Brainstem activity changes associated with restored sympathetic drive following CPAP treatment in OSA subjects; a longitudinal investigation

Linda C Lundblad¹, Rania H Fatouleh¹, David K McKenzie², Vaughan G Macefield²,³ and Luke A Henderson⁴

¹School of Medicine, University of Western Sydney, Sydney, NSW 2751, Australia;
²Department of Respiratory Medicine, Prince of Wales Hospital, Sydney;
³Neuroscience Research Australia, Sydney, Australia;
⁴Department of Anatomy and Histology, University of Sydney, Sydney, NSW, Australia, 2006;

Correspondence to: Luke Henderson, University of Sydney, Sydney, NSW, Australia, 2006; lukeh@anatomy.usyd.edu.au (email); +612 9351 7063 (Tel); +612 9351 6556 (Fax)

Short title: Brainstem changes in OSA
Abstract:

Obstructive sleep apnea (OSA) is associated with significantly elevated muscle sympathetic nerve activity (MSNA), leading to hypertension and increased cardiovascular morbidity. Although little is known about the mechanisms responsible for the sympathoexcitation, we have recently shown that the elevated MSNA in OSA is associated with altered neural processing in various brainstem sites - including the dorsolateral pons, rostral ventrolateral medulla, medullary raphe and midbrain. Given the risk associated with elevated MSNA, we aimed to determine if treatment of OSA with continuous positive airway pressure (CPAP) would reduce the elevated MSNA and reverse the brainstem functional changes associated with the elevated MSNA. We performed concurrent recordings of MSNA and Blood Oxygen Level Dependent (BOLD) signal intensity of the brainstem, using high-resolution functional magnetic resonance imaging, in 15 controls and 13 subjects with OSA, prior to and following 6 months CPAP treatment. As expected, 6 months of CPAP treatment significantly reduced MSNA in subjects with OSA, from 54±4 to 23±3 bursts/min and 77±7 to 36±3 bursts/100 heart beats. Importantly, we found that MSNA-coupled changes in BOLD signal intensity within the dorsolateral pons, medullary raphe and rostral ventrolateral medulla returned to control levels. That is, CPAP treatment completely reversed brainstem functional changes associated with elevated MSNA in untreated OSA subjects. These data highlight the effectiveness of CPAP treatment in reducing one of the most significant health issues associated with OSA, that is, elevated MSNA and its associated elevated morbidity.

Keywords: muscle sympathetic nerve activity, rostroventrolateral medulla, medullary raphe, continuous positive airway pressure, obstructive sleep apnea
Introduction:

Obstructive sleep apnea (OSA) is associated with significantly increased muscle sympathetic nerve activity (MSNA; (Carlson et al. 1996; Elam et al. 2002; Fatouleh et al. 2014a; Hedner et al. 1995; Hedner et al. 1988; Imadojemu et al. 2007; Narkiewicz et al. 1998; Somers et al. 1995) leading to hypertension and increased cardiovascular morbidity (Nieto et al. 2000; Peppard et al. 2000). Although little is known about the underlying mechanisms responsible for this increased MSNA, using concurrent recordings of MSNA and functional magnetic resonance imaging (fMRI) we have recently shown that MSNA-related functional changes occur in cortical and brainstem regions in individuals with OSA (Fatouleh et al. 2014b; Lundblad et al. 2014). Within the brainstem we found that activity within the medullary raphe, rostral ventrolateral medulla (RVLM) and dorsolateral pons was significantly reduced in OSA subjects compared with controls (Lundblad et al. 2014). Furthermore, these functional changes were associated with significantly increased grey matter volume, which was correlated to an individual OSA subject’s level of MSNA. These results provide evidence that elevated MSNA in OSA subjects is associated with changes in brainstem function and anatomy and raises the question as to whether these changes can be reversed by treatments which reduce MSNA.

Continuous positive airway pressure (CPAP) is one treatment option which is effective at reducing the elevated MSNA associated with OSA (Fatouleh et al. 2014a). If a CPAP-driven reduction in MSNA is associated with a restoration of normal brainstem function, it would provide further support for the idea that elevated MSNA in individuals with untreated OSA results from altered brainstem function. The aim of this investigation was to determine whether functional changes in brainstem activity are associated with a reduction in resting MSNA during 6 months of CPAP treatment. We used concurrent recording of MSNA and fMRI to assess regional brainstem activity associated with individual subject’s patterns of MSNA. We hypothesized that CPAP
treatment would result in a restoration in brainstem function in regions of the medullary raphe, rostral ventrolateral medulla and dorsolateral pons associated with the decrease in MSNA, providing further support to the idea that the elevated MSNA in OSA is related to disturbed brainstem function.

