Baroreflexes of the Rat. VI. Sleep and responses to aortic nerve stimulation in the dmNTS

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ABSTRACT

The sensitivity of the baroreflex determines its stability and effectiveness in controlling blood pressure (BP). Sleep and arousal are reported to affect baroreflex sensitivity, but the findings are not consistent across studies. After statistically correcting the effect of sleep on the baselines in chronically neuromuscular blocked (NMB) rats, we found that sleep affects BP and heart period (HP) baroreflex gain similarly. This finding is consistent with baroreflex modulation of HP and BP occurring at a location before the divergence of the sympathetic and parasympathetic pathways. Therefore, we hypothesized that the gain modulation occurs in the dorsal medial nucleus of the solitary tract (dmNTS). The present study used long-term dmNTS recordings in NMB rats and single pulse aortic depressor nerve (ADN) stimulation. Under these conditions, the magnitude of A-fiber evoked responses (ERs), recorded from the second- or higher-order dmNTS baroreflex neurons, was reliably augmented during high amplitude, low frequency EEG (slow-wave sleep) and reduced during low amplitude, high frequency EEG (arousal; $\Delta ER = 11\%$, $t = 9.49$, $p < 0.001$, $df = 1016$). This result has methodological implications for techniques that use changes in heart period to estimate baroreflex blood pressure gain, and general implications for understanding the relationship between sleep and cardiovascular control.

Keywords: baroreflex gain, baroreflex sensitivity, nucleus of the solitary tract, sleep, arousal, aortic depressor nerve
Introduction

The negative feedback baroreflexes have a central role in regulating blood pressure (BP), and baroreflex sensitivity or gain determines the stability and effectiveness of BP control. Sleep and arousal are reported to affect baroreflex sensitivity, but the findings are not consistent across studies that differ in experimental methodology and/or species: With the Oxford method, a vasoconstrictive drug elevates BP, and the cardiac baroreflex gain is defined as heart rate change (ΔHR) divided by the BP change (ΔBP). Pickering and others reported clear evidence of greater cardiac baroreflex gain during stages II and IV slow-wave sleep in humans, compared to the awake stage (12-13, 16). Using a non-invasive correlational method that deduces the baroreflex gain from spontaneous BP and heart rate sequences, Parati et al., (11) also found that gain increased during sleep; but, in studies in chronic animal that used a similar sequence method, but with more sophisticated criteria for sleep, Zoccoli et al., (21) found that, for rats, gain was substantially independent of states of wakefulness, active sleep, and quiet sleep.

The Oxford and the sequence methods both measure BP effects on heart rate, i.e., the baroreflex cardiac gain, but do not directly measure either the vasomotor baroreflex gain, or the total loop gain. This is because, doing the former requires implanted flowmeters, and the latter requires direct access to the baroreceptors. Thus, in practice to make inferences to total loop gain, which is the variable of interest, the cardiac reflex is taken as a surrogate for the overall reflex, and it is assumed that changes in cardiac gain proportionally represent changes in loop gain; however, the literature does not consistently support this assumption: For example, in chronic dog studies, implanted carotid sinus nerve stimulators elicit 12-22% greater bradycardia during sleep, but sleep produces no change in the net BP depressor effect (20); in baboons, during sleep, compared to the awake resting state, although the carotid occlusion (baroreceptor
inhibition) effect on heart period (HP) is increased, lower abdominal conductance is unaffected, and the renal conductance response to occlusion is attenuated (1). In an earlier study, using chronically neuromuscular blocked (NMB) rats, which have distinct diurnal and sleep-wakefulness cycles, we (4) compared the effects of electroencephalographic arousal on the open-loop HP and BP baroreflex gains: Using the power of the delta band of the electroencephalogram (EEGδ) as an index of sleep and arousal, and aortic depressor nerve (ADN) electrical stimulation as a baroreflex input, we observed that although the HP baroreflex gain increased with sleep, the open-loop BP baroreflex gain (the decrease in BP to the ADN test stimulus) did not. However, upon further analysis, we discovered that the raw correlational result was misleading: A partial-correlations analysis, which also took into account the effects of sleep on BP level, showed that because the BP level was directly related to arousal, and BP baroreflex gain was directly related to BP level, the effect of sleep on BP gain had been “masked”. But, HP gain was different: although, as with BP, HP level depended on arousal, HP baroreflex gain did not depend on HP level. By mathematically “partialing out” the BP level effects on the baroreflex BP gain, the underlying relationship between EEGδ and BP baroreflex gain was shown to be approximately the same as that for HP baroreflex gain. Because the effects of sleep on HP and BP gain were almost exactly the same, we hypothesized that the modulation occurred at a common anatomical locus, before divergence of the parasympathetic and sympathetic pathways. Testing that hypothesis led to the present study.

