Orexinergic activation of medullary premotor neurons modulates the adrenal sympathoexcitation to hypothalamic glucoprivation.

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Abstract

Glucoprivation activates neurons in the perifornical hypothalamus (PeH) and in the rostral ventrolateral medulla (RVLM), which results in release of adrenaline. The current study aimed to establish (i) whether neuroglucoprivation in the PeH or in the RVLM elicits adrenaline release in vivo; and (ii) whether direct activation by glucoprivation or orexin release in the RVLM modulates the adrenaline release. Neuroglucoprivation in the PeH or RVLM was elicited by microinjections of 2-deoxy-D-glucose or 5-thio-D-glucose in anesthetized, euglycemic, rats. We found that inhibition of neurons in the PeH abolished the increase in adrenal sympathetic nerve activity (ASNA) to systemic glucoprivation. Secondly, glucoprivation of neurons in the PeH increased ASNA. Thirdly, in vivo or in vitro glucoprivation did not affect the activity of RVLM adrenal premotor neurons. Finally, blockade of orexin receptors in the RVLM abolished the increase in ASNA to neuroglucoprivation in the PeH. The evoked changes in ASNA were directly correlated to levels of plasma metanephrine, but not to normetanephrine. These findings suggest that orexin release modulates the activation of adrenal presympathetic neurons in the RVLM.
**Introduction**

Glucoprivation is a metabolic challenge capable of eliciting adrenaline release, an important mechanism for restoration of normal blood sugar levels. Additionally, neuroglucoprivation produced by 2-deoxy-D-glucose (2-DG) is used as an experimental tool to study glucoregulatory neurons (1-4). Previous findings suggest that adrenaline release in response to glucoprivation involves activation of neurons in the perifornical hypothalamus (PeH) and rostral ventrolateral medulla (RVLM). Systemic glucoprivation using 2DG excites RVLM sympathetic premotor neurons (5; 6) and orexinergic neurons (7) in the PeH (8). Additionally, neurotropic viruses injected into the adrenal gland trans-synaptically label neurons in both the RVLM (9) and PeH (10). Disinhibition of perifornical neurons produces an increase in endogenous glucose production in the liver, which is mediated by the autonomic nervous system (11). However, it remains unknown as to whether intrinsic glucose sensitivity or projections from hypothalamic glucose-sensitive neurons (4; 12; 13) plays an important role in the excitation of RVLM adrenal premotor neurons in response to glucoprivation. In particular, if the responses evoked in RVLM neurons are modulated by orexinergic inputs (14; 15).

In this study, we hypothesize that perifornical hypothalamic neurons respond to neuroglucoprivation, and elicit adrenaline release by orexinergic activation of sympathetic premotor neurons in the RVLM. To test this hypothesis, we used a combination of *in vivo* and *in vitro* electrophysiological techniques to first examine the role played by neurons in the PeH in driving adrenal sympathetic nerve activity (ASNA). We then demonstrate for the first time that these effects are independent of any intrinsic
sensitivity of neurons in the RVLM to glucoprivation, and that the activation of orexin receptors in the RVLM modulates the adrenal sympathoexcitatory responses.
Methods

Experiments were performed according to the “Australian Code of Practice for the Care and Use of Animals for Scientific Purposes”. All experiments were approved by the Austin Health (2012/4764) and Macquarie University (2011/055) Animal Ethics Committees.

In vivo experiments:

General Procedures

Adult male Sprague-Dawley rats (250-350 g) were anesthetized with isoflurane (1.7% in 100% O₂). The left femoral vein and artery were cannulated for drug administration and arterial blood pressure (AP) recording, respectively. Body temperature was kept at 37±0.5 °C by a thermocouple-controlled heating pad. The rats were tracheostomized, paralyzed (pancuronium bromide; 1 mg*kg⁻¹, i.v.; supplemented by 0.1 mg*kg⁻¹*h⁻¹), and artificially ventilated with oxygen-enriched air (3.5 ml, 70 cycles*min⁻¹). Following completion of surgery, isoflurane was replaced by urethane (1.2 g*kg⁻¹; i.v.). The level of anesthesia was monitored by hindpaw pinch and the corneal reflex; urethane was supplemented (0.2 g*kg⁻¹ i.v.) as required. After neuromuscular blockade, anesthesia was maintained at a level in which paw pinch produced minimal changes in blood pressure (≤10 mmHg). Blood glucose was measured by withdrawing a drop of ar blood from the femoral artery and applied to a glucometer (Optium Xceed; Medisense; Abbott Laboratories, Bedford, USA), as previously described (5).
Adrenal sympathetic nerve recording