Methods:

Subjects:

Thirteen subjects with OSA (10 males, mean±SEM age 54±3, range 35-67 years) and 15 healthy control subjects (12 males, age 50±3, 35–68 years) were recruited for the study. As described previously (Fatouleh et al. 2014b), all OSA subjects were evaluated at the sleep laboratory of Prince of Wales hospital for one night. Patients were monitored continuously for 8 hours using 12-channel polysomnography: EEG, ECG and submental EMG recordings were obtained with surface electrodes, nasal and oral airflow were monitored by thermistor and chest and abdominal movements by respiratory inductive plethysmography. Oxyhaemoglobin saturation was recorded all night by finger pulse oximetry, a microphone placed on the lower neck to record snoring, and a camera sensitive to ultraviolet light recorded the patient movements during sleep. The overnight polysomnography study was analysed offline and apnoeas and hypopnoeas defined according to the international classification of sleep disorders, by using Alice (Philips Medical Systems, The Netherlands) and Somonologica (Medcare Flaga, Reykjavik, Iceland) software. Therapeutic CPAP was determined during a full night with respiratory monitoring. The treating specialist determined the CPAP pressure that resulted in the cessation of the apnoeic events. Subsequently, subjects were treated at home with a CPAP machine individually calibrated for their optimal pressure (Series 9, ResMed, Sydney, Australia) and a mask that they felt comfortable with. Compliance with prescribed CPAP therapy was based on an automated download of the CPAP machine at 6 months analysed using ResScan software. All control subjects undertook an overnight
assessment using an in-home device that monitored nasal airflow and oxygen saturation (ApneaLink™; ResMed, Sydney, Australia). All procedures were approved by appropriate Human Research Ethics Committees of the University of Western Sydney and the University of New South Wales. Written consent was obtained from all subjects in accordance with the Declaration of Helsinki.

MRI and MSNA Acquisition:

Control subjects completed one MRI session, whereas all OSA subjects completed two MRI scanning sessions. One session was conducted immediately prior to continuous positive airway pressure (CPAP) treatment and a second session was conducted after 6 months of CPAP treatment. For each MRI session, subjects lay supine on an MRI bed with their knees supported on a foam cushion, and an insulated tungsten microelectrode was inserted into a muscle fascicle of the common peroneal nerve for recording muscle sympathetic nerve activity (MSNA). The common peroneal nerve located at the fibular head by electrical stimulation through a surface probe (3-10 mA, 0.2 ms, 1 Hz; Stimulus Isolator, ADInstruments, Australia). An insulated tungsten microelectrode (FHC, Maine, USA) was inserted percutaneously into the nerve and manually guided into a muscle fascicle of the nerve while delivering weak electrical stimuli to evoke muscle twitches (0.01-1 mA, 0.2 ms, 1 Hz). A nearby subdermal microelectrode, with 1 mm insulation removed, served as the reference electrode and a surface Ag/AgCl electrode on the leg as the ground electrode. Once a muscle fascicle had been entered neural activity was amplified (gain 10⁴, bandpass 0.3-5.0 kHz) using a low-noise, electrically isolated, headstage (NeuroAmpEX, ADInstruments, Australia). The innervation territory of the muscle fascicle was identified by tapping over the muscle belly or relevant tendon, and the position of the microelectrode tip manually adjusted until spontaneous bursts of MSNA were identified. Neural activity was acquired, RMS-processed (moving average, 200 ms) and analysed on computer-based data acquisition and analysis system (LabChart 7, PowerLab 16S; ADInstruments, Sydney, Australia). A high-pass
digital filter at 300 Hz was applied to the recorded signal to remove artefacts picked up by the cable from the MR compatible stainless steel isolated headstage to the amplifier.

In the laboratory, ECG (0.3-1.0 kHz) was recorded with AG-AgCl surface electrodes, continuous non-invasive blood pressure (BP) using radial arterial tonometry (Colin 7000 NIBP, Colin Corp., Aichi, Japan) and respiration with a piezoelectric transducer around the abdomen (Pneumotrace, UFI). Spontaneous MSNA, heart rate, respiration and BP were recorded continuously for ten minutes of undisturbed rest, of which the final 5 minutes were used for analysis. Following this period, the ECG electrodes were removed and, the BP recording stopped and the subject wheeled to the scanner with the microelectrode in situ. During scanning, heart rate was monitored via an MR-compatible piezoelectric pulse transducer on the fingerpad and respiration was monitored via the MR-compatible piezoelectric transducer around the abdomen.

Once a stable MSNA recording was achieved, a continuous series of 200 gradient echo echo-planar images, sensitive to Blood Oxygen Level Dependent (BOLD) contrast and encompassing the entire brainstem was collected (46 axial slices, TR=8s, TE=40ms, flip angle=90°, raw voxel size=1.5mm³) using a 3 Tesla MRI scanner (Philips, Achieva, 32 channel SENSE head coil). All 46 axial slices were collected during the first 4secs of the 8sec TR. A high-resolution 3D T1-weighted anatomical image set was also collected (turbo field echo; TE=2.5 ms, TR=5600 ms, flip angle=8°, voxel size 0.8mm³).

**MSNA processing:**

All MSNA signals were RMS-processed (root mean square, moving average, time constant 200 ms). MSNA during the pre-MRI recording period was quantified according to standard time-domain analysis of the RMS-processed signal as burst frequency (bursts min⁻¹) and burst incidence (bursts per 100 heart beats). Analysis of variance, coupled with Tukey’s multiple comparisons test, was used to assess statistical significance across each group. Significant differences between
controls and OSA subjects prior to CPAP treatment were determined (two-tailed, two sample t-test, p<0.05), and between OSA subjects prior to and following CPAP treatment (two-tailed, paired t-test, p<0.05). During the fMRI scanning period, MSNA bursts were manually measured from the RMS-processed version of the filtered nerve signal during the 4s inter-scan OFF period. This period was divided into 4 x 1s intervals and the total numbers of MSNA bursts for each 1s epoch was determined.

