

# Catecholamine neurones in rats modulate sleep, breathing, central chemoreception and breathing variability

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Brainstem catecholamine (CA) neurones have wide projections and an arousal-state-dependent activity pattern. They are thought to modulate the processing of sensory information and also participate in the control of breathing. Mice with lethal genetic defects that include CA neurones have abnormal respiratory control at birth. Also the A6 region (locus coeruleus), which contains CA neurones sensitive to CO<sub>2</sub> *in vitro*, is one of many putative central chemoreceptor sites. We studied the role of CA neurones in the control of breathing during sleep and wakefulness by specifically lesioning them with antidopamine  $\beta$ -hydroxylase–saporin (DBH-SAP) injected via the 4th ventricle. After 3 weeks there was a 73–84% loss of A5, A6 and A7 tyrosine hydroxylase (TH) immunoreactive (ir) neurones along with 56–60% loss of C1 and C2 phenylethanolamine-*N*-methyltransferase (PNMT)-ir neurones. Over the 3 weeks, breathing frequency decreased significantly during air and 3 or 7% CO<sub>2</sub> breathing in both wakefulness and non-REM (NREM) sleep. The rats spent significantly less time awake and more time in NREM sleep. REM sleep time was unaffected. The ventilatory response to 7% CO<sub>2</sub> was reduced significantly in wakefulness at 7, 14 and 21 days (–28%) and in NREM sleep at 14 and 21 days (–26%). Breathing variability increased in REM sleep but not in wakefulness or NREM sleep. We conclude that CA neurones (1) promote wakefulness, (2) participate in central respiratory chemoreception, (3) stimulate breathing frequency, and (4) minimize breathing variability in REM sleep.

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Catecholamine (CA) neurones located in the brainstem project widely to forebrain, hindbrain and spinal cord (Hökfelt *et al.* 1984; Berridge & Waterhouse, 2003). Their natural activity pattern is state dependent; they fire substantially in wakefulness, and less so in NREM and REM sleep (Jacobs, 1986; Berridge & Waterhouse, 2003). They modulate the processing of sensory information in wakefulness (Foote *et al.* 1980; Aston-Jones *et al.* 2000; Berridge & Waterhouse, 2003) and participate in sleep cycle regulation, promoting wakefulness (Hobson *et al.* 1975; Saper *et al.* 2001; Ouyang *et al.* 2004). They are also involved in the control of breathing (Erickson & Milhorn, 1984; Guyenet *et al.* 1993; Oyamada *et al.* 1998; Dawid-Milner *et al.* 2001; Hilaire *et al.* 2004; Viemari *et al.* 2004) and cardiovascular function (Morilak *et al.* 1987; Levine *et al.* 1990; Curtis *et al.* 1993; Murase *et al.* 1993; Schreihofer & Guyenet, 2000; Dampney & Horiuchi, 2003; Swoap *et al.* 2004). Our interest lies in the role of CA neurones in the control of breathing, especially in central chemoreception, during both sleep and wakefulness.

CA neurones are labelled after retrograde tracing of viral vectors injected into phrenic motoneurons

suggesting their involvement in respiratory control (Dobbins & Feldman, 1994). Evidence from Rnx mutant mice (Shirasawa *et al.* 2000), neonatal rat brainstem studies (Hilaire *et al.* 2004), and adult anaesthetized rats (Jodkowski *et al.* 1997; Dawid-Milner *et al.* 2001) indicate that CA A5 neurones can affect breathing frequency. In respect to the A6 region fetal Phox2a mutant mice, which die within 24 h of birth and have absent A6 CA neurones (Morin *et al.* 1997), breathe more slowly and with more variability than heterozygous controls (Viemari *et al.* 2004). Pharmacological and lesion studies showed that A6 CA neurones stimulate breathing frequency and are essential for the development of a normal respiratory rhythm (Hilaire *et al.* 2004).

Central chemoreceptors are thought by some to reside at or near the ventral surface of the medulla (see Loeschcke, 1982; Nattie, 1999; Feldman *et al.* 2003) and recent data support involvement of neurones in the retrotrapezoid nucleus (Nattie & Li, 2002) that are glutamatergic (Mulkey *et al.* 2004) and in the medullary raphe that are serotonergic (Nattie *et al.* 2004; Richerson, 2004) as chemoreceptor cells. We adhere to the view that central chemoreception

is present at widespread sites within the hindbrain and cerebellum (Coates *et al.* 1993; Nattie, 2001; Nattie & Li, 2002; Feldman *et al.* 2003; Nattie *et al.* 2004) including regions rich in CA neurones. CO<sub>2</sub> stimulation increases *c-fos* expression in CA neurones of the A5 and A6 regions (Haxhiu *et al.* 1996) and increases the firing rate of A6 cells in reduced preparations (Pineda & Aghajanian, 1997; Oyamada *et al.* 1998; Ballantyne & Scheid, 2000; Filosa *et al.* 2002) and in anaesthetized rats *in vivo* (Elam *et al.* 1981). Focal acidification of the A6 region increases ventilatory output (phrenic nerve activity) in anaesthetized cats and rats (Coates *et al.* 1993).

We hypothesize that CA neurones (1) produce an excitatory drive that affects breathing frequency and its variability predominantly in wakefulness when these neurones are most active and (2) participate in central chemoreception. To test these hypotheses, we have specifically lesioned brainstem CA neurones by injection of DBH-SAP into the 4th ventricle of rats and measured ventilation during sleep and wakefulness over 21 days. IgG-SAP is used as a control of similar size, which is not taken up into the cells.

## Methods

### General

The rats were housed in a room with a light, rest period from midnight to noon and a dark, active period from noon to midnight. Food and water were available *ad libitum*. All the experiments were performed between 8 a.m. and 3 p.m. All procedures were within the guidelines of the National Institutes of Health for animal use and care and were approved by the Dartmouth College Institutional Animal Use and Care Committee. A total of 33 male Sprague-Dawley rats (250–350 g) were used of which 16 completed the entire 4-week protocol. There were two main experimental groups: (1) lesion with DBH-SAP injection ( $n = 7$ ); control with IgG-SAP injection ( $n = 6$ ). We also made 24 h sleep measurements in additional lesioned rats ( $N = 3$ ).

The rats were anaesthetized with ketamine (100 mg kg<sup>-1</sup> i.m.) and xylazine (15 mg kg<sup>-1</sup> i.p.). Hindlimb withdrawal and corneal reflexes were tested to monitor the depth of anaesthesia. Responses resulted in administration of supplemental anaesthesia in the form of a quarter of the initial dose. The skull and a portion of the abdomen were shaved and the skin cleansed with a povidone-iodine solution and 70% alcohol. The head was placed into a Kopf stereotaxic holder and two EEG electrodes were screwed into the right side of the skull. One was over frontal cortex (2 mm anterior to bregma and 2 mm lateral to midline), another over parietal cortex (3.5 mm posterior to bregma, 2 mm lateral) and an earth lead placed between the two 3 mm lateral to midline. For the EMG, a pair of

wire electrodes was threaded through the nuchal muscles. A sterile telemetry temperature probe (TA-F20, Data Sciences, St Paul, MN, USA) was placed in the abdominal cavity. After recovery and baseline data collection, each rat was re-anaesthetized and received a microinjection of either DBH-SAP or IgG-SAP into the fourth ventricle. A small incision was made on the top of the skull, and a ~2 mm diameter hole was drilled 2.3–3.0 mm caudal from lambda in the midline. The microinjection was made by using a 5  $\mu$ l Hamilton syringe with a 28 gauge needle inserted 7.1–7.4 mm below the dorsal surface of the skull. Each microinjection lasted at least 5 min and the needle remained in position for another 5 min before removal. The incision was sutured.