The right adrenal sympathetic nerve was prepared for recording via a retroperitoneal approach. Fibers emerging from the ganglion projecting towards the adrenal gland were carefully dissected free from connective tissue and fat. The fibers were tied together using 10-0 surgical nylon, cut distally, and mounted on bipolar silver wire electrodes. The nerve was covered with paraffin oil or embedded in a silicone elastomer (Kwik-Cast Sealant, WPI, Sarasota, FL, USA). Adrenal sympathetic nerve activity (ASNA) was amplified (x10000; Grass 7P5B, Quincy, MA, USA) filtered (100 Hz-3 kHz), and sampled at 6 kHz using a CED Power1401 (Cambridge Electronic Design, UK) with Spike2 v7.02 software. ASNA was rectified and integrated (τ=1s) prior to analysis. All neurograms were normalized with reference to the resting level prior to stimulus (100%) after subtraction of the noise (0%), determined post mortem or after clonidine (200 µg*kg⁻¹, i.v.; Sigma-Aldrich). Experiments were not included for analysis if the ratio of pre-/post- ganglionic ASNA was higher than 50%, verified by intravenous hexamethonium (40 mg*kg⁻¹, Sigma-Aldrich) at the end of the experiments.

Measurement of blood catecholamines

Due to the rapid degradation of catecholamines, we measured plasma levels of metanephrines (16). Plasma (0.2 ml) was extracted from blood (0.5 ml), withdrawn from the femoral arterial cannula, to determine the levels of metanephrine and normetanephrine. Plasma metanephrines were assayed by liquid chromatography tandem mass spectrometry, modified from the method of Whiting (17). Heparinized plasma samples had deuterated internal standards for each analyte that were added prior to solid phase extraction using weak cation exchange. Extracted samples were
evaporated to dryness, reconstituted and derivatised using cyanoborohydride and acetaldehyde prior to chromatographic separation and mass spectrometric detection using multiple reactions monitoring (model 6460 Agilent Technologies, Mulgrave, Australia).

**Location of the PeH and RVLM**

The perifornical hypothalamus (PeH) was located using stereotaxic coordinates \( (18) \). These were: 2.9-3.4 mm caudal to Bregma, 1.1-1.3 mm lateral to the midline, 8.6-8.7 mm ventral to the dorsal surface.

RVLM adrenal sympathetic premotor neurons are mingled with cardiovascular premotor neurons \((5)\). Hence, the RVLM was identified by extracellular recording of cardiovascular sympathetic premotor neurons, which were inhibited by phenylephrine \((10 \mu g^*kg^{-1}; \text{i.v.; Sigma-Aldrich - Supplementary Figure}) \) \((5; 19)\). These neurons were identified following antidromic field potential mapping of the facial nucleus, elicited by electrical stimulation \((0.5 \text{ Hz}, 0.1 \text{ ms}, 0.5-1.0 \text{ mA})\) of the facial nerve. Extracellular recordings were made using glass microelectrodes \((2 \text{ mm OD, 5-9 MΩ})\) filled with 2% Pontamine Sky Blue in 0.5 M sodium acetate. Extracellular potentials were recorded using a window discriminator and amplifier \((x10000; 400-4000 \text{ Hz; Fintronics, Orange, USA})\). RVLM sympathetic premotor neurons were found at: +0.1 rostral to -0.3 mm caudal, 0.1-0.3 mm medial, and 0.1-0.3 mm ventral to the caudal pole of the facial nucleus.

**Glucoprivation and Microinjections**

All experimental procedures were conducted following establishment of a euglycemic baseline \((4.8-7.0 \text{ mM; average: 6.1±0.1 mM, N=60})\). Systemic glucoprivation was
produced by 2DG (250 mg*kg⁻¹, i.v.; Sigma-Aldrich). Microinjections were performed using multibarrel micropipettes. All drugs were diluted in a solution of latex fluorescent beads 2% (Invitrogen) in artificial cerebrospinal fluid (aCSF (in mM): NaCl, 128; KCl, 2.6; NaH₂PO₄, 1.3; NaHCO₃, 2; CaCl₂, 1.3; MgCl₂, 0.9). All microinjections were 50 nl. Neuroglucoprivation was elicited by microinjections of 2DG (0.2-20 mM) or 5-thio-D-glucose (5TG; 0.6-600 mM; Sigma-Aldrich), using doses based on previous reports (2; 20). Perifornical neurons were permanently inhibited by the GABA_A agonist muscimol (4 mM; Sigma-Aldrich) or disinhibited by the GABA_A antagonist bicuculline (1 mM; Sigma-Aldrich). Note that these agents were used primarily to inhibit or activate hypothalamic neurons, and also to determine the role of their respective GABAergic inputs in glucose homeostasis. Orexin A (0.1-10 mM; Sigma-Aldrich) was microinjected into the RVLM using doses based on a previous study (21). Orexin receptors in the RVLM were blocked using the non-selective antagonist TCS1102 (5 mM; Tocris), diluted in 50% dimethyl sulfoxide (DMSO, Sigma-Aldrich) using a dose based on a previous report (22).

