ONLINE DATA SUPPLEMENT

Unilateral ablation of the preBötzinger Complex disrupts breathing during sleep but not wakefulness

Leanne C. McKay and Jack L. Feldman

Methods

Surgical Procedures

All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles (protocol #1994-159-31E). Male Sprague-Dawley rats (*n*=12, 250-350g) were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) injected intraperitoneally, and if required Isoflurane™ (1-2%; Abbott Laboratories, North Chicago, IL, USA) in 100% O_2 during surgery. Pairs of electrodes made from insulated stainless steel wire (Cooner Wire, Chatsworth, CA, USA) with the last 2 mm uninsulated, were implanted to record: diaphragm electromyography (EMG); neck EMG; electrocardiogram (ECG); and electroencephalography (EEG; electrodes screwed into the skull, one over the frontal cortex: 2 mm anterior to bregma and 2 mm to the right of midline; a second over the parietal cortex: 3 mm posterior to bregma and 2 mm to the left of midline; and a third ground electrode). The electrode wires were tunnelled subcutaneously to a 12-pin socket secured with a skin baton between the shoulder blades.

Two weeks later a second surgery was performed to stereotaxically inject either the toxin SP-SAP (*n*=9) or SP mixed with but not conjugated to SAP (unconjugated, control, *n*=3), unilaterally into the preBötC. Bregma was positioned 5 mm below lambda, the dorsal surface of the brainstem exposed and the position of the preBötC identified (0.9 mm rostral, 2 mm lateral and 2.7 mm ventral to the calamus scriptorius (obex)). Microinjections from a glass capillary tube with a 40 µm tip diameter of SP-SAP (100-150 nl; 6.7 ng; Advanced Targeting Systems, San Diego, CA, USA) were made into the

left preBötC, resulting in a sigh and a slight, transient increase in respiratory frequency. A second microinjection was made just caudal to the first injection, eliciting a similar response. SP-SAP, but not SAP, is internalized by NKIR expressing neurons, whereupon SAP inhibits ribosomal protein synthesis causing cell death (1, 2). The electrodes were left in place for 5 minutes after each injection to minimize backflow of the toxin up the electrode track. To identify the injection site, fluorescent microspheres (Molecular Probes, Carlsbad, CA, USA) were added to the toxin solution. Post-operatively, rats received buprenorphine analgesic (0.1 mg/kg i.p., followed by 3 more injections at 12 hour intervals) and Cefipime antibiotic (6 mg/kg i.p.) for 5 days. For the first 2 days post-injection, rats appeared lethargic, with shallow breathing.

Experimental Protocol

Rats were housed in a room with a 12 hour light/dark cycle (light period from 09h30 – 21h30). During periods of data collection, rats were monitored within a plethysmograph, allowing the rats to move freely, with food and water available *ad libitum.* Post-injection, data were acquired for 9 hours/day (between 10h00 - 19h00) during the light cycle. Rats were constantly monitored for any weight loss or infection; rats showing such signs were euthanized by a fatal injection of pentobarbitone (80 mg/kg i.p.). Chemosensitivity was tested prior to SP-SAP injection and on every third day after injection (hypercapnia: 5% CO_2, 21% O_2, 74% N_2; hypoxia: 10% O_2, 90% N_2).

Data acquisition and analysis

Data were acquired using Chart 5 software (Powerlab 16SP, AD Instruments, Colorado Springs, CO, USA), amplified (Grass Model P511K, Grass Instrument Co., West Warwick, RI, USA and Animal Bioamp ML136, AD Instruments), filtered (EEG: 0.3-100 Hz, EMG: 10-100 Hz, ECG: 10-100 Hz) and sampled at 1000 Hz. Data acquired within a 2 hour period (between 12h00 and 18h00) every day post injection were analyzed.

Sleep and wakefulness were determined from visual inspection, and analysis of neck EMG activity and the fast Fourier transform of the EEG signal in 10 s epochs at delta (0.3-5 Hz), theta (6-9 Hz) and sigma (10-15 Hz) frequency bands, based on the criteria defined by Benington (3). In the awake state the EEG is a low amplitude, high frequency signal, low delta power, low theta*sigma power and high EMG activity; in NREM sleep the EEG signal is a high amplitude, low frequency signal, high delta power, moderate theta*sigma power and low EMG activity; in REM sleep the EEG is a low amplitude, high frequency signal, low delta power, high theta*sigma power and low to absent EMG activity. Movement distorted the signals and prevented reliable measurements; only data when the rat was asleep and in quiet wakefulness were analyzed.

Changes in breathing frequency and amplitude were measured on a breath-by-breath basis using whole body plethysmography (Buxco Electronics Inc., New York, NY, USA). A constant airflow (2 l/min) was delivered through the plethysmographic chamber and respiratory parameters quantified by recording pressure fluctuations, relative to a

reference chamber, that are proportional to tidal volume (differential pressure transducer, DRAL501, Honeywell Data Instruments, Golden Valley, MN, USA). Inspiratory amplitude (I_{amp}) was calculated from the plethysmographic pressure signal output (in millivolts); control (pre-injection) data were normalized to 1 and all data acquired on subsequent days were rescaled relative to control. Spontaneous respiratory disturbances were defined as hypopnea when inspiratory amplitude <0.5 of normalized control values (a.u.) and an apnea when termination of breathing >2s (~3 missed breaths). Respiratory disturbances are expressed as number of episodes normalized to 1 hour each of wakefulness (WAKE), NREM, and REM. All data are expressed as mean±S.E.M. Statistical analyses were performed using a two-way repeated measures analysis of variance (ANOVA), with post hoc Bonferoni analysis for individual comparisons (StatView software, Abacus Concepts, Berkeley, CA, USA). Differences were regarded as significant if $p<0.05$.

Histology

Rats were transcardially perfused (between days 21-51 post injection) with 4% paraformaldehyde. The brain was removed, post fixed in 4% paraformaldehyde for 4 hours, cryoprotected in 25% sucrose for 24 hours, sectioned at 40 µm using a cryostat and underwent free-floating immunohistochemistry procedures. Sections were washed in phosphate buffered saline (PBS), transferred to 3% normal goat serum and then incubated for 24 hours at 4°C with one or two of the following primary antibodies: rabbit anti-NK1R (1:20,000, Chemicon, Temecula, CA, USA); goat anti-choline acetyl transferase (ChAT 1:400, Chemicon) and mouse anti-tyrosine hydroxylase (1:500, Chemicon). The

sections were then washed in PBS and incubated in species-specific fluorescent secondary antibodies for 2 hours. Some sections were placed in a biotin conjugated species-specific secondary antibody and stained using ABC and di-aminobenzidine (Vector Laboratories, Burlingame, CA, USA). All sections were mounted in sequential order on gelatin subbed slides. Under light microscopy (Zeiss, Axioplan2, Carl Zeiss, Thornwood, NY, USA) the extent of the lesion was determined by counting NKIR immunopositive soma in a series of transverse sections, beginning with the section in which the rostral border of the lateral reticular nucleus was identified, to the section that included the caudal end of the facial nucleus (this extent encompasses the preBötC). Within each section, neurons that were positively stained for NK1R were counted within a circle of 600 µm diameter representing the extent of the preBötC (2), and in a rectangle (1600 x 1070 µm) outside this circle (Fig 4 & 5 in manuscript) to determine the extent of NKIR neuronal damage out with the preBötC. The mapped sections were at least 160 µm apart to eliminate double counting.

References

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