60 min, rinsed, and incubated in ABC solution according to manufacturer’s instructions (Vectastain “Elite”; Vector Laboratories) for 45 min, then rinsed and incubated with 3,3′-diaminobenzidine (DAB)

with nickel enhancement using the DAB kit according to the manufacturer's instructions (Vector Laboratories) resulting in a black reaction product. Sections were then rinsed and incubated in sheep anti-TH (1:1000) prepared in 0.5% TNB in 100 mM Tris-saline for 16–18 h at 4°C. Sections were then rinsed and incubated in biotinylated donkey anti-sheep IgG (Jackson Immunoresearch; 1:000) for 45–60 min, rinsed and incubated in ABC solution (Vector Laboratories) for 45 min, then rinsed and incubated in DAB using a kit according to the manufacturer's instructions (Vector Laboratories) resulting in a brown reaction product. Sections were then rinsed, mounted, and covered as described above.

Light microscopic examination of putative terminals from C1 or A1 neurons. Slides were examined using a Zeiss Axioskop2 and photographed with a Zeiss MRC camera. Computer-assisted mapping of the neurons of interest was done as previously described (Bochorishvili et al., 2012; Stornetta et al., 2013). For analysis of fluorescent terminals, 10 micron Z-stacks at 0.3 μ m increments were taken through the tissue with filter sets at appropriate wavelengths to discriminate the various fluorophores without “bleed through” (wavelengths listed as excitation, beam splitter, emission; for Cy3: 545, 570, 605; for AlexaFluor 488: 500, 515, 535; for AlexaFluor 649: 640, 660, 690). For the 100 $\times$ objective used to determine bouton double labeling and close appositions, the resulting digital images were 15.2 pixels/ μ m. A bouton was considered to be double labeled if both fluorophores were in focus and coincident through at least two levels of the Z-stack. For illustration of close appositions, Z-stack images were subjected to 3D blind deconvolution through seven iterations using the AutoQuant X3 software (Media Cybernetics). Deconvoluted stacks were then processed with Volocity software (version 4.4, Improvision) for 3D rendering and confirmation of close appositions. A close apposition was defined as two objects labeled with two separate fluorophores overlapping at least 7 pixels in each Z-level, and at least in two consecutive Z-levels (Corson and Erisir, 2013). The size of this overlap represents an apposition of $\sim$ 0.45 μ m in length.

Electron microscopy methods and analysis. For ultrastructural analysis, two Δ BH^{Cre/0} mice injected 5 weeks prior with DIO-eF1 α -ChR2-mCherry AAV2 were anesthetized as above and perfused transcardially, first with 20 ml of heparinized saline (1000 U/ml), and then with fixative (25 ml of 2% PFA with 3.75% acrolein, Electron Microscopy Sciences; followed by 30 ml of 2% PFA). Brains from these animals were then postfixed for at least 3 h in 2% PFA before sectioning with a vibrating microtome into 30- μ m-thick transverse sections. The tissues were blocked in 0.8% BSA with 0.03% Triton X-100 then incubated in rabbit anti-DsRed (Clontech) at 1:500 made in blocking solution at 4°C for 16–18 h, rinsed, and incubated for 60 min in biotinylated donkey anti-rabbit IgG (1:400, Jackson Immunoresearch). Tissues were rinsed and incubated for 60 min in ABC (Vector), rinsed and incubated in DAB (Vector Laboratories) as above. Sections for immunogold-silver staining were placed in mouse anti-TH (1:1000; ImmunoStar, catalog #22941) and incubated for 16–18 h at 4°C. Sections were rinsed and incubated for 30 min in washing buffer (PBS containing 0.8% BSA, 0.1% fish gelatin, and 3% normal goat serum). Tissues were subsequently incubated for 2 h in washing buffer containing 1 nm gold-conjugated donkey anti-mouse IgG (1:50; Aurion, Electron Microscopy Sciences). The sections were next rinsed in washing buffer, followed by a rinse in PBS, and then incubated in 2.5% glutaraldehyde in PBS for 10 min. The tissue was subsequently rinsed in PBS and transferred to sterile, untreated culture well plates for a series of 1 min rinses in PBS and then in 0.2 M sodium citrate buffer, pH 7.4. Rinses were followed by a silver enhancement reaction at room temperature using IntenSEM silver kit reagents according to manufacturer's instructions (GE Healthcare). The sections were handled with wooden applicator sticks and gently swirled throughout the silver enhancement procedure for 4–6 min. Sections were then rinsed in sodium citrate buffer and 100 mM phosphate buffer. Sections were then incubated in 1% osmium tetroxide and 1% uranyl acetate (Electron Microscopy Sciences) for 90 min. Tissues were rinsed, transferred to porcelain dishes, dehydrated through a series of increasing ethanol concentrations (30, 50, 70, and 95%), and then placed into glass vials containing 100% ethanol. Finally, the tissue sections were treated with propylene oxide (2 $\times$ 10 min) and incubated in a 1:1 mixture of propylene oxide and embedding resin Embed-812 (Electron Microscopy Sci-

ences). This mixture was then replaced with straight Embed-812 which infiltrated the sections for 16–18 h. The tissue was embedded between sheets of plastic (Aclar), flattened, and cured for at least 72 h at 62°C. Then flat-embedded sections were examined with a light microscope to detect immunogold-labeled TH cells and DAB-labeled C1 projection fields in the A1 and A2 noradrenergic cell clusters. The regions of interest were further trimmed and repolymerized at the bottom of Beem capsules. The block was trimmed to a 2 $\times$ 1 mm trapezoidal block that spanned the A1 or A2 and contained the TH-immunoreactive cells. Ultrathin sections (70–90 nm) were cut at a plane near parallel to the surface of 30 μ m sections using a Leica Ultracut UCT. This approach ensures that the top 5–10 μ m of the sections, where immunolabeling is present, are sectioned to yield a wide (100–200 μ m) strip of labeled ultrathin tissue. Ultrathin sections were collected onto copper mesh grids in series of 7–10 sections per grid. Tissue was analyzed with a JEOL 1230 transmission electron microscope, and micrographs were captured with an ultra high resolution digital imaging (4K $\times$ 4K) camera (Scientific Instruments and Applications). Adobe Photoshop was used to adjust image contrast and illumination, and then images were exported into the Canvas drawing software (Version 10, ACD Systems).

Antibody characterization. All antibodies used are listed in the *Journal of Comparative Neurology* antibody database and have been previously characterized as follows:

PNMT antibody raised in rabbits against purified rat adrenal extract was obtained from Dr. M. Bohn (Northwestern University, Chicago, IL; Bohn et al., 1987) and showed a labeling pattern similar to that seen in rats using identical conditions as previously published (Verberne et al., 1999).