**Histology**

At the end of the experiments, animals were perfused with NaCl 0.9% w/v followed by 10 % formalin. Brains were removed, fixed in formalin overnight, and cut with a vibratome in 100 μm coronal sections. Sections were mounted onto gelatin-subbed slides for identification of the injection sites. Sections were examined under epi-fluorescence to locate the fluorescent bead deposits. The center of the injections were photographed (Sony DXC-9100P; Tokyo, Japan), and plotted (Supplementary Figure1) with reference to a rat brain atlas (18).
**In vitro experiments:**

**Voltage-clamp recordings from putative RVLM sympathetic premotor neurons**

Sprague Dawley rat pups (P5–P20) were anesthetized with 2-5% isoflurane (Veterinary Companies of Australia) in oxygen and moved onto a heated pad. A dorsal laminectomy was performed and the T2 spinal cord exposed. Fluorescently conjugated cholera toxin β subunit (CTB-Alexa 555, 0.5-1%, Invitrogen) was injected bilaterally at co-ordinates corresponding to the intermediolateral cell column (100 nl injections each side). After completion of microinjections the wound was closed with cyanoacrylate glue and anesthesia discontinued. Pups were allowed to recover on a warm pad until ambulatory and were then returned to the cage with their mother and littermates. Post-operative rats were carefully monitored and treated with additional analgesia when indicated (Carprofen, 2 mg/kg s.c. Norbrook pharmaceuticals, Australia).

**Whole cell recordings from RVLM medullospinal neurons**

Solutions (mM):

- **Cutting solution:** 118 NaCl, 25 NaHCO₃, 3 KCl, 1.2 NaH₂PO₄.H₂O, 10 D-glucose, 1.5 CaCl₂, 1 MgCl₂; equilibrated with 95 % O₂ – 5 % CO₂ (23).

- **aCSF:** 125 NaCl, 21 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄.H₂O, 2 D-glucose, 2 CaCl₂, 2 MgCl₂; equilibrated with 95 % O₂ – 5 % CO₂ (pH = 7.35).

- **Potassium gluconate internal solution:** 125 K-gluconate, 10 Hepes, 11 EGTA, 15 NaCl, 1 MgCl₂, 2 MgATP, 0.25 NaGTP, 0.05% biocytin (pH = 7.3, 280 < Osmolarity < 285 mOsm).
2 - 5 days after tracer microinjection pups were anaesthetized with isoflurane and quickly decapitated. The whole brain was quickly removed and dissected in ice cold oxygenated cutting solution; the brainstem was mounted in a vibratome and 300 µm thick coronal sections were cut under ice-cold carbogen-bubbled cutting solution. 3-4 sections from the region immediately caudal the facial nucleus were retained and transferred to carbogen-bubbled aCSF containing 2 mM glucose at 34 °C for at least 1h. Recordings were performed at room temperature in the recording chamber of an Olympus microscope superfused at 1.5–2 ml/min with carbogen-bubbled aCSF.

Tracer-labeled neurons were identified under epi-fluorescence: CTB-filled neurons lying ventral to nucleus ambiguus and lateral to the inferior olive were identified as RVLM putative sympathetic premotor neurons. Whole-cell recordings were made in voltage - or current-clamp modes using borosilicate pipettes with 1.5-2 µm tip diameters (3–6 MΩ). After formation of a gigaseal, recordings were obtained using a Multiclamp 700B patch clamp amplifier (Molecular Devices, USA). Baseline recordings were made for 300 seconds prior to 2DG administration. Series resistance compensation of 70 - 80 % was used in voltage clamp recordings. Recorded parameters were digitized using Spike2 with a Power 1401 mark II (Cambridge Electronic Design, UK). Data from 3 neurons recorded with the addition of 1 µM tetrodotoxin (TTX, Jomar Bioscience) to the aCSF were included in the dataset. At the conclusion of recordings the pipette was withdrawn and slices were fixed overnight in 4% paraformaldehyde and then frozen in cryoprotectant prior to immunohistochemical processing for biocytin and tyrosine hydroxylase immunoreactivity.
Immunohistochemistry

Sections containing biocytin-labelled neurons were removed from cryoprotectant, washed, and permeabilized in PBS with 0.5% Triton X-100 for 12 hours at 4 °C. The sections were incubated in blocking solution (5% bovine serum albumin, BSA, in PBS) for 4 hours at room temperature followed by incubation in mouse anti-tyrosine hydroxylase primary antibodies (TH - 1:2000, Sigma-Aldrich) for 4 hours at room temperature in 5% BSA. Sections were then washed and incubated in secondary antibodies (Cy5 Donkey anti-Mouse and ExtrAvidin FITC (both 1:500, Jackson Immunoresearch) with 5% BSA for 4 hours at room temperature, then washed, mounted and coverslipped. Sections were visualized and photographed using a Zeiss Z1 microscope (Carl Zeiss, USA), under epi-fluorescence with appropriate filter sets.