The parasympathetic (cardiac chronotropic) and sympathetic (vascular tone; cardiac inotropic) baroreflex pathways diverge at the dorsal medial nucleus of the solitary tract (dmNTS). For the parasympathetic, the baroreflex dmNTS neurons project to the nucleus ambiguous (NA), and the dorsal motor nucleus of vagus (dmnX), then to the heart. Whereas, for
the sympathetic, the dmNTS neurons project to the caudal ventrolateral medulla (CVLM), the rostral ventrolateral medulla (RVLM), the sympathetic preganglionic neurons (SPN) at the intermediolateral column of the spinal cord, and then to the peripheral vascular, renal, and cardiac sympathetic nerves. The post-dmNTS divergence provides ample opportunity for modulating influences from sleep and arousal to have separate effects on the baroreceptor parasympathetic and the sympathetic reflexes; however, our partial correlations analysis indicated that the effects were, in fact, quite similar (see the discussion of (4) for details and references). In the present study, we extended the NMB rat preparation, to enable direct measurement of the magnitude of single pulse ADN-stimulation-elicited evoked responses (ERs) from the second- or higher-order baroreflex neurons in the dmNTS. Sleep and arousal were respectively defined as epochs of high amplitude, low frequency; and low amplitude, high frequency EEG. Using these definitions, we found clear evidence that the ER magnitude was reliably augmented during sleep.

**Methods**

Twelve female Sprague-Dawley rats, weighting 230-270g, were studied one at a time, monitored continuously, and attended around the clock. These same rats were used in (17-18). All acute surgery, or any possible irritating manipulation, such as replacement of the bladder cannula or adjustment of the tracheal cannula, was done with accurately controlled and carefully monitored >1.5% deep isoflurane anesthesia. All procedures used in the experiment were approved by the Institutional Care and Use Committee of the Pennsylvania State University College of Medicine, and comply with the American Physiological Society’s “Guiding Principles
General information about the preparation  The surgical details are in (17); briefly, neuromuscular block was induced on the first day (100 µg iv. α-cobrotoxin) and maintained by continuous infusion (250 µg/day, iv). Mechanical ventilation was at 72 breaths/min: Inspiratory: Expiratory = 1:2; positive end-expiratory pressure = 3 cmH2O; minute volume = 180-220 ml and hyperinflation (anti-atelectasis) of 18 cmH2O (2s/10min). The EEG electrodes were two 0-80 s/s screws, 2.0 mm from the midline in the left parietal bone (1.0 mm anterior to bregma, and to lambda). There was a transurethral bladder cannula, subcutaneous EKG electrodes (#30 silver wire), Teflon femoral artery and silicone vein cannulae. Core temperature (vaginal) was servo-regulated at 37°C. The left aortic depressor nerve (ADN) was dissected and set on an anodized Ta-Ta2O5 capacitance electrode (17) and imbedded in a silicone compound (Kwik-Sil, WPI, Sarasota, FL): This electrode arrangement enables thousands of stimulations without detectable damage to the ADN. The baroreflex area in the dmNTS was located using atlas coordinates, as well as the somatotopic features of the gracile nucleus, which at the search coordinates is 50 -150 µm dorsal to the baroreflex cells of the dmNTS (17). The multi-unit dmNTS recording was with 1-2 MΩ glass insulated tungsten microelectrodes (Alpha-Omega, GA) mounted in a FHC hydraulic probe drive (50-16-1, FHC, ME), held in a digital stereotaxic carrier. Signals were amplified (gain = 20k; bandpass = 0.3-3 kHz; XCELL-3 X 4, 40-#40-8B, FHC) and digitized at 10 kHz. A typical preparation was maintained for an average of 14 days; the data used in this study were acquired on days 4-9. After healing of the surgical incisions and stabilization, which typically required 48-72 hrs, the isoflurane concentration was set at ≤0.5%, which is an analgesic level consistent with patterned sleep.
**EEG sleep/arousal identification:** From the bandpass filtered (0.1-100 Hz) EEG, two kinds of sleep/arousal cycles were identified (Fig.1): from Sleep to Wakefulness (S-W), and from Wakefulness to Sleep (W-S). The S-W cycles were identified as approximately symmetrical periods of high amplitude low frequency EEG activity, followed by low amplitude high frequency activity; and correspondingly, the W-S cycles were symmetrical periods of low amplitude high frequency, followed by high amplitude low frequency activity. Because of the neuromuscular block, electromyogram (EMG) or electrooculogram (EOG) criteria could not be used to distinguish arousal from rapid eye movement (REM) sleep; thus, to assure accurate identification of sleep/arousal cycles, we imposed several additional constraints: (1) cycle start-points were set at least 2 minutes after an EEG transition was complete, and cycle stop-points at least 2 minutes before the next transition. To eliminate the need to interpret ambiguous mid-cycle EEG transitions (the dashed oval in Fig.1), we used ERs from only the first 25% of the cycle after the cycle start-point, and the last 25% before the cycle stop-point, i.e., Q1 and Q4 in (Fig. Appendix 1). (2) For a cycle to be valid, it must not have had any abrupt (>25%) change in BP or HP within the cycle, except at the mid-cycle transition. (3) Complete cycles had durations of >10 and <40 min (see details in the Appendix). Fig. 1 shows a typical recording from an individual rat of EEG, cardiac inter-beat-interval, arterial blood pressure, and venous blood pressure. Six sleep/wakefulness sequences, 3 S-W, and 3 W-S, were identified in this example record.