TH antibody raised in sheep (Millipore) against native tyrosine hydroxylase from rat pheochromocytoma labels one band of expected length in Western blots of mouse brain lysates (from the manufacturer's information). The labeling pattern is identical to that seen in mouse brain in previous publications from the lab using identical conditions (DePuy et al., 2013).

TH antibody raised as a mouse monoclonal antibody against TH purified from PC12 cells recognizes an epitope in the catalytic core region of the TH molecule where extensive species homology exists. Western blots of HEK293 cells transfected with human TH probed with the antibody show one expected 60 kDa band (the manufacturer's information). The labeling pattern is identical to that seen in mouse brain in previous publications from the laboratory using identical conditions (DePuy et al., 2013).

VGLUT2 antibody raised in guinea pig against a peptide corresponding to the C-terminal of rat VGLUT2 recognizes one band of expected size on Western blots of rat brain lysate (from the manufacturer's information). Labeling is absent in terminals from neurons where VGLUT2 is eliminated by cre-mediated recombination with a floxed *VGLUT2* allele (Kaur et al., 2013). The labeling pattern is identical to that seen in mouse brain in previous publications from the laboratory using identical conditions (DePuy et al., 2013).

GFP antibody raised in chicken against recombinant GFP protein shows labeling only in tissue injected with viral vectors expressing eYFP. The antibody labeling matches exactly with nonamplified eYFP fluorescence.

DsRed antibody raised in rabbits against DsRed-Express, a variant of *Discosoma* sp. red fluorescent protein recognizes both N- and C-terminal fusion proteins containing dsRed variants in mammalian cell extracts (from the manufacturer's information). This antibody shows labeling only in tissue injected with viral vectors expressing mCherry or in tissue from tdTomato reporter mice. The antibody labeling matches exactly with nonamplified mCherry or tdTomato fluorescence.

Statistics. Statistical significance was set at $p < 0.05$. Data that passed the D'Agostino–Pearson test for normality are expressed as mean $\pm$ SEM, whereas non-normally distributed values are described by range and median. Degrees of freedom are listed for parametric tests. To compare two groups we used a paired t test or Wilcoxon signed rank test. To compare multiple groups, we used the Kruskal–Wallis test or, for repeated measures, the Friedman test followed by Dunn's test to assess intergroup differences. All statistics were done using GraphPad Prism software (version 6).

Figure 1. Selective expression of ChR2-eYFP in C1 cells. **A**, Cluster of ChR2-eYFP-positive RVLM neurons that were immunoreactive for both TH and PNMT and therefore qualified as C1 neurons. Scale bar, 30 μ m. This transverse section was located $\sim$ 6.6 mm caudal to bregma after Paxinos and Franklin, (2004). **B**, Rostrocaudal distribution of C1 (immunoreactive for both PNMT and TH; light pink symbols with gray lines and error bars; mean $\pm$ SE of six mice). Rostrocaudal distribution of ChR2-eYFP-positive C1 neurons (PNMT- and TH-ir; purple squares with purple lines and black error bars, average of three mice) and ChR2-eYFP-positive A1 neurons (TH-ir and eYFP-ir, but PNMT-negative; dark green with black lines and error bars, same three mice). Note that the percentage of C1 cells expressing ChR2-eYFP in the RVLM is much higher than expression of ChR2-eYFP in the TH-ir only (A1) neurons at more caudal levels. Abscissa indicates the level of the transverse sections in mm caudal to bregma after Paxinos and Franklin (2004). The location of the FN and that of the LRt are indicated for orientation (for LRt, starting point of arrow defines level where the anterior portion of this nucleus ends). Arrows indicate that these structures extend beyond the levels represented.

Results

Selective expression of ChR2 by RVLM catecholaminergic neurons

We examined the distribution of neurons with TH-immunoreactive (ir), PNMT-ir, or both in the VLM of 6 mice using a 1-in-3 series of transverse sections. As in rats, the PNMT-ir neurons (C1 neurons) were predominantly confined to the RVLM, namely between the caudal end of the facial motor nucleus (FN) and the rostral pole of the lateral reticular nucleus (LRt; Fig. 1). Caudal to the rostral pole of the LRt, the vast majority of the TH-ir cells lacked PNMT and were therefore A1 noradrenergic neurons. A small number of A1 (TH-positive and PNMT-negative) neurons were also present in the RVLM (Fig. 1B).

DIO-eF1 α -ChR2-eYFP AAV2 was injected into the left RVLM of three *DBH^{Cre/0}* mice. After 5 weeks, we mapped the location of the ChR2-eYFP-expressing neurons, also in a 1-in-3 series of transverse sections. These neurons were confined to the VLM. None were found in the dorsal medulla, the contralateral VLM or the pons consistent with the lack of retrograde propagation of AAV serotype 2. Almost all eYFP-positive neurons were TH-ir (respectively 99, 100, and 94% colocalization in three mice; Fig. 1A,B) and most (69, 83, and 70%, respectively) also contained PNMT immunoreactivity (Fig. 1A,B). In short, ChR2 was

selectively expressed by RVLM catecholaminergic neurons and 74% of those catecholaminergic cells were detectably PNMT-ir hence, by definition, C1 neurons (Hokfelt et al., 1974). The balance were TH-ir neurons without detectable PNMT, therefore presumably A1 cells.

ChR2-expressing RVLM catecholaminergic neurons innervate lower brainstem noradrenergic neurons

The axonal projections of ChR2-expressing RVLM catecholaminergic neurons were examined in two mice using the DIO-eF1 α -ChR2-mCherry AAV2 vector and in three mice injected with the DIO-eF1 α -eYFP AAV2 vector which also labels catecholaminergic neurons selectively ($>98\%$) when injected into the VLM of *DBH^{Cre/0}* mice (Abbott et al., 2013; DePuy et al., 2013). A dense network of mCherry-positive axonal varicosities covered the entire LC (Fig. 2A). Dense projections also covered the soma and dendrites of A1 and A2 neurons (Fig. 2B,C). Close appositions between eYFP-ir axonal varicosities and TH-ir somata or dendrites were commonly observed using light and immunofluorescence methods. Close appositions on A1 and A2 neurons from a 3D rendering are shown in Figure 2D,E.

Ultrastructural evidence for synapses between C1 cells and LC neurons was not sought because it has already been provided in rats (Milner et al., 1989; Abbott et al., 2012) and we obtained optogenetic evidence for monosynaptic connectivity between these cells (shown below). We therefore focused our ultrastructural studies on the A1 and A2 neurons. For these experiments, we used tissue from three DIO-eF1 α -ChR2-mCherry-AAV2 injected mice processed for simultaneous detection of mCherry and TH immunoreactivity.