Data analysis

The effects of 2DG were assessed by comparing the holding current and the synaptic current frequency, averaged over 50 seconds prior to drug administration (baseline), to the mean over the last 50 seconds of drug perfusion (drug). The dose of 2DG (5 mM) was selected based on previous reports (4; 13).

Statistics

D’Agostino & Pearson omnibus test was performed to verify normal distribution of the data. Changes in ASNA are presented as mean±S.E.M., determined from a 60 second window average, compared along time. Student’s t-test, one-way ANOVA, and two-way ANOVA with the Bonferroni corrections were used for group comparisons. Correlations were determined by the Pearson or Spearman tests for parametric and non-parametric samples, respectively; with linear regression to determine confidence intervals. Data
that fit a normal distribution are presented as mean±SEM; non-parametric data are expressed as median (range). Statistical significance was determined when \( P<0.05 \). All tests were performed using the GraphPad Prism 5.0.

**Experimental protocols:**

1. ASNA was plotted against levels of circulating metanephrines to establish the relationship between nerve discharge and adrenaline release. Two samples were taken per experiment: during the resting condition when ASNA recordings had been stable for 10 minutes and \(~6-10\) minutes after intravenous injection of 2DG.

2. ASNA responses to intravenous 2DG were tested after microinjection of muscimol or bicuculline into the PeH to determine the role of GABAergic drive to perifornical neurons in adrenal sympathetic responses to glucoprivation. 2DG was also microinjected after bicuculline to determine its pharmacological effect in the absence of inhibitory tone to perifornical neurons. Lumbar sympathetic nerve activity (LSNA) and ASNA were recorded to determine if sympathetic responses to glucoprivation are differentially regulated.

3. The effects of focal PeH neuroglucoprivation on ASNA were determined by bilateral microinjections of 2DG or 5TG, according to previous reports (2; 20).

4. ASNA was compared before and after bilateral microinjections of 2DG into the RVLM to determine whether adrenal RVLM sympathetic premotor neurons were responsive to glucoprivation \textit{in vivo}. Subsequent intravenous injection of 2DG confirmed that the ASNA responses were not dependent on a direct effect on RVLM neurons.
5. The intrinsic sensitivity of RVLM sympathetic premotor neurons to glucoprivation was also tested in vitro. Following the establishment of stable recordings in aCSF containing 2 mM glucose slices were perfused for 300 s in aCSF containing 5 mM 2DG (4; 13). The effect of glucoprivation on membrane potential and spontaneous discharge frequency was assessed by comparing measurements made over the final 50 s of the control period to the final 50 s of 2DG application. Membrane resistance was monitored by measuring changes in membrane potential evoked by hyperpolarizing currents (-40 pA, 1 s) every 30 seconds and calculated using Ohm’s Law (4). The average membrane resistance measured over the final 3 steps of the control period was compared to data measured at the corresponding periods of 2DG administration. Neuronal excitability was assessed by comparing the number of action potentials generated by depolarizing current pulses (20 pA, 3 s) every 60 seconds (13). As described above data were averaged from the final 3 consecutive depolarizing steps in the control and 2DG periods. Voltage clamp ramps from 0 to -140 mV from a holding potential of -60 mV were performed to assess current-voltage relationships (4).

6. These experiments determined whether orexinergic activation of premotor neurons in the RVLM mediates the adrenal sympathoexcitaton to glucoprivation. Orexin receptors were activated using microinjections of orexin A at different doses before and after microinjection of the antagonist TCS1102. Adrenal sympathoexcitation in response to microinjection of 2DG into the PeH was also tested following microinjections of TCS1102 or vehicle into the RVLM.
Results

Correlation of ASNA and plasma metanephrines

At rest, the levels of blood glucose were 6.0±0.1 mM (N=8); systemic glucoprivation (2DG, 250 mg/kg) increased the concentration of plasma metanephrine (3.4±0.7 vs. 18.4±4.4 pmol*l⁻¹, P=0.008, N=8), a methylated metabolite of adrenaline, in direct proportion to the increase in ASNA (P<0.001, Spearman r =0.79, N=15; Fig. 1). In contrast, 2DG failed to change the levels of normetanephrine (49.1±9.9 vs. 44.3±5.6 pmol*l⁻¹, P=0.583, N=8), a methylated metabolite of noradrenaline. Hence, changes in the levels of normetanephrine were not correlated with ASNA (P=0.849, Spearman r =-0.05, N=15).

