The role of the neural sympathetic and parasympathetic systems in diurnal and sleep state related cardiovascular rhythms in the late gestation ovine fetus

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Abstract

The efferent mechanisms mediating the well known diurnal cardiovascular rhythms in the late gestation fetus are only partially understood. In the present study we evaluated the contribution of the parasympathetic and sympathetic nervous systems (SNS) to these rhythms. Chronically instrumented fetal sheep at a mean (SE) of 122 (1) days gestation (term is 147 days) underwent either chemical sympathectomy with 6-hydroxydopamine the day after surgery (n=8), vagotomy at surgery (n=8) or were sham controls (n=8). Fetal heart rate (HR), fetal HR variability (HRV), mean arterial blood pressure (MAP), carotid blood flow (CaBF), electrocorticogram (ECoG) activity, and nuchal activity were measured continuously for 24 h. Changes between sleep states were determined in a 6 h interval. Control fetal sheep showed consistent diurnal rhythms in fetal HR, HRV, MAP and CaBF, with maximal activity in the evening, but not in nuchal activity. Sympathectomy was associated with a significant reduction of both fetal HR and HRV, while vagotomy was associated with a fall in fetal HRV (p<0.05) but no change in HR. Despite this, most animals in the two intervention groups still showed diurnal rhythms for fetal HR, HRV, MAP and CaBF, although peak HR may have been delayed in the sympathectomy group (mean 02:22 vs 23:54 h in controls, p=0.06). There was no effect of either intervention on sleep state cycling, although state-related cardiovascular rhythms were significantly modulated. These data indicate that, neither the SNS nor vagal activity, in isolation at least, are essential for generating cardiovascular diurnal rhythms in the late gestation fetus.

Key words: fetal heart rate variability; diurnal rhythm; fetal sheep; sleep state; autonomic nervous system.
INTRODUCTION

Despite the common use of heart rate (HR) and HR variability (HRV) for fetal surveillance, the underlying efferent mechanisms controlling these rhythms remain incompletely understood (29). Diurnal rhythms in fetal HR and HRV are well established in late gestation in the fetal human (44), nonhuman primates (39) and sheep (3, 4, 9, 11, 42). These circadian rhythms are thought to be entrained by maternal signals (28) and coordinated in part by signals from a fetal “biological clock” in the suprachiasmatic nucleus (SCN) (5, 26, 41), although other fetal tissues may be involved (37). A study in infants and children showed a circadian rhythm in HR and HRV and confirmed progressive maturation of the autonomic nervous system with age suggesting the hypothesis that the sympathetic withdrawal associated with mature, organized sleep is largely responsible for those rhythms (24).

The SCN neurons communicate with the superior cervical ganglion, whose sympathetic terminals then release norepinephrine in end organs in a rhythmic manner. In adult rats, complete lesion of the SCN abolishes the circadian rhythm of heart rate and decreases mean HR to baseline levels, consistent with the hypothesis that the sympathetic component of the rhythm was removed (34). Similarly, in adult rats, guanethidine, which depletes norepinephrine stores in peripheral tissues but not in the brain, selectively abolishes the HR circadian rhythm (48). It has been further suggested that the presence of a day-night difference in resting HR in intact but not in SCN-lesioned adult rats is evidence that the SCN generates a daily rhythm in HR via autonomic projections (35). Data suggest that the paraventricular nucleus is the most important relay station for the transmission of SCN information via the brain stem and the spinal cord to the heart (35). In addition, the SCN has a multisynaptic contact with the stellate ganglion, which is the main sympathetic ganglion.
innervating the heart. These findings suggest that clock information from the SCN traverses the sympathetic nervous system (SNS) to influence the heart. In addition, although more limited, there is increasing evidence for a circadian rhythm of systemic parasympathetic input in adults (2, 20).

However, there is no information on the role of the SNS or parasympathetic systems in mediating cardiovascular diurnal rhythms in the fetus. Both systems are active in late gestation, and influence both baseline cardiovascular function and sleep state related changes (45, 54). In the present study, we therefore examined whether the SNS and parasympathetic systems separately contribute to the diurnal cardiovascular rhythms in chronically instrumented healthy singleton near-term fetal sheep using chemical sympathectomy and vagotomy, respectively. The effects on sleep state and related cardiovascular rhythms were evaluated for comparison.