Within the A1 noradrenergic neuron-rich region of the VLM (caudal to area postrema level) mCherry-ir (DAB-labeled) profiles consisted exclusively of unmyelinated axons or nerve terminals. Immunogold-silver labeling for TH was observed within perikarya, dendrites, and nerve terminals. A representative asymmetric synapse between an mCherry-ir varicosity and a TH-ir dendrite (A1 region) is shown in two serial sections in Figure 3A1,A2. A total of 78 mCherry-ir axonal profiles were observed within the A1 region. Of these, 63 were axonal varicosities that directly contacted dendrites. Fifty-one (80%) of these dendritic contacts showed asymmetric synaptic junctions and 12 (19%) were symmetric. The 15 other mCherry-ir profiles made close appositions but no detectable synapses.

Similar results were found in the A2 noradrenergic neuron-rich region of the nucleus of the solitary tract at and caudal to the level of the area postrema. mCherry-ir (DAB-labeled) profiles also consisted exclusively of unmyelinated axons or nerve terminals and immunogold-silver labeling for TH was observed within perikarya, dendrites, and nerve terminals. The four serial sections shown in Figure 3B1–B4 show a mCherry-ir fiber establishing two synaptic junctions with a TH-ir dendrite located in the A2 region. The synapse illustrated in Figure 3A1,A2 is a typical asymmetric synapse whereas the synapse in Figure 3B1,B2 is a perforated synapse, i.e., the cross-section of a synapse that appears as two postsynaptic densities separated by a small gap. Both presynaptic and postsynaptic profiles contained silver grains and therefore were TH-ir. We identified 79 synaptic contacts between mCherry-ir varicosities and TH-ir profiles, most of which ($N = 70$; 89%) were asymmetric and the rest symmetric ($N = 9$, 11%). Another 11 mCherry-ir synaptic varicosities made close ap-

positions with a TH-ir profile but no synapse was detected. Dense core vesicles (Fig. 3, examples marked with asterisks) consistent with catecholamine-releasing organelles were detected within the more lightly labeled mCherry-ir varicosities.

Photostimulation of Chr2-labeled C1 axons activates LC neurons monosynaptically in slices

Seventy-nine LC neurons were recorded in transverse slices from 15 mice (9 males, 6 females). The LC was identified as the medium-sized, high-neuron density population below the third ventricle and medial to the superior cerebellar peduncle and trigeminal mesencephalic nucleus (Fig. 4A). The dense innervation of LC from C1 AAV2 transfected neurons (seen as either eYFP or mCherry fluorescent terminals) could also be visualized in the slice (Fig. 4A) and helped to guide placement of the recording electrode. Seven LC neurons were recorded and filled with biocytin in slices prepared from three *DβH^{Cre/0}/ROSA26-tdTomato* mice. These neurons exhibited red (tdTomato) fluorescence, thus were catecholaminergic LC neurons. Thirty-two biocytin-filled neurons were recorded in slices prepared from 5 *DβH^{Cre/0}* mice previously injected with DIO-eF1α-ChR2-mCherry AAV2. These neurons were shown to be either TH-ir and/or surrounded by other LC neurons (Fig. 5B, left column).

Pulses of laser light (1 ms, 5 mW, 473 nm) produced PSCs in 53% (42/79) of the LC neurons sampled (Fig. 4B1). Occasionally, a laser pulse failed to evoke a PSC (Fig. 4B1, red traces; failure rate $19 \pm 4\%$, $N = 23$). The PSC amplitude, determined by event-triggered signal averaging, including failures, was 34.8 ± 5.0 pA (range 8–107 pA; $N = 23$; Fig. 4B2). Failure rates, determined by peri-event histograms (Fig. 4B3), were calculated for all photoresponsive LC neurons ($N = 23$). Failure rates from LC neurons shown to be monosynaptically innervated by RVLM CA neurons, based on the recovery of synaptic transmission after addition of 4-AP and TTX ($N = 9$, details below), never exceeded 39% and did not differ significantly from untested LC neurons (0–38.7%, median 20%, $N = 9$ vs 0–73.3%, median 6.7%; $N = 14$).

In 14 LC neurons, recordings were made with cesium-filled electrodes to enable voltage-clamp recordings at -80 and 0 mV (junction potential-corrected V_{Hold}). The EPSC evoked by the 0.5 Hz laser stimulation was greatly decreased in amplitude when the neurons were held at 0 mV (from 32.3 ± 6.9 – 1.4 ± 0.1 pA, $N = 12$, $p = 0.0005$, Wilcoxon-matched pairs) but always remained inward (Fig. 4B4). Thus, low-frequency photostimulation of the C1 axons does not produce chloride-mediated IPSCs in LC neurons.

Higher-frequency photostimulation (2, 5, 10 Hz for 10 s; K-gluconate-filled pipettes) produced sustained barrages of EPSCs in LC neurons (Fig. 4C1). The EPSC rate increased during the first 2–4 s of the train before reaching a steady-state (Fig. 4C1). The light-evoked EPSCs tended to be desynchronized from the light pulses when the stimulus was delivered at high-frequency (10 Hz; Fig. 4C2), and their rate largely ex-

Figure 2. Innervation of lower brainstem noradrenergic neurons by the C1 cells. **A**, mCherry-ir axonal varicosities (black, DAB, reaction product) cover the cell body-rich portion of the LC (light brown, DAB: TH-immunoreactivity) and the region medial to it where most LC dendrites reside. scp, Left superior cerebellar peduncle. **B**, Close appositions between mCherry-ir terminals (black, DAB) and A1 neurons (light brown, DAB: TH-immunoreactivity). **C**, Close appositions, i.e., putative synapses (arrows) between mCherry-ir terminals (black, DAB) and one A2 neuron (light brown, DAB: TH-immunoreactivity). **D**, **E**, Examples of synapse (arrow) between eYFP-ir terminal (green) and A1 neuron (TH-ir, magenta; **D**) or A2 neuron (TH-ir, magenta; **E**). **D1–D7**, **E1–E7** represent different angles of observation of the putative synaptic contact revealed through velocity 3D rendering software using deconvoluted images of either 57 (**D**) or 34 (**E**) serial $0.3 \mu\text{m}$ Z-stacks. The overlapping region of the two fluorophores is seen as a white band (arrow) and represents the putative contact zone. **D7** and **E4** are enlarged at the far right. Scale bars: **A**, $50 \mu\text{m}$; in **B**, **C**, $25 \mu\text{m}$; in **E1**, $1 \mu\text{m}$ for **D**, **E**; in enlargement for **E4**, $1 \mu\text{m}$ for enlargements of **D7** and **E4**.

ceeded the photostimulus rate (Fig. 4C1–C3). This result can probably be explained by the relatively slow kinetics of ChR2(H134R) which leads to a sustained depolarization and probable intracellular calcium build-up during high-frequency stimulation (Lin, 2011). The hypothesized intracellular calcium accumulation could explain the rapid but noninstantaneous return of EPSC frequency rate to base line values after the stimulus train ($\tau = 1.6$ ms; Fig. 4C3 and inset). In current-clamp, high-frequency photostimulation at 10 Hz reversibly activated LC neuronal firing from 1.9 ± 0.4 Hz to 3.1 ± 0.7 Hz ($N = 8$, $p = 0.019$, Friedman test; Fig. 4D).