Role of perifornical neurons in driving sympathetic responses to glucoprivation

ASNA responses to systemic 2DG (250mg/kg) in intact rats were compared to those measured following inhibition of perifornical neurons with microinjections of muscimol (4 mM). Prior to 2DG administration, blood glucose was at 6.0±0.2 mM (N=14). Systemic glucoprivation increased ASNA (165±12%, P<0.001, N=17), which peaked at ~6 min (Fig. 2A&B). Bilateral microinjections of muscimol into the PeH abolished the ASNA increase to systemic 2DG (88±9%, P<0.001, N=6; Fig. 2C&D). By contrast, following establishment of a stable glucose baseline (6.0±0.2 mM; N=8) unilateral microinjection of bicuculline (1 mM) into the PeH increased ASNA (199±14%, P<0.001, N=8) while subsequent microinjection of 2DG reduced ASNA (143±12%, P<0.001, N=8; Fig. 2E&F). Systemic glucoprivation (2DG; 250mg/kg) selectively increased ASNA (162±9%, P<0.001, N=6), but did not affect LSNA (101±6%, P=0.22, N=6). By contrast,
elevation of blood pressure (phenylephrine, 10 µg·kg⁻¹) or blockade of sympathetic ganglionic transmission (hexamethonium, 40 mg·kg⁻¹) reduced only LSNA (Fig. 2G&H).

**Effects of neuroglucoprivation in the PeH**

Perifornical focal microinjection of 2DG or 5TG evoked adrenal sympathoexcitation (Fig. 3). Resting levels of blood glucose prior to 2DG and 5TG administration were 6.6±0.3 mM (N=10) and 6.7±0.1 mM (N=6), respectively. Bilateral microinjections of 2DG into the PeH (20) dose-dependently augmented ASNA (175±10%, \( P<0.001 \), N=10). Bilateral 5TG also increased ASNA (145±11%, \( P<0.01 \), N=6). The increases in ASNA in response to either 2DG or 5TG were similar in magnitude (\( P>0.05 \), N=6), and correlated with the increases in arterial blood glucose (Fig. 3D&F).

**Glucoprivation of RVLM sympathetic premotor neurons in vivo**

At a blood glucose baseline of 6.4±0.2 mM (N=6), bilateral microinjections of 2DG (2 mM) into the RVLM evoked no effect on ASNA (90±12%, \( P=0.33 \), N=6; Fig. 4). Subsequent systemic injection of 2DG (250 mg/kg; i.v.) increased ASNA (162±18%, \( P<0.001 \), N=6).

**Glucoprivation of RVLM sympathetic premotor neurons in vitro**

16 sympathetic premotor neurons were recorded in 11 brainstem slices from 5 rats (Fig. 5). In all but three cases current- and voltage-clamp data were obtained from the same neurons. In no case did 2DG evoke any clear effect on any parameter recorded. In current clamp the resting membrane potential was -52.7±1.6 mV (N=15 including 3 neurons recorded with TTX), with spontaneous action potentials occurring at 3.7±0.8 Hz (N=12). At the end of the 2DG superfusion the membrane potential (-53.3±1.6 mV,
$P=0.55$, $N=15$), spontaneous discharge frequency ($3.6\pm0.8$ Hz, $P=0.46$, $N=12$) and input resistance ($335\pm38$ vs $327\pm41$M$\Omega$, $P=0.35$, $N=14$) were unchanged from baseline values. There was no significant change in the number of action potentials evoked by depolarizing current pulses by addition of 2DG to the perfusate ($11.8\pm1.7$ vs $10.9\pm1.6$ spikes, $P=0.48$, $N=12$, Fig. 5D). In voltage clamp mode no changes in holding current ($-54.4\pm7.5$ vs $-55.9\pm7.4$ pA, $P=0.54$, $N=13$) or response to voltage ramps were noted following addition of 2DG to the perfusion fluid.