**MATERIALS AND METHODS**

*Surgical procedures*

Twenty-four Romney/Suffolk sheep (116-122 days gestation) were operated on using sterile techniques under halothane anesthesia (2%). All procedures were approved by the Animal Ethics Committee of the University of Auckland. Food, but not water, was withdrawn 18 h before surgery. Ewes were given 5 mls of Streptopen (procaine penicillin (250,000 IU) and dihydrostreptomycin (250 mg/ml), Pitman-Moore, Wellington, New Zealand) intramuscularly for prophylaxis 30 min prior to the start of surgery. Anesthesia was induced by intravenous (i.v.) injection of Saffan (Alphaxalone and Alphadolone; 3 mg/kg, Schering-Plough Animal Health Ltd, Wellington, New Zealand), and general anesthesia maintained using 2-3% halothane in O₂. The depth of anesthesia and maternal respiration
were constantly monitored by trained anesthetic staff. A 20 gauge i.v. catheter was placed in a maternal front leg vein, and the ewes were placed on a constant infusion of saline to maintain maternal fluid balance.

Catheters were placed in the left fetal femoral artery and vein, right axillary artery and the amniotic sac. Two pairs of electrodes (AS633-5SSF, Cooner Wire Co., Chatsworth, CA, USA) were placed on the dura over the parasagittal parietal cortex (5 mm and 10 mm anterior to bregma and 5 mm lateral) and secured with cyanoacrylate glue to measure the electrocorticogram (ECoG). A reference electrode was sewn over the occiput. Electrodes were placed in the nuchal muscle to measure electromyogram (EMG) nuchal activity, and electrocardiogram (ECG) electrodes were sewn across the chest to record HR. A 4S ultrasound blood flow probe (Transonic Systems Inc., Ithaca, NY, USA) was placed around the left carotid artery to measure carotid artery blood flow (CaBF) (1, 43). In the vagotomy group the vagus nerve was exposed through bilateral 2 cm incisions, carefully dissected free from the carotid artery, and a 1 cm section was removed to ensure that there could be no residual contact between the ends. All fetal leads were exteriorized through the maternal flank and a maternal long saphenous vein was catheterized to provide access for post-operative care and euthanasia. Antibiotics (80 mg gentamicin, Rousell, Auckland, New Zealand) were administered into the amniotic sac prior to closure of the uterus.

Antibiotics were administered daily for 5 days i.v. to the ewe (600 mg benzylpenicillin sodium (Crystapen) and 80 mg gentamicin). Fetal catheters were maintained patent by continuous infusion of heparinized isotonic saline (20U/ml at 0.2 ml/h), and the maternal catheter maintained by daily flushing. Animals were housed in a temperature and humidity room with a 12 h light:dark cycle (6:00 AM-6:00 PM light phase), and ad libitum access to food and water.
Experimental protocol

Fetuses were randomly assigned prior to surgery to either sham controls (n=8), sympathectomy (n=8), or vagotomy (n=8). Experiments were conducted at 119 to 126 days gestation, after 3-4 days post-operative recovery. Fetuses were studied for 24 h. On completion of the experiment the ewes and fetuses were killed by an overdose of sodium pentobarbitone (9 g i.v. to the ewe, Pentobarb 300, Chemstock International, Christchurch, New Zealand).

Sympathectomy

The day after surgery a 20 mg/ml infusion (2.5 mL/h) of 6-hydroxydopamine (6-OHDA) (Sigma, St Louis, MO, USA) was administered i.v. over approximately 3 h to the fetuses (18). To test that the sympathectomy was effective a bolus of the norepinephrine releasing agent, tyramine (0.2 mg i.v., Sigma), was administered 48 h later, prior to beginning recording. None of the sympathectomy group showed an increase in blood pressure after tyramine, whereas all sham controls showed an immediate increase of greater than 5 mmHg.