**fMRI processing:**

Using SPM8 (Friston et al. 1995), the functional image sets for each individual subject were realigned, co-registered to their T1-weighted image set and global signal intensity drifts removed using a linear de-trending method. Manual correction of the images was performed to create an accurate match between the functional and anatomical image sets. Using the SUIT toolbox (Diedrichsen 2006), the brainstem and cerebellum were isolated and the images spatially normalized into Montreal Neurological Institute space using a spatially unbiased atlas template of the cerebellum and brainstem. Note that we did not spatially smooth the images in order to maintain fine spatial detail.

For each of the 200 fMRI image volumes, the 4 seconds during which brainstem BOLD signals were recorded, were related to the MSNA burst in the preceding 4 second period. This is possible because (i) neurovascular coupling delays mean that changes in BOLD signal intensity lag the actual neuronal events in the brain by ~5 seconds (Logothetis et al. 2001), (ii) slow conduction along unmyelinated peripheral axons means it takes ~1 second for an individual sympathetic burst to travel from the brain to the peripheral recording site (Fagius and Wallin 1980); by taking these two factors into account one can see that (iii) an increase in BOLD signal intensity should appear ~4 seconds following an increase in neuronal activity within the brain. Moreover, since the scanning sequence was conducted in a caudal to rostral direction, extending from the upper cervical
spinal cord to the thalamus, we could target specific regions in the brainstem on the basis of the
temporal relationship between scanning slice and scanned structure (Figure 1).

Therefore, in each subject, the brainstem was divided into 4 separate sections from caudal to
rostral corresponding to the 1st, 2nd, 3rd and 4th second epochs and a brain mask created for each of
the 4 x 1 second epochs. The fourth second epoch was removed since this encompassed the region
rostral to the brainstem. The MSNA recording was also then divided into corresponding 1-second
epochs. For each 1 second epoch, if a MSNA burst occurred a “1” was entered into an fMRI search
model and if no burst occurred a “0” was entered. This was repeated for the entire 200-volume
scanning period, resulting in a 200-volume fMRI search model for each of the remaining 3 x 1
second epochs in each individual subject. An example of this model creation is shown in Figure 1.

A general linear model approach was used to determine changes in BOLD signal intensity
that matched each individual subject’s MSNA burst pattern for each of the 3 x 1 second epochs. The
hemodynamic delay function and associated microtime resolution were removed, since we had
already accounted for the hemodynamic delay in our methodological setup. Furthermore, in each
individual subject’s analysis, the 6-directional movement parameters derived from the realignment
step were added as nuisance variables. To eliminate the effects of heart rate, given the pulsatile
nature of cerebrospinal fluid, we included signal changes derived from a 2mm sphere placed in the
centre of the 4th ventricle in each individual subject as a nuisance variable. Following this, second
level, random effects analyses were performed to compare signal intensity changes during each
MSNA burst in OSA subjects prior to and following 6 months CPAP treatment (random effects,
displayed using a threshold of p<0.005, uncorrected, minimum cluster size 3 voxels). During this
second level analysis, the resulting statistical maps were masked with the brainstem region
corresponding to the 1st, 2nd, or 3rd second epochs. As we hypothesized that CPAP treatment would
result in signal change restoration in regions of the medullary raphe, rostral ventrolateral medulla
and dorsolateral pons we then employed small volume (p<0.05, corrected for multiple comparisons).

Since we were essentially correlating on-going fluctuations in signal intensity with spontaneous fluctuations in MSNA, and given that bursts of MSNA were significantly more frequent in OSA subjects prior to CPAP treatment than after treatment and in controls, it is possible that differences in brainstem activation patterns may have been partially due to differences in the number of MSNA bursts and the resulting MSNA fMRI model. That is, differences in the number of “ON” and “OFF” periods could potentially influence the overall significance of the final contrast maps, which may have in turn influenced the second level analyses. To ensure that this was not the case, for each significant cluster we extracted the raw signal intensity changes in OSA subjects prior to and following CPAP treatment, as well as in control subjects, and compared signal intensity during bursts of MSNA to signal intensity during periods where there were no bursts. Significant differences in signal intensity between controls and OSA subjects pre-CPAP treatment and between controls and OSA subjects post-CPAP were determined (two-tailed, two sample t-test, p<0.05), and also between OSA subjects prior to and following CPAP treatment (two-sample, paired t-test, p<0.05). Finally, for each significantly different cluster, linear relationships between percentage change in fMRI signal intensity pre-CPAP compared with post-CPAP and the associated percentage change in MSNA total bursts during the fMRI session were determined (p<0.05).

T1 image processing:

The T1-weighted image from each subject was segmented and spatially normalized with a dedicated symmetrical brainstem template. In brief, the image was cropped and the brainstem masked before normalisation such that no supratentorial grey matter can bias the results using the SUIT toolbox. The subsequent normalisation and re-slicing process produces brainstem "maps" of grey matter probabilities, spatially normalised into the brainstem template space, and modulated by
the volume changes due to the normalisation. Finally, the images were re-sliced into the new atlas space and smoothed (FWHM 3mm). Significant differences in grey matter between OSA subjects prior to and following 6 months of CPAP treatment were determined using a voxel-by-voxel analysis (paired t-tests; p<0.05, false discovery rate corrected, minimum cluster size 3 voxels). Significant volume differences were then overlaid onto a T1-weighted template for visualization.

