MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON.....21November 2001.....

.....[redacted].....

for Sec. Research Graduate School Committee

Under the copyright Act 1968, this thesis must be used only under the normal conditions of scholarly fair dealing for the purposes of research, criticism or review. In particular no results or conclusions should be extracted from it, nor should it be copied or closely paraphrased in whole or in part without the written consent of the author. Proper writter acknowledgement should be made for any assistance obtained from this thesis.

H24/3053

Prostaglandin D_2 and the Development of Sleep in the Ovine Fetus

by

Brenda Lee

B.Sc. (Hons) (Monash University)

A thesis submitted to Monash University
in fulfilment of the requirements for the
degree of Doctor of Philosophy

Department of Physiology

Faculty of Medicine, Monash University, Clayton, Australia

October 2000

For my father

"Real knowledge is to know the extent of one's ignorance."

Confucius, 551 BC-479

Addendum

Abstract

A) Page vi, paragraph 3:

The overall aim of this thesis was to determine if prostaglandin D_2 has a role in the maintenance of prenatal sleep, as proposed for the adult. Firstly, experiments were performed where selenium chloride was infused into the lateral ventricle of the late gestation ovine fetus *in utero*. It was found that selenium chloride induced a dose dependent increase in the incidence of arousal ($P<0.05$), whereas control infusions of artificial cerebrospinal fluid had no effect. Administration of 500 pmole/10 µl/min of prostaglandin D_2 immediately after intracerebroventricular infusion of 500 pmole/10 µl/min of selenium chloride immediately abolished the selenium chloride-induced increase in arousal below control levels ($P<0.05$). Infusion of prostaglandin alone appeared to decrease fetal arousal, although this effect did not quite reach significance. These results suggest that selenium chloride increased fetal arousal by inhibiting the synthesis of endogenous prostaglandin D2. Therefore, it was concluded that endogenous prostaglandin D_2 may play a significant role in the maintenance of fetal sleep, as hypothesized in the adult.

Amend to:

The overall aim of this thesis was to determine if prostaglandin D_2 has a role in the maintenance of prenatal sleep, as proposed for the adult. Firstly, experiments were performed where selenium chloride was infused into the lateral ventricle of the late gestation ovine fetus *in utero*. It was found that selenium chloride induced a dose dependent increase in the incidence of arousal ($P<0.05$), whereas control infusions of artificial cerebrospinal fluid had no effect. Administration of 500 pmole/10 µl/min of prostaglandin D_2 immediately after intracerebroventricular infusion of 500 pmole/10 µl/min of selenium chloride immediately abolished the selenium chloride-induced increase in arousal below control levels ($P<0.05$). Infusion of *500 pmole/10 µl/min of* prostaglandin alone *caused a significant decrease in the incidence fetal arousal compared to pre-treatment levels ($P<0.05$). Although experiments that directly show the inhibitory effect of selenium chloride on prostaglandin D_2 in the fetus were not performed in this thesis, these results* suggest that selenium chloride increased fetal arousal *possibly* by inhibiting the synthesis of endogenous prostaglandin D_2. Therefore, it was concluded that endogenous prostaglandin D_2 may play a significant role in the maintenance of fetal sleep, as hypothesized in the adult.

Chapter Two

B) Insert into Page 90, paragraph 2 as last sentence:

Furthermore, it appears that treatment of $SeCl_4$ did not have an effect on the increase in heart rate and MAP that is associated with episodic periods of arousal. Nor did it change these cardiovascular parameters during REM and NREM sleep (Table 2.7a).

C) Page 96, paragraph 4:

Upon examination of the effect of i.c.v infusion of PGD_2 on fetal arousal, statistical analysis showed that there was a significant effect of time period ($P<0.01$), but no significant interaction between animal and dose ($P=0.146$). However, administration of PGD_2 greater than 100 pmole/10 µl/min into the lateral ventricle of the ovine fetal sheep appeared to decrease the incidence of arousal below the incidences observed when aCSF and 25 pmole of PGD_2/10 µl/min where infused (Fig 2.15c). It seems administration of 100pmole/10 µl/min almost halved the incidence of arousal within the first four hours of treatment. The decrease in arousal continued in the 5th and 8th post-infusion period to gradually return to pre-infusion control levels by the end of recording. Similar trends were also observed after 500 pmole and 1 nmole of PGD_2/10 µl/min administration.

Amend to:

Upon examination of the effect of i.c.v infusion of PGD_2 on fetal arousal, statistical analysis *using two way repeated measures analysis of variance* showed that there was a significant effect of time period ($P<0.01$), but no significant interaction between period and dose *with animal* ($P=0.146$). However, administration of PGD_2 greater than 100 pmole/10 µl/min into the lateral ventricle of the ovine fetal sheep appeared to decrease the incidence of arousal below the incidences observed when aCSF and 25 pmole of PGD_2/10 µl/min where infused (Fig 2.15c). It seems administration of 100 pmole/10 µl/min almost halved the incidence of arousal within the first four hours of treatment. The decrease in arousal continued in the 5th and 8th post-infusion period to gradually return to pre-infusion control levels by the end of recording. Similar trends were also observed after 500 pmole and 1 nmole of PGD_2/10 µl/min administration. *Since there was an apparent significant interaction between time and dose, it was decided to remove the additional interactive variable of treatment and test each dose independently using a one way repeated measures ANOVA. As expected, treatment with aCSF and 25 pmole/10 µl/min did not have a significant effect. However, the results show there was a significant effect of 500 pmole/10 µl/min on incidence of arousal compared to pre-treatment values ($P = 0.0147$) and that the power of the test was extremely sensitive (0.7289). Treatment of 1 nmole/10 µl/min was not statistically significant ($P = 0.108$). However, bearing in mind the $P = 0.10$ means that 90% of the time a response would be observed, this result is relatively promising considering only a group of 4 was tested. Furthermore, the treatment of 100 pmole/10 µl/min did not show a significant effect between pre and post-treatment, this is likely to be a result of the large SEM of the control period. Thus when isolating the different doses it appears that 500 pmole of PGD_2/10 µl/min had a significant effect on the incidence of arousal, producing a significant decrease in incidence, and doses 100 and 1000 pmole/10*

µl/min exhibited a trend toward a decrease in the incidence of arousal. However, according to the power of the test, several more animals would be required to show this.

Table 2.7a: The effect of i.c.v. administration of artificial CSF (aCSF) and $SeCl_4$ (25, 100, 500, 1000 pmole/10 µl/min) on mean arterial pressure (MAP; above) and heart rate (below) during rapid eye movement (REM) and non REM sleep and arousal in each animal. In each experiment, MAP associated with REM/NREM sleep was measured on an hourly basis, whereas MAP associated with arousal was measured during each wakeful minute. The mean±SD of each experimental dose is given.

Treatment 10 µl/min	Animal	Change in MAP		
		NREM vs REM	Arousal vs NREM	Arousal vs REM
ACSF	1	0.91	4.07	4.99
	2	0.74	5.07	4.34
	Mean	**0.83**	**4.57**	**4.66**
25 pmole	1	0.57	6.15	6.70
	2	1.55	9.07	7.51
	3	0.97	4.39	3.48
	4	5.3	3.25	8.57
	mean±SD	**2.09±2.19**	**5.71±2.53**	**6.58±2.20**
100 pmole	1	0.99	10.94	9.94
	2	0.43	3.34	2.91
	3	0.51	7.11	7.62
	4	1.19	3.43	2.24
	mean±SD	**0.78±-0.37**	**6.20±3.61**	**5.68±3.71**
500 pmole	1	4.5	6.2	10.8
	2	0.37	3.60	3.97
	3	0.53	5.16	5.70
	4	0.71	9.04	8.33
	mean±SD	**1.54±2.30**	**6.02±2.29**	**7.02±2.99**
1 nmole	1	0.89	5.13	6.02
	2	0.54	3.70	4.24
	3	0.44	4.58	5.02
	mean±SD	**0.62±0.23**	**4.47±0.72**	**5.11±0.89**

Treatment 10 µl/min	Animal	Change in Heart Rate		
		NREM vs REM	Arousal vs NREM	Arousal vs REM
ACSF	1	0.08	62.20	62.29
	2	5.86	2.93	8.79
	mean	**2.97**	**32.57**	**36.54**
25 pmole	1	10.24	46.34	56.77
	2	1.88	44.39	42.50
	3	11.64	34.52	46.15
	4	24.49	33.39	57.89
	mean±SD	**12.11±9.33**	**39.66±6.65**	**50.83±7.67**
100 pmole	1	3.6	69.50	65.90
	2	5.37	2.09	7.45
	3	13.70	46.60	32.90
	4	11.07	65.83	76.91
	mean±SD	**8.44±4.47**	**46.00±30.95**	**45.79±31.66**
500 pmole	1	3.89	39.16	43.01
	2	2.21	55.03	52.82
	3	0.65	32.10	31.44
	4	1.74	77.23	75.48
	mean±SD	**2.13±1.34**	**50.86±20.10**	**50.68±18.70**
1 nmole	1	5.08	25.32	30.40
	2	8.42	27.06	18.64
	3	1.66	12.94	14.6
	mean±SD	**5.03±3.38**	**21.78±7.70**	**21.21±8.21**

Table 2.10a: The effect of i.c.v. administration of artificial CSF (aCSF) and PGD_2 (25, 100, 500, 1000 pmole/10 µl/min) on mean arterial pressure (MAP; above) and heart rate (below) during rapid eye movement (REM) and non REM sleep and arousal in each animal. In each experiment, MAP and heart rate associated with REM/NREM sleep was measured on an hourly basis, whereas MAP associated with arousal was measured during each wakeful minute. The mean±SD of each experimental dose is given.

Treatment 10 µl/min	Animal	Change in MAP		
		NREM vs REM	Arousal vs NREM	Arousal vs REM
ACSF	1	0.91	4.07	4.99
	2	0.74	5.07	4.34
	mean	**0.83**	**4.57**	**4.66**
25 pmole	1	0.63	5.49	6.12
	2	1.47	5.07	3.59
	mean	**1.05**	**5.28**	**4.86**
100 pmole	1	0.17	1.95	2.12
	2	0.41	4.13	4.54
	mean	**0.29**	**3.04**	**3.33**
500 pmole	1	1.41	11.41	12.82
	2	3.81	12.88	9.06
	3	1.77	5.21	3.44
	mean±SD	**2.67±1.70**	**12.14±1.03**	**10.94±2.66**
1 nmole	1	0.31	5.90	6.21
	2	2.30	2.72	4.92
	3	0.76	3.26	4.02
	mean±SD	**1.10±1.00**	**3.96±1.70**	**5.06±1.10**

Treatment 10 µl/min	Animal	Change in Heart Rate		
		NREM vs REM	Arousal vs NREM	Arousal vs REM
ACSF	1	0.08	62.20	62.29
	2	5.86	2.93	8.79
	mean	**2.97**	**32.57**	**36.54**
25 pmole	1	3.11	35.39	38.51
	2	3.57	66.99	70.56
	mean	**3.34**	**51.19**	**54.53**
100 pmole	1	25.51	83.00	57.48
	2	0.38	29.30	29.69
	mean	**12.95**	**56.15**	**43.58**
500 pmole	1	2.87	17.10	14.20
	2	5.56	45.91	51.46
	3	9.38	22.54	13.17
	mean±SD	**4.22±1.70**	**31.50±20.38**	**32.83±26.34**
1 nmole	1	4.60	48.00	52.60
	2	3.11	16.16	19.27
	3	6.64	17.53	24.17
	mean±SD	**4.78±1.77**	**27.23±17.99**	**32.01±17.99**

D) Page 99, paragraph 1, insert as last sentence:

Furthermore, it appears that treatment of PGD_2 did not have an effect on the increase in heart rate and MAP that is associated with episodic periods of arousal. Nor did it change these cardiovascular parameters during REM and NREM sleep (Table 2.10a).

E) Page 108, paragraph 1:

Inhibition of PGD_2 production by i.c.v infusion of the specific PGDS inhibitor $SeCl_4$ caused a significant dose-dependent increase in the incidence of arousal in the late gestation ovine fetus. This increase in fetal arousal was abolished by subsequent administration of exogenous PGD_2, which then reduced the incidence of arousal to significantly lower levels compared to control. The infusion of PGD_2 alone into the lateral ventricle decreased the incidence of arousal. This decline in arousal was not dose-dependent, as all doses higher than 100 pmole of PGD_2/10 µl/min reduced the incidence of arousal by the same amount (i.e. not statistically different from each other) compared to lower doses. Therefore, from these observations it can be said that $SeCl_4$ increased the amount of time the ovine fetus spent 'awake' presumably by inhibiting of endogenous production of PGD_2.

This suggests that PGD_2 may have a role in the maintenance of sleep-wake cycles *in utero*, as is it hypothesised during adult life (Hayaishi 1988).

Amended to:

It was observed that administration of 100 and 500 pmole/10 µl/min of $SeCl_4$ caused a significant increase in the incidence of fetus arousal. This evident increase in arousal was significantly higher than control infusions of aCSF and lower doses of $SeCl_4$. This *$SeCl_4$-induced* increase in fetal arousal was abolished by subsequent administration of exogenous PGD_2, which then reduced the incidence of arousal to significantly lower levels compared to control. The infusion of *500 pmole/10 µl/min of* PGD_2 into the lateral ventricle *produce a significant decrease in the incidence of arousal.* Therefore, from these observations it can be said that $SeCl_4$ increased the amount of time the ovine fetus spent 'awake'. *Based on previous studies (Islam et al. 1991; Matsumura et al. 1991) it is conceivable that the observed effects on arousal was due to $SeCl_4$ inhibition of endogenous PGD_2 production. However, it is important to note that there were no experiments undertaken to elucidate this.* These observations suggests that PGD_2 may have a potential role in the maintenance of sleep-wake cycles *in utero*, as is it hypothesised during adult life (Hayaishi 1988).

F) Insert into Page 110, paragraph 2:

It is not certain why doses of 100 and 500 pmole/10 µl/min of $SeCl_4$ elicited an increase in nuchal EMG but the dose of 1 nmole pmole/10 µl/min of $SeCl_4$ did not – perhaps this dose was too high and beyond normal dose range to elicit a normal physiological response. This phenomenon is observed in other ligand-enzyme interactions, such as the arachidonic acid suicide reaction, where it is suggested that high concentrations and rapid turnover of the arachidonic acid substrate produces an unstable protein that induces inactivation of the cyclooxygenase enzyme (Smith and Marnett 1991). Further pharmacological studies examining the interaction between $SeCl_4$ and the PGDS enzyme are required for greater elucidation. The threshold dose of $SeCl_4$ cannot be established from this study. In preliminary studies, doses of 5 nmole and 10 nmole of $SeCl_4$/10 µl/min administered into the fetal lambs produced seizure like activities (FBM, EOG and nuchal EMG occurring in HV ECoG). It is hard to compare exactly the doses given in this experiment with other studies that show toxicological effects of selenium, considering the differences in treatment regime. Doses of 1 to 10 nmole of $SeCl_4$/10 µl/min is equivalent to a total of 3.04 to 30.4 ng of selenium over the four hour infusion period. Humans with an average daily intake of 4.99 mg of selenium develop lesions on the nails and central nervous system and suffer loss of hair, symptoms of selenosis (Yang et al. 1983). In comparison, lambs that were given an intramuscular injection of 4.55 mg of selenium faced certain death by convulsive spasms and respiratory failure, one to seven days after treatment (Caravaggi et al. 1970). Therefore, it appears that there is a marked difference between responses elicited by low doses and high doses of selenium. However, the exact ranges of dose that differentiate a normal physiological response and toxic pharmacological side effects are not know and require further elucidation.

G) Page 110, paragraph 3:

The results also suggest that administration of PGD_2 into the lateral ventricle of fetal sheep increased the time spent asleep. The decline in arousal was not dose dependent, so that all doses above 100 pmole of PGD_2/10 µl/min (i.e. 100, 500 and 1000 pmole/10 µl/min) had a similar effect. Furthermore, PGD_2 was shown to reduce the incidence of arousal to below the level observed in the control periods, and below that present when aCSF was infused. The observed reduction in arousal was due to the decrease, although not significant in itself, in nuchal muscle activity during LV ECoG rather than a reduction in the total amount of EMG activity. This suggests that PGD_2 synthesis in the fetal brain has a role in determining the incidence of 'wakefulness' or arousal-like behaviour in late gestation in the sheep. These observations are consistent with studies that show excess production of PGD_2, stimulated by acute pain and inflamation (Smith and Wagner 1991), in transgenic mice that overexpress the PGDS gene (Pinzar *et al* 2000) result in increased incidence of NREM sleep.

Amended to:

The results also suggest that administration of PGD_2 into the lateral ventricle of fetal sheep *caused an observed reduction in arousal. When the data was originally analysed collectively using a two way repeated measures ANOVA the analysis showed that there was not a significant dose dependent decrease in arousal. However, when each of the doses were analysed in isolation it was found that the dose of 500 pmole of PGD_2/10 µl/min had a significant effect on arousal, decreasing it below pre-treatment levels. Furthermore, the results also show that subsequent infusion of PGD_2 immediately following $SeCl_4$ not only abolished the $SeCl_4$-induced increase in arousal but also significantly reduced the incidence of arousal below control levels. It is important to note, however, that no concomitant increase in sleep with the observed decrease in arousal. While it is not possible to definitively conclude from the results that PGD_2 decreases arousal behaviour in an untreated fetus, they do implicate PGD_2 in the role of the maintenance of sleep during late gestation. These observations in the fetus are consistent with adult studies that show the relationship between PGD_2 and sleep. For example, it has been demonstrated that* excess production of PGD_2, stimulated by acute pain and inflamation (Smith and Wagner 1991), in transgenic mice that overexpress the PGDS gene (Pinzar *et al* 2000) result in increased incidence of NREM sleep. *Other in vivo experiments performed in the fetus, such as receptor antagonist studies, should be the basis of future work.*

H) Page 111, paragraph 2:

Since $SeCl_4$ was able to induce a dose-dependent response, it could be said the observed increase in arousal was a specific response to the inhibition of PGDS in the brain. Administration of PGD_2 immediately after $SeCl_4$ infusion significantly

abolished the $SeCl_4$-induced increase in arousal, reducing it to levels significantly lower than control incidences. Therefore it is likely that the observed increase in arousal is specifically caused by the depletion of endogenous PGD_2 levels, which can be reversed by addition of PGD_2 into the brain.

Amended to:

Administration of PGD_2 immediately after $SeCl_4$ infusion significantly abolished the $SeCl_4$-induced increase in arousal, reducing it to levels significantly lower than control incidences. *Culminating this observation with other previous evidence (Islam et al. 1991; Matsumura et al. 1991), it is therefore* likely that the *apparent* increase in arousal is caused by the depletion of endogenous PGD_2 levels, which can be reversed by addition of PGD_2 into the brain. *However, further evidence that clearly shows $SeCl_4$ inhibitory effect on PGDS in the fetus is required to definitively conclude that $SeCl_4$ increased arousal via PGD_2 mechanisms.*

I) Insert into page 111, as paragraph 4 & 5:

The results of the second chapter are based upon changes in the pattern of ECoG, EOG and nuchal EMG activity recorded at slow chart speeds, which was done specifically to display shifts in behavioural activity in response to treatment of $SeCl_4$ and PGD_2. One of the pitfalls of using this technique is that it is unable to display changes in the frequency of these parameters. The frequency of the recorded parameters can be examined by subjecting digital recordings to power spectral analysis using the fast Fourier transform (Szeto et al 1992, Vo et al 1986). Thus, it is agreed that there are additional methods to analyse more subtle changes in ECoG and EOG that may attribute to alterations in sleep-wake behaviour. Unfortunately, the resources required for spectral analysis were not at my disposal. Hence, I was limited by the equipment and experimental methods that were available. The pitfalls of trying to interpret modulations in behaviour based on analysing changes in the separate descriptive parameters, especially in a fetus that can not actually be observed, were taken considered. The problem of trying to differentiate whether the effect of $SeCl_4$ was a drug-induced change in behavioural state or simply an increase in incidence of nuchal EMG during LV ECoG was recognised. To overcome problem, it was endeavoured to measure as many behavioural parameters as possible and include the presence of FBM and EMG in the definition of arousal. In addition, an increase in blood pressure and heart rate associated with the incidence of arousal was observed after treatment, these results suggest that the treatment elicited a generalised behavioural response inducing changes in the autonomic system rather than a pharmacological modulation that specifically increases nuchal muscle activity during LV-ECoG.

Another limitation of using traditional polygraphic criteria to define complex sleep-wake states is that not all recorded parameters can be defined as a behavioural state. It is evident in the results since the three individual behavioural states do not add up to the 240 minutes, as one may expect in theory. This apparent result is due to time periods that do not contain descriptive recordings that fall into the three behavioural states, as defined by ECoG, EOG, nuchal EMG and FBM. These unidentified periods are considered non-defined behavioural states i.e. do not qualify to be either NREM, REM and arousal behavioural states. Despite the pitfalls mentioned here and above, this method that employs polygraphic recording to monitor changes in sleep-wake behaviour, particularly arousal, is still an acceptable experimental technique that has been employed in recent literature (Nicol et al. 1999). Nicol et al's recent observation that evoked EEG amplitude increased after treatment (inhibition of 5α-reductase activity finasteride) that also increased arousal incidence, does much to confirm polygraphic recordings as the 'gold standard', which other methods (such as fast Fourier Transform) will help to establish. Therefore, based on previous literature and the resources available, it is believed for the purposes of the study that these polygraphic techniques are sufficient to identify arousal and changes in its incidence.

J) Page 111, paragraph 4:

To analyse the data, the incidence on arousal was analysed within four-hour sequential epochs.

Amend to:

Unfortunately as a result of both time and resource constraints some data points are arbitrarily low. It is important to note that this it was not a source of bias that some data points are n=3 versus n=5. No animal data was edited nor omitted unless it was impossible to analyse the data. Observations could not used for several reasons such as catheters not always patent, or because recordings were not able to be analysed due to poor signal quality or it was discovered at post mortem that the cannular was not inserted correctly. When analysing the data, the incidence of arousal was analysed within four-hour sequential epochs.

K) Page 114, paragraph 2:

Consistent with previous studies, transient increase in both blood pressure and heart rate occurred simultaneously with episodes of arousal (Szeto 1983). These results indicate that both treatments did not have any adverse side effects and that the fetus remained in good health through out the experiment.

Amend to:

Consistent with previous studies *(Szeto 1983)*, transient increase in both blood pressure and heart rate occurred simultaneously with episodes of arousal *during the experiment.* These results indicate that *that the incidence of arousal induced by treatment was more likely to be normal physiological response exhibiting changes in the autonomic system. Further more, that* both treatments did not have any adverse side effects and that the fetus remained in good health through out the experiment.

Chapter Three

L) Insert into Page 149, paragraph 2 as last sentence:

Alternatively, another reason for the inconsistency between PGE_2 and PGES concentrations may be due to the pitfalls in the experimental protocol, since the synthesised prostanoid was not converted into a stable methyloxime derivative until after extraction. It is conceivable that the PGE_2 synthesised during the incubation time could be rapidly metabolised before methoximation, reducing the measured concentrations.

M) Page 150, insert as new paragraph 3:

It is important to consider the activity of hypothalamic PHGS in the ovine fetus when measuring the specific activity of PGDS and PGES. A recent study has demonstrated an increase in PGHS-1 and PGHS-2 immunoreactivity in the ovine fetal hypothalamus during late gestation (Deauseault et al 2000). Although Deauseault et al measured only expression of the enzyme, it more than likely these results suggest that the activity of the enzyme increases and peaks in late gestation in parallel with expression. In this thesis, the significant increase in PGES activity at 135 days gestation did not occur in parallel with a significant increase of PGDS. These results suggest that mechanisms that independently increase PGES and PGHS activity, but not PGDS activity. This differential change in PGHS, PGDS and PGES activities can be induced by other co-factors or substrates present which specifically stimulate activity. For example, recent studies have shown that pentobarbital causes a concurrent up regulation of both PGHS and PGDS, but a concomitant and down regulation of PGES (Forsberg et al. 2000).

N) Page 150, original paragraph 3:

However, this does not completely rule out PGD_2 and PGE_2 in fetal sleep-wake maturation of regulation, as other mechanisms such as prostaglandin receptor affinity, density or distribution density or distribution may occur in the hypothalamus as part of the mechanisms required for the onset of sleep-wake cycles.

Amend to:

However, this does not completely rule out PGD_2 and PGE_2 in fetal sleep-wake maturation of regulation, as *discrete changes in concentration may occur but are masked by the gross measurements taken in the experiment. Furthermore, other* mechanisms such as prostaglandin receptor affinity, density or distribution density or distribution may occur in the hypothalamus as part of the mechanisms required for the onset of sleep-wake cycles. *However, since only the tritiated PGD_2 ligand of low specific activity was available at the time, these autoradiography and receptor binding experiments were not undertaken because of time constraints.*

General Discussion

O) Page 200, insert into the 13th line of paragraph 2:

In fact subsequent statistical analysis showed that, when examining the each PGD_2 treatment in isolation, 500 pmole/10 µl/min of PGD_2 caused a significant decrease in arousal compared to pre-treatment levels.

References

Insert:

Page 207 **Caravaggi, C., Clarke, F.L. and Jackson, A.R.B.** (1970) Acute selenium toxicity in lambs following intramuscular injection of sodium selenite. *Research Veterinary Science* **11**: 146 – 149.

Page 208 **Deauseault, D., Giroux, D. and Wood, C.E.** (2000) Ontogeny of immunoreactive prostaglandin endoperoxide synthase isoforms in ovine fetal pituitary, hypothalamus and brainstem. *Neuroendocrinology* **71**: 287 –291.

Page 209 **Forsberg, L., Leeb, L., Thoren, S., Morgenstern, R., and Kakobsson, P.,** (2000) Human glutathione dependent prostaglandin E synthase: gene structure and regulation. *Federation of European Biochemical Societies Letters* **471**: 78-82.

Page 212 **Haberl, C., Hultner, L., Flugen, A., Falk, M., Geuenich, S., Wilmanns, W. and Denzlinger, C.** (1998) Release of prostaglandin D_2 by murine mast cells: importance of metabolite formation for antiproliferative activity. *Mediators of Inflammation* **7(2)**: 79-84.

Page 218 **Mitchell, MD.** (1986) Pathways of arachidonic acid metabolism with specific application to the fetus and mother. *Seminars in Perinatology* **10(4)**: 242 – 54.

Page 226 **Szeto, H.H., Cheng, P.Y., Decena, Y.C, Cheng Y., Wu, D.-L. and Dywer J.A.** (1992) Fractal properties in fetal breathing dynamics. *American Journal of Physiology* **263**: R141-R147.

Page 229 **Vo, T.D., Dwyer, G. and Szeto, H.H. (1986)** A distributed micro-controlled system for data acquistion and power spectral analysis of EEG. *Journal of Neuroscience Methods* **16(2)**: 141-8.

Page 231 **Yang, G., Wang, S., Zhou, R. and Sun, S.** (1983) Endemic selenium intoxication of humans in China. *The American Journal of Clinical Nutrition* **37**: 872-881.

Table of Contents

Declaration

I hereby declare that, to the best part of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text. No part of this thesis has been submitted to any other university for any degree or diploma.

Brenda Lee

Acknowledgements

I'd like to thank

David Walker
for his
wisdom, encouragement, guidance and inspiration,

Jonathan Hirst, Milton Hearn, Reinhard Boysen,
Hooi Hong, Robert Andrews, Megan Wallace, Alan Tilbrook,
Luis Garcia-Fernandez, Ross Young, Nancy Nichols,
Tracey Warner, Jan Loose and Alex Santragno
for their
intellectual input
and technical advice,

my family
for their
unconditional support,

Kel, Marcus, Nicole, Amanda,
Andrea, Francis and Louie
for
making me laugh
and keeping me sane,

and especially
John
for everything.

Preface

Publications arising from these studies:

Publications:

B. Lee, J.J. Hirst, D.W. Walker,
Inhibition of prostaglandin D synthase by selenium chloride induces behavioural arousal in the ovine fetus.
American Journal of Physiology: Regulatory, Integrative and Comparative Physiology (accepted 2000)

B. Lee, J.J. Hirst, D.W. Walker,
Developmental changes in prostaglandin D and E synthase activities in the ovine hypothalamus of fetal and newborn sheep.
Pediatric Research (submitted 2000)

Abstracts:

B. Lee, D.W. Walker, J. Hirst,
Inhibition of prostaglandin D synthase in fetal sheep brain with selenium chloride induces arousal.
Federation of American Societies for Experimental Biology, San Diego, CA, USA, April 2000.

B. Lee, J.J. Hirst, D.W. Walker,
Prostaglandin D synthase inhibitor, selenium chloride induces arousal in fetal sheep.
The Perinatal Society of Australia and New Zealand, 8th Annual Conference, Melbourne, Australia, March 1999.

B. Lee, R. Boysen, M.T.W. Hearn, J.J. Hirst, D.W. Walker.
Measurement of β-trace expression and specific activity in the hypothalamus of fetal sheep.
Proceedings of the Australian Neuroscience Society, vol 10, 19th Meeting, Hobart, January 1999.

B. Lee, R. Boysen, M.T.W. Hearn, J.J. Hirst, D.W. Walker.
Identification of β-trace protein in cerebrospinal fluid and hypothalamus of fetal sheep.
Fetal and Neonatal Physiological Society, 25th Annual Meeting, Lake Arrowhead, CA, USA, September 1998.

B. Lee, J.J. Hirst, D.W. Walker,
A role of prostaglandin D and E synthase as regulators of sleep during ovine fetal development?
Journal of Sleep Research, vol 7(2), 14th European Congress on Sleep Research, Madrid, Spain, September 1998.

B. Lee, J. Hirst and D.W. Walker.
Prostaglandin D_2 and E_2 in the hypothalamus and cortex of fetal and neonatal sheep.
Proceedings of the Australian Neuroscience Society, vol 9:190, 18th Meeting, Canberra, January 1998.

Abstract

It has been proposed in the adult that endogenous prostaglandin D_2 acts on sleep-inducing centres within the ventrolateral preoptic area of the anterior hypothalamus to induce sleep (Scammell *et al.* 1998). In contrast, prostaglandin E_2 acts on the tubermammillary nucleus of the posterior hypothalamus to promote activation of the cortex, and so produce vigilance (Onoe *et al.* 1992). Prostaglandin D_2 is synthesised by the enzyme prostaglandin D synthase in the brain parenchyma (Narumiya *et al.* 1982), choroid plexus and leptomeningeal membranes covering the brain (Urade *et al.* 1987). It is thought that prostaglandin D_2 is secreted into the cerebrospinal fluid to act on a population of prostaglandin D_2-sensitive receptors on the ventral surface of the hypothalamus, adjacent to the ventrolateral preoptic area to promote sleep (Matsumura *et al.* 1994). The observation that prostaglandin D_2 concentrations in the cerebrospinal fluid (Pandey *et al.* 1995a) and prostaglandin D synthase activity in the brain (Ueno *et al.* 1985a) exhibit circadian fluctuations in parallel with sleep-wake behaviour in adult rats further indicates a role for this prostanoid in the regulation of natural sleep. Specific inhibition of PGD_2 synthesis, by intracerebroventricular infusion of reversible inhibitor selenium chloride, was shown to suppress sleep and increase the incidence of the time spent awake in adult rats (Matsumura *et al.* 1991).

Prostaglandin D synthase purified from the rat brain is structurally (Hoffmann *et al.* 1993) and functionally (Watanabe *et al.* 1994) homologous to β-trace, a major protein constituent of cerebrospinal fluid. Although the functional significance of this finding remains obscure, it is hypothesised that prostaglandin D synthase/β-trace is a protein in cerebrospinal fluid that has enzymatic activity, and may synthesise prostaglandin D_2. Thus in the adult, prostaglandin D_2 may be synthesised *in situ* at a number of sites in the brain, or from prostaglandin D synthase/β-trace that circulates throughout the ventricular system in

cerebrospinal fluid, to act at the putative sleep centres in the ventral lateral preoptic area of the hypothalamus, and globally facilitate sleep (Hayaishi *et al.* 1993).

During prenatal development in the sheep, sustained electrical activity in the cortex first appears at about 90 days gestation (term, 147 days). Cortical activity indicative of a sleep-like behaviour first appears at about 110 - 120 days gestation. Epochs of non-rapid and rapid eye movement sleep stages are partly differentiated by 120 days gestation and become fully developed by 130 days gestation. Thereafter, the fetus has a high propensity to sleep and spends only ~5% of the time 'aroused', as defined by the presence of nuchal muscle activity, fetal breathing and eye movements during low voltage electrocortical activity (Clewlow *et al.* 1983).

The overall aim of this thesis was to determine if prostaglandin D_2 has a role in the maintenance of prenatal sleep, as proposed for the adult. Firstly, experiments were performed where selenium chloride was infused into the lateral ventricle of the late gestation ovine fetus *in utero*. It was found that selenium chloride induced a dose-dependent increase in the incidence of arousal ($P<0.05$), whereas control infusions of artificial cerebrospinal fluid had no effect. Administration of 500 pmole/10 µl/min of prostaglandin D_2 immediately after intracerebroventricular infusion of 500 pmole/10 µl/min of selenium chloride abolished the selenium chloride-induced increase in arousal to below control levels ($P<0.05$). Infusions of prostaglandin D_2 alone appeared to decrease the incidence of fetal arousal, although this effect did not quite reach significance. These results suggest that selenium chloride increased fetal arousal by inhibiting the synthesis of endogenous prostaglandin D_2. Therefore, it was concluded that endogenous prostaglandin D_2 may play a significant role in the maintenance of fetal sleep, as hypothesised in the adult.

To further investigate the role of endogenous prostaglandin D_2 in the ontogenesis of sleep, the capacity of the ovine hypothalamus to synthesise prostaglandin D_2 and E_2 during prenatal life was then determined. The concentration of prostaglandin D_2 and E_2 and the activities of their respective synthetic enzymes, prostaglandin D and E synthases, were

determined in homogenates prepared from samples of the anterior and posterior hypothalamus obtained from 90, 125, 135 day fetuses, and from 8 week old juvenile lambs. Prostaglandin D and E synthase activities were calculated from the conversion rate of arachidonic acid to prostaglandin D_2 and E_2 respectively, both of which were measured by specific radioimmunoassay techniques. Prostaglandin D and E synthase activities were present from 90 days gestation, and detectable concentrations of prostaglandin D_2 and E_2 could be measured in the hypothalamus throughout fetal development. However, the concentrations of prostaglandins and the specific activity of prostaglandin D synthase did not change distinctly in association with the appearance of sleep patterns in the fetus. Other factors, such as changes in prostaglandin receptor affinity, which may occur in parallel with the ontogenesis of sleep need to be explored. The activity of prostaglandin E synthase was higher at 135 days gestation compared to other developmental ages ($P<0.05$). Whether this increase in enzyme activity may be linked to the appearance of arousal episodes in late gestation or other hypothalamic functions regulated by prostaglandin E_2, such as increased release of adrenocorticotrophic hormone (Morimoto *et al.* 1989), requires further investigation.

Finally, the temporal expression of prostaglandin D synthase/β-trace protein and mRNA was followed using immunoblot analysis and mRNA hydrisation techniques. Ovine hypothalamic and cerebrospinal fluid samples were assayed at 90, 120 and 135 days gestation, in parallel with the ontogenesis of fetal sleep. Two different antibodies were employed in this part of the study: the first was raised from the entire β-trace protein purified from human cerebrospinal fluid, and the second was raised from a peptide sequence common to the N-terminal of prostaglandin D synthase and β-trace protein derived from rat brain and human cerebrospinal fluid respectively.

Using the first antibody, it was shown that β-trace protein was not detectable in ovine cerebrospinal fluid at 90 days gestation, but was clearly present from 120 days gestation. Therefore, it is suggested the synthetic and secretory mechanisms that govern the release of β-trace into cerebrospinal fluid develop at this fetal age. Since the cardinal signs of sleep also first appear at ~110 - 120 days gestation, the observations suggest that β-trace in the

ovine cerebrospinal fluid may be associated with the ontogensis of non-rapid and rapid eye movement sleep. If so, these results may further substantiate the proposal that β-trace protein and prostaglandin D synthase enzyme may be not only structurally homologous but also share the same function *in vivo*. Further studies in this thesis show that the antibody that detected β-trace in the cerebrospinal fluid was not immunoreactive with prostaglandin D synthase in the ovine brain. It is conceivable that variations in antiserum immunogenicity to lower concentrations ofprostaglanidn D synthase in the brain could account for the differences in antigen immunoreactivity with this antibody. Whether the maturation of β-trace secretory mechanism is definitively linked to the onset of fetal sleep at 120 days gestation requires further investigation. *In vitro* studies, such as knockout mice that have the β-trace gene deleted would help elucidate this relationship.

The second antibody used had been raised against the peptide fragment of prostaglandin D synthase/β-trace, and successfully detected prostaglandin D synthase/β-trace in human cerebrospinal fluid but was not immunoreactive with a β-trace-like protein in ovine cerebrospinal fluid or the hypothalamus. These results suggest that the ovine N-terminal sequence of prostaglandin D synthase/β-trace protein is not homologous to that of the human and rat. This heterogenity between proteins was further substantiated by poor hybridrisation of a rat prostaglanidn D synthase probe to samples prepared from ovine cerebrospinal fluid and hypothalamus.

In conclusion, these results demonstrate that inhibition of endogenous prostagladin D_2 caused a change in fetal behaviour, increasing the incidence of arousal. Furthermore, the fetal hypothalamus has the capacity to synthesise prostaglandin D_2 and E_2 and measurable amounts of these prostanoids are present as early as 90 days gestation. While these results suggest that prostaglandin D_2/prostaglandin D synthase system could play a role in the ontogensis of sleep in the fetus, it is evident that other factors specific to the fetus, such as interation of neurosteriods with the $GABA_A$ receptor (Crossley *et al.* 2000), and placental release of adenosine (Karimi *et al.* 1996) and opioids (Szeto *et al.* 1988), should not be ruled out. It was observed that the secretion of β-trace protein into the cerebrospinal fluid coincided with the development of prenatal sleep. These findings suggest, but do not

definitively implicate, prostaglandin D synthase/β-trace protein in the ontogenesis and maintenance of sleep in the fetus.

Abbreviations

A	Ampere
ACh	acetylcholine
ACN	acetonitrile
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotrophin hormone
AU	arbitrary unit
Asn	asparagine
Å	Armstrong
BBB	blood brain barrier
BF	basal forebrain
B_{max}	maximum binding
bp	base pair
BP	blood pressure
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine 5'monophosphate
cDNA	complementary deoxyribonucleic acid
ChAT	choline acetyl transferase
Ci	Curie
CSF	cerebrospinal fluid
CNS	central nervous system
CP	choroid plexus
cpm	counts per minute
Cys	cysteine
°C	degrees Celsius
Da	Dalton
DEPC	diethyl pyrocarbonate
DDC	diethyl dithiocarbamate
dd-H_2O	double distilled water
DIEA	N,N'-diisopropyl-ethylamine
DMF	N,N'-dimethyl-formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSIP	δ-sleep-inducing peptide
DR	dorsal raphe
ECoG	eletrocorticogram
EDTA	ethylenediamine tetra-acetic acid
EEG	electroencephalogram
ELISA	enzyme linked immunosorbent assay
EMG	electromyogram
EOG	electro-oculogram
FBM	fetal breathing movements
Fmoc	9-fluorenylmethoxy-carboxyl
g	force of gravity
g	gram
G	gauge
GABA	γ -aminobutyric acid
HBTU	O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate
HCl	hydrochloric acid
HOBT	N-Hydroxybenzotriaole
h	hour
HV	high voltage
Hz	Hertz
H_2SO_4	sulfuric acid
H_2O	water
H_2O_2	hydrogen peroxide
Kb	kilobase pair
IL-1	interleukin-1
i.c.v.	intracerebroventricular
I.D.	inner diametre
IPTG	isopropylthio-β-D-galactoside
i.v.	intravenous
KCl	potassium chloride
KLH	keyhole limphete hemocyanin
Km	Michaelis constant
l	litre
LC	locus coeruleus
LDT	laterodorsal pontine tegmentum
LGN	lateral geniculate nucleus

LV	low voltage
m	metre
M	molar
MAP	mean arterial pressure
MBS	M-maleimidobenzol-N-hydroxyssuccinimide
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minute
mmHg	millimetres of mercury
mole	moles
MOPS	^{3}N[-Morpholino] propanesulfonic acid
mRNA	messenger ribonucleic acid
NA	noradrenaline
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	disodium hydrogen orthophosphate
NaH_2PO_4	sodium phosphate
NaN_3	sodium azide
Na_2SeO_3	sodium selenite
NO_2	nitrous oxide
nt	nucleotide
NREM	non-rapid eye movement
O.D.	outer diametre
O_2	oxygen
PB	parabrachial
PBS	phosphate buffered solution
pCO_2	partial pressure of carbon dioxide
PG	prostaglandin
PGDS	prostaglandin D synthase
PGD_2	prostaglandin D_2
PGES	prostaglandin E synthase
PGE_2	prostaglandin E_2
PGO	ponto-geniculo-occipital
PMSF	phenylmethylsulpholfluoride
POAH	preoptic area of the anterior hypothalamus
pO_2	partial pressure of oxygen
PPT	pedunoculo-pontine tegmentum
P1	antiserum raised against 20-mer peptide fragment
P2	antiserum raised against 13-mer peptide fragment
REM	rapid eye movement
RIA	radioimmunoassay
RNA	ribonucleic acid
RP-HPLC	reverse phase-high performance liquid chromatography
rpm	rotations per minute
SCN	suprachiasmatic nuclei
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
$SeCl_4$	selenium chloride
sem	standard error of the mean
sO_2	oxygen saturation
TFA	trifluroacetic acid
TFMSA	trifluoromethane-sulphonic acid
tHb	total haemoglobin
TMN	tubermammillary nucleus
TNBSA	trinitrobenzenesulfonic acid
Tris-Base	Tris[hydroxymethyl]-aminomethane
Tris-HCl	Tris[hydroxymethyl]-aminomethane hydrochloride
UV	ultraviolet
VLPO	ventrolateral preoptic
V	volt
v/v	volume/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galatoside
5-HT	serotonin

Chapter 1

Literature Review

1.1 Introduction

The behavioural state of sleep is a complex phenomenon, requiring the co-ordination of several brain regions. It is postulated that the principle 'sleep-centre', localised in the ventrolateral preoptic (VLPO) area of the anterior hypothalamus plays an imperative role in the orchestration of the integrated neural mechanisms that govern somnolence (Sherin *et al.* 1996). Amidst the long history of proposed sleep-inducing substances, it has been more recently hypothesised that endogenously synthesised prostaglandin D_2 (PGD_2) exerts a potent hypnogenic effect in adults by acting at the VLPO site (Scammell *et al.* 1998).

PGD_2 and its' synthesising enzyme prostaglandin D Synthase (PGDS) are ubiquitously distributed through out the body, but generally found in high levels within specific regions of the central nervous system (CNS), including the hypothalamus (Narumiya *et al.* 1982; Ogorochi *et al.* 1984). Recent studies have shown that PGDS is structurally (Hoffmann *et al.* 1993) and enzymatically (Watanabe *et al.* 1994) identical to the prevalent cerebrospinal fluid (CSF) protein named β-trace. The physiological significance of the structural and functional homology between the PGDS enzyme and the β-trace protein is not known. It is conceivable that the two previously identified proteins may be functionally the same, and thus PGDS may be present in high concentrations in the CSF. Therefore it is possible that somnolence may be globally facilitated by increased concentrations of PGDS/β-trace

circulating throughout the ventricular system and exerting a soporific effect by acting at the putative 'sleep centres' of the VLPO area.

The exact neural processes and cellular substances that dictate sleep behaviour in the adult are not entirely understood. Even less is known about the neurological mechanisms that govern the development of sleep prenatally. In the ovine fetus, like other precocial animals and perhaps the human fetus, the manifestations of somnolent and vigilant behavioural states develop *in utero* prior to birth (Dawes *et al.* 1972). Since PGD_2 and PGDS/β-trace have been strongly implicated in the regulation of sleep in the adult, this thesis is an investigation of the role that these substances may play in the ontogenesis of sleep-wake behaviours during ovine fetal development.

1.2 The Sleeping Phenomena

The concept that sleep is absolutely essential is somewhat of a truism. In fact humans spend at least a third of their lives in this slumberous state. Sleep, however, is not merely the result of physical fatigue or decrease in activity, instead it is a complicated behavioural state requiring the integration of several neuronal processes. Despite the necessity for habitual somnolence, the underlying mechanisms that regulate sleep and the definitive physiological functions of sleep are still not exactly elucidated.

1.2.1 What is Sleep and Wakefulness?

When studying sleep, it is imperative that one also understands the physiology of wakefulness, as sleep is a behavioural state that alternates with arousal. Generally, a recumbent posture, an increased threshold to sensory stimulation and a decreased level of motor output accompanies sleep. Uniquely, sleep is also associated with the fascinating behaviour of dreaming. In comparison, wakefulness is characterised by the highest, most

complicated conscious experience that makes us acutely aware of and thus interactive with our immediate surroundings and general environment (reviewed in Hobson 1999).

As far as we know, there are essentially three behavioural states observed in all mammals and birds, traditionally defined by electrocorticogram (ECoG) activity and associated behavioural features; they are rapid-eye movement (REM) sleep, non-rapid eye movement (NREM) sleep and wakefulness. It is critical to note that the parameters that define these behavioural states are merely semantic; a more accurate description and definition is presently limited by our lack of knowledge.

During sleep, two individual states can be clearly discerned based strictly on ECoG activity. At the onset of sleep, the brain deactivates and conscious awareness of the external world slips away as we drift into NREM sleep. NREM sleep is characterised by a diminution of autonomic and regulatory functions, including heart rate, respiration rate, blood flow, and more importantly a progressive change of ECoG activity to synchronised high voltage (HV), low frequency spindle activity occurs. This distinct cortical activity is the basis to terms used to describe NREM sleep: such as slow-wave sleep, synchronised sleep, quite sleep or HV sleep. NREM sleep is also associated with a reduction, but not a complete loss, of postural muscle tone and absence of motor-ocular movements (Hobson 1999).

During sleep, adults alternate between NREM sleep and REM sleep on an approximately 90 minute cycle, starting with 80 minutes NREM sleep and followed by a 10 minute REM sleep interval. REM sleep is identified by the presence of desynchronised, fast, low voltage (LV) ECoG. The LV ECoG activity that occurs during the REM sleep state is very similar to the thalamocortical activity exhibited during wakefulness. To scientists, the notion of an 'active' brain during periods of sleep seemed both an intriguing and paradoxical concept. Thus from these characteristics, REM sleep is also alternatively termed as paradoxical sleep, active sleep, desynchronised sleep and LV sleep. During REM sleep, LV ECoG activity occurs simultaneously with periodic bursts of rapid eye movements and changes in respiratory rate. It is also characterised by the inhibition of sensory input and absence of antigravity muscle activity, i.e. the subject experiences atonia within the major muscle

systems, with the exception of the diaphragmatic and ocular muscles (Siegel 1990). Other associated phenomena observed during REM sleep reside subcortically; the presence of phasic ponto-geniculo-occipital (PGO) spikes and highly synchronised hippocampal eletroencephalogram (EEG) activity (Sakai 1985). Generally dreams which occur during REM sleep are particularly vivid, accompanied with bizarre and apparently illogical thought and can be intense in emotion (Hobson 1999).

1.2.2 The Functions of Sleep

Freud, in his seventh and most theoretical chapter from his magnum opus of psychoanalytical theories *The Interpretation of Dreams* (1900), hypothesised that the process of dreaming served as a guardian of sleep: preventing the intrusion of forbidden wishes into the conscious waking. Unfortunately, Freud did not benefit from the fundamental knowledge that we have today, considering at that time basic phenomena such as REM sleep had not yet been discovered. Since then, extensive scientific research has attempted to unravel the mysteries of sleep. However, despite 100 years of rapid scientific progress and exploration, the definitive physiological function of sleep still remains a biological enigma.

1.2.2.1 Function of Non-Rapid Eye Movement Sleep

Intuitively it would be reasonable for one to assume that sleep serves a restorative purpose. Classically, it is believed that the function of sleep is to regenerate the energy that is expended during wakefulness and to maintain physiological homeostasis. This theory is generally accepted, especially when taking into account the detrimental consequences of sleep deprivation on normal behavioural functioning. Sleep deprived rats experience a variety of symptoms that are consistent to those exhibited in idiopathic mania. Furthermore, the observed manic symptoms of these insomniac rats were significantly attenuated following treatment with lithium (Gessa *et al.* 1995). Sleep deprivation is also recognised to have a detrimental impact on vital biological processes that are considered necessary for

cognitive processing and physical health (reviewed in Everson 1995). However, the exact mechanisms by which sleep, or alternatively the absence of sleep, profoundly effects our physical and mental welfare are not completely clear and require further elucidation. On a cellular level NREM sleep is characterised by a global reduction in brain activity and energy consumption (Steriade and McCarley 1990), the diminution of cerebral blood flow (Maquet *et al.* 1997) plus a decrease in glucose production and utilisation (Scheen *et al.* 1996). These observed decreases in activity are consistent with the hypothesis that NREM sleep serves as a restorative purpose. Furthermore, the onset of sleep is associated with the pulsatile release of growth hormone. In fact two-thirds of the daily secretion of this hormone occurs during the NREM sleep period, thus implicating synchronised sleep in the regulation of cellular regeneration and growth (Mendlewicz *et al.* 1999). Whether this association between development regulated by endocrine release of growth hormones and NREM sleep is causal or coincident remains unclear and requires further elucidation (Salzarulo and Fagioli 1995).

1.2.2.2 Function of Rapid Eye Movement Sleep

The high incidence of REM sleep in precocial fetuses and immature altricial neonates (see Section 1.2.4.2) compared to that present in the adult suggest that it may play a critical role in the development of neuronal connections and synaptogenesis (reviewed in Marks *et al.* 1995; Mirmiran 1995). It is uncertain whether the positive correlation between the high incidence of REM sleep with periods of neuronal plasticity is by definition a passive or active relationship. The association between the presence of REM sleep and increased neuronal activity could be interpreted as the emergence of behavioural states that reflect the degree of brain maturation; i.e. the capacity of neuronal circuitry to execute REM sleep behaviour. Alternatively REM sleep could play a crucial role in neuronal plasticity, providing a dynamic function in the ontogenesis of the CNS and subsequent long-term physiological and cognitive development. Episodes of REM sleep are characterised by intense endogenous cortical activation occurring simultaneously with all sensory input and motor output inhibition. Thus it is proposed that REM sleep may be necessary for the

provision of endogenous neuronal activation during a time when cortical stimulation is essential for neuronal plasticity. Thus, REM sleep may be crucial for satisfying the early-stimulation requirements of a growing brain and with the increase in postnatal age this need decreases as the brain matures and requires less auto-stimulation (Roffwarg *et al.* 1966). The subsequent promotion of long term development by REM sleep has been illustrated by suppression of PGO spiking in kittens by bilateral lesions of PGO pathway, which subsequently resulted in morphological and functional changes in the lateral geniculate nucleus (LGN) and thus long term damage to the visual system (Davenne *et al.* 1989). These results suggest that REM sleep may be essential for the onset of normal vision. Previous studies have also demonstrated that REM sleep deprivation in developing rats may retard normal functional development, causing marked alterations in adaptive behaviour including increased anxiety, reduced sexual activity and disturbed sleeping patterns (Mirmiran *et al.* 1983). In humans, REM sleep deprivation has also been linked to the onset of endogenous depression in adulthood (Vogel *et al.* 1990). REM sleep has also been implicated in normal cognitive function, playing a role in long term memory consolidation and perceptual learning, acquisition and retention of procedural knowledge (Karni *et al.* 1994).

Not every hypothesis explaining the function of sleep has been described. Unfortunately, despite the plethora of theories that exist, there is not one single hypothesis can exclusively account for all the data present.

1.2.3 Sleeping Patterns and Circadian Rhythms

Mammals are classified into two groups based upon habitual sleeping patterns; diurnal mammals which sleep during the day, as opposed to nocturnal mammals that rest at night. Despite this contrast in behaviour, the propensity of mammalian sleep is essentially synchronised to the same biological pattern; the circadian rhythm, that oscillates one complete cycle within a period of a day (Latin, *Circa*, around; *Dies*, a day).

The circadian system in the mammal is comprised of three important elements: the photoreceptor and visual pathway, the neural pacemaker, and the efferent projections that couple the pacemaker to afferent physiological activities. The circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is considered an intrinsic biological clock that synchronises the circadian rhythm. Visual pathways are required to entrain or reset the intrinsic biological clock with environmental stimuli, formally recognised as Zietgebers (German, *Zeit*, time; *Geber*, giver). The light-dark cycle is the principal entraining stimulus, which imposes a rhythm on a variety of different processes including behaviour such as motor activity, feeding, drinking, sensory discrimination, thermo-regulation, metabolism, hormone secretion (corticosterone and vasopressin) and sleep (for review see Moore 1990).

1.2.3.1 Influence of the Circadian Cycle on Sleep

The circadian system is critical for the expression of normal sleep-wake synchronicity. 'Free running' experiments show that sleep occurring in environments that are totally isolated from time cues follows an endogenous 24.5 - 25.5 hour rhythm (Wever 1975). Furthermore, total ablation of the SCN results in random distribution of sleep period through out a 24 hour period (Ibuka *et al.* 1977). Circadian rhythms have the most influence on the incidence of REM sleep, whereas NREM sleep is relatively unaffected (Broughton *et al.* 1990). Therefore it appears circadian rhythms are able to effect the propensity of sleep; where habitual sleeping patterns are superimposed upon an intrinsic biological rhythm. The two synchronised systems are tightly integrated and thus sleeping patterns can be altered by circadian rhythm manipulation. This is demonstrated by melatonin, a substance diurnally released from the pineal gland, and used to treat patients who suffer from jet lag and other sleeping disorders (Dawson and Encel 1993).

1.2.4 The Development of Sleep

The sleep-wake cycling patterns exhibited during fetal and newborn life are markedly different compared to those present in the adult. Throughout the wide cross section of mammalian species that have been explored it has been discovered that the ontogenesis of somnolent and vigilant behaviour in different mammals follow a similar developmental pathway, where the organisation of neurological activity into distinct behavioural states reflects of the maturation of the CNS.

1.2.4.1 The Developmental Course of Sleep Patterns in Human Life-span

REM and NREM sleep state patterns continuously change through out the entire human life span. After birth, REM sleep accounts for a high proportion of total sleep of the neonate. This amount sharply diminishes as maturation proceeds, falling from 5 to 2 hours per day between newborn infancy to 3 years of age. The total amount of REM sleep steadily decreases from childhood to old age. In comparison to the rapid diminution of REM sleep, NREM sleep gradually decreases from 8 to 5 hours per day from birth to old age, with a small increase during adolescence. The decrease in both REM and NREM sleep contributes to an overall decline in the total amount of time spent asleep which individuals experience during a lifetime (Roffwarg *et al.* 1966). In addition to the total amount of time spent asleep, the distribution of somnolent episodes also change during early maturation. During infancy the neonate experiences epochs of sleeping and waking which are short and randomly distributed throughout the day. Over the first 6 months, periods of sleep and wakefulness gradually consolidate into two distinct periods which progressively lengthen in time and form two distinct wake and sleep periods as a result of the establishment of circadian rhythm of sleep-wake cycles (Coons and Guilleminault 1984; de Roquefeuil *et al.* 1993). Interestingly, at old age sleep patterns revert back to infant-like sleep behaviour, being light and fragmented (Miles and Dement 1980). Therefore sleep-wake cycles continuously evolve during the human life span, with the most rapid changes occurring sequentially with the maturation of the CNS during early infancy.

1.2.4.2 Ontogenesis of Fetal and Newborn Sleep in Mammals

Upon examination of the ontogenesis of fetal and newborn sleep, mammals can be associated into two distinct categories. At birth, precocial species exhibit well developed sleeping patterns, in addition to other physiological systems including thermo-regulation and vision re also relatively well developed. In comparison, altricial animals exhibit relatively immature regulatory systems, where sleep patterns are not fully established at birth and develop postnatally. Humans (Roffwarg *et al.* 1966), pigs (Scott *et al.* 1990), sheep (Dawes *et al.* 1972) and monkeys (Balzamo *et al.* 1972) are recognised to be precocial animals as they are born with relatively mature CNS, thus the onset of somnolence and vigilance occurs *in utero* prior to birth. On the contrary, adult-like ECoG aspects of sleep and wakefulness are not fully developed in altricial animals such as the rat (Jouvet-Mounier *et al.* 1970), rabbit (Thoman *et al.* 1979) and cat (Shimizu and Himwich 1968) at the time of birth. Therefore it is hypothesised that the degree of sleep maturation is correlated with neurological maturity of CNS (Szeto 1992).

1.2.4.3 Identification of Distinct Sleep States in the Ovine Fetus

Since humans and sheep are precocial mammals sharing similar ontogenic pathways, the ovine fetus is considered to be an appropriate animal model used to explore the prenatal development of somnolence and vigilance. The behavioural states of both the human and ovine fetus undergo considerable modification as prenatal maturation of the CNS proceeds.

One of the early experiments that described the existence of behavioural states in the ovine fetus were made by Dawes, who monitored fetal ECoG by measuring cortical activity *in utero* via indwelling bi-parietal electrodes. He cited observations that distinguished three distinct behavioural states in fetal lambs from approximately 90 days gestation (term 147 days gestation). These behaviours were defined by variations in ECoG, breathing movements associated with REM, nuchal muscle activity and large variations in heart rate,

blood pressure (BP) and aortic blood flow, which appeared to be unrelated to maternal or fetal blood gas status (Dawes *et al.* 1972).

It is now well established that REM/NREM sleep and arousal develop in the ovine sheep prenatally, and that these can be defined by concurrent polygraphic recordings of ECoG, electromyogram (EMG), electro-oculogram (EOG) and fetal breathing movements (FBM). NREM sleep is identified as EMG activity occurring exclusively during HV ECoG, with the complete lack of changes in intra-thoracic pressure and eye movements, whereas REM sleep is defined as periods of LV ECoG occurring simultaneously with ocular and breathing movements, in the absence of nuchal muscle tone. In comparison, fetal arousal is characterised by episodes of EOG, FBM and EMG activity occurring together in the presence of LV ECoG. Essentially the fetus oscillates between the REM and NREM sleep states for the majority of time with intermittent bursts of arousal manifesting less frequently (see Fig. 1.1; Szeto and Hinman 1985).