**Blockade of orexin receptors in the RVLM during neuroglucoprivation of the PeH**

Microinjection of orexin A into the RVLM produced an increase in ASNA ($162\pm16\%$, $P<0.001$, $N=6$) that was blocked by the non-selective antagonist TCS 1102 ($99\pm3\%$, $P<0.001$, $N=6$; Fig. 6A-D). Bilateral microinjections of 2DG (2 mM) into the PeH increased ASNA ($151\pm16\%$, $P<0.01$, $N=6$), following microinjections of vehicle into the RVLM. By contrast, TCS 1102 in the RVLM abolished the increase in ASNA ($95\pm5\%$, $P<0.001$, $N=6$) produced by microinjection of 2DG into the PeH (Fig. 6E-G).
Discussion

The principal finding in this study is that orexin modulates the activity of RVLM adrenal sympathetic premotor neurons resulting in excitation of adrenal chromaffin cells. We showed that local glucoprivation or disinhibition of PeH neurons increased ASNA while inhibition of PeH neurons abolished the ASNA response following systemic glucoprivation. Conversely, glucoprivation of perifornical neurons subsequent to activation by the GABA_A agonist bicuculline reduced the adrenal sympathoexcitatory response. In addition, local neuroglucoprivation in the RVLM failed to activate premotor neurons in vivo or in vitro suggesting that RVLM neurons are not intrinsically glucose sensitive. Finally, ASNA was directly correlated with plasma metanephrine levels but not normetanephrine levels confirming that adrenal sympathoexcitation coincides with adrenaline release into the circulation. The ASNA, noradrenaline, and adrenaline responses to glucoprivation noted in our study were consistent with previous reports of the effects of glucoprivation on sympathetic preganglionic neurons (24).

In this study, microinjection of 2DG/5TG or bicuculline into the PeH increased ASNA, whereas microinjection of the GABA_A agonist muscimol into the PeH abolished the ASNA response to systemic injection of 2DG. Reports by others have shown that 2DG exerts a glucomimetic inhibition of orexinergic and GABAergic perifornical neurons (4; 13; 25). Thus, direct excitation of perifornical neurons by 2DG in our study is unlikely to be the mechanism underlying the increase in ASNA. Alternatively, adrenal sympathoexcitation could result from disinhibition of perifornical neurons that receive GABAergic drive (11). Orexinergic neurons express GABA receptors (26), and may receive inhibitory inputs from adjacent interneurons (25) or from the ventromedial
hypothalamus (27; 28). In our study, microinjection of 2DG into the PeH decreased the ASNA response evoked by prior administration of bicuculline into the same site, confirming the glucomimetic inhibitory effect of 2DG seen in vitro (4; 13; 25). One interpretation of this result is that 2DG acts at some location adjacent to the PeH. If so, this could explain the onset (~1min) of the ASNA response to microinjection of 2DG into the PeH. Consistent with this notion, are previous observations that injection of 2DG into the ventromedial hypothalamus (1) or into the ventrolateral portion of the lateral hypothalamus (29) elicits glucoprivic effects resulting in adrenaline release and adrenal sympathoexcitation, respectively. While we have demonstrated that 2DG can exert inhibitory effects on PeH neurons, consistent with previous observations in vitro (4; 13; 25), the inevitable conclusion is that in our in vivo study an excitatory response predominates.

Blockade of orexin receptors in the RVLM by microinjection of TCS1102 eliminated the adrenal sympathoexcitatory response to injections of 2DG into the PeH. The dose of the orexin antagonist used was sufficient to block the effects of microinjection of orexin into the RVLM on ASNA. Based on the density of the extracellular milieu (30) and histology, our injections extended for ~400 µm, and so targeted the majority of C1 neurons (6). The ASNA response to microinjection of orexin into the RVLM concurs with previous observations (21). Glucoprivation activates slow-conducting (<1 m/s) RVLM adrenal premotor neurons, which are intermingled with the cardiovascular premotor neurons (5). The slow-conducting axons suggest that they are C1 catecholaminergic cells (31). Glucoprivation also elicits Fos expression (6) and phosphorylation (32) in C1 neurons. Orexinergic neurons project to the C1 region of the RVLM (14; 15) and their terminals
make close appositions with C1 neurons (33). Moreover, neurotoxic ablation of C1 neurons eliminates the glucoregulatory response to 2DG (34). Together, the evidence suggests that orexinergic activation of adrenal sympathetic premotor neurons modulates the adrenal sympathoexcitatory response to glucoprivation. Although previous studies (35; 36) have shown that selective glucoprivation of hindbrain neurons increases blood glucose; local application of 2DG failed to activate the RVLM adrenal premotor neurons. Thus, hindbrain glucose-sensitive neurons (6; 37) are presumably located outside the RVLM, but project to (38) and excite the adrenal C1 neurons.