*Average EEG power:* is the root-mean-square (RMS) of the bandpass filtered (0.1-100 Hz) EEG signal.

*Magnitude of the A-fiber ERs:* Fig. 2 is a typical example of a stimulus triggered ensemble-averaged A-fiber ER trace from a dmNTS recording site. Using electrophysiology,
pharmacology, and histology, we verified that the recordings were from second- or higher-order dmNTS baroreflex neurons and not from ADN fibers in the solitary tract (see details in (17-18)). The A-fiber ER is the activity in the post-stimulus latency range of 4-20 ms. Because the Pythagorean distance between the dmNTS recording and ADN stimulation sites is >11.5 mm, and only A-fibers conduct at >2 m/sec (5), it is virtually certain that any activity arriving (at the dmNTS) within 20 ms is propagated in A-fibers, see detail in (17).

The magnitude of A-fiber ERs was determined (from a stimulus-triggered, at $t_s$ ($t_s=0$ in Fig. 2), ensemble-averaged ER trace) as follows: After removing DC and rectifying, the signal in the interval of $(t_s+4)$ to $(t_s+20)$ ms, and in the corresponding interval symmetrically reflected around the trigger, $((t_s-20)$ to $(t_s-4)$ ms; i.e., the baseline) were integrated. The A-fiber ER magnitude on a trial was defined as the difference between the ER area and the corresponding baseline area:

$$\text{Mag}_{ER} = \int_{t_s+0.04}^{t_s+0.2} |\text{Signal}| - \int_{t_s-0.2}^{t_s-0.04} |\text{Signal}|$$

**Experimental Protocol:** Using a transduction curve analysis (see detail in (17)), we characterized the relationships of the ADN stimulus amplitude to the magnitude and latency of the ER complex, and chose a test amplitude near the center of the linear range of the amplitude-magnitude curve. To evaluate the effects of sleep/arousal on the magnitude of A-fiber ERs, we stimulated the ADN, with single current pulses (300 µs, pseudorandom interpulse interval = 6 ± 1 s) at the test amplitude, and recorded the A-fiber ERs at a constant location in the dmNTS.

**Data Acquisition and Analysis:** Data were acquired using Spike2® software and Power 1401 hardware (Cambridge Electronic Design, Cambridge, UK). For EEG and ER results, to
pool results across sleep/arousal cycles and rats, the data were represented as percentages of the cycle mean activity: Each cycle was divided into 4 quarters, the magnitudes of ensemble averaged A-fiber ERs and RMS powers of the EEG were calculated for each quarter; the mean of the 4 quarters in the cycle was determined, and the ratios of the measurement from each quarter to the mean for the 4 quarters were calculated for each variable, and multiplied by 100 (see detail in Appendix). The average value of each cardiovascular variable, i.e., systolic blood pressure (sBP), diastolic blood pressure (dBP), heart period (HP), was determined for each quarter cycle. Data were pooled across all rats, and means and SE calculated for each variable.