The ontogenesis of the two sleep states is a highly organised procedure, where the arrangement of activity follows a very specific temporal pathway broken into three distinct developmental stages (Clewlow *et al.* 1983). Fetal spontaneous cortical potentials first appear from 90 days gestation, and begin to differentiate into HV and LV ECoG, where the superimposition of synchronised HV ECoG over desynchronised LV ECoG occurs at approximately 110 days gestation. By 120 days gestation the two different cortical activities can be clearly discerned and the fetus alternates between the desynchronised and synchronised electrocortical activity. The manifestation of episodic EOG, EMG and FBM occur in parallel with the differentiation of HV and LV ECoG. Fetal ocular, nuchal muscle and breathing movements are almost continuous until approximately 110 days when breathing movements gradually become episodic and are associated with ocular activity. From 130 days gestation when ECoG has distinctly differentiated into HV and LV ECoG, EMG, FBM and EOG are clearly episodic and exclusively occur in the appropriate behavioural state. The development of fetal sleep oscillating between synchronised and

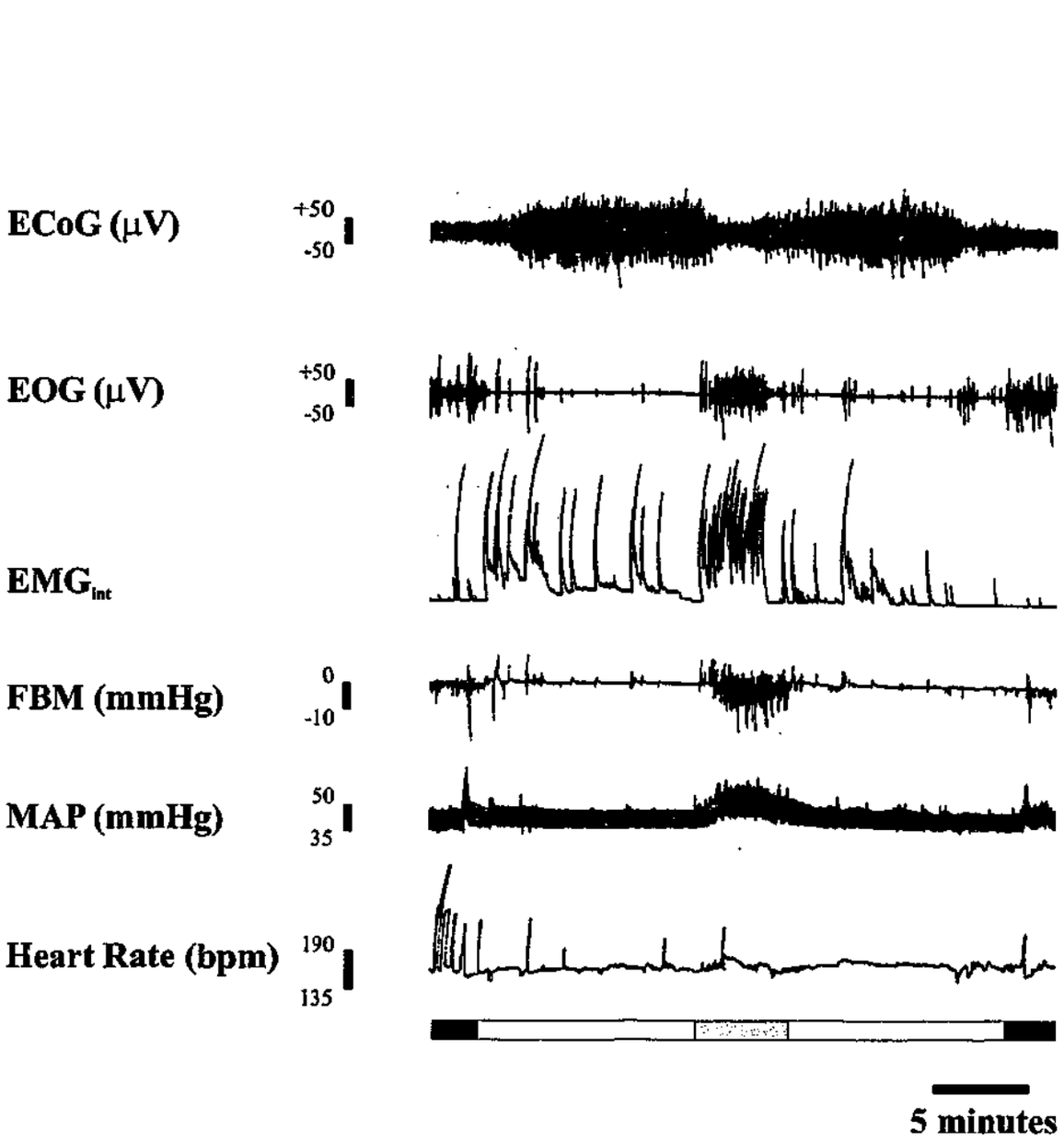


Figure 1.1
The three behaviourial states in the ovine fetus are defined by the electrocorticogram (ECoG) and associated electro-ocular (EOG) and integrated nuchal electromyographic (EMG) activities and fetal breathing movements (FBM). Rapid eye movement (REM) sleep is defined by low voltage (LV) ECoG occurring simultaneously with eye and breathing movements (black bar). In comparison, non-REM sleep is considered to be high voltage ECoG in the presence of nuchal muscle activity but the absence of FBM and EOG (white bar). During arousal the fetus exhibits burst of LV ECoG, EMG and EOG activity with FBM, which coincides with a transitional increase in mean arterial pressure MAP) and heart rate (grey bar).

desynchronised ECoG activity in a cyclical rhythm is an endogenous process and persist independently from maternal and environmental input (Ruckebusch *et al.* 1977).

1.2.4.4 Identification of Arousal in the Ovine Fetus

Remarkably, amidst the predominate quiescence of the prenatal existence, the fetus experiences behaviour which is consistent with wakefulness exhibited in the adult. The concept of fetal arousal was first deduced in premature lambs (115 - 147 days gestation) that were delivered under maternal epidural anaesthesia into a warm saline bath with the umbilical cord still intact (Dawes *et al.* 1972). Although the fetuses spent the majority of time in quiet or active sleep, each lamb exhibited infrequent periods where they appeared to be aware of their surroundings and were able to react to external stimuli. It was described that during this condition the fetuses could move their limbs, raise their heads and open their eyes to look around.

It is generally excepted that the fetus experiences episodes of arousal, which is consistent with postnatal wakefulness in terms of cortical activation and capacity to respond to external stimuli and executing motor activity. On average the fetus spends approximately 2 hours per day the in the wakeful state (Ruckebusch *et al.* 1977). The pattern of arousal progressively changes with age in parallel with the heterogenous sleep states, in that the incidence of arousal gradually increases at the expense of REM sleep significantly during the last trimester, i.e. REM sleep initially constituted as much as 60% of total sleep time and gradually decreased to 45% by parturition. As the 20 minute cycle of sleep (11 minutes REM sleep and 8 minutes NREM sleep) does not significantly change before parturition but the incidence of arousal does, it is evident the length of arousal periods increased between REM and NREM sleep episodes (Szeto and Hinman 1985). The state of arousal is also associated with transient increase in heart rate and BP, suggesting that the behavioural state is a complex phenomena, integrating cardiovascular, respiratory and metabolic fluctuations (see Fig. 1.1; Szeto 1983).

In summary, the cardinal signs of the three behavioural states of sleep and wakefulness in the ovine fetus are absent prior to 110 days gestation and considered to be poorly organised between 115 - 120 days. As the CNS matures, fetal ECoG begins to differentiate. By 130 days gestation the fetal behaviour is consolidated into distinct states, where the fetus alternates between long periods of quiescence and intermittent episodes of activity. Despite the unequivocal evidence that demonstrates the development of the sleep-wake states, the physiological significance of these behavioural states in the fetus is not fully understood. The functional significance of the high incidence of fetal REM sleep compared to adult REM sleep is not exactly clear. Nor is it known why the fetal experiences episodes of arousal *in utero*. In fact the concept of the fetus being awake is quite paradoxical, especially since the prenatal environment is not conducive to conscious behaviour and also requires long periods of quiescence, which is necessary to conserve energy for a rapidly growing fetus. Furthermore, the underlying mechanisms that govern this high propensity of fetus sleep are still not completely understood; whether sleep is regulated by the same neural substances and pathways in the fetus as it is in the adult also requires further elucidation.

1.2.5 Endogenous Sleep Substances

The existence of an endogenous sleep substance was originally proposed by Henri Pieron (1913). He postulated that during the course of the waking hours a 'sleep poison' or 'hypnotoxin', presumably derived from metabolic waste, accumulated in the brain to eventually induce sleep. In 1939, Schnedorf and Ivy further explored this theory by removing 8 ml of CSF by aseptic cisternal puncture from dogs that had been sleep deprived for 7 to 16 days. The CSF was transfused into naïve dogs, which had 8 ml of CSF previously removed. After treatment the recipient dogs exhibited a gradual reduction of vigilance, which eventually induced sleep 45 minutes to 3 hours after administration (Schnedorf and Ivy 1939). Although the authors did not observe sleep that was considered to be normal, essentially Schnedorf and Ivy confirmed Pieron's hypothesis and initiated the widespread search for an endogenous sleep-promoting substance.

Since then as many as 30 substances have been demonstrated to have sleep-promoting properties (reviewed in Borbely and Tobler 1989). Most of these hypnogenic factors are hormones or substances involved in the endocrine or immune response. One of the most extensively studied somnogenic substances includes δ-sleep-inducing peptide (DSIP), a blood-derived peptide that induces δ-delta-wave sleep, and Factor S, which was purified from the CSF of sleep-deprived animals and later identified to be muramyl peptide. Immune response modifiers that are reported to exert soporific effects include interleukin-1 (IL-1; Section 1.2.5.2) and PGD_2 (Section 1.3), which will also be discussed later. Steroid metabolites may also be implicated in maintaining fetal sleep and are mentioned (Section 1.2.5.3)

1.2.5.1 δ-Sleep-Inducing Peptide

Kornmuller is considered to be the pioneer who first discovered a sleep-inducing factor which was derived from the blood (1961). He and his co-workers performed cross-circulation studies between two cats, where the blood received from a cat induced to sleep by electrical stimulation increased δ-wave-sleep in the naïve cat. Fractional and purification procedures have led to the identification of a blood-borne sleep inducing nonapeptide that is believed to be responsible for the observed phenomena - Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu – designated as DSIP (Schoenenberger and Monnier 1977). Extensive research into the role of DSIP as a sleep promoting substance has been fraught with contradictory and inconsistent conclusions, mostly depending on several independent variables such as species, dose and administration route. It has been recently suggested that the peptide may likely represent only a fragment of a larger degraded protein molecule (Graf *et al.* 1985). Nevertheless, whether DSIP is a single entity or a integral component of a larger protein, it is generally accepted that it is essentially a potent stimulator of sleep.

1.2.5.2 Muramyl Peptides and Other Immunogens

When Pappenheimer and his co-workers extracted large quantities of CSF from sleep deprived goats and reintroduced the sample into the ventricle of rats, physiological NREM sleep was induced (Pappenheimer *et al.* 1967). In early years, the unknown hypnogenic substance was designated as Factor S. As sufficient amounts of Factor S were unavailable in CSF, the hypnogenic substance was alternatively extracted, purified and characterised from human urine (Garcia-Arraras and Pappenheimer 1983). Factor S was identified as N-acetylglucosaminyl-1-6-anhydro-N-acetylmuramyl-alanyl-glutamyl-diaminopimelyl-alanine or muramyl peptide, a common monomeric constituent of gut-derived bacterial cell walls (for review see Krueger 1985). It is these microbial products that are considered potent promoters of slow-wave sleep as well as immunomodulatory and pyrogenic activities (Krueger *et al.* 1982).

The cellular mechanisms by which muramyl peptide promotes somnolence is not completely elucidated. It has been hypothesised that sleep may be a controlled inflammatory state that employs immunogenic factors that were linked by co-evolution to gut flora such as muramyl peptides. The novel theory postulates that during wakefulness the permeability of the blood brain barrier increases due to exposure of gut-derived bacteria allowing subsequent accumulation of muramyl peptides in the brain, causing an increased propensity of sleep. Therefore sleep serves as a period which allows for the reconstitution of the blood brain barrier and clearance of accumulated sleep-inducing muramyl peptides (Korth 1995).

Alternatively, muramyl peptides may produce their somnogenic effect via production of cytokines and other immunogenic modifiers that are stimulated during an immune response. Substances including endotoxin and lipid A, double stranded polyriboinosinic:polyribocytidylic acid and IL-1, which initiate systemic host defence reactions, have also been shown to exert somnogenic effects (Krueger *et al.* 1990). It has been demonstrated that IL-1 maximally induces slow wave sleep when infused into the subarachnoid space underlying the ventral surface of the BF, situated adjacent to the

hypnogenic centres of the preoptic area of the anterior hypothalamus (POAH; Terao *et al.* 1998). Furthermore, levels of IL-1 mRNA were affected by sleep patterns and sleep deprivation (Taishi *et al.* 1998). Subsequently IL-1 was also shown to elicit the release of a host of somniferous factors that induce sleep (Krueger 1990), including PGD_2 (Yamamoto *et al.* 1988). Thus somnolence may be a self-perpetuating behavioural state generated from positive feedback mechanisms of immmunological substances.

1.2.5.3 Suppression of Fetal Arousal by Pregnanolone Metabolites in Utero

Since the sleeping patterns exhibited in the fetus are markedly different to that experienced by an adult, it is not certain whether the same mechanisms underlie both fetal and adult sleep. Perhaps alternative factors unique to or predominant in the fetus are required to maintain the high propensity of fetal sleep. During pregnancy increased levels of progesterone are synthesised and are believed to maintain uterine quiescence (Dolling and Seamark 1979), thus making the fetus invariably exposed to elevated levels of progesterone and progesterone metabolites, especially during late gestation. It has been demonstrated that pregnanolone, a major 5α reduced metabolite of progesterone, has potent sedative effects in the adult by interacting with the γ-aminobutyric acid $(GABA)_A$ receptor (Turner *et al.* 1989), inhibiting CNS activity. Studies have revealed that elevation (Nicol *et al.* 1997) and inhibition (Crossley *et al.* 1997) of maternal progesterone levels decreased and increased the incidence of ovine fetal arousal respectively. Furthermore pregnanolone administration directly into the fetus decreased fetal arousal, compared to $GABA_A$ antagonist, picrotoxin, which increased arousal episodes (Nicol *et al.* 1999). These results suggest that progesterone and progesterone metabolite production may tonically suppress fetal wakefulness, and thus be contributing factors that sustain the prenatal state of perpetual somnolence.

Despite years of effort, a single substance derived from the CNS that is solely accountable for inducing somnolence has not been described. Rather several non-exclusive factors have been shown to possess sleep-promoting qualities. It may be naïve to assume that one

specific substance is completely responsible for the onset and maintenance of such a complex behaviour that is associated with widespread physiological and neurological changes (see Section 1.5). Alternatively, it is conceivable that sleep is a shift of complex behavioural states, requiring integration of several substances.

1.3 Enzyme Prostaglandin D Synthase and Prostaglandin D_2 Regulate Adult Mammalian Sleep

Prostaglandins (PGs) are a family of naturally occurring lipid compounds that contain a unique 5 membered ring structure. They are ubiquitously distributed in mammalian tissues, exerting a variety of physiological and pathological effects such as disaggregation of blood platelets (Schror 1997), relaxation of smooth muscle (Walch *et al.* 1999) and pain and inflammation (Adelizzi 1999). It is generally excepted that PGD_2 is one of the major PGs unique to the CNS, when compared to the relatively low concentrations present in peripheral tissue (Narumiya *et al.* 1982). However, despite the relative abundance of PGD_2, little is known about its metabolism and exact function in the brain. It was only very recently demonstrated that PGD_2 possesses sedative effects in the mammalian adult brain, thereby initiating extensive research exploring the role of PGD_2 as a potent endogenous sleep-promoting substance in the brain (comprehensively reviewed by Hayaishi 1988; Hayaishi 1991; Hayaishi *et al.* 1993; Hayaishi 1997; Urade *et al.* 1995c; Urade *et al.* 1996; Urade and Hayaishi 1999).

1.3.1 Prostaglandin D Synthase and the Synthesis of Prostaglandin D_2

1.3.1.1 Arachidonic Cascade

The biosynthesis of the positional isomer PGD_2 is clearly defined (reviewed by Smith and Marnett 1991). PGD_2 is produced after arachidonate is converted to endoperoxides PGG_2

and PGH_2 by prostaglandin endoperoxide synthase (PGHS, commonly known as cyclooxygenase, E.C. 1.14.99.1) and then formed by a non-oxidative rearrangement of PGH_2 regulated the by enzyme PGDS [E.C.5.3.99.2] (Fig. 1.2).

1.3.1.2 Brain-type Prostaglandin D Synthase

Brain-type PGDS has been characterised from the adult rat brain, and has a molecular weight of 26 kDa (Shimizu *et al.* 1979b; Urade *et al.* 1985a). It differs markedly from PGDS derived from other tissues, in not having an absolute requirement for glutathione for activity. The cDNA of human (Nagata *et al.* 1991) and rat (Urade *et al.* 1989) brain-type PGDS have been cloned and sequenced. The amino acid sequence, deduced from the isolated cDNA, shows that the human and rat PGDS consist of 190 and 189 amino acid residues respectively with 71% identity and 83% similarity ratios. The structural organisation and chromosomal localisation for both the human (White *et al.* 1992) and rat (Igarashi *et al.* 1992) gene for the brain form PGDS has been established. The PGDS gene spans 3600 and 3000 bp in the human and rat genomic DNA library respectively, both consisting of seven extrons and six introns (Igarashi *et al.* 1992; White *et al.* 1992).

1.3.1.3 Member of Lipocalin Protein Superfamily

The deduced the tertiary structure of PGDS, based on observed amino acid sequence of the enzyme, is believed to consists of 8 stranded anti-parallel β-barrel structure forming a hydrophobic pocket (Fig.1.3; Nagata *et al.* 1991). The enzyme is also glycosylated exhibiting N-linked oliogosaccharide chains at two sites (Urade *et al.* 1989). Results from a data base search of proteins homologous in tertiary structure show that the PGDS enzyme appears to be a member of the lipocalin protein superfamily (Doolittle 1989; Peitsch and Boguski 1991). Essentially, lipocalin proteins are secretory hydrophobic carrier proteins, which transport lipophilic ligands within the unique hydrophobic pocket through humoral media (Nagata *et al.* 1991). Other protein members of this protein superfamily include β-

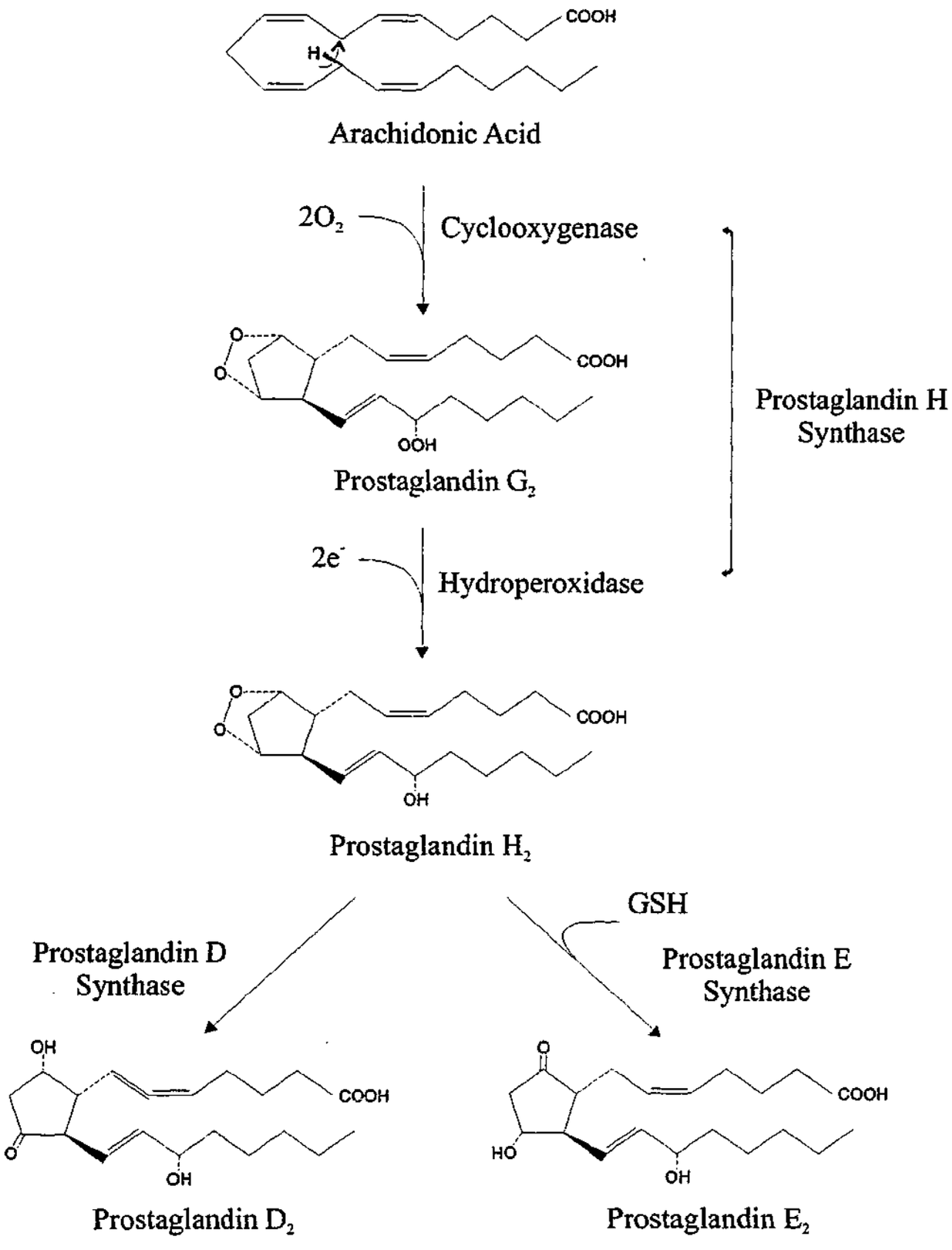
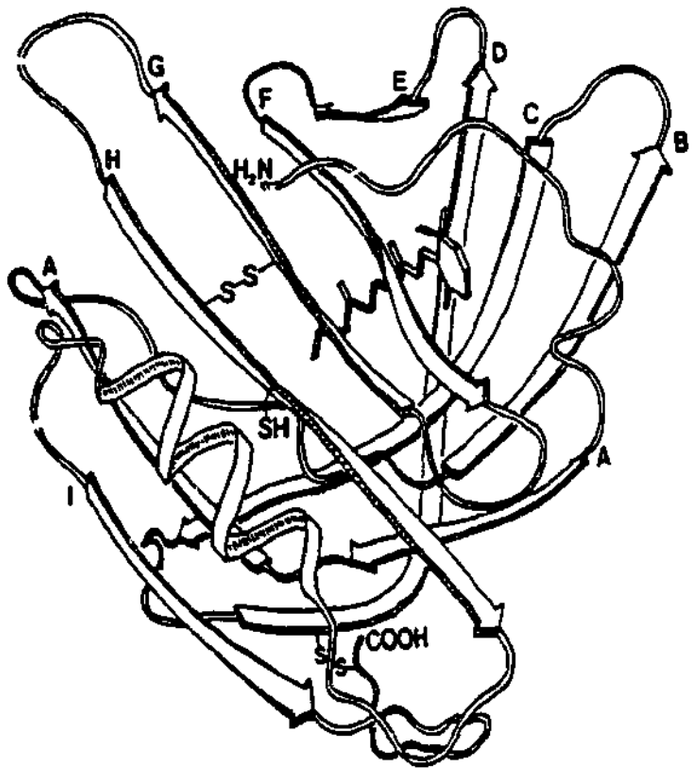


Figure 1.2
Production of prostaglandin D_2 and prostaglandin E_2 from common arachidonic acid precursor.



Prostaglandin D Synthase

Figure 1.3
Schematic representation of the deduced tertiary structure of prostaglandin D synthase (Nagata *et al.* 1991). Arrows indicate the β-sheets that form the hydrophobic calix, which is a unique characteristic exhibited by lipocalin proteins.

lactoglobulin (Godovac-Zimmermann 1988) and retinol-binding protein (Newcomer *et al.* 1984). Furthermore, the gene structure of PGDS is also remarkably analogous to other lipocalin proteins in terms of number and sizes of exons and phase of slicing of introns (Igarashi *et al.* 1992).

Interestingly, embedded within the hydrophobic pocket of the PGDS resides the unique cysteine[65] (Cys) residue which is not conserved within the other lipocalin proteins (Nagata *et al.* 1991). Therefore the Cys residue is proposed to be the active site of the enzyme, since it is absent from other lipocalin proteins that do not have enzymatic properties. This hypothesis was later substantiated by studies that performed specific site mutagenesis and chemical modification of the thiol group on the Cys residue, which demonstrated that the Cys was essential for the normal formation of PGD_2 from the PGH_2 precursor (Urade *et al.* 1995b).

Therefore based upon tertiary structure homology, it was postulated that PGDS is evolutionarily linked to lipocalin protein superfamily, but unlike other lipocalin proteins, PGDS exhibits a unique Cys residue embedded within the hydrophobic calix, enabling it to execute enzymatic activities. Together these results suggest that PGDS has differentiated from the hydrophobic-ligand carrier ancestry to become a unique protein and the only recognised enzyme that is a member of the lipocalin superfamily.

1.3.2 Prostaglandin D_2 and Prostaglandin D Synthase in the Central Nervous System

PGD_2 is generally considered to be one of the major PGs in the CNS. Past results have shown that PGD_2 is an predominate PG present in the adult rat brain (Abdel-Halim *et al.* 1977) but in contrast was barely detectable in the late gestation fetal ovine brain (Pace-Asciak and Rangaraj 1978). These discrepancies in results might be attributable to differences in age and species, or more importantly differences in assay technique, especially since PGD_2 is a considerably labile substance (Kelly *et al.* 1986). Nevertheless,

in the human (Ogorochi *et al.* 1984) and rat (Narumiya *et al.* 1982) it is excepted that both PGD_2 and PGE_2 are considered the major cyclooxgenase products of in the brain.

1.3.2.1 Regional Distribution in the Central Nervous System

PGD_2 derived from neural tissue was once thought to be an inert non-enzymatic decomposition product of PGH_2 It was not until relatively high concentrations of both synthesising and metabolising enzymes, PGDS (Shimizu *et al.* 1979a; Urade *et al.* 1985a) and 15-hydroxy PGD_2 dehydrogenase (Tokumoto *et al.* 1982; Watanabe *et al.* 1980) respectively, were purified and characterised in the rat and swine brain that attention was directed to the biological significance of the PG. It is now well established that PGD_2 is a major PG present in the rat brain, which is actively synthesised in neural tissue (Chiabrando *et al.* 1984; Islam *et al.* 1990). It exhibits significant regional differences in concentration in specific areas, with high concentrations present in the hypothalamus of the human (Ogorochi *et al.* 1984) and rat (Narumiya *et al.* 1982). The significant distribution and distinct regional localisation of PGD_2 suggest that it may have potential role in physiological functions, particularly those regulated by hypothalamic mechanisms.

PGDS is also constituitively expressed within the other areas of the CNS. PGDS appears to be mainly synthesised and secreted by the leptomeningeal lining enveloping the brain and the choroid plexus (CP; see Section 1.6.1.2). Immunohistochemical and *in situ* hybridisation studies have shown that the enzyme and transcript are predominately expressed in the leptomeninges and CP and the specific activity of these sites are respectively 3 and 7 fold higher than the activity exhibited in the brain (Urade *et al.* 1993). Additional studies have shown the leptomeninges and CP also actively secrete PGD_2 into cultured medium in vitro (Urade *et al.* 1995a). Both these sets of observations agree that PGDS is synthesised within the leptomeninges and CP.

1.3.2.2 Cellular Distribution in the Central Nervous System

On a cellular level, PGDS is mainly associated with the non-neuronal component of brain tissue in the adult rat. PGDS immunoreactivity was observed on the rough endoplasmic reticulum and the outer nuclear membrane of the glial cells in adult rat parietal cortex (Urade *et al.* 1987). More specifically, recent work found that in the adult rat PGDS mRNA is highly expressed in oligodendrocytes (Urade *et al.* 1993). In comparison, studies have demonstrated that adult (Giacomelli *et al.* 1996) and newborn (Seregi *et al.* 1987) rat astrocyte cell cultures have the capacity to actively synthesis and secrete PGDS *in vitro*. These studies suggest PGDS is specifically localised in the glial component of central nervous tissue in the adult rat. In comparison, PGDS immunoreactivity was also detected in neurons of 2 week old rat pups rather than in glial cells as demonstrated in adults (Urade *et al.* 1987). These results suggest during development PGDS plays a role in growth of the neuronal process, whereas in the adult PGDS may be alternatively involved in specific glial cell function, such as neuronal-glial interaction or myelin maintenance.

Therefore, it appears that PGD_2 and its synthesising enzyme PGDS are abundantly located in specific regions of the CNS, including the hypothalamus. This topographic distribution of both enzyme and product provides clues to the potential functional role that PGD_2 may have in physiological processes regulated by the hypothalamus. Studies which are described below (Section 1.3.3), hypothesise that PGD_2 could be an endogenous neuromodulator involved in the hypothalamic control of sleep.

1.3.3 Prostaglandin D_2 Induces Sleep in the Mammalian Adult

As previously discussed the putative substance that is solely responsible for the global induction of sleep remains to be identified. Unlike classical sleep substances that have been studied extensively, the soporific effect of PGD_2 has only been described relatively recently.

1.3.3.1 Prostaglandin D_2 and Prostaglandin D Synthase Role as Sleep Substances

The sleep-inducing effect of PGD_2 was discovered almost serendipitously after PGD_2 was injected into the POAH to determine the effect on body temperature and an unexpected increase in sleep was observed (Ueno *et al.* 1982a). Later studies confirmed these findings, where continuous microinfusion of PGD_2 into the third ventricle for six hours during active-nocturnal period induced a dose-dependent increase in REM/NREM sleep (Ueno *et al.* 1983). As little as 1 fmole/min of PGD_2 produced excess sleep indistinguishable to physiological sleep, judged by ECoG, EMG, EOG, brain temperature, heart rate and general behaviour. In addition, the rats were easily aroused by auditory stimulation, suggesting the PGD_2 induced sleep was physiological rather than a generalised pharmalogical anaethestic depression. This study was also replicated in conscious rhesus monkeys (Onoe *et al.* 1988), where 6 hour infusions of PGD_2 into the third and lateral ventricle during light period produced excess sleep without disturbance of sleep-wake circadian rhythms. Therefore, the evidence strongly suggests that PGD_2 may have a potential role in the regulation of sleep in adult mammals.

In contrast, it had been previously reported that a bolus intracerebroventricular (i.c.v.) administration of PGD_2 had little effect on sleep in rabbits (Krueger *et al.* 1992). Although the brain can readily take up PGD2 from the systemic circulation (Suzuki *et al.* 1986) it is possible that the promotion of sleep is dependent upon route of administration and, more importantly, the duration of PGD_2 exposure, requiring slow infusion to achieve long-term exposure. PGD_2 is actively metabolised, the half-life of PGD_2 in the brain and the blood is 1.1 and 0.9 minutes respectively (Suzuki *et al.* 1986). Increased sleep is also associated with sustained changes of PGD_2 production. For instance the Asian chipmunk shows seasonal variations of PGD_2 levels in the brain, revealing a strong correlation of increased PGD_2 with the hibernation phenomena (Takahata *et al.* 1996). Furthermore, high levels of PGD_2 in CSF are exhibited in the CSF of hypersomniac patients suffering African sleeping sickness (Pentreath *et al.* 1990) and malaria (Kilunga Kubata *et al.* 1998).

More discrete changes in PGD_2 and PGDS levels associated with somnolent behaviour have also been demonstrated, in addition to the gross or pathological behavioural alterations mentioned. Studies have shown that the specific activity of PGDS of the adult rat brain fluctuates in parallel with circadian rhythms, peaking during sleep periods and falling during wake periods, whereas levels of the dehydrogenase remain the same (Ueno *et al.* 1985a). Subsequent studies consistent with these results show that levels of PGD_2 in the CSF in conscious (Pandey *et al.* 1995a; Ram *et al.* 1997) and unconscious (Pandey *et al.* 1995b) rats exhibited diurnal fluctuations in parallel with sleep-wake cycles. More recently, studies show that transgenic mice that over-express the PGDS gene experience prolonged somniac periods in response to 'tail clipping', compared to wild-type counterparts (Pinzar *et al.* 2000). These investigators propose that increased levels of PGD_2, resulting from immunological response to the 'tail clipping' assault (Smith and Marnett 1991), caused the excess sleep experienced by the mice. Therefore, the evidence suggests that elevated levels of PGD_2 for prolonged duration may be required to initiate and maintain increased episodes of sleep.

1.3.3.2 Inhibition of Sleep by Prostaglandin D Synthase Inhibitor Selenium Chloride

Inhibition of PGD_2 production has been shown to decrease the amount of time spent asleep, suggesting the PGD_2-induced sleep is a consequence of an induction of physiological mechanism rather than a pharmacological side effect. Sleep suppression by PGH_2 inhibitors indomethacin and diclofenac sodium was first demonstrated in rats which received doses orally, intravenously and into the cerebral ventricle (Naito *et al.* 1988). Later studies showed that inorganic selenium chloride ($SeCl_4$) and sodium selenite (Na_2SeO_3) specifically and reversibly inhibited PGDS by interacting with the free Cys thiol group, the putative active enzymatic site of PGDS (Islam *et al.* 1991). Administration of $SeCl_4$ or Na_2SeO_3 intravenously (Takahata *et al.* 1993) and into the third ventricle (Matsumura *et al.* 1991) of free moving rats for 8 hours caused a marked cessation of sleep within two hours. Rats did not experience an elevation of body temperature, indicating the decrease in sleep occurred independently of thermo-regulatory mechanisms. It was thought that the observed

changes were not a result of pharmalogical toxicity from the inhibitors as general behaviour and eating patterns did not change. After cessation of treatment, the rats recovered from the insomniac period and normal REM/NREM sleep was fully restored.

1.3.3.3 Localisation of Prostaglandin D_2 Binding in the Hypothalamic Sleep Centres

PG receptors are widely distributed though out various tissues and organs, and mediate a variety of physiological responses. However, there is little knowledge regarding PGD_2 binding in the CNS. First evidence of PGD_2 binding in CNS tissue was demonstrated in pig, where high affinity binding of PGD_2 sites was observed in the Purkinjie cell layer of the cerebellum. This study also demonstrated that 15-hydroxy PGD_2 dehydrogenase is also localised at this same site, providing further evidence PGD_2 is actively bound and degraded in the brain and may be significantly involved in neural processes and functions (Watanabe *et al.* 1983). The binding of PGD_2 in human brain homogenates (Watanabe *et al.* 1985), and rat brain sections mounted on slides (Yamashita *et al.* 1983) was of high affinity, saturable, reversible and time dependent. PGD_2 binding was also found to be rich in the synaptic membranes or the synaptosomal fraction of rat (Shimizu *et al.* 1982) and human brain homogenates (Watanabe *et al.* 1985). Generally, in the human (Watanabe *et al.* 1985), monkey (Watanabe *et al.* 1989) and rat (Yamashita *et al.* 1983) brain PGD_2 binding is abundantly distributed in the cerebral and cerebellar cortex, hypothalamic, thalamic and limbic areas. In particular, distinct localisation of PGD_2 binding is exhibited in the POAH, the neural region closely linked with the autonomic control of the cardiovascular system and the putative centre of sleep regulation.

The close correlation of binding sites and localisation of PGD_2's central actions at the hypothalamus further indicates a role of PGD_2 for a hypothalamic regulator of sleep. Using a microdialysis probe, PGD_2 was administered into 200 different sites of the rat brain (Matsumura *et al.* 1994). It was observed that the site of action that elicited the greatest PGD_2-induced sleeping effect was localised in the medial ventral surface of the rostral BF, directly rostral to and in the immediate vicinity of the POAH and diagonal bands of Broca,

the putative sites of sleep regulation (see Section 1.5.2.1). In addition, PGD_2 administration into the subarachnoid space below the rostral forebrain of the rat not only increased sleep but also induced marked FOS protein expression in the VLPO optic area (Scammell *et al.* 1998). Nucleic FOS expression in the VLPO area, an index of neuronal activity, was positively correlated with amount of sleep (See Section 1.5.2.1). Electrophoretic studies in head-restrained unanaethesised rats have shown that PGD_2 applied to the POAH generally has an excitatory effect on sleep-active neurons (Koyama and Hayaishi 1994; Osaka and Hayaishi 1995). Studies have demonstrated that this response could potentially be modulated by acetylcholine (ACh; Inokuchi and Oomura 1986), whereas the inhibitory effect on the waking-active neurones is possibly modulated by noradrenaline (NA; Osaka and Matsumura 1995). However, the exact mechanism which governs the PGD_2-induced somnolence at the chemosensitive area of the rostral forebrain and the sleep centres of the anterior hypothalamus is unclear. Other substances such as nitric oxide (Matsumura *et al.* 1995), IL-1 (Terao *et al.* 1998), serotonin (5-HT; Hollingsworth and Patrick 1985) and human chronic gonadatrophin (Toth *et al.* 1994) also enhance the promotion of sleep by PGD_2 at this area. Thus PGD_2 may not act exclusively, but requires the involvement of other sleep substances and neurotransmitters to exert a soporific effect.

Therefore it is hypothesised that PGD_2 may act as a classical neurotransmitter to facilitate changes in behaviour. The exact cellular mechanisms by which PGD_2 produces these neural changes are not known. PGD_2 may bind to receptors of the POAH to increase intracellular calcium stores via cAMP (Okuda-Ashitaka *et al.* 1993) or by NA (Namima and Okamoto 1987), which are both are involved in neuronal modulation at a pre- and postsynatic level. Alternatively, PGD_2 may be a neurohormone, synthesised by the leptomeninges and CP and secreted into the CSF that acts at the PGD_2 chemosensitive zone at the ventral surface of the rostral BF and the putative sleep centres of the POAH to exert a somnogenic effect. Although the exact mechanisms are not exactly known, unequivocal evidence supports the hypothesis that PGD_2 is a potent sleep substance in the adult.

1.3.4 Prostaglandin E_2 as a Wake Substance

The awaking effect of PGE_2 was first demonstrated in chronically catheterised adult rats. PGE_2 was administrated into the POAH by microinjection and caused a significant dose-dependent decrease in both NREM and REM sleep (Matsumura *et al.* 1988), and that only the maximum dose induced an increase in rectal temperature suggesting that for lower doses the increase in vigilance was not a secondary effect of hyperthermia. Identical results were observed after PGE_2 was infused for six hours into the third ventricle of freely moving rats (Matsumura *et al.* 1989b). Conversely i.c.v. administration of the PGE_2 anatagonist, AH6809, into unanaethetised rats caused a decrease in the incidence of wakefulness (Matsumura *et al.* 1989a). The precise neural population responsible for the promotion of PGE_2-induced wakefulness was found to be localised in the tubermammillary nucleus (TMN) of the posterior hypothalamus (Onoe *et al.* 1992), the specific pool of neurons designated as the wake centre (see Section 1.5.1.3). Microdialysis administration of PGE_2 into the TMN elevated the incidence of arousal above other probed sites, and had negligible effects on heart rate and body temperature. In addition PGE_2 concentrations, sampled by microdialysis technique, increase and decrease in the posterior hypothalamus (Gerozissis *et al.* 1995) and prefrontal cortex (Gerozissis *et al.* 1998) in parallel with the occurrence of wakefulness and of slow-wave sleep respectively. These results further substantiate the role PGE_2 may play in the maintenance of wakefulness and cortical activation.

These studies propose that PGE_2 potently promotes vigilant behaviour in the adult rat by acting on TMN, the putative wake centre of the posterior hypothalamus. In contrast, extensive research demonstrates the hypnogenic effect of PGD_2, mediated by a neuronal network governed by the POAH. Therefore PGD_2 and PGE_2 may have a reciprocal relationship in regulating somnolent and attentive behaviour (Fig. 1.4). Simple administration of the prostanoids can initiate a shift in between the complex behavioural states of somnolence and vigilance; where changes in the precise balance of PGD_2 and PGE_2 concentrations can induce sleep and arousal by acting on the POAH and TMN respectively (see Section 1.5.2.4).

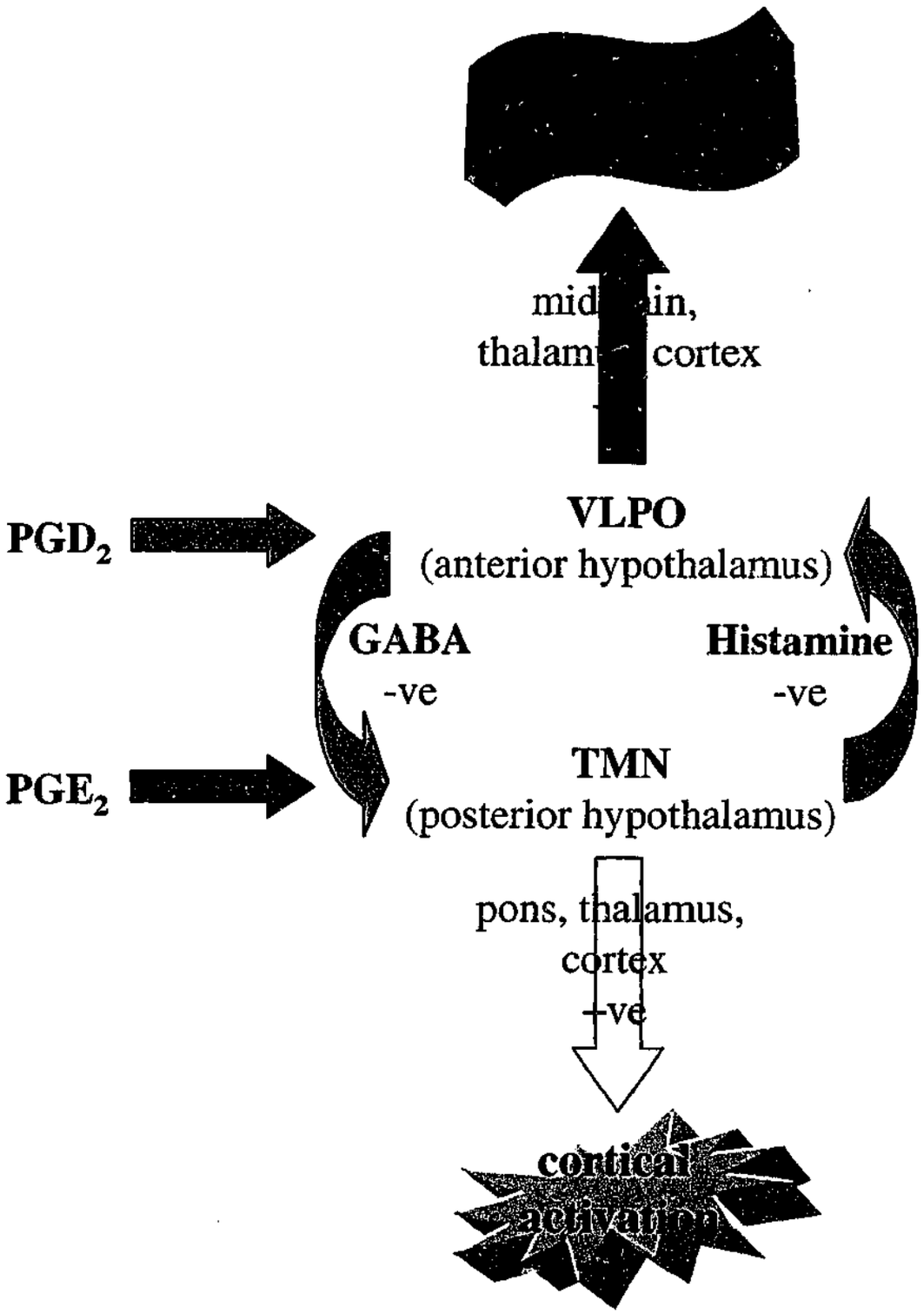


Figure 1.4
Schematic diagram illustrating the hypothesised interaction between prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) and the sleep-wake centres of the anterior and posterior hypothalamus respectively. PGD_2 exerts a soporific effect by acting on the ventrolateral preoptic (VLPO) area, which makes inhibitory projections (presumably GABAergic) to the arousal centre of the tubermammilary nucleus (TMN) and mesencephalic, thalamic and cortical nuclei involved with cortical activation. Conversely, PGE_2 induces wakefulness at the level of the TMN by reciprocally inhibiting the VLPO sleep-centre using histaminergic mechanisms and promoting cortical activation via pontine, thalamic and cortical afferents.

1.3.5 Other Physiological Functions of Prostaglandin D_2 and Prostaglandin E_2

PGD_2 and PGE_2 have both been implicated in other roles of neural regulation. For instance PGE_2 has been shown to increase newborn cerebral blood flow (Li *et al.* 1997), decrease fetal breathing (Kitterman *et al.* 1983) and promote the secretion of luteinizing hormone-releasing hormone (Rage *et al.* 1997). In comparison, PGD_2 may be involved in the processing of odorous stimuli at the olfactory bulb (Watanabe *et al.* 1986).

1.3.5.1 Hypothalamic Functions of Prostaglandin D_2 and Prostaglandin E_2

Specifically, PGs appear to be involved in several hypothalamic regulations, as suggested by the high density of PGD_2 (Yamashita *et al.* 1983) and PGE_2 (Matsumura *et al.* 1990) binding in the region encompassing the anterior wall of the third ventricle, especially the POAH and surrounding areas. PGD_2 and PGE_2 have been implicated in a reciprocal modulation of central cardiovascular and thermal regulation at the level of the anterior hypothalamus, since the high density of PG binding in the hypothalamus. Administration of PGE_2 into the POAH not only induces direct effects on the cardiovascular system by increasing blood pressure but has also been implicated in the role of hyperosmotic-evoked vasopressin secretion. Microinjections of PGE_2 and PGE_2 inhibitor, meclofenamate, into the preoptic and periventricular regions of the hypothalamus the have been shown to augment and attenuate plasma vasopressin respectively (Yamaguchi *et al.* 1997). On the contrary, administration of PGD_2 into the lateral ventricle caused opposite effects on the cardiovascular system, revealing an initial hypotensive effect (Siren 1982).

Traditionally, the induction of fever is believed to be initiated by pyrogenic cytokines, specifically PGE_2. The febrile action of PGE_2 has been distinctly localised in studies where microdialysis of PGE_2 into the POAH caused a significant hyperthermic response compared to other sites of administration (Onoe *et al.* 1992). Furthermore, microinjections of PGE_2 have shown to stimulate FOS-protein in the POAH. This topographic production of FOS mimics the expression of the early immediate gene induced by systemic immune

stimulation (Scammell *et al.* 1996), suggesting that the observed PGE_2-mediated elevation in body temperature was a physiological febrile response. In comparison, microinjections of PGD_2 into the POAH caused marked hypothermia in conscious adult rats, whereas infusions administered into the posterior hypothalamus had little effect on colonic temperature (Ueno *et al.* 1982b). This study also revealed that concentrations of PGD_2 significantly increased in POAH one hour after lipopolysaccharide-induced hypothermia.

Therefore, PGD_2 and PGE_2, although derived from the same precursor and structurally very similar, are capable of eliciting opposing physiological actions within the hypothalamus. PGD_2 and PGE_2 modulate fundamental systems such as the cardiovascular system and thermo-regulation, but can also induce complex changes in behavioural state. Alterations in sleep and arousal are also associated with variations in BP, heart rate and temperature, thus it is fair to speculate that the PG-induced sleep and arousal may be a secondary effect of these changes. However sleep-wake behaviour can be differentiated from cardiovascular and thermo-regulation variations by administering different doses that elicit each response. Therefore, the physiological processes are considered inter-related, and are most likely governed by independent mechanisms.

1.4 β-TRACE: PROPOSED REGULATOR OF SLEEP

β-trace is a single polypeptide chain, which was first identified by immunophoretic techniques in 1961 (Clausen 1961). Although found in a variety of tissues, β-trace is a major protein of CSF, with concentrations of ~2.6 mg/100 ml, constituting ~7% of total CSF proteins (Olsson *et al.* 1973). It is detected in other fluid media, such as urine and serum (Hoffmann *et al.* 1997). β-trace has been detected in various tissues; the highest concentrations of the protein are found in the white matter of the brain and stroma of the epididymus (Olsson and Nord 1973). Despite the early identification and widespread distribution of the β-trace, little is actually understood regarding the exact function of the protein. More recent studies have associated β-trace with thyroid hormone regulation in

growth (Garcia-Fernandez *et al.* 1993), transportation of lipophilic ligands (Hoffmann *et al.* 1996a), and more importantly the regulation of sleep in the adult (Hayaishi 1988).

1.4.1 Purification and Chemical Characterisation of β-trace

The β-trace protein isolated from human CSF is a unique single polypeptide chain with apparent relative molecular mass ranging of 25 - 31kDa (Harrington *et al.* 1993; Link 1967). The N-terminal sequence of the β-trace protein from pooled human CSF samples has been purified, and the amino acid sequence shown to be - APEAQVSVQPNFQQDKFLG (Kuruvilla *et al.* 1991; Zahn *et al.* 1993). The entire sequence of the polypeptide was generated by tryptic digest from human CSF (Hoffmann *et al.* 1993). Furthermore, the complete amino acid sequence was also deduced from cDNA sequence in porcine CP epithelia cells (Hoffmann *et al.* 1996b) and murine embryonic and adult tissue (Hoffmann *et al.* 1996a).

The primary structure of β-trace consists of 190 amino acids in length and carries exclusively N-linked oligosaccharides at two sites (Asn^{29} and Asn^{56}; Hoffmann *et al.* 1993). The carbohydrate structure of β-trace has been investigated in detail; there is a high degree of fucosylation and a bisecting *N*-acetylglucosamine is present, as well as terminal *N*-acetulglucosamine and galactose residues, and significant amounts of *N*-acetylneuraminic acid (Hoffmann *et al.* 1994).

1.4.2 Regional Expression of β-trace in the Central Nervous System

Studies have demonstrated that β-trace mRNA is predominately expressed in the epithelia cells of human CP, thus elucidating the CP to be the major site of β-trace synthesis (Blodorn *et al.* 1996). *In vitro*, β-trace is secreted from cultured porcine CP epithelia cells (Hoffmann *et al.* 1996b) and rat leptomeningeal cells (Ohe *et al.* 1996). Therefore the

evidence suggests that β-trace is primarily derived from the CP, and secondly from the leptomeningeal lining, rather than the brain parenchyma itself.

1.4.3 Identification of β-trace as Prostaglandin D Synthase, Implications in Sleep Regulation

To establish a possible function for this CSF protein, potential proteins that were homologous in structure were identified from a protein database. It was discovered, upon alignment of the 28 amino acids of the N-terminus, that human β-trace exhibited a high degree of homology to what had been previously and independently identified to be the enzyme PGDS. β-trace was 93% and 68% structurally identical to PGDS in the human and the rat brain respectively (Zahn *et al.* 1993). Alignment of entire amino acid sequences of human β-trace and rat PGDS confirmed positive conservation of amino acid sequence between the two proteins (Fig. 1.5), suggesting a strong relationship if not the identity of the protein (Hoffmann *et al.* 1993). Furthermore, β-trace purified from human CSF exhibited almost identical enzymatic properties to PGDS purified from the rat brain in terms of molecular weight, optimum pH, Km value and sulfhydryl requirement. In fact, β-trace appeared to have a higher specific activity compared to PGDS, 20 - 130 nmole/min/mg versus 2 - 7 nmole/min/mg protein (Watanabe *et al.* 1994).

Despite the unequivocal evidence that demonstrates the structural identity between the CSF-derived β-trace protein and the brain-type PGDS enzyme, whether this discovery has any functional significance requires further elucidation. The enigmatic question arises, can β-trace protein exert enzymatic activity in vivo like PGDS? Alternatively, does PGDS possess protein carrier-like abilities similar to that of other lipocalins while circulating in the CSF? Recent studies have revealed PGDS can bind to retinoids, suggesting it may have a potential role in retinoid transport *in vivo* (Tanaka *et al.* 1997). This could be another possible example of gene sharing, by which a single gene sequence codes for a protein that serves two completely different functions (Urade *et al.* 1995b).

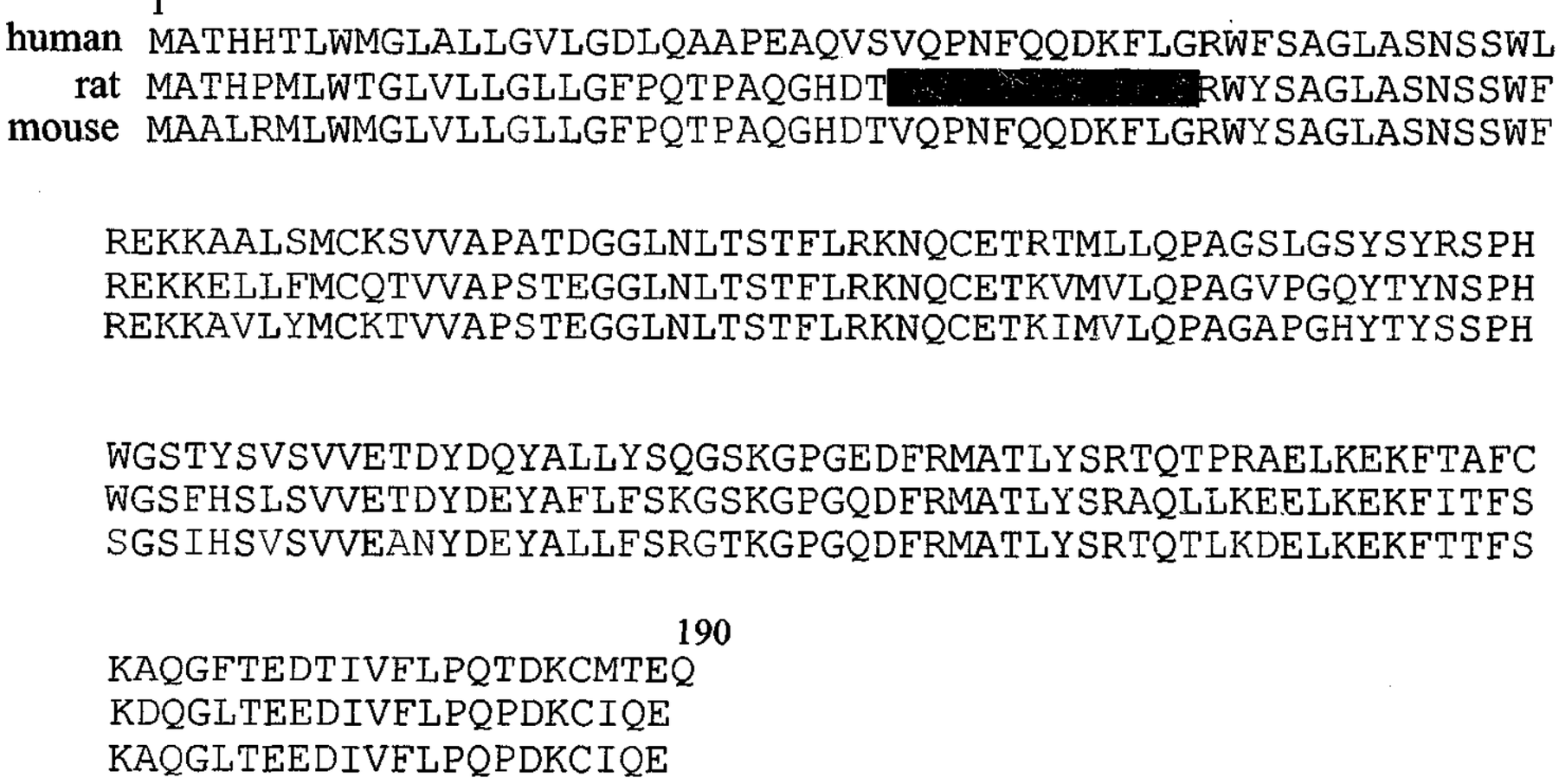


Figure 1.5

Comparison of amino acid sequence of human β-trace protein purified form cerebrospinal fluid (blue; Hoffman. *et al.* 1993), rat brain prostaglandin D synthase (pink; Urade *et al.* 1989) and murine β-trace (purple; Hoffman *et al.* 1996). Common residues are coloured in black. The synthetically synthesised peptide fragments P1 and P2 are demarcated in yellow and orange respectively.

Thus the concept that β-trace and PGDS are structurally and functionally identical precipitates many questions as to the role that β-trace may have in the CNS. If β-trace is enzymatically active in CSF it is conceivable that it may be involved in the global induction of sleep. After synthesis by the CP and then release into the CSF, β-trace may elevate PGD_2 concentrations in the CSF. Prolonged increased endogenous concentrations of PGD_2 may subsequently stimulate the PGD_2-chemosensitive area on the ventral surface of the POAH and thus facilitate behavioural somnolence via hypothalamic mechanisms (Loeschcke 1982). There is the possibility that the synthesis and release of β-trace is modulated by serotonergic, noradrenergic and cholinergic innervation of the CP (Section 1.6.2). Therefore it is postulated that β-trace and PGD_2 both may play a role as a potent sleep-promoting substance via 'volume transmission', where large quantities of neurohormones or neurosubstances are required to initiate fundamental shifts in complex integrated behaviour, such as consciousness and sleep (Fuxe and Agnati 1991).

1.4.4 Other proposed functions of β-trace

1.4.4.1 Blood-Fluid Barrier Mechanisms

Proteins demonstrated to be 100% homologous with CSF derived β-trace have also been identified in other specific blood-tissue barriers (BBB) and bodily fluids. β-trace has been localised in seminal plasma (Gerena *et al.* 1998), Leydig cells of the testis, prostate and epithelial cells of the epididymus (Tokugawa *et al.* 1998). It was also shown to be synthesised in the epithelial cells of the retina and actively accumulated in the interphotoreceptor matrix (Beuckmann *et al.* 1996), iris, ciliary bodies and eye fluids (Gerashchenko *et al.* 1998). During murine embryonic development, there is specific temporal expression of β-trace in specific blood-tissue barriers such as blood-CSF, blood-retina, blood-aqueous humor and blood-testis barrier. Thus, this study indicates that β-trace

may have a potential role in transport and or maintenance of these barriers attributing to their development (Hoffmann *et al.* 1996a).

1.4.4.2 Implications in Thyroid Hormone Function

Severe thyroid hormone deficiency during ontogenesis of the nervous system produces adverse effects on normal development and in the extreme form has been shown to cause mental retardation (Porterfield and Hendrich 1993). Hypothyroidism greatly reduces the temporal and spatial expression of β-trace in the brain during embryonic development (Garcia-Fernandez *et al.* 1997), suggesting that β-trace may play an integral role in normal synaptogenesis and maturation of neurons and glia. Further studies have shown that thyroid modulation on β-trace expression is directly regulated because the β-trace gene contains promoter regions specifically responsive to thyroid hormone (Garcia-Fernandez *et al.* 1998; White *et al.* 1997).

β-trace has also been implicated in numerous pathologies, where β-trace concentrations decrease in CSF during bacterial meningitis (Tumani *et al.* 1998) and increase in the hearts of angina patients (Eguchi *et al.* 1997). Thus, the specific function of β-trace appears to be ambiguous, emerging in a variety of diverse physiological and pathological situations. Whether the β-trace protein has a split biological role, functioning as both a protein transporter and a synthesising enzyme exclusively, or is simply evolutionarily related to PGDS and has an independent function, is not completely understood and requires further elucidation.

1.5 Neural Mechanisms that Control Sleep and Wakefulness

All associated phenomena that define arousal, REM and NREM sleep can be explained in terms of changes in neuronal activity. Over the last century the neurobiology of sleep has been explored at a cellular, systemic and molecular level. Although this research has

provided great insight to the physiology of sleep, the definitive answers to the adaptive significance of this complex behaviour still remain to be fully elucidated.

The following sections provide a brief insight into the complex neuronal activities that regulate vigilance and somnolence. Each behavioural state requires the orchestration of numerous neural regions extending from the brainstem to the cortical mantle. A recent hypothesis describes PGD_2 and PGE_2 as proposed sleep and wakefulness promoting substances by action at the putative somnolence and vigilance centres in the anterior and posterior hypothalamus respectively (Section 1.3). This section briefly describes the specific hypothalamic nuclei involved in the regulation of arousal and quiescence, and illustrates the pivotal role of these nuclei have within the entire integrated neuronal network that dictate these complex behavioural states.

1.5.1 Where is Wakefulness Generated?

1.5.1.1 The Brainstem Reticular Formation

The state of waking and consciousness is dependent upon the activity of the ascending reticular formation located in the brainstem (see Steriade and McCarley 1990). The brainstem reticular formation is an extensive network of neurons, which extends throughout the core of the medulla, pons and midbrain. It is roughly divided into two functional systems; the more rostral mesencephalic part called the midbrain reticular formation and the medullar part known as the medullar or bulbar reticular formation (reviewed in Coenen 1998).

Overwhelming evidence suggest that the ascending medullar reticular formation is vital for normal wakefulness, by maintaining tonic cortical activation via projecting pathways to the thalamus and basal forebrain from the midbrain reticular formation (Szymusiak 1995). The thalamic pathways terminate mainly at intrathalamic structures, particularly the intrathalamic and reticularis nuclei, that give rise to widespread reciprocal cortical

protections, named the thalamocortical loop. Thalamocortical excitation exhibits two distinct states in EEG activity: synchronised rhythmic activity in the form of delta, spindle and other slow waves apparent in NREM sleep, and desynchronised tonic activity during waking and REM sleep (see reviews by McCormick and Bal 1997; Steriade 1993). A ventral pathway project to nuclei in the hypothalamus and basal forebrain, primarily involved with sleep-wake activity (Szymusiak and McGinty 1989). These continue rostrally and dorsally to continue to make diffuse projections to the neocortex (Fisher *et al.* 1988) and thalamus (Asanuma and Porter 1990).

Therefore the brainstem reticular formation is considered to be an assembly of interconnecting neurons, which facilitates the excitability of a diffuse population of neurons in the thalamus, hypothalamus and the entire cortical mantle. It receives and integrates sensory afferents from visceral, somatic and sensory input, to exert widespread effects over most cortical fields through projections via the thalamus and hypothalamus, thus bringing the brain into a behavioural state associated with a heightened level of consciousness (see Fig. 1.6; also reviewed in Coenen 1998).

1.5.1.2 Neurotransmitters of Brainstem Reticular Formation

The brainstem reticular formation is primarily composed of a series of chemically specified groups which maintain specific connections and integrative roles; that is, noradrenergic projections originating from the locus coeruleus (LC), serotonergic projections derived from the dorsal raphe (DR) nucleus, cholinergic projections from the laterodorsal pontine tegmental (LDT) nucleus and pedunculo-pontine tegmental (PPT) nucleus (Kayama and Koyama 1998). The role of each of these neurochemical (and others including dopamine and histamine) involved in the behavioural regulation of cortical activation has been extensively studied and reviewed in Gottensman (1999).