### Protocol

After at least 7 days recovery from the EEG/EMG placement, baseline ventilation ( $\dot{V}_E$ ), tidal volume ( $V_T$ ), frequency ( $f$ ), and oxygen consumption were measured at least twice in each rat while breathing room air, 3 and 7% CO<sub>2</sub> in air during sleep and wakefulness. Each rat then received one injection (2.2  $\mu$ l, 5  $\mu$ g) of (a) IgG-SAP or (b) DBH-SAP (Advanced Targeting Systems, San Diego, CA, USA). Ventilatory measurements were made on days 7–9, 14–16 and 21–23 after the injection. For this protocol after the rat was acclimatised to the plethysmograph chamber data were obtained over 30–45 min of breathing air and each of the CO<sub>2</sub> mixtures. This allowed ample time for awake and NREM periods but REM periods were not consistently observed. In order to obtain breathing data in REM, sleep cycling and breathing in air were separately measured in five control and four lesioned rats during 4 h prior to and ~10 days after the injection. We also analysed the variability of breathing in wakefulness, NREM and REM sleep using 400 breaths in each state. In a separate set of rats ( $N = 3$ ) sleep cycling was recorded over 24 h prior to and ~21 days after the injection.

### Data analysis

We determined sleep and wakefulness using EEG and EMG electrode signals, the fast Fourier transform (FFT) of the EEG signal analysed in 3.6 s epochs at delta (0.3–5 Hz), theta (6–9 Hz) and sigma (10–17 Hz) frequency bands, and behavioural observations. We used criteria as previously described (Nattie & Li, 2002; Nattie *et al.* 2004). In our case, we measured breathing only during quiet wakefulness. In active wakefulness the activity of the rat in the plethysmograph interferes with reliable measurement of breathing. For 4 h and 24 h sleep cycling data SleepSign sleep analysis software (Kissei Comtec America, Inc.) was used. We analysed the amount of time the rat spent in NREM and REM sleep and in wakefulness

during each experiment. Ventilation was measured using a flow-through whole-body plethysmograph as previously described (Nattie & Li, 2002; Nattie *et al.* 2004). The volume of the plethysmograph was 7.6 l with a 3.5 l top to protect the head pedestal. The plethysmograph was connected by a high resistance leak to a similarly sized reference chamber. The inflow gas for the plethysmograph chamber was humidified and controlled by a flow meter at a minimum of  $1.4 \text{ l min}^{-1}$  to prevent rebreathing of exhaled gas. The outflow was matched to the inflow via a flow meter connected to a vacuum system. Approximately  $100 \text{ ml min}^{-1}$  of outflow gas served  $\text{O}_2$  and  $\text{CO}_2$  analysers (Applied Electrochemistry). We measured chamber pressure by transducer and calibrated the plethysmograph with multiple 0.3 ml injections. We measured chamber temperature by thermometer and rat body temperature by telemetry continuously.

For calculation of ventilatory data, we used the DataPac 2000 system to determine the pressure deflections and the respiratory cycle time for each of 100–300 breaths at defined sleep and wake periods breathing air, 3% and 7%  $\text{CO}_2$ . Sighs, sniffing and recording artifacts were edited from analysis. These data were exported to SigmaPlot 6.0 (SPSS, Inc., Chicago, IL, USA) with  $f$ ,  $V_T$  and  $\dot{V}_E$  per 100 g of body weight calculated for each breath using plethysmograph and body temperatures for that time period. We obtained two to four measurement periods for NREM sleep and wakefulness as a baseline prior to 3% or 7%  $\text{CO}_2$  exposure and one or two periods during the 30–40 min of  $\text{CO}_2$  exposure. In our two experimental groups, we compared  $\dot{V}_E$ ,  $V_T$  and  $f$  breathing air, 3% or 7%  $\text{CO}_2$  in NREM sleep and wakefulness on days 7–9, 14–16 and 21–23 to the baseline data for that treatment in each rat. We defined the ‘ $\text{CO}_2$  response’ as the  $\Delta\dot{V}_E$  breathing 7% (or 3%)  $\text{CO}_2$  minus that in air breathing. Oxygen consumption was calculated using the Fick principle and the difference in  $\text{O}_2$  content between gas entering and leaving the plethysmograph and the flow rate and normalized to  $\text{ml (g body weight)}^{-1} \text{ h}^{-1}$ . We monitored  $\text{CO}_2$  content of the outflow gas continuously to ensure that no build-up of  $\text{CO}_2$  took place within the chamber.

For breathing variability, we applied the Poincaré analysis in which the duration of each breath is plotted *versus* the duration of the next breath. We calculated the width of the variation perpendicular to (SD1) and along the line of identity (SD2) and calculated the area of the ellipse that describes the distribution of the points (Brennan *et al.* 2001).

## Anatomy

Three weeks after injection following all physiological experiments the rats were anaesthetized with ketamine and xylazine then transcardially perfused with

200 ml saline followed by 300–500 ml of chilled 4% paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). The brain was removed and post-fixed overnight in 4% paraformaldehyde at  $4^\circ\text{C}$ , then cryoprotected for 48 h in 30% sucrose. The brains were sectioned at  $30 \mu\text{m}$  thickness on a Leica cryostat for immunohistochemical staining of tyrosine hydroxylase (TH) or phenylethanolamine-*N*-methyltransferase (PNMT). For the individual reactions (TH, PNMT) we used the diaminobenzidine (DAB) reaction for visualization. All immunohistochemical procedures were performed by using free-floating sections at room temperature. We used 0.1 M phosphate buffer (PB) for TH and PNMT quenched by 3%  $\text{H}_2\text{O}_2$  and then blocked with 5% normal goat serum (NGS). The sections were incubated with either a rabbit polyclonal antibody against PNMT (1 : 10 000, Chemicon), or a mouse monoclonal antibody against TH (1 : 10 000, Sigma) for 48 h at  $4^\circ\text{C}$  followed by a biotinylated goat anti-rabbit or anti-mouse IgG overnight at  $4^\circ\text{C}$  (1 : 500, Vector Laboratories). An avidin–biotin–horseradish peroxidase procedure with DAB was used to visualize PNMT and TH staining. These sections were used for all cell counts. All the sections were mounted and dehydrated with graded alcohol (25–100% EtOH), cleared with xylene and cover-slipped.