Recordings

Fetal mean arterial blood pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure (Novatrans II, MX860; Medex Inc., Hilliard, OH, USA)(21), CaBF (T208 Ultrasonic Flowmeter; Transonic Systems Inc), ECG, ECoG and EMG were recorded continuously. The blood pressure signal was collected at 64 Hz and low pass filtered at 30 Hz. The raw ECG was analog filtered between 0.05 and 80 Hz and digitized at 512 Hz (51). The nuchal EMG signal was band-pass filtered between 100 Hz and 1 kHz, and the signal was then integrated using a time constant of 1 sec. The analogue ECoG
signal (recorded using amplifier filter modules kindly supplied by Brainz Ltd, Auckland, New Zealand) was processed with a first-order high-pass filter at 1.6 Hz (the signal is attenuated below 1.6 Hz, but not completely absent) and a 6th order Butterworth low-pass filter with a cut-off frequency of 50 Hz and then digitally stored at a sampling rate of 64 Hz. Mean ECoG intensity from both hemispheres was derived from the intensity spectrum signal between 1 and 20 Hz. Data were collected by computer and stored to disk for off-line analysis.

Fetal HR, MAP, CaBF, ECoG, and nuchal activity were recorded over a 24 h period from 10 am to 10 am. Electrocortical activity was analyzed by visual inspection and divided into periods of low-voltage high frequency activity (“low-voltage”) and high-voltage low frequency activity (“high-voltage”) over a representative 6 h period from 4 am to 10 am, and HR, HRV (as calculated below), MAP and CaBF were determined during each behavioral state.

pH and blood gas determination (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, MA., USA) and glucose and lactate measurements (YSI model 2300, Yellow Springs, Ohio, USA) were performed at the time recording was started, and fetal blood samples were taken for analysis of catecholamines in the control and sympathectomy groups. One ml of blood was collected into a cold EDTA tube, centrifuged immediately at 4,000 rpm at 4°C and the resulting plasma stored at -80°C. For assay, the plasma was extracted on alumina and catecholamines were eluted with acetic acid. The extracted catecholamines were separated and measured by reverse phase HPLC with electrochemical detection (13).

Data analysis and statistics
Fetal blood gases and glucose, lactate and catecholamine concentrations were analyzed by one-way ANOVA. One min averages of MAP were calculated for each fetus. One min averages of R-R intervals were used to calculate fetal HR. Fetal HRV was calculated as described by Dawes et al. (10) to obtain the mean minute range (MMR, the difference between the maximum and minimum R-R intervals every min) and the short term variability (STV) measure of HRV (the average of R-R interval differences for each 3.75 epoch). Since there was no difference in the pattern of change between MMR and the STV measure of HRV, we only report MMR values.

The presence of circadian rhythms in fetal MAP, HR, HRV, CaBF and nuchal activity were assessed by fitting a cosine with a fixed period of 24 h to a single day’s data for each variable (27). The number of animals was not the same for all variables because some signals were missing for individual cases. Hourly averages were plotted against time using Sigma Plot (v10, Systat Software Inc., San Jose, CA). Using Sigma Plot’s built in curve fitter, a cosine of the form in equation 1 was fitted to the data of each animal. The parameters: mesor or mean (m), amplitude of cosine (a) and the time of peak (b), were optimized to minimize the sum of the squared residuals. A significant 24-h rhythm was accepted when \( p<0.05 \) for the best fit of the data.

\[
f = m + a \times \cos\left(\frac{2\pi(t-b)}{24}\right) \tag{Equation 1}
\]

Fisher’s exact test was used to compare the incidence of rhythms between groups. Rayleigh’s test was used to determine the existence of a unimodal peak within groups and the Wheeler-Watson-Mardia test was used to test for a difference in the time of the peak between the groups (STATA Statistical Software, StataCorp, Texas)(12). The hourly averages over 24 h for HR, HRV, MAP, CaBF, and nuchal activity were analyzed by repeated measures two-way ANOVA. The effects of sleep state (high-voltage versus low-
voltage activity) and time between controls and the treatment groups were analyzed by
repeated measures two-way ANOVA. The paired t-test was used to compare data (HR, HRV,
MAP and CaBF) from sequential episodes of high-voltage activity and low-voltage activity.
The magnitude of sleep state related changes was calculated as the mean differences between
sequential low-voltage and high-voltage states over 6 h and analyzed by one-way ANOVA
followed by Dunnett’s test. The mean number of sleep states was compared between groups
by one-way ANOVA. Statistical significance was accepted when p<0.05. All data are
presented as mean ± SEM.
RESULTS

Fetal blood gases and catecholamines

Fetal arterial PO\textsubscript{2} was lower (p<0.05) in the vagotomy group compared to controls (Table 1). There were no significant differences for fetal arterial PCO\textsubscript{2}, pH, glucose or lactate concentrations. There was no significant change in circulating levels of epinephrine or norepinephrine in the sympathectomy group compared with sham controls (Table 1).