Bath application of the broad-spectrum glutamatergic antagonist kynurenate (1 mM) reversibly decreased the amplitude of the postsynaptic current (4 cells, 67.0–82.1% attenuation, median 76.7%; $p = 0.0046$; Fig. 4E1,E2). To determine whether the excitatory C1 input to the LC was mono- or poly-synaptic, we blocked voltage-gated sodium (Na_v) channels with $1 \mu\text{M}$ TTX, then applied 100 – $200 \mu\text{M}$ 4-AP to block K_v channels (Shu et al., 2007). TTX eliminates action potential-dependent EPSCs, and thus eliminates any polysynaptic event, whereas 4-AP augments the light-induced, direct depolarization of ChR2-positive nerve terminals (Petreanu et al., 2009). TTX eliminated evoked EPSCs (Fig. 4F1,F2), indicating that these events were action-potential-dependent. The addition of 4-AP reinstated the EPSCs, though these events were typically broader (decay time constant, 20.5 ± 5.4 ms vs 10.0 ± 1.4 ms; $p = 0.0547$, Wilcoxon matched pairs), their onset latency delayed (14.1 ± 1.3 ms vs 5.1 ± 0.4 ms; $p = 0.0039$, Wilcoxon matched pairs), and their latency to peak longer (26.7 ± 0.3 ms vs 9.5 ± 0.7 ms; $\text{df} = 8$, $p = 0.006$, paired t test) than before drug application (Fig. 4F1). Evoked EPSCs

Figure 3. Ultrastructural evidence for synaptic contacts between putative C1 cells and A1/A2 catecholaminergic neurons. Electron micrographs display a number of asymmetric synapses formed between mCherry-ir terminals and catecholaminergic dendrites (TH-d) in adjacent sections. The terminal is identified by the presence of mCherry revealed by immunohistochemistry with a DAB reaction product (fine DAB precipitate) and the TH-d by black grains indicating the TH immunoreactivity revealed by a silver-gold reaction (silver-intensified immunogold particles, white arrows). The presynaptic terminal is also TH-ir as evidenced by the presence of immunogold reaction product (white arrows). **A1, A2**, Labeled A1 (TH-ir) neuronal dendrite shown in serial sections contains immunogold-silver particles (white arrows) and receives an asymmetric synapse (white rimmed arrows point to postsynaptic densities) from an mCherry-ir terminal. **B1–B4**, Four adjacent serial sections with labeled dendritic profiles forming one conventional synaptic junction (1) and a perforated synapse (2) from labeled terminals with small round vesicles and dense core vesicles (asterisks) in the A2 catecholaminergic cell group. The perforated synapse exhibits postsynaptic densities profiles (white rimmed arrows) that have a discontinuity. Synapses were also formed between unlabeled terminals with small clear round vesicles and TH-ir dendrites (example in **B3, B4** indicated by white arrowhead). Scale bar, 0.5 μ m.

were virtually eliminated by blocking ionotropic glutamatergic receptors with CNQX and AP5 (Fig. 4F1,F2).

In 11 LC neurons, high-frequency stimulation voltage-clamp experiments were done with cesium-filled electrodes. At -80 mV V_{HOLD} , spontaneous EPSCs were observed, and the response to 10 Hz trains of stimuli was identical to that recorded with potassium gluconate-filled electrodes. At 0 mV V_{HOLD} , however, IPSC frequency was very low and remained unchanged by the 10 Hz stimulus (Fig. 6A1,B). Thus, even high-frequency photostimulation of the C1 input did not pro-

duce chloride-mediated IPSCs in LC neurons. As previously, sIPSCs had much slower decay kinetics than sEPSCs ($N = 18$ and $N = 20$, 44.4 ± 4.1 ms vs 5.2 ± 0.4 ms; $p < 0.0001$, Mann–Whitney test; Fig. 6A2).

Optical stimulation of ChR2-expressing RVLM-CA neurons in brain slices

Whole-cell current and voltage-clamp recordings of ChR2-eYFP-positive RVLM neurons were made in slices from adult $D\beta H^{\text{Cre/0}}$ mice to verify that these cells could be activated by light pulses. Recorded neurons (11 neurons, 5 mice) were directly visualized by the presence of eYFP fluorescence. Shortly after patching, these cells were either silent or had a slow tonic discharge pattern (0.68 ± 0.23 Hz, 0–2.3 Hz, $N = 11$). Each light pulse produced a depolarization leading to a single action potential ~ 4 –8 ms after the onset of the light pulse (Fig. 7A,D). This long delay occurred despite the fact that the light-evoked current, observed in voltage-clamp, was instantaneous (Fig. 7B1, B2). The latency to the peak of the action potential (5.94 ± 0.51 , $N = 11$) was the same as the latency of the EPSCs evoked in LC neurons by photostimulating the ChR2-expressing axons of the RVLM CA neurons (5.0 ± 0.4 ms, $N = 23$, Mann–Whitney Test, NS; Fig. 7A,C,D).

All the recorded cells were filled with biocytin and, after histological processing, every recovered biocytin-positive neuron ($N = 6$) was found to contain both eYFP and tyrosine-hydroxylase immunoreactivity demonstrating that they were ChR2-expressing RVLM catecholaminergic neurons (Fig. 7E).