## Cell counting methods

Brainstem sections were cut at  $30 \mu\text{m}$  thickness in a cryostat with two of every four successive sections stained for TH or PNMT. To evaluate the effects of our DBH-SAP injections on brainstem CA neurones we counted the number staining for TH in the A5, A6 and A9 regions and the number staining for PNMT at the C3 and C1 regions. We counted all somatic cell profiles in each area of interest. A5, A6, A7, A9 and C1 regions were counted on one side only. The C3 region is in the midline. Each section counted for any marker was  $30 \mu\text{m}$  in thickness and separated from the next counted section by  $90 \mu\text{m}$ . For the A5 region we applied the approach of Byrum *et al.* (1984) as shown in their Fig. 1. TH-positive cells were counted in a small rectangle in the ventrolateral pons and medulla over the entire length of this cell group, a rostral-caudal range of just over 2 mm. A6 cells were counted over the entire length of this cell group in the dorso-lateral pons along the lateral margin of the fourth ventricle and extending caudally for approximately 1.5 mm (Hökfelt *et al.* 1984). A7 TH-ir neurones were counted in six to nine sections rostrally starting at the first evidence of TH-ir cells ventral to the most rostral aspect of A6. A9 TH-positive cells were counted in four successive sections beginning at about  $P = 5.8$  (Hökfelt *et al.* 1984). The C1 PNMT-ir cells were counted in the ventrolateral medulla for their entire rostral-caudal extent, which is approximately 2.7 mm (Hökfelt *et al.*

1984). The C3 PNMT-ir cells were counted in the centre of the dorsal medulla for their entire rostral–caudal extent, a distance of approximately 1.5 mm (Hökfelt *et al.* 1984).

## Statistics

Counts of somatic cell profiles of TH-ir and PNMT-ir neurones were compared at each CA neuronal location between the IgG-SAP and DBH groups by *t* test. For analysis of ventilatory data we used a two-way repeated measures ANOVA with  $\dot{V}_E$ ,  $V_T$ , and  $f$  as the repeated measures variable and state (awake, NREM sleep), treatment (IgG-SAP, DBH-SAP) and CO<sub>2</sub> (room air, 3% CO<sub>2</sub>, 7% CO<sub>2</sub>) as categorical variables. To evaluate the 'CO<sub>2</sub> response' we calculated the  $\Delta\dot{V}_E$  ( $\Delta V_T$  and  $\Delta f$ ) comparing values in 3 or 7% CO<sub>2</sub> to the corresponding air breathing values. These were analysed with the same two-way repeated measures ANOVA approach. For analysis of breathing variability we applied a two-way repeated measures ANOVA for each parameter of the Poincaré analysis (SD1, SD2, area) with baseline and 10 days as repeated measures and treatment (IgG-SAP, DBH-SAP) and state (awake, NREM, REM) as categorical variables. For analysis of sleep–wake time within the 4 h measurement periods we applied a two-way repeated measures ANOVA with baseline and 10 days as the repeated measure and treatment (IgG-SAP, DBH-SAP) and state (awake, NREM, REM) as categorical variables. In each case, *post hoc* comparisons of within-group effects were made using Dunnett's test and the *t* statistic. For analysis of treatment effects on sleep–wake time in the

24 h group, we applied a two-way repeated measures ANOVA with baseline and 21 days as repeated measures and treatment (IgG-SAP, DBH-SAP) and time (each 2 h period over the 24 h) as categorical factors.

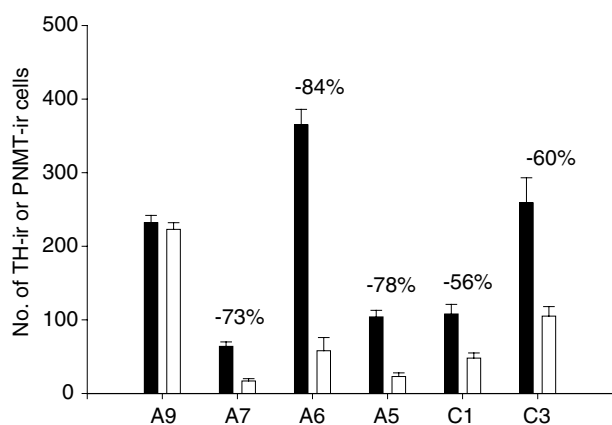
## Results

### Cell counts

Figure 1 shows the mean  $\pm$  s.e.m. of the total number of counted immunoreactive cell profiles for each region in IgG-SAP injected controls ( $N = 5$ ) and DBH-SAP lesioned rats ( $N = 6$ ). For each region of interest the number of sections counted and the rostral-to-caudal length were not different between the groups.

In the A6 region, we counted cell profiles over  $1248 \pm 61 \mu\text{m}$  in the control group and  $1480 \pm 67 \mu\text{m}$  in the lesion group. DBH-SAP decreased the number of TH-ir cell profiles by 84% on average from  $365 \pm 21$  to  $58 \pm 18$  ( $P < 0.001$ ). In the A5 region, we counted cell profiles over  $2232 \pm 111 \mu\text{m}$  in the control group and  $2180 \pm 147 \mu\text{m}$  in the lesion group. DBH-SAP injections decreased the number of TH-ir cell profiles by 78% on average from  $104 \pm 9$  to  $23 \pm 5$  ( $P < 0.001$ ). In the A7 region, we counted cell profiles in six to nine sections per rat. DBH-SAP decreased the number of TH-ir cell profiles from  $64 \pm 6$  to  $17 \pm 3$ , a 73% decrease ( $P < 0.01$ ). In the more rostral A9 region, we counted cell profiles in four consecutive sections. DBH-SAP injections had no significant effect on the number of TH-ir cell profiles with  $232 \pm 10$  and  $223 \pm 9$  being the counts.

In the C3 region, we counted cell profiles over  $1440 \pm 114 \mu\text{m}$  in the control group and  $1640 \pm 106 \mu\text{m}$  in the lesion group. DBH-SAP injections decreased the number of PNMT-ir cell profiles by 56% on average from  $108 \pm 13$  to  $48 \pm 7$  ( $P < 0.003$ ). In the C1 region, we counted cell profiles over  $2722 \pm 222 \mu\text{m}$  in the control group and  $2640 \pm 161 \mu\text{m}$  in the lesion group. DBH-SAP injections decreased the number of PNMT-ir cell profiles by 60% on average from  $259 \pm 34$  to  $105 \pm 13$  ( $P < 0.005$ ).



**Figure 1.** DBH-SAP injections (open bars;  $N = 6$ ) decrease the number of TH-ir neurones in A7, A6 and A5 regions and the number of PNMT-ir neurones in C1 and C3 regions as compared to IgG-SAP injections (filled bars;  $N = 5$ )

Mean  $\pm$  s.e.m. values are shown. The number of immunoreactive cell profiles was significantly lower in treated compared to control rats in A6 ( $P < 0.001$ ; *t* test), A5 ( $P < 0.001$ ), A7 ( $P < 0.001$ ), C3 ( $P < 0.003$ ) and C1 ( $P < 0.005$ ).