Diurnal rhythms

A diurnal rhythm for fetal HR was found in 6/7 animals over 24 h in the control group (Figs. 1 and 2). The sympathectomy group also had a diurnal rhythm (6/8 animals) for HR (N.S. vs. controls). We observed a diurnal rhythm in 6/8 animals for HR in the vagotomy group, similar to controls (N.S. vs. controls). Rayleigh’s test showed a significant unimodal peak time for fetal HR in all three groups (Table 2, p<0.01). There was a trend for the peak to be different among the groups (p=0.06). Peak time appeared to be later in the sympathectomy group compared to controls. There was no significant difference in amplitude for the diurnal rhythm among the groups. Mean HR was significantly lower overall in the sympathectomy group than in controls (p<0.05), with a significant increase over time (p<0.0001, Fig. 2), whereas there was no significant effect of vagotomy on fetal HR.

Similarly to HR, a diurnal rhythm was observed for HRV in 6/7 control animals, with a slightly earlier peak than for HR (Table 2, Figs. 1 and 2). Four out of 8 sympathectomy animals showed a significant rhythm for HRV (N.S. vs. controls, Fisher Exact test). Three out of 7 animals showed a significant rhythm for HRV in the vagotomy group (N.S. vs. controls). Rayleigh’s test showed a significant unimodal peak time for FHRV in controls
(p<0.01) and the vagotomy group (p<0.05). There was no significant difference in peak time or amplitude for the diurnal rhythm for HRV among the groups. Overall, HRV in the sympathectomy group was approximately half that observed in controls (p<0.05) and there was an increase in HRV over time (p<0.001). HRV in the vagotomy group was approximately half that observed in controls (p<0.05), similar to the sympathectomy group, and there was a significant decrease over time (p<0.001).

Four out of 7 animals in the control group showed a diurnal rhythm for MAP. The sympathectomy group showed a diurnal rhythm for MAP in 7/8 animals (N.S. vs. controls). The vagotomy group showed a diurnal rhythm for MAP in 5/8 animals (N.S. vs. controls). The existence of a unimodal peak could not be demonstrated within any of the groups, and therefore, it was not appropriate to test for a difference in time of the peak among the groups. There was no significant difference in amplitude in the diurnal rhythm for MAP among the groups. There were no significant time or group effects for MAP (Fig. 3).

Two out of 7 animals in the control group had a diurnal rhythm for nuchal activity. Two out of 6 animals in the sympathectomy group and 2/8 animals in the vagotomy group had a diurnal rhythm for nuchal activity (N.S. vs. controls). The existence of a unimodal peak could not be demonstrated within any of the groups, and therefore, it was not appropriate to test for a difference in time of the peak among the groups. There was no significant difference in amplitude in the diurnal rhythm for nuchal activity among the groups. There were no changes over time in nuchal activity in controls (Fig. 3). There was no effect of sympathectomy or vagotomy on nuchal activity.

Four out of 6 animals in the control group had a diurnal rhythm for CaBF. All (7/7) of the sympathectomy animals and 4/8 animals in the vagotomy group had a diurnal rhythm for CaBF (N.S. vs. controls). The existence of a unimodal peak could not be demonstrated
within any of the groups, and therefore, it was not appropriate to test for a difference in time of the peak among the groups. There was no significant difference in amplitude in the diurnal rhythm for CaBF among the groups. There were no changes over time for CaBF in controls (Fig. 3). There was no effect of sympathectomy or vagotomy on CaBF.

There was no diurnal rhythm for the number of high vs low voltage sleep states in any of the groups. There was no significant difference in the number of sleep states per hour in the 6 h period from 4.00 am to 10.00 am among controls (4.0 ± 0.3/h), sympathectomy (4.4 ± 0.2/h) and vagotomy groups (3.8 ± 0.3/h). There was also no difference in the total mean number of sleep states over 24 h among controls (102 ± 7), sympathectomy (112 ± 5) and vagotomy groups (100 ± 8).

**Effect of behavioral state on fetal cardiovascular variables**

*Fetal heart rate*

Fetal HR was significantly higher during high-voltage activity than during low-voltage activity in controls (Fig. 4, p<0.001) Fetal HR was also significantly higher during high-voltage activity than during low-voltage activity in the sympathectomy group (p<0.01). However, this increase during high-voltage activity was less than in controls (p<0.05) such that the difference in HR between sleep states was approximately half that in controls (p<0.001).