C1 rather than A1 neurons are the most probable source of the glutamatergic PSCs recorded in the LC

After AAV2 injection into the RVLM, $\sim 75\%$ of the ChR2-expressing catecholaminergic neurons were C1 (PNMT-ir), and the remainder were A1 neurons (TH-ir but PNMT-negative; Fig. 1). To determine which catecholaminergic cells (A1 or C1) were primarily responsible for the glutamatergic EPSCs elicited in LC neurons, ChR2-eYFP AAV2 was injected unilaterally (left side) into the caudal VLM, where no TH-ir neuron expresses PNMT. After 4 weeks, the LC was examined, and the proportion of eYFP-positive axonal varicosities that were also VGlut2-ir were counted in 1–3 coronal sections containing the LC per mouse. Injections resulted in eYFP-ir neuronal somata located no more rostral than 7.3 mm caudal to bregma, where few if any PNMT-ir neurons could be identified (Fig. 1B). For comparison, the same experiment was executed in three other mice in which the vector was injected into the RVLM and therefore predominantly

Figure 4. Monosynaptic glutamatergic input from C1 neurons to LC. **A**, Experimental design. The LC was identified in transverse slices by its location medial to the superior cerebellar peduncle (scp) and by the presence of a dense network of mCherry or eYFP fluorescent axons emanating from the C1 neurons (small red dots represent individual boutons originating from C1 neurons after Abbott et al., 2013). **B**, EPSCs evoked in LC neurons by low-frequency photostimulation of the axons of C1 cells (**B1**, single responses; red traces are failures). Blue vertical line or filled box represents the period of laser stimulation throughout the figure. **B2**, Average of 75 consecutive stimulations. **B3**, Event triggered histogram showing that 61 of 75 stimuli resulted in an EPSC; (**B1–B3**, same neuron). **B4**, Recording made with cesium-filled electrode at $V_{\text{hold}} = -85$ and 0 mV. Average of 75 stimuli. Note lack of outward current at $V_{\text{hold}} = 0$ mV. **C**, responses elicited by higher frequency photostimulation of C1 axons (2–10 Hz). **C1**, Representative example: bottom, original traces; top EPSC frequency binned every 0.5 s. **C2**, Expanded scale traces. Note that at 10 Hz, the evoked EPSCs are no longer synchronized with the light pulses. **C3**, EPSC frequency at rest, during the last 5 s of a 10 s, 10 Hz stimulation train and at three recovery times in 7 LC neurons (red horizontal lines indicate the median response; nonparametric ANOVA, Dunn's *post hoc* test; group identified by an asterisk is significant from all others at $p < 0.05$). **C3**, Inset, Blue box represents the mean EPSC frequency (per seconds) during the stimulation period and the black boxes represent the mean EPSC frequency binned in each 1 s interval following the end of the stimulus. Decay time constant was 1.6 s with a 95% interval of 1.1–2.8 s. **D**, Current-clamp recording: 10 Hz stimulation (1 ms pulses) more than doubles the firing rate of the neuron. **E**, Reversible attenuation of the evoked EPSC by 1 mM kynurenic acid. **E1**, Representative example. **E2**, Summary data from four neurons; red horizontal lines indicate the median response, black horizontal bars at top indicate $p < 0.05$ between control and kynurenic acid by Dunn's test following nonparametric ANOVA. **F**, Test for monosynaptic connection. **F1**, Representative experiment. Each trace is the average of 75 evoked responses. **F2**, Group data from nine neurons/six mice; red horizontal lines indicate the median response; black horizontal bars at top link groups that are significantly different at $p < 0.05$ using nonparametric ANOVA followed by Dunn's test.

labeled C1 cells (subset of the mice used for Fig. 1). eYFP fibers were present in the LC in both groups of animals; however, the proportion of eYFP varicosities that were also VGluT2-ir was much greater when the vector was injected into the C1 dense RVLM ($78\% \pm 1.5\%$; Fig. 8, example of double-labeled terminal) than when the vector was injected into the C1 sparse caudal VLM ($10.2 \pm 1.1\%$ double-labeled terminals). Thus, although the A1 cells do innervate the LC, few such cells express VGluT2. This suggests that the glutamatergic

Figure 5. Identification and location of recorded brainstem noradrenergic neurons. **A**, Identification of noradrenergic neurons in *DBH^{Cre/0}/ROSA26-tdTomato* mice. Fluorescence photomicrograph centered on the left dorsal vagal complex (transverse 30- μ m-thick section). Left, TH; Middle, tdTomato; Right, merged image. Middle, White-rimmed arrows show catecholaminergic tdTomato-labeled neurons. Right, White arrows show TH-ir neurons devoid of tdTomato fluorescence. This representative section contained 17 TH-ir neurons, 12 of which were positive for tdTomato. Some of the TH-ir neurons devoid of tdTomato could be dopaminergic. Scale bar, 100 μ m. **B**, Top left, Low-power photomicrograph showing a recorded LC neuron filled with biocytin (blue) and its location in the nucleus (LC neurons identified by TH immunoreactivity, green). Bottom, Higher-magnification showing more clearly that the recorded neuron was TH-ir. **B**, Middle, A1 neuron located in the caudal VLM, labeled with biocytin (top, blue) and identified as noradrenergic by the presence of tdTomato fluorescence (second from top, red). The neuron was surrounded by axonal processes labeled with ChR2-eYFP which originated from C1 neurons (green, third down). Bottom shows the merged image. **B**, Right, A2 neuron located in the nucleus of the solitary tract, filled with biocytin after recording (top, blue) and identified as noradrenergic by the presence of tdTomato fluorescence. The neuron is surrounded by axonal processes originating from C1 neurons (eYFP, green). Bottom, Merged image. Scale bars: **A**, 190 μ m in left column, 30 μ m in other panels; **B**, 500 μ m. **C**, location of the recorded noradrenergic (tdTomato-positive) A1 and A2 neurons (represented by blue-filled circles) recovered following histology plotted on representative sections that also illustrate the typical location of noradrenergic (TH + PNMT⁺ cells represented by red squares) and adrenergic neurons (PNMT⁺ cells represented by gray diamonds). Drawings are computer-assisted plots; numbers next to sections indicate the location of the transverse plane in relation to bregma after Paxinos and Franklin, (2004). IO, inferior olive; LRT, lateral reticular nucleus; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; 12, hypoglossal nucleus.

Figure 6. Photostimulation of C1 cell axons does not elicit IPSCs in LC neurons. Whole-cell recordings were obtained with cesium-filled electrodes in 11 neurons from five mice. Pulses of laser light (1 ms, 5mW, 10 Hz) were applied for 10 s (blue box). **A1**, EPSCs were elicited when the neuron was clamped at -80 mV but no detectable current was elicited at 0 mV. **A2**, High-resolution example of single EPSC recorded at -80 mV (left) and single IPSC recorded at 0 mV (right). The red lines are a single exponential fit of the PSC decay. EPSC decay 4.3 ms, IPSC decay 21.2 ms. **B**, Left, EPSC frequency before (Ctrl) during (stim) and after photostimulation at 10 Hz (rec). Red horizontal lines indicate the median response in both graphs. Asterisk indicates $p < 0.05$ relative to the control and recovery period (nonparametric ANOVA, Dunn's *post hoc* test). **B**, Right, IPSCs were very infrequent and their frequency was unaffected by photostimulation.