To explore the direction and overall grey matter volume differences, individual grey matter volumes (probability*volume) were extracted from clusters of difference from the OSA subjects prior to and following CPAP treatment as well as from the control subjects and the means compared between groups (p<0.05, paired t-test).

Results:

OSA subject characteristics:

Based on overnight polysomnography OSA patients were diagnosed based on their apnea-hypopnea index (AHI) values (apnea-hypopnea events per hour) as mild, moderate or severe (mild: AHI 5-15; moderate: AHI 15-30; severe: AHI>30). Of the 13 OSA subjects, one subject had mild OSA, one subject had moderate OSA and 11 had severe OSA (mean±SEM AHI 41 ± 4; range 7-6). The minimum SaO2 during sleep was 82±3% (range 67-93%); the baseline SaO2 during wakefulness was 95±1% (range 91-99%) and the Baseline Epworth Sleep Scale score was 8±1 (range 3-14). We monitored compliance during the 6 months of CPAP treatment period and found that OSA patients used CPAP for an average of 5.0±0.4 h/night, as reported by the ResScan software. Also, as reported by the software, there was a significant reduction in AHI after 6 months of compliant treatment (AHI range 1-21; AHI 3±2).

The mean AHI for the control subjects following an in-home overnight assessment of sleep patterns was 3±1. Overnight monitoring of sleep was made at variable times after the scanning had been conducted, and revealed that, while the majority had an AHI of 1-3, two of the control subjects
had an AHI of 8 and 10. We did not exclude these subjects because they were asymptomatic normotensive and did not report being tired during the day or snoring during sleep; we did not consider it necessary to undertake a full polysomnographic assessment in these two subjects. Although there was no significant difference in age between OSA and control subject groups (two sample t-test; \( p > 0.05 \)), those with OSA had a significantly higher body mass index (BMI: pre-CPAP \( 30 \pm 2 \), controls \( 25 \pm 1 \), \( p = 0.007 \)). There was no significant difference in BMI between MRI sessions pre-CPAP and post-CPAP in the OSA subjects (pre-CPAP: \( 30 \pm 2 \); post-CPAP: \( 31 \pm 2 \), \( p = 0.6 \)).

**Physiology:**

During the laboratory recording session, compared to controls, OSA subjects had significantly elevated systolic (\( 143 \pm 5 \) vs \( 121 \pm 4 \) mmHg; \( p = 0.007 \)) and diastolic (\( 84 \pm 2 \) vs \( 68 \pm 4 \) mmHg; \( p = 0.02 \)) pressures before CPAP. Furthermore, MSNA burst frequency (\( 54 \pm 4 \) vs \( 23 \pm 3 \) bursts/min; \( p < 0.0001 \)) and burst incidence (\( 77 \pm 7 \) vs \( 36 \pm 3 \) bursts/100 heart beats; \( p < 0.0001 \)) were both significantly elevated prior to CPAP. Although there was no significant difference in heart rate before and after CPAP (\( 72 \pm 3 \) vs \( 70 \pm 2 \) beats/min; \( p = 0.55 \)), the respiratory period was significantly longer (i.e. respiratory rate was slower) following CPAP (\( 3.5 \pm 0.4 \) vs \( 4.3 \pm 0.2 \) s; \( p = 0.038 \)). Six months of CPAP treatment resulted in a significant reduction in resting MSNA, both when measured as burst frequency (\( 36 \pm 2 \) bursts/min; \( p < 0.0001 \)) and burst incidence (\( 54 \pm 4 \) bursts/100 heart beats; \( p = 0.003 \)) in all 13 OSA subjects. Despite this impressive fall in MSNA (a decrease of 31 bursts/min and 41 bursts/100 heart beats), CPAP treatment did not have a significant effect on systolic blood pressure (\( 143 \pm 5 \) vs \( 131 \pm 6 \) mmHg, \( p = 0.2 \)), or heart rate (\( 74 \pm 4 \) vs \( 69 \pm 2 \) beats/min; \( p = 0.11 \)), but did cause a significant fall in diastolic pressure (\( 84 \pm 2 \) vs \( 73 \pm 3 \) mmHg; \( p = 0.02 \)). While levels of resting MSNA cannot be predicted from baseline arterial pressure in healthy normotensive subjects below 40 years of age, there is a linear relationship between MSNA and mean arterial
pressure in subjects above 40 (Narkiewicz et al. 2005). Nevertheless, despite the majority of our OSA subjects being over 40 there were no significant correlations between MSNA, expressed either as burst frequency or burst incidence, and systolic or diastolic pressure, either before or after CPAP.

Experimental records from one subject with OSA, before and after 6 months of CPAP are shown in Fig. 2A. It is clear that MSNA was lower following CPAP in this subject. Fig. 2B shows the total number of bursts of MSNA counted during the scanning sequence in all OSA subjects, before and after CPAP, and in the controls. With the exception of two subjects, MSNA declined towards control levels in OSA subjects following 6 months of CPAP treatment.