### Oxygen consumption and body temperature

Oxygen consumption and body temperature did not differ between the IgG-SAP control and DBH-SAP lesion groups at any of the four measurement days nor did they differ with time. In controls mean ( $\pm$  s.e.m.) oxygen consumption at baseline was  $1.18 \pm 0.02 \text{ ml h (g body wt)}^{-1}$  and at 21 days was  $1.15 \pm 0.04 \text{ ml h (g body wt)}^{-1}$  while in the lesion group it was  $1.21 \pm 0.11$  and  $1.14 \pm 0.01 \text{ ml h (g body wt)}^{-1}$ , respectively. Body temperature in controls at baseline was  $38.0 \pm 0.08^\circ\text{C}$  and at 21 days was  $37.8 \pm 0.12^\circ\text{C}$  while in the lesion group it was  $38.1 \pm 0.11$  and  $37.9 \pm 0.09^\circ\text{C}$ , respectively.

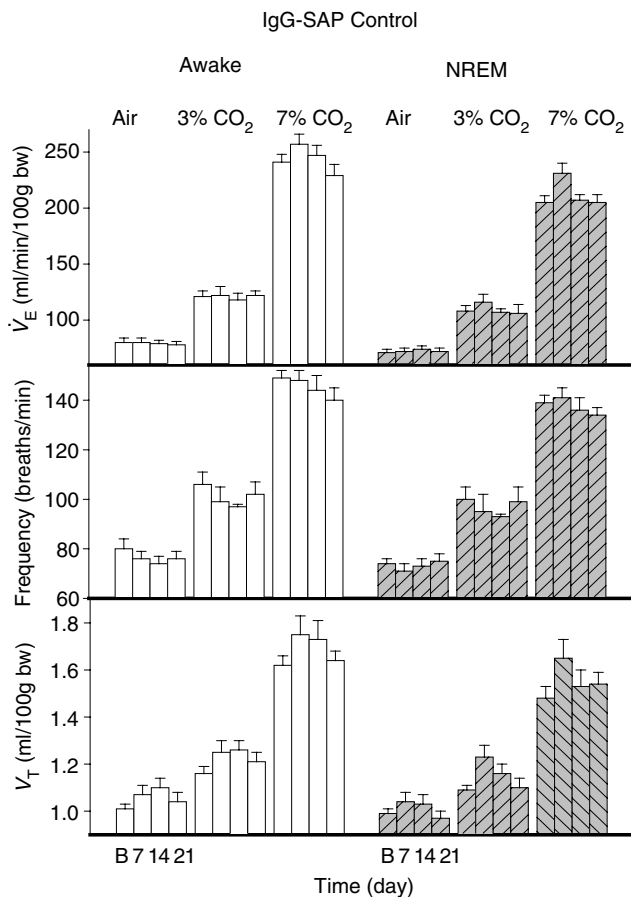
**Ventilation**

$\dot{V}_E$  (Figs 2 and 3) decreased significantly over the time course of the experiment ( $P < 0.001$ ) with significant interactions with treatment ( $P < 0.001$ ),  $\text{CO}_2$  ( $P < 0.001$ ) and with treatment and  $\text{CO}_2$  together ( $P < 0.002$ ). There was no interaction of the  $\dot{V}_E$  effect with state but there was a significant effect of state as a categorical variable ( $P < 0.001$ ). Thus  $\dot{V}_E$  was significantly less in NREM sleep overall without any variation in this sleep effect over the post-baseline time course of the experiment. In 7%  $\text{CO}_2$   $\dot{V}_E$  was significantly decreased at 7, 14 and 21 days in wakefulness ( $P < 0.01$ ) and at 14 ( $P < 0.05$ ) and 21 days ( $P < 0.01$ ) in NREM sleep. *Post hoc* comparison showed no treatment effect in 3%  $\text{CO}_2$  or in air breathing.

Breathing frequency ( $f$ ) (Figs 2 and 3) decreased significantly over the time course of the experiment ( $P < 0.01$ ) with significant interactions with treatment ( $P < 0.001$ ) and  $\text{CO}_2$  ( $P < 0.001$ ). There was no interaction of the  $f$  effect with state but there was a significant effect of state as a categorical variable ( $P < 0.001$ ). Thus  $f$

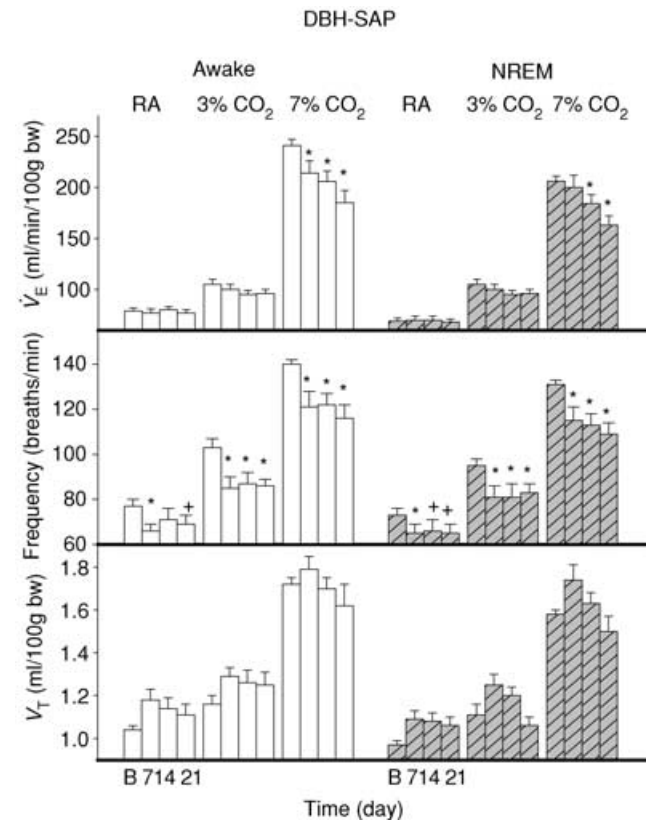
was significantly less in NREM sleep overall without any variation in this sleep effect over the post-baseline time course of the experiment. The absence of a significant interaction between the effect on  $f$ , treatment and  $\text{CO}_2$  indicates that the effect on  $f$  is present at all levels of  $\text{CO}_2$  (air, 3%  $\text{CO}_2$  and 7%  $\text{CO}_2$ ). Given the a priori hypothesis that treatment would decrease  $f$  at all levels of  $\text{CO}_2$ , we made *post hoc* comparisons. Frequency was significantly decreased in 7%  $\text{CO}_2$  at 7, 14 and 21 days in wakefulness and NREM sleep ( $P < 0.01$ ), in 3%  $\text{CO}_2$  at 7, 14 and 21 days in wakefulness and NREM sleep ( $P < 0.01$ ) and in air breathing during wakefulness at 7 ( $P < 0.01$ ) and 21 ( $P < 0.05$ ) but not 14 days and in NREM sleep at 7 ( $P < 0.01$ ), 14 and 21 ( $P < 0.05$ ) days.