Briefly, NA is localised in wake-activated cells of the LC (Tononi *et al.* 1994). Activity of noradrenergic LC cells appears to be associated with aroused processes compared to

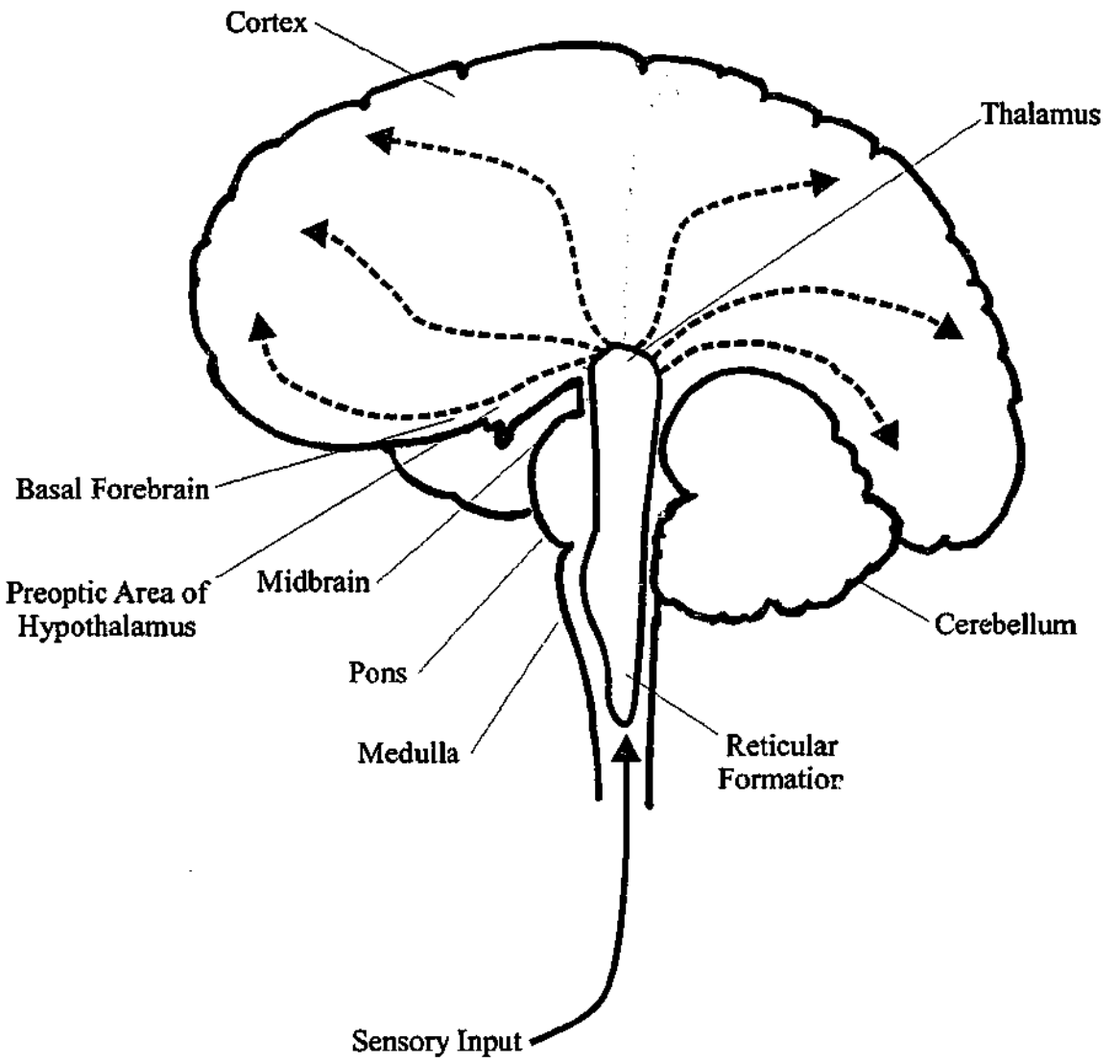


Figure 1.6
The state of consciousness and waking is dependent upon the activity of the ascending brain stem reticular formation. This interconnecting network of neurons extends throughout the core of the medulla, pons and midbrain and makes diffuse aborisation with the entire cortical mantle via projections through the thalamus, hypothalamus and basal forebrain. The brain stem reticular formation receives and integrates sensory afferents to exert widespread cortical activation to enable a heightened level of awareness of the external environment.

sedative behaviours, thus implicating the LC system in the control of vigilance (see Section 1.5.3.3 and review Aston-Jones 1985). More recent studies have shown that single-pulse stimulation of the LC inhibited sleep-related neurons of the POAH (Osaka and Matsumura 1994). Furthermore, application of NA into the POAH through a multibarrel pipette inhibited the activity of sleep-neurons present (Osaka and Matsumura 1995). These results further substantiate the role of noradrenergic LC cells in the promotion of wakefulness by inhibiting sleep-active neurons (see Section 1.5.2.1)

The classical neurotransmitter ACh is an important component of the reticular activating system as it is strongly associated with cortical activation during behavioural arousal and REM sleep (Steriade and McCarley 1990). ACh resides predominately in the LDT and PPT, and is believed to sustain widespread cortical activation by diffuse projections to the entire forebrain via neurons in the basal forebrain (see Section 1.5.3.4).

The serotonergic cells of the DR lie along the midline of the brainstem reticular formation. The involvement of the serotonergic cells in the maintenance of arousal has been long and controversial; with a myriad of lesion, electrophysiology and microdialysis studies showing conflicting results since 1964 (reviewed by Jouvet 1999). However, today serotonergic innervation is generally accepted to be associated with an increase in behavioural arousal and motor activity (Rueter *et al.* 1997). It has recently been shown that serotonergic cells of the DR nucleus make direct projections to cholinergic cells of PPT nuclei (Steininger *et al.* 1997), which are considered REM-on cells of the pons (see Section 1.5.3.2). Furthermore, microinfusions of 5-HT into REM-on cells of the LDT nucleus, which lie adjacent to the PPT, exerts an inhibitory effect somnolent behaviour, reducing the incidence of REM sleep (Horner *et al.* 1997).

The role of dopamine in sleep-waking behaviour is unclear. It has been shown to exhibit dual mode of actions; having a sedative effect at low doses compared to inducing cortical activation at high doses (Kropf and Kuschinsky 1991). Nevertheless, substantial evidence suggests that dopamine helps regulate several cognitive and behavioural functions that are involved with cortical arousal, such as temporal information processing and motor

execution (Rammsayer 1997). Histamine is also strongly implicated in the maintenance of cortical arousal and is discussed later (Section 1.5.1.3).

In short, during consciousness constant levels of ACh, NA, 5-HT, dopamine and histamine released from the brainstem reticular formation and ascending projections to the hypothalamus and cortex. These neurotransmitters maintain a heighten level of cortical activation thus producing awareness and enabling us to process data from our external environment. When the transition between wakefulness and NREM sleep is made, the reticular formation deactivates and changes the availability of these neurotransmitters thus altering the level of cortical activation. During REM sleep, the ACh concentrations return to waking levels to maintain a higher level of cortical activation. However, in contrast to the behavioural state of waking, during REM sleep external stimuli are not registered and replaced by internal representations of the surrounding environment.

1.5.1.3 Histaminergic Neurons of the Posterior Hypothalamus

The first clinical observations describing the significance of the posterior hypothalamus in the maintenance of wakefulness were made by Constantin von Economo, a volunteer lieutenant, in the Austrian Army (1931). He observed that several of his patients who suffered from influenza developed encephalitis lethergica or hypersomnia. Upon post-mortem he discovered that these patients exhibited pathological lesions in the posterior walls of the third ventricle. Conversely, patients who suffered insomniac syndrome exhibited lesions in the anterior hypothalamus. Therefore von Economo postulated that the posterior and anterior hypothalamus contained the vigilant and somnolent centres of the brain and that these lesions to these specific sites caused a disruption of normal sleep-wake behaviour.

Since then studies have confirmed the role of the posterior hypothalamus in the induction of arousal. These wake mechanisms have been localised in a tuberal region, which is collectively named the TMN (Lin *et al.* 1989). Studies have shown that a dense population of cells in this area are histaminergic (Watanabe *et al.* 1984), which are active during

waking, decrease in firing during NREM and become silent during REM sleep (Vanni-Mercier *et al.* 1984). Further pharmacological (Lin *et al.* 1988) and H_1 and H_2 antagonist receptor studies (Nicholson *et al.* 1985) support the hypothesis that posterior hypothalamic histaminergic cells are necessary for the maintenance of an aroused cortical state (reviewed by Monti 1993).

The mechanisms by which the histaminergic cells elicit the behavioural arousal are unclear. However studies have demonstrated activity of hypnogenic neurons of the POAH may be inhibited by posterior hypothalamic arousal centres mediated via H_1- and H_2-receptors (Lin *et al.* 1994). In addition, histaminergic cells in the TMN make widespread projections to the thalamus and cortex (Staines *et al.* 1987), and descending projections to the mesopontine cholinergic nuclei (Lin *et al.* 1996). Therefore histaminergic facilitation may promote cortical desynchronisation of thalamocortical firing and behavioural vigilance directly or indirectly via the cholinergic mechanisms residing in the pons.

1.5.2 Where is Non-Rapid Eye Movement Sleep Generated?

NREM sleep is a complex behavioural state requiring the co-ordination of multiple generators located throughout the brain; extending rostrally from the forebrain to more caudal regions in the brainstem. It is hypothesised that PGD_2 acts as an endogenous sleep-inducing substance by potentially stimulating somnolent mechanisms residing in the hypothalamus. Therefore this section primarily focuses on the integrated role of the POAH, within the entire neural network, in the maintenance of sleep. Although it is not completely understood, it is proposed that these hypothalamic mechanisms elicit sleep by inhibiting areas of the midbrain reticular formation, thalamus and cortex that are generally involved in cortical activation and arousal. Thus, it is via these processes that PGD_2 is presumed to induce a soporific effect.

1.5.2.1 Sleep Centres of the Anterior Hypothalamus

Originally it was believed that sleep was simply a state where all cognitive processing and functioning ceased. It was not until early this century after a series of clinical observations forced scientists to believe that sleep, like wakefulness, is a dynamic process, actively controlled by specific neuronal areas. Constantin von Economo first observed that many patients suffering influenza-induced insomnia exhibited pathological lesions in the anterior hypothalamus upon post-mortem (Von Economo 1931). Twenty years later, Nauta conducted experiments on albino rats that strengthened von Ecomono's hypothesis, which postulated a discrete area in the anterior hypothalamus was responsible for the generation of somnolence (Nauta 1946). He examined the effect of various transections made throughout the brain on sleep and behavioural patterns, and found that transections in the supraoptic levels of the hypothalamus produced severe insomnia. These were the first set of observations which ignited extensive research into what is now the putative sleep centre in the anterior hypothalamus and basal forebrain.

Since then, distinct neuronal pools confined to the POAH and basal forebrain (BF) have been specifically implicated in the role of sleep. For simplicity the term BF refers to the magnocellular nuclei of the BF, which include the horizontal and vertical limb of the diagonal band of Broca, the substantia innominata, globus pallidus, medial septum, peripallidal nucleus and lateral preoptic area. In adult cats the selective damage of the cell bodies by neurotoxic lesions in the BF (Szymusiak and McGinty 1986a) and POAH (Sallanon *et al.* 1989) have been shown to cause severe insomnia for several weeks. Conversely, the incidence of NREM sleep dramatically increased after electrical stimulation of these areas (Sterman and Clemente 1962). Electrophysiological recordings performed in adult cats demonstrate that individual neurons also located in the POAH (Kayama and Koyama 1998; Mallick *et al.* 1983) and BF (Szymusiak and McGinty 1986b) maximally discharge during the transition from waking to NREM sleep, which were comparatively inactive during wakefulness.

Recent studies using immunohistochemistry have shown a subpopulation of cells in the POAH, which coincide with the VLPO area, increase in FOS protein expression during periods of NREM sleep. This observed increase in FOS expression in the VLPO area was higher compared to other hypothalamic regions during NREM sleep and specifically declined during waking (Sherin *et al.* 1996). The amount of FOS protein accumulated in the nucleus is used as an index of the increased neuronal activity, thus further substantiating the essential involvement of the POAH-BF in the generation of the sleeping behaviour. The electrophysiological profile of these cells also delineate the role of the VLPO area in the regulation of sleep; upon examination this specific cell cluster exhibited an increase in neuronal discharge during both NREM and REM sleep, which fired in parallel to the depth of sleep (Szymusiak *et al.* 1998). Furthermore, specific cell damage by microinjection of ibotenic acid caused a decrease in FOS-immunoreactive neurons in the VLPO cell cluster and a parallel decrease in the incidence of NREM sleep (Lu *et al.* 2000). These studies reinforce the primary effect of the VLPO area on regulation of sleep.

1.5.2.2 GABAergic Cells of the Anterior Hypothalamus

It is widely accepted that GABA possesses inhibitory properties; studies have shown that enhancement of brain GABA levels, by inhibiting its catabolism by GABA transaminase, increases the incidence of behavioural sleep (Scherschlicht 1985). Immunohistochemical techniques have shown the presence of a high concentration of GABAergic synthesising cells in the VLPO area are thought to be involved with cortical activation (Gritti *et al.* 1993). These GABAergic VLPO cells have been shown to be active during waking and are inhibited by neurotransmitters classically involved in cortical activation: NA, ACh, 5-HT. However, interestingly their activity was unaffected by histamine (Gallopin *et al.* 2000). Therefore it is postulated that the classical neurotransmitter GABA localised in the VLPO area may play a significant role in the promotion of NREM sleep, and that these cells may be reciprocally inhibited by neurotransmitters of the reticular formation during arousal.

1.5.2.3 Ascending and Descending GABAergic Projections from the Preoptic Area of the Anterior Hypothalamus

POAH GABAergic cells make descending and ascending projections through the hind- and forebrain to neural areas involved in heightening consciousness and cortical activation. Although the exact nature of some of these connections are not completely elucidated, there is evidence to suggest these cells play an important role in the regulation of somnolent behaviour by providing GABAergic ascending and descending inhibition.

Briefly, neurons in the lateral BF which discharge selectively during NREM sleep have been identified with descending projections to the midbrain reticular formation (Szymusiak and McGinty 1989), which is considered vital for the maintenance of wakefulness (see Section 1.5.1). The study showed that single units within the midbrain reticular formation responded to stimulation of the POAH. These findings suggest that the POAH-BF may indirectly control state-dependent changes in thalamocortical excitability via inhibitory GABAergic descending afferents to the midbrain reticular formation.

GABAergic POAH-BF could also directly control thalamocortical activity through the descending projections to the thalamus. The thalamic reticularis nucleus receives afferents from the BF, which contain a GABAergic component as well as cholinergic neurons (Asanuma and Porter 1990). Despite the fact that the exact discharge profiles of these projections have not been characterised, potentially these connections could represent an additional and direct source of thalamocortical inhibition exhibited during NREM sleep.

Subsets of sleep-activated neurons from the BF have been identified to make diffuse aborisation directly to the neocortex (Szymusiak and McGinty 1989). Although these projections are thought to be mainly cholinergic (Detari and Vanderwolf 1987), GABAergic cortical projections have also been identified (Fisher *et al.* 1988). Anatomical studies show that terminals of GABAergic BF projections make diffuse aborisations with GABAergic interneurones distributed throughout the entire cortical mantle (Freund and Gulyas 1991) that are reportedly most active during NREM sleep (Steriade and Hobson

1976). This suggests these GABAergic projections from the BF may have a potential role in cortical disinhibition during NREM sleep.

1.5.2.4 Intra-hypothalamic Sleep-Wake Regulation

It is postulated that GABAergic cells of the POAH-BF also make monosynaptic connection to the arousal centres of the posterior hypothalamus. VLPO neurons, which accumulate FOS protein during sleep, provide direct input to the histaminergic neurons of the TMN in the posterior hypothalamus (Sherin *et al.* 1996). Thus it is hypothesised that upon sleep, the hypnogenic cells of the POAH-BF provide tonic inhibition of the wake-activated neurons of the TMN. This inhibition is believed to be GABAergic, since injections of the GABA agonist muscimol into both the TMN (Lin *et al.* 1989) and posterior hypothalamus (Sallanon *et al.* 1989) has shown to restore sleep in pre-treated insomniac cats. Therefore, it appears that an intra-hypothalamic regulation of sleep-wake behaviour exists. That is, the sleep and wake promoting centres in the anterior and posterior hypothalamus respectively play a reciprocal role in the modulation of sleep-wake cycles, via direct monosynaptic descending tonic GABA inhibition between the two regulatory bodies (see Fig. 1.4).

1.5.3 Where is Rapid Eye Movement Sleep Generated?

REM sleep, like NREM sleep, requires the dynamic interaction between forebrain and brainstem systems. Considerable evidence suggests that a distinct area of the pons, which is just a few cubic millimetres in volume, critically orchestrates the co-ordination of these widespread behavioural processes. These cells are located in the LDT nucleus, are cholinergic in natur ıd are considered to be the principal site required for the generation of REM sleep. However the exact mechanisms by which this discrete population of cells elicit such widespread neural changes that resulted in behavioural REM sleep are not completely clear and requires further elucidation (reviewed in Jones 1989).

1.5.3.1 Early Studies Investigating Pontine Rapid Eye Movement Sleep Regulation

The earliest studies showed that separation of the hindbrain from the forebrain in a *cerveau isole* cat by coronal transections abolished any REM sleep-like characteristics exhibited in the forebrain. The same study also demonstrated that muscle atonia, rapid eye movements, PGO spikes, as well as REM sleep-like activation of the reticular formation units still occurred in a regular ultradian rhythm caudal to the lesion (Jouvet 1962). Coronal transection between the medulla and spinal cord caused no disruption of the cardinal signs of REM sleep rostral to the transection (Adey *et al.* 1968). Therefore these results suggest that spinal as well as cerebral mechanisms are not necessary for the stimulation of complex behaviour exhibited during REM sleep.

More recent studies have shown that the generation of REM sleep can be localised to a specific region in the pons. Comprehensive experiments in adult cats, which destroyed various areas of the pontine tegmentum by radiofrequency heat, revealed that particular lesions situated in the lateral regions permanently eliminated REM sleep (Friedman and Jones 1984). This suggests that the lateral pontine tegmentum contain the nuclei responsible for the generation of REM sleep. These particular lesions, although restricted to the pontine tegmentum, were relatively diffuse and generally extended through the entire length of the pons (P 3.1 to P 6.0 in the cat), and encompassed several pontine nuclei.

1.5.3.2 Cholinergic Dorsalateral Potine Tegmental Nuclei Regulate Rapid Eye Movement Sleep

More recent electrophysiological and immunohistochemical techniques have been used to distinctly localise a discrete cluster of cholinergic cells that is proposed to be responsible for the genesis of REM sleep.

Electrophysiological recordings have demonstrated the presence of REM-on cells in the medial pontine reticular formation which discharge at a high rate throughout REM sleep but

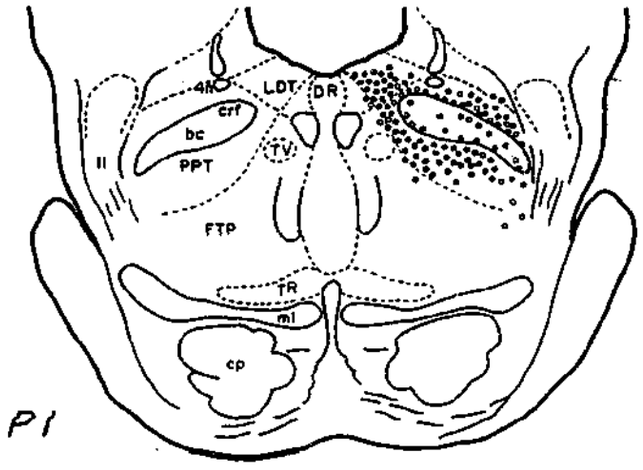
exhibit little activity during NREM sleep (Shiromani *et al.* 1987). In addition, the location of the reticular REM-on cells coincides with the lesions of the lateral pontine tegmentum that has also been previously shown to completely abolish REM sleep (Friedman and Jones 1984).

Immunohistochemical studies that labelled choline acetyl transferase (ChAT), the synthetic enzyme responsible for the production of ACh, have identified the presence of a dense population of cholinergic cells localised in the LDT nucleus (see Fig. 1.7; Jones and Beaudet 1987). Selective destruction of these neuronal bodies caused a decrease in the incidence of REM sleep, where the size of lesioned cholinergic LDT nuclei negatively correlated with the percent of REM sleep epochs, frequency of PGO spikes, and positively correlated with the presence of EMG activity (Webster and Jones 1988). Furthermore, cholinergically induced REM sleep has shown to increase FOS-like immunoreactivity in LDT (Yamuy *et al.* 1993) and PPT nuclei (Shiromani *et al.* 1992; Shiromani *et al.* 1995). Together these studies illustrate a discrete pool of cholinergic nuclei with the pontine tegmentum, namely the LDT nuclei, to be critical for the generation and maintenance of REM sleep.

1.5.3.3 Intra-pontine Sleep-Wake Regulation; REM-on & REM-off cells

It is hypothesised that the cholinergic cells of the LDT nuclei and the noradrenergic cells of the LC play a reciprocal role in the regulation of vigilance and somnolent behaviour. Although these clusters of cells lie adjacent to each other; almost overlapping, each population displays their own markedly different electrophysiological profiles during the sleep-wake cycle. ChAT-positive cells are clustered within the LDT and PPT nuclei and extend caudally to the LC and parabrachial (PB) nuclei, whereas tyrosine-hydroxylase positive cells are concentrated within the LC and PB nuclei and extend rostrally towards the LDT and PPT nuclei (see Fig. 1.7; Jones and Beaudet 1987). The noradrenergic cells of the LC, together with the serotonergic cells of the DR system, exhibit a similar discharge pattern throughout the sleep-wake cycle. In that, during wakefulness these cells discharge at

Rostral Pons



Caudal Pons

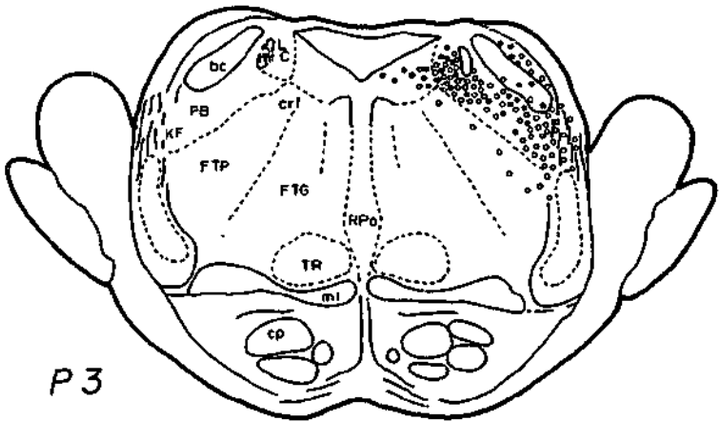


Figure 1.7
Closed circles indicate the location of choline acetyltransferase containing neurons (presumably cholinergic) of the laterodorsal pontine tegmental (LDT) nucleus and the pedunculopontine tegmental (PPT) nucleus, which are considered to be the principle generators of rapid eye movement (REM) sleep. Situated adjacent to these REM-on cells are tyrosine hydroxylase positive REM-off cells (presumably noradrenergic; open circles) of the locus coeruleus (LC) and parabrachial (PB) nuclei that are silent during REM sleep and thus believed to be involved in the maintenance of wakefulness. Therefore, it is hypothesised that the pontine REM-off and -on cells have a reciprocal role in the regulation of REM sleep.

Abbreviations; bc, brachium conjunctiva; cp, central peduncle; crf, central reticular fasciculus; DR, dorsal raphe nucleus; FTG, gigantocellular tegmental field; FTP, paralemniscal tegmental field; KF, Kolliker Fuse nucleus; 5m, tract of the mesencephalic trigeminal nucleus; ml, medial lemniscus; 4n, 4th nerve; RPo, raphe pontis nucleus; TR, tegmental reticular nucleus; TV, ventral tegmental nucleus. Illustrations modified from Jones and Beaudet (1987).

a regular rate, between the transition from wakefulness to NREM sleep the firing rate dramatically decreases, and throughout REM sleep activity is almost completely silent (Aston-Jones and Bloom 1981). Furthermore the neural clusters show no changes in FOS expression during REM sleep (Merchant-Nancy *et al.* 1992), and thus are designated REM-off cells. In comparison, cells of the LDT and PPT nuclei are considered to be REM-on cells, which increase in firing rate during REM sleep and exhibit little activity during NREM sleep (Shiromani *et al.* 1992). Thus, located in close proximity within the pons are two populations of neurons that demonstrate completely opposing functions. It is proposed that REM-off cells may have a significant interactive role with REM-on cell in the regulation of sleep-wake cycles, perhaps by gating or disinhibition. However, the exact mechanisms that govern this reciprocal relationship are unclear and require further elucidation.

1.5.3.4 Ascending and Descending Projections from Laterodorsal Pontine Tegmental Nuclei

The destination of the cholinergic projections from the LDT nuclei also provides further evidence for the role of the pontine reticular nuclei as the principle generator of REM sleep. Immunohistochemical studies show that neurones from this pontine centre make prominent ascending innervations to the intralaminar nuclei of the thalamus, as well as providing descending projections to the ventral and dorsal horn of the spinal cord (Jones and Yang 1985). Thus it can be seen how pontine cholinergic cells have widespread influences on the hind- and forebrain to produce the cardinal signs associated with REM sleep. During REM sleep innervation by cholinergic projections can promote cortical synchrony, as exhibited in arousal, via the thalamic intralaminar cells. In addition intralaminar cells also aborize with the dorsal lateral geniculate nucleus, which is responsible for the generation of PGO spikes classically and uniquely observed in REM sleep (Sakai 1985).

Therefore a small region of the pons, namely the LDT nucleus, appears to be critical for the generation of the widespread behavioural changes associated with REM sleep. The activity

of these pontine cells continually change with shifts in the behavioural state; in that, they are active during wakefulness, silent during NREM sleep and firing through out REM sleep. The exact processes underlying REM sleep are not completely understood, although they are presumed to be cholinergic in nature.

1.6 PHYSIOLOGY OF CEREBROSPINAL FLUID

Within the confinements of the skull and vertebral column, the brain and spinal cord are securely suspended in an aqueous environment provided by the CSF. For normal functioning, it is imperative that the brain exists in a well-controlled internal environment isolated from the general condition of the body. Barriers between the blood-brain and blood-CSF help achieve this stable state by selective filtration and active secretion of specific substances into the internal environment. Therefore, the CSF of the ventricular system provides a means to maintain a controlled environment. It is also proposed that CSF serves as a medium for the communication of many endocrine substances essential for regular brain functioning, including behavioural regulation such as sleep induction. The following section gives a brief overview of the physiology of CSF in hope to provide a better understanding of how PGD_2 (Section 1.3), β-trace/PGDS (Section 1.4) and other CSF derived sleep-inducing substances previously mentioned (Section 1.2.5) may promote a soporific effect.

1.6.1 Structure of the Ventricles, Subarachnoid Space and Choroid Plexus

1.6.1.1 Ventricular System

The CSF is synthesised and secreted by the CP and circulates throughout the CNS via the ventricles and subarachnoid space. Ventricles are the vestiges of the hollow tube from which the CNS arose in early embryonic stages. The ventricular system consists of four

hollow interconnecting cavities within the brain that are hard to visualise on a single 2D plane (Fig. 1.8). The two lateral ventricles situated inside the cerebral hemispheres are the most complicated of all the cavities. It is divided into three anatomical parts designated as horns. The left and right anterior horns reside within the each frontal lobe respectively. These extend caudally to the splenium of the corpus callosum where they divide into the posterior horn of the occipital lobe and the inferior horn of the temporal lobe. Both anterior horns open by the interventricular foramina of Monro into a single third ventricle found in the midline dorsal to the thalamus. The third ventricle has a preoptic recess between base of lamina terminalis and optic chiasm and also communicates with the fourth ventricle via aqueduct of Sylvius. The fourth ventricle is situated between the dorsal surface of the pons and ventral base of the cerebellum and continues caudally as the spinal canal (Davson *et al.* 1987).

1.6.1.2 Lining of the Brain and the Subarchanoid Spaces

The brain is covered by three membranous linings; the dura, arachnoid and pia mata. The dura mata is a tough fibrous membrane which lines the inner walls of the cranium whilst the finer pia mata follows the contours of the brain sulci. In between the two linings is the arachanoid mata. The space between the apposing dura and arachnoid mata is called the subdural space and is only a potential gap containing a thin film of moisture. Since the arachnoid mata does not follow the convolutions of the brain surface, the subarachnoid space between the arachnoid and pia mata holds a considerably greater amount of CSF compared to the volume of the ventricles. The subarachnoid space makes direct communication with the fourth ventricle via the foramen of Magendie and foramina of Luschka (Brodal 1992). The arachnoid and pia mata are collectively recognised as the leptomeningeal lining and are usually considered to provide a protective barrier function. However, it has more recently been implicated in more dynamic role in many potential biological events since it has been shown to secrete many proteins including PGDS,

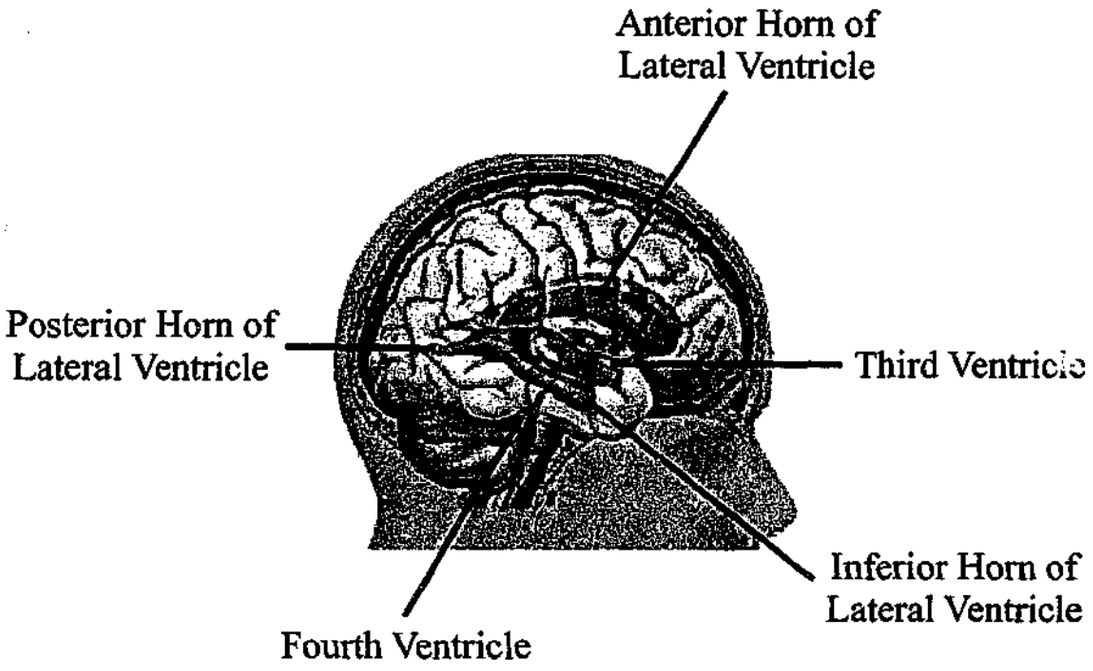


Figure 1.8
Picture illustrating the ventricular system of the central nervous system.
Modified image by Pat Thomson taken from the Internet.

insulin-like growth factor and binding protein apolipoprotein E, cystatin C, transferrin and collagen (Ohe *et al.* 1996).

1.6.1.3 Choroid Plexus

The pia mata, which lines the roof of the third and fourth ventricle and the walls of the lateral ventricle, become highly vascularised; it is this morphological modification which constitutes the CP. The surface of the CP is increased by numerous villi consisting of single cuboidal epithelial cells, characterised by abundant mitochondria and rough endoplasmic reticulum that are a typical feature of protein synthesising cells, covering highly vascularised connective tissue stroma. The capillaries of the CP are fenestrated, hence contain gaps that favour the passage of lipophilic substance while excluding hydrophilic and macromolecules. In comparison, the cuboidal epithelium of the CP is sealed by unique tight junctions between the epithelial cells, it is these specialised tight junctions that form the blood-CSF barrier (Nilsson *et al.* 1992).

1.6.2 Functional Aspects of Choroid Plexus and Cerebrospinal Fluid

For many years, CSF production and absorption was thought to function primarily as a means of removing metabolites from the brain: i.e. CSF acted as a 'sink' to remove waste substances. It is now established that CSF and CP have a more specific role in maintaining a homeostatic environment by providing a selective barrier between the blood and brain matter. Furthermore, the CSF and CP have also been recently implicated in neurogenic and endocrinological mechanisms.

Both BBB and CP act as a protective physical interface between the internal environment of the CNS and the rest of the body. To provide optimal conditions for neural functioning, these barriers facilitate selective inclusion or exclusion of blood-derived substances into the CSF and subsequently brain tissue by either diffusion or active transport (reviewed in Saunders *et al.* 1999b). Many of these substances are essential nutrients necessary for

normal brain functioning such as glucose whose entry mainly occurs at the BBB (Davson *et al.* 1987).

Analysing the composition of CSF, it is clearly evident that it cannot simply be a by-product formed as a result of plasma ultra-filtration. Alternatively CSF requires modification by active secretion of the CP. It is generally accepted that the CP contributes to 60 - 90% of CSF production, and turns over 4 - 5 volumes per day (Davson *et al.* 1987). The CP also actively secretes plasma proteins into the CSF, and is considered to be the major source of transthyrethin, serum retinol binding protein, transferrin, insulin-like growth factors and binding proteins and PGDS (Aldred *et al.* 1995).

In addition to providing a regulated chemical environment for neural functions, the CP has been implicated in playing a role in a variety of neurogenic and endocrine regulatory mechanisms. Studies have shown that the CP vasculature and secretory epithelium are stimulated by cholinergic, adrenergic, peptidergic and serotonergic innervation (Lindvall and Owman 1981), suggesting that the CP may be able to synthesise or secrete CSF or proteins, or induce transport functions upon autonomic stimulation. Receptor binding, autoradiography, immunohistochemical and *in situ* hybridisation studies have shown the presence of putative autonomic neurotransmitters and neuro-humoral substances in the epithelium of the CP, including 5-hydroxytryptamine, atriuretic peptide, vasopressin and angiotensin II (see Table 1.1), suggesting the CP-CSF system could contribute to neuroendocrine signalling in the brain, although these precise pathways require further elucidation (Nilsson *et al.* 1992).

Thus the CSF is continually synthesised and secreted into the ventricular system by the CP. Cilia lining the ventricles contribute to the flow of CSF around the ventricular system and subarachnoid space to eventually drain into the venous system. The dynamic movement of CSF washing throughout the CNS allows the CSF to percolate through the brain carrying with it proteins and other molecules necessary for a homeostatic environment and other neuroendocrine functions.

Table 1.1
Receptors or binding sites demonstrated in the choroid plexus epithelium. Modified from Nilsson *et al.* 1992.

Receptor	*Endogenous Ligand*
β_1 and β_2	Noradrenaline
D_1	Dopamine
H_2	Histamine
5-HT_{IC}	Serotonin
melatonin	Melatonin
muscurinic	Acetylcholine
V_1 and AVP_{4-9}	Arginine vasopressin
Ang-II	Angiotensin II
IGF-I	Insulin growth factor I and II
insulin	Insulin & IGF-II
Growth hormone	Growth hormone
VIP	Vasoactive intestinal peptide
endothelia	Endothelia
prolactin	Prolactin
$GABA_A$	γ-aminobutyric acid
Benzodiazepine	?
T-2	Trytamine

1.6.3 Ontogeny of Cerebrospinal System in the Ovine Fetus

The development of the blood-brain and blood-CSF barrier is not a straight forward sequential set of events, since several properties of the CP develop at different times. The most fundamental diffusion restraint of the barriers is provided by the tight junctions of the capillary endothelium and CP epithelium, which is superimposed several active transport and synthesising mechanisms. Therefore, when describing the ontogenesis of the barriers

one must account for each process separately. It is presumed the composition of fetal CSF is related closely to development of BBB and CP, and that the establishment of adult-like concentrations reflect maturation of these barriers. This is the case for many developing mechanisms, such as protein transport, but the fetal brain also possesses unique restrictive mechanisms that are not present in the adult.

1.6.3.1 Cerebrospinal Fluid Production

It is well established that the both CP and BBB develop prenatally. In the human embryological evidence of the CP first appears at 6 weeks (Jacobsen *et al.* 1982). In the ovine fetus the brain and CP are well vascularised with the presence of tight junctions in the capillary endothelium and CP epithelium by 60 days gestation (Mollgard *et al.* 1987). The capacity of the CP to produce CSF increases as prenatal maturation proceeds, where secretion in the ovine fetus is calculated to be 3 μl/min at 69 days gestation, 21.4 μl/min by 125 days gestation (Fossan *et al.* 1985) and 77 μl/min by ~138 days gestation (Bissonnette *et al.* 1981).

1.6.3.2 Passive Diffusion

The passive diffusion of lipid soluble substances across the tight junctions of the blood-brain and blood-CSF barriers has shown to decrease during prenatal development. The permeability of these barriers to radiolabelled sucrose (Evans *et al.* 1974), glucose (Saunders 1977), inulin, albumin and IgG (Dziegielewska *et al.* 1979) decrease with the increase in age in the ovine fetus. The uptake of these lipophilic substances, such as inulin, into the brain and CSF from the blood is maximal at 60 days gestation but progressively decreases to adult levels by approximately 125 days gestation. Since the tight junctions of the barriers are histologically well established by 60 days gestation and the permeability of extremely large proteins like IgG does not change with age, the observed decrease in effective penetration may be accounted for by a reduction in pore number rather than pore

size of the tight junctions (Dziegielewska *et al.* 1979). Another factor which may influence the net passive diffusion constant is the decrease in surface area of the CP, where the decrease in permeability surface area occurs during the later part of gestational development when the CP ceases to develop and 'shrinks' relative the to the size of the rapidly growing brain (Davson *et al.* 1987).

1.6.3.3 Active Transport

It is evident that the movement of peptides and amino acids are appreciably higher in the fetus compared to that in the adult (reviewed in Saunders *et al.* 1999a). In addition the developing brain appears to transfer a greater number of amino acids between the different aqueous compartments prenatally than postnatally (for review see Lefauconner 1992), suggesting that the developing brain may possess qualitatively different transport mechanisms compared to the adult brain. It is believed the generally higher active transport of peptides and amino acids may be a reflection of the increased nutritional requirements essential for the developing brain.

1.6.3.4 Protein Concentration of Cerebrospinal Fluid

It is clearly evident that changes in the composition of protein in the fetal CSF occurs with the increase in fetal age. Total protein, albumin, fetuin and alpha-fetoprotein concentration in CSF of fetal sheep are highest at 35 days gestation, (840 mg/ml) and gradually fall to 260 mg/ml at 60 days gestation and 50 mg/ml at 125 days gestation, which is equivalent to twice the adult value (Dziegielewska *et al.* 1980). These observations suggest the CP is functional at a very early age, actively synthesising or transporting proteins into the CSF compartment. It has also recently been demonstrated that at 60 days gestation the fetal brain exhibits a unique barrier not present in the adult that prevents the entry of proteins into brain parenchyma. Thus it is thought retention of proteins in the CSF contributes to the observed elevation in CSF protein concentrations (Fossan *et al.* 1985). The physiological significance of the high concentrations of protein in fetal CSF is not entirely understood. It

has been postulated that the high concentration of proteins in the CSF provides a major driving force for the mechanical tension required for tension-based morphogenesis and compact wiring of the CNS during development (Van Essen 1997). Alternatively, since maximal size of the CP plus CSF protein levels in the CSF coincide with a critical developmental period of neurogenesis and formation of neocortical plate, it is again proposed that the high protein levels are required to meet the nutritional demands of the developing brain (Davson *et al.* 1987).

Therefore the barriers of the brain not only develop in early gestation but also may have specialised functions that are not present in the adult. These significant differences act to provide a specific prenatal environment that is modified to meet the high demands of CNS ontogensis.

1.7 SUMMARY

Contrary to early beliefs, sleep is a dynamic behaviour requiring the co-ordination of several brain regions and integration of numerous neurotransmitters. Studies have revealed a variety of endogenous substances that convincingly induce sleep. However, not one single substance can account for all the data, thus a sole putative sleep substance has not been unequivocally identified.

Among the multitude of sleep-promoting substances, PGD_2 has been described as a somnolence promoting substance in the adult rat by acting on the traditional sleep centres of the VLPO area. PGD_2 is produced from PGH_2 precursor by enzyme PGDS that is predominately synthesised in the leptomeningeal layers and CP of the brain. Brain-type PGDS has been shown to be both structurally and functionally homologous to β-trace protein, an abundant protein of the CSF, sharing the same primary and tertiary structure and exhibiting similar enzymatic properties. Thus, it is postulated that PGD_2 and β-trace/PGDS may play a role in the regulation of sleep. It is hypothesised that β-trace/PGDS is synthesised in the leptomeningeal lining and CP and secreted into the CSF on a diurnal

rhythm. The global induction of sleep may be facilitated by increased concentrations of PGD_2 in the CSF that is detected by PGD_2-chemosensitive area of the ventral surface of the POAH-BF. This stimulation is proposed to subsequently modulate the activity of the sleep centres of the VLPO area, which are located directly adjacent in the PGD_2-chemosensitive site.

The manifestation of sleep and arousal patterns continuously change throughout a life span. In precocial animals, such as humans and sheep, sleep develops prenatally and by the end of gestation the fetus spends the majority of time in a quiescent state broken only by short episodes of aroused activity. Based on observation of the cardinal signs of episodic sleep (i.e. ECoG, EMG, EOG and breathing movements) sleep first appears at ~110 - 120 days gestation in the ovine fetus (term c. 147 days), and become fully diffentiated into adult-like sleep patterns by 130 days gestation.

The exact mechanisms that underlie the onset and regulation of sleep in the fetus is unclear. It is not known whether factors which are unique to the fetus, e.g. placental substances such as pregnane steroid metabolites, contribute to the maintenance prenatal sleep. Alternatively, substances that preserve sleep in the adult may play a role in the initiation of sleep-behaviour in the *in utero*. This thesis will investigate whether PGD_2 and PGDS/β-trace play a role in the regulation of sleep in the fetus as it is proposed in the adult. I hoped to determine whether specific inhibition of the PGDS enzyme has any effects on the sleep-wake status of the fetus. Furthermore, I set forth to elucidate whether the PGDS enzyme is present prenatally and to establish whether enzyme activity and PGD_2 concentration in the fetal brain change in parallel with the ontogenesis of fetal sleep. Finally, I planned to map the temporal expression of β-trace/PGDS mRNA and protein through out development, again to ascertain whether changes in expression occurred in correlation with the onset of fetal sleep.

Chapter 2

Intracerebroventricular administration of Selenium Chloride, Prostaglandin D Synthase inhibitor, induces arousal in the ovine fetal sheep

2.1 INTRODUCTION

Selenium is a trace element ubiquitously distributed in both prokaryotic and eukaryotic cells (reviewed in Stadtman 1990). At low levels, selenium is considered to be an essential trace nutrient in humans and rats (Schwarz and Foltz 1989). Selenium is also recognised to be a highly specific and essential component of many hepatic, renal, plasma and brain-derived enzymes including glutathione peroxidase (Arthur *et al.* 1987), nicotinic acid hydroxylase (Gladyshev *et al.* 1994) and iodothyronine deiodinase (Arthur *et al.* 1991; Beckett *et al.* 1992). The mineral element also occurs naturally in amino acid transfer nucleic acids present in certain strains of bacteria (Stadtman 1983). During selenium deficiency, uptake is prioritised to the brain (Behne *et al.* 1988) thus suggesting the element has significant functions in neural tissue. Selenium containing glutathione peroxidase protects polyunsaturated fatty acid in synaptosomal membrane against peroxidation and thus it is believed to be involved in the maintenance of neuronal regulation (Clausen 1991; Huang *et al.* 1994). The element is also considered to possess antiviral (Balansky and Argirova 1981) and anticarcinogenic (Ip and Ganther 1991) properties.

It is well established that some inorganic selenium compounds specifically and reversibly inhibit PGDS by interacting at the sulfhydryl radical (Islam *et al.* 1991) presumably of the essential 65th Cys amino acid residue of the enzyme (Urade *et al.* 1995b). The synthesising enzyme, PGDS, regulates the non-oxidative rearrangement of PGH_2 to PGD_2. Extensive evidence suggests that PGD_2 may act as an endogenous substance synthesised by the adult mammalian brain to induce physiological sleep (Hayaishi 1988). It has been demonstrated that a 6 hour infusion of PGD_2 administered into the third or lateral ventricle of conscious male rhesus monkeys induced somnolence indistinguishable from physiological sleep (Onoe *et al.* 1988). The response appeared to be dose-dependent (500 pmole - 2250 pmole/min), and dependent of the infusion route, where treatment into the third ventricle induced a greater response than the lateral ventricle. These results are consistent with those performed in unrestrained adult rats, where doses as small as 60 fmole/min of PGD_2, administered into the third ventricle for 10 hours during the nocturnal active periods, increased sleep (Ueno *et al.* 1983). Maximum time spent asleep was observed after doses of 600 fmole/min of PGD_2. These results demonstrate that addition of exogenous PGD_2 produced excess sleep. In addition to these studies, it has been demonstrated that endogenous levels of PGD_2 (Pandey *et al.* 1995a) fluctuate with normal circadian rhythms, with significantly higher levels present in the CSF of the adult rat during sleep periods compared to wake periods. Microdialysis of PGD_2 into the subarachnoid space adjacent to the ventral surface of the rostral forebrain induced larger increases in sleep compared to other sites in the brain (Matsumura *et al.* 1994). Thus, it is proposed in the adult that the PGD_2 is diurnally released into the CSF, and circulates throughout the ventricular system to act on a PGD_2-sensitive zone of the rostral forebrain to induce a somnogenic effect.

Conversely, administration of inorganic selenium compounds, such as $SeCl_4$ or Na_2SeO_3, have shown to increase wakefulness, presumably by inhibiting the enzyme PGDS and reducing the sleep-promoting effects of PGD_2. Administration of $SeCl_4$ over 6 hours into either the venous system (Takahata *et al.* 1993) or into the third ventricle (Matsumura *et al.* 1991) of freely moving adult rats reduced the time spent asleep. Obviously, lower doses were required for the microdialysis of $SeCl_4$ into the third ventricle (60 - 200 pmole/0.2

µl/min) compared to intravenous (i.v.) administration (5 - 20 nmole/µl/min) to induce a dose dependent inhibition of sleep. Both treatments produce normal behaviour associated with wakefulness, including an increase in active behaviour plus elevation of food and water intake. The increased amount of wakefulness produced by $SeCl_4$ appeared to be a specific physiological response to the inhibition of PGDS because the effect was reversed by the addition of excess dithiothreitol (Matsumura *et al.* 1991), a compound that specifically and competitively binds to the sulfhydryl groups of PGDS and thus prevents $SeCl_4$ producing an inhibitory effect on PGD_2 synthesis (Islam *et al.* 1991). Therefore, it can be said the observed increased state of wakefulness produced by $SeCl_4$ was a specific physiological response arising from the inhibitory effect on PG synthesis, rather than a response resulting from a more generalised effect on the brain.

In the ovine fetus, REM and NREM sleep states are fully established by 135 days gestation (Clewlow *et al.* 1983). During this time the fetus experiences episodes of REM and NREM sleep similar to that exhibited in the adult (as defined by ECoG, EMG and EOG, see 1.2.4.3). The fetus, however, is not 'asleep' the entire duration *in utero*, and spends approximately to 5% of the time 'awake' (Szeto and Hinman 1985). These periods of fetal arousal are characterised by episodes of LV ECoG activity occurring simultaneously with eye and nuchal muscle activity and breathing movements (Szeto 1992). This combination of parameters is consistent with those present during wakefulness in adults animals, including humans.

Little is known regarding the neural substances which precisely regulate sleep *in utero* and how it is ensured that the propensity of sleep remains high in the fetus until birth, or perhaps *in utero* until shortly after the onset of labour (Dawes 1984). We hypothesised that PGD_2 is an endogenous sleep substance in the fetus in late gestation, as it is in the adult. This study was designed to investigate whether PGD_2 is involved the maintenance of fetal sleep by infusing $SeCl_4$ into the lateral ventricle of the ovine fetus, thus effectively removing endogenously produced PGD_2. Additional experiments infusing PGD_2 alone, or $SeCl_4$ followed by PGD_2 into the lateral ventricle were also performed, to establish whether

the inhibition of sleep induced by decreased levels of PGD_2 could be reversed by the addition of exogenous PGD_2.

In this study PGD_2 and $SeCl_4$ were infused into the lateral ventricle of chronically catheterised late gestation fetuses a device recently developed in our laboratory (Hirst *et al.* 2000).

2.2 METHODS

2.2.1 Animals

Merino-Border Leicester cross bred ewes, which carry fetuses to 147 gestational days, were used in accordance to the Standing Committee on Ethics in Animal Experimentation of Monash University. The ewes were brought to the department animal house and held together under artificially lit conditions on a 12 hour light/dark cycle for at least 5 days prior to surgery. All animals underwent surgery for the chronic implantation of catheters and electrodes as given in detail below.

2.2.2 Electrode Preparation

Three wire ECoG electrodes and two wire EMG and EOG electrodes were made and sterilised by ethylene oxide prior to surgical implantation into the fetus. Bundles of two or three lengths (1.2 metres long) of insulated multistranded stainless steel wire electrode (Cooner Wire Co., Chatsworth, CA, USA) were threaded through 1 m of polyvinyl tubing (ID 1.50 mm, OD 2.5 mm; Cat# 1116, Critchley Electrical Products NSW, Australia), leaving excess Cooner Wire at each end of the tubing for the soldering of pin contacts and preparation of appropriate attachments. The wires were secured in the tubing by forcing silastic sealant into the first 8 - 10 cm at each end and left overnight to dry. The plastic sheath was stripped from one end of the wire, allowing 1 mm diameter gold-plated pins

(Radiospares, France) to be attached by solder. At the opposite end of the wire, a small knot was made in the wire approximately 4 cm from the end for EOG and EMG electrodes. A small area of wire was then exposed by removing the plastic sheath directly below the knots to allow direct contact of the wires with the tissue. For the ECoG three wire electrode, one wire was prepared as above to be attached subcutaneously under the skin of the scalp, which would therefore act as the electrical 'common' electrode for all other electrodes. A rubber disk (7 mm diameter) was threaded onto the remaining two wires; this was used to hold the electrode onto the skull using cyanoacrylate glue. A knot was made approximately 1 cm from the end and the plastic sheaths below the knot to were removed. These exposed bare wires were later inserted into drilled holes made in the parietal plates of the fetal skull.

2.2.3 Fetal Surgical Procedure

The animals were fasted 24 hours prior to the commencement of surgery. All fetuses were 125 or 126 days gestation and either of singleton or twin status at the time of surgery.

2.2.3.1 Pre-operation Preparation

At the time of operation, a region on the left neck of the ewe was shorn to expose the jugular vein. The ewe was initially anaethesised with 1 g sodium-thiopentone dissolved in 20 ml distilled water (Pentathol, Bomac Laboratories, Ltd., Asquith, NSW, Australia) administered intravenously. With the aid of the stylet, the trachea was intubated with an endotracheal tube (I.D.. 8.0 mm, O.D.. 11.0 mm, Portex Ltd., UK) and connected to an anaethesic apparatus (Midget, CIG, Australia), which delivered 3% halothane anaesthesia in a gas mixture of 50:50 O_2/NO_2. The halothane mixture was reduced to 1 - 2.5% once a fully anaesthetised state was established. A mechanical ventilator (Campbell, ULCO Engineering, NSW, Australia) was used to assist respiration. The right side of the ewe's neck, right flank and ventral abdomen, extending from the ribcage to behind the udder and inner legs, were shaved and washed with Savlon antiseptic soap (Cetrimide 15%, chlorohexidine gluconate 1.5%; ICI, Australia), sterilised with three washes with Betadine

surgical scrub (Povidine iodine 7.5%; Faulding Pharmaceuticals, Australia), and then saturated with Betadine antiseptic solution (Povidine iodide; Faulding Pharmaceuticals).

Surgery was performed using aseptic techniques. All gowns, drapes and instruments were sterilised in an autoclave. Catheters, electrodes and the cannula plus Delrin cap were sterilised using ethylene oxide. Hands were scrubbed with Hibiclens Antiseptic Solution (chlorohexidine gluconate 4%, isopropyl alcohol, ICI) and covered with sterile latex gloves (Gammex, Ansell, Australia). Those who participated in the surgery wore gowns, face masks and a bonnet to cover the hair.

2.2.3.2 Catheterisation of Fetal Carotid Artery, Jugular Vein

To exteriorise the uterus, an incision was made down the midline from the umbilicus to the udder of the ewe. Care was taken to avoid cutting superficial vessels. The uterus was opened and the fetal head and neck were exposed. The cut edges of the uterus, together with the fetal membranes were secured around the neck of the fetus with Babcock forceps to prevent loss of amniotic fluid. The carotid artery and jugular vein were exposed by blunt dissection. The vessels were ligated rostrally, and occluded caudally with a bulldog clip to prevent blood loss. Each vessel was catheterised by inserting a heparinised saline-filled polyvinyl catheter (SV65, I.D. 0.86 mm, O.D. 1.52 mm; Dural Plastics & Engineering, NSW, Australia), caudally into a small incision. When the position of the catheter was secured by caudal and rostral ties and the bulldog clip was removed.

2.2.3.3 Cannulation of Trachea and Amniotic Cavity

The fetal trachea, below the larynx, was exposed by blunt dissection and was cannulated in a caudal direction to a distance of 5 cm using a heparinised saline filled catheter (SV116, I.D. 1.50 mm, O.D. 2.70 mm; Dural Plastics and Engineering, NSW, Australia) inserted between two cartilaginous rings. A long tie, knotted 5 cm from the end of the catheter, was used to sew the cannula to the tracheal wall. The skin incision was sutured closed and all

the catheters were secured to the skin at the base of the ear, together with an additional open-ended catheter (SV116) filled with saline which was used for recording amniotic pressure.

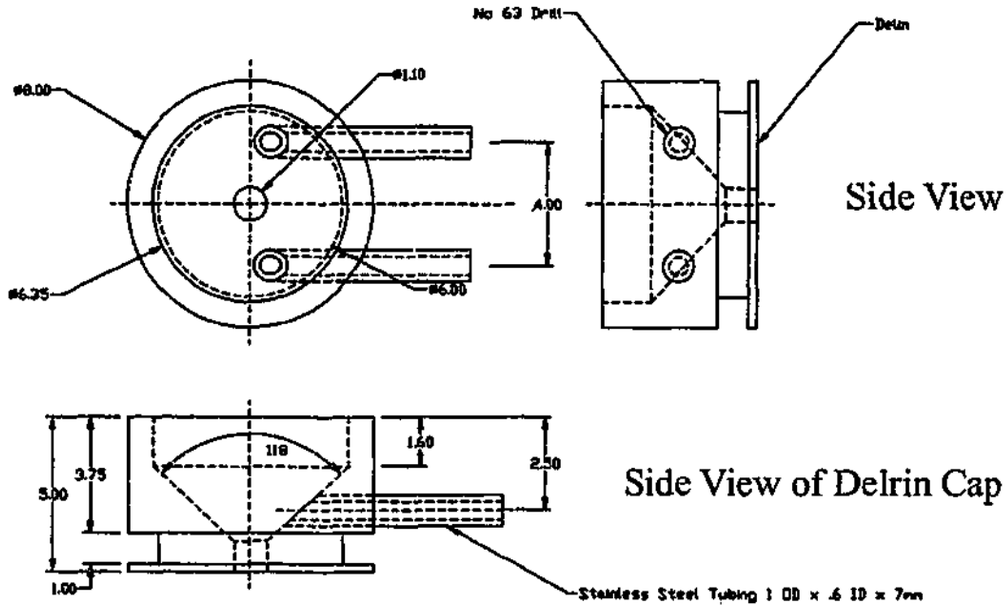
2.2.3.4 Implantation of Fetal Electrodes

Electrodes used to measure ECoG activity were attached to the dura over the parietal and frontal cortex. The skull was exposed through a midline scalp incision and cleaned of connective tissue. The exposed end of two wires were inserted into burr holes drilled into the parietal plates approximately 1 cm either side of the midline. The holes passed through the bone but the dura mata was left intact. The wires were secured to the skull by sliding the two rubber discs along the wires until they rested on to the parietal plates and were secured together with cyanoacrylate adhesive (Vetbond, 3M Animal Care Products, MN, USA). The remaining third wire was attached to the subcutaneous connective tissue at the caudal end of the skin incision to acts as a common electrode. A two wire EOG electrode, used to measure electro-ocular activity was attached subcutaneously through two small skin incisions located immediately above and below one of the eyes. The two wire EMG electrode was attached bilaterally into the posterior nuchal muscles to record electromyogram activity.

2.2.3.5 Implantation of Intracerebroventricular Cannula

Infusions of fluids into a lateral ventricle of the fetal brain was accomplished by using a small indwelling catheter introduced into the left lateral ventricle, and attached to the skull via a two-way Delrin cap. The Delrin cap, custom made for this purpose, had dimensions of 5 mm in height and 8 mm in diameter and contained a centre cavity which created a reservoir, by which two polyvinyl catheters (SV65) at 30° apart were attached via two short metal tubes inserted into the walls of the Delrin cap (Fig. 2.1a). A 1.1 mm hole in the centre

a) Aerial View of Delrin Cap



b) Aerial View of Rubber Disk

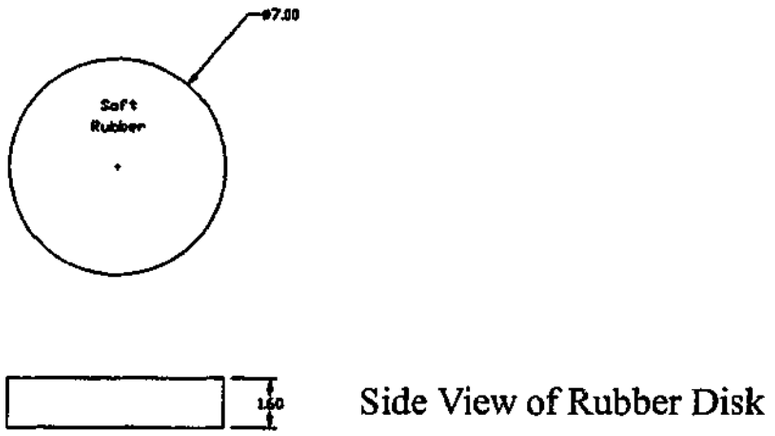


Figure 2.1
a) Line diagram showing the aerial and side views of the Delrin cap. Two stainless steel tubes were inserted into the walls of the Delrin cap and used for the attachment of the inflow and outflow catheters. A 1.1 mm hole was also drilled in the base of the Delrin cap for the insertion of the intracath.
b) Line diagram showing the aerial and side view of the rubber disk that was inserted into the top of the Delrin cap to create 15 µl internal space.