Statistical Analysis: Two-tailed Student’s t-tests were used for statistical analysis (SAS, version 9.13).

Results:

Twelve rats were analyzed; one rat was excluded, because of insufficient data; 1017 sleep cycles (505 S-W; 512 W-S) were identified in the 11 rats. The average number of cycles (mean ± SE) per rat was 46 ± 5 for S-W and 47 ± 6 for W-S; and average duration was 27 ± 1.73 min and 24 ± 1.23 min for S-W and W-S cycles, respectively. We found no effect of cycle sequence order, i.e., S-W vs. W-S, on the sleep/arousal effects on any cardiovascular variable or on the magnitude of the A-fiber ERs (Table 1); thus, we combined the S-W and W-S data. Over the 1017 cycles, during sleep (high-amplitude low-frequency EEG) as compared to arousal (low-amplitude high-frequency EEG), the EEG power was higher (ΔEEG = 38%, \( t=93.66 \), \( p<0.001 \), \( df=1016 \)); the magnitude of single pulse ADN stimulation elicited A-fiber dmNTS ERs was larger (ΔER = 11%, \( t=9.47 \), \( p<0.001 \), \( df=1016 \); systolic and diastolic blood pressures, (sBP and dBP) were lower (ΔsBP = -14 mmHg, \( t=-33.67 \), \( p<0.001 \), \( df=1016 \); ΔdBP = -10 mmHg, \( t \)
= -28.1, \( p < 0.001, \text{df} = 1016 \); and the heart period (HP) was longer (\( \Delta \text{HP} = 9 \text{ ms}, t = 14.4, P < 0.0001, \text{df} = 1013 \) (Fig. 3).

Table 2 summarizes the sleep/arousal effects for all 11 rats on sBP, dBP, HP, and A-fiber dmNTS ERs. Fig. 3 is the graphic representation of the data in Table 2. In 9 of the rats (i.e., rats 1-9 in Table 2), taken individually, the magnitude of A-fiber dmNTS ERs was reliably larger during the high amplitude low frequency EEG (sleep) compared to the low amplitude high frequency EEG (arousal) periods; in two rats (i.e., rats 10 and 11 in Table 2), the A-fiber ERs did not change significantly.

Fig. 4 depicts the effects of sleep/arousal on (a) baroreflex gain for heart period (HP), (b) baroreflex gain for blood pressure (BP), and (c) magnitude of A-fiber dmNTS ERs. Fig 4 (a) and (b) are from (4), and each point represents the RMS (root mean squared) amplitude of 1 min EEG \( \delta \) band activity (EEG\( \delta \)) and the relative ADN-HP, and ADN-BP open-loop gain ratios during the same minute. The open-loop gain ratio was calculated as the systolic BP or HP change from baseline during the ADN stimulus, divided by the mean change for all responses to that kind and amplitude of stimulus, e.g., 35 impulses/s, 20 \( \mu \)A ADN. Variables were converted to “z-scores” by subtracting the individual rat’s mean from each measure and dividing by the corresponding standard deviation (see (4)). Each point in (c) represents the EEG activity of each quarter period and the magnitude of A-fiber dmNTS ERs of that same quarter period, and all sleep, arousal, as well as the mid-cycle transitional quarter periods were included. Fig. 4 shows that (a) HP baroreflex gain increased with sleep (\( r = 0.315; \text{df} = 424; p<0.0001 \); (b) the uncorrected BP baroreflex gain was independent of sleep/arousal (\( r = 0.001; \text{df} = 424; \text{NS} \); and (c) as predicted in (4), the magnitude of A-fiber dmNTS ERs was increased with sleep (\( r = 0.2; \text{df} = 4067; p< 0.0001 \)).
Discussion:

Studies of modulation by sleep and arousal of baroreflex sensitivity, across different species, and/or experimental methodologies (10, 19, 21) do not agree. To understand why, it is necessary to know where and how the modulation occurs. In the present study we have, for the first time, directly measured the effects of naturally occurring cycles of sleep and arousal on the open-loop baroreflex sensitivity at the dmNTS, and have found clear evidence that sensitivity increases during slow wave sleep.