Tidal volume ( $V_T$ ) (Figs 2 and 3) increased over the post-baseline time course of the experiment ( $P < 0.001$ ) but there was no significant interaction with treatment,



**Figure 2. IgG-SAP effects**

IgG-SAP injections have no effect on ventilation ( $\dot{V}_E$ ), tidal volume ( $V_T$ ), and frequency ( $f$ ) while breathing air, 3%  $\text{CO}_2$  or 7%  $\text{CO}_2$  in wakefulness (open bars) or NREM sleep (hatched bars) over 7, 14 and 21 days after baseline (B).

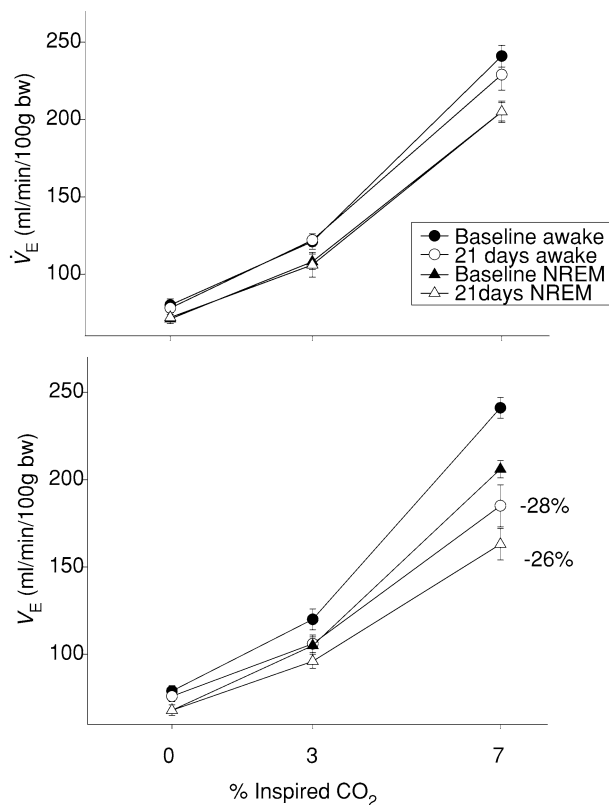


**Figure 3. DBH-SAP effects**

DBH-SAP injections: (1) had no effect on ventilation ( $\dot{V}_E$ ) (top row) in wakefulness (open bars) or NREM sleep (hatched bars) in air breathing (left panels) or 3%  $\text{CO}_2$  (middle panels) but significantly decreased  $\dot{V}_E$  ( $*P < 0.01$ ,  $+P < 0.05$ ; *post hoc* Dunnett's test after two-way repeated measures ANOVA; see text) in 7%  $\text{CO}_2$  (right panels) at 7, 14 and 21 days in wakefulness and at 14 and 21 days in NREM sleep; (2) decreased breathing frequency ( $f$ ) (middle row) at all times in 3 and 7%  $\text{CO}_2$  and at 7 and 14 days breathing room air awake and at 7, 14, and 21 days in NREM sleep; and (3) had no significant effect on tidal volume ( $V_T$ ; bottom row) in any condition.

state or CO<sub>2</sub>. There was a significant effect of state as a categorical variable. Thus  $V_T$  was significantly less in NREM sleep overall without any variation in this sleep effect over the post-baseline time course of the experiment.

To emphasize the effect of DBH-SAP lesions on the CO<sub>2</sub> response, we show CO<sub>2</sub> response curves of  $\dot{V}_E$  for the 21-day data in wakefulness and NREM sleep in control and lesion groups (Fig. 4). The 'CO<sub>2</sub> response' ( $\Delta\dot{V}_E$ ) decreased over the post-baseline time course of the experiment ( $P < 0.001$ ) and there was a significant interaction with treatment ( $P < 0.001$ ), CO<sub>2</sub> ( $P < 0.02$ ), and with treatment and CO<sub>2</sub> together ( $P < 0.05$ ). There was no interaction of the  $\Delta\dot{V}_E$  repeated measures effect with state but there was a significant effect of state as a categorical variable indicating that  $\Delta\dot{V}_E$  was less in NREM sleep. *Post hoc* analysis showed that in 7% CO<sub>2</sub>,  $\Delta\dot{V}_E$  was significantly decreased in wakefulness at 7 ( $P < 0.05$ ) and at 14 and 21 days ( $P < 0.01$ ) and in NREM sleep at 21 days ( $P < 0.01$ ). At 21 days the 'CO<sub>2</sub> response' breathing 7%



**Figure 4. CO<sub>2</sub> response before and after IgG-SAP and anti-DBH-SAP**

IgG-SAP injections had no effect on the  $\dot{V}_E$  CO<sub>2</sub> response (top panel) in wakefulness or NREM sleep examined at 21 days after injection. DBH-SAP injections decreased the  $\dot{V}_E$  CO<sub>2</sub> response in wakefulness or NREM sleep examined 21 days after injection. ●, baseline data for wakefulness; ▲, NREM sleep; ○ and △, data obtained at 21 days. The percentage values indicate the percentage decrease compared for 7% CO<sub>2</sub> at 21 days.

CO<sub>2</sub> was decreased by 28% in wakefulness and by 26% in NREM sleep. In 3% CO<sub>2</sub> the 'CO<sub>2</sub> response' was decreased by 33% in wakefulness and NREM sleep. These effects did not reach statistical significance so while we conclude that there is no treatment effect at the lower stimulus level we can warn that this conclusion is tentative. Analysis of  $\Delta f$  showed a significant effect over the post-baseline time course of the experiment ( $P < 0.001$ ) but there was no significant interaction with state, CO<sub>2</sub> or treatment ( $P = 0.06$ ). Analysis of  $\Delta V_T$  showed no significant effect.

### The variability of breath duration

Figure 5 shows for a single animal the Poincaré plots of the duration of a breath,  $T_{TOT}$ , versus the duration of the subsequent breath,  $T_{TOT}(N+1)$ , for 400 breaths in each state (awake, NREM sleep and REM sleep) at baseline and at day 10 after injection. After DBH-SAP injections, the variability of breath duration increased in REM sleep but not in wakefulness or NREM sleep. To quantify the distribution of points we used the parameters SD1 and SD2 as shown on the upper left panel and the area of the ellipse that fits the distribution of points (not shown) (Brennan *et al.* 2001). In this animal SD1, SD2 and area did not differ between baseline and day 10 in the awake and NREM states. In REM sleep, SD1, SD2 and ellipse area were significantly greater at day 10 by 23, 32 and 131%, respectively. Average data for five lesion and four control rats are shown in Table 1. For each parameter SD1, SD2 and ellipse area, there was no significant lesion effect in wakefulness or in NREM sleep. In REM sleep all parameters showed a significant increase after the lesions. For SD1 there was a significant interaction of state, treatment, and SD1 ( $P < 0.01$ ). SD1 at day 10 was greater than baseline in the lesion group ( $P < 0.05$ ) and greater than day 10 in controls ( $P < 0.05$ ). For SD2 the interaction term was not significant but a *post hoc* comparison based on the a priori hypothesis showed day 10 greater than baseline in the lesion group ( $P < 0.01$ ) and greater than day 10 in the control group ( $P < 0.01$ ). For the area, the interactive term was significant ( $P < 0.01$ ) and *post hoc* analysis showed day 10 greater than baseline in the lesion group ( $P < 0.01$ ) and greater than day 10 in the controls ( $P < 0.01$ ). We repeated the lesions in four additional rats and extended the variability analysis out for 3 weeks and found no further disruption of variability. In REM sleep, there were also significantly more breaths longer than 1.5 s, a long breath duration for the rat. The number of breaths out of 400 that were longer than 1.5 s was  $6.4 \pm 0.7$  and  $3.0 \pm 0.6$  at baseline and day 10 in the control group and  $2.4 \pm 0.7$  and  $11.3 \pm 2.3$  in the lesion group (day 10 in lesion group significantly different from baseline and from day 10 in control group;  $P < 0.001$  for interaction term;  $P < 0.05$  *post hoc*).