Figure 2 near here

fMRI signal intensity changes:

Voxel-by-voxel comparison of MSNA-related BOLD signal intensity changes pre- and post-CPAP treatment revealed that the reduction in resting MSNA was coupled with significant changes in signal intensity in a number of brainstem regions (Figure 3, Table 1a). Significantly increased signal intensity changes post-CPAP compared with pre-CPAP occurred in the region of the caudal medullary raphe, left rostral ventrolateral medulla (RVLM), left and right dorsolateral pons (dl pons) and in the ventral midbrain. Significantly decreased signal intensity changes occurred in the region of the right nucleus tractus solitarius (NTS). Direct comparison of percentage changes in signal intensity during MSNA bursts, compared with periods of no bursts, showed that CPAP significantly increased signal intensity changes within the medullary raphe, RVLM, dl pons and ventral midbrain (mean±SEM signal intensity pre-CPAP vs post-CPAP: raphe: -0.04±0.06 vs 0.39±0.14, p=0.009; left RVLM: -0.22±0.13 vs 0.13±0.15, p=0.02; left dl pons: -0.10±0.08 vs 0.50±0.20, p=0.008; right dl pons: 0.04±0.10 vs 0.37±0.12, p=0.03; ventral midbrain: -0.13±0.12 vs 0.52±0.28, p=0.02); and decreased signal intensity in the NTS (right NTS: 0.15±0.10 vs -0.24±0.15, p=0.009) (Figure 4). Additionally, the percentage increases in signal intensity within the medullary raphe and left RVLM during CPAP treatment were significantly correlated to the
percentage decrease in MSNA bursts during the fMRI scanning session (*raphe*: \( r=0.80, \ p=0.001 \);

*left RVLM*: \( r=0.56, \ p=0.04 \); *left dl pons*: \( r=0.12, \ p=0.68 \); *right dl pons*: \( r=0.20, \ p=0.51 \); *ventral midbrain*: \( r=0.02, \ p=0.95 \); *right NTS*: \( r=0.28, \ p=0.36 \)).

*Figure 3 near here*

Within the medullary raphe, left RVLM and left dl pons, signal intensity was significantly decreased in OSA subjects pre-CPAP compared with controls (controls vs OSA pre-CPAP: *raphe*: 0.17±0.09 vs -0.04±0.06, \( p=0.04 \); *left RVLM*: 0.26±0.14 vs -0.22±0.13, \( p=0.01 \); *left dl pons*: 0.19±0.07 vs -0.10±0.08, \( p=0.007 \)). No significant difference between controls and OSA pre-CPAP occurred in the NTS, right dl pons or ventral midbrain (controls vs OSA pre-CPAP: *NTS*: 0.23±0.09 vs 0.15±0.10, \( p=0.32 \); *right dl pons*: 0.19±0.05 vs 0.04±0.10, \( p=0.11 \); *ventral midbrain*: 0.12±0.12 vs -0.13±0.12, \( p=0.08 \)). Furthermore, within all of these brainstem regions, apart from the NTS, signal intensity returned to control levels post-CPAP (controls vs OSA post-CPAP: *NTS*: \( p=0.01 \); *raphe*: \( p=0.11 \); *left RVLM*: \( p=0.27 \); *right dl pons*: \( p=0.09 \); *left dl pons*: \( p=0.09 \); *ventral midbrain*: \( p=0.11 \)).

*Figure 4 near here*

**Functional overlap:**

To determine if there was a restoration of brainstem function in those brainstem regions that we have previously shown to be significantly reduced in OSA subjects prior to CPAP treatment compared with controls, we overlapped two statistical maps: controls > OSA pre-CPAP and OSA post-CPAP > OSA pre-CPAP. This resulted in three brainstem regions, the medullary raphe, left RVLM and left dl pons (Figure 5). Extraction of signal intensity changes during MSNA bursts compared with periods of no bursts confirmed that signal intensity returned to controls levels following CPAP treatment (mean±SEM signal intensity: controls, OSA pre-CPAP, OSA post-CPAP: *raphe*: 0.16±0.08, -0.11±0.06, 0.27±0.15; *left RVLM*: 0.24±0.10, -0.13±0.11, 0.13±0.09; *left
dl pons: 0.18±0.08, -0.12±0.08, 0.48±0.19). For each of these three regions, signal intensity was significantly reduced in OSA subject pre-CPAP compared with controls (raphe: p=0.008; left RVLM: p=0.01; left dl pons: p=0.007), increased significantly in OSA post-CPAP compared with pre-CPAP (raphe: p=0.04; left RVLM: p=0.02; left dl pons: p=0.006) to levels that were not significantly different to controls (raphe: p=0.27; left RVLM: p=0.22; left dl pons: p=0.09).

Figure 5 near here

Grey matter volume changes:
Comparison of grey matter volumes in OSA subjects revealed that CPAP treatment resulted in a significant reduction in grey matter volume in the medullary raphe extending into the RVLM and in the right and left dl pons (probability*volume±SEM: pre-CPAP vs post-CPAP: raphe: 0.30±0.02 vs 0.18±0.01, p=0.00001; right dl pons: 0.24±0.02 vs 0.13±0.01, p=0.00006; left dl pons: 0.20±0.01 vs 0.10±0.01, p=0.00009) (Figure 6, Table 1b). Furthermore, within all of these brainstem regions, grey matter was significantly different between controls and OSA pre-CPAP treatment (controls vs OSA pre-CPAP: raphe: 0.25±0.02 vs 0.30±0.02, p=0.04; right dl pons: 0.19±0.02 vs 0.24±0.02, p=0.04; left dl pons: 0.16±0.02 vs 0.20±0.01, p=0.047) and significantly reduced in OSA subjects post-CPAP compared with controls (raphe: p=0.01; right dl pons: p=0.01; left dl pons: p=0.003).