EPSCs elicited in LC neurons by photostimulation of axons from the catecholaminergic neurons located in the RVLM were most likely caused by activation of C1 cells. This interpretation assumes that the A1 neurons located in the RVLM have the same phenotype as those located in the caudal VLM.

Photostimulation of RVLM-CA neurons activates A1 and A2 neurons in slices

To visualize A1 and A2 neurons in live slices, we used adult *DBH^{Cre/0}/ROSA26-tdTomato* mice. Preliminary histological experiments performed in three such mice showed that tdTo-

mato was expressed by TH-ir neurons ($94 \pm 1\%$ in the A1 region of the VLM, $92 \pm 1\%$ in the nucleus of the solitary tract, i.e., A2/C2 region; Fig. 5A). Neurons immunoreactive for tdTomato and TH lacked PNMT in regions caudal to the rostral pole of the lateral reticular nucleus (Fig. 5C). Therefore, we targeted this region to record A1 neurons (location of biocytin-filled recorded neurons also shown in Fig. 5C). In the dorsal medulla, neurons immunoreactive for both tdTomato and TH lacked PNMT in regions caudal to the area postrema. These cells were therefore A2 neurons. We recorded preferentially from tdTomato-positive neurons located in this caudal region but a fraction of the recorded neurons could have been PNMT-ir C2 neurons (Fig. 5C). TdTomato was detected in $86 \pm 1\%$ of A1 and $66 \pm 13\%$ of A2 neurons, therefore the sampled neurons may not have been totally representative of this population of neurons.

For slice recordings, we injected DIO ChR2-eYFP AAV2 (8 mice) or DIO ChR2-mCherry AAV2 (4 mice) into the C1 region of *DBH^{Cre/0}/ROSA26-tdTomato* mice (6 males, 6 females). Five to 14 weeks later, 15 tdTomato-labeled A1 and 21 tdTomato-labeled A2 neurons were recorded in transverse medulla oblongata slices. Cells that received monosynaptic input from C1 neurons were filled with biocytin and processed histologically. We verified that the recorded neurons contained tdTomato and plotted the location of the recorded neurons (Fig. 5C). In some cases, the catecholaminergic nature of the recorded neurons was also verified by the presence of TH immunoreactivity in addition to the presence of tdTomato in the biocytin-labeled neuron (6 A1 neurons, 13 A2 neurons).

Low-frequency photostimulation (1 ms, 5mW, 0.5 Hz) elicited EPSCs in 60% (9/14) of A1 neurons sampled with a $36.3 \pm 11.7\%$ failure rate. Average EPSC amplitude (including failures) was 24.6 ± 10.5 pA. As in the LC, the EPSCs were virtually eliminated by the addition of TTX ($N = 5$ neurons; Fig. 9A1,A2), were reinstated after addition of 4-AP, and were again eliminated in the presence of CNQX and AP-5 (Fig. 9A1,A2). Responses of the same five A1 neurons to a high-frequency photostimulation (1 ms, 5mW, 10 Hz, 10 s train), applied in alternation with the periods of low-frequency stimulation, were also examined. The 10 Hz trains produced the same pattern of response as in the LC (compare Figs. 4C1 and 9B1), namely an increase in EPSC frequency that reached a steady-state within 5 s. As in the LC, the EPSCs elicited by high-frequency stimulation were largely random relative to the light pulses (not shown), and their steady-state frequency was several-fold higher than the stimulus frequency (2.5-fold in Fig. 9B1; range: 1.1- to 3.8-fold, median 2.8-fold; $N = 4$). TTX reduced but did not eliminate the EPSCs evoked by high-frequency stimulation (Fig. 9B1,B2), although EPSC frequency rose more slowly during the photostimulation than in control. Adding 4-AP increased the frequency of EPSCs evoked by the stimulus and changed the temporal pattern of response (Fig. 9B1,B2) such that the maximal EPSC frequency was

Figure 7. ChR2-mediated excitation of RVLm catecholaminergic neurons. **A**, Action potential elicited in a ChR2-expressing RVLm neuron by a 1 ms laser pulse (blue bar). Note the long latency of the action potential. Inset, Higher resolution shows that the slow depolarization begins rapidly after laser onset. **B1**, Voltage-clamp recording of the same neuron showing that the inward current generated by the laser pulse (0.1 ms long) is virtually instantaneous and decays exponentially (red line; $\tau = 23$ ms). **B2**, Higher resolution of the initial response showing that the inward current is instantaneous. **C**, Typical EPSC elicited in a LC neurons by photoactivating the severed axons of RVLm catecholaminergic neurons (1 ms pulse). **A** and **C** are aligned to emphasize the similarity between the latency of the action potential recorded at the level of a cell body and the latency of the EPSC evoked by stimulating the axons of the same cells. **D**, Group data (latency to peak of action potentials recorded from the somata of 11 C1 cells from five mice and EPSC latencies recorded in nine LC neurons from six mice). **E**, Example of a recorded ChR2-expressing RVLm catecholaminergic neuron (white arrow). Scale bar, 20 μ m. TH (red), eYFP (green), and biocytin (blue). Inset, Individual channels.

Figure 8. Terminals from RVLm catecholaminergic neurons in locus coeruleus are glutamatergic. **A1, B1**, Serial 0.3 μ m Z-stack sections of axonal varicosities from RVLm catecholaminergic neurons. **A1, B1**, eYFP immunoreactivity (green); **A2, B2**, VGLUT2 (magenta); **A3, B3**, merged image. Scale bar, 5 μ m.

elicited at the onset of the stimulus train and tended to decay thereafter. Following addition of CNQX and AP5, all observable PSCs were eliminated and the stimulus train produced no observable current (Fig. 9B1,B2).