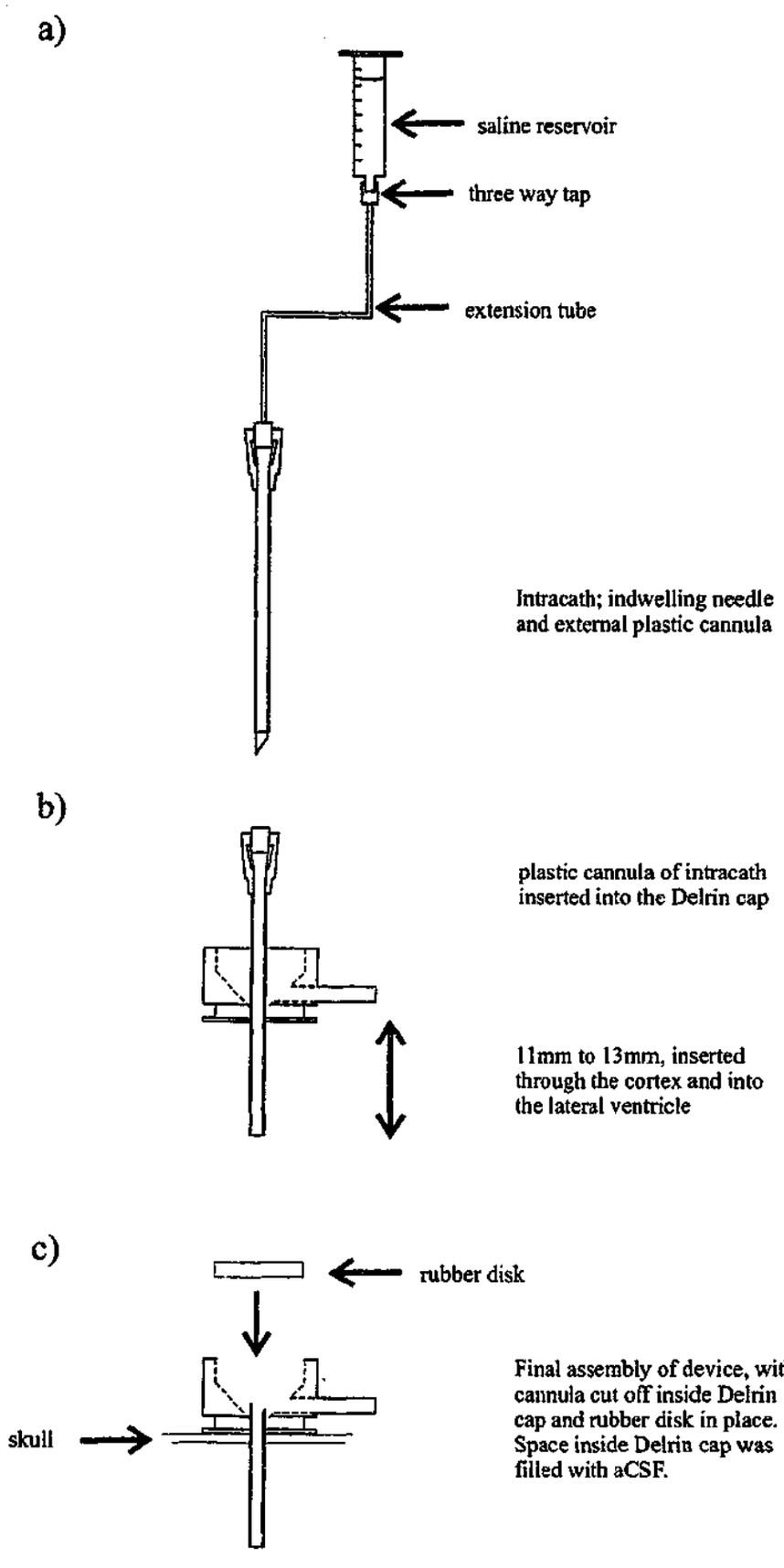


Figure 2.2
a) Schematic diagrams illustrating the extension tube, three-way tap and syringe vessel, filled with sterile non-heparinsed saline, attached to the indwelling needle of the intracath. When the saline reservoir was elevated above the level of the fetal head, the saline rapidly fell under approximately 30 cm of hydrostatic pressure and flowed into the lateral ventricles, if inserted correctly.
b) Diagram of the external plastic cannula of the intracath, with the indwelling 22G needle removed, inserted correctly into the Delrin cap.
c) Diagram showing the Delrin cap fixed to the skull with cyanoacrylate adhesive. The plastic cannula of the intracath was cut near to the bottom of the internal surface of the cap, and the rubber disk was used to seal the top of the Delrin cap.

of the floor of the cap allowed the passage of a dwelling i.v. plastic cannula (I.D.. 0.80 x 51 mm) and 22 G needle (Intracath: Terumo Medical Corporation, MD, USA; Fig. 2.2). Prior to insertion into the lateral ventricle, the intracath was attached to a vessel containing sterile non-heparinised saline via three way tap and an extension tube (Tuta Lab. Pty. Ltd., Lane Cove, NSW, Australia; Fig. 2.2a). The needle and plastic cannula were inserted through the hole in the base of the Delrin cap and, depending on the size of the fetus, between 11 to 13 mm of the intracath was pushed passed the ventral surface of the Delrin cap (Fig. 2.2b). For insertion into the brain, a 1.1 mm hole was drilled through the left frontal plate, 5 mm rostral and 5 mm lateral to bregma, and the intracath combined with Delrin cap was carefully inserted into the drilled hole. Special attention was made to ensure the intracath was passed perpendicularly to the surface of the skull and through the cortex (Fig 2.2c). To ensure the intracath was positioned correctly into the left lateral ventricle, the reservoir of non-heparinised saline was elevated above the level of the fetal head and the 3-way stopcock opened. If the saline fell rapidly under approximately 30 cm of H_2O hydrostatic pressure, it was assumed the intracath was correctly inserted into the lateral ventricle, and that the saline was flowing freely into the ventricular system. If saline movement was impeded by brain tissue, the intracath was repositioned by either increasing or decreasing the depth of penetration. Once positioned correctly the inner needle of the intracath was removed leaving the plastic cannula in place, which was then secured to the Delrin cap with cyanoacrulate glue. The Delrin cap was then fixed to the surface of the skull with cyanoacrylate adhesive. The part of the plastic cannula that protruded above the inner surface of the Delrin cap was then cut off using the point of a #11 scapel blade. Both the chamber and catheters were filled with non-heparinised saline. A rubber disk (1.2 mm thick, 8 mm diameter; Fig. 2.1b) was then inserted into the top of the Delrin cap to seal the reservoir, and was secured with cyanoacrylate adhesive (Fig. 2.2c). This created an inner chamber with a volume of approximately 15 µl. The skin edges were then sutured over the cap, catheters and wires, ensuring all wires and catheters were covered and not entangled in the stitches.

All electrodes and catheters were bundled and tied together and secured approximately 6 cm beyond the fetal ear to avoid tangling in the amniotic sac. Care was taken to not occlude the catheters. Before the fetus was returned to the uterus, 2 ml of a mixture of procaine penicillin (200 mg/ml) and dihydrostreptomycin (250 mg/ml) antibiotic (Depomycin, Intervet Ptd, Ltd., NSW, Australia) was injected subcutaneously into the neck. The uterus and fetal membranes were sutured in one layer and all catheters and electrodes were exteriorised through an incision in the ewe's right flank. The linea alba, subcutaneous tissue and the skin of the abdomen of the ewe were closed as separate layers. A catheter was also implanted into the maternal jugular vein. Upon completion of surgery, the halothane anaesthesia was withdrawn and mechanical ventilation ceased when spontaneous breathing began. The endotracheal tube was removed when the regular breathing and the swallowing reflex returned. Two-way stopcocks (Distofix-3, B. Braun, Belgium) were attached to the end of the catheters to allow flushing and the taking of blood and fluid samples. The ewe was dressed in an elastic net (Setonet, size 7, Seton Health Care Group, UK) to hold the catheters against the ewe. Catheters and electrodes were placed in plastic bags tied under the netting. The ewe was returned to a metabolic cage and given access to food and water *ad libitum*.

2.2.4 Recordings

FBM, BP, heart rate, ECoG EMG and EOG were recorded to measure the behavioural changes of the fetuses induced by treatment. All transducer and electrical leads were cleaned with absolute ethanol, and pressure transducers were sterilised internally with aqueous Hibitane (Zeneca Pharmaceuticals, Melbourne, Australia) and then rinsed with sterile saline before use.

2.2.4.1 Respiratory and Cardiovascular Parameters

FBM and blood pressure was measured directly from the fetal tracheal and carotid artery catheter respectively. These catheters were connected to fluid-filled pressure transducers

(Pa23, Gould-Statham, USA). Prior to use pressure transducers were calibrated to 100 mmHg using a water-filled manometer connected to a mercury column. Amniotic pressure was subtracted electronically from both the tracheal and carotid blood pressure recordings by a differential amplifier to account for any pressure changes caused by movement of the ewe or non-labour contractions of the uterus. The pressure output was passed directly into DC driver amplifiers and displayed on Grass 8 Channel Polygraph recorder (Model 7D Polygraph, Grass Instruments Co., Quincy, Mass., USA). The baseline positions of the pens were established while the transducers were opened to air, and the sensitivity adjusted so that 100 mmHg gave 1.0 V of output signal. For the fetal arterial pressure, 100 mmHg was set to move the pens 5 cm distance on the trace, while FBM were recorded as 50 mmHg/5 cm trace. FBM were considered to be a rapid fall in intra-thoracic pressure, which was transduced into repetitive negative deflections on the recording trace. Systolic and diastolic arterial pressure could be accurately measured from the highest and lowest points of the oscillating blood pressure recordings. Heart rate was derived from the arterial pulse using a tachometer circuit (Electronic Workshop, Department of Physiology, Monash University, Clayton, Australia). The heart rate record was calibrated electronically so that a range of 25 to 300 beats per minute spanned a 5 cm height on the record trace.

2.2.4.2 Electrocortical, Electro-ocular and Electromyographic Recordings

Electrical activity from the surface of the cortex was amplified using a Wide Band AC EEG Pre-amplifier (Model 7P5B, Grass Instrument Co.) with low frequency cut-off set at 1 Hz. The signal was then passed to the DC pen driver amplifier that had the ½ amplitude high frequency set at 15 Hz before being displayed directly on the polygraph. The two wire EOG electrodes secured above and below the eye socket detected movement of the eyeball. This electrical signal was also amplified bandpass filtered (0.3 – 15 Hz) and displayed on the polygraph. The EMG signal was initially passed through a Wide Band AC Pre-amplifier with low frequency cut-off (10 or 30 Hz) and displayed as the running mean using a "leaky' integrator with the time constant set at 0.2 seconds. The signal was passed to the DC pen driver amplifier set at 15 Hz ½ amplitude high frequency. This form of signal display tends

to emphasise tonic muscular contraction, rather than phasic muscle twitching. The driver amplifiers were calibrated before use whereby 1 mV equalled 1cm vertical deflection on the trace. See Figure 2.3.

2.2.5 *Experimental Procedure*

The fetuses were allowed at least four post-operative days for recovery from the anaesthesia and surgery. Once adequate recordings of the fetal behavioural parameters were established, the experimental procedures commenced.

2.2.5.1 Equipment and Apparatus

A double syringe infusion pump (Model 70134, B.Braun Melsungen AG, West Germany) which infused and withdrew the same volume simultaneously was used. On the day of the experiment one of the catheters connected to the cap fixed to the fetal skull was designated the inflow catheter, and was connected to a 0.20 μm microfilter (Minisart, Sartorius AG, Gottingen, Germany) to maintain sterility. Both this inflow cathether and the remaining outflow catheter were then attached to 5ml glass syringes (B.Braun Melsungen AG, West Germany), and placed in the appropriate position on the pump and secured. The treatment solutions were then perfused through the cathethers and the Delrin cap. Since the cannular connecting the cap with the ventricle was a much smaller diameter plus a push-pull system was used, no infusate entered the entered the brain when the catheters were filled until commencement of experiment. Injection of known volumes of fluid into the ventricle was achieved by closing a stopcock on the outflow catheter for a known period of time, causing the fluid, which continued to be infused through the inflow catheter, to be forced through the cannula into the ventricle. Infusion into the ventricle was done at a flow rate of 10 μl/min over 4 hours, so that the volume delivered over this time was 2.4 ml.

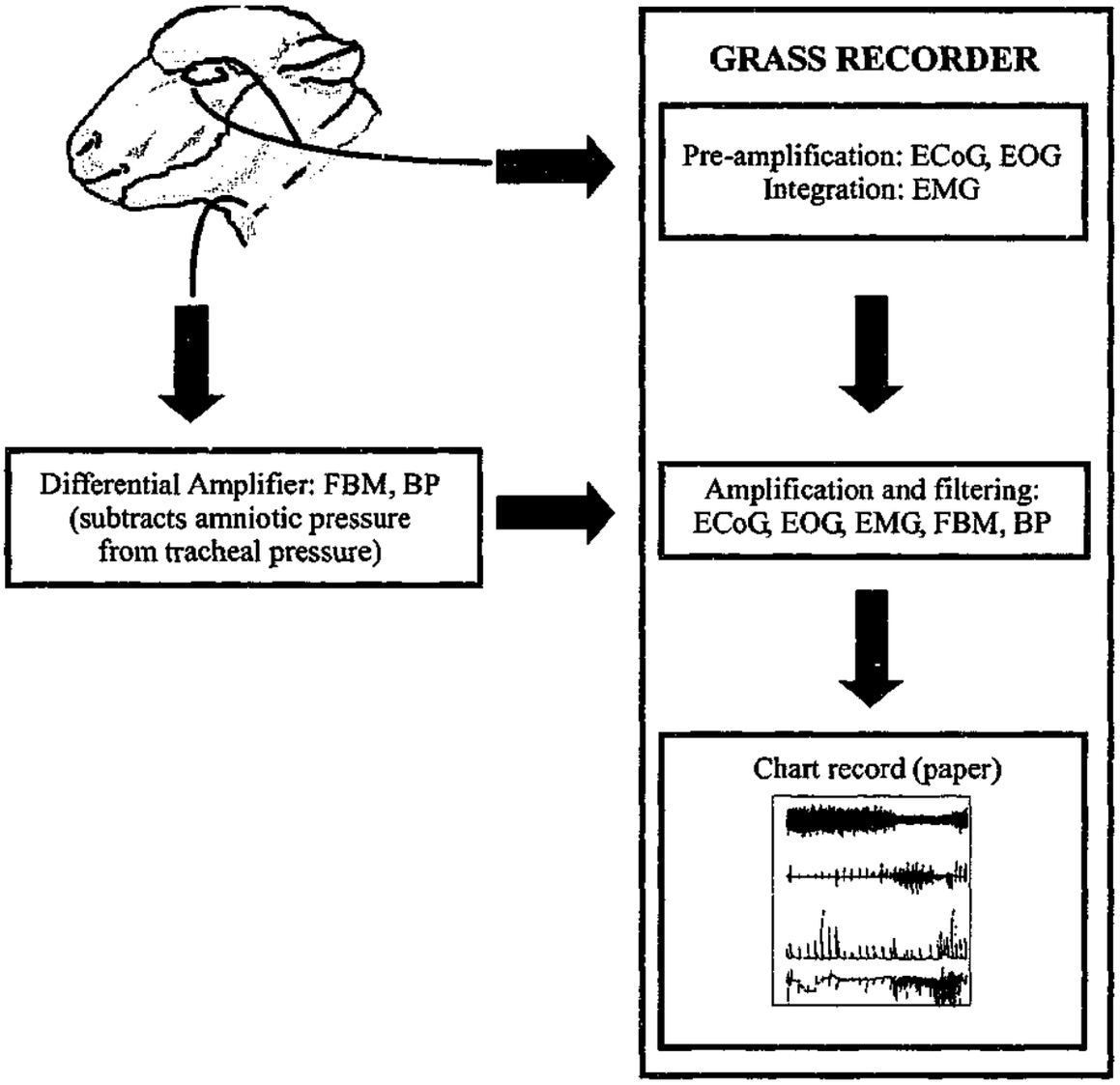


Figure 2.3
Diagrammatic representation of polygraph set up. The fetus was chronically catheterised in the trachea and carotid artery for fetal breathing movements (FBM), blood pressure (BP) and heart rate respectively. Electrodes were attached to the orbital margins of the eye, the nuchal muscle and the dural lining covering the parietal cortex to measure electro-ocular (EOG), eletromyogram (EMG) and electrocorticogram (ECoG) activities respectively. Signals were transduced, amplified or integrated as explained in the text to produce the final chart record.

The quantity of $SeCl_4$ or PGD_2 delivered into the ventricle was calculated as the product of the concentration of each substance in the infusate multiplied by both the flow of infusion and time of administration.

i.e. quantity infused (pmole) = concentration (pmole/ml) x flow (ml/min) x time (min)

At the end of each treatment with $SeCl_4$ or PGD_2 the catheters and caps were re-filled with aCSF.

2.2.5.2 Experimental Protocol

Four different infusion protocols were completed at the same infusion rate of 10 µl/minute.

1. control treatment of artificial cerebrospinal fluid (aCSF), see Table 2.1 and 2.2.
2. treatment with $SeCl_4$ (Sigma Chemical Co., St. Louis, MO, USA)
3. treatment with PGD_2 (Cayman Chemical Co., Ann Arbor, MI, USA)
4. treatment with $SeCl_4$, subsequently followed with PGD_2.

Each study, with the exception of the last, consisted of a four hour infusion of experimental or control treatment, with hourly arterial blood samples taken one hour prior to, and for the remaining duration of the infusion for the measurement of partial pressure of oxygen (pO_2), carbon dioxide (pCO_2), oxygen saturation (sO_2), haemoglobin (tHb) and pH corrected for body temperature of 38.5°C using an ABL5 blood gas analyser (Radiometer, Copenhagen, Denmark). The maximum volume of blood taken at each time did not exceed 0.6 ml; the total volume taken during the experiments was approximately 3.5 ml. In the final study, a four hour infusion of $SeCl_4$ was immediately followed by another four hour infusion of PGD_2 to determine whether the effect of $SeCl_4$ treatment could be reversed by exogenous PGD_2. The polygraph recorded FBM, BP, heart rate, ECoG, EMG and EOG activity four

hours prior to the commencement of treatment and for the following twelve hours at a trace speed of 5 mm/min.

2.2.5.3 Infusion Solutions: Calculations and Doses

Artificial CSF contained the constituents shown in Table 2.1 dissolved in 1 litre of dd-H_2O (double distilled water). The solution was made fresh every 4 weeks. The composition of aCSF gave the calculated ionic concentrations shown in Table 2.2.

Table 2.1
Mass of cerebrospinal constituents dissolved in 1 litre of double distilled water.

	mass (g)
$NaH_2PO_4.2H_2O$	0.049
$NaHCO_3$	1.00
NaCl	8.0
Glucose	0.802
$CaCl_2.2H_2O$	0.106
$MgCl_2.6H_2O$	0.109
KCl	0.204

Table 2.2
Osmolarities (meq/kg H_2O) of ions in artificial cerebrospinal fluid.

Ion	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-
Osmolality	149	2.7	0.7	0.5	142	12

On the day required, appropriate concentrations of $SeCl_4$ infusate was diluted from a stock solution of 5 mM $SeCl_4$. The $SeCl_4$ stock solution with aCSF as the solvent was made fresh every two weeks and stored at 4°C. A 1 mM stock of PGD_2 in absolute ethanol was stored at -20°C. On the day of the experiment appropriate dilution of PGD_2 treatment solution was

freshly made in aCSF. Originally, treatments varying from 25 pmole to 1000 pmole/10 µl/minute was administered to establish the optimum dose. For animal numbers see Table 2.3. These treatments were given randomly between the ages of 130 to 140 days gestation to eliminate any behavioural changes associated with an increase in developmental age. It was found administration of approximately 500 pmole/10 µl/minute of both $SeCl_4$ and PGD_2 produced maximal changes in fetal behaviour. Thus, in the final study 500 pmole/10 µl/minute of $SeCl_4$ was followed by infusion of 500 pmole/10 µl/minute of PGD_2.

Table 2.3
Summary of doses administered given and the corresponding number of fetuses receiving each dose.

Treatment	*Dose (pmole/10 µl /min)*	*Number of fetuses*
aCSF	10 µl/min	5
$SeCl_4$	25	3
	100	4
	500	4
	1000	3
PGD_2	25	4
	100	4
	500	5
	1000	4
$SeCl_4$ + PGD_2	500	4

To ensure that baseline conditions were achieved, it was required that the fetus exhibited at least 12 hours of normal behaviour before any treatment was given. Therefore, the fetuses were left 24 to 48 hours before beginning another experiment with a different treatment.

2.2.6 *Post-mortem*

Immediately prior to post-mortem at approximately 142 days gestation, the lateral ventricle was infused with 100 µl of Indian Ink (Winsor & Newton, London, UK). This procedure was performed to establish whether the i.c.v cannula was inserted correctly into the lateral ventricle. The ewe and fetus were then killed by a 20 ml i.v. injection of sodium pentobarbitone (325 mg/ml, Lethobarb, Virbac, NSW, Australia) and the fetus was immediately removed and weighed. To validate whether the cannula was positioned correctly, the frontal area of the fetal skull was exposed and carefully removed rostral to the position of the Delrin cap. Coronal slices of the brain were removed in a caudal direction using a blade, until the area containing the lateral ventral, hypothalamus and optic chiasm was reached. In the majority of cases the cannula passed through cortex with the tip positioned correctly into the lateral ventral. The presence of the ink in the lateral ventricle verified that the $SeCl_4$ and PGD_2 solutions had been infused into the lateral ventricle. There were no sign of any tissue damage nor necrosis within the lateral ventricle and surrounding cortical tissue, which suggests the behavioural responses exhibited was not due to toxicity of each treatment. If it was not evident that the cannula was inserted correctly into the lateral ventricle, the data generated from that fetus was not included in the final analysis.

2.2.7 Analysis of Polygraph Records

The entire 16 hour record of each experiment was analysed on a minute-to-minute basis. In each minute ECoG, EMG, EOG and FBM was coded and then used to determine the particular behavioural state that was present at the time. ECoG was coded as either HV (1) or LV (0), and the presence or absence of nuchal EMG, EOG and FBM activities were coded as 1 or 0 respectively for each minute. These values were entered into a spreadsheet and each minute was scored as either NREM and REM sleep and arousal according to the criteria meet on the combination of behavioural parameters present (Table 2.4). NREM sleep was defined as HV ECoG occurring simultaneously with nuchal EMG and the absence of EOG and FBM activities. In comparison REM sleep was considered to be LV

ECoG in the presence of EOG and FBM activities without nuchal EMG. As it has been described previously, in this study arousal was described as LV ECoG with nuchal EMG, EOG and FBM activity (Szeto and Hinman 1985). States that did not fall within these criteria were considered non-defined. A sample of a polygraph trace during control recordings is shown in Figure 2.4a. Blood pressure and heart rate recordings were measure at every ten minutes to generate a representative value for a four hour epoch.

Table 2.4
An example of the spreadsheet used to determine the behavioural sleep state experience by a 131 day gestation ovine fetus, upon a minute-to-minute basis. See above text for detailed explanation.

Hour	*Minute*	*HV ECoG*	*EMG*	*EOG*	*FBM*	*REM*	*NREM*	*Arousal*
2	47	0	1	0	1	1	0	0
2	48	0	1	0	1	1	0	0
2	49	0	1	0	1	1	0	0
2	50	0	1	0	1	1	0	0
2	51	0	1	1	1	0	0	1
2	52	0	1	1	1	0	0	1
2	53	0	1	1	1	0	0	1
2	54	0	1	0	1	1	0	0
2	55	0	1	1	1	0	0	1
2	56	1	0	1	0	0	1	0
2	57	1	0	1	0	0	1	0
2	58	1	0	1	0	0	1	0
2	59	1	0	1	0	0	1	0
2	60	1	0	1	0	0	1	0

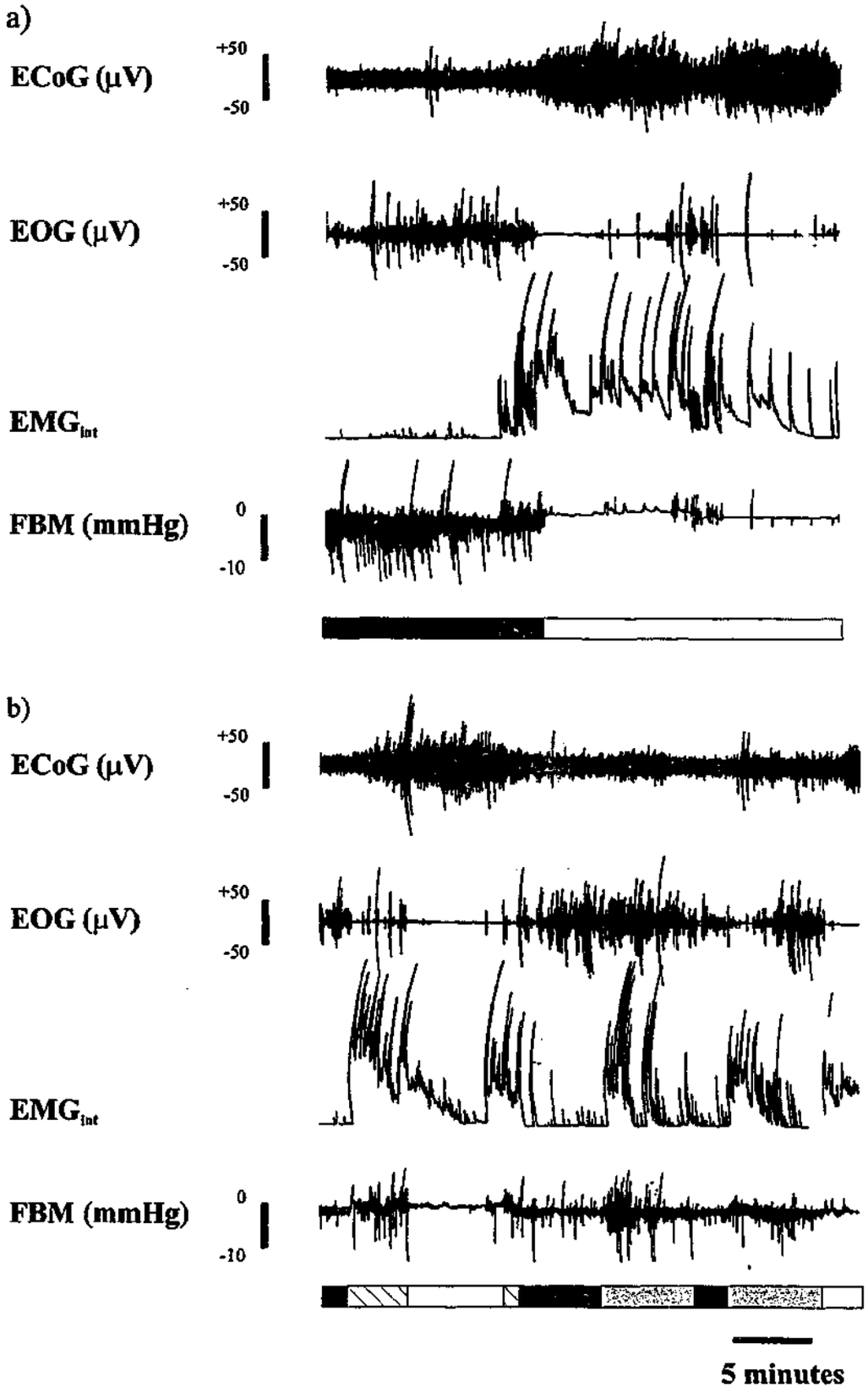


Figure 2.4

a) An example of control trace record showing the three distinct behavioural states in a 135 day gestation fetal sheep: non-rapid eye movement (NREM; white bar) sleep, rapid eye movement (REM; black bar) sleep and arousal (grey bar), as defined by electrocorticogram (ECoG), electro-oculogram (EOG), electromyogram (EMG) activities and fetal breathing movements (FBM). NREM sleep is defined by the presence of nuchal muscle EMG in high voltage ECoG, whereas REM sleep is considered present when EOG and FBM occur in the presence of low voltage (LV) ECoG. Arousal is defined by the presence of EOG, EMG and FBM during LV ECoG. States that do not fall into these definitions were thought to be non-defined states (hatched bar). Trace remained at a constant speed of 5 mm/min

b) An example of experimental trace recording showing the effect of infusing 500 pmole/10 μl/min of $SeCl_4$ into the lateral ventricle of a 135 day gestation fetal sheep. $SeCl_4$ increased the incidence of EMG activity during LV ECoG, thus increasing the overall occurrence of defined arousal.

2.2.8 Statistical Analysis

All data are presented as the mean±standard error of the mean (sem). Data was analysed by repeated measures analysis of variance to compare the effect of treatment with between subject factors which were doses (aCSF, 25, 100, 500 and 1000 pmole/10 µl/min of $SeCl_4/PGD_2$) and the repeated variable (within subject factor) was period (4 hour epoch). When a significant interaction between treatment and time was identified, paired comparisons were made between the two points using the least significant difference test, where $P<0.05$ was considered to be statistically significant. All data were checked for homogeneity of variance using a Levene's test of equality of error variances. If the data was not normally distributed, it was transformed by square root or natural log.

2.3 RESULTS

2.3.1 Normal Fetal Behavioural States

By 130 days gestation the ECoG had clearly differentiated into synchronised and desynchronised activity, thus the heterogenous sleep states could be readily discriminated. During recording periods before and after each experiment, all fetuses exhibited normal breathing movements, eye and neck muscle activity, and behavioural states (Fig. 2.4a). For the majority of time, the fetus exhibited periods of REM and NREM sleep (mean REM duration 4.78±0.81 minutes; mean NREM duration 7.76±0.92 minutes), but these could be as short or long as 1 and 20 minutes, respectively. Intermittent periods of arousal usually occurred during the transition between the two sleep states, when nuchal muscle activity, which is generally prevalent during NREM, persisted for a brief period beyond the time that the ECoG had changed from HV to LV activity, and was present when the FBM and EOG activity commenced (Fig. 2.4a). The mean duration of the individual arousal-like episodes was 1.56±0.2 minutes, and the total amount of arousal activity in the 4 hour control epoch

was 10.75±4.09 minute. Arousal was generally associated with a transient elevation on blood pressure and heart rate (Fig. 1.1). Administration of aCSF into the lateral ventricle did not significantly alter these behavioural patterns.

2.3.2 Selenium Chloride Infusions

2.3.2.1 Effect of Selenium Chloride on Recorded Fetal Behavioural Parameters

Both the incidence of HV and LV ECoG activity remained unaffected by the four hour i.c.v infusions of $SeCl_4$ (Fig. 2.5a). Treatment of $SeCl_4$, at all doses (25, 100, 500 and 1000 pmoles/10 µl/min), did not change HV and LV ECoG activity compared to control pre-infusion and aCSF infusion levels throughout the 12 hour period.

Four hour administration of 500 pmole of $SeCl_4$/10 µl/min into the lateral ventricle significantly increased the incidence of EMG activity ($P<0.05$) during the 9 - 12 hour recording epoch above control levels (Fig. 2.6c). There was no significant effect of $SeCl_4$ on the EMG activity at other doses (25, 100 and 1000 pmole/10 µl/min; Fig. 2.6a, b and d respectively). Unlike nuchal muscle activity, the incidences of EOG (Fig. 2.7) and FBM (Fig. 2.8) activities were not influenced by the 4 hour administration of $SeCl_4$ at any of the doses infused into the lateral ventricle.

2.3.2.2 Effect of Selenium Chloride Infusion on Electromyographic and Electro-ocular Parameters and Fetal Breathing Movements during Low Voltage Electrocorticogram Activity

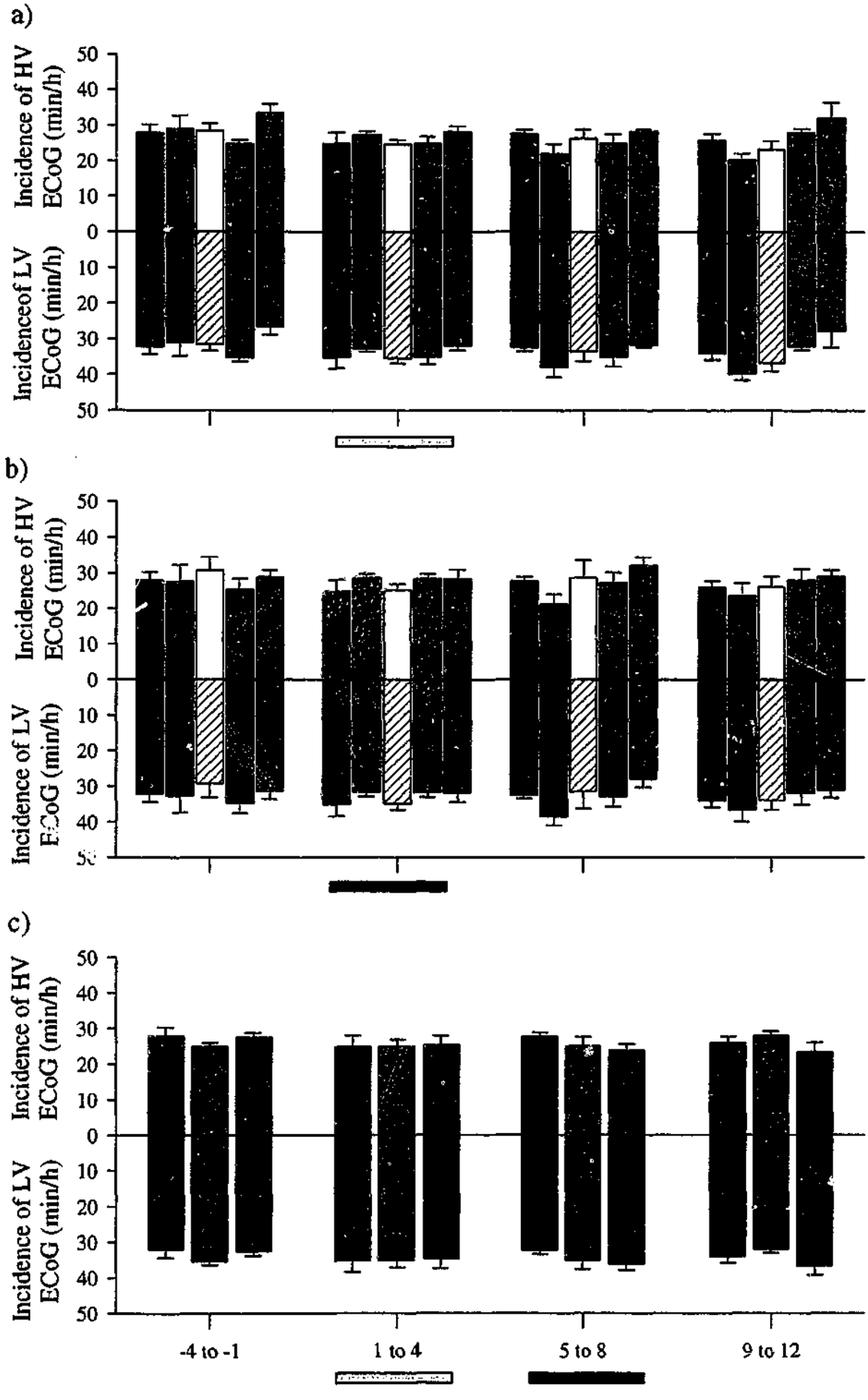
There was a significant effect of $SeCl_4$ treatment on the EMG activity which specifically occurred during LV ECoG ($P<0.05$), but no significant interaction between the time periods and the different doses of $SeCl_4$ ($P=0.116$). This suggests the different doses of $SeCl_4$ do not solely contribute to the apparent significant variation in the increase of EMG activity in

Figure 2.5

a) The effect of infusing artificial cerebrospinal fluid (aCSF; blue) and different doses of $SeCl_4$; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of high (clear) and low (hatched) voltage electrocorticogram (HV and LV ECoG) activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (grey bar). All data represent the mean±sem, n = 3, 4 or 5.

b) The effect of infusing aCSF (blue) and different doses of PGD_2; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of HV ECoG (clear) and LV ECoG (hatched) activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represent the mean±sem, n = 4 or 5.

c) Compares the effect of infusing of aCSF (blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of HV ECoG (clear) and LV ECoG (hatched) activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of artificial CSF or $SeCl_4$, whereas the black bar represents administration of PGD_2. All data represent the mean±sem, n = 4 or 5.


a)
b)
c)
Incidence of HV ECoG (min/h)
Incidence of LV ECoG (min/h)
50
40
30
20
10
0
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

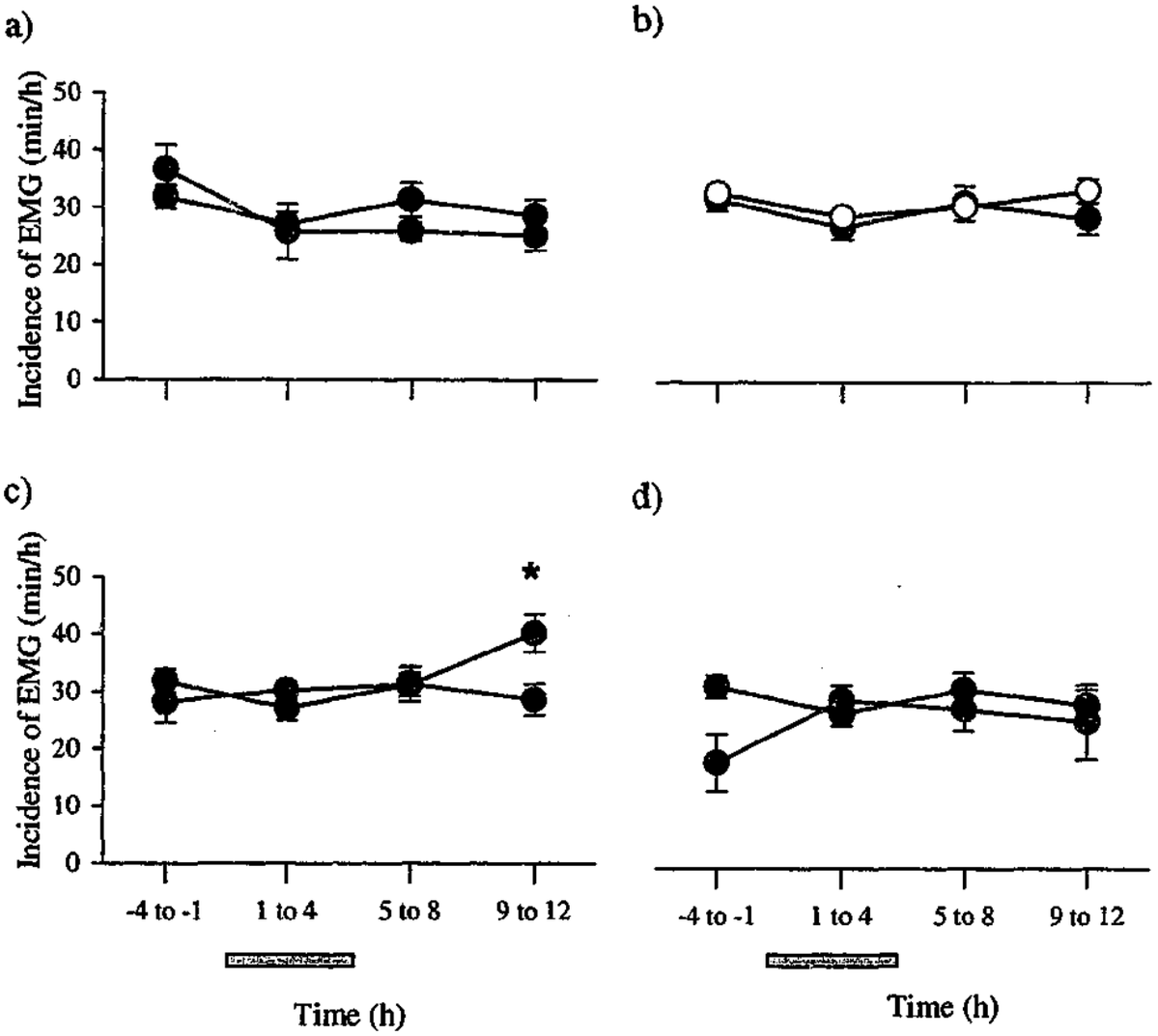


Figure 2.6

The effect of infusing of artificial cerebrospinal fluid (blue) and different doses of $SeCl_4$; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of electromyogram (EMG) activity. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (grey bar). Administration of 500 pmole of $SeCl_4$/10 µl/min induced a significant increase in EMG activity higher than control levels (*, $P<0.05$). All data represent the mean±sem, n = 3, 4 or 5.

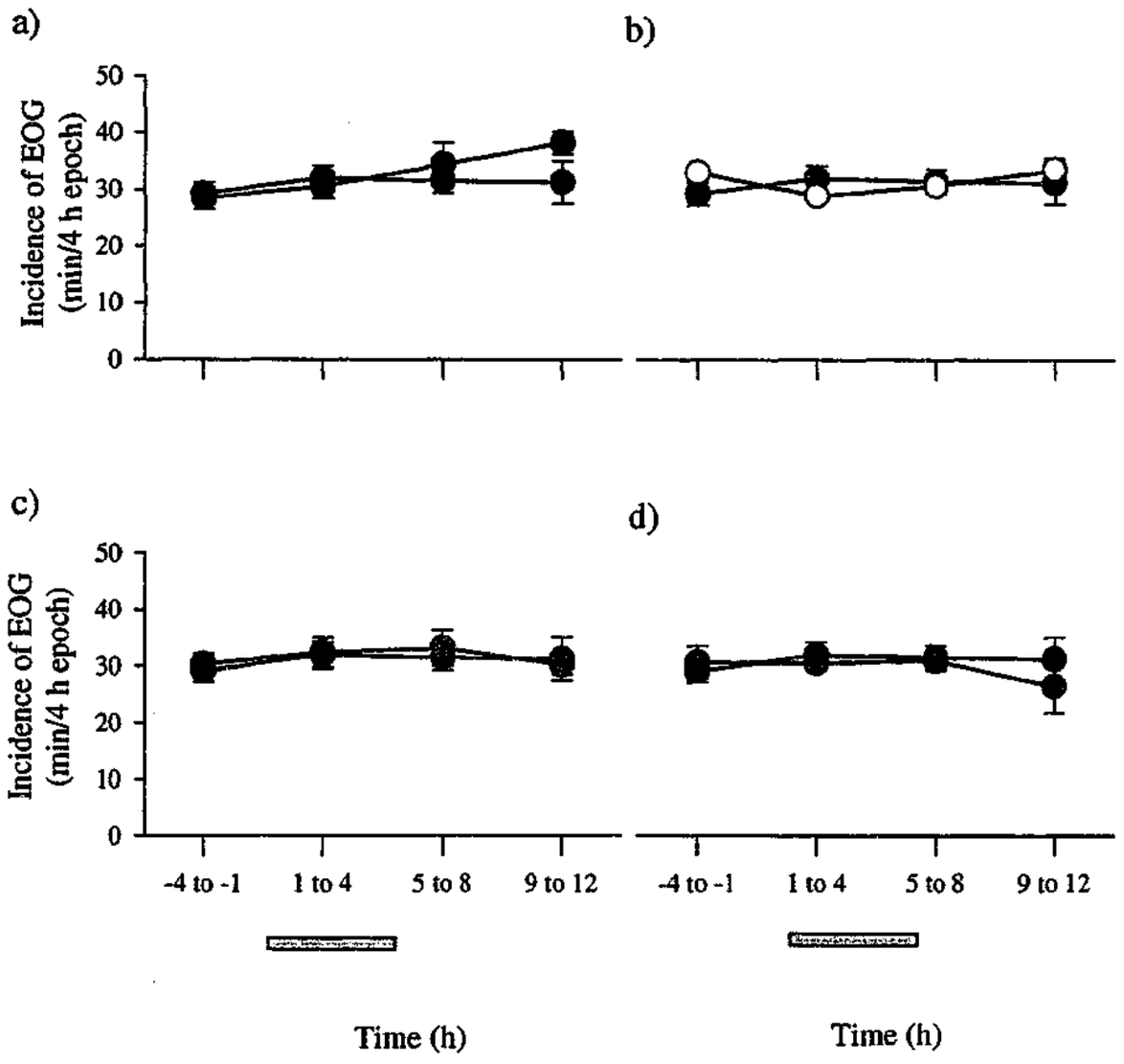


Figure 2.7
The effect of infusing artificial cerebrospinal fluid (blue) and different doses of $SeCl_4$; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of electro-ocular (EOG) activity. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (grey bar). All data represent the mean±sem, n = 3, 4 or 5.

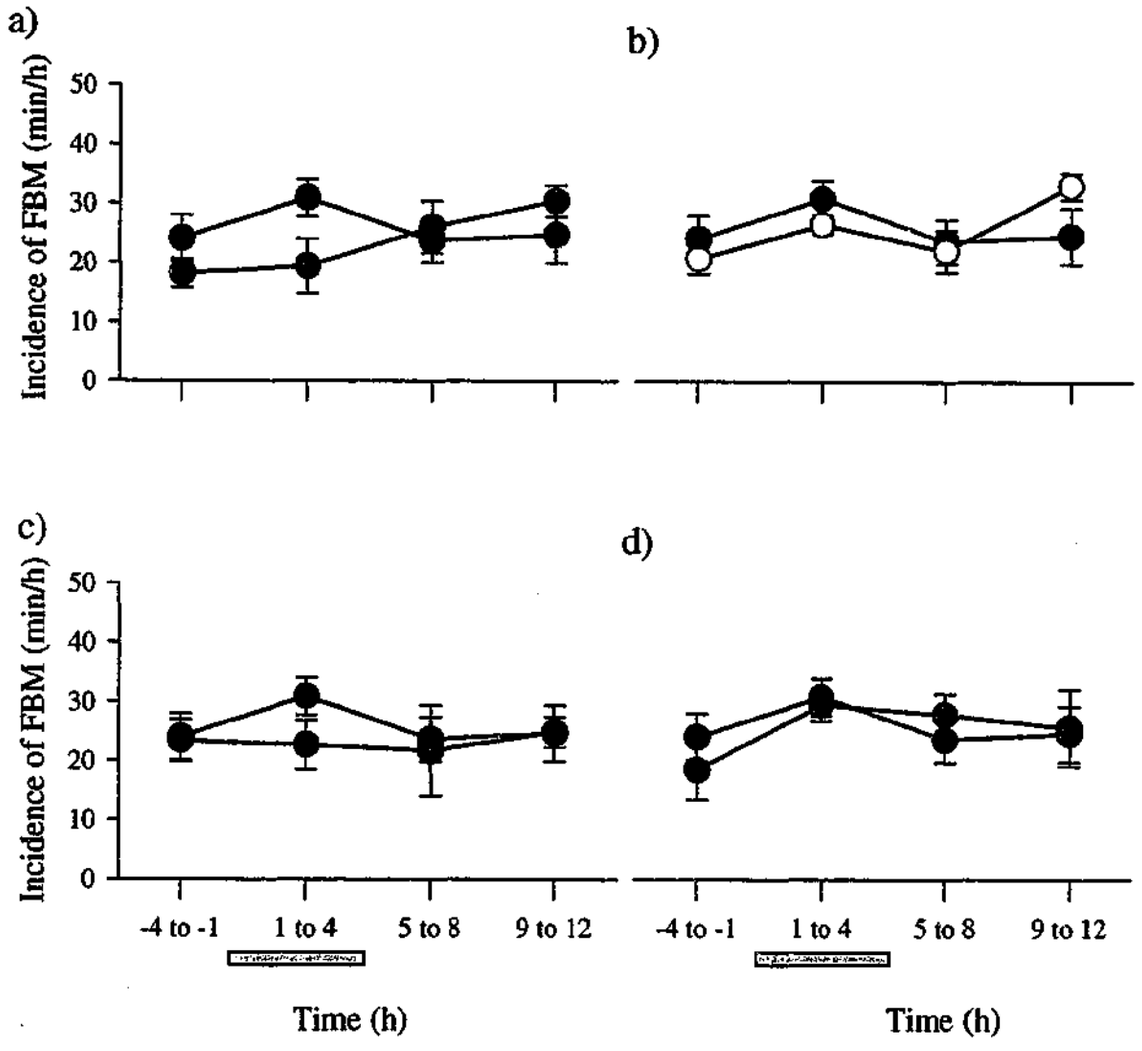


Figure 2.8
The effect of infusing artificial cerebrospinal fluid (blue) and different doses of $SeCl_4$; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of fetal breathing movements (FBM). All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (grey bar). All data represent the mean±sem, n = 3, 4 or 5.

LV ECoG. Despite the absence of statistical significance, the data does appear to suggest a trend between the different doses of $SeCl_4$ administrated and the amount of EMG activity which occurred during LV ECoG (see Fig. 2.4). Administration of 100 pmole of $SeCl_4$/10 µl/min into the lateral ventricle appeared to be associated with a greater increase in EMG activity during LV ECoG activity during the last 4 hour epoch, compared to infusions of aCSF and 25 pmole $SeCl_4$/10 µl/min (Fig. 2.9a). Furthermore, administration of 500 pmole of $SeCl_4$/10 µl/min induced the greatest increase in EMG activity during LV ECoG at 9 hours post-infusion, compared to all other treatments. In comparison, the incidence of EOG (Fig. 2.9b) and FBM (Fig. 2.9c) activity during LV ECoG were not significantly changed upon administration of $SeCl_4$ at any varying dose examined.

2.3.2.3 Effect of Selenium Chloride Infusion on Fetal Behavioural States

Administration of $SeCl_4$ into the lateral ventricle for four hours did not produce any significant changes in the incidence of NREM (Fig. 2.10a) and REM (Fig. 2.10b) sleep. All doses of $SeCl_4$ (25, 100, 500 and 1000 pmole/10 µl/min) did not significantly alter the incidence of both sleep states from control levels.

The lowest dose of 25 pmole of $SeCl_4$/10 µl/min did not appear to increase arousal throughout the four hour infusion period or during the 8 hour post-infusion period (Fig. 2.10c). In contrast, administration of 100 pmole of $SeCl_4$/10 µl/min significantly increased in arousal during the the 9 - 12 hour post-infusion period ($P<0.05$) compared to treatment with either aCSF, 25 pmole and 1 nmole of $SeCl_4$/10 µl/min. The dose of 500 pmole of $SeCl_4$/10 µl/min also caused a significant increase in the incidence of arousal between the 5^{th} and 8^{th} hour period ($P<0.05$) compared to vehicle alone or $SeCl_4$ at 25 and 1000 pmole/10 µl/min. This increase in arousal continued into the remaining duration of the 12 hour period and was significantly greater than all other treatments ($P<0.05$). Interestingly, administration of 1 nmole $SeCl_4$ did not increase the incidence of arousal. One fetus (omitted from the aggregate results) which received this dose exhibited abnormal seizure-like periods (fetal breathing, ocular and nuchal muscle movements during HV ECoG)

Figure 2.9

a) The effect of infusing artificial cerebrospinal fluid (aCSF; blue) and different doses of $SeCl_4$; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of electromyogram (EMG) activity during low voltage electrocorticogram (LV ECoG) activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 μl/min for four hours (grey bar). All data represent the mean±sem, n = 3, 4 or 5.

b) The effect of infusing aCSF (blue) and different doses of $SeCl_4$; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of electro-ocular (EOG) activity during LV ECoG activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 μl/min for four hours (grey bar). All data represent the mean±sem, n = 3, 4 or 5.

c) The effect of infusing aCSF (blue) and different doses of $SeCl_4$; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of fetal breathing movements (FBM) during LV ECoG activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 μl/min for four hours (grey bar). All data represent the mean±sem, n = 3, 4 or 5.

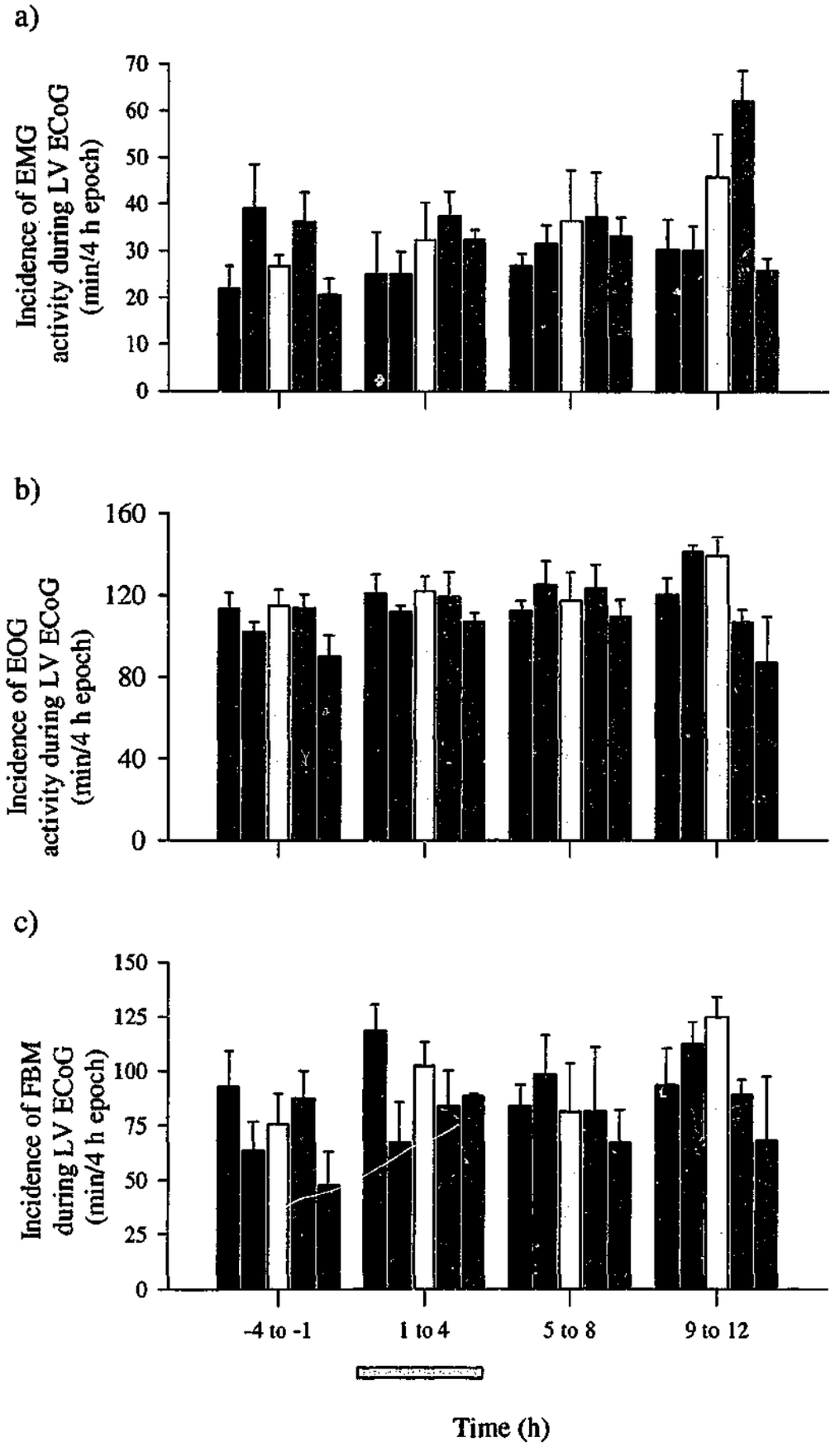
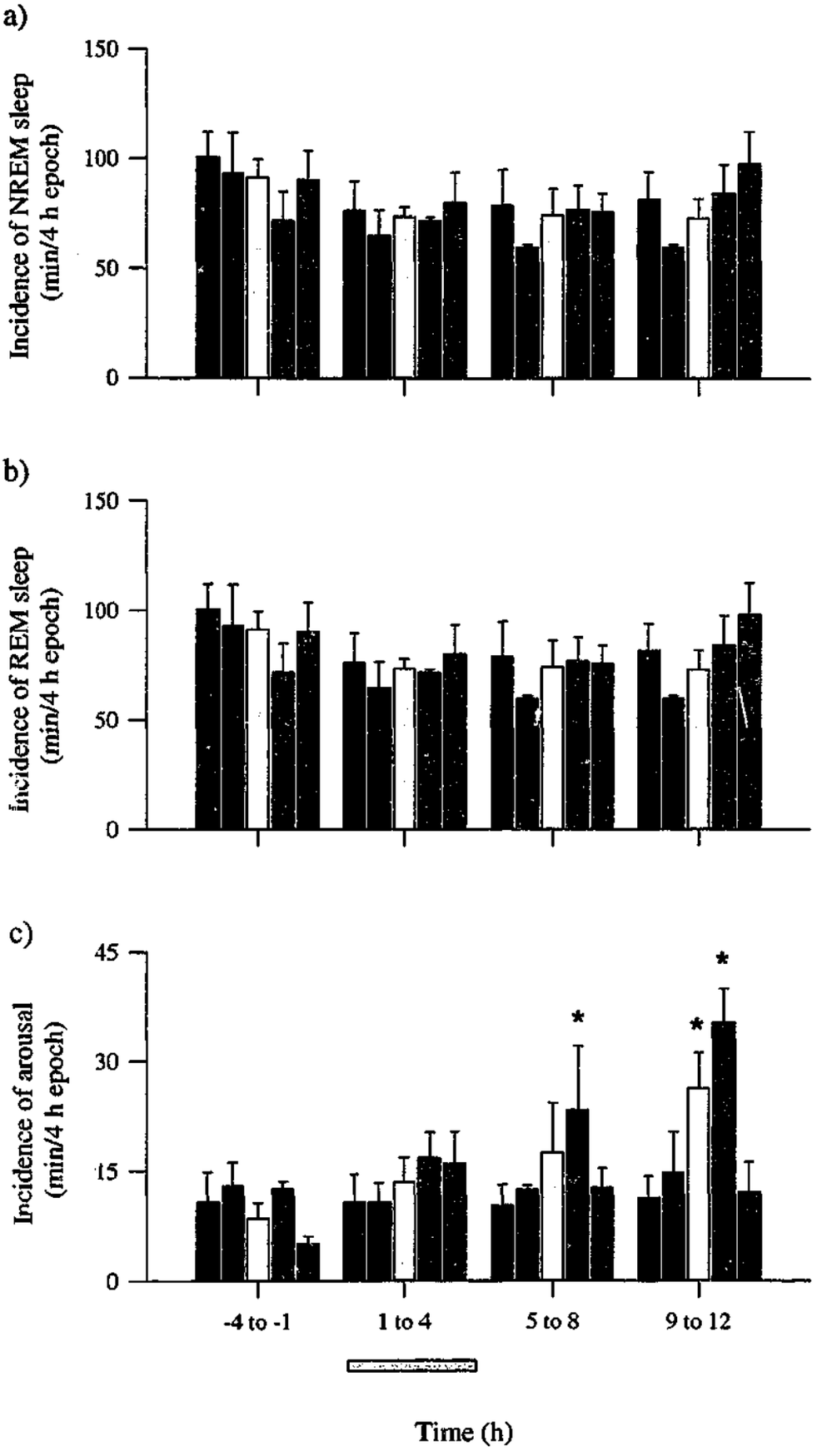

a)
Incidence of EMG activity during LV ECoG (min/4 h epoch)
70
60
50
40
30
20
10
0
b)
Incidence of EOG activity during LV ECoG (min/4 h epoch)
160
120
80
40
0
c)
Incidence of FBM during LV ECoG (min/4 h epoch)
150
125
100
75
50
25
0
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

Figure 2.10

a) The effect of infusing artificial cerebrospinal fluid (aCSF; blue) and different doses of $SeCl_4$; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of fetal non-rapid eye movement (NREM) sleep in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130-140 days gestation, at a flow rate of 10 µl/min for four hours (grey bar). All data represents the mean±sem, n=3, 4 or 5.

b) The effect of infusing aCSF (blue) and different doses of $SeCl_4$; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of fetal rapid eye movement (REM) sleep in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (grey bar). All data represents the mean±sem, n = 3, 4 or 5.

c) The effect of infusing aCSF (blue) and different doses of $SeCl_4$; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of fetal arousal in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours (grey bar). $SeCl_4$ induced a dose-dependent increase in the incidence of arousal, where administration of 100 and 500 pmole/10 µl/min significantly increased arousal above control and lower dose levels (*, $P<0.05$). All data represents the mean±sem, n = 3, 4 or 5.


a)
Incidence of NREM sleep (min/4 h epoch)
150
100
50
0
b)
Incidence of REM sleep (min/4 h epoch)
150
100
50
0
c)
Incidence of arousal (min/4 h epoch)
45
30
15
0
*
*
*
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

which spasmodically occurred during the 12 hour recording period. Doses greater than 10 nmole $SeCl_4$ (n = 4) induced seizure-like activity which persisted over 12 hours.

2.3.2.4 Effect of Selenium Chloride Infusion on Blood Gas Values, Blood Pressure and Heart Rate

Treatment with $SeCl_4$ (25, 100, 500, 1000 pmole/10 µl/min) did not significantly change the blood-gas status (Table 2.5), mean arterial pressure (MAP; Table 2.6) or heart rate of in any of the fetuses examined (Table 2.7).

2.3.3 Prostaglandin D_2 Infusions

2.3.3.1 The Effect of Prostaglandin D_2 Infusion on Recorded Fetal Behavioural Parameters

The incidence of HV and LV ECoG were not significantly altered by the four hour i.c.v infusions of PGD_2 at doses varying from 25 - 1000 pmoles/10 µl/min compared with control pre-infusion period values, or when compared to the 12 hour period when aCSF was infused (Fig. 2.5b). The same PGD_2 infusion regime did not produce any significant changes to the incidence of EMG (Fig. 2.11), EOG (Fig. 2.12) and FBM (Fig. 2.13) compared to pre-infusion control and infusion of aCSF.