Similar to the other groups, there was a significant difference between the high- and low-voltage states in the vagotomy group (p<0.05). However, HR during low-voltage activity in the vagotomy group was higher than in controls (p<0.05), such that the difference in HR between sleep states in the vagotomy group was approximately half that in controls (p<0.001).
Fetal heart rate variability

HRV was not significantly different between the high- and low-voltage states in controls (Fig. 5) and there were no significant differences in the sympathectomy group. HRV was significantly higher during low-voltage than high-voltage activity in the vagotomy group (p<0.05), although it tended to be lower (p=0.09) than in controls even during low-voltage.

Mean arterial blood pressure

MAP was significantly lower during low-voltage than during high-voltage activity in controls (Fig. 6, p<0.01) and in the sympathectomy group (p<0.01). MAP tended to be lower in the sympathectomy group during high-voltage activity than in controls (p=0.06) The difference in MAP between sleep states observed in the other groups was no longer apparent after vagotomy.

Carotid blood flow

CaBF was significantly higher (p<0.01) during low-voltage than high-voltage activity in controls and in the sympathectomy group (Fig. 7). However, this difference between states was significantly greater (p<0.01) in the sympathectomy group than controls. CaBF was also significantly higher during low-voltage compared to high-voltage activity in the vagotony group (p<0.01). This difference between states was significantly greater in the vagotony (p<0.01) group than controls and was similar to the difference in sympathectomy animals.
DISCUSSION

The present study demonstrates directly for the first time that neither SNS nor vagal input are essential for expression of fetal diurnal cardiovascular rhythms in late gestation. Although there was trend for a later acrophase of HR after sympathectomy this was not significant. In contrast with this lack of effect on diurnal rhythms, sympathectomy was associated with a marked reduction of mean fetal HR and HRV, while vagotomy was associated with a fall in HRV but no change in HR. Further, sleep state related changes were modulated, consistent with a pattern of greater sympathetic tone during the high-voltage state (45, 47, 54) and greater vagal tone during the low-voltage state (45, 47).

Peripheral sympathectomy achieved by 6-OHDA leads to loss of sympathetic nerve terminals, and thus of peripheral norepinephrine release (18, 22). We found no effect of sympathectomy on norepinephrine levels, which indicates that norepinephrine was still being released from other sources, such as the adrenal gland. In the present study, sympathectomy and vagotomy were associated with an approximately 50% reduction in HRV, confirming that the rapid changes in heart rate that underlie HRV are primarily a function of direct neural mechanisms. These results are highly consistent with previous findings of a close correlation between directly measured SNS activity and short-term HR changes and that ganglionic blockade reduced the coefficients of variation of HR (36). Further, in the present study sympathectomy was associated with a significant fall in fetal HR compared to controls, consistent with significant background SNS activity. Although there are some older conflicting data (19), this finding is broadly consistent with the fall in HR reported during infusion of a nonselective beta blocker, propranolol (8, 52), and with previous reports that chemical sympathectomy was associated with a trend for reduced HR in late gestation fetal sheep (18, 40); this difference from the present findings may reflect shorter recording times.
in the previous studies.

The parasympathetic system is also important in cardiovascular control. In the present study, vagotomy was associated with a marked reduction in HRV comparable with that of sympathectomy in the present study, and with acute atropine infusion (8, 52). The highly similar 50% reduction in HR variability after both sympathectomy and vagotomy suggests that in each case residual variation reflects remaining autonomic function from the complementary system. Although we did not test whether combined sympathectomy and vagotomy would abolish fetal HRV, Segar et al have shown that HRV was reduced to less than 15% of baseline variation by ganglionic blockade with trimethaphan (36). The finding that HR was not changed after vagotomy is in contrast with the reported increase in HR after atropine infusions in the near-term fetal sheep (8, 46, 52). This difference may in part reflect the longer recovery period in the present study, which will have allowed time for autonomic adjustment. These data suggest that basal fetal HR is primarily modulated by sympathetic activity. As discussed below, it may also reflect the differential effects between sleep states, since in the present study vagotomy was associated with increased fetal HR only during low-voltage activity sleep.