Figure 6 near here

Discussion:
Given that resting MSNA is driven primarily from brainstem structures, it is not surprising that altered brainstem function is associated with elevated MSNA in individuals with OSA. In a previous investigation we found that OSA is associated with altered MSNA-related activity changes in brainstem regions: the medullary raphe, rostral ventrolateral medulla and dorsolateral pons (Lundblad et al. 2014). We extend these earlier findings by showing that altered brainstem function
in OSA subjects can be reversed by 6 months of CPAP treatment, which is also associated with a significant reduction in resting MSNA. Signal intensity within the medullary raphe, rostral ventrolateral medulla and dorsolateral pons, which was significantly reduced in OSA subjects prior to CPAP treatment compared with controls, returned towards control levels following 6 months of CPAP treatment. Furthermore, grey matter increases that were observed in these brainstem regions in OSA subjects prior to CPAP treatment, reduced to control levels and even below following CPAP treatment. These data strongly suggest that functional and anatomical changes within the brainstem, which we believe underlies the elevated sympathetic activity in individuals with untreated OSA, can be restored to healthy levels by CPAP treatment.

We found that burst frequency fell by 31 bursts/min compared to only 10 bursts/min in a previous investigation by Narkiewitz et al (1999). We suspect we achieved a more impressive fall in MSNA because our subjects were more compliant with their use of CPAP, using them on average for five hours per night, as documented from the software within the machines. Moreover, great care was taken to ensure that all patients had individually fitted masks that they felt comfortable with, and were treated with pressures that were individually titrated. Compliance in the Narkiewitz et al (1999) study was self-reported; although the authors state that patients reported >75% compliance they do not know the number of sleeping hours each patient received CPAP therapy.

The MSNA changes associated with CPAP treatment were associated with functional and anatomical restoration in the medullary raphe encompassing the nucleus raphe obscures and pallidus. In anaesthetized animals, direct chemical stimulation of this raphe region evokes significant changes in arterial pressure and sympathetic nerve activity (Coleman and Dampney 1995; Henderson et al. 1998), which is likely mediated by direct projections to the RVLM (Ellenberger and Feldman 1994; Zagon 1995) or the sympathetic preganglionic neurones in the intermediolateral cell column (Aicher et al. 1994; Allen and Cechetto 1994; Bacon et al. 1990). Although caudal medullary raphe activity does not appear to regulate arterial pressure at rest, our
data strongly suggests that it regulates resting MSNA under pathological conditions, since signal intensity within this region is elevated at rest in controls and returns to an elevated level following CPAP treatment in OSA subjects. Furthermore, the activity restoration within this region during CPAP treatment was significantly correlated to the decrease in MSNA, strengthening our argument for a critical role of the raphe in OSA-related MSNA changes. Our data suggests that in individuals with untreated OSA, reduced medullary raphe activity results in elevated MSNA.

A similar pattern of signal intensity and anatomical changes also occurs in the region of the left and right dorsolateral pons. That is, signal intensity is reduced and grey matter elevated in untreated OSA and return to control levels following CPAP treatment. Similar to the medullary raphe, although chemical stimulation of the same region can evoke increases or decreases in arterial pressure and sympathetic nerve activity (Dampney 1994; Hade et al. 1988; Miyawaki et al. 1991), inactivation of the dorsolateral pons does not alter either resting arterial pressure or heart rate (Shafei et al. 2011). These dlPons neurons do not appear to project directly to spinal preganglionic sympathetic neurons (Barman et al. 1999), although they do project directly to the RVLM (Dampney 1994). It appears that both the dlPons and medullary raphe contain neurons which can modulate sympathetic activity and whose activity corresponds to a 10 Hz resting sympathetic activity discharge (Barman et al. 1995). Altered activity within these two brainstem regions would almost certainly affect resting sympathetic outflow and potentially underlie the increases in MSNA that occur in OSA.

The region of the RVLM also displayed a return to control levels following 6 months CPAP treatment and this return was significantly correlated to the reduction in resting MSNA. In addition to its role in baroreflex function, the RVLM is the major output nucleus from which almost all brain regions, including the cerebral cortex, control arterial pressure (Dampney et al. 2002; Gabbott et al. 2005). We had previously shown that BOLD signal intensity within the human homologue of the RVLM covaries with MSNA in healthy subjects (Macefield and Henderson 2010), as well as
patients with OSA (Lundblad et al. 2014). Curiously, however, while we had predicted that BOLD signal intensity would be higher in OSA - which would fit with the elevated MSNA seen in OSA - it was actually lower than in controls. Nevertheless, as we had discussed previously (Lundblad et al. 2014), given that BOLD signal intensity is considered to reflect synaptic activity (Logothetis et al. 2001) our counterintuitive observation of a decrease in signal intensity within RVLM in patients with OSA may indicate that active inhibition of RVLM is lower in OSA. If this is the case, then the increase in MSNA-coupled signal intensity within RVLM following successful treatment with CPAP must reflect an increase in active inhibition of the RVLM, bringing its total activity and hence MSNA down towards control levels. Furthermore, since the medullary raphe and dorsolateral pons project directly to the RVLM, it could be the case that RVLM functional restoration results from restoration of afferent inputs from these brainstem regions.