**Table 1. The variability of breath duration (mean ± s.e.m.) in wakefulness, NREM and REM sleep at baseline conditions (B) and at 10 days (10) after DBH-SAP (n = 4) or IgG-SAP (n = 5) injections**

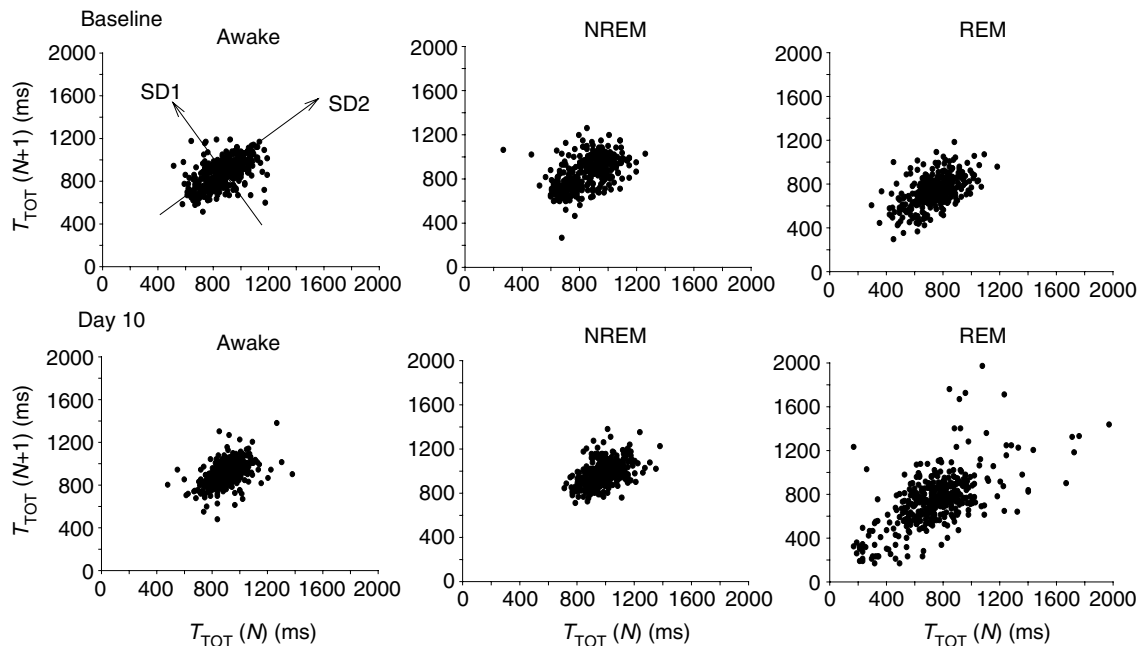
	Awake				NREM				REM			
	IgG-SAP		DBH-SAP		IgG-SAP		DBH-SAP		IgG-SAP		DBH-SAP	
	B	10	B	10	B	10	B	10	B	10	B	10
SD1	81 (6)	77 (10)	76 (4)	73 (4)	71 (9)	77 (7)	78 (8)	70 (8)	93 (5)	87 (2)	85 (3)	108*# (8)
SD2	175 (25)	142 (17)	150 (13)	127 (9)	110 (16)	128 (20)	139 (16)	144 (30)	170 (5)	152 (9)	166 (13)	223*# (31)
Area (× 10 <sup>3</sup> )	43.9 (5.6)	35.7 (8.2)	35.8 (3.9)	29.0 (2.7)	25.6 (5.9)	31.1 (5.8)	34.9 (7.3)	32.2 (7.5)	50.1 (3.7)	41.4 (2.4)	44.0 (2.5)	75.0*# (12.2)

SD1, SD2 (both in ms) and area (defined in Fig. 5) were determined from 400 breaths in each case. \*Significantly different from control value at day 10; #significantly different DBH-SAP value at baseline (B); P < 0.05.

**Sleep**

We analysed the effects of the DBH-SAP lesions on sleep-wake state during 4 h data collections periods obtained at baseline and 10 days after injection while breathing air. The lesioned rats spent less time awake and more time in NREM sleep. At 10 days the time awake

for the lesion group (N = 9) was 30 ± 2% (s.e.m.) versus 37.1 ± 2.7% for baseline (P < 0.05) while the time in NREM sleep was 66 ± 2% versus 58 ± 2.2% for baseline (P < 0.05). In the control group the time awake was 38 ± 2.3% at baseline and 41 ± 3% at 10 days while the NREM sleep time was 54 ± 1.4% at baseline and 56 ± 1.7% at 10 days. In three rats we obtained 24 h



**Figure 5. Poincaré plots**

Poincaré plots graph for one rat the duration of a breath,  $T_{TOT}$ , versus the duration of the subsequent breath,  $T_{TOT}(N + 1)$ , for 400 breaths in each state (awake, NREM sleep, REM sleep) at baseline (top row) and at day 10 (bottom row) after injection. The variability of breath duration increases in REM sleep (right panels) but not in wakefulness (left panels) or NREM sleep (middle panels) after DBH-SAP lesions. SD1 and SD2 shown on the upper left panel and the area of the ellipse that fits the distribution of points (not shown) are parameters that quantify the point spread (Brennan *et al.* 2001). In this animal SD1, SD2 and area do not differ significantly between states in the baseline condition nor do they vary between baseline and day 10 in wakefulness and NREM sleep. In REM sleep, SD1, SD2 and area are significantly greater at day 10 by 23, 32 and 131% greater, respectively, as compared to awake or NREM at day 10 or to REM at baseline. See Table 1 for average data.