Similar results were obtained with photostimulation of C1 terminals onto the A2 neurons. Low-frequency photostimulation (0.5 Hz) elicited EPSCs in 57% (12/21) of A2 cells sampled (amplitude 22.86 ± 5.9 pA) with occasional failures (failure rate, $27.5 \pm 8.8\%$; $n = 8$). The EPSCs were eliminated by the addition of tetrodotoxin, reinstated by addition of 4-AP, and again abolished after addition of CNQX and AP-5 ($N = 8$; Fig. 10A1,A2). The responses of A2 neu-

Figure 9. A1 neurons receive monosynaptic glutamatergic input from C1 neurons. **A**, Effect of TTX, 4-AP and glutamate receptor antagonists on the EPSCs elicited by low-frequency (0.5 Hz) photostimulation of the axons of RVLM catecholaminergic neurons. **A1**, Representative example; 0.5 Hz, 1 ms photostimulation at blue arrows, each trace is the average of 75 stimuli. **A2**, Group data from five neurons and four mice. Red horizontal lines indicate the median response; black horizontal bars at top of figure link groups that are significantly different at $p < 0.05$ determined using a nonparametric ANOVA with Dunn's *post hoc* test. **B**, Effect of TTX, 4-AP and glutamate receptor antagonists on the responses elicited by high-frequency photostimulation (10 Hz, 1 ms pulses; photostimulation periods represented by the blue boxes). **B1**, Representative example; bottom traces, original recordings, $V_{\text{hold}} = -85$ mV. **B2**, Group data; red horizontal lines indicate the median response; black horizontal bars at top of figure link groups that are significantly different at $p < 0.05$ determined using a nonparametric ANOVA with Dunn's *post hoc* test.

Figure 10. A2 neurons receive monosynaptic glutamatergic input from C1 neurons. **A**, effect of TTX, 4-AP and glutamate receptor antagonists on the EPSCs elicited by low-frequency (0.5 Hz) photostimulation of the axons of RVLM catecholaminergic neurons. **A1**, representative example; 0.5 Hz, 1 ms photostimulation at blue arrows, each trace is the average of 75 stimuli. **A2**, Group data from eight neurons and seven mice; red horizontal lines represent the median response; black horizontal bars at top of figure link groups that are significantly different at $p < 0.05$ determined using a nonparametric ANOVA with Dunn's *post hoc* test. **B**, effect of TTX, 4-AP and glutamate receptor antagonists on the responses elicited by high-frequency photostimulation of the axons of RVLM catecholaminergic neurons (10 Hz, 1 ms pulses; photostimulation period represented by the blue boxes). **B1**, Representative example; bottom traces, original recordings, $V_{\text{hold}} = -85$ mV. **B2**, Group data; red horizontal lines represent the median response; black horizontal bars at top of figure link groups that are significantly different at $p < 0.05$ determined using a nonparametric ANOVA with Dunn's *post hoc* test.

rons to 10 Hz, 10 s trains of 1 ms laser pulses were identical to those observed in A1 neurons. The high-frequency stimulus produced a gradual increase in PSC frequency that reached steady state within 3 s and were largely random with respect to the light stimuli (not

shown). The steady-state frequency of the evoked EPSCs was several fold higher than the stimulus frequency (4.5-fold in Fig. 10B1; range, 1.7- to 5.7-fold, median 3.4-fold, $N = 8$). TTX again reduced but did not eliminate the EPSCs (Fig. 10B1,B2), which were increased after

Figure 11. The C1 cells regulate peripheral and CNS noradrenaline release. C1 cells (green) directly innervate and activate sympathetic preganglionic neurons (black) plus every major group of CNS noradrenergic neurons (orange). The C1 cells thus have the capacity to activate noradrenergic release throughout the body and the brain under conditions such as, hypotension, hypoxia, etc. Basal f. brain, Basal forebrain; cereb, cerebrum; dienceph, diencephalon; hippo, hippocampus; olf, olfactory bulb.

addition of 4-AP and underwent a similar temporal change as described by A1 cells (Fig. 10*B1,B2*). The addition of CNQX eliminated all observable EPSCs before and during the photostimulus (Fig. 10*B1,B2*).

One catecholaminergic neuron, not included in the above description, was recorded in the intermediate reticular nucleus (Fig. 8*C*, asterisk), and therefore could not be classified as either A1 or A2. C1 axon photostimulation also elicited monosynaptic, glutamatergic EPSCs in this neuron.

Discussion

We show that RVLM catecholaminergic neurons, most likely C1 neurons, establish glutamatergic synapses with LC, A1 and A2 noradrenergic neurons. The C1 neurons activate the sympathetic nervous system in response to hypoglycemia, hypotension, pain, hypoxia, infection, and psychological stress (Ericsson et al., 1994; Jansen et al., 1995; Guyenet, 2006, 2013; Marina et al., 2011; Abbott et al., 2012). The C1 cells presumably also broadly increase CNS noradrenaline release under the same conditions (summarized in Fig. 11).

Glutamatergic activation of lower brainstem noradrenergic neurons by the C1 cells

After AAV injection into the RVLM, ChR2 was almost exclusively expressed by catecholaminergic neurons, 75% of which were TH- and PNMT-ir, thus C1 (Hokfelt et al., 1974). A similar proportion of ChR2-labeled axonal varicosities (78%) were VGluT2-ir whereas caudal VLM catecholaminergic neurons (A1 cells) had generally VGluT2-negative varicosities. Accordingly, C1 rather than A1 neurons were the principal source of the glutamatergic EPSCs observed presently. However, we confirm that A1 neurons also innervate the LC (Robertson et al., 2013) and find that ~10% of these cells may also express VGluT2 (Stornetta et al., 2002a). A subset of A1 cells may therefore be glutamatergic and may have contributed to the observed glutamatergic EPSCs along with C1 cells. Approximately 10% of C1 cells express the noradrenaline transporter, representing yet another example of a potential A1/C1 intermediate phenotype (Lorang et al., 1994; Comer et al., 1998).

C1 axon stimulation elicited EPSCs in a majority of LC (53%) and other noradrenergic neurons sampled (62%). These percentages could be underestimated because the number of ChR2-expressing varied between animals, as did the level of expression of ChR2.

The test used to determine whether the EPSCs evoked by C1 cell stimulation were monosynaptic was based on the effects of TTX and 4-AP (Petreanu et al., 2009). When short light pulses are used, ChR2-induced depolarization, hence calcium influx and vesicular release, is critically dependent on voltage-gated sodium current, and therefore blocked by TTX (Boyden et al., 2005; Zhang and Oertner, 2007; Petreanu et al., 2009). Addition of 4-AP reinstates transmitter release by facilitating the depolarization of the stimulated nerve terminals (Petreanu et al., 2009). The long EPSC latency observed without TTX (~5 ms) almost certainly reflects the gradual depolarizing action of ChR2, not the conduction velocity of the stimulated fibers. Indeed, ChR2-generated action potentials elicited by direct somatic illumination, antidromic axonal stimulation or monosynaptic transmission have very similar latencies in tissue slices although the inward current generated in ChR2-expressing cells is instantaneous (Petreanu et al., 2007, 2009; Zhang and Oertner, 2007). We verified that these assumptions apply to the C1 cells by showing that the latency to produce a spike in ChR2-expressing C1 somata was identical to the EPSC latency observed in the noradrenergic neurons by photostimulating the axons of the C1 cells. Under TTX and 4-AP, the latency of the EPSCs was longer still, likely reflecting that when fast sodium channels are inoperative, sufficient terminal depolarization to produce calcium influx is principally dependent on ChR2 and is delayed. In sum, the observed latencies were fully compatible with monosynaptic transmission.