Slow wave sleep is distinguishable from wakefulness by EEG criteria; however, distinguishing wakefulness from desynchronized, rapid eye movement, sleep is more problematical. Conventionally, electromyogram (EMG) and/or electrooculogram (EOG) signals are used to help distinguish the states; but, paralysis by NMB precluded those criteria; thus, to minimize possible mis-identification, we restricted sampling to only stable, non-transitional, periods of sleep and wakefulness: It is known that, in cat, the end of a desynchronized episode in one cycle through the end of desynchronized episode in the subsequent sleep cycle includes a regular progression of component states: Arousal, slow-wave sleep, transition state and desynchronized sleep (8); a similar pattern, including slow-wave sleep, rapid eye movement sleep and arousal states, is seen in freely moving rats (15). Similarly, over days or weeks, NMB rats have stable regular alternating sleep-arousal patterns, within which, we were able to identify clear sleep-to-wakefulness (S-W), and wakefulness-to-sleep (W-S) sequences. To control for possible sequence order effects, we first analyzed the S-W and W-S sequences separately, and, finding no reliable difference, pooled the S-W and W-S data. Irrespective of absolute cycle length, each sequence was divided into quarters; wherein the first and last quarters were the S or
W phases used in the analyses, and the two mid-cycle quarters were designated as transitional, and except for Fig 4(c), the transitional quarters were excluded from the analysis. Although our criteria possibly might be seen as too stringent, they offered, in exchange for a loss of half of the data, a high degree of confidence that the identification of the states was as objective as possible.

Because the baroreflex is a closed loop, the overall loop gain, which is the decrease in BP per unit increase in receptor pressure (mmHg/mmHg), cannot be directly measured without opening the loop and isolating the receptors. An open loop requires surgical interventions, which are technically difficult, and in humans ethically unacceptable. Instead, most studies have estimated relative baroreflex gain from heart rate, either by manipulating receptor pressure and measuring corresponding changes in rate (12-13); or by statistically analyzing the sequential relationship of natural pressure and rate fluctuations (11). Both of these methods assume that the cardiac rate response is representative of, and affected in parallel with, the overall baroreflex depressor response; however, some studies suggest that this assumption may not be correct (20).

Previously (4), using NMB rats, with ADN electrodes, but without dmNTS brainstem recordings, we found that the heart period (HP) gain was greater during periods of greater EEGδ (Fig. 4 (a)), but that the BP gain was unaffected by the EEGδ (Fig. 4 (b)); this result appeared to show differential effects of sleep on the two efferent baroreflex mechanisms. A true differential effect would imply that HP gain was not a good surrogate for BP gain. Although the raw variables gave different results, sleep also lowers both the heart rate and BP baseline, and a partial correlations analysis showed that the apparent difference could be explained by an inverse dependence of the BP gain, but not the HP gain, on the corresponding baseline level (4). The difference between the HP and BP gains was an artifact of measurement, and not a physiologically relevant effect: the lowered baseline BP is closer to the hydrostatic ‘floor’ and
responses at lowered baseline pressure have a higher probability of being ‘clipped’ or limited by the ‘floor’. In (4; see Fig. 7), after partial correlations correction for BP baseline, the relationship between BP gain and “EEG slow wave activity” was 0.26, which is comparable to the 0.20 value that we have found for the correlation between EEG and the NTS neural ER in the present study. The partial correlations analysis thus showed that the HP and BP gains were affected approximately in parallel.

If the underlying effect of sleep on both HP and BP gain is similar, this would be consistent with a common site of modulation; and indeed, other physiological and neurophysiological evidence suggested that the dmNTS was the common site: Hypothalamic defense area stimuli inhibit carotid or ADN elicited baroreflex bradycardia and depressor responses (2, 6-7), and dmNTS cells with excitatory inputs from the baroreceptor sinus nerve and inhibitory inputs from hypothalamic defense area, are hyperpolarized by hypothalamic defense area stimulation in a way that shunts sinus nerve evoked excitatory postsynaptic potentials (9). These data led us to hypothesize that arousal attenuates baroafferent transmission through the dmNTS (4).