2.3.3.2 Effect of Prostaglandin D_2 Infusion on Electromyographic and Electro-ocular Parameters and Fetal Breathing Movements during Low Voltage Electrocorticogram Activity

PGD_2 infusion had a significant effect on EMG activity that specifically occurred during LV ECoG with time period ($P<0.02$). However, there was no significant interaction

Table 2.5

The effect of i.c.v infusion of artificial cerebrospinal fluid (aCSF; 10 µl/min) or $SeCl_4$ (25, 100, 500, 1000 pmole/10 µl/min) on pH, partial pressure of carbon dioxide (pCO_2) and oxygen (pO_2), oxygen saturation (sO_2), and total haemoglobin (tHb) taken at one hour prior to and at every hour during the four hour infusion period. Data represents mean±sem, n = 3 - 5.

		Time (h)					
		Pre-Infusion		***Post-Infusion***			
	10 µl/min	***-1***	***1***	***2***	***3***	***4***	***5***
pH	*aCSF*	7.37±0.01	7.37±0.01	7.36±0.002	7.38±0.001	7.38±0.01	7.37±0.01
	25 pmole	7.37±0.01	7.36±0.01	7.38±0.004	7.38±0.008	7.38±0.01	7.37±0.01
	100 pmole	7.37±0.01	7.37±0.01	7.37±0.01	7.38±0.01	7.36±0.02	7.38±0.01
	500 pmole	7.4±0.003	7.4±0.004	7.4±0.007	7.4±0.007	7.4±0.003	7.4±0.004
	1 nmole	7.37±0.01	7.38±0.01	7.38±0.01	7.37±0.01	7.37±0.01	7.36±0.01
pCO_2	*aCSF*	40.0±3.0	42.0±1.1	41.8±2.3	38.5±2.8	39.7±2.3	40.6±2.0
	25 pmole	44.6±3.9	42.2±1.3	43.0±0.7	42.1±2.7	42.8±1.1	40.7±1.6
	100 pmole	41.7±1.0	42.5±2.4	42.0±1.6	39.2±0.8	40.7±1.2	40.1±0.8
	500 pmole	42.2±0.9	39.9±1.7	40.4±0.8	42.5±1.8	41.1±2.7	36.5±2.6
	1 nmole	41.9±1.4	37.9±5.6	37.9±0.9	40.2±0.1	39.7±0.9	37.9±1.2
pO_2	*aCSF*	23.4±2.3	22.8±2.5	22.5±1.9	24.5±3.2	25.3±2.6	23.2±2.3
	25 pmole	25.0±2.0	25.4±1.6	25.8±2.9	26.9±3.9	26.2±2.7	26.3±3.2
	100 pmole	25.3±1.8	24.9±1.9	25.6±2.8	23.6±1.8	24.6±1.8	24.7±2.1
	500 pmole	26.4±1.3	26.7±1.0	26.1±1.2	27.1±1.1	26.9±0.9	27.7±0.9
	1 nmole	23.9±0.7	23.7±0.6	24.6±0.3	23.5±0.5	23.8±0.2	23.0±0.9
sO_2	*aCSF*	64.5±6.9	64.1±8.3	58.0±3.9	65.7±7.2	67.9±7.4	63.3±7.0
	25 pmole	72.3±3.7	72.2±2.8	72.1±7.3	73.9±6.6	73.8±5.6	72.4±6.4
	100 pmole	70.4±3.1	67.1±6.2	66.5±6.2	67.1±5.0	66.7±4.3	67.3±5.7
	500 pmole	73.9±2.3	74.4±1.1	72.9±1.1	75.1±1.8	75.1±2.5	77.1±1.7
	1 nmole	67.9±3.7	69.1±7.0	69.4±6.0	67.3±3.8	67.5±5.6	67.1±8.0
tHb	*aCSF*	8.8±0.5	9.3±0.5	9.6±0.5	8.7±0.6	8.8±0.5	8.7±0.4
	25 pmole	9.3±0.3	9.0±0.4	9.6±0.7	8.5±0.2	9.3±0.5	9.1±0.9
	100 pmole	9.1±0.1	9.6±0.4	9.5±0.6	9.2±0.5	9.2±0.7	9.1±0.4
	500 pmole	10.5±0.8	9.4±0.5	9.5±0.8	10.4±0.6	9.0±1.2	9.4±0.7
	1 nmole	9.0±0.8	9.9±1.1	9.2±0.8	9.8±1.0	9. ±10.7	9.1±0.7

Table 2.6
The effect of i.c.v infusion of artificial cerebrospinal fluid (aCSF; 10 µl/min) or $SeCl_4$ (25, 100, 500, 1000 pmole/10 µl/min) on mean arterial pressure (MAP; mmHg). MAP was measured every ten minutes during four hour pre-infusion period and for 12 hours immediately following administration of treatment. The data was averaged over four hour epochs. Values are mean±sem, n = 3 - 4. Absence of sem, n = 2.

	Time (h)			
	Pre-Infusion		***Post-Infusion***	
10 µl/min	***-4 to –1***	***1 to 4***	***5 to 8***	***9 to 12***
aCSF	48.22±7.52	48.87±8.01	46.90±5.34	35.53±11.40
25 pmole	40.30	41.00	42.58	41.59
100 pmole	41.84	42.53	43.47	44.25
500 pmole	43.97±10.28	43.02±10.55	46.34±9.80	47.81±10.56
1 nmole	49.68±8.72	49.09±8.28	49.24±8.60	49.01±7.94

Table 2.6
The effect of i.c.v infusion of artificial cerebrospinal fluid (aCSF; 10 µl/min) or $SeCl_4$ (25, 100, 500, 1000 pmole/10 µl/min) on heart rate (beats/min). Heart rate was measured every ten minutes during four hour pre-infusion period and for 12 hours immediately following administration of treatment. The data was averaged over four hour epochs. Values represents mean±sem, n = 3 - 4. Absence of sem, n = 2.

	Time (h)			
	Pre-Infusion		***Post-Infusion***	
10µl/ min	***-4 to –1***	***1 to 4***	***5 to 8***	***9 to 12***
aCSF	163.97±4.80	168.64±18.17	175.84±11.98	179.59±12.14
25 pmole	144.08	140.33	148.84	156.72
100 pmole	160.38	156.92	151.26	162.09
500 pmole	175.60±12.85	167.22±11.50	174.20±7.05	170.60±12.68
1 nmole	160.35±16.63	164.98±16.98	158.12±16.57	154.89±11.54

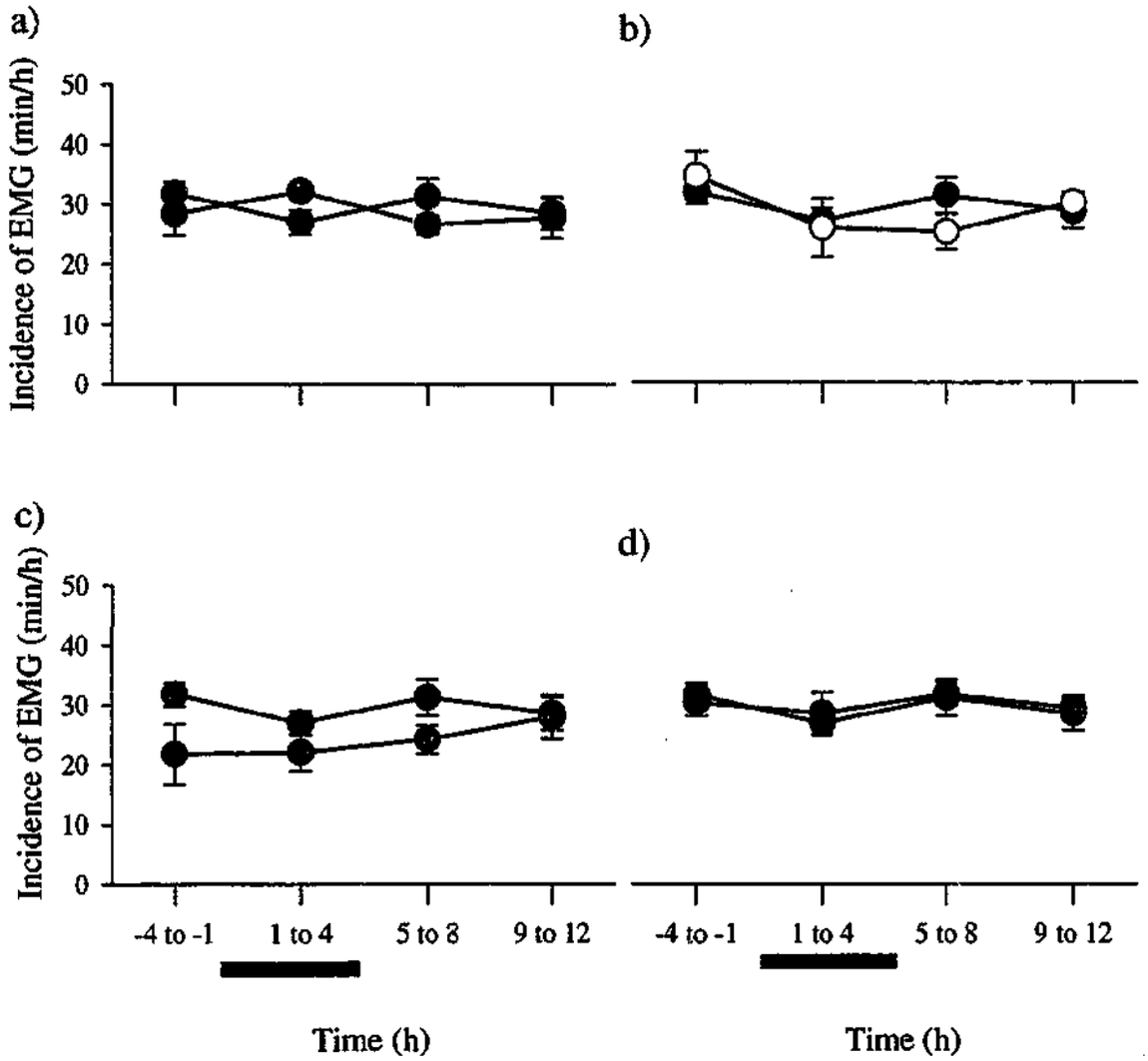


Figure 2.11
The effect of infusing artificial cerebrospinal fluid (blue) and different doses of PGD_2; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of electromyogram (EMG) activity. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represent the mean±sem, n = 4 or 5.

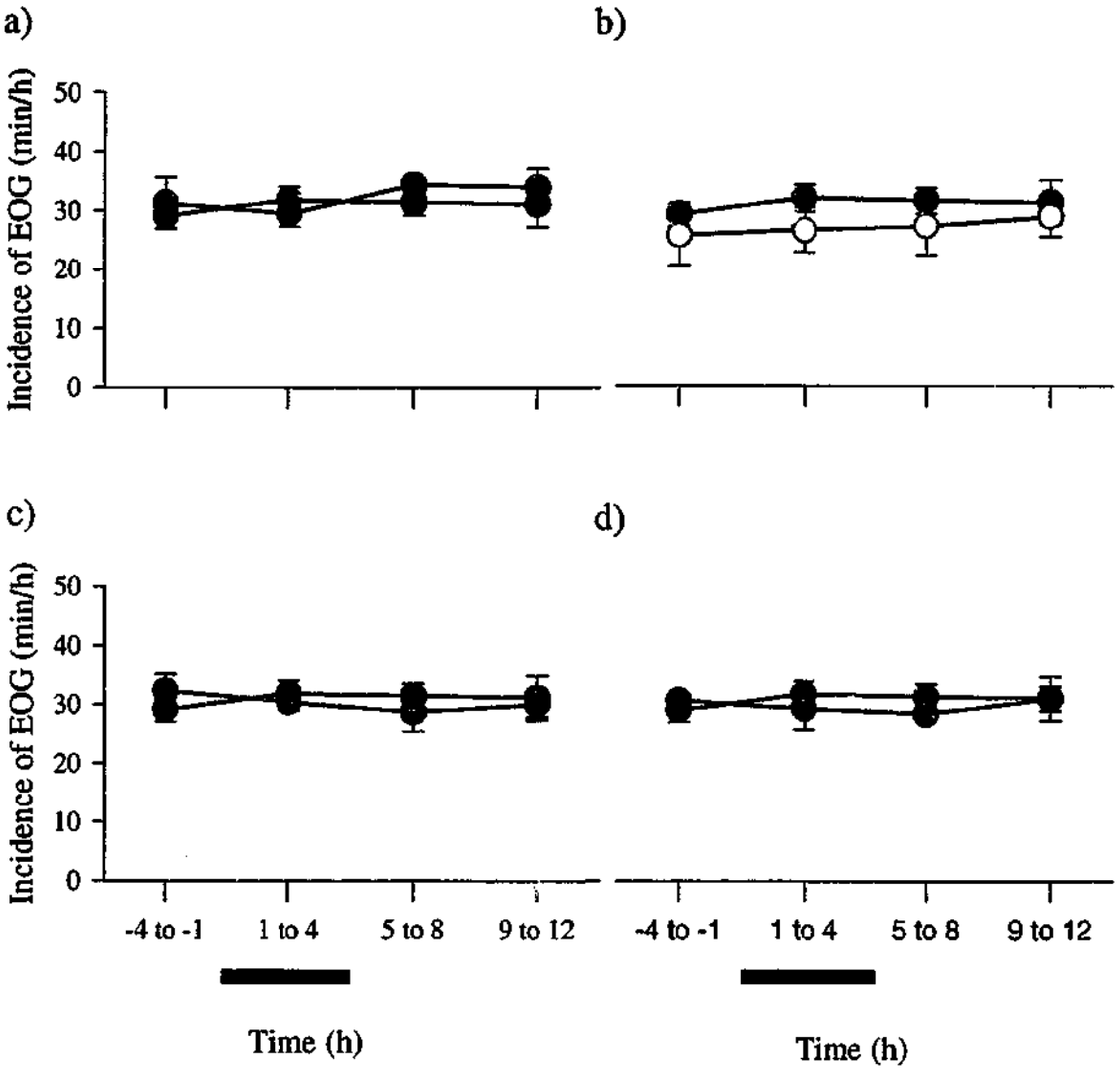


Figure 2.12
The effect of infusing artificial cerebrospinal fluid (blue) and different doses of PGD_2; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of electro-ocular (EOG) activity. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represent the mean±sem, n = 4 or 5.

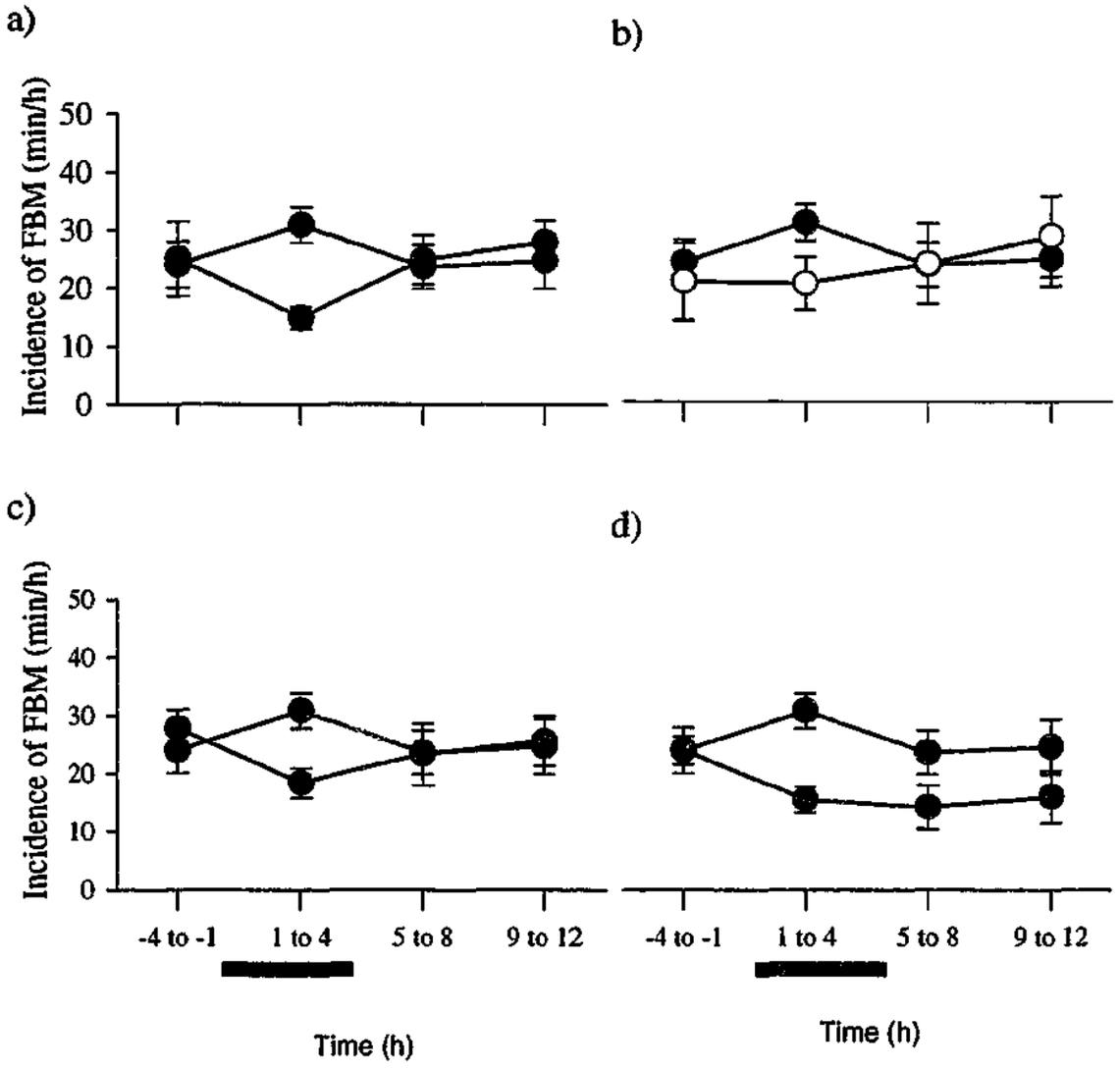


Figure 2.13
The effect of infusing artificial cerebrospinal fluid (blue) and different doses of PGD_2; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of fetal breathing movements (FBM). All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represent the mean±sem, n = 4 or 5.

between the time periods and the different doses of PGD_2 (P=0.130). This suggests the different doses of PGD_2 were not responsible for the apparent significant variation in the increase of EMG activity in LV ECoG. Despite the absence of statistical significance, there appears to be a decrease in the incidence of EMG activity during LV ECoG associated with administration of 100 and 500 pmole of PGD_2/10 µl/min during the duration of the infusion compared to treatment with aCSF and 25 and 1000 pmole of PGD_2/10 µl/min (Fig. 2.14a).

There were no significant effects of PGD_2 treatment on the incidence of EOG (Fig. 2.14b). Although all doses of PGD_2 appeared to decrease the incidence of FBM during LV ECoG compared to aCSF treatment (Fig. 2.14c), the observed decrease was not significant. This decline in activity continued for the remainder of experimental period at higher doses of PGD_2 (500 and 1000 pmole/10 µl/min).

2.3.3.3 *Effect of Prostaglandin D_2 Infusion on Fetal Behavioural States*

Four hour infusions of PGD_2 into the lateral ventricle at different doses (25, 100, 500 and 1000 pmole/10 µl/min) had no significant effect on the incidences of NREM (Fig. 2.15a) and REM (Fig. 2.15b) sleep.

Upon examination of the effect of i.c.v infusion of PGD_2 on fetal arousal, statistical analysis showed that there was a significant effect of time period (P<0.01), but no significant interaction between time period and dose (P=0.146). However, administration of PGD_2 greater than 100 pmole/10 µl/min into the lateral ventricle of ovine fetal sheep appeared to decreased the incidence of arousal below the incidences observed when aCSF and 25 pmole of PGD_2/10 µl/min where infused (Fig. 2.15c). It seems administration of 100pmole of PGD_2/10 µl/min almost halved the incidence of arousal within the first four hours of treatment. The decrease in arousal continued in the 5^{th} and 8^{th} post-infusion period to gradually returned to pre-infusion control levels by the end of recording. Similar trends were also observed after 500 pmole and 1 nmole of PGD_2/10 µl/min administration.

Figure 2.14

a) The effect of infusing artificial cerebrospinal fluid (aCSF; blue) and different doses of PGD_2; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of electromyogram (EMG) activity during low voltage electrocorticogram (LV ECoG) activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represents the mean±sem, n = 4 or 5.

b) The effect of infusing aCSF (blue) and different doses of PGD_2; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of electro-ocular (EOG) activity during LV ECoG activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represents the mean±sem, n = 4 or 5.

c) The effect of infusing aCSF (blue) and different doses of PGD_2; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of fetal breathing movements (FBM) during LV ECoG activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represents the mean±sem, n = 4 or 5.

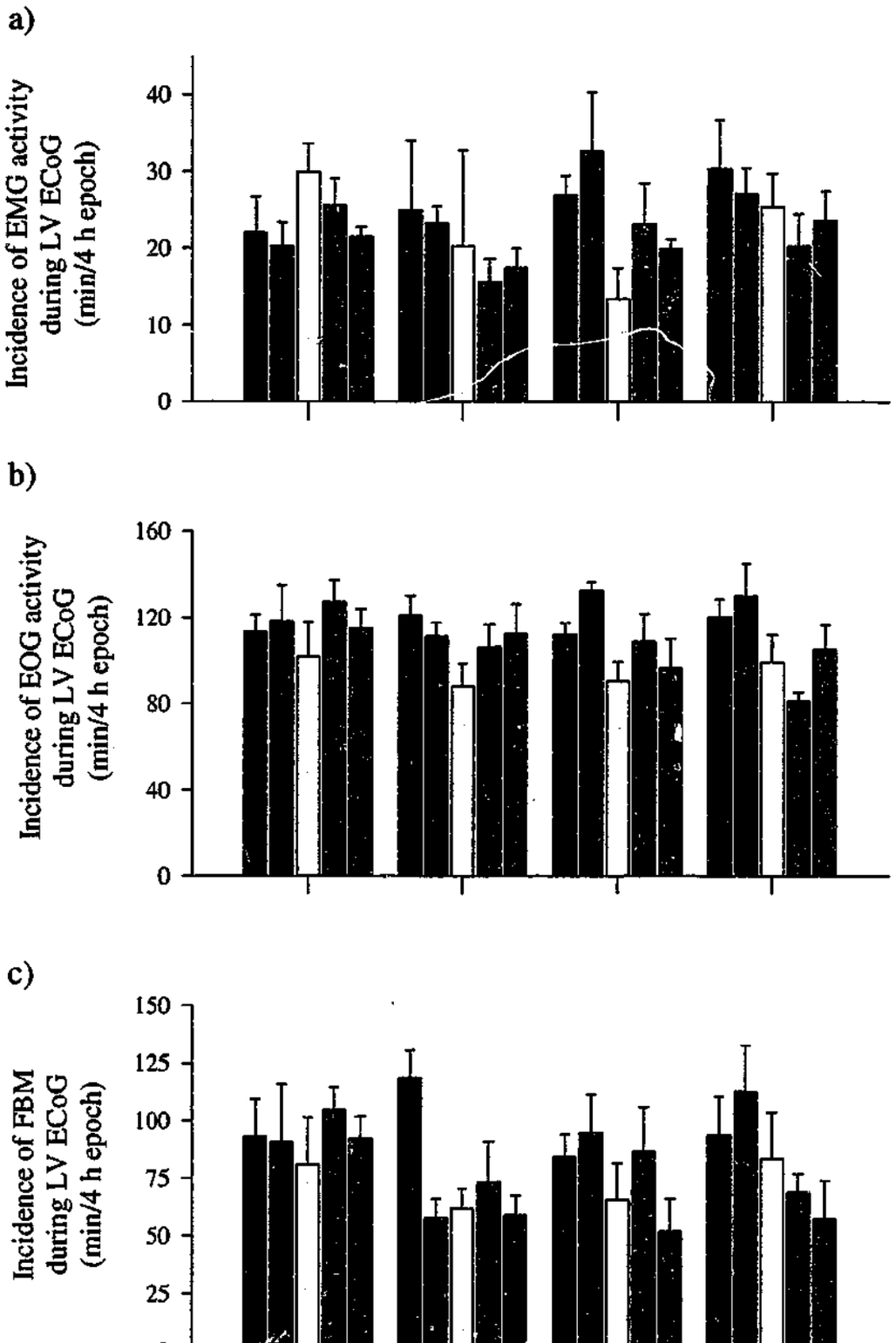
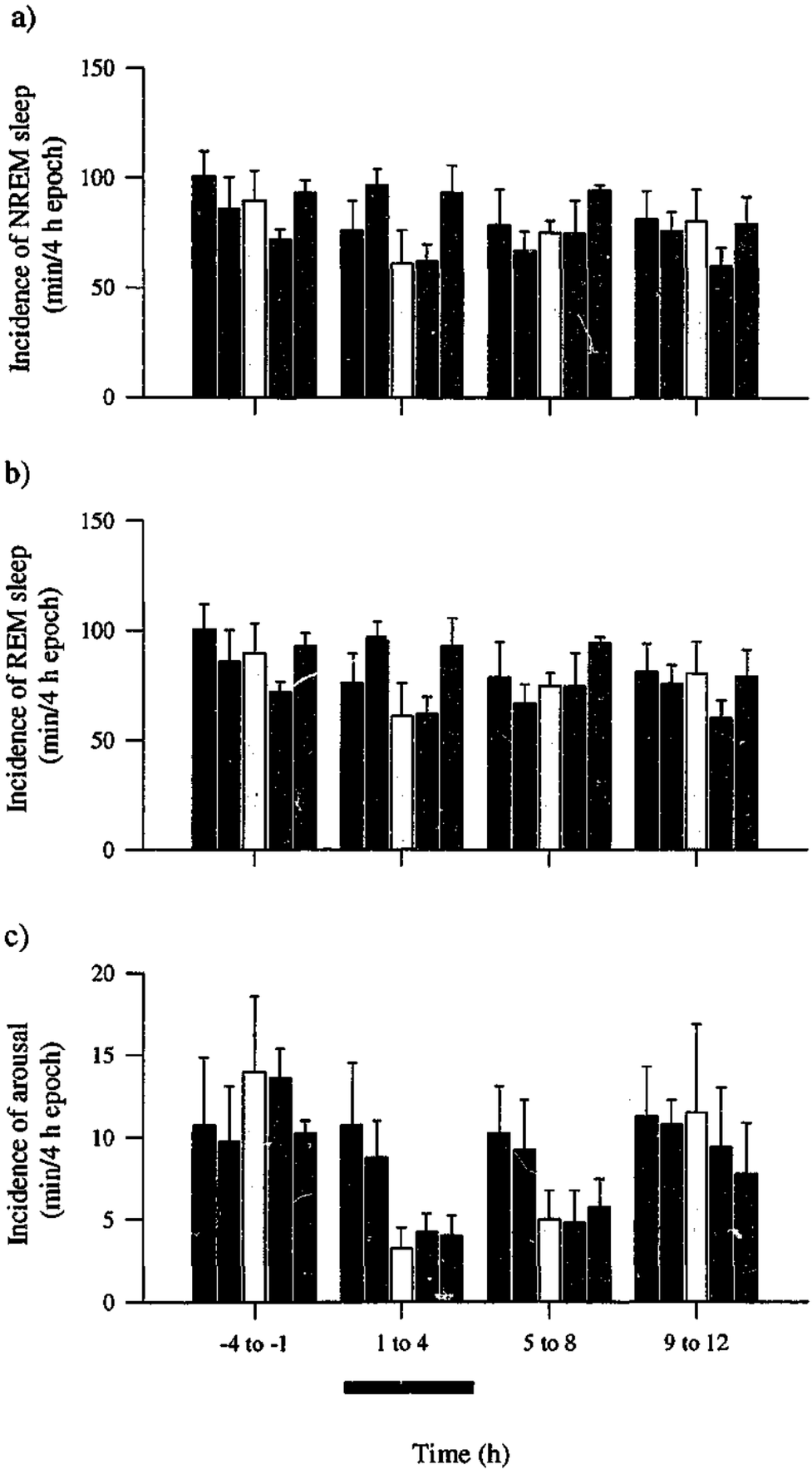

a)
Incidence of EMG activity during LV ECoG (min/4 h epoch)
40
30
20
10
0
b)
Incidence of EOG activity during LV ECoG (min/4 h epoch)
160
120
80
40
0
c)
Incidence of FBM during LV ECoG (min/4 h epoch)
150
125
100
75
50
25
0
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

Figure 2.15

a) The effect of infusing artificial cerebrospinal fluid (aCSF; blue) and different doses of PGD_2; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of fetal non-rapid eye movement (NREM) sleep in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represents the mean±sem, n = 4 or 5.

b) The effect of infusing aCSF (blue) and different doses of PGD_2; 25 pmole (a; green), 100 pmole (b; yellow), 500 pmole (c; red) and 1 nmole (d; purple), on the incidence of fetal rapid eye movement (REM) sleep in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represents the mean±sem, n = 4 or 5.

c) The effect of infusing aCSF (blue) and different doses of PGD_2; 25 pmole (green), 100 pmole (yellow), 500 pmole (red) and 1 nmole (purple), on the incidence of fetal arousal in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours (black bar). All data represents the mean±sem, n = 4 or 5.


a)
Incidence of NREM sleep (min/4 h epoch)
150
100
50
0
b)
Incidence of REM sleep (min/4 h epoch)
150
100
50
0
c)
Incidence of arousal (min/4 h epoch)
20
15
10
5
0
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

2.3.3.4 Effect of Prostaglandin D_2 Infusion on Blood Gas Values, Blood Pressure and Heart Rate

Different doses of PGD_2 (25, 100, 500 and 1000 pmole/10 µl/min) infused into the lateral ventricle did not induce any changes in the fetal blood gas status (Table 2.8), MAP (Table 2.9) nor heart rate (Table 2.10).

2.3.4 Selenium Chloride followed by Prostaglandin D_2 Infusions

2.3.4.1 The Effect of Selenium Chloride followed by Prostaglandin D_2 Infusion on Fetal Behavioural Parameters

Four hour treatment with 500 pmole $SeCl_4$/10 µl/min followed by 500 pmole of PGD_2/10 µl/min had no significant influence of the incidences of the measured behavioural parameters; HV and LV ECoG (Fig. 2.5c), EOG (Fig. 2.16b) and FBM (Fig 2.16c). However, administration of 500 pmole of $SeCl_4$/10 µl/min induced a significant increase in EMG activity in the 9^{th} to 12^{th} hour period ($P<0.05$), this elevation of EMG activity did not occurred when 500 pmole of PGD_2/10 µl/min was administered (Fig. 2.16a).

2.3.4.2 Effect of Selenium Chloride followed by Prostaglandin D_2 Infusion on Electromyographic and Electro-ocular Parameters and Fetal Breathing Movements during Low Voltage Electrocorticogram Activity

A significant effect to time period of EMG activity during LV ECoG ($P<0.005$) was observed upon infusion of $SeCl_4$ followed by PGD_2 (500 pmole/ 10 µl/min), but no significant interaction between time period and dose ($P = 0.096$) exist. However, despite the statistical insignificance the data does suggest that administration of 500 pmole $SeCl_4$/10

Table 2.8

The effect of i.c.v infusing artificial cerebrospinal fluid (aCSF; 10 µl/min) or PGD_2 (25, 100, 500, 1000 pmole/10 µl/min) on pH, partial pressure of carbon dioxide (pCO_2) and oxygen (pO_2), oxygen saturation (sO_2), and total haemoglobin (tHb) taken at one hour prior to and at every hour during the four hour infusion period. Data represents mean±sem, n = 4 or 5.

		Time (h)					
		Pre-Infusion		*Post-Infusion*			
	Treatment	*-1*	*1*	*2*	*3*	*4*	*5*
pH	*aCSF*	7.37±0.01	7.37±0.01	7.36±0.01	7.38±0.01	7.38±0.01	7.37±0.01
	25 pmole	7.37±0.01	7.37±0.01	7.36±0.01	7.37±0.2	7.37±0.01	7.37±0.01
	100 pmole	7.36±0.01	7.36±0.01	7.36±0.01	7.36±0.01	7.37±0.01	7.37±0.01
	500 pmole	7.37±0.01	7.38±0.01	7.36±0.03	7.37±0.01	7.40±0.01	7.37±0.01
	1 nmole	7.38±0.01	7.37±0.01	7.37±0.01	7.38±0.01	7.38±0.01	7.38±0.01
pCO_2	*aCSF*	40.0±3.0	42.0±1.1	41.8±2.3	38.5±2.8	39.7±2.3	40.6±2.0
	25 pmole	43.5±3.7	43.6±3.4	46.8±2.2	44.6±3.2	47.1±1.6	46.6±2.2
	100 pmole	47.0±1.4	44.8±4.7	45.4±3.5	46.5±2.5	44.9±3.3	45.9±3.4
	500 pmole	43.7±2.3	43.9±2.0	41.3±2.3	44.1±2.1	39.9±1.8	41.2±2.7
	1 nmole	44.9±1.6	44.4±3.4	45.3±2.6	46.2±1.9	44.8±1.2	43.3±1.4
pO_2	*aCSF*	23.4±2.3	22.8±2.5	22.5±1.9	24.5±3.2	25.3±2.6	23.2±2.3
	25 pmole	22.6±1.0	23.8±0.9	24.2±1.4	24.7±2.2	27.3±3.3	25.0±1.6
	100 pmole	22.9±2.4	24.5±2.6	23.5±1.5	22.7±2.0	22.8±2.5	21.3±3.0
	500 pmole	22.4±1.0	23.3±0.9	23.7±1.1	23.8±10.0	24.0±2.2	23.7±1.4
	1nmole	20.5±0.6	19.0±0.9	20.5±0.7	20.4±0.7	20.8±0.4	20.0±0.5
sO_2	*aCSF*	64.5±6.9	64.1±8.3	58.0±3.9	65.7±7.2	67.9±7.4	63.3±7.0
	25 pmole	71.3±3.0	71.1±3.6	71.9±4.4	71.3±2.2	73.4±3.7	72.0±4.7
	100 pmole	72.1±3.9	72.0±3.7	71.4±3.0	69.7±4.4	69.3±3.3	65.3±6.2
	500 pmole	65.5±3.5	69.4±3.0	69.0±2.8	69.1±1.9	71.0±5.4	68.1±3.7
	1 nmole	59.3±4.1	53.5±5.2	57.8±2.4	56.7±3.7	59.4±3.9	54.8±4.8
tHb	*aCSF*	8.8±0.5	9.3±0.5	9.6±0.5	8.7±0.6	8.8±0.5	8.7±0.4
	25 pmole	8.8±0.5	8.9±1.0	9.5±0.5	9.8±0.8	9.8±0.7	9.5±0.3
	100 pmole	10.2±0.6	8.9±0.3	9.2±0.2	9.7±0.6	9.3±0.5	10.0±0.3
	500 pmole	10.2±0.4	9.6±1.1	9.7±0.7	9.7±0.8	9.3±0.5	9.00±.7
	1 nmole	10.9±0.5	9.9±0.7	10.4±0.6	10.9±0.6	10.4±0.6	10.4±0.5

Table 2.9
The effect of i.c.v infusion of artificial cerebrospinal fluid (aCSF; 10 µl/min) or PGD_2 (25, 100, 500, 1000 pmole/10 µl/min) on mean arterial pressure (MAP; mmHg). MAP was measured every ten minutes during four hour pre-infusion period and for 12 hours immediately following administration of treatment. The data was averaged over four hour epochs, thus represents mean±sem, n = 3 or 4.

	Time (h)			
	Pre-Infusion	***Post-Infusion***		
Treatment	***-4 to -1***	***1 to 4***	***5 to 8***	***9 to 12***
aCSF	48.22±7.52	48.87±8.01	46.90±5.34	35.53±11.40
25 pmole	35.47±1.54	34.54±0.96	35.71±0.72	31.82±2.84
100 pmole	34.29±1.35	34.91±0.74	35.08±0.80	35.66±1.16
500 pmole	38.81±1.85	41.08±3.10	42.11±3.99	40.23±5.55
1 nmole	49.23±5.68	49.87±6.17	50.53±6.16	51.15±6.64

Table 2.10
The effect of i.c.v infusion of artificial cerebrospinal fluid (aCSF; 10 µl/min) or PGD_2 (25, 100, 500, 1000 pmole/10 µl/min) on heart rate (beats/min). Heart rate was measured every ten minutes during four hour pre-infusion period and for 12 hours immediately following administration of treatment. The data was averaged over four hour epochs, thus represents mean±sem, n = 3 or 4.

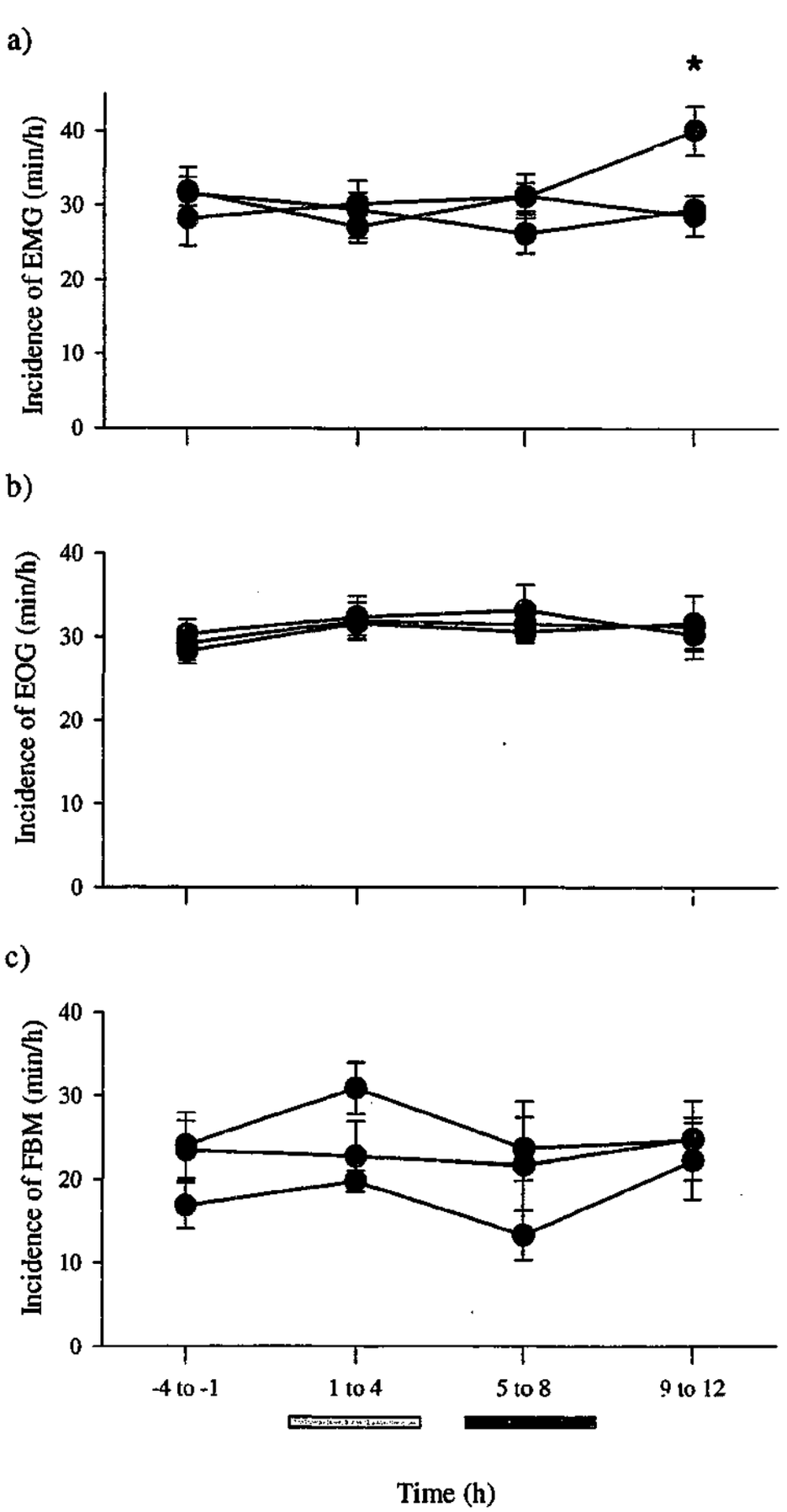
	Time (h)			
	Pre-Infusion	***Post-Infusion***		
Treatment	***-4 to -1***	***1 to 4***	***5 to 8***	***9 to 12***
aCSF	163.97±4.80	168.64±18.17	175.84±11.98	179.59±12.14
25 pmole	144.30±15.15	137.18±13.85	136.71±13.10	145.14±12.32
100 pmole	122.20±8.30	132.61±8.90	132.91±7.76	135.60±5.40
500 pmole	129.30±6.00	134.90±8.50	130.30±6.00	145.10±7.30
1 nmole	145.88±8.00	148.26±5.92	139.90±2.46	145.40±5.13

Figure 2.16

a) Compares the effect of infusing artificial cerebrospinal fluid (aCSF; blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of electromyogram (EMG) activity. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion. Administration of 500 pmole of $SeCl_4$/10 µl/min induced a significant increase in EMG activity higher than control levels (*, $P<0.05$). Subsequent administration of 500 pmole of PGD_2/10 µl/min abolished the observed $SeCl_4$-induced significant increase in EMG activity. All data represent the mean±sem, n = 4 or 5.

b) Compares the effect of infusing of aCSF (blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of electro-ocular (EOG) activity. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion. All data represent the mean±sem, n = 4 or 5.

c) Compares the effect of infusing aCSF (blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of fetal breathing movement (FBM). All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion. All data represent the mean±sem, n = 4 or 5.


a)
*
Incidence of EMG (min/h)
40
30
20
10
0
b)
Incidence of EOG (min/h)
40
30
20
10
0
c)
Incidence of FBM (min/h)
40
30
20
10
0
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

µl/min increase the incidence of EMG activity during LV ECoG, and that this response is not observed when 500 pmole PGD_2/10 µl/min is infused immediately after (Fig. 2.17a). The same infusion regime did not significantly change in incidences of EOG (Fig. 2.17b) and FBM (Fig. 2.17c) during LV ECoG.

2.3.4.3 Effect of Selenium Chloride followed by Prostaglandin D_2 Infusion on Fetal Behaviour

Administration of 500 pmole $SeCl_4$/10 µl/min followed by 500 pmole of PGD_2/10 µl/min did not significantly alter the incidence of NREM (Fig. 2.18a) and REM (Fig. 2.18b) sleep exhibited in the fetuses either.

In comparison, treatment of 500 pmole $SeCl_4$/10 µl/min induced a significant increase in the incidence of arousal above pre-infusion and control aCSF levels during the 5^{th} to 12^{th} hour ($P<0.05$). This significant increase in arousal induce by 500 pmole of $SeCl_4$ was abolished by 500 pmole PGD_2/10 µl/min administration during the 5^{th} and 8^{th} hour period (Fig. 2.18c). The infusion of 500 pmole of PGD_2/10 µl/min not only reversed the effect of $SeCl_4$ treatment but also significantly reduced the incidence of arousal below control levels ($P<0.05$). The reduction in arousal occurred during the four hour infusion period of PGD_2, and returned to normal levels by the 9^{th} to 12^{th} hour period.

2.3.4.4 Effect of Selenium Chloride followed by Prostaglandin D_2 infusion on Blood Gas Values, Blood Pressure and Heart rate

Subsequent administration of PGD_2 after $SeCl_4$ (500 pmole/10 µl /min) did not produce any significant changes to MAP and heart rate (Table 2.11), blood gases, pH, O_2 saturation or haemoglobin concentration status (Table 2.12) of the fetus.

Figure 2.17

a) Compares the effect of infusing artificial cerebrospinal fluid (aCSF; blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of electromyogram (EMG) activity during low voltage electrocorticogram (LV ECoG) activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion. All data represents the mean±sem, n = 4 or 5.

b) Compares the effect of infusing aCSF (blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of electro-ocular (EOG) activity during LV ECoG activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion.. All data represents the mean±sem, n = 4 or 5.

c) Compares the effect of infusing aCSF (blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of fetal breathing movements (FBM) during LV ECoG activity in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion. All data represents the mean±sem, n = 4 or 5.

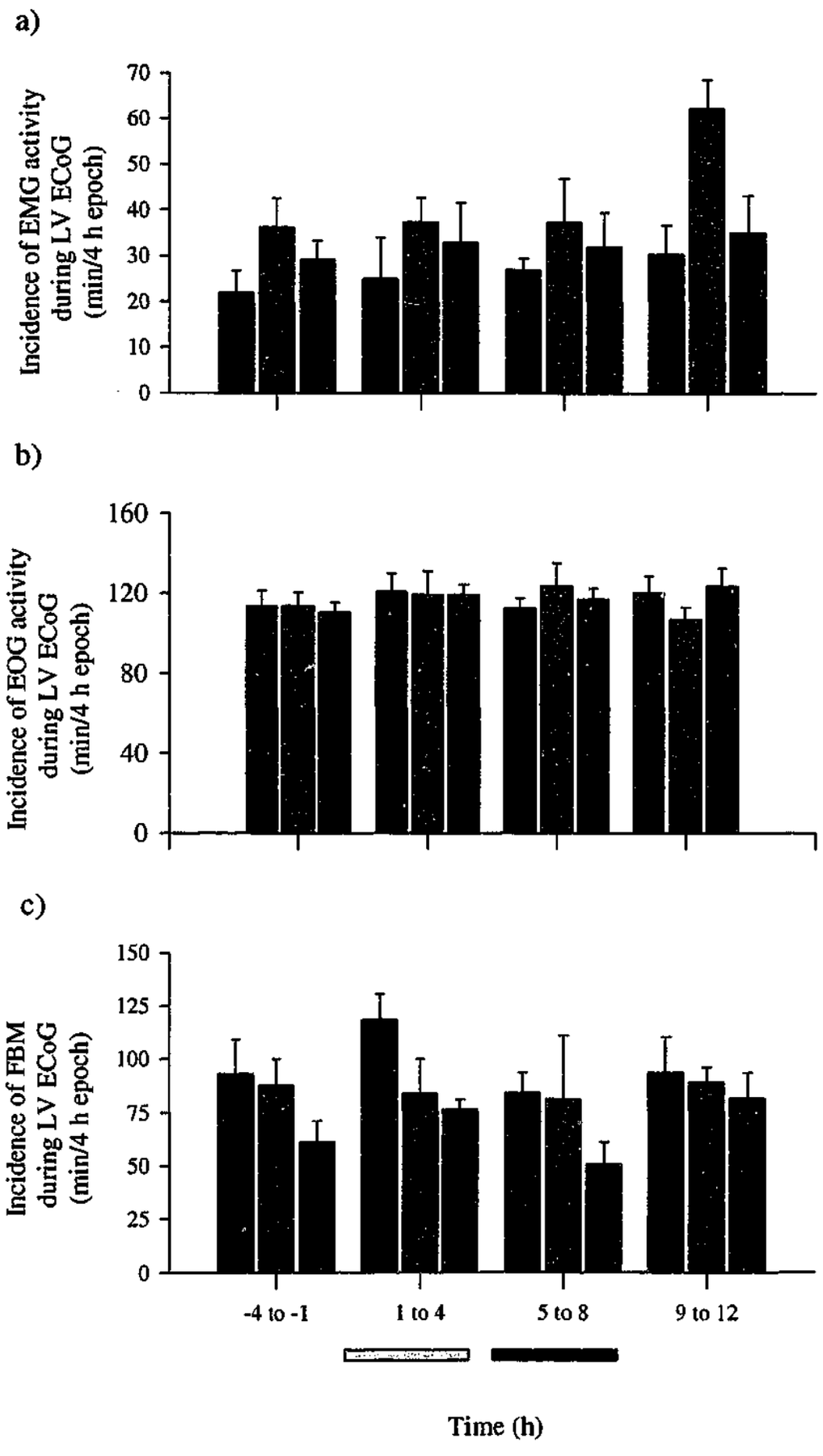

a)
Incidence of EMG activity during LV ECoG (min/4 h epoch)
70
60
50
40
30
20
10
0
b)
Incidence of EOG activity during LV ECoG (min/4 h epoch)
160
120
80
40
0
c)
Incidence of FBM during LV ECoG (min/4 h epoch)
150
125
100
75
50
25
0
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

Figure 2.18

a) Compares the effect of infusing artificial cerebrospinal fluid (aCSF; blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of non-rapid eye movement (NREM) sleep. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion. All data represents the mean±sem, n = 4 or 5.

b) Compares the effect of infusing aCSF (blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of rapid eye movement (REM) sleep. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 days gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2 given immediately after $SeCl_4$ infusion. All data represents the mean±sem, n = 4 or 5.

c) Compares the effect of infusing aCSF (blue), 500 pmole of $SeCl_4$ (red) and 500 pmole of $SeCl_4$ followed by 500 pmole PGD_2 (aqua) on the incidence of arousal in four hour epochs. All infusions were administered into the left lateral ventricle of fetuses of 130 - 140 day gestation, at a flow rate of 10 µl/min for four hours. The grey bar represents administration of aCSF or $SeCl_4$, whereas the black bar represents administration of PGD_2. Administration of 500 pmole of $SeCl_4$/10 µl/min significantly increase arousal above aCSF control levels between the 5^{th} to 12^{th} hour, (*, $P<0.05$). Subsequent administration of 500 pmole PGD_2/10 µl/min abolished this $SeCl_4$-induced increase in arousal to incidences significantly lower than levels exhibited in control infusions of aCSF (*, $P<0.05$). All data represents the mean±sem, n = 4 or 5.

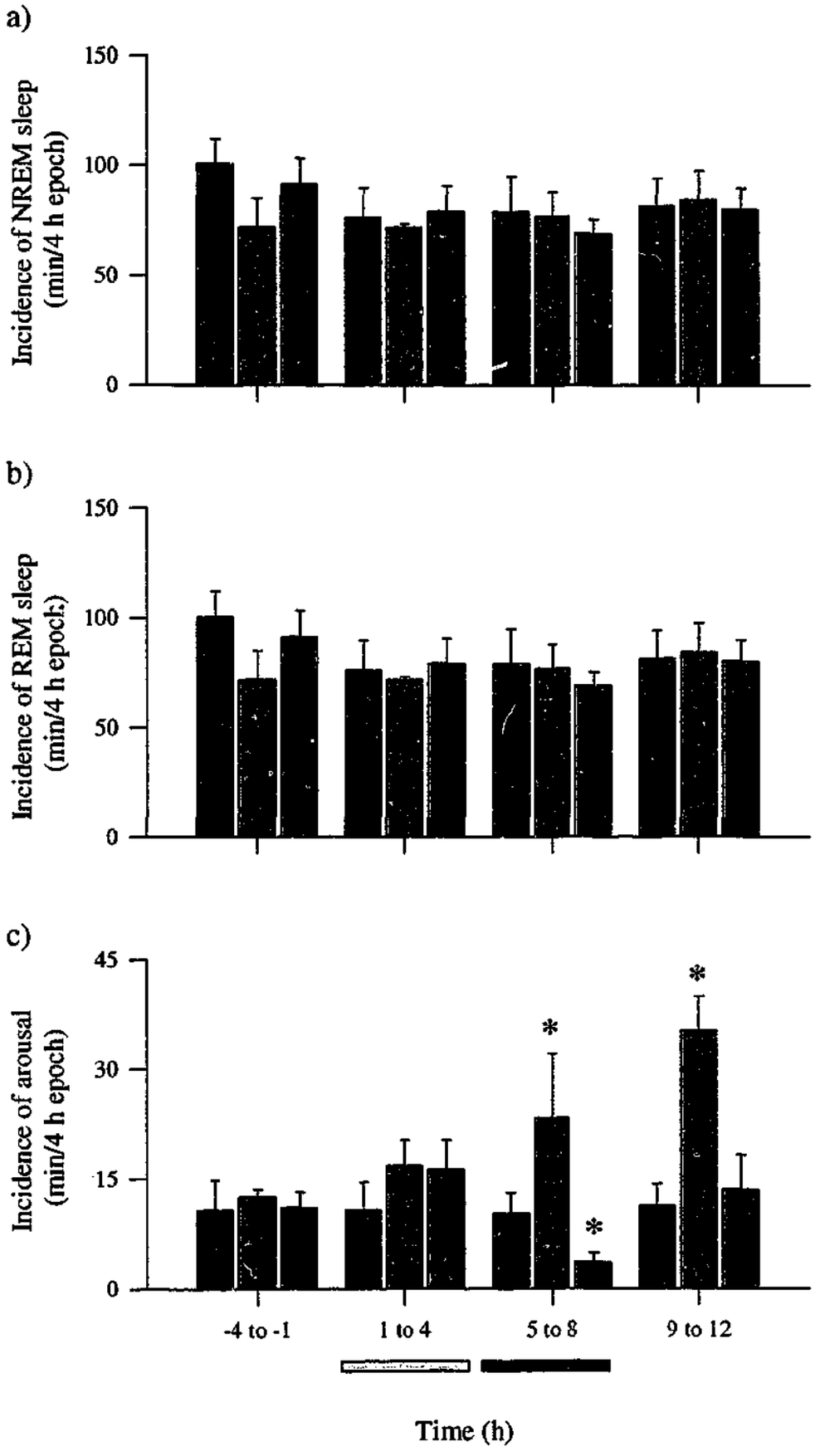

a)
Incidence of NREM sleep (min/4 h epoch)
150
100
50
0
b)
Incidence of REM sleep (min/4 h epoch)
150
100
50
0
c)
Incidence of arousal (min/4 h epoch)
45
30
15
0
*
*
*
-4 to -1
1 to 4
5 to 8
9 to 12
Time (h)

Table 2.11

The effect of i.c.v administration of 500 pmol/10 µl/min of $SeCl_4$ followed by 500 pmol/10 µl/min of PGD_2 on mean arterial pressure (MAP; mmHg) and heart rate (beats/min). MAP and heart rate was measured every ten minutes during four hour pre-infusion period and for 12 hours following administration of treatment. The data was averaged over four hour epochs, thus represents mean±sem, n = 3 or 4.

	Time (h)			
	Pre-Infusion	***Post-Infusion***		
	-4 to -1	***1 to 4***	***5 to 8***	***9 to 12***
MAP	39.09±6.11	38.13±5.94	37.85±6.00	38.19±6.02
heart rate	157.40±.75	160.56±5.30	191.71±3.05	158.71±2.39

Table 2.12

The effect of i.c.v administration of $SeCl_4$ (500 pmole/10ul/min) followed by PGD_2 (500 pmole/10µl/min) infusion on pH, partial pressure of carbon dioxide (pCO_2) and oxygen (pO_2), oxygen saturation (sO_2), and total haemoglobin (tHb) taken at one hour prior to and at every hour during the four hour infusion of $SeCl_4$ followed by 4 hour infusion of PGD_2. Data represents mean±sem, n=4.

	Time (h)									
	Pre-Infusion						***Post-Infusion***			
	-1	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***
pH	7.37±0.01	7.36±0.01	7.37±0.01	7.38±0.01	7.37±0.01	7.36±0.02	7.36±0.02	7.37±0.01	7.37±0.01	7.36±0.02
pCO_2	40.3±1.7	44.4±3.0	39.9±0.3	41.4±0.8	42.6±1.3	42.1±3.0	42.9±2.0	39.0±2.6	40.8±1.5	42.4±2.6
pO_2	23.3±3.0	23.2±3.3	26.0±1.1	25.9±1.5	25.6±1.6	24.5±1.5	24.4±1.2	27.3±1.4	26.0±1.4	25.0±1.2
sO_2	70.5±3.1	65.1±8.4	72.6±0.8	73.2±2.8	73.5±1.3	68.2±2.3	68.6±1.8	72.6±2.3	69.8±1.1	70.3±0.5
tHb	9.8±0.2	10.7±0.6	9.9±0.4	10.3±0.1	10.2±0.2	9.7±0.4	9.8±0.5	9.4±0.3	9.9±0.3	9.9±0.6

2.4 DISCUSSION

Inhibition of PGD_2 production by i.c.v infusion of the specific PGDS inhibitor $SeCl_4$ caused a significant dose-dependent increase in the incidence of arousal in the late gestation ovine fetus. This increase in fetal arousal was abolished by subsequent administration of exogenous PGD_2, which then reduced the incidence of arousal to significantly lower levels compared to control. The infusion of PGD_2 alone into the lateral ventricle decreased the incidence of arousal. This decline in arousal was not dose-dependent, as all doses higher than 100 pmole of PGD_2/10 µl/min reduced the incidence of arousal by the same amount (i.e. not statistically different from each other) compared to lower doses. Therefore, from these observations it can be said that $SeCl_4$ increased the amount of time the ovine fetus spent 'awake' presumably by inhibiting of endogenous production of PGD_2. This suggests that PGD_2 may have a role in the maintenance of sleep-wake cycles *in utero*, as is it hypothesised during adult life (Hayaishi 1988).

A Delrin cap, attached to the fetal skull, contained a sealed chamber that provided two way infusion of solution. Closure of output flow path allowed constant flow of solution at slow rate into the lateral ventricle via a plastic cannula secured into the base of the Delrin cap. Delrin plastic was specifically chosen instead of metal material, to reduce interference with ECoG activity. Numerous difficulties were encountered when attempting chronic implantation of catheter into the brain. The two main problems encountered were; firstly, maintaining a sterile environment to ensure the health of the fetus and secondly, ensuring the cannula was correctly inserted into the lateral ventricle. Correct insertion of cannula could only be verified at post-mortem after all experiments and recordings had been performed. If the cannula was correctly inserted into the lateral ventricle, data from the experiments were performed between125 to 135 days gestation were analysed. The cannula tip remained in the lateral ventricle over this time despite the continuing growth of the brain (Hirst *et al.* 2000).

The administration of aCSF into a lateral ventricle at 10 µl/min for 4 hours did not alter the normal ECoG, EOG or nuchal muscle EMG activities, nor did it alter the patterns of activities from which the behavioural states of NREM and REM sleep and wakefulness are deduced (Szeto and Hinman 1985). The lateral ventricles are occupied by the CP, which synthesises and secretes CSF into the ventricular system. The production rate of CSF in late gestation fetal sheep has been estimated to be 77 µl/min (Bissonnette *et al.* 1981) at c.125 days gestation is likely to be turned over 4 - 5 times/day, as for most adult mammals (Davson *et al.* 1987). Therefore, upon administration into the lateral ventricle, it is likely that the $SeCl_4$ and/or PGD_2 would have been distributed throughout the CSF volume by the end of the 4 hour infusion period. In the ovine fetus both substances would need to travel a distance less than 5 mm to reach what is considered to the target site. The proposed PGD_2-sensitive, 'somnogenic' area is located on the ventral surface of the rostral basal forebrain in adult rats between 0.7 and 2.7mm rostral to bregma on the midline (Matsumura *et al.* 1994), situated adjacent to the defined site of the sleep centres in the VLPO area of the anterior hypothalamus (Lu *et al.* 2000). It is proposed that PGD_2 acts at this site to induce a soporific effect. In comparison, $SeCl_4$ would be required to reduce the amount of endogenous PGD_2 by inhibition of PGDS before inducing any changes in fetal behaviour. If the pre-optic hypothalamic area is the main site of the synthesis and action of PGD_2 in the fetal sheep brain, as in the adult rat (Scammell *et al.* 1998), it might be expected that the $SeCl_4$ would have access to this site from the lateral ventricle in minutes, rather than hours. The delayed onset of the increase of arousal is consistent with observations made in previous studies (Matsumura *et al.* 1991) and with the inhibition of an enzyme system and the subsequent slow decline in the amount of PGD_2 in the brain. In comparison, when PGD_2 was administered the suppression of arousal was observed during the time the prostanoid was being infused, which is consistent with it being presented to the POAH relatively quickly, and being able to act directly at the sites that influence arousal activity.

The increase in the incidence of arousal behaviour elicited by $SeCl_4$ occurred primarily due to an increase in the amount of nuchal muscle EMG activity. At the dose of 100 pmole/10 µl/min, $SeCl_4$ did not alter the total amount of nuchal EMG activity present but re-

distributed the amount of nuchal muscle activity so that a greater incidence occurred when the ECoG was in LV activity, when it is otherwise is generally absent. At a dose of 500 pmole/10 µl/min $SeCl_4$ there was a significant increase in the total amount of EMG activity present in addition to an increase in the incidence of EMG activity during LV ECoG. Administration of $SeCl_4$ did not evoke any significant changes in the other parameters used to characterise fetal behavioural states; viz., HV and LV ECoG activities, fetal breathing and ocular movements, or the occurrence of these breathing and eye movements during LV ECoG.