The question in the present study was whether the SNS or parasympathetic systems mediated fetal cardiovascular diurnal rhythms. We did not find any diurnal rhythm in sleep states (i.e. the proportion of the two states). This is in contrast to a previous study in late gestation fetal sheep that found a decrease in the number of sleep states with a greater proportion of the low voltage sleep state at night (33). This difference may be because the previous study was performed just before full term, at 138 to 140 days gestation, significantly older than in the present study. Consistent with findings in the human fetus (44) and other precocial species such as the sheep (3, 4, 9, 42), in the present study the majority
of control fetal sheep showed clear diurnal cardiac rhythms, with the peak in HR at approximately 2400 h and a slightly earlier peak in HRV and MAP. Neither sympathectomy nor vagotomy significantly reduced the magnitude or incidence of these diurnal rhythms, although there was an apparent trend for the peak in HR to be delayed after sympathectomy. Further studies with a larger sample size will be required to validate this finding. These findings are in contrast to limited data from the adult rat, which suggest that the SNS mediates the central SCN driven diurnal rhythm in HR and MAP (17, 48). A limitation of the present study is that the effect of the SNS and vagal input were tested separately. Thus, it remains possible that the diurnal rhythm is mediated by both arms of the autonomic system or that additional mechanisms are involved, as discussed in detail below.

The present study also suggests that the late gestation fetal sheep has a diurnal rhythm for CaBF, with greatest flow in the evening. There was no effect of either sympathectomy or vagotomy on the diurnal rhythm, which was expected since both interventions target the peripheral, not central autonomic effectors. To our knowledge, this is the first study to demonstrate such a rhythm in the fetus, but it is consistent with the reported circadian periodicity for cerebral blood flow in the adult rat (49). Since CaBF is greatest during the low-voltage sleep state, which is increased both in duration (38) and in the percentage of cycles in the evening (33), potentially, sleep state could contribute to the diurnal rhythm in CaBF. However, in the present study we did not find a change in the proportion of low voltage, high frequency sleep over night. Further, as discussed below, HR was decreased during low-voltage activity and there were no differences in HRV between sleep states (see below), and thus state cannot contribute to the HR and HRV diurnal rhythms.

Since the autonomic systems did not seem to mediate the cardiovascular diurnal rhythms in the fetal sheep, the question remains what other factors might be involved? Older
data have related body movements and fetal breathing movements to short-term variation in HR in the fetal sheep (9, 44), and to changes in renal sympathetic activity (23). There are no data on the relationship with long-term variation, such as circadian rhythms. We did not measure fetal breathing in the present study and thus are unable to assess whether it was associated with diurnal rhythms. However, in the present study, only two animals in each group showed a significant diurnal rhythm for nuchal activity, although when it was present, this activity also peaked in the evening. Furthermore, the large reduction in short-term variability after both interventions was not associated with any change in nuchal activity. Thus, it seems unlikely that body movements are a significant factor. This finding is supported by the finding by Segar and colleagues that HRV was not reduced by muscle paralysis in near-term fetal sheep (36).

Speculatively, the different results of the present study and previous data in adult rats may be a function of differences between fetal and postnatal environments. Before birth, maternal signals including feeding time, which may act through metabolic or hormonal signals, and melatonin entrain the fetal circadian rhythms (37). In particular, fetal circulating melatonin is derived from maternal-fetal transfer in nonhuman primates (31) and sheep (25, 53), and there are data that the daily melatonin rhythm in pregnant sheep entrains the daily rhythm of fetal breathing activity (15). Thus, the residual cardiac rhythms could be mediated directly or indirectly by these maternal influences. However, direct injections of melatonin in fetal sheep and postnatally in adult women do not appear to significantly affect HR (6, 50), supporting indirect mechanisms at least in part.

One possible mechanism may be seen in the evidence that in addition to the fetal clock in the SCN, there are peripheral clocks in many fetal tissues, including the liver and adrenal gland that are also entrained by maternal feeding and melatonin (28, 37). Further, there is
emerging evidence that at least in some species, the fetal SCN does not necessarily determine the rhythm of the peripheral tissue clocks (37). Thus, the present finding of limited effects of the autonomic nervous system on fetal diurnal rhythms supports the hypothesis that these putative peripheral clocks contribute to synchronizing cardiovascular rhythms before birth.