To examine that hypothesis, we added dmNTS recordings to the cardiovascular and EEG measures, and found that natural sleep modulates transmission of baroreceptor activity in the dmNTS: as predicted, the magnitude of A-fiber ERs from the second- or higher-order dmNTS baroreflex neurons, elicited by single pulse ADN A-fiber stimulation, was reliably augmented during high amplitude low frequency EEG, and reduced during low amplitude high frequency EEG (Fig. 4(c)). We have thus confirmed the conjecture of the partial correlations analysis (4) by direct physiological measurement.
**Perspectives:** Doba and Reis (3) found that lesions in the solitary tract nucleus (NTS), produced “fulminating hypertension”, which was “abolished by mid-collicular decerebration”. Reis initially thought that he had found an important locus of integration of excitatory and inhibitory influences on blood pressure (14), but as evidence of a more extensive and anatomically distributed baroreflex began to emerge, his supposition appeared to be wrong. With time, the NTS came to be regarded by most physiologists as a “relay”, rather than an important integrative center. However, in concordance with studies in acute and slice preparations, our recent report in NMB rats, of long-term potentiation (LTP) of the baroreflex input, and now, of sleep/wakefulness dependence of ADN elicited evoked responses in the NTS, affirms that there is functionally important modulation of the baroreceptor input at or before the nucleus of the solitary tract.

**Grants:**

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Appendix: Identification and analysis of sleep cycles: Because we did not find a reliable automated procedure for paralyzed rats, individual sleep cycles were identified by manual scanning of the EEG record. Our specific goal was limited to comparison of sleep and wakefulness; thus, by rigorously defining a procedure for parsing the cycles into sleep and wakefulness periods, we sought to eliminate systematic bias that could influence the results. When a putative EEG sleep cycle was identified, to minimize ambiguity at the transitions, the start and end points, designated as $t_0$ and $t_{\text{end}}$, were set at 120s later and earlier than the visually apparent start and end points of the cycle, i.e. the first and last two minutes of data were discarded (Fig. Appendix 1). Sleep cycles have variable durations (Fig. 1), and the cycle duration was defined as $(t_{\text{end}} - t_0)$. Somewhere in the mid-section of a cycle there is a transition from sleep to wake, or wake to sleep, but the precise point of transition is often not distinct; thus, to eliminate the need for a judgment, we divided the cycle into quarters (i.e., Q1-Q4); discarded the data from the second and third quarters, and took the data for analysis only from the first and fourth quarters. Thus, given that the duration of a quarter cycle was $(t_{\text{end}} - t_0)/4$, the data were taken from the intervals, $[t_0]$ to $[t_0 + (t_{\text{end}} - t_0)/4]$, and $[t_{\text{end}} - (t_{\text{end}} - t_0)/4]$ to $[t_{\text{end}}]$. The corresponding EEG activity was calculated as follows:

\[
EEG_{Q1} = \sqrt{\frac{4}{(t_{\text{end}} - t_0)} \int_{t_0}^{t_{\text{end}} - (t_{\text{end}} - t_0)/4} [V_{\text{EEG}}]^2 dt} \quad \text{(for the first quarter, Q1)}
\]

and

\[
EEG_{Q4} = \sqrt{\frac{4}{(t_{\text{end}} - t_0)} \int_{t_{\text{end}} - (t_{\text{end}} - t_0)/4}^{t_{\text{end}}} [V_{\text{EEG}}]^2 dt} \quad \text{(for the fourth quarter, Q4)}
\]

where $V_{\text{EEG}}$ is the instantaneous EEG amplitude.

To enable pooling of EEG data across cycles and rats, the EEG activity for each quarter was converted to a percentage using the mean of the 4 quarters as the base,
Thus, $EEG_{Qn} \%$, is a measure of the relative magnitude of EEG activity during quarter $Q_n$ as compared to the average EEG magnitude of the four quarters, and $EEG_{Qn} \%$ is a random variable, which is $>100\%$ when the magnitude of EEG activity during $Q_n$ is greater than the average EEG magnitude of the four quarters, and $<100\%$ when the magnitude of EEG activity during $Q_n$ is less than the average EEG magnitude of the four quarters.