The results also suggest that administration of PGD_2 into the lateral ventricle of fetal sheep increased the time spent asleep. The decline in arousal was not dose-dependent, so that all doses above of 100 pmole of PGD_2/10 µl/min (i.e. 100, 500 and 1000 pmole/10 µl/min) had a similar effect. Furthermore, PGD_2 was shown to reduce the incidence of arousal to below the level observed in the control periods, and below that present when aCSF was infused. The observed reduction in arousal was due to the decrease, although not significant in itself, in nuchal muscle activity during LV ECoG rather than a reduction in the total amount EMG activity. This suggests that PGD_2 synthesis in the fetal brain has a role in determining the incidence of the 'wakefulness' or arousal-like behaviour in late gestation in the sheep. These observations are consistent with studies that show excess production of PGD_2, stimulated by acute pain and inflammation (Smith and Wagner 1991), in transgenic mice that overexpress the PGDS gene (Pinzar *et al.* 2000) result in increased incidence of NREM sleep.

Interestingly, PGD_2 appeared to have an effect on FBM, where the overall incidence of FBM appeared to decrease following 1 nmole of PGD_2, and the incidence of FBM during LV ECoG decreased after all doses of PGD_2. Although these observations were not statistically significant, they are worthy of mention as administration of PGE_2, a closely related prostaglandin synthesised from the same PGH_2 precursor, has been shown to decrease FBM (Kitterman *et al.* 1983). Conversely treatment of indomethacin (cyclooxygenase inhibitor) stimulated FBM in late gestation fetuses (Adamson *et al.* 1997).

The observed association between PGD_2 in the brain and spontaneous muscle activity has been noted in studies in the adult rat. PGD_2 (2 μg/i.c.v.) was shown to increase the latency period of pentylenetetrazol-induced seizures in rats, whereas Na_2SeO_3 (2 μg/i.c.v.) potentiated the incidence and intensity of the chemically-induced convulsion (Akarsu *et al.* 1998). This relationship could also explain the seizure-like activities observed when the fetuses were given doses greater than 1 nmole $SeCl_4$/10 μl/min. Therefore it appears that administration of 1 nmole of $SeCl_4$ may be at the boundaries of physiological dose range where an arousal response can be observed. Doses above 1 nmole appear beyond the normal physiological range, and elicited pharmacological convulsant effects.

Since $SeCl_4$ was able to induce a dose-dependent response, it could be said the observed increase in arousal was a specific response to the inhibition of PGDS in the brain. Administration of PGD_2 immediately after $SeCl_4$ infusion significantly abolished the $SeCl_4$-induced increase in arousal, reducing it to levels significantly lower than control incidences. Therefore it is likely that the observed increase in arousal is specifically caused by the depletion of endogenous PGD_2 levels, which can be reversed by addition of PGD_2 into the brain.

Treatment with $SeCl_4$ or PGD_2, individually or sequentially, did not alter the overall incidence of REM/NREM sleep. Previous studies have demonstrated that inhibition of PGD_2 synthesis caused a marked reduction of both sleep states in the adult rat (Matsumura *et al.* 1991), thus directly suggesting a causal effect of PGD_2 with the occurrence of sleep. However, as the fetus already spends at least 95% of the time asleep (Szeto and Hinman 1985) it would be difficult to discriminate addition significant increases in the incidence of REM/NREM sleep *in utero*. Since arousal occupies the remaining 5% of the time, any slight variations in the time spent 'awake' can be readily identified.

To analyse the data, the incidence in arousal was analysed within four-hour sequential epochs. Low incidences of arousal occurred randomly at varying intervals within each hour. When examining the incidence of arousal per hour, arousal appeared to occur in a cyclical fashion, oscillating between hours which exhibited considerably high incidences of arousal

in some hours, in contrast to other hours where arousal was virtually absent (Fig. 2.19). This rhythm seemed to be based on a four hour time frame; therefore, it was considered necessary to analyse in blocks of time that were four hours long.

The mechanism by which $SeCl_4$ produces the increase of arousal in the fetus remains to be fully elucidated. In adult rats it has been proposed that a group of PGD_2-sensitive neurons reside in the POAH (Matsumura *et al.* 1994). These neurons coincide with the VLPO area, a neuronal pool which exhibit FOS activation during sleep (Sherin *et al.* 1996). The sleep-active GABAergic VLPO area inhibit a population of histaminergic TMN of the posterior hypothalamus (Sherin *et al.* 1996) that are thought to maintain excitability of the cortex during states of wakefulness (Lin *et al.* 1989). Furthermore, bilateral lesions of the VLPO area in adult rats produced by ibotenic acid microinjection caused insomnia (Lu *et al.* 2000). The reciprocal inhibitory relationship between the VLPO area and the putative wake-centres has been further substantiated, where *in vitro* activity of GABAergic VLPO cells were inhibited by addition of neurotransmitters associated with vigilance, including NA, ACh and serotonin (Gallopin *et al.* 2000). Thus, inhibition of PGD_2 production by $SeCl_4$ could results in decreased inhibition of the posterior hypothalamic neurons and increased excitability of cortical neurons. While immunocytochemical studies have not yet been done to identify either PGDS-positive cells, or neurons in the fetal hypothalamus that possess the PGD_2 receptor, it has been shown that PGD_2 is produced by the hypothalamus of fetal sheep from at least 90 days gestation (see Chapter 3). An alternative explanation would be that the reduced PGD_2 production allowed a redirection of arachidonate catabolism to increase PGE_2 production from the common prostanoid precursor PGH_2. In adult rats it has been proposed that PGE_2 can act directly on the posterior hypothalamic neurons to induce wakefulness (Matsumura *et al.* 1988). It should be noted however, that systemic (Kitterman *et al.* 1983) and central (Walker and Pratt 1998) administrations of PGE_2 do not alter fetal sleep states or increase the incidence of arousal behaviour. While treatment of fetal sheep with the PG synthesis inhibitor indomethacin also does not produce increased arousal, it may be that a change in the ***ratio*** of PGD_2 and PGE_2 concentrations in the hypothalamus is critical to determining the propensity for sleep and wakefulness. This

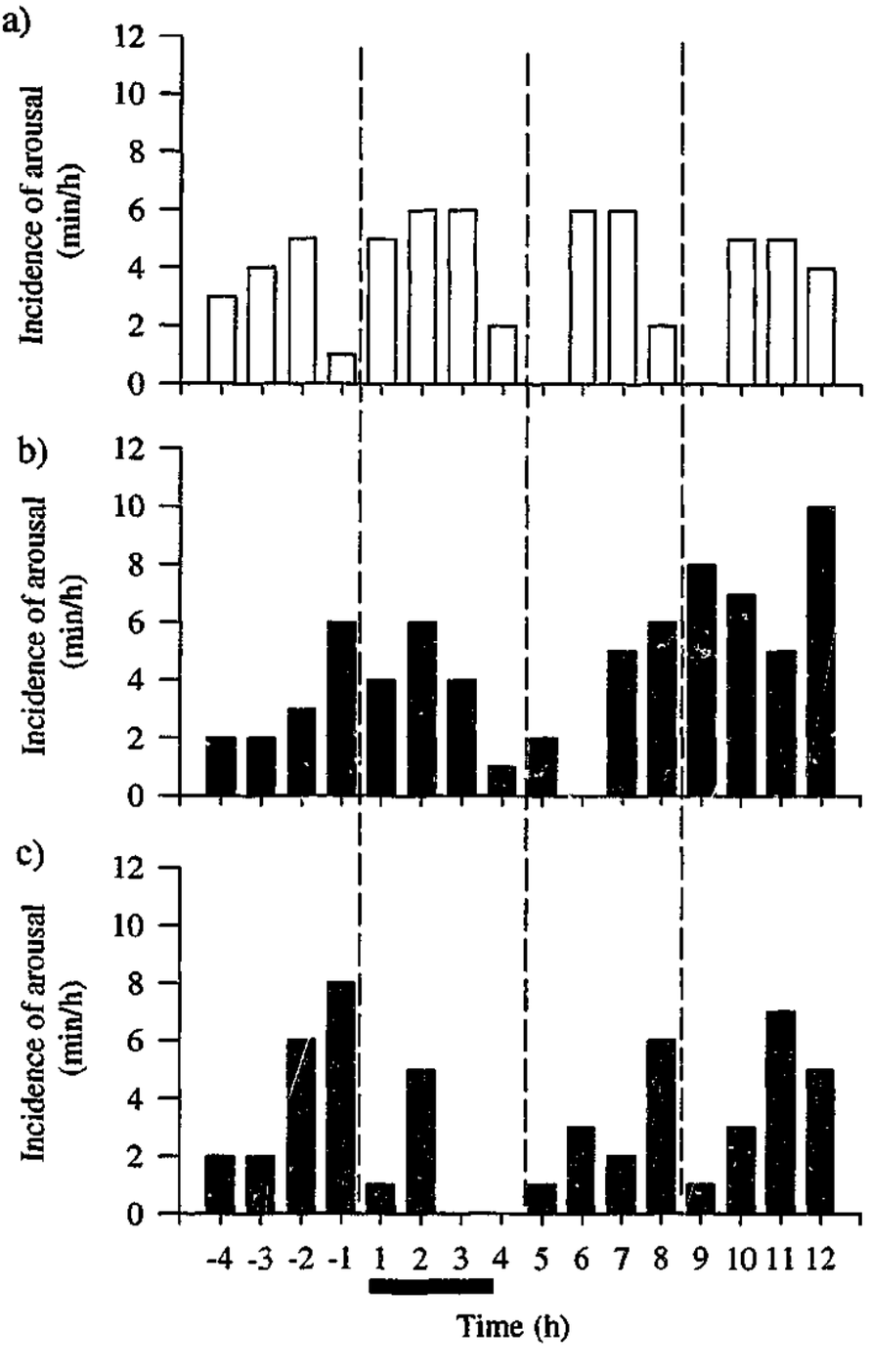


Figure 2.19
Graphs illustrating examples of the cyclical pattern of the incidence of arousal, based on a four hour rhythm, in late gestation ovine fetuses that received 4 hour intracerebroventricular infusions (green bar) of artificial cerebrospinal fluid (yellow), 500 pmole/10 µl/min of $SeCl_4$ (blue) and PGD_2 (red), n=1.

would be consistent with the view that PG mechanisms modulate sleep and wakefulness, which are themselves determined by other mechanism residing in the reticular activating system of the midbrain and brainstem.

Infusions of PGD_2 or $SeCl_4$ did not induce any significant changes in the blood gas status of the fetus (i.e. pH, pCO_2, pO_2, sO_2 and tHb) or in long term changes of blood pressure or heart rate. Consistent with previous studies, transient increases of both blood pressure and heart rate occurred simultaneously with episodes of arousal (Szeto 1983). These results indicate that both treatments did not have any adverse side effects and that the fetus remained in good health throughout the experiment.

Intracerebroventricular infusion of sleep promoting substances has been demonstrated to be a reliable technique to alter the sleep-wake patterns in the adult rat and monkey. The incidence of physiological somnolence has been increased by administering a variety of recognised sleep substances including DSIP, muramyl peptides, PGD_2, uridine and sleep promoting peptide (Inoue *et al.* 1984). This is the first study that has demonstrated the effect of i.c.v infusion of PGD_2 and its enzymatic inhibitor $SeCl_4$, on the sleep-wake status in the fetus. However, whether the observed increase of nuchal EMG activity during LV ECoG represents true arousal in the fetus must also be considered. Consistent with previous studies, transient increases in fetal blood pressure and heart rate were observed to occur simultaneously with the episodes of increased activity identified as arousal (Szeto 1983). Similar cardiovascular and autonomic changes have been observed in the newborn infant on arousal from sleep (Sibley *et al.* 1982). In a recent work from this laboratory, it was shown that evoked somato-sensory responses were increased in amplitude after treating fetal sheep with the 5α-reductase inhibitor finasteride, a treatment that also increased the incidence of fetal arousal (Nicol *et al.* 1999), as defined from the behavioural parameters used in this study. These observations are consistent with the episodes of arousal being periods when there is increased cortical excitability in the fetus. It is also of interest that even after $SeCl_4$ treatment arousal episodes were extended but did not totally obliterate sleep, suggesting that other powerful sleep-promoting mechanisms remain active in the fetal sheep until the time of birth. The results of this study suggest that a PGD_2 mechanism

contributes to the suppression of wakefulness in the fetus, but is not the sole mechanism that maintains the sleep that accounts for most of the behavioural state in fetal life.

Chapter 3

Changes in the specific activity of Prostaglandin D and E Synthase in the ovine hypothalamus

3.1 Introduction

The biosynthesis of the PGD_2 and PGE_2 is clearly defined (see review Smith and Marnett 1991). PGD_2 and PGE_2 are produced from the common precursor arachidonic acid, which is a polyunsaturated fatty acid component of the phospholipid membrane (Katsuki and Okuda 1995). Arachidonic acid is mobilised from the cell membrane or triglycerides and converted to the endoperoxides PGG_2 and PGH_2 by prostaglandin endoperoxide synthase (or cyclooxygenase; E.C. 1.14.99.1). PGD_2 and PGE_2 are subsequently formed by a non-oxidative rearrangement of PGH_2 regulated by enzymes PGDS [E.C.5.3.99.2] and PGES [E.C.5.3.99.3.] respectively.

PGDS (Shimizu *et al.* 1979b; Urade *et al.* 1985a) and PGES (Ogorochi *et al.* 1987) present in the CNS have both been characterised. Brain-type PGDS differs from the PGDS found in other tissues by not having an absolute requirement for glutathione. Glutathione-independent PGDS purified from the rat brain has been reported to have a molecular mass of approximately 26 kDa (Urade *et al.* 1985a). The cDNA of brain-type PGDS have been isolated in the human (Nagata *et al.* 1991) and rat (Urade *et al.* 1989), encoding 190 and 189 amino acid residues respectively, with 71% identity and 83% similarity. Less is known about the properties of PGES present in the CNS; brain-type PGES is generally considered

to be a glutathione-dependent enzyme, although it has been shown to produce PGE_2 without the addition of the cofactor (Watanabe *et al.* 1997). Two isoforms of soluble PGES have been purified from brain tissue, and both have shown to be associated with glutathione S-transferase activity (Ogorochi *et al.* 1987). PGES purified from human brain was associated with the cytosolic fraction and had a molecular weight of 15 to 16 kDa (Jakobsson *et al.* 1999).

PGD_2 and PGE_2 are found in high concentrations in the hypothalamus compared to other regional areas of the brain (Ogorochi *et al.* 1984). This finding suggests that these two PGs have a central role in regulating hypothalamic function. PGE_2 has been implicated in thermo-regulation and the onset of fever (Onoe *et al.* 1992), the release of adrenocorticotrophin hormone (ACTH; Morimoto *et al.* 1989) and in the behavioural state of arousal (Matsumura *et al.* 1989b). In contrast, strong evidence exists substantiating the role for hypothalamic PGD_2 in the maintenance of sleep in adults (Scammell *et al.* 1998). Seasonal variations in levels of PGD_2 in the brain of Asian chipmunk have been observed, where levels increase and decrease significantly in parallel with the hibernation phenomenon (Takahata *et al.* 1996). In addition, marked elevations of endogenous PGD_2 concentrations in CSF occur in patients who suffer African sleeping sickness (Pentreath *et al.* 1990).

It could be argued that these observed increases in PGD_2 are a direct result of a generalised response associated with increased PGD_2 due to inflammation or alterations in thermo-regulation (Forstermann *et al.* 1983). However, empirical evidence specifically links PGD_2 to the physiological sleep-wake rhythms. PGD_2 in the supernatant fraction of rat brain exhibits circadian fluctuations, with significantly higher levels present during sleep periods (Ueno *et al.* 1985a). Furthermore, concentrations of PGD_2 in CSF also exhibit diurnal rhythms in rats (Pandey *et al.* 1995a; Pandey *et al.* 1995b). Recent studies have also shown that continuous micro-infusions of PGD_2 into the third ventricle of conscious adult rats produced a dose-dependent increase in sleep that was indistinguishable from physiological sleep in terms of brain electrocortical activity, electro-ocular activity, muscle tone, body temperature, heart rate and general behaviour (Ueno *et al.* 1983). In contrast, when a

specific reversible inhibitor of PGDS, $SeCl_4$, was infused into the third ventricle of freely moving rats, the amount of time spent asleep was reduced (Matsumura *et al.* 1991). The sleep inducing effect of PGD_2 has been localised to the POAH (Matsumura *et al.* 1994). Conversely, infusion of PGE_2 into the third ventricle of adult sleeping rat has been shown to reduce the time spent in REM/NREM sleep (Matsumura *et al.* 1989b). PGE_2 is believed to act on 'arousal centres' within the posterior hypothalamus (Onoe *et al.* 1992). Therefore, it has been hypothesised that both PGD_2 and PGE_2 play a significant role in the regulation of the normal sleep-wake cycle in the adult by acting on separate sleep- and arousal-promoting centres in the anterior and posterior hypothalamus respectively (Hayaishi 1988).

Electrocortical signs of sleep first appear at approximately 120 days of gestation in fetal sheep, and become fully differentiated into episodes of adult-like REM and NREM sleep by 130 days (term is 147 gestational days; Clewlow *et al.* 1983). Since the cellular mechanisms which govern this predominant sleeping state are unknown, the question therefore arises as to whether the appearance of definitive sleep during fetal development is a result of the maturation of hypothalamic cells which synthesise and release PGD_2 and PGE_2.

Since the previous chapter's results found that $SeCl_4$ changed arousal levels in the fetus, and because PGD_2 and PGE_2 are found in high concentrations in the adult hypothalamus (Section 1.3.2.1), it was decided that an investigation of PGDS and PGES activities in the hypothalamus during fetal development was warranted. The purpose of this study was to use an enzyme assay to describe the specific activities of PGDS and PGES in hypothalamic tissue during ovine fetal development (90, 125 and 135 gestational days) and in the juvenile lamb, and to assess whether the changes in activity correlate with the time that sleep states become evident in the ovine fetus at about late gestation. It was hoped that fetal hypothalamic tissue had the capacity to produce endogenous PGD_2 and PGE_2 from early gestation, which could be potentially used in the maintenance of prenatal sleep. In addition, it was hypothesised that the synthetic rate of the respective enzymes increased in parallel with the ontogenesis of fetal sleep, hence directly reflecting an increase in need and utilisation of the PGs for the establishment of PGD_2 and PGE_2-induced behavioural states.

Basal concentrations of both PGD_2 and PGE_2 in the specific sleep-wake centres of the anterior and posterior hypothalamus respectively were also determined by radioimmunoassay (RIA).

3.2 METHODS

3.2.1 Materials

The following items were obtained from Sigma Chemical Co. (St Louis, MO, USA); citrate (trisodium salt), reduced glutathione, diethyldithiocarbamate (DDC), ethylenediamine tetra-acetic acid (EDTA), leupeptin, arachidonic acid, L-tryptophan, indomethacin, phenylmethylsulpholfluoride (PMSF), methoxyamine HCl and the protein assay kit. PGD_2 and PGE_2 were bought from Cayman Chemical Co. (Ann Arbor, MI., USA) [5,6,8,9,12,14,15-^{3}H(N)] PGD_2 (168 Ci/mmole) and [5,6,8,11,12,14,15-^{3}H(N)] PGE_2 (200 Ci/mmole) were obtained from Amrad Pharmacia Biotech (Melbourne, Australia).

3.2.2 Tissue Collection

Merino-Border Leicester cross bred ewes and young (juvenile) sheep used in all procedures were in accordance with the Code of Practice for the core use of animals for scientific purposes and approved by the Standing Committee on Ethics in Animal Experimentation of Monash University, Australia. The pregnant ewe or young sheep were humanely killed by sodium pentobartitone overdose (130 mg/kg i.v.) at fetal ages of 90, 125 and 135 days gestation, and at 8 weeks of age.

3.2.2.1 Tissue Collected for Enzyme Activity

The cortex and spinal cord were exposed by carefully opening the cranium and the first 2 - 4 cervical vertebrae above the spinal column. After the severing the pituitary stalk and the cranial nerves with fine scissors, the spinal cord was cut caudal to the medulla, the entire brain and upper spinal cord was removed immediately. The hypothalamic tissue was blocked by making two transverse cuts; the first was made caudal to the mammillary bodies which separated the hypothalamus from the midbrain, and the second was made approximately 4 mm rostral to the optic chiasm, which included the relevant parts of the BF. Two longitudinal cuts were then made approximately 1 cm either side of the midline, and the block was undercut at a depth of approximately 1 cm. Surrounding blood vessels and leptomeningeal layers were removed. The tissue was dissected and blocked within 5 minutes of the death of the animal and immediately frozen in liquid nitrogen and stored at -80°C until the enzyme activity was measured.

3.2.2.2 Tissue Collected for Prostaglandin D_2 and E_2 Concentration Studies

The brain tissue collected for the analysis of PGD_2 and PGE_2 concentration was pretreated by transcardiac perfusion of the fetus with 2 L of indomethacin (1 mg/ml in saline, 5% ethanol) to remove plasma and prevent the post-mortem accumulation of PGs (Narumiya *et al.* 1982). Upon exposure of the beating heart, the perfusion cannula was inserted into the base of the left ventricle and secured in the carotid artery with a tie. The descending aorta was occluded with forceps and the right atrium was cut open to allow unobstructed outflow of the perfusate and direct flow of the indomethacin into the circulation of the brain. The tissue was considered completely perfused when the indomethacin solution, which had passed through the circulation, was clear and free of residual blood. The hypothalamus was removed as described above, and then cut into an anterior and posterior section by a transverse cut placed just caudal to the tuber cinereum. A sample of the parietal cortex was

also taken from the pre- and post-central sulcal lobe. The blocks of tissue were immediately frozen in liquid nitrogen and kept at -80°C until further processing on the same day.

3.2.3 Preparation of Homogenates for the Assay of Enzyme Activity

The entire hypothalamic block was required in the enzyme activity assay to provide sufficient tissue needed for the incubation procedure. Each individual tissue block taken from each animal was prepared and analysed separately, the data were subsequently pooled after the enzyme activity for each tissue block was determined to calculate a mean±sem value for each group.

Table 3.1
Number of animals from which tissue was obtained to analyse the enzyme activity of PGDS and PGES in hypothalamic homogenates over development.

	Enzyme Activity Assay	
	Animal Numbers	
Age Groups	*PGDS*	*PGES*
90 days gestation fetus	4	5
125 days gestation fetus	5	5
135 days gestation fetus	5	5
8 week lamb	4	4

The frozen tissue was pulverised with a pre-chilled air gun on dry ice. The powdered tissue was collected and weighed. Tissue samples were added to a buffer (50 mM Tris-HCl, pH 8.0) containing 2 mM EDTA, 2.4 mM tryptophan, 0.25 M sucrose, 1 mM DDC, and protease inhibitors leupeptin (2 µg/ml) and 1 mM PMSF. The tissue slurry was then homogenised with an Ultra-Turrax homogeniser (Jenke & Henkell, IKA, Labortechnik, Germany) on the maximum setting for 3 x 30 seconds bursts, with a 15 seconds interval between each burst. The samples were kept on ice at all times to prevent tissue degradation. After the homogenate was centrifuged for 15 minutes at 2,000 *g* and 4°C, the supernatant

was removed immediately and maintained at 4°C to avoid loss of enzymatic activity. A 40 µl aliquot was removed for total protein content determination using the Lowry method, as adapted by Sigma Chemical Co.

3.2.4 Enzyme Activity Assay

The enzyme activity assay essentially followed the protocol developed by Smieja (1993) and was performed immediately after the homogenate had been prepared. The reaction was performed in 12 x 75 mm borosilicate glass tubes. Each tube contained 50 µl incubation buffer (50 mM Tris-HCl, 2 mM EDTA, pH 8.0) with 90 µM arachidonic acid substrate, and 175 µl of each of the cofactors tryptophan (8.5 mM) and reduced glutathione (4.2 mM), at 37°C. The reaction was started by adding 50 µl of the brain supernatant, so that the total reaction volume was 275 µl. The reaction was stopped after five minutes with the addition of 4 ml of chilled sodium citrate (50 mM, pH 3.0) containing 15% (v/v) ethanol and placed onto ice.

All assay runs included a reagent blank where 50 µl of reagent blank buffer (50 mM Tris-HCl, 2 mM EDTA, 2.4 mM tryptophan, pH 8.0) was added rather than the supernatant. The reagent blank value was subtracted from the enzyme activity in final calculations. Zero controls in which the supernatant was added after the stop solution (sodium citrate/ethanol) were also performed for each tested homogenate. All reaction points were performed in triplicate with two dilutions of supernatant. Appropriate concentrations of arachidonate and glutathione were prepared freshly from stock solutions each day using incubation buffer.

3.2.5 Extraction by Sep Pak Cartridges

PGD_2 and PGE_2 that were synthesised in the enzyme incubation assay were extracted from the assay solution using Sep Pak C_{18} cartridges (Waters, Milford, MA, USA) as previously described (Powell 1980), where acidified samples were passed through the column of

octadecylsilyl silica, which is recognised to retain oxygenated metabolites of arachidonic acid.

For rapid extraction of PGs from the incubation medium the samples were eluted under vacuum. Clean syringe barrels were attached to the cartridges that provided a vesicle reservoir for the addition of sample and wash solutions. The cartridges were secured to a plastic manifold that was sealed onto a glass chamber and connected to a vacuum. Under vacuum, the negative pressure generated within the chamber increased the flow of assay solution and eluents through the cartridge. The cartridges were preconditioned with 4 ml of 95% ethanol followed by 4 ml of dd-H_2O; 100 µl of [^{3}H]PGD_2 or [^{3}H]PGE_2 (10,000 cpm/ml dd-H_2O) was added to the 4 ml volume of each sample before it was passed through the cartridge. The cartridges were subsequently washed with 2 ml of citrate ethanol to elute phospholipids, proteins and very polar materials and then washed with 2 ml of dd-H_2O. Non-polar lipids and monohydroxyl fatty acids were eluted with 3 ml of petroleum ether. The retained PGs were finally eluted from the column with 3 ml of ethyl acetate. All eluent washes were discarded except the ethyl acetate fraction, which was kept and evaporated under vacuum. When completely evaporated the residue was reconstituted with 250 µl of 0.1 M phosphate buffer solution (PBS, pH 7.4) and 250 µl of methoximating reagent (1% methoxyamine in 10% ethanol, and 0.1 mM sodium acetate, pH 5.6) and incubated overnight at room temperature. From the 500 µl methoximated sample, 2 x 200 µl was taken and analysed by RIA for each of PGD_2 and PGE_2 concentration, as described below.

The remaining 100 µl of sample was added to 1 ml of EcoScint (National Diagnostics, USA) and counted using a β-counter (Beckmann Coulter, USA) to determine the recoveries of each sample. The recovery of the PGD_2 and PGE_2 were monitored for each extraction and the appropriate correction was made for any loss calculated. The average recovery for all extractions was 95.8 ± 0.46%.

3.2.6 Prostaglandin D_2 and E_2 Concentration Studies

PGD_2 and PGE_2 concentration was determined in each block of tissue (anterior and posterior hypothalamus and cortex), obtained from each animal. After the content of each block of tissue was established the data were pooled and statistical analysis performed as described below.

Table 3.2
Number of animals from which tissue was obtained to analyse the PGD_2 and PGE_2 concentration of the anterior and posterior hypothalamus and cortex during development.

PGD_2 and PGE_2 Concentration Analysis

Age Group	*Animal Numbers*
90 days gestation fetus	4
125 days gestation fetus	4
135 days gestation fetus	6
8 week lamb	4

Each block of tissue was weighed and homogenised separately on ice in pre-cooled ethanol (5 ml/g) with an Ultra-Turrax homogeniser. The tissue was homogenised for 6 x 15 seconds on the maximum setting, with 1 minute cooling intervals in between, and then subsequently centrifuged at 2,000 *g*, 4°C for 15 minutes (Narumiya *et al.* 1982). The supernatant was removed and mixed with an equivalent volume of methoximating reagent and left overnight at room temperature.

The samples were acidified to pH 5 using HCl and the PGD_2 and PGE_2 were extracted using Sep Pak C_{18} cartridges as previously described (Powell 1980). The average recovery for all extractions was 60.37 ± 1.7%; and the appropriate correction was made for each individual extraction. The final ethyl acetate elution fraction was completely evaporated under vacuum and reconstituted in 500 µl of 0.1 M PBS, pH 7.0, and then analysed by RIA.

3.2.7 Prostaglandin D_2 and E_2 Radioimmunoassays

PGD_2 and PGE_2 methyloxime derivatives were measured using antibodies raised against PGD_2 and PGE_2 as their methyloximes by direct RIA procedure, previously described by Fowden (1987) and Kelly (1986). Since PGD_2 and PGE_2 are considerably labile, conversion of PGs to their methyloximes allows easy and reliable analysis without decomposition during storage (Kelly *et al.* 1986).

3.2.7.1 Methoximation of Trace and Standards

Labelled and unlabelled PGD_2 and PGE_2 were methoximated by incubation with methoxyamine HCl (final concentration 0.12 mM in 1 nM sodium acetate buffer, pH 5.6) at room temperature overnight. The methoximated samples of labelled and unlabelled PGD_2 and PGE_2 were extracted with ether:ethyl acetate (3:1, v/v) and subsequently evaporated with air to dryness. The residue was reconstituted in absolute ethanol and stored at -20°C until required.

3.2.7.2 Methoximated Prostaglandin D_2 and E_2 Antibodies

The PGD_2 methyloxime antiserum, raised in New Zealand White rabbits against the methyloxime of PGD_2 conjugated with Freud's complete adjuvant, was a kind gift from Dr. R.W. Kelly (MRC Reproductive Biology Unit, Edinburgh, UK). The cross-reactivity of the PGD_2 methyloxime antiserum with other various methyloximated compounds was previously established (Kelly *et al.* 1986) and is summarised (Table 3.3). The PGD_2 methyloxime antiserum exhibited invariably low cross-reactivity with closely related methyloxime compounds; the cross-reactivity of PGD_2 methyloxime antiserum with PGE_2 methyloxime was <0.01% (Kelly *et al.* 1986).

The PGE_2 methyloxime antiserum raised in goats against the methyloxime of PGE_2 conjugated to bovine serum albumin (BSA) was generously supplied by Dr. R.I. Cox

Table 3.3
Percent cross-reactivities of the PGD_2 methyloxime antiserum with closely related compounds (from Kelly *et al.* 1986).

PGD_2 Methyloxime Antiserum Cross-Reactivities

Methyloximated Compound	*%*
Thromboxane B_2	7.0
PGA_1	<0.01
PGB_2	<0.01
PGD_2	100
13, 14, dihydro-15-oxo $PGF_{2\alpha}$	<0.01
15-oxo $PGF_{2\alpha}$	<0.01
$PGF_{3\alpha}$	<0.02
$PGF_{2\alpha}$	<0.02
$PGF_{1\alpha}$	<0.02
16, 15-dioxo-13, 14 dihydro $PGF_{1\alpha}$	<0.01
16, 15-dioxo $PGF_{1\alpha}$	<0.01
6-oxo $PGF_{1\alpha}$	<0.01
6-oxo PGE_1	<0.01
13, 14 dihydro-15-oxo PGE_2	<0.01
15-oxo PGE_2	<0.01
20 methyl PGE_2	<0.01
20-OH PGE_2	<0.01
19-OH PGE_2	<0.01
19-OH PGE_1	<0.01
8 iso PGE_2	<0.01
PGE_3	<0.63
PGE_2	<0.01
PGE_1	<0.01
13,14 dihydro $PGF_{2\alpha}$	<0.01
13, 14 dihydro $PGF_{1\alpha}$	<0.01

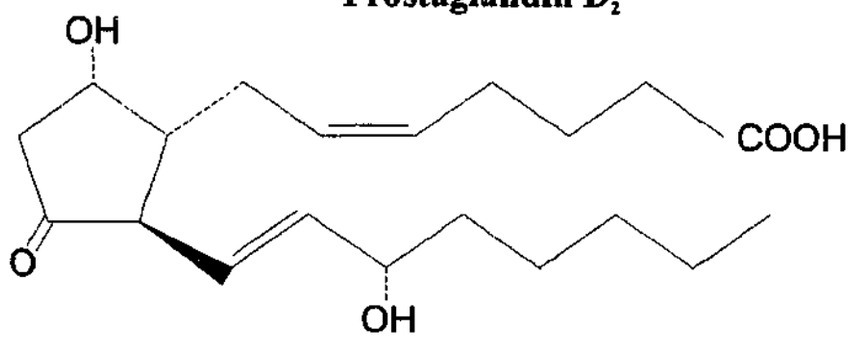
(CSIRO, Blacktown, NSW, Australia). The cross-reactivity of the PGE_2 methyloxime antiserum with a variety of methyloximated compounds previously investigated (Fowden et al. 1987) is summarised below (Table 3.4). It was found that the antiserum exhibited consistently low cross-reactivity with closely related methyloxime compounds except PGE_1, which showed a cross-reactivity of 270%. PGE_2 and PGE_1 are structurally identical except that the double bond is absent on the carboxyl carbon chain in PGE_1 (Fig. 3.1). As far as we are aware there is no literature available regarding the distribution of basal levels of PGE_1 in the brain, although the precursor of PGE_1, eicosatrienonate is considered to be less abundant compared to the PGE_2 precursor arachidonate (Lands 1989). More importantly, the cross-reactivity of PGE_2 methyloxime antiserum with PGD_2 methyloxime was <0.1% (Fowden et al. 1987).

3.2.7.3 Radioimmunoassay Procedure

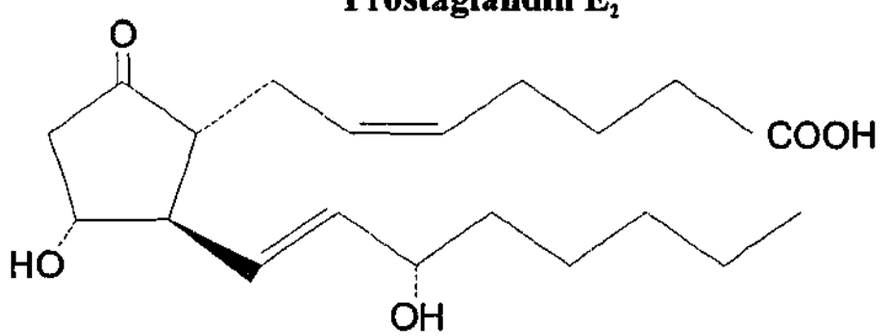
Both PGD_2 and PGE_2 assays followed the same direct RIA protocol. The optimal dilution of PGE_2 antisera was previously validated in this Department, and the optimal dilution of PGD_2 antiserum was established prior to utilisation (see Results, Section 3.3). The labelled PGs were diluted in 0.1 M PBS (pH 7.4) to a final concentration of 5,000 cpm/100 µl. Appropriate dilutions of the methyloxime standards in ethanol were aliquoted to give a standard curve ranging from 0.05 to 20.0 pmole/ml. B_{max} tubes devoid of any standards and non-specific binding tubes containing excess standards were also included. After extraction, 100 µl of each sample was assayed in duplicate by the following procedure.

The standards and samples were incubated with the methyloximated [^{3}H]PGD_2 or [^{3}H]PGE_2 (100 µl) and respective antisera (100 µl) overnight at 4°C. The free and antibody-bound substrate were separated by precipitation of bound substrate with 50 µl of bovine-γ-globulin (1 mg; Calbiochem, La Jolle, CA, USA) and 1.0 ml of 22% polyethylene glycol 6000 (BDH, Poole, England). Following centrifugation at 4,000 *g* for 15 minutes at 4°C,

Prostaglandin D_2



Prostaglandin E_2



Prostaglandin E_1

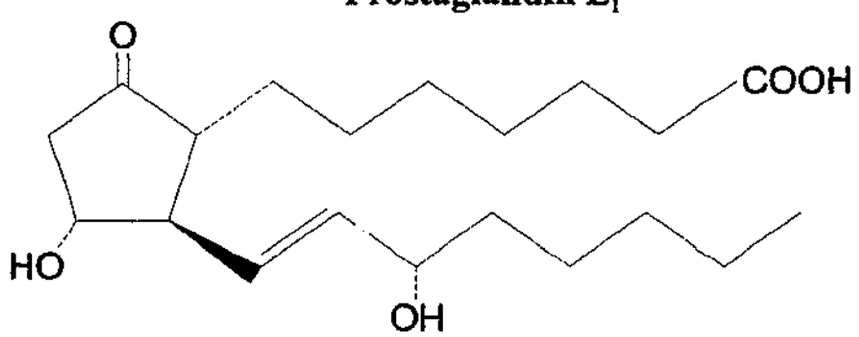


Figure 3.1
Chemical structures of prostaglandin D_2 (above), prostaglandin E_2 (middle) and

Table 3.4
Percent cross-reactivities of the PGE_2 methyloxime antiserum with closely related compounds (from Fowden *et al.* 1987).

PGE_2 Methyloxime Antiserum Cross-Reactivities

Methyloximated Compound	*%*
$PGF_{2\alpha}$	<0.10
15-keto-$PGF_{2\alpha}$	<0.10
13,14-dihydro-$PGF_{2\alpha}$	<0.10
13,14-dihydro-15-keto-$PGF_{2\alpha}$	<0.10
$PGF_{2\beta}$	<0.10
6-keto- $PGF_{1\alpha}$	<0.10
PGE_1	270
PGE_2	100
PGD_1	<0.10
PGD_2	<0.10
TXB_2	<0.10
15-keto-PGE_2	0.30
PGB_2	<0.10

the supernatant was aspirated and discarded. After the remaining pellet was carefully resuspended with 50 µl of ethanol, 1 ml of EcoScint was added to all tubes plus four total count (5,000 cpm of labelled ligand) and sonicated for 30 minutes and then counted on the scintillation counter.

The intra- and inter-assay co-efficients of variation of the PGE_2 assay were 17.1% and 17.8% respectively, and for the PGD_2 assay they were 23.4% and 9.2% respectively.

3.2.8 Statistical Analysis

All data are presented as the mean±sem. One way analysis of variance was used to compare the specific enzyme activity or PG content between the age groups. When significant effects were found between groups, the Student-Newman-Keuls test was used to determine significant differences between each group. $P<0.05$ was considered to be statistically significant.

3.3 RESULTS

3.3.1 Validation of Prostaglandin D_2 Radioimmunoassay

Serial dilutions of the PGD_2 methyloximated antiserum (1:750 to 1:3 x 10^6) were incubated with a fixed amount of methyloximated [^{3}H]PGD_2 (5,000 cpm). Optimum amount of the antisera was estimated to be 1:7,500 dilution (Fig 3.2), which corresponded to the concentration sufficient to bind to ~50% of the tracer ligand. This concentration of antiserum was sensitive enough to allow significant shifts in the bound and free fractions to occur upon addition of unlabelled ligand.

Varying concentrations of methyloximated PGD_2 samples (500 fmole to 2 pmole/assay tube) were incubated in the fixed amount of tracer (5,000 cpm) and the optimum dilution of antiserum (1:7,500) to establish a standard curve of sigmoidal shape (Fig. 3.3a). Standard curves were also performed in higher dilutions of antiserum (Table 3.5; 1:10,000 and 1:20,000). While higher dilutions exhibited low B_{max} values and were more sensitive, intra-assay variation would increase, therefore 1:7,500 diltuion was considered most suitable. In comparison, higher concentrations of antiserum would decrease the variability of the assay but at the same time reduce the sensitivity of the system, and these were therefore not used.

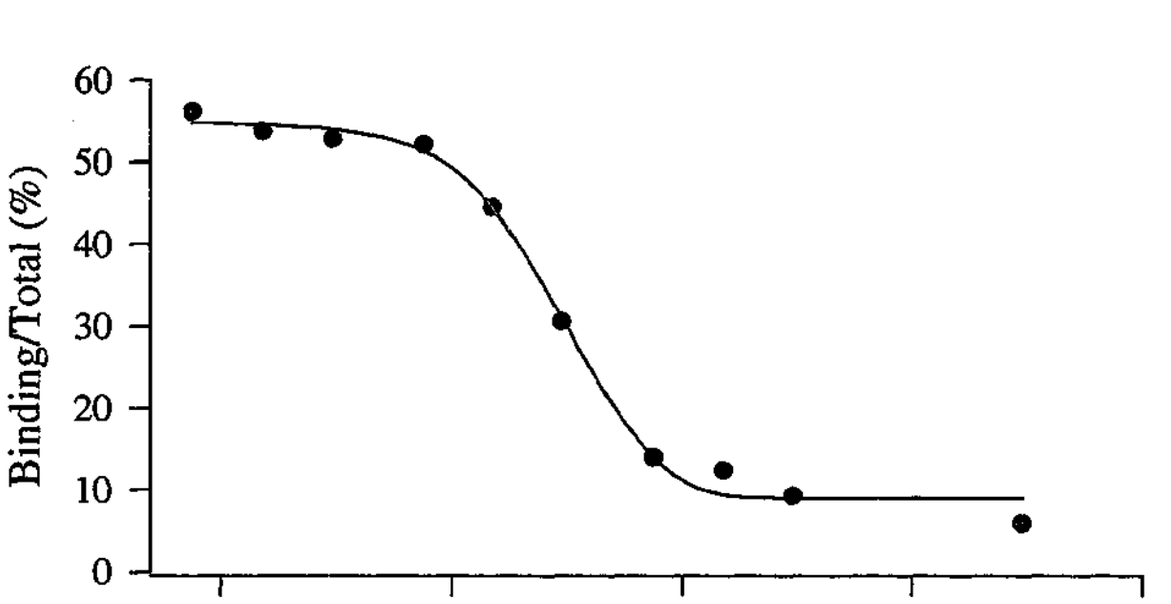


Figure 3.2
Antibody dilution curve, showing serial dilutions of the antiserum incubated with fixed amount of [^{3}H]PGD_2 methyloxime. The percentage of tracer ligand bound to the antiserum decreases with the increase in antiserum dilution used. The appropriate dilution of antiserum used in the radioimmunoassay (1:7,500) corresponds to the concentration that binds to 50% of the tracer.

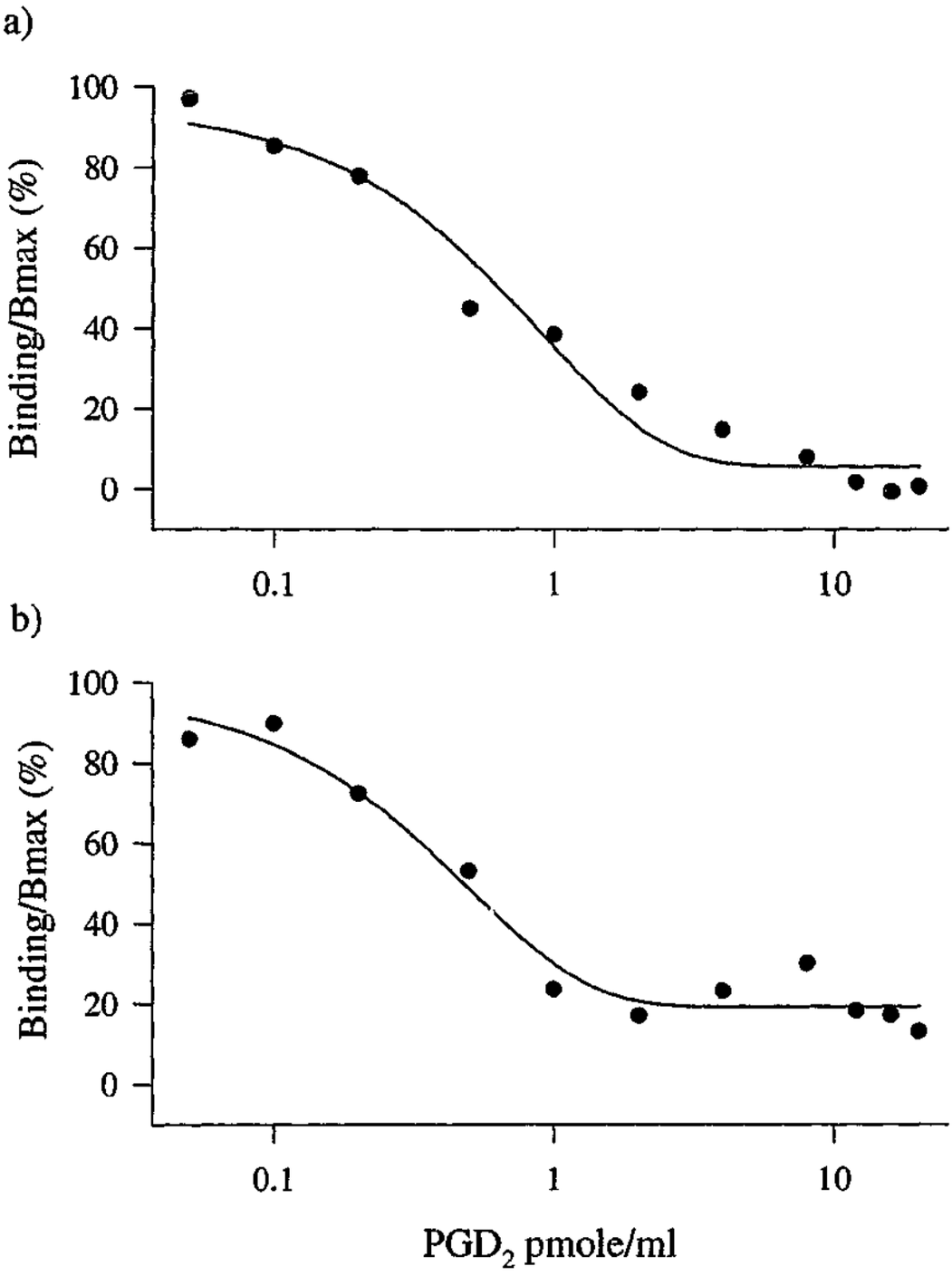


Figure 3.3
Fixed amount of [^{3}H]PGD_2 methyloxime (5,000 cpm/100ml) and PGD_2 methyloxime antiserum (a; 1:7,500, b; 1:10,000) incubated with increasing concentrations of unlabelled PGD_2 methyloxime standards (0.05 to 20.0 pmole/ml). The percentage of the tracer bound to antiserum is progressively reduced with increasing concentrations of the unlabelled ligand, showing simple, first order competition for binding to the antibody.

Table 3.5
Exhibited B_{max} values decreased upon incubation of set amount of methyloximated [^{3}H]PGD_2 (5,000 cpm) and increasing dilutions of methyloximated PGD_2 antiserum (1:7,500, 1:10,000 and 1:20,000). Subsequent tests were performed using antiserum dilution of 1:7,500, which achieved sensitivity with low variability.

Antiserum Dilution	1:7,500	1:10,000	1:20,000
B_{max}	30.43%	28.02%	16.56%

Therefore, using the optimal 1:7,500 dilution of antiserum, optimal assay conditions were established. It was found that maximal binding of antibody to occurred after incubating the antiserum, labelled and unlabelled ligand for 24 hours at 4°C (Fig 3.4a). Optimal concentrations of ligand fraction binding to antiserum were precipitated with 22% polyethylene glycol (Fig. 3.4b) and 20 mg of bovine γ globulin (Fig. 3.4c).

3.3.2 Homogenate Preparation

In initial experiments the brain tissue was homogenised and the supernatant from the 2,000 *g* centrifugation was separated into cytosolic and microsomal fractions by a subsequent 100,000 *g* centrifugation procedure. The microsomal pellet exhibited approximately 2.5 times more PGES activity than the cytosol in the presence of 80 μM arachidonic acid. Both the microsomal pellet and cytosolic fraction contained approximately the same PGDS activity (Table 3.6). Thus, in order to measure the activities of both enzymes in the same sample, the tissue was homogenised and the supernatant from the 2,000 *g* centrifugation was used in the subsequent PGDS and PGES activity determinations.

Figure 3.4

(a) Changes in B_{max} value (red) with increasing incubation time at 4°C. Using fixed amount of [^{3}H]PGD_2 methyloxime (5,000 cpm/100 ml) and PGD_2 methyloxime antibody (1:7,500), maximal binding occurred after 24 hours incubation and the subsequently decreased. Non-specific binding (blue) did not change significantly with incubation time.

(b) Changes in B_{max} value (red) with increasing concentration of polyethylene glycol. Using fixed amount of [^{3}H]PGD_2 methyloxime (5,000 cpm/100 ml) and PGD_2 methyloxime antibody (1:7,500), maximal binding occurred using concentration above 20%. Non-specific binding (blue) did not change significantly with increasing concentration.

(c) Changes in B_{max} value (red) with increase in concentration of bovine γ globulin. Using fixed amount of [^{3}H]PGD_2 methyloxime (5,000 cpm/100 ml) and PGD_2 methyloxime antibody (1:7,500), maximal binding using concentrations above 20 mg/ml. Non-specific binding (blue) did not change significantly with bovine γ globulin concentration.

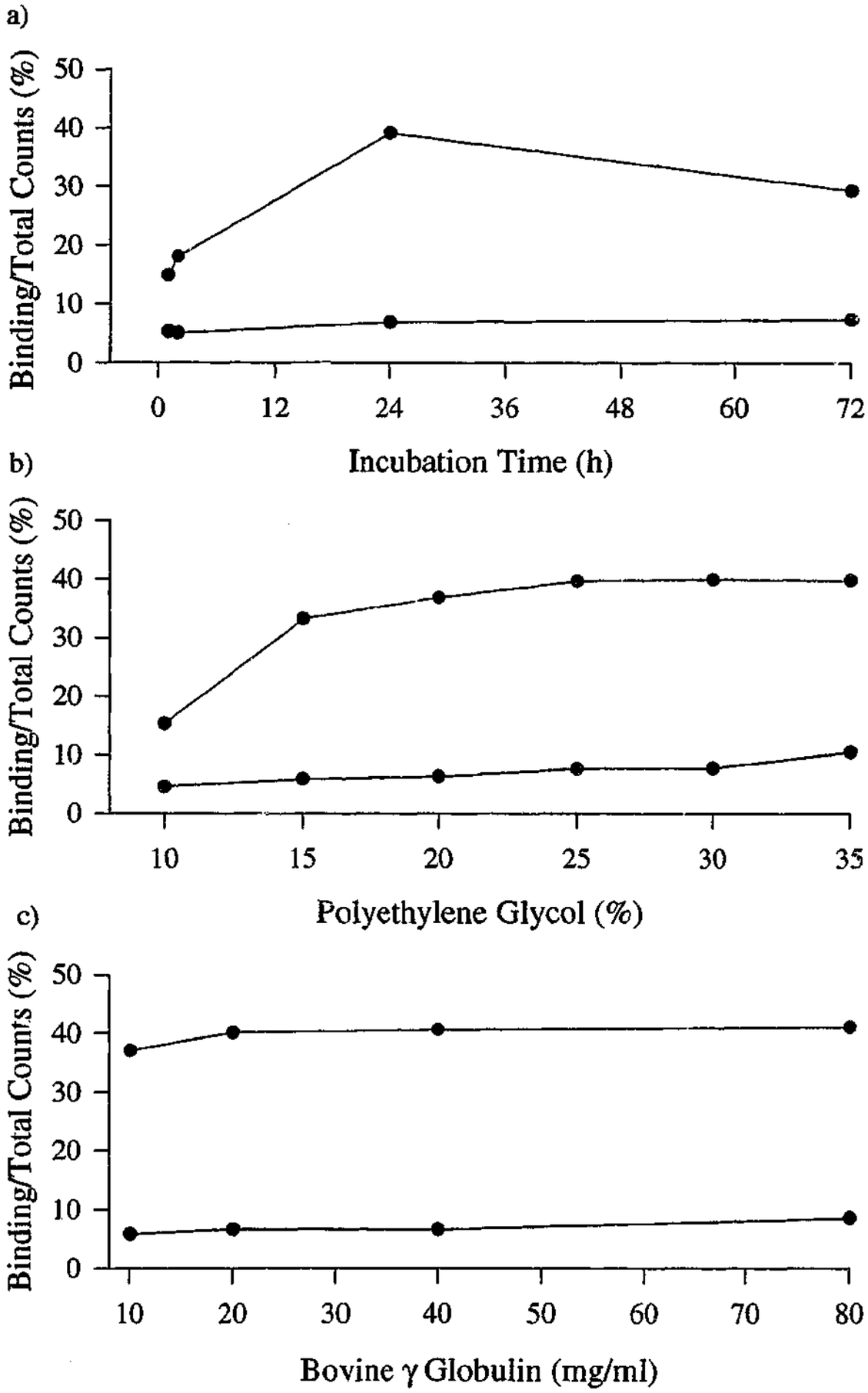

a)
Binding/Total Counts (%)
50
40
30
20
10
0
0
12
24
36
48
60
72
Incubation Time (h)
b)
Binding/Total Counts (%)
50
40
30
20
10
0
10
15
20
25
30
35
Polyethylene Glycol (%)
c)
Binding/Total Counts (%)
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Bovine γ Globulin (mg/ml)

Table 3.6
PGDS and PGES activity in the fetal ovine hypothalamic cytosolic and microsomal fractions after 100,000 g centrifugation, incubated in 0 - 80 μM of exogenous arachidonic acid, 4.3 mM reduced glutathione, 8.5 mM tryptophan, pH 8, at 37°C for 5 minutes. The microsomal fraction exhibited greater PGES activity, whereas no difference in PGDS activity was observed. Data represent the mean of triplicate samples and their standard deviation (SD).

	PGE_2 (pmole/tube)		*PGD_2 (pmole/tube)*	
Arachidonic Acid (μM)	*Cytosol*	*Microsome*	*Cytosol*	*Microsome*
0	0.000	0.000	0.000	0.000
20	1.104±0.037	1.083±0.104	0.385±016	0.1.33±0.05
40	1.857±0.83	2.293±0.709	0.848±0.72	0.854±0.45
80	1.653±0.18	4.205±0.802	0.948±0.32	0.880±0.72

3.3.3 Optimisation of Prostaglandin D and E Synthase Assay Conditions

3.3.3.1 pH, Temperature, Cofactor Concentrations and Incubation Time Variables

Preliminary studies were performed to determine optimal conditions required for analysing the specific enzyme activity of PGDS and PGES using hypothalamic homogenates prepared from 135 day gestation fetuses with 20 μM of exogenous arachidonic acid. The pH dependence of the enzymes was examined over the range 6.0 - 9.0, where pH 8.0 yielded optimal activity (Table 3.7). Optimum temperature for incubation was examined over a range of 22 - 45°C; as expected the specific activities of PGDS and PGES was maximal at 37°C (Table 3.8). The cofactors glutathione and tryptophan were added to the incubation media following a previously published protocol (Smieja *et al.* 1993). Enzyme incubations performed without the addition of these cofactors lead to an overall reduction in both PGDS and PGES activities by approximately four-fold (Table 3.9). Therefore, the cofactor concentrations described above were used in all subsequent incubations.

Table 3.7
PGDS and PGES enzyme activity dependence on the pH of incubation buffer, in ovine hypothalamic homogenates at 135 days gestation, in the presence of 20 μM of exogenous arachidonic acid, 4.2 mM reduced glutathione and 8.5 mM tryptophan, at 37°C. Conditions at pH 8 increased PGD_2 and PGE_2 production above pH 7 and pH 9. Data represent two experimental trials performed in triplicate, and the mean of the two experiments.

	Experiment	*pH 7*	*pH 8*	*pH 9*
PGD_2	*1*	0.028	0.061	0.040
(fmole/μg prot/min)	*2*	0.032	0.066	0.054
	Mean	*0.030*	*0.064*	*0.047*
PGE_2	*1*	0.510	0.730	0.502
(fmole/μg prot/min)	*2*	0.602	0.982	0.368
	Mean	*0.556*	*0.856*	*0.435*

Table 3.8
PGDS and PGES enzyme activity dependence on incubation temperature in ovine hypothalamic homogenates at 135 days gestation, in the presence of 20 μM of exogenous arachidonic acid, 4.2 mM reduced glutathione and 8.2 mM tryptophan at pH 8. Temperatures at 37°C increased PGD_2 and PGE_2 production above 22°C and 40°C. Data represent two experimental trials performed in triplicate, and the mean of the two experiments.

	Experiment	*22°C*	*37°C*	*40°C*
PGD_2	*1*	0.101	0.190	0.121
(fmole/μg prot/min)	*2*	0.140	0.248	0.160
	Mean	*0.121*	*0.219*	*0.141*
PGE_2	*1*	0.664	1.006	1.040
(fmole/μg prot/min)	*2*	0.723	1.434	1.240
	Mean	*0.694*	*1.220*	*1.140*

Table 3.9
PGDS and PGES enzyme activity dependence on the addition of exogenous cofactors; 4.2 mM reduced glutathione (Glu), 8.2 mM tryptophan (Tryp), and reduced glutathione and tryptophan combined (Glu+Tryp), in ovine hypothalamic homogenates at 135 days gestation, in the presence of 20 μM of exogenous arachidonic acid, pH 8 at 37°C. Addition of combined Glu + Tryp treatment increased PGD_2 and PGE_2 production above control homogenates that was without cofactor treatment (blank) and isolated Glu and Tryp treatment. Data represent two experimental trials performed in triplicate, and the mean of the two experiments.

	Experiment	*Blank*	*Glu*	*Tryp*	*Glu+Tryp*
PGD_2	*1*	0.068	0.100	0.096	0.1904
(fmole/μg prot/min)	*2*	0.056	0.098	0.120	0.2484
	Mean	*0.062*	*0.099*	*0.108*	*0.2194*
PGE_2	*1*	0.300	0.600	0.800	1.006
(fmole/μg prot/min)	*2*	0.480	0.880	1.160	1.434
	Mean	*0.390*	*0.740*	*0.980*	*1.220*

Using the determined optimal pH, temperature and cofactor conditions, the time dependence of PGDS and PGES activities was examined at a substrate concentration of 20 μM arachidonic acid. Maximum production of both PGD_2 and PGE_2 had occurred at 5 minutes and declined by 10 minutes (Fig. 3.5a). Interestingly, PGDS activity declined after 7.5 minutes falling by 50%, compared to PGES activity that reached a plateau. Thus, incubation time of 5 minutes was used in following experiments. This time period was on the upward slope of the curve, but also provided the maximum time for PG production and hence minimum level of variability between incubates.

3.3.3.2 Substrate Concentration

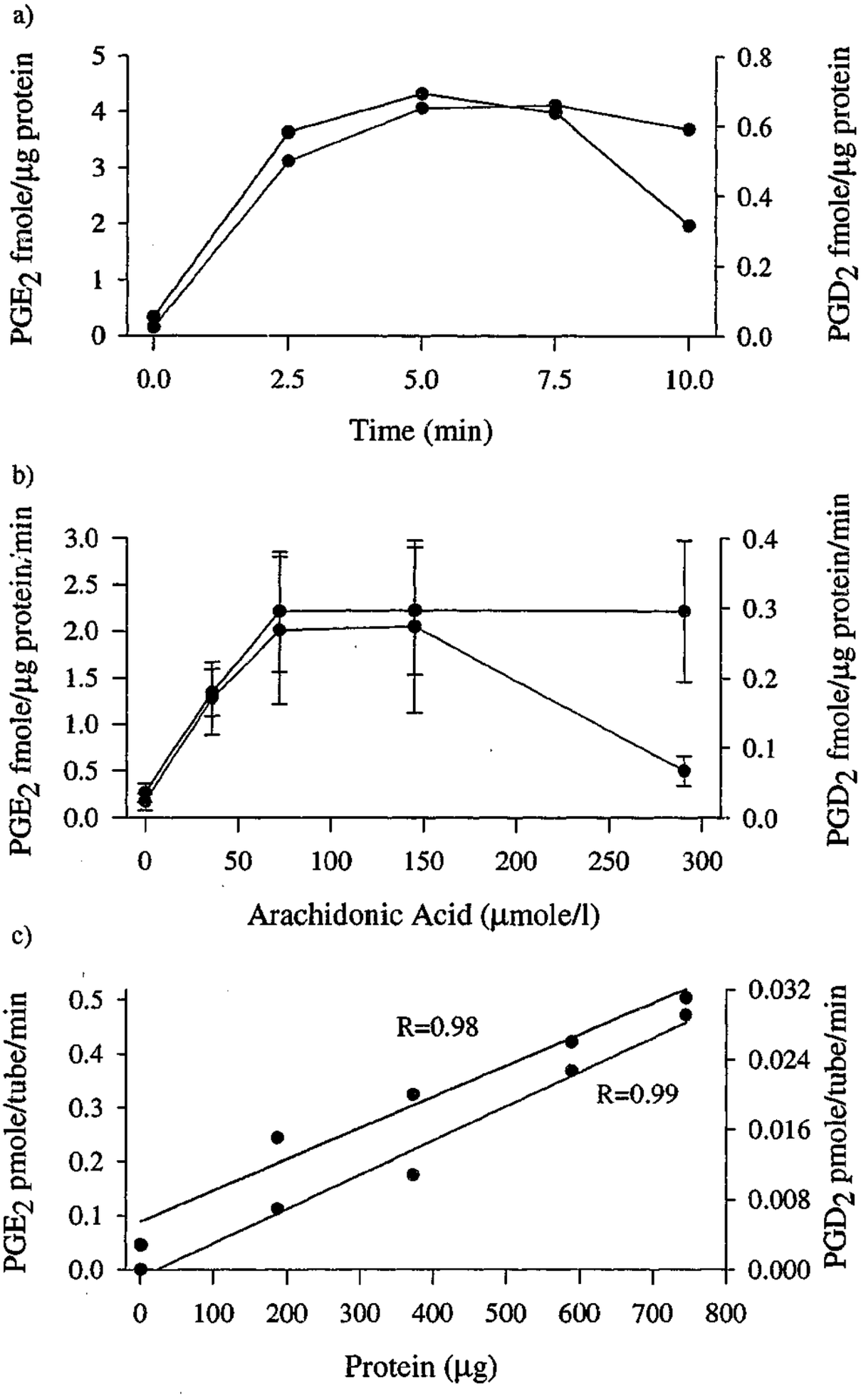
The substrate dependence of PGDS and PGES activity was tested over the range 0 - 300 μM arachidonic acid; the effect of substrate concentrations on PGD_2 and PGE_2 production was similar for all fetal and postnatal samples. PGDS and PGES activities increased with the additional levels of substrate for concentration up to 80 μM of arachidonic acid (Fig.