Data in adults suggest that the mechanisms affecting long-term cardiovascular rhythms are distinct from short-term control (16). It is well established that ECoG activity in the mature fetal lamb cyclically changes between high-voltage activity (comparable to deep, non-rapid eye movement sleep) and low-voltage activity, which resembles rapid eye movement sleep in postnatal life (11). Consistent with previous reports, in the current study HR and MAP were higher in the high-voltage state than in low-voltage in controls (7, 23), and CaBF was increased during low-voltage activity (30, 32).

Sympathectomy was associated with lower HR during high-voltage activity than controls, similar to the effect of propranolol infusion (45, 54). It has been suggested that propranolol can abolish the cyclic variation in HR (54), whereas in the present study sympathectomy only reduced this difference. Given that there is significant variation between successive sleep cycles (e.g. see Fig. 4) most likely this simply reflects the much larger number of sleep cycles examined in the present study than previously. Conversely, in the present study, vagotomy was associated with an increase in HR during low-voltage activity, with loss of the normal cyclic variation in HR, consistent with studies of atropine infusion (45, 47), suggesting a tonic increase in vagal activity in the low-voltage state.

Further, vagotomy was associated with an increase in both MAP and CaBF in the low-voltage state. In the fetal sheep, stroke volume is relatively constrained by extracardiac factors and increased fetal HR increases combined ventricular output (14). Thus, it is likely
that increased MAP in this sleep state after vagotomy was directly mediated by increased HR.

In contrast with changes in other cardiovascular variables, HRV was not significantly different between the sleep states in controls, similar to limited previous data (9). This implies that although tonic sympathetic and parasympathetic activity vary cyclically with sleep state (23), the rapid fluctuations in autonomic activity mediating short-term HRV are present at similar levels in both sleep states. Intriguingly, the only differential effect in the present study was a relative smaller reduction in HRV during low-voltage activity compared with high-voltage activity in the vagotomy group. The mechanism underlying this small difference is not known, but could reflect compensatory changes in the SNS, or other regulatory systems such as the renin-angiotensin system.

Perspectives and Significance

In contrast with the important contribution of peripheral sympathetic and parasympathetic activity to short-term and sleep state related cardiovascular rhythms, the present study does not support the hypothesis that SNS and vagal tone have major roles in mediating cardiac diurnal rhythms in the late gestation fetal sheep, although we cannot exclude a potential combined effect of these systems. Rather, these findings support emerging data that in the fetus peripheral clocks may be synchronized directly by maternal signals such as melatonin and feeding and thus mediate at least some peripheral rhythms before birth (37). The interplay between the central clock and these putative peripheral clocks requires further research.
This study was supported by grants from the Health Research Council of New Zealand, the March of Dimes Birth Defects Foundation, the Auckland Medical Research Foundation and the Lottery Grants Board of New Zealand.
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FIGURE LEGENDS

Fig. 1. Representative examples from individual animals of fetal HR and fetal HRV hourly means depicted as the best fitting cosine function for control, sympathectomy and vagotomy groups. Note that despite marked reduction in mean HRV in both intervention groups, and in HR after sympathectomy, diurnal rhythms were preserved in both intervention groups. Open bars above the graph indicate the time of the light cycle and the grey bar indicates the dark cycle.

Fig 2. Time sequence of changes (30 min averages) in HR and HRV over 24 h. There was a diurnal rhythm for HR over 24 h in all 3 groups. Sympathectomy was associated with a significant reduction in HR. All groups showed a diurnal rhythm in HRV, as measured by MMR (the difference between the maximum and minimum R-R intervals every min). Sympathectomy and vagotomy were both associated with an overall reduction in variability compared to controls. HRV increased over the 24 h interval in the sympathectomy group and decreased over time in the vagotomy group. Open bars above the graph indicate the time of the light cycle and the grey bar indicates the dark cycle. Closed circles, controls; closed triangles, sympathectomy group; open circles, vagotomy group. *p<0.05, ***p<0.001, ****p<0.0001 for comparison of time within groups. a p<0.05, compared to controls.

Fig. 3 Time sequence of changes (30 min averages) in MAP, CaBF and nuchal activity over 24 h. There were no significant differences among the groups for MAP, CaBF or nuchal activity. Open bars above the graph indicate the time of the light cycle and the grey bar indicates the dark cycle. Closed circles, controls; closed triangles, sympathectomy group; open circles, vagotomy group.