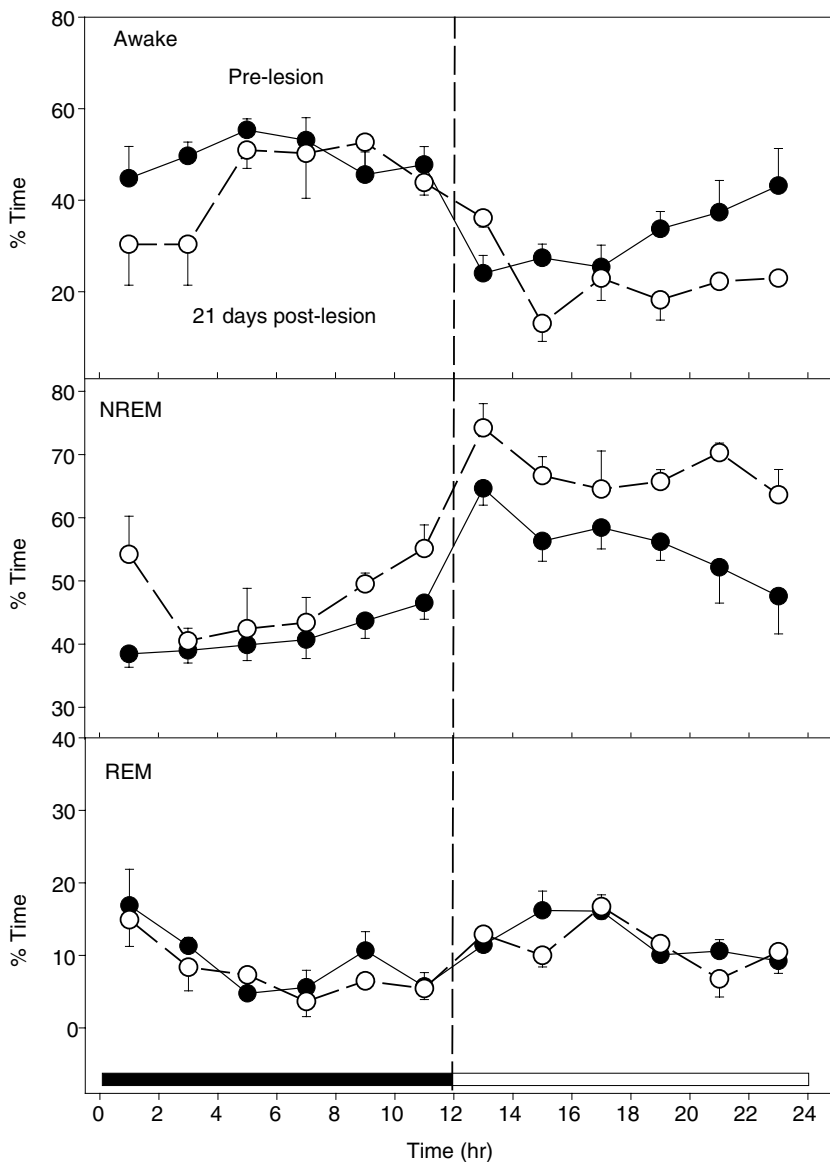
sleep-wake data at baseline and at 21 days after injection of DBH-SAP (Fig. 6). At 21 days compared to baseline these rats were awake less ( $P < 0.04$ ) and in NREM sleep more ( $P < 0.001$ ). REM time was unaffected. This effect on sleep-wake time was small. Over the 24 h average time awake went from  $37 \pm 3\%$  at baseline to  $32 \pm 3\%$  at day 21; NREM sleep went from  $51 \pm 2\%$  at baseline to  $58 \pm 2\%$  at day 21.

## Discussion

### CA-cell-specific lesions

Our goal was to produce a widely distributed and significant loss of CA neurones by injection of this toxin (DBH-SAP) into the ventricular system. This is in essence similar to a knock-out mouse for CA neurones but produced in the adult thus avoiding the

complications of any CA neuronal effects on development or unpredictable effects of gene deletion. We substantially reduced the numbers of brainstem CA neurones while avoiding non-specific tissue damage by injection into the 4th ventricle of a conjugate of the ribosomal toxin saporin with a monoclonal antibody to the CA-specific enzyme dopamine  $\beta$ -hydroxylase (DBH). Direct injection or dialysis into brain tissue by insertion of a needle or probe produces tissue damage visible at low power; this was not the case in the present experiments. The antibody binds to DBH in exposed vesicular membranes, the complex is endocytosed and saporin is released to kill the cell (Wrenn *et al.* 1996; Madden *et al.* 1999). Our lesion effects are similar to those of Wrenn *et al.* (1996) who injected the DBH-SAP into the lateral ventricle. The questions are: do CA neurones participate in chemoreception and/or in the determination of the breathing frequency and its variability in a state-dependent manner?



**Figure 6. Percentage time in wakefulness, NREM and REM sleep**

DBH-SAP injections decrease the percentage of time spent in wakefulness (top panel) and increase the percentage of time spent in NREM sleep (middle panel) over 24 h measured 21 days after the injections. There was no effect on the percentage time spent in REM sleep (bottom panel). ●, baseline pre-lesion data; ○, data 21 days after injection. Mean  $\pm$  s.e.m. values are shown for each 2-h period. The active, dark 12 h is at left; the quiet, light 12 h at the right. Data are from 3 rats. ( $P < 0.04$  for wakefulness;  $P < 0.001$  for NREM sleep).



### Brainstem CA neurones and breathing

Based on prior published observations from anaesthetized rats (Guyenet *et al.* 1993; Jodkowski *et al.* 1997; Oyamada *et al.* 1998; Dawid-Milner *et al.* 2001) and various mutant mice (Shirasawa *et al.* 2000; Hilaire *et al.* 2004; Viemari *et al.* 2004) we expected an effect on breathing frequency. Hilaire *et al.* (2004) concluded that A6 noradrenergic neurones provide a tonic excitatory stimulus that maintains breathing frequency while A5 neurones probably slow breathing frequency. Our results show that substantial lesions of brainstem CA neurones slow breathing frequency during air and CO<sub>2</sub> breathing in adult rats and that this effect is present in both wakefulness and in NREM sleep. We conclude that brainstem CA neurones provide a tonic excitatory drive that maintains a normal frequency in both states. The size of the lesion effect on frequency in air breathing was small being ~6% but in 3% and 7% CO<sub>2</sub> the decrease in frequency was ~16–17%.  $\dot{V}_E$  was unchanged in air breathing as was oxygen consumption so the effect of the lesions on frequency probably did not disturb alveolar ventilation or arterial  $P_{CO_2}$ .

Part of our hypothesis was that any effect of our lesions on breathing frequency would be greater in wakefulness when these neurones are most active. This was not the case. Our lesions reduced  $f$  equally in quiet wakefulness and NREM sleep.

### Chemoreception

There is evidence indicating that brainstem CA neurones participate in central chemoreception. For A5 neurones, CO<sub>2</sub> stimulation increases *c-fos* expression (Haxhiu *et al.* 1996) and CA release (Rentero *et al.* 1997). For A6 neurones CO<sub>2</sub> stimulation increases *c-fos* expression (Haxhiu *et al.* 1996) and A6 neurones are chemosensitive in reduced preparations (Pineda & Aghajanian, 1997; Oyamada *et al.* 1998; Ballantyne & Scheid, 2000; Filosa *et al.* 2002). In anaesthetized rats, systemic hypercapnia increases A6 neurone firing even after carotid body denervation (Elam *et al.* 1981) and focal acidification of the A6 region increases ventilatory output (phrenic nerve activity) indicating a direct role in chemoreception (Coates *et al.* 1993).