The present study has explored the neural pathway(s) that relay the adrenal sympathoexcitatory response to neuroglucoprivation. We used 2DG as a glucoprivic agent because it allows the investigator to produce localized glucoprivation when injected into the brain parenchyma. Importantly, systemic 2DG produces secretion of adrenaline, glucagon, cortisol, and growth hormone (39; 40). Since 2DG is also detected by most glucometers, we were unable to determine blood glucose changes after systemic 2DG. Nonetheless, glucoprivation elicits hyperglycemia via activation of glycogenolysis and gluconeogenesis in the liver (3; 11; 41). General anesthesia was essential for measurement of ASNA and eliminated the influence of stress, respiration, or body temperature (42-44). Anesthesia can alter neural metabolism and modulate glycemia and it is known that intraperitoneal urethane causes hyperglycemia (45). However, under the conditions of our experiment we found that urethane produced normoglycemic animals (~6.1 mM). Comparison of different methods for determining catecholamine levels indicated that plasma metanephrines determined by mass spectrometry is the most reliable method (16). Finally, the age of rat pups used the in
vitro experiments correspond to previous electrophysiological studies (46) and the catecholaminergic neurons are likely to be mature and functional (47).

In conclusion, our findings suggest a key role for orexin in modulating the sympathetic drive to the adrenal chromaffin cells during glucoprivation. It is possible that during arousal orexin changes the electrophysiological properties of adrenal premotor neurons facilitating adrenaline release in response to glucopenia – a mechanism that may be compromised when hypoglycemia unawareness develops in response to recurrent glucoprivation (3).

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Legends

Figure 1. Glucoprivation elicits increases in ASNA correlated with the levels of metanephrines

Systemic injection of 2DG increased adrenal sympathetic nerve activity (ASNA). The increase in ASNA correlated with plasma levels of metanephrine, a methylated metabolite of adrenaline. However, 2DG failed to alter the levels of normetanephrine, the corresponding methylated metabolite of noradrenaline. All data are presented as mean ± S.E.M. ** P<0.01; n.s. non-significant.

Figure 2. Selective effects of glucoprivation on ASNA depends on perifornical neurons

A. Neurograms of adrenal sympathetic nerve activity (ASNA), top: arbitrary units (a.u.) bottom: rectified, integrated, and normalized to % of baseline. Systemic glucoprivation with 2-deoxy-D-glucose (2DG) increased ASNA, which was abolished by bilateral microinjections of muscimol into the perifornical hypothalamus (PeH). B. Pooled increases in ASNA following 2DG. C. Group data of the response to 2DG after inhibition of the PeH. D. Muscimol in the PeH reduced the maximum increase in ASNA to systemic 2DG in the group. E. Microinjection of bicuculline into the PeH increased ASNA, and subsequent microinjection of 2DG reduced the evoked increase in ASNA. F. Group data of maximum ASNA increases to microinjection of bicuculline and 2DG into the PeH. G. Systemic 2DG increased only ASNA, but did not affect lumbar sympathetic nerve activity (LSNA). H. The differential sympathetic response to 2DG replicated within the group. All data are presented as mean ± S.E.M. * P<0.05, ** P<0.01, *** P<0.001 to
baseline; ++ $P<0.01$, +++ $P<0.001$ to control group; n.s. non significant. AP, arterial blood pressure.

Figure 3. Glucoprivation in the PeH increases ASNA

A. Neurograms of raw and rectified, smoothed, and normalized adrenal sympathetic nerve activity (ASNA). Bilateral microinjections of 2-deoxy-D-glucose (2DG) into the periformal hypothalamus (PeH) elicited dose-dependent increases in ASNA. B. Bilateral microinjections of 5-thio-D-glucose (5TG) into the PeH also augmented ASNA. C. Group data of sympathetic responses to microinjections of 2DG and 5TG. D. Pooled data of maximum increases in ASNA to 2DG or 5TG into the PeH. E. Increases in ASNA in response to microinjections of 2DG and 5TG into the PeH were directly correlated with levels of blood glucose. All data are presented as mean ± S.E.M. ** $P<0.01$, *** $P<0.001$ to baseline; +++ $P<0.001$ to 2DG (2 mM); n.s. non-significant. aCSF, artificial cerebrospinal fluid.