Fig 4. Fetal heart rate during high-voltage and low-voltage activity (sleep states). Events 1-
10 correspond to sequential episodes of high-voltage and low-voltage activity over 6 h (4 am to 10 am). Open bar above the graph indicates the time of the light cycle and the grey bar indicates the dark cycle. ***p<0.001, **p<0.01, *p<0.05 for comparison between sleep states within groups; c p<0.001, for comparison between sleep states between groups (compared to controls); †p<0.05, for comparison between high or low voltage state compared to controls.

Fig 5. Fetal heart rate variability during high-voltage and low-voltage activity (sleep states). Events 1-10 correspond to sequential episodes of high-voltage and low-voltage activity over 6 h (4 am to 10 am). Open bar above the graph indicates the time of the light cycle and the grey bar indicates the dark cycle. *p<0.05 for comparison between sleep states within groups.

Fig 6. Mean arterial pressure during high-voltage and low-voltage activity (sleep states). Events 1-10 correspond to sequential episodes of high-voltage and low-voltage activity over 6 h (4 am to 10 am). Open bar above the graph indicates the time of the light cycle and the grey bar indicates the dark cycle. **p<0.01 for comparison between sleep states within groups.

Fig 7. Carotid blood flow during high-voltage and low-voltage activity (sleep states). Events 1-10 correspond to sequential episodes of high-voltage and low-voltage activity over 6 h (4 am to 10 am). Open bar above the graph indicates the time of the light cycle and the grey bar indicates the dark cycle. **p<0.01 for comparison between sleep states within groups, b p<0.01 and c p<0.001 for comparison between sleep states between groups (compared to controls).
### TABLES

#### Table 1. Fetal arterial blood gases, glucose, lactate, and catecholamine concentrations.

<table>
<thead>
<tr>
<th></th>
<th>PO(_2) (mmHg)</th>
<th>PCO(_2) (mmHg)</th>
<th>pH</th>
<th>Glucose (mM)</th>
<th>Lactate (mM)</th>
<th>Norepinephrine (pg/ml)</th>
<th>Epinephrine (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>22.5±1.0</td>
<td>45.5±2.2</td>
<td>7.40±0.01</td>
<td>0.76±0.08</td>
<td>0.73±0.06</td>
<td>1318±261</td>
<td>119±33</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>24.3±1.1</td>
<td>44.2±1.5</td>
<td>7.41±0.01</td>
<td>0.85±0.11</td>
<td>0.74±0.07</td>
<td>939±219</td>
<td>176±47</td>
</tr>
<tr>
<td>Vagotomy</td>
<td>18.5±1.2*</td>
<td>44.5±1.0</td>
<td>7.39±0.01</td>
<td>0.82±0.06</td>
<td>0.87±0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are mean±SE for controls (n=6), sympathectomy (n=7) and vagotomy (n=8) groups. Samples were taken between 8:00 AM to 10:00 AM. Note; samples for catecholamines were not taken in the vagotomy group. *p<0.05 compared to controls.
**Table 2.** Summary of cosinor analysis of fetal cardiovascular variables and nuchal activity.

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>HRV</th>
<th>MAP</th>
<th>CaBF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude</td>
<td>Peak</td>
<td>Mesor</td>
<td>Amplitude</td>
</tr>
<tr>
<td></td>
<td>(beats/min)</td>
<td>(h:min±h)</td>
<td>(beats/min)</td>
<td>(msec)</td>
</tr>
<tr>
<td>Controls</td>
<td>8.6±1.0</td>
<td>23:54±0.8**</td>
<td>181±5</td>
<td>5.9±0.8</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>5.9±0.8</td>
<td>02:22±0.4**</td>
<td>158±2</td>
<td>7.1±1.5</td>
</tr>
<tr>
<td>Vagotomy</td>
<td>7.5±0.8</td>
<td>24:30±1.0**</td>
<td>179±5</td>
<td>6.8±1.3</td>
</tr>
</tbody>
</table>

All values are mean±SE for controls (n=4-6), sympathectomy (n=4-7) and vagotomy (n=3-6) groups.*P<0.05, **P<0.01; significant unimodal peak within groups (Rayleigh’s test).
Mean arterial blood pressure (mmHg)

Controls

Sympathectomy

Vagotony

**
Controls

Sympathectomy

Vagotony

Carotid blood flow (ml/min)

Sleep state #