The EPSCs elicited by stimulating C1 axons at 10 Hz had a frequency higher than the light pulses and tended to occur randomly. This characteristic likely indicates the gradual accumulation of intracellular calcium within the photostimulated axons rather than some form of polysynaptic transmission. Because of the relatively slow kinetics of ChR2(H134R), high-frequency stimulation produces a tonic depolarization. Cytoplasmic calcium rise probably also accounted for the brief persistence of EPSCs after a high-frequency photostimulus in the presence of TTX (Figs. 9*B1*, 10*B1*). The fact that asynchronous EPSCs could be elicited in the presence of TTX, albeit after a longer delay than without TTX (Figs. 9*B1*, 10*B1*) also suggests that they could be mediated by a presynaptic rise in intracellular calcium rather than by some form of polysynaptic transmission.

The EPSCs elicited by low or high-frequency stimulation in the presence of TTX and 4-AP were virtually eliminated by standard ionotropic glutamatergic receptor antagonists demonstrating their predominantly glutamatergic nature. This result agrees with the fact that ChR2-labeled catecholaminergic varicosities were VGluT2-ir and formed conventional excitatory-like synapses with the brainstem noradrenergic neurons examined. Neither C1 nor A1 cells express GABAergic or glycinergic markers (Comer et al., 1999; Schreierhofer et al., 1999; Stornetta and Guyenet, 1999; Stornetta et al., 2002a). In agreement, IPSCs were never evoked by C1 axon stimulation, even at high-frequency. Rat LC neurons may receive input from nearby GABA interneurons (Aston-Jones et al., 2004) but these interneurons are probably not targeted by C1 cells because C1 cell stimulation did not produce IPSCs in LC neurons.

Electrical stimulation of the RVLM produces a biphasic response in LC neurons *in vivo* (excitation-inhibition; Aston-Jones et al., 1992). The excitation was originally attributed to a noncatecholaminergic input and the inhibition supposedly due to C1 neurons. When rat ChR2-expressing RVLM catecholaminergic cells are selectively photoactivated *in vivo*, the LC response also consists of an excitation-inhibition sequence but both components are eliminated by the administration of a glutamate antag-

onist (Abbott et al., 2012). The inhibition could therefore be a polysynaptic effect or an intrinsic property of LC neurons. It could also result from catecholamine release by LC dendrites or by the A1 input to the LC (Egan et al., 1983; Williams et al., 1984; Huang et al., 2012).

In summary, the EPSCs evoked in LC, A1 and A2 neurons by photostimulating RVLM catecholaminergic neurons were glutamatergic, monosynaptic, and most probably originated from C1 rather than A1 cells. The electrophysiological evidence is consistent with the presence of VGluT2 in the terminals of RVLM catecholaminergic neurons and with ultrastructural evidence for synapses between these neurons and lower brainstem noradrenergic neurons.

Is the glutamatergic phenotype of the C1 cells an artifact caused by AAV2 transfection?

VGluT2 is downregulated postnatally in most CNS dopaminergic neurons and upregulated when dopaminergic neurons are lesioned or maintained in culture (Dal et al., 2008; Bérubé-Carrière et al., 2009; El Mestikawy et al., 2011). Exposure to AAV2 may reinstate VGluT2 expression in mature dopaminergic neurons (for review, see El Mestikawy et al., 2011). However, in adult rats never exposed to AAV, most C1 neurons express VGLUT2 mRNA and VGluT2 immunoreactivity is detectable in most PNMT-immunoreactive varicosities. In addition, in TH-Cre rats, the AAV2 vector used presently does not change the VGluT2 immunoreactivity present in C1 neuron axonal varicosities (Stornetta et al., 2002a; DePuy et al., 2013). For technical reasons we could not replicate this evidence in mice. However, unless VGluT2 up-regulation by AAV2 vectors is unique to the mouse and unless, among VLM catecholaminergic neurons it is unique to the RVLM, the glutamatergic component of C1 cell transmission observed in the present study is not an AAV-induced artifact.

Do the C1 cells release catecholamines?

The C1 cells express vesicular monoamine transporter-2 (Sevigny et al., 2008). They also synthesize numerous neuropeptides (for review, see Guyenet et al., 2013) and their terminals contain dense-core vesicles likely mediating the release of these substances (present data). Our failure to detect any residual synaptic event after glutamate receptor blockade, even during high-frequency stimulation, is consistent with our prior study on vagal motor neurons (DePuy et al., 2013) and likely means that our tests were not suited to detect responses mediated by peptides and catecholamines. In subcortical regions, noradrenaline often produces long-term potentiation or even metaplasticity (Neve-rova et al., 2007; Inoue et al., 2013). These phenomena develop over a much longer time period than the acute responses that we examined. Finally, the catecholamines released by the C1 cells could have limited effects on neurons and instead activate glia (O'Donnell et al., 2012).

Control of CNS noradrenergic neurons by the C1 cells: functional significance

The C1 cells are activated by hypoglycemia, hypotension, hypercapnia, infection, hypoxia, and pain, and contribute to the increased sympathetic tone and corticosterone release elicited by these stimuli (Madden and Sved, 2003; Abbott et al., 2009; Marina et al., 2011; Guyenet et al., 2013). These stimuli also activate LC neurons (Elam et al., 1981; Morilak et al., 1987a,b; Curtis et al., 1993; Murase et al., 1994; Guyenet et al., 2013). The present results suggest that the C1 cells likely relay these stimuli to LC, A1 and A2 noradrenergic neurons as well as to sympathetic pregan-

glionic neurons (Fig. 11). The A5 neurons, not examined in the present study, are excited by C1 neurons *in vivo* and are probably also directly innervated by these cells (Card et al., 2006; Abbott et al., 2012, 2013).

C1 cell activation by hypotension or by any of the above-mentioned stresses is therefore likely to produce widespread increases of CNS noradrenaline release with broad consequences on neuronal excitability, glial cell metabolism, and potentially, cerebral blood flow (Bekar et al., 2008; Attwell et al., 2010; Gordon et al., 2011).

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