Figure 3.5

(a) Time dependence of PGDS (red) and PGES (blue) activity. Hypothalamic homogenates were incubated with 20 μM arachidonic acid from 0 to 10 minutes at 37°C. The data represent the mean of triplicate incubation from a fetus at 135 days gestation. The rate of PGDS and PGES increased until about 5 and 7.5 minutes respectively, then reached a plateau or decline for PGES and PGDS respectively.

(b) Arachidonic acid dependence of hypothalamic homogenates PGDS (red) and PGES (blue) specific activity. Hypothalamic homogenates were incubated in assay buffer with increasing concentrations of arachidonic acid (0 to 300 μM). Results shown are a combination of four hypothalamic samples, one tissue sample collected from each age group (90, 125 and 135 days gestation and 8 postnatal weeks) i.e. n = 4. The data represent the mean±sem.

(c) Protein concentration-dependence of PGDS (red; regression co-efficient = 0.99) and PGES (blue regression co-efficient = 0.98) activity. Hypothalamic homogenate were incubated in assay buffer with 90 μM arachidonic acid with increasing concentrations (0 to 744 μg) of protein. Production of PGD_2 and PGE_2 increased linearly with increasing amounts of protein added to the incubation. Data represent mean of triplicate incubation from a fetus at 135 days gestation.


a)
PGE2 fmole/µg protein
PGD2 fmole/µg protein
Time (min)
b)
PGE2 fmole/µg protein/min
PGD2 fmole/µg protein/min
Arachidonic Acid (µmole/l)
c)
PGE2 pmole/tube/min
PGD2 pmole/tube/min
R=0.98
R=0.99
Protein (µg)

3.5b). PGDS activity decreased at levels higher than 150 mM of substrate, whereas PGES activity remained relatively constant at these high substrate concentrations. The activity of PGES and PGDS was approximately 8.8 and 11.6 times respectively greater at 80 μM exogenous arachidonic concentration compared to the activity present with endogenous substrate alone (i.e. no arachidonic acid added). The optimum substrate concentrations were between 80 - 150 μM arachidonic acid, and subsequent assays were performed using 90 μM arachidonic acid.

3.3.3.3 Linearity with Protein Concentration

Using the optimal conditions described, the enzyme activity was found to increase linearly with the amount of protein over the range of 0 - 744 μg of protein per incubation (Fig. 3.5c). Approximately 450 μg (451.82±31.65 μg, mean±sem) was added to each assay tube in subsequent assays.

3.3.4 Enzyme Activity in Hypothalamic Homogenates during Fetal and Newborn Development

PGDS and PGES activity was detected in the fetal hypothalamus from as early as 90 days gestation, when the amount of PGD_2 and PGE_2 formed in one minute was 0.11±0.03 and 0.96±0.18 fmole/μg protein respectively (Fig. 3.6). At each age (fetal and postnatal), the hypothalamus produced approximately ten times more PGE_2 than PGD_2. The specific activity of PGDS tended to be greatest at 125 days gestation, but this did not quite reach significance ($0.05<P<0.10$) when compared to the other age groups (Fig. 3.6a). The specific activity of PGES was significantly higher at 135 days gestation compared to younger fetal ages and the postnatal group ($P<0.05$; Fig. 3.6b).

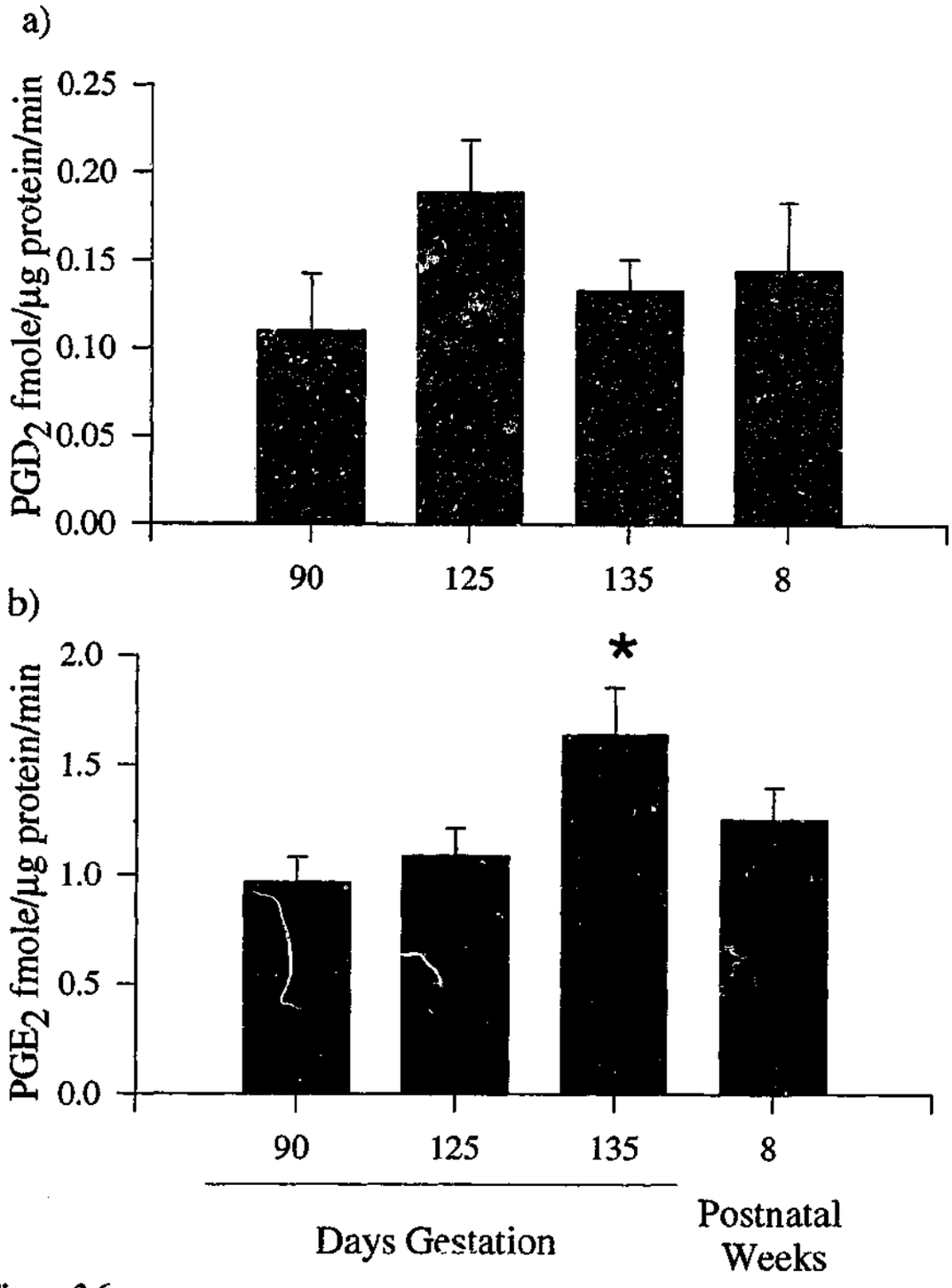


Figure 3.6
The specific activity of PGDS (a; red) and PGES (b; blue) in both posterior and anterior ovine hypothalamic homogenate obtained at 90, 125 and 135 days of gestation and 8 postnatal weeks. PGES activity increased significantly at 135 days gestation above other pre- and postnatal ages (*P<0.05). The data represent mean±sem, n = 4 or 5.

3.3.5 Prostaglandin Concentrations in the Anterior and Posterior Hypothalamus and Cortex

The PGD_2 concentration in the anterior and posterior hypothalamus during fetal development is shown in Figure 3.7. A significant increase in the posterior hypothalamus was observed at 135 days gestation compare to other groups ($P<0.05$; Fig. 3.7b). The anterior hypothalamus (Fig. 3.7a) and cortical homogenates (Fig. 3.7c) did not quite reach followed a similar trend but did not quite reach significance ($0.05<P<0.10$).

Upon analysis of basal levels of PGE_2, no consistent trend was revealed between the anterior and posterior hypothalamus and parietal cortex with an increase in age. Concentrations of PGE_2 in the cortex were significantly higher at 8 postnatal weeks compared to all fetal ages ($P<0.05$; Fig. 3.8c). The concentrations of PGE_2 in the anterior hypothalamus tended to increase progressively with age (Fig. 3.8a), but this change was not statistically significant. In comparison, concentrations of PGE_2 in the posterior hypothalamus appeared to decrease after 125 days gestation (Fig. 3.8b).

The ratio between PGE_2 and PGD_2 concentrations in each brain region did not change significantly with age (Fig. 3.9). However, the ratio did appear to fall during 125 to 135 days gestation from high values at 90 days gestation. The ratio of PGE_2:PGD_2 concentration in the anterior hypothalamus and cortex was greater in the juvenile sheep compared to the fetuses at 125 and 135 days gestation, whereas the concentration ratio for the posterior hypothalamus did not change between the fetal and postnatal age groups, and remained lower than the value obtained at 90 days gestation.

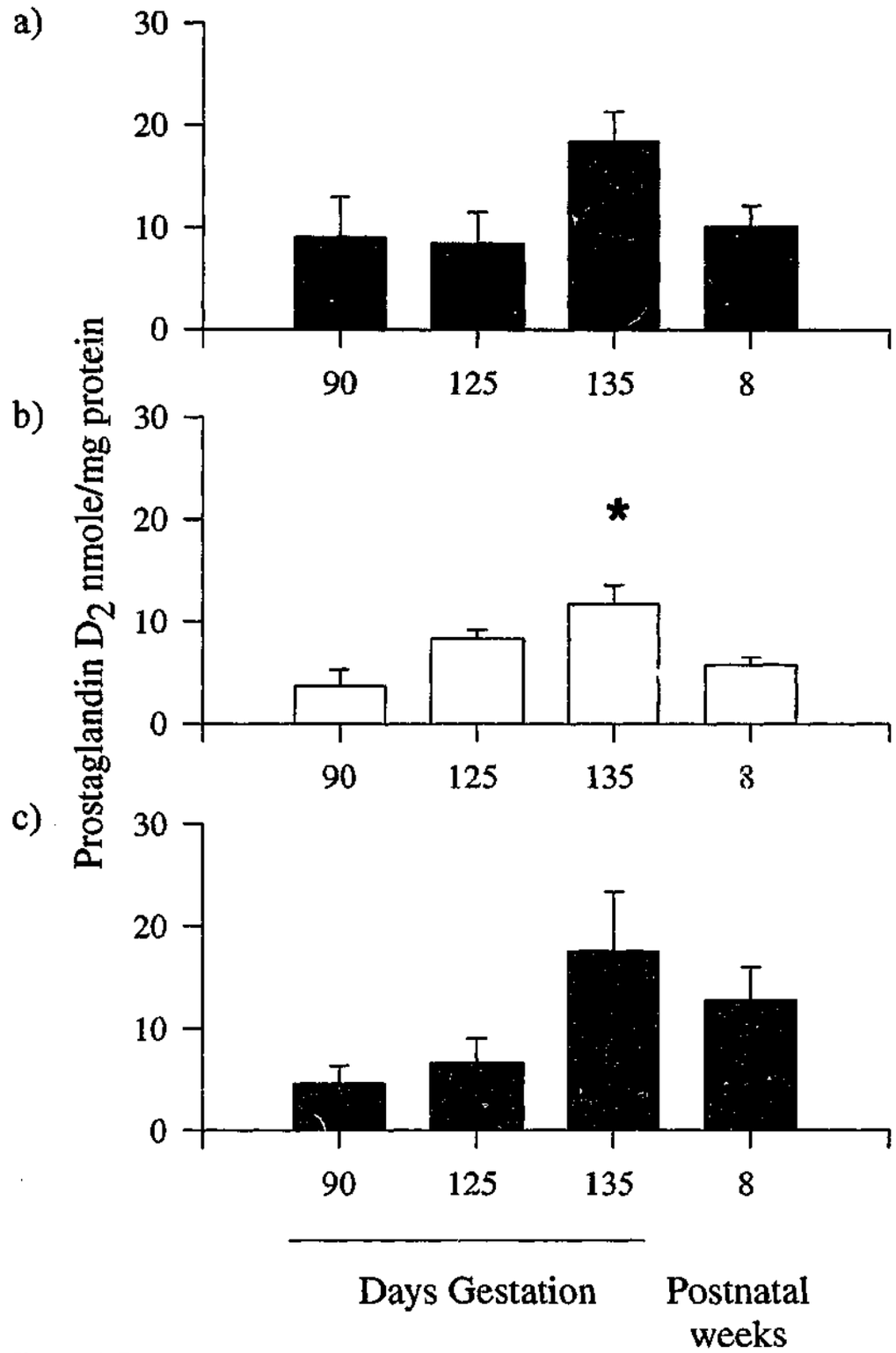


Figure 3.7
The PGD_2 content in the ovine anterior (a; green) and posterior (b; yellow) hypothalamus and parietal cortex (c; purple) at 90, 125 and 135 days of gestation and 8 postnatal weeks. PGD_2 concentration increased significantly in the posterior hypothalamus at 135 days gestation compared to all other pre- and postnatal ages (*$P<0.05$). The data represent mean±sem, n = 4 - 6.

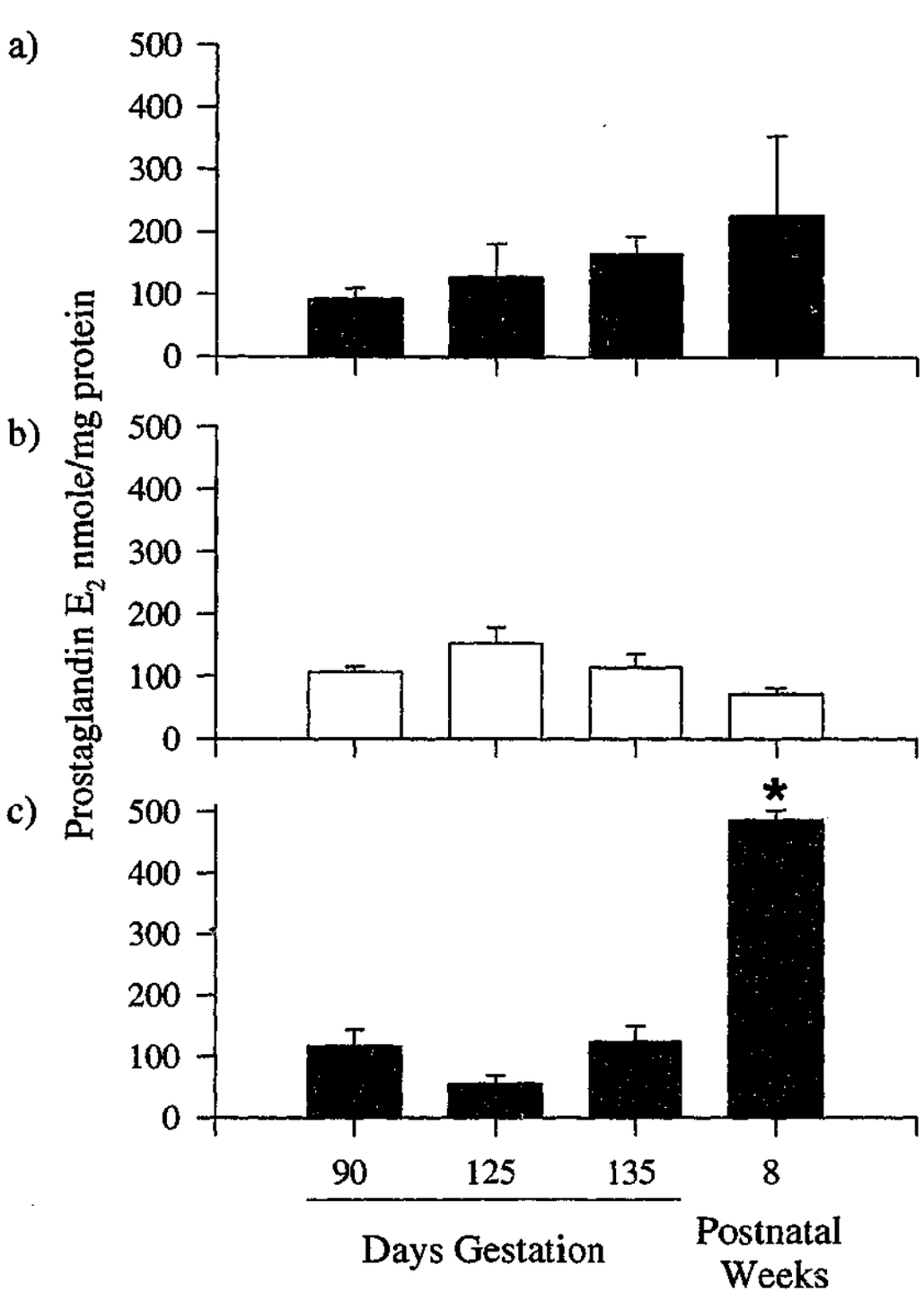


Figure 3.8
The PGE_2 content in the ovine anterior (a; green) and posterior (b; yellow) hypothalamus and parietal cortex (c; purple) at 90, 125 and 135 days gestation and 8 postnatal weeks. The PGE_2 concentration was markedly higher at 8 postnatal weeks in the parietal cortex above prenatal levels (*$P<0.05$). The data represent mean±sem, n = 4 - 6.

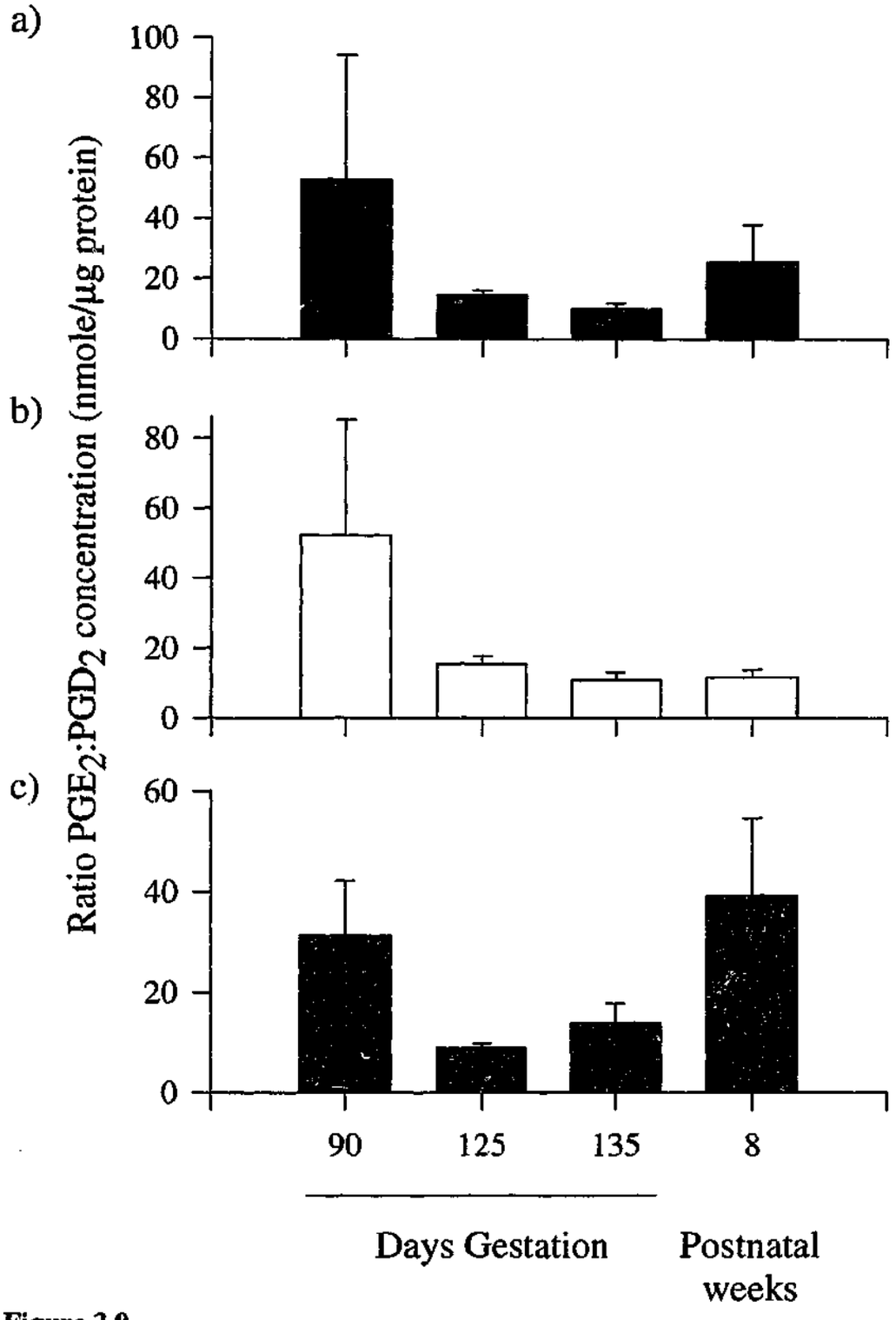


Figure 3.9
The ratio of PGE_2:PGD_2 concentration in the ovine anterior (a; green) and posterior (b; yellow) hypothalamus and parietal cortex (c; purple) at 90, 125 and 135 days gestation and 8 postnatal weeks. The data represent mean±sem, n = 4 - 6.

3.4 DISCUSSION

It has been hypothesised that PGD_2 and PGE_2 have specific and somewhat opposing roles in the regulation of sleep-wake cycles by acting on specific neuronal pools within the anterior and posterior hypothalamus respectively in adult rats (Hayaishi 1988). PGD_2 appears to induce sleep (Ueno *et al.* 1983), whereas the hypothalamic action of PGE_2 promotes wakefulness (Matsumura *et al.* 1988). In the ovine fetus REM and NREM sleep, as defined by conventional parameters (e.g. ECoG, EMG and EOG activities) are not present at 90 days gestation and partly developed by approximately 120 days gestation. By 130 days electrocortical activity has fully differentiated into REM and NREM-like activities as exhibited in adults (Clewlow *et al.* 1983). This study investigated whether changes in the content and synthetic capacity of PGD_2 and PGE_2 might correlate with the appearance and maintenance of prenatal sleep.

The results show that homogenates made from fetal sheep hypothalamus, upon the addition of arachidonic acid, were capable of producing considerable amounts of both PGD_2 and PGE_2 from as early as 90 days gestation, thus indicating that the enzymes PGDS and PGES are active in the hypothalamus from approximately 0.6 of gestation in sheep. Consistent with these observations on enzyme activities, there were measurable and significant concentrations of PGD_2 and PGE_2 in the ovine anterior and posterior hypothalamus from 90 days gestation. Concentrations of PGD_2 appeared to increase in the anterior hypothalamus at 135 days gestation above other ages but this increase was not quite significant ($P=0.08$). In the posterior hypothalamus, concentrations of PGD_2 increased significantly between 90 and 135 days, despite the absence of a significant parallel increase in PGDS activity. In comparison, the specific activity of PGES was significantly greater at 135 days gestation compared to other fetal ages and subsequently fell at postnatal ages. However, there was no increase in the associated PGE_2 content in the anterior or posterior hypothalamus.

These ontogenic variations of PGE_2 and PGD_2 concentration and synthase activities in the hypothalamus suggests differences in the maturation of cellular systems for which each

prostaglandin have an important role. The significant increase in PGES activity at this specific stage of development, 135 days gestation, suggests that PGE_2 may play an important role in the maintenance of some hypothalamic functions which develop or peak at this time, including arousal (Clewlow *et al.* 1983) and ACTH release (Brooks *et al.* 1992). However, further elucidation is required to substantiate a causal link between enzyme activity and basal concentration with neural functioning of these hypothalamic systems.

Despite the well-established acceptance that PGES is a membrane bound microsomal enzyme (Jakobsson *et al.* 1999; Watanabe *et al.* 1997), previous studies have reported PGES activity in the cytosolic fraction prepared from samples of human (Ogorochi *et al.* 1984; Ogorochi *et al.* 1987) and rat (Shimizu *et al.* 1979b) brain. In comparison, PGDS appears to be associated with the membrane in the rat brain (Shimizu *et al.* 1979b; Urade *et al.* 1985b). Our preliminary results show that the microsomal fraction of ovine fetal hypothalamus exhibited greater PGES activity than the cytosolic fraction. In contrast, PGDS activities were similar in both cytosolic and microsomal fractions. Thus, to permit the measurement of integrated synthetic capacity of both cellular compartments, and to ensure that no specific changes in either fraction would be over looked, the homogenate containing both the cytosolic and microsomal fractions was used in this study to concurrently determine the specific activity of PGDS and PGES during ovine fetal life. This also reduced preparation time and handling of the tissue, and therefore minimised the risk of enzyme degradation.

Since this study endeavoured to compare the synthetic capacities of PGDS and PGES hypothalamic homogenates at different ages, all enzyme assays were incubated for 5 minutes to make relative comparisons between the amounts of PGD_2 and PGE_2 produced in over this time, as an indication of the enzyme activity in the hypothalamic homogenates. It is important to note that, as shown from Figure 1c, there is a linear relationship between the amount of protein and production of PGs, and therefore the activity of the enzyme is directly proportional to the amount of protein in the system in the conditions used. In addition, since cyclooxygenase reaction is irreversible and the enzyme is inactivated during

catalysis leading to the loss of activity (Marshall *et al.* 1987), the maximal product is therefore dependent on the amount of enzyme in the tube at the start of the reaction.

As expected, enzyme activity was dependent on the dose of exogenous arachidonic acid. Unlike PGES activity, which was maximum in the presence of 75 μM arachidonic acid or more, PGDS activity decreased at concentrations of arachidonic acid greater than 150 μM. This is consistent with the recognised 'suicide' phenomenon, where high concentrations and rapid turnover of the arachidonic acid substrate produces an unstable protein intermediate that induces inactivation of the cyclooxygenase enzyme (Smith and Marnett 1991). However, since PGES was not concurrently affected, high concentrations of the fatty acid precursor may specifically inhibit PGDS activity by alternative mechanisms.

PGDS activity in the fetal hypothalamus was found to be glutathione-dependent, in that the activity decreased by 50% in the absence of glutathione in the incubation medium. This suggests the active isoform of PGDS in the fetal ovine brain may not be the glutathione-independent isoform of the enzyme as previously characterised in the adult rat brain (Urade *et al.* 1985a). Whether differences in developmental age or variations in species account for this difference requires further elucidation.

The specific activities of PGDS and PGES were measured in hypothalamic homogenates over approximately the last third of fetal development. We showed that PGES activity was consistently 10-fold greater than that of PGDS. These results are consistent with previous studies that show PGES in the human cerebrum has a higher specific activity than PGDS (Ogorochi *et al.* 1984), However, they are markedly different to those using homogenates made from whole fetal rat brain (Ueno *et al.* 1985b), which showed that PGDS activity was significantly greater than PGES. The specific activity of PGES determined in the present study was comparable with those for the adult rat (Watanabe *et al.* 1997) and human (Ogorochi *et al.* 1984) brain. In contrast, the specific activity of PGDS was relatively low compared to that measured in the whole adult (Urade *et al.* 1985b) and fetal (Ueno *et al.* 1985b) rat brain homogenates. This discrepancy could be simply due to a difference in species or age between the studies. However, it is likely that there are regional differences

in enzyme activity throughout the brain, and that homogenates made from the whole brain, as in the rat studies cited above, may mask important differences that are present in specific brain regions, such as the hypothalamus.

It is most likely that the measured concentrations of PG were endogenously produced and not derived from plasma. PGD_2 concentrations in fetal serum have not yet been measured but plasma concentrations in the adult rat are almost negligible (Suzuki *et al.* 1986), whereas large amounts of PGE_2 are produced by the placenta in late gestation and released into the fetal circulation (Olson *et al.* 1984). Since both PGD_2 (Suzuki *et al.* 1987) and PGE_2 (Jones *et al.* 1993) have been demonstrated to cross the blood brain barrier and enter the brain, it is conceivable that brain levels of PGE_2 are derived from blood-borne sources. However, it is more than likely the prostanoids measured were synthesised by the hypothalamic tissue and not derived from blood since the brain tissue was perfused with saline prior to analysis and that these current findings showed that the fetal hypothalamic homogenates have the capacity of synthesise both PGD_2 and PGE_2. Furthermore PGD_2 and PGE_2 are metabolised remarkably rapidly in blood (half-life of 0.9 and 0.29 minutes respectively) (Forstermann and Neufang 1983; Suzuki *et al.* 1986), hence it is believed the measured concentrations of PGs are most likely to be endogenous levels synthesised by brain tissue rather than from plasma PGs.

The observed changes in endogenous concentrations of PGD_2 may reflect the maturational changes in the cellular component in the brain. The non-neural astrocytic (Giacomelli *et al.* 1996; Seregi *et al.* 1987) and oligodentrocytic (Urade *et al.* 1987) component of brain tissue are considered the principle synthesiser of PGDS in the adult rat. However, the localisation of the enzyme changes with development, predominantly expressed in the neurons of 2 week old rat pups (Urade *et al.* 1987). The physiological significance of shift in localisation during development is unclear, but suggest that PGD_2 is associated with neural differentiation or synaptogenesis.

In this study PGDS activity was measured in the fetal hypothalamus and cortex from at least 90 days gestation. The significance of this relatively early expression of PGDS activity

in the developing brain is unclear. It is possible that the enzyme may be associated with the early maturation of the CNS, and may be one of the developmental processes regulated by thyroid hormones (Garcia-Fernandez *et al.* 1993), since hypothyroidism causes both spatial and temporal increase in PGDS mRNA expression in the developing rat brain (Garcia-Fernandez *et al.* 1997). It is well established that thyroid hormone, which is produced as early as the 17th day gestation in the prenatal rat (Kawaoi and Tsuneda 1985), is essential for normal brain development and that severe thyroid deficiency lead to irreversible mental retardation and neurological deficiencies (Porterfield and Hendrich 1993). These results are also consistent with the observed increase in the PGD_2 concentrations in both the anterior and posterior hypothalamus and parietal cortex with fetal maturation, where the maximal levels were detected at 135 days gestation. Although not all trends exhibited were statistically significant, the endogenous levels of PGD_2 tended to increase gradually in all tested regions during fetal development, suggesting it may be implicated in the regulation of CNS ontogensis and maturation.

PGES activity was also present from as early as 90 gestational days. This activity increased significantly between 90 and 135 days gestation, and subsequently decreased postnatally. The significant increase in PGES specific activity in the hypothalamus may be related to the appearance of episodes of wakefulness as defined by ECoG, EOG and EMG activities (Szeto 1992). Although such periods of arousal only constitute 5% of the total time in the fetal sheep, the amount of time spent in such epochs increases with gestational age and peaks around 135 - 140 days gestation (Szeto and Hinman 1985). The increase in PGES specific activity at this time may be related in the up-regulation of the incidence of 'arousal activity'. The concentration of basal PGE_2 in the hypothalamus did not increase in parallel with the elevation of PGES activity observed at 135 days gestation, and may suggest an increased need and utilisation of the PG for PGE_2 regulated functions, such as arousal. However, these results are not conclusive and require further studies to ascertain whether a functional relationship between hypothalamic PGE_2 levels and fetal arousal exists.

It is important to note that the prenatal increase in PGES activity may not exclusively relate to sleep and arousal alone. The increase of PGES activity may also be associated with other

mechanisms regulated by the hypothalamus that develop at this stage in precocial mammals prior to parturition. For example, it may be involved in the up-regulation of ACTH which occurs at late gestation (Brooks *et al.* 1992). The fall in hypothalamic PGES activity during the transition between pre- and postnatal life is consistent with previous reports, where a dramatic decreased in PGE_2 levels in the CSF (Jones *et al.* 1994) coincides with an increased uptake and catabolism of PGE_2 by the CP (Krunic *et al.* 1997). It has been hypothesised that this event may contribute to the establishment of continuous breathing (Kitterman *et al.* 1983).

The functional significance of the increase in cortical PGE_2 content in lambs 8 weeks after birth is unclear. These findings conflict with previous adult data which revealed that cortical areas yielded a much lower distribution of PGE_2 compared to hypothalamic regions (Ogorochi *et al.* 1984). The shift in PGE_2 and PGD_2 ratios over gestational age reflect the relative changes in concentration of the two PGs. Although the ratio changes were statistically insignificant, there was a trend for an increase in PGD_2 content relative to PGE_2 between 90 to 125 days gestation in all brain regions tested. This relative increase is sustained throughout the remainder of fetal life and decreases again in the anterior and parietal cortex. This trend is consistent with the current hypothesis that PGD_2 and PGE_2 may play a reciprocal role in the control of somnolence and vigilance in the fetus as it does in the adult, whereby perhaps the alternations in the homeostatic balance between the two regulating factors rather than basal levels contributes to the maintenance of fetal sleep which is predominant in the late gestation fetus.

These observations do not indicate that marked changes in PGDS and PGES enzyme activity nor basal levels of PGD_2 and PGE_2 occur in the hypothalamus just prior to, or at the time when sleep is definitively established in fetal life, i.e., at about 120-125 days gestation. PGD_2 may be perhaps related to neural functions such as synatogenesis or glial migration (Urade *et al.* 1987). However, this does not completely rule out the involvement of PGD_2 and PGE_2 in fetal sleep-wake maturation or regulation, as other mechanisms such as prostaglandin receptor affinity, density or distribution may occur in the hypothalamus as part of the mechanisms required for the onset of sleep-wake cycles. Significant changes in

prostaglandin synthesis and release from sites other than the hypothalamus (e.g., secreted by leptomeninges or choroid plexus), or alterations in other mechanisms involved in prostaglandin action, such as receptor binding, need to be determined to elucidate further the potential regulatory roles of PGD_2 and PGE_2 in the development of prenatal sleep-wake cycles.

Chapter 4

Expression of β-trace protein in the fetal sheep hypothalamus and cerebrospinal fluid

4.1 INTRODUCTION

β-trace is a glycoprotein with a molecular mass of 25 - 31 kDa (Harrington *et al.* 1993; Link 1967) and a primary structure consisting of 190 amino acids (including a 22 amino acid signal peptide) bearing N-linked oligosaccharides at two sites (Hoffmann *et al.* 1994). β-trace is a major protein constituent of human CSF where it represents almost 3% of total CSF proteins (Zahn *et al.* 1993). Elsewhere, β-trace is present in the central nervous tissue, particularly white matter, and also genital tissues, such as the testis and epididymus (Olsson and Nord 1973). β-trace is also present in various tissue fluids such as serum, tear fluid, urine and seminal plasma (Hoffmann *et al.* 1997; Olsson and Link 1973; Tokugawa *et al.* 1998).

The tertiary structure of β-trace protein, consisting of a conserved 8 stranded anti-parallel β-barrel, strongly suggests that it is a member of the lipocalin superfamily, a group of small secretory proteins involved in the binding and transportation of lipophilic molecules (Nagata *et al.* 1991). β-trace mRNA levels in specific cells associated with blood-tissue barriers has been shown to increase during murine embryonic development, implicating the involvement of β-trace in the maturation of these barriers (Hoffmann *et al.* 1996a). Other studies have shown that hypothyroidism causes both temporal and spatial alterations in β-

trace mRNA expression in the brain during postnatal development, suggesting that changes in synthesis and secretion of β-trace may contribute to the complex effects of thyroid hormone on brain development and function (Garcia-Fernandez *et al.* 1997). However, despite recognition of β-trace as a lipocalin protein and being implicated with thyroid hormone regulation of neural development, the exact function of the protein has not been clearly elucidated.

Based on the homology of its amino acid sequence deduced from the corresponding cDNA sequences (Igarashi *et al.* 1992; Urade *et al.* 1993; White *et al.* 1992), β-trace has been structurally identified as glutathione-independent, brain-type PGDS (Hoffmann *et al.* 1993). PGDS is the synthesising enzyme responsible for the production of PGD_2 from the precursor PGH_2 in the arachadonic acid cascade. It has been hypothesised that endogenous levels of PGD_2 may play a role in the regulation of sleep in a variety of mammalian species including human, monkeys and rats (Hayaishi *et al.* 1993), by acting at the POAH (Matsumura *et al.* 1994) the topographic area considered as the putative centre of sleep regulation. β-trace isolated from human CSF exhibited almost identical enzymatic properties to PGDS purified from the rat brain (Watanabe *et al.* 1994). Thus, based on these structural and functional homologies, the terms β-trace and PGDS have been used interchangeably in many recent publications (Beuckmann *et al.* 1996; Blodorn *et al.* 1996; Eguchi *et al.* 1997; Garcia-Fernandez *et al.* 1998; Gerashchenko *et al.* 1998; Tanaka *et al.* 1997). However, whether β-trace circulating in the ventricular system of the adult CNS definitively contributes to the maintenance of physiological sleep requires further elucidation.

The aim of the present study was two fold: firstly, to determine if changes in PGDS/β-trace expression occur in the CSF and hypothalamus of sheep during fetal development; and secondly, to establish if changes are related to the development of physiological sleep. In sheep (term, 147 gestational days) the electrocortical signs of sleep first appear at approximately 120 days gestational age and fully differentiate into distinct REM/NREM sleep states by 130 days of gestation (Clewlow *et al.* 1983). We therefore measured the level of PGDS/β-trace protein expression by immunoblot analysis before and after the onset

of sleep in fetal sheep, using polyclonal antibodies raised against either human β-trace protein, or an antiserum raised against a 20 amino acid peptide synthesised on the basis of the N-terminal sequence homology between human β-trace and rat PGDS protein. In addition, an attempt was made to measure the expression of β-trace mRNA in the ovine hypothalamus during this developmental period using a cDNA probe based on the known nucleotide sequence of the rat lipocalin/PGDS gene.

4.2 Methods for Antibody Production and Immunoblot Analysis

4.2.1 Materials

The following items were obtained from Sigma Chemical Co., St Louis, MO, USA: keyhole limpet hemocyanin (KLH), complete and incomplete Freund's adjuvant, PMSF, Tris base and Tris-HCl. Resin p-MeBHA, trifluroacetic acid (TFA), N,N-dimethylformamide (DMF), piperidine, O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and all L-α-9-fluorenylmethoxycarboxyl (Fmoc)-protected amino acids were purchased from Auspep (Melbourne, Australia). Trifluoromethanesulphonic acid (TFMSA), thianisole, acetic anhydride and 1,3-diisopropylethylamine (DIEA) were obtained from Aldrich Chemical Co. (Milwaukee, USA). Trinitrobenzenesulfonic acid (TNBSA) was purchased from Fluka (USA). M-maleimidobenzoly-N-hydroxysuccinimide (MBS) ester was purchased from Pierce, Rockford, IL, USA. Protein assay kits were purchased from Biorad Laboratories, Mississauga, Ontario, Canada. Unless otherwise stated all other chemicals and solvents were analytical grade and of the highest purity available.

4.2.2 Peptide Synthesis and Purification

4.2.2.1 Selection of Peptide Fragment

Segments of β-trace protein sequence were utilised as immunogens for the production of a polyclonal β-trace antiserum. Initially two peptide fragments were employed to increase the probability of producing a successful antiserum. The first peptide fragment (P1) consisted of 20 amino acids (CAPEAQVSVQPNFQQDKFLG), whilst the second peptide fragment (P2) was 13 amino acids in length (CVQPNFQQDKFLG). The individual peptides were specifically selected for several reasons. Firstly, the longer peptide P1 was homologous to the N-terminal sequence of the β-trace protein purified from human CSF (Kuruvilla *et al.* 1991). This 20-mer peptide also included the amino acid sequence of peptide P2 that was identical to the N-terminal sequence of rat PGDS (Watanabe *et al.* 1994) and murine β-trace (Fig 1.5; Hoffmann *et al.* 1996a). Thus these distinct peptide sequences were chosen because they are highly conserved sequences of the β-trace protein between different species, therefore increasing the likelihood of producing an antiserum which would be antigenic across several species including sheep. Secondly, the selection of each peptide was also guided by the instrinsic hydrophobicity of each peptide fragment. Generally, it is assumed that very hydrophobic peptides are internalised with the core of the polypeptide tertiary structure and hence are not ideal antigens. The hydorphobic moment of each peptide was calculated using the Hydropath computer program (care of Prof M.T.W Hearn, Monash University, Clayton, Australia), which indicated that each synthetic peptide was in a region of the protein exposed to aqueous environment, and thus would theoretically be more accessible when probed by the raised antibody.

4.2.2.2 Solid Phase Peptide Synthesis Using Fmoc Amino Acids

Peptide fragments P1 and P2 were manually prepared by solid phase peptide synthesis, which is considered to be the most appropriate method for the production of peptides and small proteins of known sequence (Fields and Noble 1990). In these studies, the method employ L-α-amino acids with the N-terminal protected by an Fmoc chemical group, while other groups such as acetamidomethyl, *tert.*-butyloxycarbonyl, *tert.*-butyl and triphenylmethyl are used to protect the appropriate side chains. During this procedure the amino acid is loaded onto the resin, which acts as an insoluble solid support, where individual amino acids could be sequently coupled to produce an entire peptide chain. Deprotection of the Fmoc N-α-protection group of the amino acid attached to the resin permits the coupling of the pre-activated carboxyl group of the subsequent amino acid. The deprotection and coupling cycles were repeated until the desired amino acid sequence was generated (see Fig. 4.1).

A clean dry plastic 50 ml syringe barrel with a filter base attached to a three-way tap and vacuum manifold was used as a synthesis vessel. The synthesis was performed using p-MeBHA resin (244 mg, Batch 792084, 0.88 mmole/g) with a loading capacity of 0.2 mmole and an estimated 3-fold excess of synthesised peptide product using the Fmoc strategy. The resin was weighed, loaded into the vessel and neutralised with 4 ml of 10% DIEA in DMF. Typically 0.6 mmole of Fmoc amino acid was weighed and dissolved in 4ml of DMF. Activation of the carboxyl group at the C-terminal was initiated with the addition of activating agents; 228 mg HBTU, 81 mg of HOBT and 0.25 ml of DIEA. The reaction mixture was stirred thoroughly for 30 seconds, then added directly in the resin. The activated amino acid and resin complex was incubated for 90 minutes with occasional mixing. The coupling procedure was repeated for the lysine residue, to ensure adequate binding to the adjacent amino acid. After the amino acid was sufficiently coupled to the resin, the Fmoc protecting group was removed from the N-α-moiety by three five minute incubations with 20% piperidine in DMF. Deprotection of the N-terminal permits subsequent coupling with the next pre-activated amino acid. The resin-amino acid complex

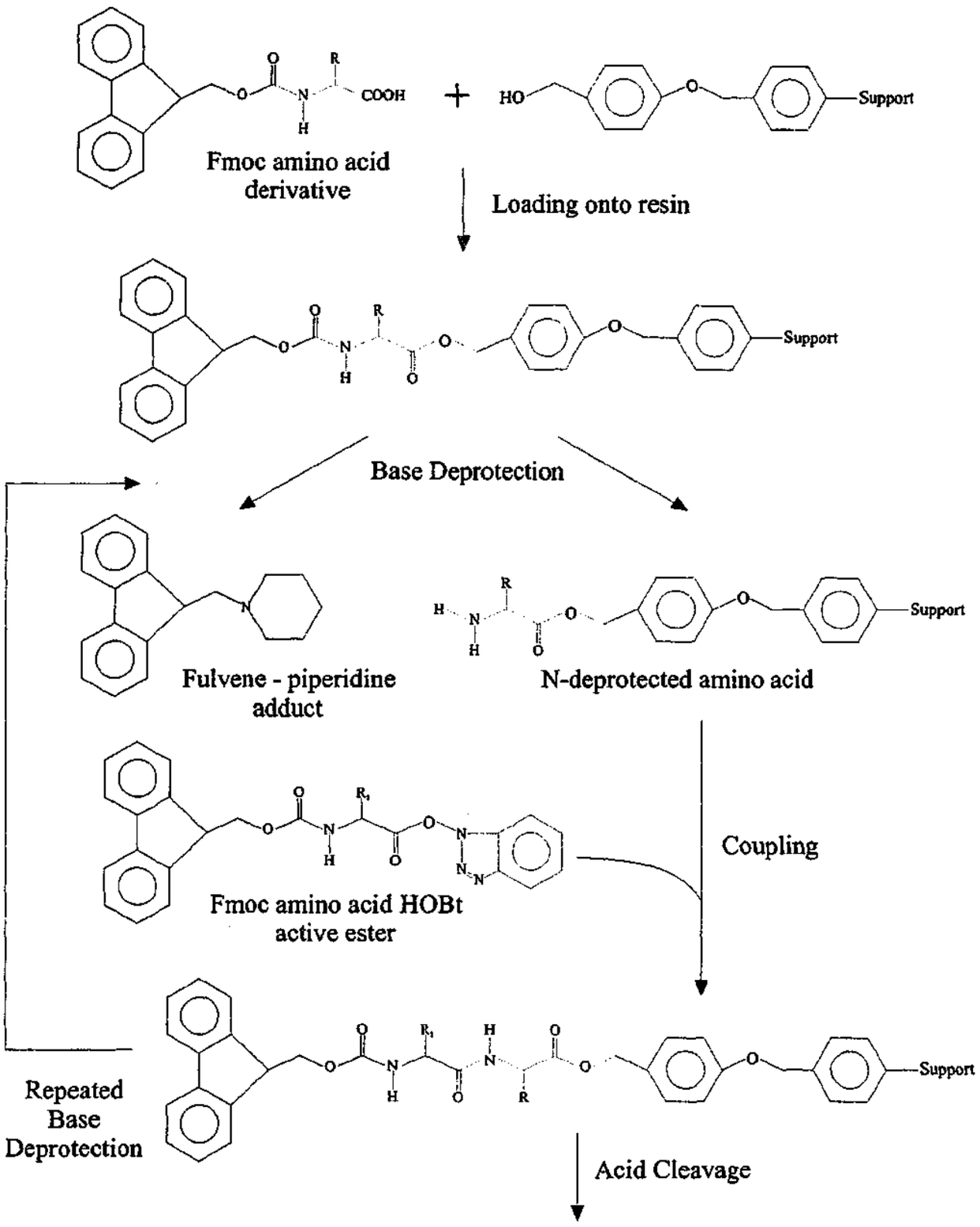


Figure 4.1
Solid phase peptide synthesis utilising L-α-9-fluorenylmethoxycarboxyl (Fmoc) amino acids (Fields and Noble 1989).

was washed thoroughly with excess volumes DMF and filtered between each step. All reactions were performed in the fume hood. The deprotection and coupling procedure was repeated until all the designated amino acids were attached to produce the final peptide chain.

It was imperative that all the amino acids were deprotected and coupled completely to ensure the production of complete peptide chains of desired sequence and to reduce the risk of producing 'deletion peptides' of incorrect amino acid sequence. After each deprotection and coupling step, a small sample of resin was removed and tested with 2 drops of both DIEA and TNBSA in 0.25 ml of DMF. TNBSA reacts with the free amino group to produce a chromophoric compound. Therefore, after the deprotection procedure, formation of orange resin beads suspended in a clear solution verified the presence of free amino acids of the N-terminal and thus detachment of the Fmoc protection group. In comparison, after coupling of the new amino acid, the resin beads were expected to remain clear indicating the absence of free unprotected amino groups. Deprotection and coupling procedures were repeated if the respective step failed. If coupling of the amino acids repeatedly failed, the complex was washed three times with 2% DIEA, 10% acetic anhydride in DMF to covalently block the free N-terminal and prevent the formation of unwanted side peptides.

Finally, the acetamidomethyl-protected Cys residue was coupled to the N-terminal position of the completed peptide chains. The presence of this residue facilitated the coupling of the peptide to the KLH carrier protein. Upon completion the finished peptide-resin complex was dried in a desiccator overnight, weighed and stored until cleaved.

4.2.2.3 Peptide-Resin Cleavage

The completed peptide was cleaved from the resin and the side chain protection groups were removed using standard TMSFA cleavage conditions (Cavallaro *et al.* 1998). All glassware was dried overnight to evaporate any residual trace of water, which interferes

with the acidolysis of the resin and side chain protection groups. Appropriate eye wear and protective clothing were also worn when handling this strong acid.

One hundred and fifty mg of peptide-resin was weighed and placed into a small round bottom flask. The side chain protective groups were deactivated with the addition of thioanisole:ethanedithiol (2:1, total volume 375 µl) and then cleaved with 2.5 ml TFA; each incubation procedure required ten minutes of mixing. Two hundred and fifty µl of TFSMA was added drop wise while continuously stirring the resin, and was then left to mix for two hours to allow for complete cleavage of peptide from resin. Since considerable heat was generated from acidolysis of the protection groups and resin, all cleavage steps were performed on ice.

The scavenger side chain protection groups were removed by the addition of 35 ml of cold diethylether and filtered through a scintered funnel. The peptide was extracted from the resin and washed through the fritted filter with excess washes of TFA. The excess TFA was then evaporated using a Rotovapor for 60 minutes. Forty-five ml of ice cold ether was added to the remaining 2 ml of TFA and left overnight at 4°C. The following day the ether was added and the mixture was filtered again to ensure the adequate removal of scavengers. The remaining peptide was washed through the filter using 25 ml of 50% acetronitrile (ACN) in water. Fifty µl of crude peptide was removed for the analysis by reverse phase-high performance liquid chromatography (RP-HPLC), while the remaining sample was lyophilised overnight in a freeze drier and stored at -20°C until further analysis.

4.2.2.4 Peptide Purification by Reverse-Phase High Performance Liquid Chromatography

The profile of peptide fragments contained in the crude peptide sample was first obtained by analytical RP-HPLC using a TSK-ODS-120T column (150 x 4.6 mm I.D., Tosoh Corp., Yamaguchi, Japan), packed with 5 µm octadecylsilica. The column was initially washed with eluent B (60% ACN, 0.1% TFA in dd-H_2O) to remove any hydrophobic contaminants

and then gradually preconditioned with eluent A (0.1% TFA in dd-H_2O). Fifty µl of crude peptide sample dissolved in 100% ACN was eluted through the column by a linear gradient of eluent A to B, at a flow rate of 1 ml/min, for 60 minutes. Different peptides were eluted from the column at various percentages of ACN, depending on the hydrophobicity of the peptide. It was assumed the highest peak in the eluted profile, detected by absorbance set at 214 nm, was the desired peptide sequence and the remaining peaks were deletion peptides that were subsequently removed in following preparative RP-HPLC.

Fifty mg of the crude lyophilised peptide sample was dissolved in 3 ml of eluent B and purified using preparative RP-HPLC using a TSK-ODS-120T column (300 x 21.5 mm I.D., Tosoh Corp.) packed with 10 µm, 300Å ocetadecylsilica. The column was pre-washed and preconditioned as described above and the fractions were eluted with a linear gradient of 0 - 100% eluent B, at a flow rate of 7.5 ml/min for 105 minutes. The UV detection was set at the standard wavelength of 254 nm. Three ml fractions were taken when the absorbance exceeded 0.10 AU, and were further examined by the analytical RP-HPLC, as described above.

Sixty µl samples were taken from each of the fractions obtained when the absorbance measurements exceeded 0.10 AU, and placed in individual analytical tubes to be analysed by the analytical RP-HPLC procedures as previously described. Crude sample and eluent A sample were also analysed for comparison. Fractions that contained purer quantities of the target peptide, with profiles of a single sharp peak, were lyophilised overnight, stored in Eppendorf tubes and frozen at -20°C until required for immunisation.

The molecular mass of the synthetic peptide was confirmed by electrospray mass spectrometry to be equivalent to the theoretical molecular mass, 2.2765 kDa.

4.2.3 Production of Anti-β-trace Antiserum

4.2.3.1 Coupling Peptides to Keyhole Limphete Hemocyanin Carrier

The successful production of antiserum relies on the *in vivo* immunogenic response elicited in an animal. Typically small synthetic peptides are generally unrecognised by the mammalian immune system, or induce weak immune responses. Therefore, short peptides are generally conjugated to a larger carrier protein, such as KLH, to ensure the generation of an immune response. P1 and P2 were both conjugated to KLH via the Cys residue using a heterobifunctional reagent MBS ester as the linker (Lerner *et al.* 1981). Briefly, 200 µl of MBS (10 mg/ml stock solution dissolved in DMSO) was added drop-wise to 20 mg of KLH dissolved in 2 ml 0.05 M Na_2HPO_4 (1 mg/ml, pH 6.0), and continuously mixed for 30 minutes at room temperature. The uncoupled MBS was separated from the KLH-MBS conjugate by Sephadex G25m gel filtration (PD-10 column, bed volume 9.0 ml, Pharmacia, Buckinghamshire, England) eluted with 0.05 M Na_2HPO_4, pH 7.0. After a 3ml void fraction, the peak KLH-MBS fraction (2 ml) was collected and added to 2 mg of peptide which had been previously dissolved in 2 ml of 0.05 M Na_2HPO_4, pH 7.0. The peptide-KLH-MBS mixture was incubated for 3 hours at room temperature, divided into aliquots, and stored at -80°C .

4.2.3.2 Immunisation and Bleeding Protocol

An immune response is the culmination of a series of interactions between macrophages, T lymphocytes and B lymphocytes responding to the presence of a foreign antigen, resulting in the generation of antibodies that can specifically bind to the antigen and provide rapid removal from the animal. Twelve white New Zealand rabbits were used for immunisation with either the P1 or P2 conjugated peptides. The rabbits were used in accordance to the Standing Committee on Ethics in Animal Experimentation of Monash University. Pre-

immunisation bleeds were taken from each rabbit to establish control pre-immune titres. Before administration into the animal, the peptide was emulsified in Freund's complete adjuvant (containing dead *Mycobacterium Tuberculosis*), that protects the antigen from rapid dispersal and stimulates the secretion of local host factors that recruit macrophages and hence increases the efficiency of phagocytosis. One ml of peptide-KLH conjugate solutions, containing approximately 2 mg of conjugate peptide, and an equivalent volume of Freund's complete adjuvant were emulisfied and injected intradermally into the back and neck of the rabbit at 4 - 6 different sites. Subsequent secondary boosts of conjugate administered in Freund's incomplete adjuvant were given at 3 week intervals to induce further antibody production by the immune system.

A blood sample was taken from the ear vein 10 days after the second injection of antigen; typically, this corresponds with the peak of antibody titres. Further blood samples were taken every two weeks thereafter. The rabbits were bled from the marginal ear because it was easily accessible and does not have high numbers of nerve endings. To obtain blood, the rabbit was placed in a restraining device and the marginal vein was gently shaven and placed under a warming lamp to stimulate blood flow. A 22 gauge i.v. catheter (Instyte, Becton Dickinson, Utah, USA) was carefully inserted into the vein. The vessel was continuously stroked to increase blood flow from the vein, which was collected in a clean 50 ml plastic tube. Up to 35 ml of blood was taken at any one time. The blood was allowed to coagulate at 37°C for one hour. The clot was then separated from the sides of the tube using a Pasteur pipette ('ringing') and refrigerated overnight and then removed the following day. Any remaining insoluble debris was separated from the serum by centrifugation at 10,000 *g* for 15 minutes at 4°C. The serum was stored in individual aliquots at -80°C until further analysis by enzyme linked immunosorbent assay (ELISA). The immunisation regime ceased if the antiserum did not produce appropriate titres after four bleeds. If titres above 1:25,000 dilution were achieved, the maximum volume of blood was collected at each bleed. By the fifth bleed the animal was finally sacrificed by exsanguination.

4.2.3.3 Enzyme Linked Immunosorbent Assay

The titre of each post-bleed was tested against the pre-bleed of the corresponding animal by indirect ELISA. Microtitre plates were coated with the 1 µg of the tested synthetic peptide (P1 or P2) dissolved in coating buffer (20 µg/ml, 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.3 mM NaN_3, pH 9.6). One column of wells remained uncoated and provided a negative control to check the tested peptides. The two wells that were used as positive controls were coated with a previously synthesised peptide that produced antiserum of known titre (generously supplied by Prof. M.T.W. Hearn). Following 4 hour incubation at 22°C, the plates were washed three times with 0.1 M PBS pH 7.4, containing 0.05% Tween 20 and each well was subsequently blocked with 100 µl 0.1M PBS containing 0.05% Tween plus 1% BSA, covered in plastic and left overnight at 4°C.

The following day, the plate was washed three times with the same washing buffer. A 1:50 dilution of the pre-immune serum and antiserum were made in 0.1 M PBS containing 0.05% Tween plus 0.1% BSA and added to the first well. Subsequent serial double dilutions were applied to the all remaining wells excluding one row which provided another negative control to check reagents. A 1:50 dilution of positive control antiserum with previously established titre was made and added to positive control wells. The plates were incubated for 30 minutes at 22°C and then were washed six times with washing buffer to remove any non-specific antibodies that did not bind to the antigen peptide. Fifty µl of swine anti-rabbit IgG conjugated to horseradish peroxidase (1:500 dilution in 0.1 M PBS, 0.05 % Tween, 0.1% BSA) was added to each well and incubated for 30 minutes at 22°C to detect the bound antibody. After washing the plates six times with washing buffer, 100 µl of the enzyme substrate (200 µg/ml of orthophenylenediamine, 0.006% H_2O_2 in milliQ water) was added to each well and incubated for one hour at 22°C for the colour reaction to develop. The colour development was terminated by the addition of 25 µl of 8 M H_2SO_4 into each well, and measured at 490 nm on a multiplate reader (Model 3550, BioRad, CA, USA).

4.2.4 Identification of β-trace Protein by Immunoblot Analysis

4.2.4.1 *Tissue Collection*

Fetal hypothalamic, parietal cortex and CSF samples were collected at post-mortem at approximately 90, 125 and 135 gestational days (n = 3, each group) after the fetuses had been killed by an overdose of sodium pentobarbitone (130 mg/kg i.v.). Each brain was immediately removed, blocked into hypothalamic and cortical sections (as described in Section 3.2.2.1), frozen in liquid nitrogen and stored at -80°C until assayed for β-trace. Samples of CP taken from the lateral ventricle and samples of liver and muscle were also collected from 135 day gestation fetuses and frozen. Whole cortices from Sprague-Dawley rats (n = 3) were obtained after euthanasia by intraperitoneal administration of sodium pentobartitone (10 ml/kg), and were used as a positive control. The Animal Ethics Committee of Monash University, Clayton, approved the use of these animals. Samples of human CSF collected by spinal lumbar puncture (kindly provided by Dr. Samathatha Richardson, Department of Biochemistry, University of Melbourne, Parkville, Australia) was also used as a positive reference.

4.2.4.2 Tissue Preparation

Microsomal preparations of the adult rat and ovine fetal tissues were prepared as follows: each sample was weighed and pulverised with an air hammer on dry ice. The powdered tissue was homogenised in fresh buffer (0.1 M PBS, 0.32 M sucrose, pH 6.0, 100 mM PMSF; 5 ml/g of tissue) using an Ultra-Turrax homogeniser, and centrifuged at 1,500 *g* at 4°C for 15 minutes (Nichols *et al.* 1996). The supernatant from all tissue samples were removed and then centrifuged at 20,000 *g* for 60 minutes at 4°C. CP samples were homogenised using the procedures described previously but were not centrifuged. Ovine CSF samples were concentrated using microconcentrators (molecular weight cut off 10,000 kDa; Amicon, Danvers, MA, USA). The protein content in each sample was quantified by

the method of (Bradford 1976), to determine the volume of sample to be loaded in each lane. The cytosolic fraction of the brain, liver and muscle, the CP and CSF samples were denatured by boiling for 5 minutes in an equivalent volume of a loading buffer (0.125 M Tris-HCl, 4% sodium dodecyl sulphate, 20% glycerol, 10% β-mercaptoethanol, pH 6.8), and stored at -80°C until separation by electrophoresis.

4.2.4.3 Sodium Dodecyl Sulphate-Polyacrylamine Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most common technique employed for the separation of proteins. During boiling the polypeptides denature and bind to the strongly anionic detergent SDS, contained in the loading buffer, to become negatively charged. The amount of SDS binding to the polypeptide is independent of amino acid sequence, rather directly proportional to the size of the protein chain. Therefore, when a potential difference is placed across a gel, the migration rate of the denatured polypeptides towards the positive electrode is determined by the size of the protein.