Somewhat surprising were the signal changes within the region of the NTS, another brainstem region known to influence RVLM activity through direct excitatory projections. Unlike the RVLM, dorsolateral pons or medullary raphe, signal intensity within the region of the NTS was not altered in individuals with OSA prior to CPAP treatment, but was significantly reduced by CPAP treatment to levels well below both control and pre-CPAP levels. Although this study provides the first evidence of restored brainstem function following CPAP, a number of studies have reported brainstem functional changes in the chronic intermittent hypoxia (CIH) model of OSA. More specifically, the elevated sympathetic activity and increased arterial pressure in CIH (Bao et al. 1997; Fletcher et al. 1992) is associated with neural activation in the NTS and RVLM (Greenberg et al. 1999; Knight et al. 2011).

Our data cannot determine the underlying cellular changes that result in grey matter volume restorations and indeed decreases relative to controls in the medullary raphe, RVLM and dorsolateral pons in OSA subjects following CPAP treatment. Since it has been observed that increased use can lead to an increase in grey matter volume within a relevant cortical region
(Amunts et al. 1997; Maguire et al. 2000; Sluming et al. 2002), it is possibly that the elevated MSNA in OSA subjects prior to CPAP treatment reflects a similar process. While we cannot determine the mechanisms responsible for increased and subsequent decrease in grey matter volume that occurs in OSA subjects, the finding that the medullary raphe, RVLM and dorsolateral pons display both functional and anatomical changes that subsequently reverse following CPAP treatment along with MSNA, strongly suggests that these changes are coupled.

Limitations:

There are some limitations to the present investigation. We did not follow control subjects over a 6 months period to examine reproducibility and we did not include OSA subjects that did not undergo CPAP treatment; though others have shown no MSNA change in OSA patients who did not receive CPAP when followed up over a year (Narkiewicz et al. 1999). Although the effects of such limitations cannot be examined, given the strong relationship in OSA subjects between the reduction in MSNA and functional and anatomical changes, we are confident that our results are not unduly influenced by these issues. Additionally, while we did not measure SaO₂ or end-tidal CO₂ in the OSA subjects before and after CPAP, we know clinically that they are neither hypercapnic nor hypoxic in the awake state. Accordingly, we are confident that levels of carbon dioxide or oxygen in the blood did not unduly influence our results. Finally, although diastolic pressure fell significantly, systolic did not. The elevated systolic pressure may reflect long-term vascular remodelling since aortic stiffness increases (Cortuk et al. 2014) and arteriolar endothelium-dependent vasodilatation is reduced in OSA (Kato et al. 2000); both of these mechanisms may contribute to the persistent increase in systolic pressure despite a marked fall in neurally-mediated vasoconstriction.

Conclusions:
Our results show that 6 months of CPAP treatment significantly reduces the elevated MSNA and the associated brainstem functional and anatomical changes associated with OSA. These data show that the underlying mechanisms responsible for elevated MSNA in OSA are indeed reversible and highlight the effectiveness of CPAP treatment in reducing one of the most significant health issues associated with OSA.
Acknowledgments:

All MRI scanning was conducted at Neuroscience Research Australia. This work was supported by the National Health and Medical Research Council of Australia (Grant 1007557). We are grateful to the assistance provided by Elie Hammam in these experiments, and to ResMed (Australia) for donating the CPAP machines for this study. The authors state that there are no conflicts of interest.
References:


Table 1: Location, T-score and cluster size for regions showing significant differences in (a) signal intensity changes coupled to spontaneous muscle sympathetic nerve activity and (b) grey matter volume in OSA subjects prior to and following 6 months of CPAP treatment. Cluster locations are given in Montreal Neurological Institute space.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>MNI Co-ordinate</th>
<th>cluster size</th>
<th>t-score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>y</td>
<td>z</td>
</tr>
<tr>
<td>(a) MSNA coupled fMRI signal intensity differences</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSA post CPAP&gt;pre-CPAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>medullary raphe</td>
<td>2</td>
<td>-38</td>
<td>-58</td>
</tr>
<tr>
<td>left rostral ventrolateral medulla</td>
<td>-6</td>
<td>-40</td>
<td>-46</td>
</tr>
<tr>
<td>right dorsolateral pons</td>
<td>6</td>
<td>-32</td>
<td>-28</td>
</tr>
<tr>
<td>left dorsolateral pons</td>
<td>-4</td>
<td>-36</td>
<td>-28</td>
</tr>
<tr>
<td>ventral midbrain</td>
<td>2</td>
<td>-20</td>
<td>-12</td>
</tr>
<tr>
<td>OSA post CPAP&gt;pre-CPAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>right nucleus tractus solitarius</td>
<td>-4</td>
<td>-46</td>
<td>-58</td>
</tr>
<tr>
<td>(b) Grey matter volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSA post CPAP&gt;pre-CPAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>medullary raphe</td>
<td>1</td>
<td>-34</td>
<td>-44</td>
</tr>
<tr>
<td>right dorsolateral pons</td>
<td>12</td>
<td>-39</td>
<td>-38</td>
</tr>
<tr>
<td>left dorsolateral pons</td>
<td>-12</td>
<td>-40</td>
<td>-37</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1: A) A typical microneurographic recording in an individual subject during concurrent functional magnetic resonance imaging (fMRI). Brain images were collected during a 4 seconds at which time muscle sympathetic nerve activity (MSNA) activity was not distinguishable. However, due to the fMRI hemodynamic delay (approximately 5 seconds) and the delay for MSNA traffic to travel from the brain to the recording electrode (approximately 1 second), brain activity during the MSNA collection period was reflected in signal intensity changes during the subsequent 4 second period. The vertical grey shading represents a 1 second period of MSNA recording and the associated 1 second period of fMRI image collection. Note the MSNA burst during the first second of the MSNA collection period. RMS: root mean square. B) During each 1 second epoch of the collection period (4 seconds), if a MSNA burst occurred, a “1” was entered into an fMRI search model. This was repeated for each of the 200 fMRI volumes to create a 200 point fMRI model. The MSNA burst in the above recording is entered into the fMRI search model (vertical grey bar). C) The MSNA burst that occurred during the first second of the collection period arose during collection of fMRI images through the medulla. This brainstem region is represented by the grey horizontal shading on a sagittal section of an individual’s fMRI images set.