**Analysis of ERs:** Similar to the EEG analysis, to pool ER data across cycles and rats, the magnitude of ER for each quarter was converted to a percentage using the mean of the 4 quarters as the base:

$$Mag_{ER_{Qn}} \% = \left( \frac{\sum_{i=1}^{4} Mag_{ER_{Qn_i}}}{4} \right) \times 100\% \quad (n = 1, 2, 3, \text{ and } 4)$$

Thus, $Mag_{ER_{Qn}} \%$, is a measure of the relative magnitude of ER activity during $Q_n$ as compared to the average ER magnitude of the four quarters, and $Mag_{ER_{Qn}} \%$ is $>100\%$ when the magnitude of ER during $Q_n$ is greater than the average ER magnitude of the four quarters, and $<100\%$ when the magnitude of ER during $Q_n$ is less than the average ER magnitude of the four quarters.
The only exception to restricting the analysis to Q1 and Q4 was for the correlational analysis in Fig. 4, which compares the neural evoked responses of the present study with the cardiovascular evoked responses in (4). Because, in the earlier study, we did not classify the sleep state, using all four quarters for this figure better aligned the evoked response with the cardiovascular data.
Figure Legends:

Fig. 1 A typical recording from an individual rat of electroencephalogram (EEG), heart period (HP), femoral arterial blood pressure (ABP), and venous blood pressure (VBP). Three sleep to wakefulness (S-W) and wakefulness to sleep (W-S) cycles were identified in the figure: The S-W cycles were identified as approximately symmetrical periods of high amplitude low frequency EEG activity, followed by low amplitude high frequency activity; and correspondingly, the W-S cycles were symmetrical periods of low amplitude high frequency, followed by high amplitude low frequency activity. The start and end points of each cycle were defined at 120s after and before the visually apparent start and end points of the cycle. The dashed oval designates a typical mid-cycle EEG transition (c.f. Fig. Appendix 1).

Fig. 2 A typical example of a stimulus triggered ensemble averaged (n = 179) A-fiber ER trace from a dmNTS recording site. The ADN was stimulated with single pulses (300 µs, interpulse interval = 6 ± 1 s) at 10 µA. The Pythagorean distance between the dmNTS recording and ADN stimulation sites is >11.5 mm, because only A-fibers conduct at >2 m/sec (5), it is virtually certain that any activity seen at the dmNTS electrode <20 ms post-stimulus was propagated in A-fibers. For additional details see (17).

Fig. 3 A summary of sleep/wake differences in (A) EEG activity and A-fiber dmNTS ERs (see Appendix for calculation procedure of percentages), (B) sBP and dBP and (C) HP for the 11 rats. EEG: electroencephalogram; sBP: systolic blood pressure; dBP: diastolic blood pressure; HP: heart period; ERs: evoked responses. Open columns represent measurement variables during
sleep, and filled columns during wakefulness. Compared with wakefulness, during sleep, magnitude of EEG activity, and ERs were higher, sBP and dBP were lower, and HP was longer. Measurements: mean ± SE. “*” indicates that the $p \leq 0.0001$.

Fig. 4  Effect of sleep/wakefulness on (A) relative baroreflex gain for heart period; (B) “uncorrected” relative baroreflex gain for blood pressure (see details in text and (4)); and for (C) single pulse ERs in the dmNTS. Each point in (A) and (B) represents the root mean squared (RMS) amplitude of 1 min EEG$_{\delta}$ and the averaged evoked cardiovascular responses to a standard stimulus test train during the same minute ((A): $r = 0.315$; $df = 424$; $p<0.0001$). In (B), the gray dashed line shows the slope of the partial correlations corrected relationship between EEG$_{\delta}$ and blood pressure gain, and the black solid line shows the slope of uncorrected relationship between EEG$_{\delta}$ and blood pressure gain (r = 0.26; $df = 424$; $p<0.0005$, for the gray dashed line; and r = 0.001; $df = 424$; NS, for the black solid line). Each point in (C) represents the magnitude of EEG activity for each quarter period of a sleep/arousal cycle and the ER magnitude of the same quarter period in the cycle ($r = 0.2$; $df = 3387$; $p<0.0001$). (A) and (B) are from Fig. 7 in (4) with the gray dashed line newly added in (B) to indicate the difference in relationship between EEG$_{\delta}$ and blood pressure gain before and after the partial correlations correction: After partial correlations correction for blood pressure baseline, the relationship between BP gain and “EEG slow wave activity” was 0.26, which is comparable to the 0.20 value that we have found for the correlation between EEG and the NTS neural ER in the present study.