Figure 4. RVLM neurons are not glucose-sensitive in vivo

A. Neurograms of raw and rectified, smoothed, and normalized (% of baseline) adrenal sympathetic nerve activity (ASNA). Bilateral microinjections of 2-deoxy-D-glucose (2DG) into the rostral ventrolateral medulla (RVLM), where premotor neurons are found, did not alter ASNA. However, subsequent intravenous injection of 2DG in the same animal increased ASNA. B. Group data of ASNA responses to microinjections of 2DG into the RVLM, followed by systemic 2DG. All data are presented as mean ± S.E.M. + $P<0.05$, ++ $P<0.01$, +++ $P<0.001$ to 2DG into the RVLM; n.s. non significant.
Figure 5. 2DG exerts no direct effect on medullospinal RVLM neurons recorded in vitro

A. Whole-cell current clamp recording performed in the presence of TTX: bath application of 2DG exerts no effect on resting membrane potential or resistance. Deflections indicate responses to current injection. B1. Current clamp recording showing effect of 2DG on spontaneous discharge of medullospinal RVLM neuron. Regular increases in firing frequency indicate responses to depolarizing current injections. Breaks in recordings in panels A and B1 indicate recording mode switch. B2. Raw data excerpts at points denoted (arrows) in panel B1. C. 2DG exerted no effect on current-voltage relationships recorded in voltage clamp mode. D. 2DG exerted no effect on responsiveness to depolarizing currents (see also panel B1). E. In some cases neurons were filled with biocytin during recording and subsequently examined histologically. i. Low power photomicrograph showing distribution of CTB labeling. Field of view of high powered images denoted by box. ii. Two neurons were recorded and recovered in the experiment shown; raw data from each is shown in panels C & D. Both were CTB-labeled (iii) and under close examination C was lightly TH-positive whereas D was TH-negative (iv). v. Schematic diagram showing locations of recorded neurons.

Figure 6. Orexin in the RVLM mediates the ASNA increase to glucoprivation.

A. Neurograms of rectified, smoothed, and normalized (% of baseline) adrenal sympathetic nerve activity (ASNA). Microinjection of orexin A (OxA) into the rostral ventrolateral medulla (RVLM) produced sympathoexcitation, which was blocked by the non-selective antagonist TCS1102. B. The blockade of the response replicated in a
group of animals. C. Neurograms of raw ASNA. OxA into the RVLM evoked a dose-dependent increase in ASNA that was antagonized by TCS1102. D. The dose-response effect replicated in a group of animals. E. Neurograms of raw (Top) and rectified, smoothed, and normalized (Bottom) ASNA. Bilateral microinjections of 2DG into the perifornical hypothalamus (PeH), following vehicle (50% dimethyl sulfoxide; DMSO) microinjection into the rostral ventrolateral medulla (RVLM), increased ASNA. However, bilateral injections of the antagonist TCS1102 into the RVLM abolished the rise elicited by 2DG in the PeH. F. Blockade of the ASNA response replicated in a group of animals. G. Grouped data of maximum increases in ASNA to 2DG in the PeH, subsequent to DMSO or TCS1102 in the RVLM. All data are presented as mean ± S.E.M. ** P<0.01, *** P<0.001 to baseline; + P<0.05, +++ P<0.001 to control group; ++ P<0.01 to OxA (1mM); n.s. non-significant.
Figure 1. Glucoprivation elicits increases in ASNA correlated with the levels of metanephrines. Systemic injection of 2DG increased adrenal sympathetic nerve activity (ASNA). The increase in ASNA correlated with plasma levels of metanephrine, a methylated metabolite of adrenaline. However, 2DG failed to alter the levels of normetanephrine, the corresponding methylated metabolite of noradrenaline. All data are presented as mean ± S.E.M. ** P<0.01; n.s. non-significant.

118x99mm (300 x 300 DPI)
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(1mM); n.s. non-significant.

196x276mm (300 x 300 DPI)
Supplementary Figure 1

A. Coronal photomicrograph of the perifornical hypothalamus (PeH) showing a deposit of fluorescent beads as a result of a microinjection. Microinjection sites were of muscimol, bicuculline, 2-deoxy-D-glucose (2DG), and 5-thio-D-glucose (5TG) have been plotted in schematic drawing taken for the Paxino & Watson rat brain atlas. B. Recording of a rostral ventrolateral medulla (RVLM) neuron inhibited by baroreceptor loading, photomicrograph of an RVLM coronal slice, and a schematic drawing showing the distribution of injection sites. C. Distribution of orexin A (OxA) and TCS1102 injections at the level of the RVLM. The right hand panel depicts the 2DG injection sites in the PeH, and dimethyl sulfoxide (DMSO) or TCS1102 in the RVLM. Abbreviations: mt, mammillothalamic tract; fx, fornix; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; LH, lateral hypothalamus; V, trigeminal nucleus; Amb, nucleus ambiguous; Py, pyramidal tract.

233x342mm (300 x 300 DPI)
Supplementary Figure 2

Diagram showing the neural connections activated by systemic injection or local microinjections of 2-deoxy-D-glucose (2DG) and 5-thio-D-glucose (5TG). Note that in the hypothalamus, these compounds may exhibit glucomimetic or glucoprivic effects on different subsets of neurons.

98x65mm (300 x 300 DPI)