Figure 2: A) Multiunit recording of muscle sympathetic nerve activity (MSNA) from a 45-year-old male patient with obstructive sleep apnea (OSA) during scanning of the brain, obtained prior to (upper panel) and following (lower panel) 6 months of CPAP treatment. The mean-voltage neurogram is shown in the nerve RMS (root mean square) trace; this was used to quantify the number of sympathetic bursts. The black areas represent the scanning artefacts. MSNA burst amplitudes were measured during the OFF periods. Asterisks indicate individual bursts of MSNA. Resting levels of MSNA were greatly reduced following continuous positive airway pressure
(CPAP) treatment. Heart rate was calculated from a piezoelectric pulse transducer on the fingerpad; respiration was monitored via a piezoelectric transducer around the abdomen. B) Total MSNA burst count during the 200-volume functional magnetic resonance imaging scan in controls, and in OSA subjects prior to and following 6 months of CPAP treatment. Note that CPAP results in a reduction in MSNA in all but 2 OSA subjects.

Figure 3: Brainstem regions in which fMRI signal intensity was significantly different in subjects with obstructive sleep apnoea (OSA) prior to, compared to following 6 months of continuous positive airway pressure (CPAP) treatment. Hot and cool colour scales indicate regions in which signal intensity changes were significantly increased or reduced following CPAP treatment, respectively. Significant clusters are overlaid onto a T1-weighted anatomical template image. Slice location in Montreal Neurological Institute space are indicated at the top right of each image. Note that CPAP treatment resulted in signal intensity increases in regions of the medullary raphe, rostral ventrolateral medulla (RVLM), dorsolateral pons (dlpons) and ventral midbrain. Signal decreased occurred in the dorsal medulla in the region of the nucleus tractus solitaries (NTS).

Figure 4: Plots of percentage signal intensity (SI) changes in regions in which signal intensity changes correlated to muscle resting sympathetic nerve activity (MSNA) were significantly different in subjects with obstructive sleep apnea (OSA) prior to, compared with following 6 months continuous positive airway pressure (CPAP) treatment. Graphs show mean (±SEM) SI changes during MSNA bursts compared with periods of no bursts in controls (black), OSA pre-CPAP (white) and OSA post-CPAP (grey). Note that, CPAP treatment results in a significant reduction in signal intensity during each MSNA burst in all six brainstem regions. Furthermore, these signal reductions return to control levels in all regions except for the nucleus tractus solitaries (NTS). * indicates p<0.05.
Figure 5: Brainstem regions in which signal intensity (SI) changes correlated to muscle sympathetic activity (MSNA) were significantly reduced in obstructive sleep apnoea (OSA) subjects prior to continuous positive airway pressure (CPAP) treatment compared with controls, which then increased significantly to control levels following CPAP treatment. Significant clusters are overlaid onto a T1-weighted anatomical template image. Slice location in Montreal Neurological Institute space are indicated at the top right of each image. Graphs show mean (±SEM) SI changes during MSNA bursts compared with periods of no bursts in controls (black), OSA pre-CPAP (white) and OSA post-CPAP (grey). * indicates p<0.05.

Figure 6: Brainstem regions in which grey matter volume (GM) was significantly greater in subjects with obstructive sleep apnea (OSA) prior to continuous positive airway pressure (CPAP) treatment compared to following 6 months CPAP treatment. Significant clusters are overlaid onto a T1-weighted anatomical template image. The cerebellum is shaded since it was not included in the analysis. Slice location in Montreal Neurological Institute space are indicated at the top right of each image. dl pons: dorsolateral pons; RVLM: rostral ventrolateral medulla. To the lower right are plots of GM in controls (black), OSA pre-CPAP (white) and OSA post-CPAP (grey) subjects. * indicates p<0.05.
A

MSNA (raw nerve)

10µV

MSNA (RMS nerve)

1µV

MSNA collection period (4 secs)
fMRI collection period (4 secs)

MSNA burst

1 sec

B

MSNA fMRI model (second 1)

C

fMRI image region (second 1)
A

OSA pre-CPAP

nerve

20 μV

nerve RMS

1 μV

heart rate

100 bpm

pulse

85

respiration

OSA post 6 months CPAP

nerve

20 μV

nerve RMS

1 μV

heart rate

75 bpm

pulse

65

respiration

B

MSNA bursts (total during fMRI scan)

controls OSA pre-CPAP OSA post-CPAP