Fig. Appendix 1  Identification of cycles and isolation of sleep and wakeful stages for analysis. The S-W cycles were defined by approximately symmetrical periods of high amplitude low
frequency EEG activity, followed by low amplitude high frequency activity; W-S cycles were symmetrical low amplitude high frequency, followed by high amplitude low frequency activity.
The typical S-W cycle above shows the defined components of sleep and wakefulness stage (see details in the Appendix): Transitions are often ambiguous; thus, to minimize errors of judgment, the start and end points, designated as \( t_0 \) and \( t_{\text{end}} \), were truncated at 120s after and 120s before the visually apparent start and end points of a cycle (dashed vertical lines). The truncated cycle was divided into four equal time intervals. Visual identification of a cycle depends upon observing a transition; thus somewhere in Q2 or Q3, a transition occurs from sleep to wake, or wake to sleep; however, it is sometimes difficult to designate exactly where; thus, to eliminate errors of judgment, and make the data selection as objective as possible, we discarded the data from Q2 and Q3, and used only those from Q1 and Q4 for analysis (Fig. 4 was an exception: because identification of the mid-cycle transition was unnecessary, all four quarters were used).
In the example, Q1 is a sleep stage (S), and Q4 is wakefulness stage (W). \( Q_n \) represents the \( n^{th} \) quarter. S-W is the visually identified sleep to wakeful cycle.

**Table Legends:**

Table 1  ERs: (ADN A-fiber dmNTS) evoked responses; sBP: systolic blood pressure; dBP: diastolic blood pressure; HP: heart period. Measurements: (mean ± SE). The magnitude of A-fiber ERs during a quarter period of sleep or arousal was given as a percentage of the mean magnitude of A-fiber ERs for the 4 quarters in that S-W or W-S cycle (see detailed calculation in the Appendix). NS: not significant.

Table 2  The sleep/arousal effects on A-fiber ERs in the dmNTS, sBP, dBP and HP were evaluated in 11 rats. Individually, compared to arousal (low amplitude high frequency EEG
periods), the magnitude of A-fiber dmNTS ERs was significantly increased during sleep (high amplitude low frequency EEG periods) in rats 1-9; and decreased (not significantly) for rats 10 and 11 (p values are in the last column of Table 2). Over all 11 rats, sleep and arousal had significant effects on every variable, i.e. the A-fiber dmNTS ERs, sBP, dBP, and HP. * indicates that $P \leq 0.0001$. EEG: electroencephalogram; sBP: systolic blood pressure; dBP: diastolic blood pressure; HP: heart period; ERs: (ADN A-fiber dmNTS) evoked responses.
Stimulation Artifact

A-fiber group ERs

Sweep #: 179
Table 1. Cycle sequences of S-W and W-S have no influence on the sleep/arousal effects on the magnitude of dmNTS A-fiber ERs, and measured cardiovascular variables over the 11 rats.

<table>
<thead>
<tr>
<th></th>
<th>ERs (%)</th>
<th>sBP (mmHg)</th>
<th>dBP (mmHg)</th>
<th>HP (ms)</th>
<th>ERs (%)</th>
<th>sBP (mmHg)</th>
<th>dBP (mmHg)</th>
<th>HP (ms)</th>
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<td><strong>S-W</strong></td>
<td>106±0.93</td>
<td>131±0.74</td>
<td>80±0.7</td>
<td>164±1</td>
<td>95±0.94</td>
<td>143±0.8</td>
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<td><strong>W-S</strong></td>
<td>105±1.02</td>
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<td>78±0.85</td>
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The High Amplitude Low Frequency EEG Periods (Sleep) | The Low Amplitude High Frequency EEG Periods (Arousal)
Table 2: The sleep/wakefulness effects on the magnitude of A-fiber ERs in the dmNTS.

<table>
<thead>
<tr>
<th>Rats</th>
<th>Cycles Length (min)</th>
<th>Sleep EEG (%)</th>
<th>Wake EEG (%)</th>
<th>Sleep sBP (mmHg)</th>
<th>Wake sBP (mmHg)</th>
<th>Sleep dBp (mmHg)</th>
<th>Wake dBp (mmHg)</th>
<th>Sleep HP (ms)</th>
<th>Wake HP (ms)</th>
<th>Sleep ER (%)</th>
<th>Wake ER (%)</th>
<th>S vs. W</th>
<th>P &lt;</th>
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S vs. W: t = 19.13; p < 0.0001

W: t = 7.63; p < 0.0001

HP: t = 6.39; p < 0.0001

ER: t = 9.52; p = 0.0001