Putative chemoreceptor neurones at or near the ventral medullary surface have been identified as glutamatergic (Mulkey *et al.* 2004), serotonergic (Richerson, 2004), and other (Okada *et al.* 2002; Ribas-Salgueiro *et al.* 2005). We contend that central chemoreception is present at many hindbrain locations and involves many different neuronal phenotypes (see Nattie, 2001; Nattie & Li, 2002; Feldman *et al.* 2003; Nattie *et al.* 2004). Our lesion data support the hypothesis that CA neurones participate in central chemoreception. Lesions of brainstem CA

neurones reduced the ventilatory response to systemic hypercapnia in wakefulness and in NREM sleep. That our large lesions produced a 26–28% decrease in the CO<sub>2</sub> response indicates that other central chemoreceptor sites and the carotid body also contribute. Our findings were significant only with the larger stimulus of 7% CO<sub>2</sub> breathing and not with 3% CO<sub>2</sub> exposure. We can firmly conclude that at high CO<sub>2</sub> stimulus levels brainstem CA neurones are important in central chemoreception. We note that at 3% CO<sub>2</sub> exposure there was a similar per cent decrease in the  $\Delta\dot{V}_E$  value comparing CO<sub>2</sub> with room air baseline. While this did not reach statistical significance we suggest caution in rejecting the idea that CA neurones also participate in chemoreception at lower stimulus intensities. Studies with more animals in each group may uncover an effect at the lower stimulus level. We also expected a greater effect of our lesion on the CO<sub>2</sub> response in wakefulness than in NREM sleep given the higher firing rate of CA neurones in wakefulness. This was not observed suggesting that CA neurones are important in chemoreception in both wakefulness and NREM sleep even at the lower firing rate of NREM sleep. It may be that the CO<sub>2</sub> stimulus increases the firing rate of CA neurones by a similar amount in both states.

Note that we cannot conclude from these data that CA neurones are themselves the chemoreceptors, although they may well be CO<sub>2</sub>/pH sensitive as suggested by studies of A6 neurones in slices (Pineda & Aghajanian, 1997; Oyamada *et al.* 1998; Ballantyne & Scheid, 2000; Filosa *et al.* 2002) and by the observation that focal acidification at A6 increases respiratory output (Coates *et al.* 1993). CA neurones could also modulate inputs from other neurones and from the carotid body, which are chemosensitive.

### Variability of breathing

Heart rate variability reflects non-linear control system output and the balance of sympathetic and parasympathetic activity in heart rate control (Kamen *et al.* 1996; Brennan *et al.* 2001). The normal variability of breath duration is affected by many factors and in general reflects some optimal control system function that lies between an absence of any variability at one extreme and a clearly periodic breathing pattern at the other (Bruce & Daubenspeck, 1995; Khoo, 2000; BuSha & Stella, 2002). That is, some level of variability is indicative of a normal, healthy control system with many feed-back loops and many sources of excitatory and inhibitory inputs.

We reasoned that CA neuronal activity, which is state dependent, would alter the variability of breath duration in a state-dependent manner, and that our lesions would affect variability less in sleep. This hypothesis is based on the assumption that CA neurones provide input to the respiratory control system that contributes to the normal variability of breath duration. CA neurone lesions

had no effect on variability in wakefulness or NREM sleep indicating that in these states other inputs maintain normal variability. However, in REM sleep, the state normally associated with the lowest firing rate of CA neurones, the lesions increased variability even at this low firing rate. We suggest that in REM sleep breathing variability is directly influenced by brainstem CA neurones. For example, it may be that in REM sleep, breathing rate and rhythm are determined by the pre-Bötzinger complex (McKay *et al.* 2005), which is responsive to CA inputs, whereas in wakefulness and NREM sleep, rate and rhythm are determined by more complex circuits, which are affected by non-CA inputs. Alternatively, central chemoreceptor afferent inputs during REM could provide a needed drive for normal variability and these could be reduced in our lesioned rats.

### CA neurones and sleep

CA neurones have long been thought to be important in sleep regulation (Hobson *et al.* 1975). DBH<sup>-/-</sup> knock-out mice with a total absence of CA neurones (Thomas *et al.* 1998) are awake less and in NREM sleep more (Ouyang *et al.* 2004). Our results support this. Substantial and specific depletion of CA neurones results in less wakefulness and more sleep. CA neurones promote wakefulness but the small quantitative effect of these substantial lesions suggests that they play an accessory role in sleep regulation; they are one of three groups of neurones that promote wakefulness (Saper *et al.* 2001).

### Significance – CA neurones, CCHS and SIDS

Central congenital hypoventilation syndrome (CCHS) is an autosomal dominant disorder with a high likelihood of a mutation in *Phox2b*, a strong determinant of the noradrenergic system (Weese-Mayer *et al.* 2003, 2004). CCHS patients hypoventilate in sleep, requiring ventilatory support at night, and have reduced or absent responses to CO<sub>2</sub> and hypoxia. Mice heterozygous for *Phox2b* have sleep-disordered breathing and have been suggested as a partial animal model for CCHS (Durand *et al.* 2005). Insofar as specific lesions of brainstem CA neurones decrease breathing frequency and chemosensitivity and in REM sleep increase the variability of breathing with a higher incidence of prolonged breath durations, one important defect in CCHS may be attributed to the loss of CA neurones.

The Sudden Infant Death Syndrome (SIDS) has been linked to CA neurone abnormalities (Obonai *et al.* 1998). Genes related to the embryological development of the autonomic nervous system including the CA neurones are abnormal in 15.2% of SIDS cases (Weese-Mayer *et al.* 2004). SIDS may arise from genetic, or other, alterations

in the function of brainstem serotonergic (Kinney *et al.* 2003) and/or CA neurones, both of which are important in the development and maintenance of normal autonomic function and breathing.

### What do brainstem catecholaminergic neurones do?

CA neurones are of importance in normal blood pressure regulation in awake mice (Swoap *et al.* 2004) and during physiological stress in anaesthetized rats (Schreihofer & Guyenet, 2000). In unanaesthetized cats (see Levine *et al.* 1990) A6 CA neurones were activated by stresses like noise, restraint, hypoglycaemia, increased temperature and as part of the 'defense reaction' precipitated by exposure to a threatening situation. More recent studies have linked the firing of A6 CA neurones to the presence, in wakefulness, of new sensory stimuli (Aston-Jones *et al.* 2000; Berridge & Waterhouse, 2003). These studies suggest that LC neurones modulate behaviours based on novel sensory input – that the LC governs attentiveness.

It is of interest that A6 CA neurones respond both to physiological stimuli that arise within internal milieu and to sensory stimuli from the external environment. Their efferent projections modulate behaviour and homeostatic physiology. Our results indicate their involvement as modulators of breathing frequency in wakefulness and in NREM sleep, and breathing variability in REM sleep and as one of many central chemoreceptor sites. We suggest that brainstem CA neurones may link the control of breathing, and perhaps blood pressure, to demands imposed by both internal stresses and external environmental changes.

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