SDS-PAGE electrophoresis was performed using standard protocols using 40% acrylamide/bisacrylamide 37.5:1 solution (Laemmli 1970). Briefly, aliquots of the samples equivalent to 40 μg of total protein were loaded into vertical 4% stacking gel (0.125 M Tris-HCl, 0.1% SDS, 0.05% ammonium persulfate, 0.05% tetramethylethylenediamine, pH 6.8) and 12% running gel (0.375 M Tris-HCl, 0.1% SDS, 0.05% ammonium persulfate, 0.05% tetramethyl-ethylenediamine, pH 8.8), casted immediately before use of the mini-gel casting apparatus (SE 260, Mighty Small, Hoefer Scientific Instruments, CA, USA). Low range molecular weight standards diluted 1:20 in loading buffer (BioRad), was used to determine the expected position of proteins in the gel between 97.4 kDa and 14 kDa. The samples and standards were subjected to SDS-PAGE in a discontinuous buffer system (0.02 M Tris Base, 0.192 M glycine, 0.1% SDS, pH 8.3), run at a constant current of 16 mA for approximately 150 minutes.

4.2.4.4 Immunoblot Analysis

The separated proteins were transferred eletrophoretically onto a nitrocellulose membrane (Schleicher and Scheull, Protan, 0.2 μm, Keene, N.H., Germany) in a tank (TE 52X, Hoefer Transfer Tanks, Hoefer Scientific Instruments) to allow access of the antigens to immunodetection reagents. Transfer of the proteins followed a conventional protocol (Ausubel *et al.* 1997) using appropriate buffer (50m M Tris Base, 0.384 M glycine, 40% methanol) for 2 hours at a constant voltage of 30 V, on ice. The gel was subsequently stained with a traditional Coomassie Blue (0.25% Coomassie Blue, 40% methanol, 7% glacial acid) to test whether the proteins were completely transferred.

Upon completion, the membrane was blocked with blocking buffer (0.1 M PBS, 0.05% Triton X-100, 3% non-fat powdered milk) overnight at 4°C. Membranes where either incubated with an affinity purified antiserum raised against the complete β-trace protein purified from human CSF (1:5,000 dilution in 0.1 M PBS, 0.05% Triton X-100, 3% non fat milk powder; a generous gift from Prof. Mader, Georg-August-University, Gottingen, Germany), or with the antiserum raised against the synthetic peptide (P1; 1:2,000 dilution in 0.1 M PBS, 0.05% Triton X-100, 3% non fat milk powder), for one hour at room temperature. After washing in blocking buffer the membrane was incubated in goat anti-rabbit secondary antibody conjugated to horse radish peroxidase (1:10,000; Boehringer Mannheim Corp., IN., USA) for one hour at room temperature, and then thoroughly washed again with 0.1 M PBS with 0.05% Triton X-100 to reduce background signal. The bound antibody was detected using an enhanced chemiluminescence technique (ECL Kit, Amersham, Buckinghamshire, England), where the horseradish peroxidase acted as a catalyst for the oxidation of the substrate luminol, which subsequently emits small quantities of light. The treated membrane was exposure to film (Biomax Light, Kodak, Rochester, NY, USA) for 20 minutes. Levels of detected β-trace protein were quantitatively analysed by computer software that measured the density of each positive signal using the UTHSCSA Image tool program (developed at the University of Texas Health Science Centre at San Antonia, Texas, USA). The total protein in the membrane was later stained

with conventional Ponceau S (0.5% Ponceau S, 1% glacial acetic acid). The membrane was then air dried and stored in a sealed plastic bag.

4.2.5 Validation of Anti-P1 Antiserum

To ensure the antiserum raised against P1 did not bind to proteins other than the desired antigen non-specifically, the antiserum was subjected a series of validation tests.

4.2.5.1 Column Purification of Anti-P1 Antiserum

The coupling to the column solid phase Protein-Affi-gel 10/15 (Biorad) was via the accessible amino groups on the peptide and the peptide-BSA conjugate to reduce any steric hinderance between epitotes of the antiserum and peptide fragment coupled to the resin (Lerner *et al.* 1981).

To couple the synthetic peptide P1 to BSA, 200 µl of MBS (10 mg/ml in DMSO) was added drop-wise to 10 mg of BSA dissolved in 2 ml of 0.05 M Na_2HPO_4, pH 6.0. After 30 minutes incubation at room temperature on a rotating platform, the uncoupled MBS was separated from coupled BSA-MBS by column filtration (PD10 Sepadex G25, Pharmacia bed; volume 9 ml). After the column was washed with 0.05 M Na_2HPO_4, pH 7.0, a 3 ml void fraction was passed and then the following 2 ml peak of BSA-MBS was collected. The eluted 2 ml fraction was incubated with 0.5 mg of P1 previously dissolved in 2 ml of 0.05 M Na_2HPO_4 (pH 7.0) for 2 hours with gentle rocking.

Approximately 0.5 mg of both peptide and peptide conjugated to BSA were each coupled to 5 ml of 1:1 mixture of Protein-Affi-gel 10/15 separately and later pooled together. Briefly, 10 ml of resin was washed in a sintered glass funnel with one bed volume of ice cold isopropanol, then twice with ice cold dd-H_2O. Air was not drawn into the resin with the filtration disconnected when the liquid reached the top resin bed. The resin was added immediately to the peptide and gently rocked overnight at 4°C. Any unreacted sites of the

resin were blocked with 10 mM ethanolamine, and the resin was washed several times with column buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4) by centrifugation for 10 minutes at 2,700 rpm.

Twenty ml of serum from the first and second bleed were pooled together and loaded into a 10 x 1 cm column of Protein-Affi-gel 10/15 at 20 ml/hour and then washed with column buffer until the absorbance (280 nm) returned to baseline. The flow-through eluent was collected in 10 ml fractions. The column was then eluted with 0.1 M glycine, pH 2.8 at an increased flow rate of 50 ml/hour. Four ml fractions were collected into tubes containing 1 ml of 1 M Tris, pH 8.5, which was used to neutralised the eluted antibody. The column was regenerated with 50 ml of column buffer containing 0.02% sodium azide and stored at 4°C. The absorbance of each fraction was measured (280 nm) to determine the antibody concentration, and then dialysed with 0.01 M PBS containing 0.15 M NaCl, pH 7.4 to remove any unwanted salts and reduce the 4 ml volume. The purified antibody was stored in 500 µl aliquots and stored at -80°C. Peak fractions and column wash (at 1:12.5 & 1:25 dilution) were tested on 20 µg of protein from human CSF and 40 µg of protein from ovine cortex and rat brain to show immunoreactivity to β-trace protein by immunoblot analysis and chemiluminescence as previously described.

4.2.5.2 Pre-adsorption Experiment

To verify whether the antiserum bound exclusively to the peptide antigen, the antiserum (1:2,000 dilution) was incubated with 50 and 500 µg/ml the of P1 peptide for two hours at room temperature. Five µg of protein from adult human CSF and 40 µg of protein obtained from a fetal sheep hypothalamus (135 days gestation) was screened using the antiserum pre-adsorbed with two different concentrations of P1 peptide by immunoblot analysis and chemiluminescence as previously described. 'Virgin' antiserum that had not been incubated with additional P1 peptide was used as a positive control.

4.2.6 Statistical analysis

All data are presented as mean±sem. A one way analysis of variance was used to compare differences in levels of β-trace at different fetal ages. Where significant effect of age was detected, the Student-Newman-Keuls test was used to determine differences at each time point.

4.3 Results for Antibody Production and Immunoblot Analysis

4.3.1 Peptide Synthesis and Purification

Under the conditions described, the purified P1 peptide was eluted from the analytical RP-HPLC column at approximately 35 minutes as a single sharp peak (see Fig. 4.2). Peptide P2 was eluted at approximately 34.5 minutes. Mass spectrometry analysis of the P1 and P2 provided monisotopic molecular weights (averaged over isotopes) of 2,276.12 and 1,594.74 Da respectively. These molecular weights were comparable to the expected molecular masses (2,276.75 and 1,593.89 Da respectively) calculated by the addition of the specific molecular weights of the individual amino acid residues. Thus, it was confidently concluded that the synthetic peptides P1 and P2 had been successfully assembled by Fmoc chemistry and possessed the desired amino acid sequences. The synthesis and purification procedures yielded 21.9 mg of P1 and 65.0 mg of P2 which were subsequently used for immunisation, screening of serum by ELISA, and column purification.

4.3.2 Enzyme Linked Immunoabsorbent Assay

Every bleed obtained from the New Zealand White rabbits was evaluated by ELISA for immunoreactivity towards the original peptides. Serial dilutions of the antiserum were made and showed a dose dependant decrease of binding to the synthetic peptide. Only one of the twelve rabbits which was immunised against peptide fragment P1 produced a

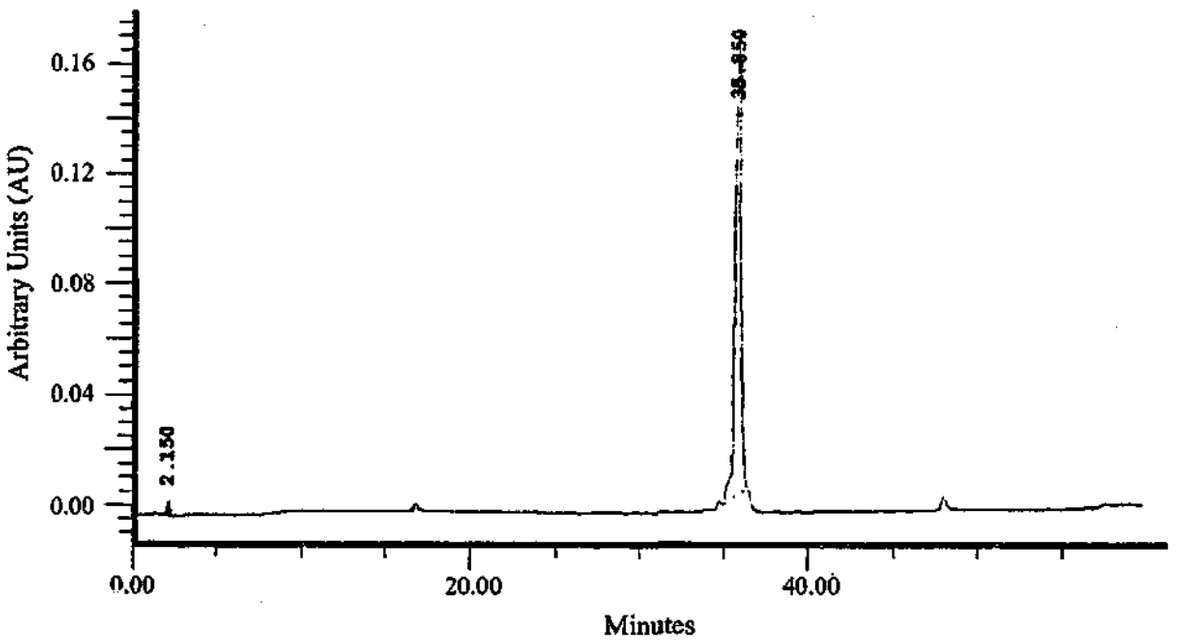


Figure 4.2
Sample of the crude synthesised peptide was purified using an preparative reverse phase-high performance liquid chromatography (RP-HLPC) column. Fractions exceeding an absorbance of 0.10 AU were collected and analysised using an analytical RP-HPLC column. The profile of the purified P1 peptide exhibited a single sharp peak eluted at approximately 35 minutes. The molecular weight of the purified P1 peptide obtained by mass spectrometry analysis (2,276.12 Da) was almost identical to the calculated molecular weight calculated (2,276.75 Da), confirming the peptide was correctly synthesised with the desired amino acid sequence.

significant antibody titre to the respective peptide, giving a titre of approximately 1 in 12,800 (Fig. 4.3a). No immunoreactivity was observed between peptide and pre-immune serum, suggesting the pre-immune serum was free of immunoglobulins that reacted non-specifically with the peptide. Bleeds from rabbits immunised with P2 when screened by ELISA using respective peptide exhibited antiserum titres which did not exceed 1 in 6,400 (Fig. 4.3b), which suggests that the 13-mer peptide was too short to produce a sufficient quantity of antibodies.

4.3.3 Immunoblot Analysis

4.3.3.1 Antiserum Validation

Optimal dilutions of the primary anti-P1 antiserum and secondary antibody conjugated to horseradish peroxidase were established (Fig. 4.4). Various dilutions of primary anti-P1 antiserum (1:200 and 1:2,000) were tested on human CSF and ovine fetal hypothalamus using a secondary antibody dilution of 1:20,000 (Fig. 4.4a). Using the optimal primary antiserum dilution of 1:2,000, different dilutions of the secondary antibody (1:5,000, 1:10,000 and 1:20,000) was tested on the same tissue samples. The optimal dilution of primary antiserum was 1:2,000 and secondary antibody was 1:10,000, and used in subsequent immunoblot analyses.

Figure 4.5 shows the detection of β-trace protein in several samples using the antiserum raised against the entire human β-trace protein (Fig. 4.5a) and the P1 peptide fragment (Fig. 4.5b). Different tissues were screened by immunoblot analysis to validate each antiserum; adult human CSF and rat brain tissue samples were selected as positive controls. Ovine liver and muscle were tested as negative controls since β-trace has been reported to be virtually undetectable in these tissues (Olsson and Nord 1973). Ovine CSF, CP, hypothalamus and cortex were examined as test tissues.

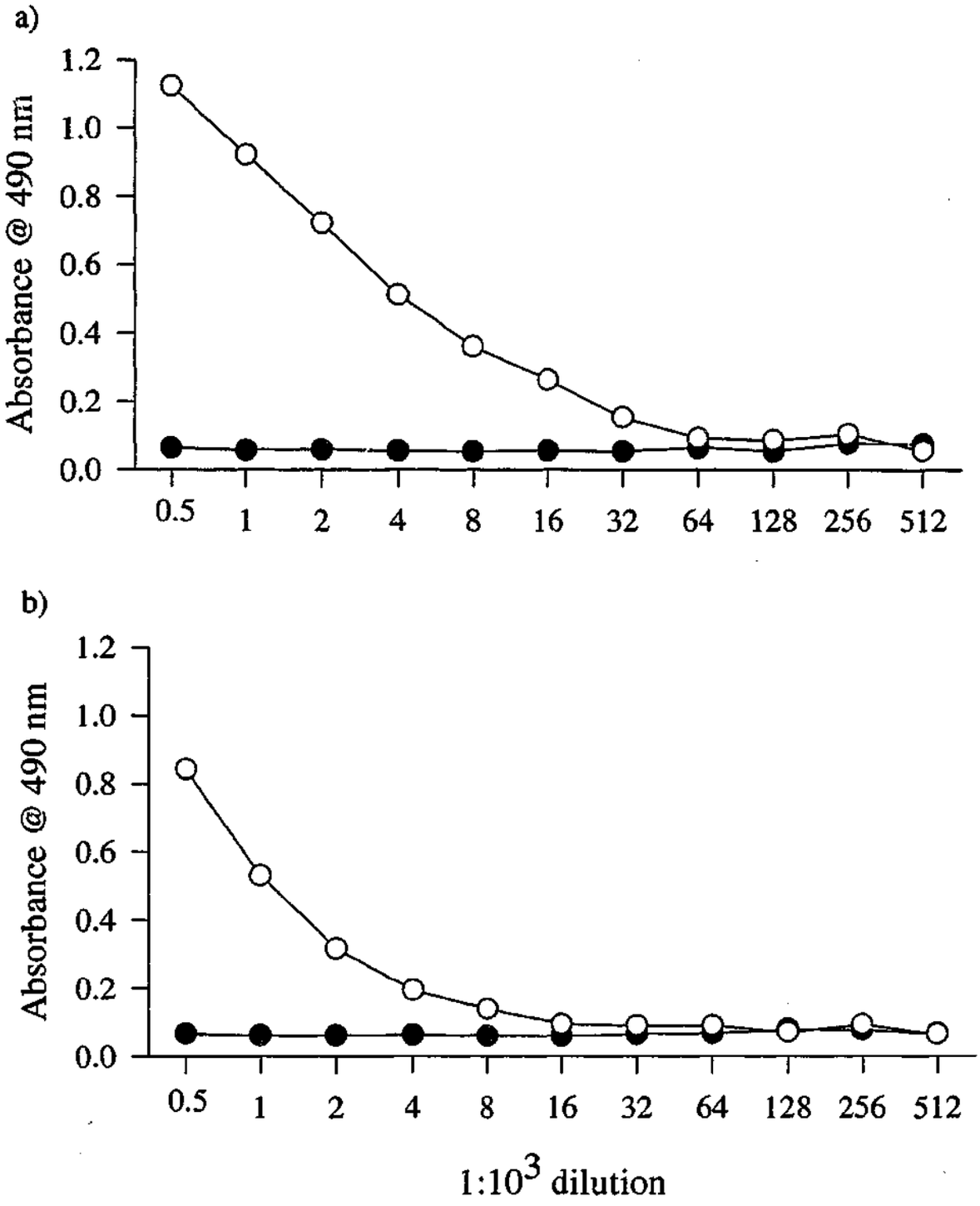


Figure 4.3
Results of enzyme linked immunosorbent assay testing the titre of post-immune serum (yellow) against pre-immune serum (blue) from a rabbit immunised with peptide P1 (a) and a rabbit immunised with peptide P2 (b).

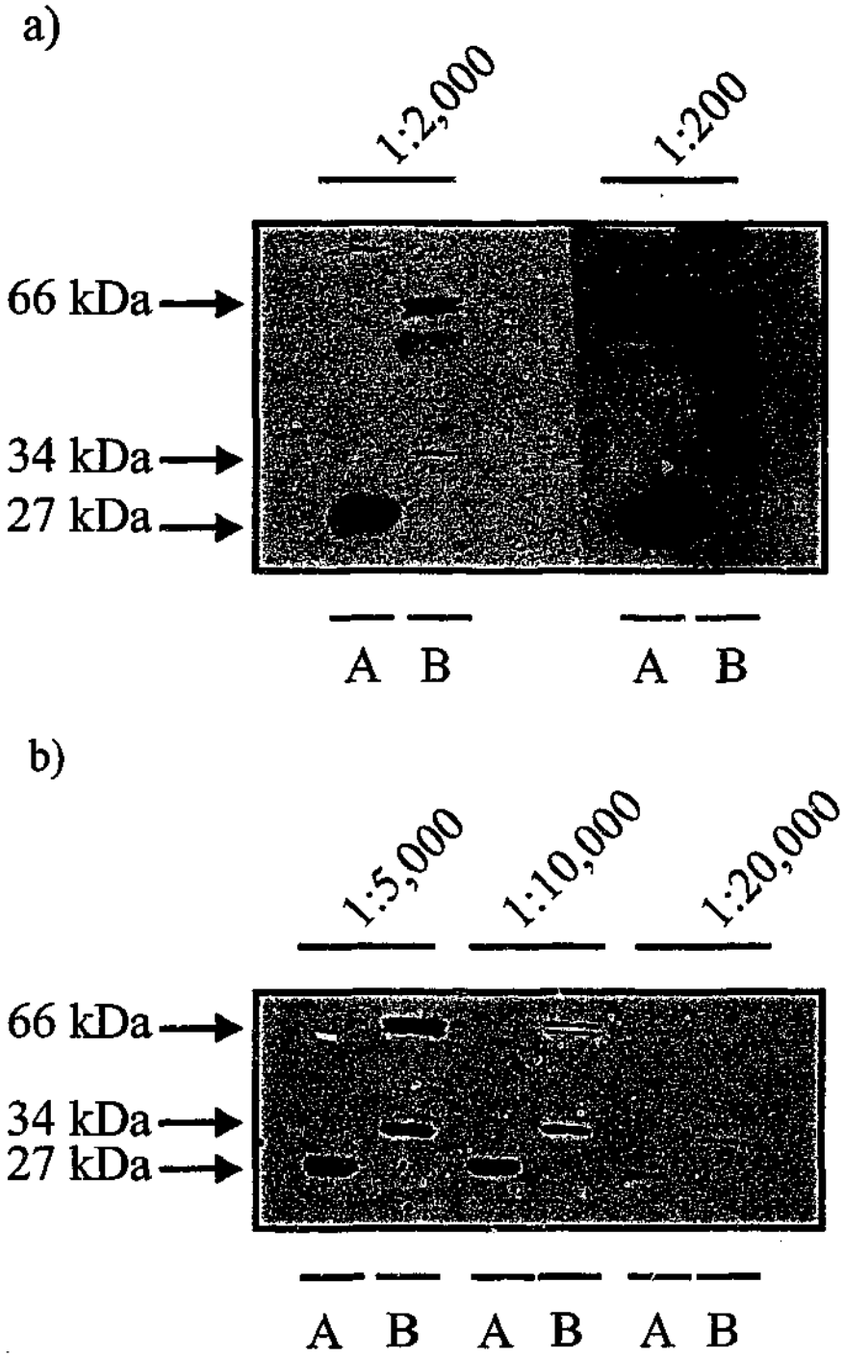


Figure 4.4

(a) Various dilutions of anti-P1 antiserum (1:2,000 and 1:200) were tested on 20 μg of protein from human cerebrospinal fluid (Lane A) and 40 μg of protein from ovine fetal hypothalamus at 135 days gestation (Lane B) by immunoblot analysis. Results showed the optimal primary antiserum dilution to be 1:2,000. The secondary antibody was used at a 1:20,000 dilution.

(b) Using the determined optimal primary antiserum dilution, various dilutions of the secondary antiserum conjugated to horse radish peroxidase (1:5,000, 1:10,000 and 1:20,000) were tested on 5 μg of protein from human cerebrospinal fluid (Lane A) and 40 μg of protein from ovine fetal hypothalamus (Lane B). Optimal secondary antibody dilution was shown to be 1:10,000.

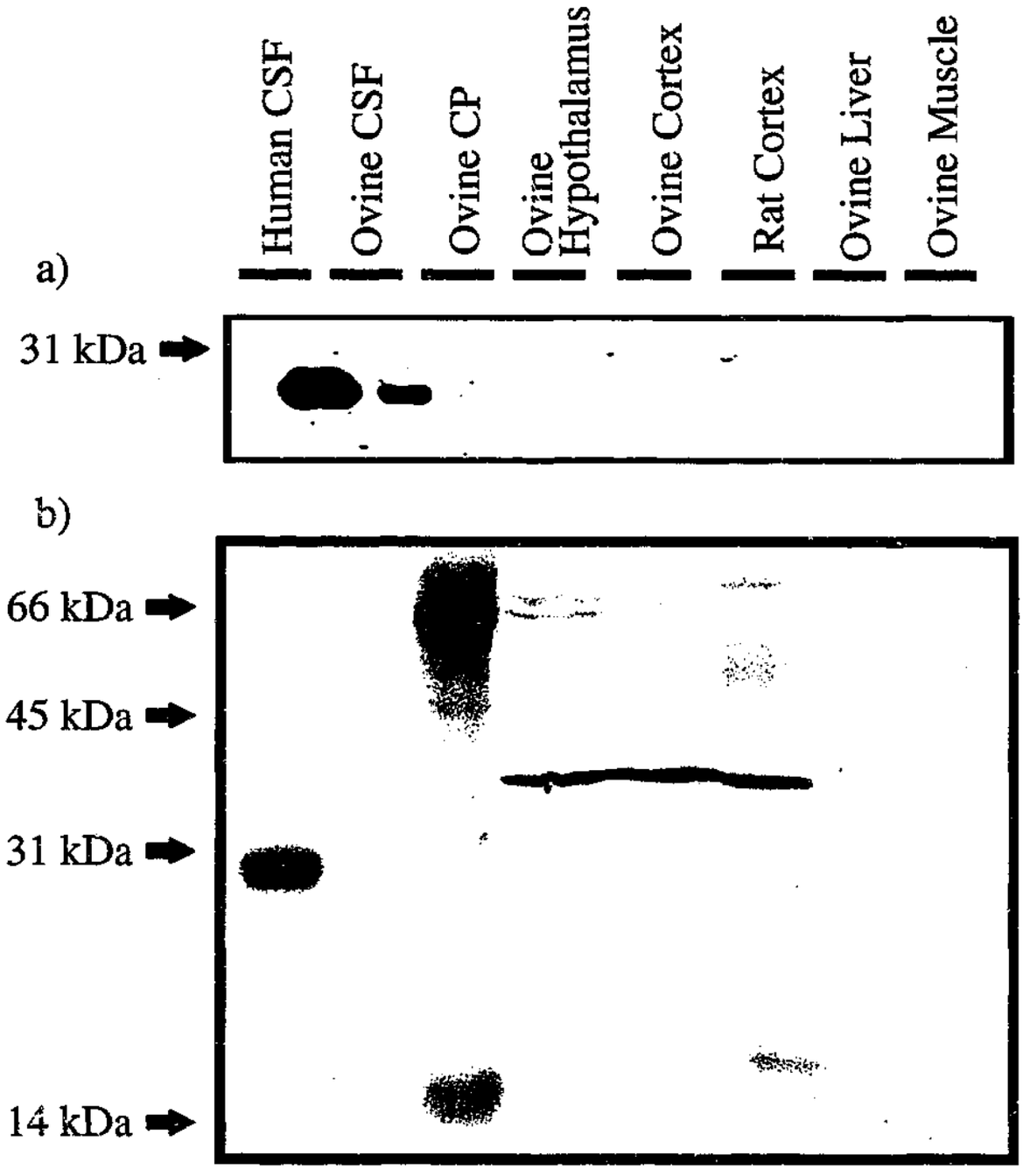


Figure 4.5
(a) β-trace was detected at 27 kDa in 20 μg of protein in adult human cerebrospinal and 40 μg of protein in ovine cerebrospinal fluid using affinity purified antiserum raised against entire β-trace protein isolated from human cerebrospinal fluid, no non-specific binding was observed in other tested tissue.
(b) In comparison, β-trace protein was expressed at 27 kDa in human but interestingly not in ovine cerebrospinal fluid using antiserum raised against the synthetic β-trace peptide fragment (P1). High levels of binding to an unknown protein at 34 kDa was observed in ovine hypothalamus, ovine cortex and adult rat cortex. The non-specific binding was observed in ovine CP at 66 kDa and 14 kDa. All ovine and rat samples contained 40 μg of total protein. Ovine tissues were sampled from 135 day gestation fetus.

Using the human β-trace antiserum, an immunoreactive protein band was detected in both adult human CSF and ovine fetal CSF. The signal occurred at a molecular weight of approximately at 27 kDa, which corresponds to previously documented molecular weight of the investigated protein (Fig. 4.5a; Harrington *et al.* 1993). However, no β-trace immunoreactivity was detected in ovine fetal CP, hypothalamus, and cortex, or in the cortex of the adult rat brain using this antiserum (Fig. 4.5a).

The same tissues were then screened by immunoblot analysis using the β-trace antiserum raised against the synthetic P1 peptide (Fig. 4.5b). Consistent with the observations using the human β-trace antiserum, the anti-P1 antiserum detected a protein of approximately 27 kDa molecular weight in human CSF. However, contrary to previous results, the anti-P1 antiserum did not detect detect a 27 kDa protein in the ovine CSF. In the rat cortex, the anti-P1 antiserum reacted with a protein of a higher molecular weight (~ 34 kDa). A distinct band at the same molecular weight was also detected in the fetal sheep hypothalamus and cortex at 135 days gestation. In contrast, no signal was observed in the ovine fetal CP, liver or muscle at 135 days gestation. Other signals were also observed in the ovine and rat brain tissue samples at ~66 kDa and ~14 kDa. The signals at these molecular weight were particularly high in ovine fetal CP.

All remaining pre- and post-immune bleeds from this rabbit were tested on ovine hypothalamic tissue from a 135 day gestation fetus (Fig. 4.6). All bleeds, excluding the pre-immune and the last bleed 5, exhibited a strong signal at 34 kDa.

The absence of a signal at 27 kDa (the expected size of the β-trace protein) and the unexpected presence of the 34 kDa protein, suggested that further analysis of the anti-P1 antiserum was required. Samples of the antiserum were either purified by affinity column or subjected to pre-adsorption with different concentrations of the P1 peptide. After affinity column purification, two peak fractions (fraction 11 & 12) were eluted containing protein concentrations of 115.0 μg/μl and 60.7 μg/μl, respectively, ideally 100 - 500 μg/μl is expected. The immunogenicity of each purified fraction was tested (Fig. 4.7) on 20 μg of protein in adult human CSF (Lane A), 40 μg of protein in ovine fetal hypothalamus (Lane

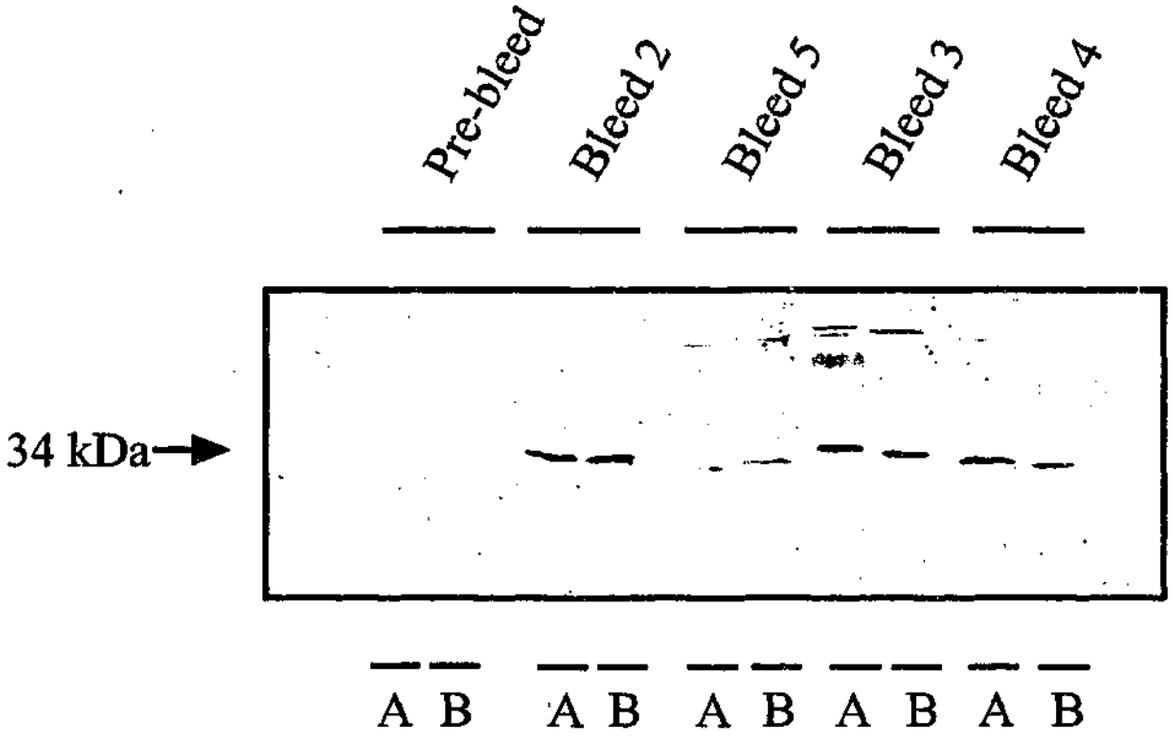


Figure 4.6
Immunoblots comparing the immunoreactivity of the pre-and post-immune serum from rabbit (immunised with P1 peptide fragment) in 40 μg of protein in fetal ovine hypothalamus of 135 days gestation (Lane A) and 40 μg of protein in rat cortex (Lane B). An unidentified protein of 34 kDa was immunoreactive with the post-immune serum but not with the pre-bleed, suggesting the antibody specific to the 34 kDa antigen was absent from the serum until after the immunisation procedure commenced.

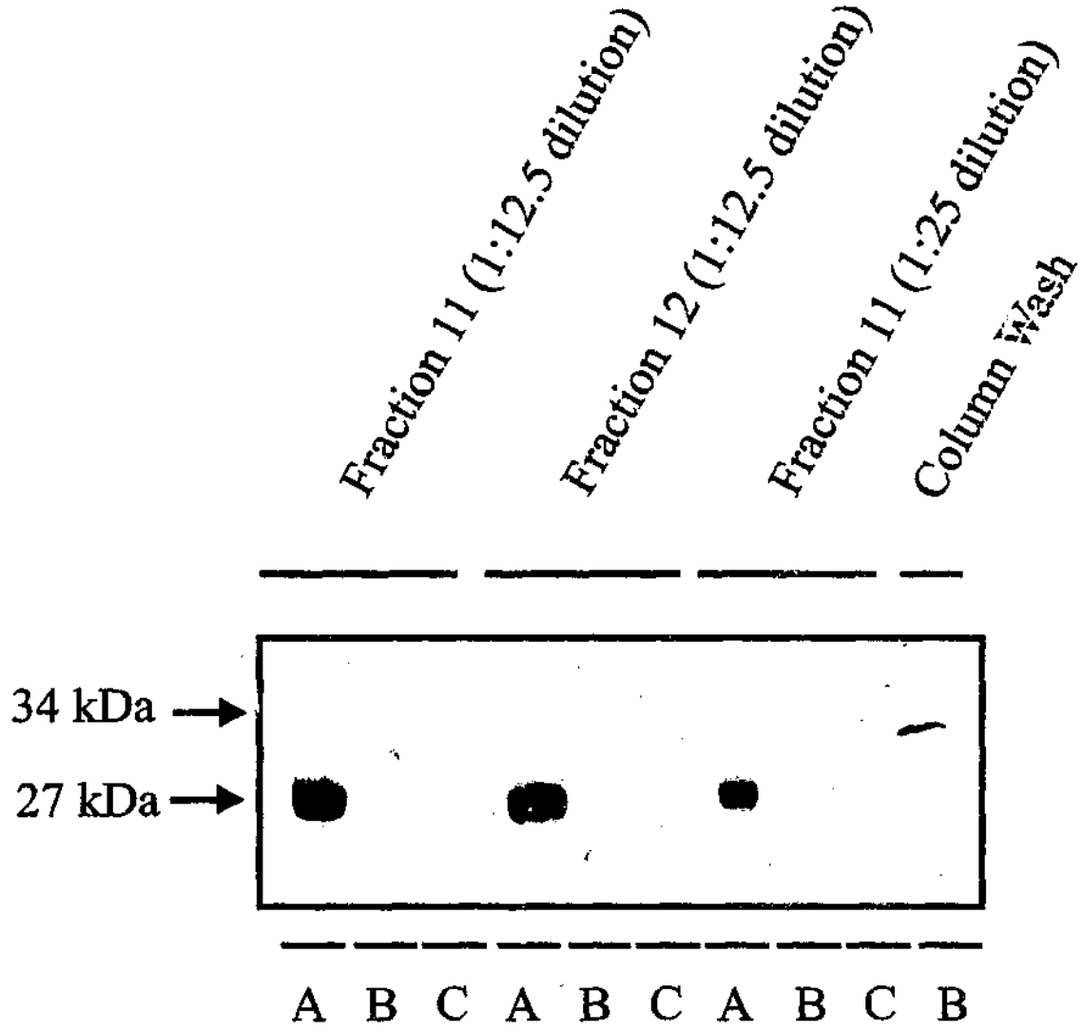


Figure 4.7
Immunoblot showing the expression of β-trace protein at 27 kDa in 20 μg of protein in adult human cerebrospinal fluid (Lane A) using purified anti-P1antiserum eluted from affinity column in Fraction 11 and 12 at different dilutions (1:12.5, 1:25). The purified antiserum did not detect any β-trace-like proteins specific to the P1 peptide in 40 μg of protein in ovine fetal hypothalamus of 135 days gestation (Lane B) nor 40μg of protein in adult rat cortex (Lane C).
An unidentified protein was detected at 34 kDa in ovine hypothalamus using the column wash as the primary antiserum, suggesting the antiserum prior to column purification contained non-specific antibodies that are immunogenic to antigens other than the immunised peptide.

B) and 40 μg of protein in adult rat cortex (Lane C). As anticipated a distinct signal was observed at 27 kDa in human CSF; furthermore the intensity of each signal was consistent with the corresponding dilution of the antibody (Fraction 11 at 1:12.5 and 1:25 and Fraction 12 at 1:12.5 dilution). However, no immunogenic proteins were observed in the brain tissue homogenates from the adult rat cortex and fetal hypothalamus.

Comparative immunoblot analysis was also performed using the column wash that unexpectedly bound to a polypeptide at approximately 34 kDa (Fig. 4.7). This suggests that the column wash contained an antibody that did not bind to the peptide conjugated in the resin column, and therefore the antiserum contained non-specific antibodies that are reactive with antigens other than the peptide used as the antigen.

The anti-P1 antiserum was pre-adsorbed with 500 and 50 μg/ml of P1 peptide, and then tested on 5 μg of protein in adult human CSF (Fig. 4.8, Lane A) and 40 μg of protein in ovine fetal hypothalamus at 135 days gestation (Fig. 4.8, Lane B). Consistent with previous results, the pre-adsorbed antiserum did not bind to any proteins present in the human CSF, whereas control antiserum bound strongly to a protein that had the molecular weight of 27 kDa. However, in fetal ovine hypothalamic tissue both the pre-adsorbed antiserum and control antiserum not pre-absorbed with peptide exhibited signal at approximately 34 kDa. Since an observed signal was unexpected using antiserum pre-absorbed with immunogenic peptide, these results suggest that the antiserum was immunoreactive against proteins other than the desired peptide, and that the observed proteins at 66 kDa and 34 kDa are non-specific signals.

All serum taken from rabbits immunised with P2 peptide were tested on human CSF, fetal ovine CSF and hypothalamus and adult rat brain by immunoblot analysis and produced no positive signals (data not shown).

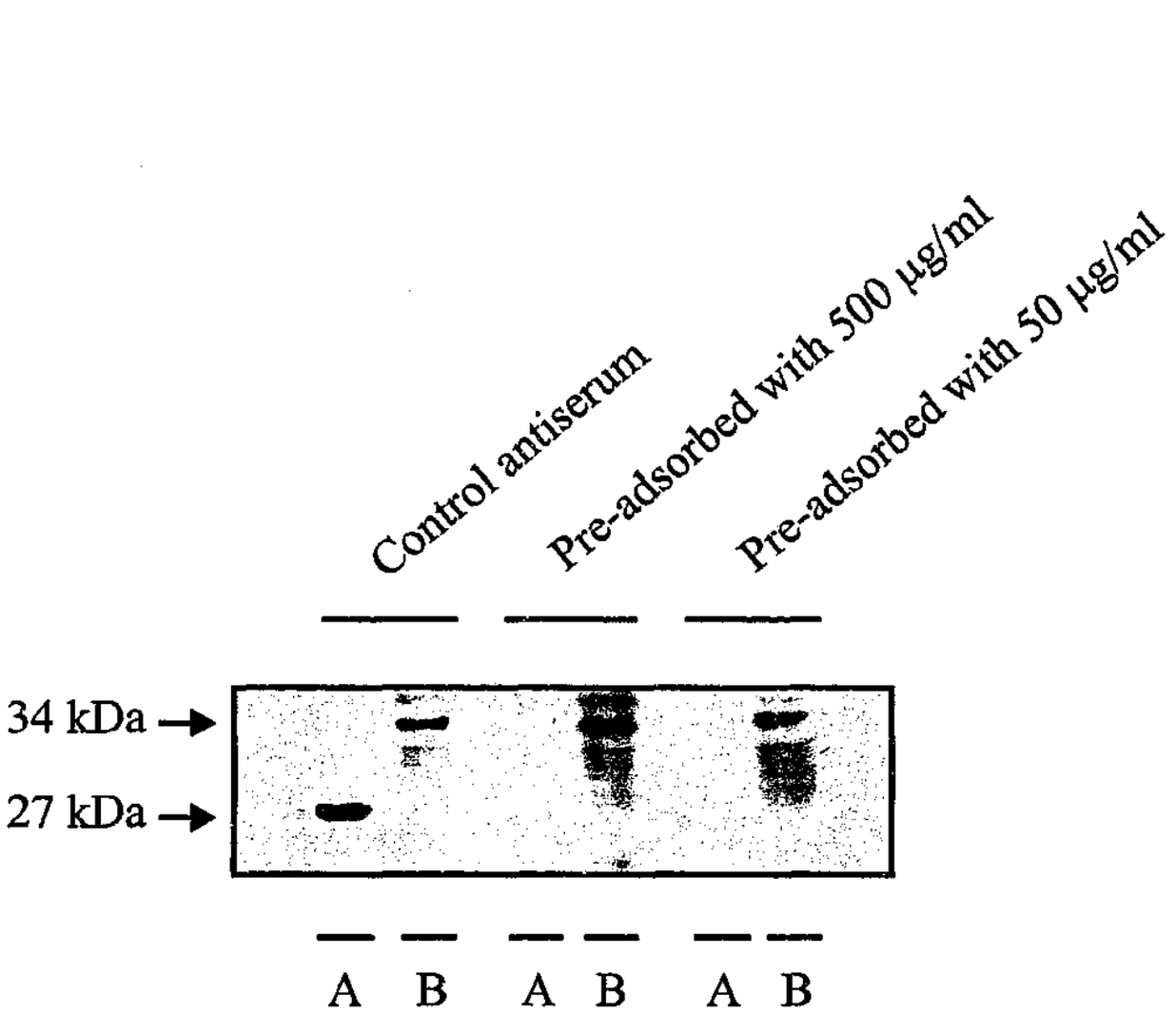


Figure 4.8
Immunoblot comparing the immunoreactivity of control anti-P1antiserum with anti-P1 antiserum pre-absorbed with 50 and 500 µg/ml of P1 peptide in 5µg of protein in human cerebrospinal fluid (Lane A) and 40 µg of protein in fetal ovine hypothalamus of 135 days gestation (Lane B). The control antiserum detected proteins of 27 kDa and 34 kDa in molecular weight in human cerebrospinal fluid and ovine hypothalamus respectively. As expected the 27 kDa protein in human cerebrospinal fluid was no longer detected after the antiserum was saturated with peptide. However, the signal at 34 kDa was still evident suggesting the antiserum is immunoreactive to other antigens that are not specific to the P1 peptide.

4.3.3.2 Ontogenetic Study

Further work was therefore done using only the human β-trace antiserum. β-trace protein was detected at 27 kDa in ovine fetal CSF at 125 and 135 days gestation, but not at 90 days gestation (Fig. 4.9a). The level of β-trace present at 125 and 135 days gestation was significantly greater than at 90 days gestation (Fig. 4.9b; *P<0.05), which showed only signal background. Unexpectedly, no β-trace-like protein was recognised by this antiserum in the ovine CP at any fetal age (data not shown).

4.4 Methods for Probe Preparation and RNA Blot Hybridisation Analysis

4.4.1 Preparation of Prostaglandin D Synthase Probe

A 426 bp fragment corresponding to the nucleotides 304 - 730 of the cDNA for rat lipocalin-type PGDS gene was generously provided by Dr. Luis Garcia-Fernandez (Instituto de Investigaciones Biomedicas, Madrid, Spain). The sequence of cDNA was inserted into the plasmid vector pCR™-II (TA Cloning Kit, Invitrogen, Groningen, Netherlands) and was to be used as a template for RNA probes.

The cloning vector pCR™-II is a ~4.4 Kb phagemid, which features 21 unique restriction sites, a ColE1 replicon, genetic markers for selection by ampicillin and kanamycin resistance, *lacZ* gene for blue/white screening and T7 RNA polymerase promoters for *in vitro* transcription (Fig. 4.10).

The plasmid DNA was originally received absorbed onto Whatman filter paper. A small sample of the filter paper was cut using a sterile blade and the plasmid was eluted with 50 µl of autoclaved 10 mM Tris, pH 7.6. The paper and buffer mixture was vortexed and the

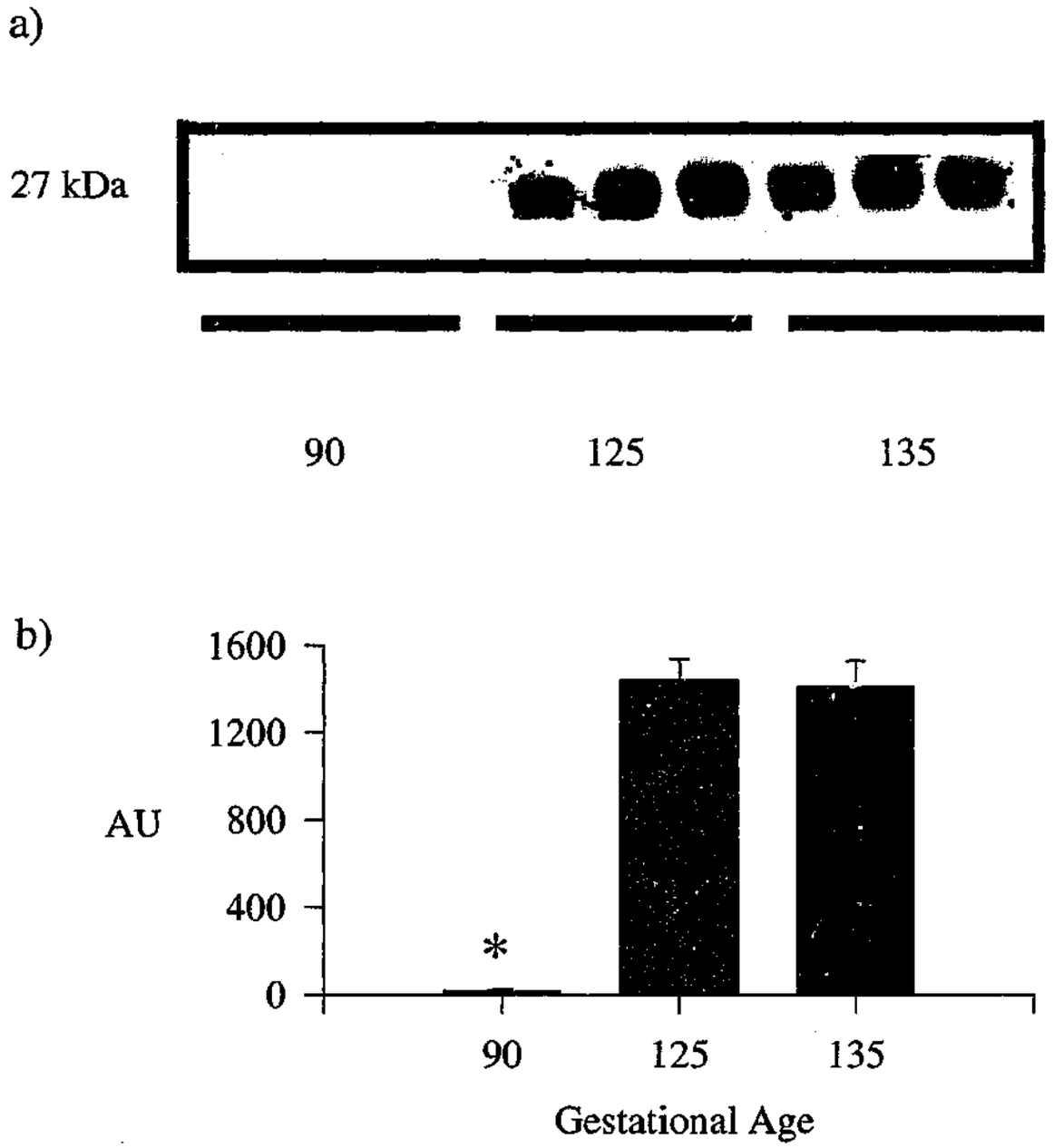


Figure 4.9
(a) Expression of β-trace protein (27 kDa) in ovine cerebrospinal fluid samples at 90, 125 and 135 days gestation by immunoblot analysis using affinity purified antiserum raised against the entire β-trace protein isolated from human cerebrospinal fluid.
(b) Densitometric analysis of β-trace in ovine cerebrospinal fluid samples at 90, 125 and 135 days gestation. β-trace was hardly detectable at 90 days gestation and significantly increased at 125 days gestation (*$P<0.05$). Results shown as mean±sem. AU = arbitrary units.

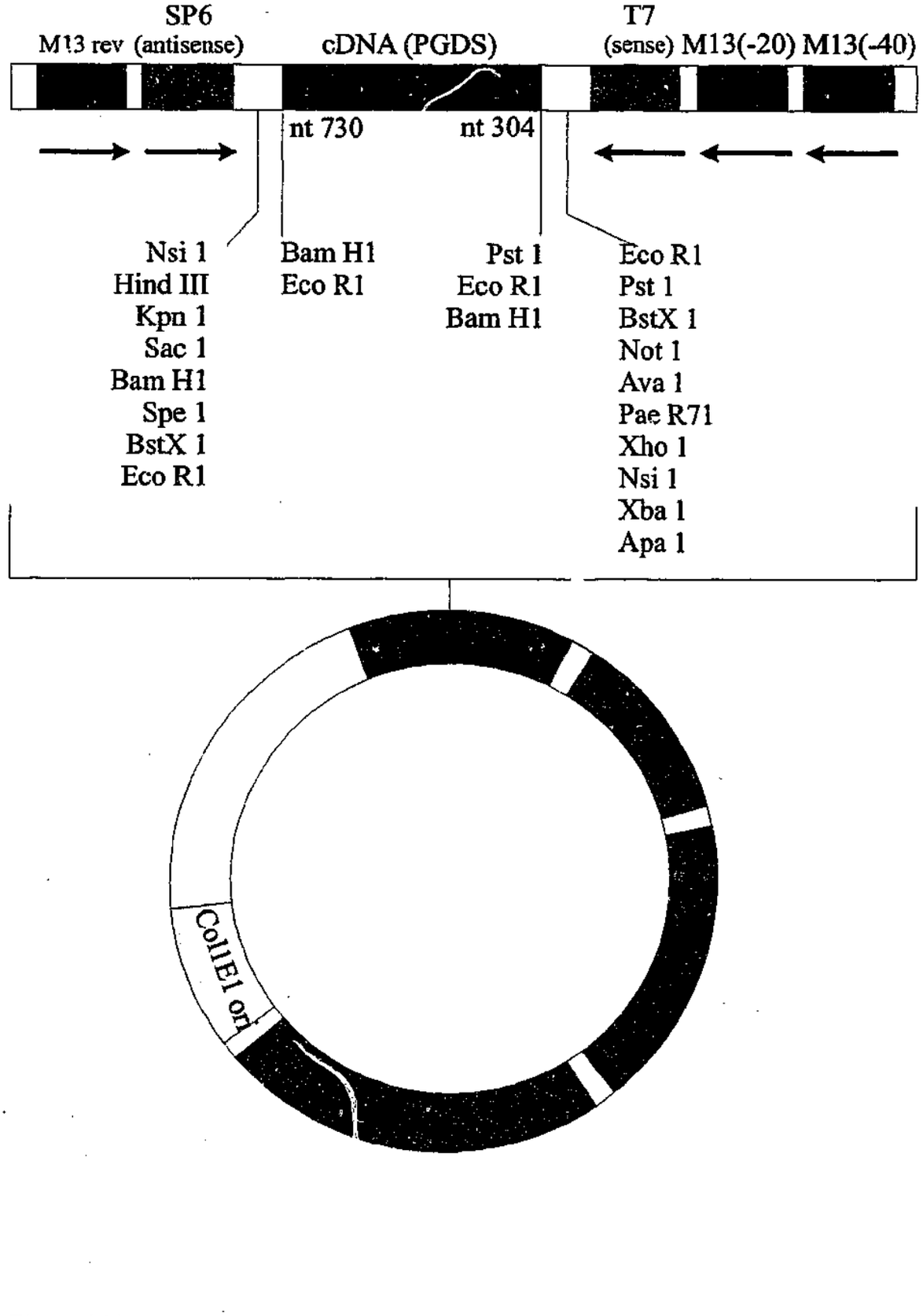


Figure 4.10
Recombinant pCR™ - II (Invitrogen) plasmid with 426 base pair Prostaglandin D Synthase (PGDS) cDNA insert was transformed with supercompetent cells for the preparation of the PDGS probe. nt=nucleotide.

filter was allowed to re-hydrate for 30 minutes. The filter paper was removed and solution evaporated under vacuum. The residual DNA was reconstituted with 10 µl of sterile water and stored at -20°C until used for transformation.

4.4.1.1 Transformation of Competent Cells

Epicurian Coli® XL1-Blue Supercompetent Cells (Stratagene Cloning Systems, CA, USA) were transformed with recombinant pCR™-II DNA using standard protocols. Epicurian cell DNA encoded a tetracycline resistance gene for selection by antibiotic resistance.

Agar culture plates were prepared to enable the selection of transformed colonies by blue/white colour and antibody resistance. Plates were prepared using 121 µl of previously autoclaved LB broth (0.2 M NaCl, 1% tryptone, 0.5% yeast extract) containing 12 µg/ml tetracycline and 60 µg/ml ampicillin. After the plates were set, they were coated with a solution of 75 µl 5-bromo-4-chloro-3-indolyl-β-D-galatoside (X-gal; 20 mg/ml in dimethylformamide, Progen Industries Ltd, QLD, Australia) and 4 µl of isopropylthio-β-D-galactoside (IPTG; 200 mg/ml in sterile water, Progen). The plates were dried in a 37°C incubator for 3 - 4 hours.

For the introduction of phagemid into bacterial cells, the frozen supercompetent cells were firstly thawed on ice. When completely thawed 50 µl of the cells was added to 15 ml polypropylene tubes (Falconer 29059) containing 0.85 µl of denaturing agent β-mercaptoethanol (freshly diluted with dd-H_2O to 1.42 M). The reaction tubes were incubated for ten minutes on ice with gentle swirling every two minutes. The competent cells were incubated on ice for 30 minutes after the addition of 2.5 µl vector/plasmid. Control tests was performed by incubating the reaction tubes in the absence of DNA plasmid, or with 1 µl of control DNA (pUC18). To increase the permeability of the cell membrane and permit entry of the plasmid into the bacterium, the competent cells were heat-shocked for exactly 45 seconds in the water bath set at 42°C. The transformed bacteria were placed on ice for 2 minutes, and then allowed to warm at room temperature. The

contents of each tube were added to 450 µl of autoclaved SOC medium (10 mM $MgCl_2$, 10 mM $MgSO_4$, 8 mM NaCl, 2% tryptone, 0.5% yeast extract, 0.4% sucrose, preheated to 42°C) and incubated at 225 - 250 rpm for 1 hour at 37°C, in an orbital incubator (Gallenkamp, Leighbrough, UK). Two hundred µl of the transformed DNA were spread on the ampicillin plates using a sterile spreader, allowed to dry and then left inverted overnight at 37°C.

The pCR™-II vector carries a segment of DNA for the amino-terminal fragment of β-galactosidase which reacts with the chromogenic substrates of the coating buffer (X-gal and IPTG) to form blue colonies. However, successful insertion of the foreign DNA into the polycloning site of the circular plasmid inactivates the fragment of β-galactosidase, and therefore bacteria carrying recombinant plasmids and inserts give rise to white colonies. Hence a single white colony was selected for the growth of the bacterial phage in 25 ml of LB broth containing tetracycline and ampillicin. After inoculation the medium was incubated in an unsealed aerated flask at 37°C, overnight in an orbital incubator at 250 rpm.

4.4.1.2 Extraction of Recombinant DNA Plasmid and Restriction Enzyme Digest of Vector

After amplification of the recombinant pCR™-II and PGDS insert by inoculation of transformed bacterial cells in LB broth, the plasmid DNA containing the insert was extracted from the culture using a Wizard™ Miniprep DNA Purification System (Promega, WI, USA). In brief, 3 ml of the broth was centrifuged, and the medium was discarded. The remaining pellet was resuspended and the membranes lysed using an alkaline lysis solution (0.2 M NaOH plus 0.1% SDS). The DNA ligand was neutralised (4.09 M guanidine HCl, 0.795 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) and dried down under vacuum and the purified DNA was reconstituted with 50 µl of sterile water and stored at -20°C.

Samples of purified DNA (2 μg) were subjected to 3 hour incubations at 37°C with different combinations of restriction enzymes. The digested DNA fragments were analysed by size relative to a DNA ladder (D-15, 2645 – 36 bp, Novex, CA, USA) by electrophoresis (HE33, Hoefer Scientific Instruments) using 1% agarose minigel (2 M Tris base, 50 mM EDTA, 5.71% glacial acetic acid) stained with ethidium bromide (5 μg/ml). This showed that the insert was excised from the plasmid with the restriction enzyme EcoR1 (Gibco, Life Technologies, MD, USA), as confirmed by the presence of two DNA bands under UV light corresponding to the size of the plasmid and insert, respectively.

The remaining culture of transformed cells in LB broth were boosted by adding 400 ml of fresh tetracycline and ampicillin resistant LB broth and grown overnight at 37°C. After incubation the DNA was isolated from 350 ml of the inoculated broth using the Wizard™ Maxiprep DNA Purification System (Promega), which purified the recombinant DNA from the bacteria by employing the same separation principles mentioned above. The extracted DNA was resuspended in 1 ml of sterile water. The concentration of DNA was determined and then stored at -20°C. Bacterial stocks were made from remaining 50 ml of LB broth, by centrifugation at 2,000 rpm for 10 minutes. The pellet was resuspended with 3 ml of supernatant and separated into 700 μl aliquots. Three hundred μl of glycerol was added, the stock was mixed and stored at -20°C.

The concentration of extracted DNA was determined using a GBC UV/VIS 918 Spectrophotometer (GBC Scientific Equipment, USA). Five μl of isolated DNA purified by Maxiprep was diluted to 100 μl with TE Buffer (10 mM Tris, 1 mM EDTA) and read at wavelengths of 260 and 280 nm. Since the optical density of 50 μg/ml of DNA is equivalent to 1 at 260 nm, the concentration of DNA tested can be determined by formula,

$$\text{DNA } (\mu g/ml) = 50 \times \text{dilution factor} \times OD_{260nm}.$$

The relative purity of a sample was estimated by the ratio,

$$OD_{260nm}/OD_{280nm}.$$

The isolated DNA vector and insert yielded a relative purity of 1.8. This calculated ratio was comparable to the ideal relative purity ratio of 1.8 – 2.0 (Sambrook *et al.* 1989).

One hundred µg of the purified vector and insert was treated with 20 µl of EcoR1, incubated at 37 °C for two hours. The digested PGDS insert was separated from the phagemid vector by electrophoresis, in five combined wells in a high melting point 1% agrose gel stained with ethium bromide (5 µg/100 ml). After electrophoresis at 60 V for 1.5 hours, two distinct bands corresponding to the plasmid and insert DNA were observed under UV light.

Under UV light the gel slice containing the ~440 bp insert was excised, weighed and then placed in a dialysis bag pre-soaked with TAE buffer (50 mM EDTA, 2 M Tris-base, 6% glacial acetic acid). All air bubbles were removed and the bag was sealed with clips and electroeluted at 50 V for 30 minutes. The polarity of the electrodes were reversed to prevent DNA adhering to the interior walls of the dialysis bag. Diffusion of DNA from the gel into the buffer was checked under UV light. The buffer was then carefully transferred to an eppendorf tube using a pipette. Two equivalent volumes of 100% ethanol and 3 M sodium acetate was added, mixed thoroughly and stored at -20°C. The following day the PGDS probe was concentrated and desalted using a ethanol precipitation procedure. Briefly, the DNA was centrifuged for 10 minutes at 14,000 rpm. The supernatant was discarded and the pellet was reconstituted with 750 µl of 70% ethanol and subsequently centrifuged again for another two minutes. The ethanol was completely evaporated and the final pellet was resuspended in 50 µl of TE buffer and stored at -20°C until future oligolabelling.

4.4.2 Identification of Prostaglandin D Synthase mRNA by RNA Blot Hybridisation

To prevent mRNA degradation all working solutions need to be autoclaved for sterility and glassware baked at 180°C to remove residual RNAse enzymes.

4.4.2.1 Tissue Collection and Preparation

Adult rat brain and ovine fetal hypothalamic and cortical tissue were collected using procedures previously described (Section 3.2.2). The total mRNA was extracted from the tissue blocks using a standard Tri Reagent™ (Sigma) protocol. This product, a mixture of guanidine thiocynate and phenol separates homogenised tissue into three phases: an aqueous RNA phase, interphase containing DNA and organic phase containing proteins. In short, the 300 mg of the tissue pulverised on dry ice was homogenised using an Ultra-Turrax with 6 ml of Tri-reagent and left at room temperature for five minutes. To remove the proteins 1.2 ml chloroform was added and mixed by inversion and then incubated for 3 minutes at room temperature. The solution was placed in sterile Cortex™ (Beckmann Coulter) tubes and centrifuged at 12,000 *g* for 15 minutes at 4°C. Once the aqueous layer was removed, placed into a clean Cortex tube and incubated at room temperature for 10 minutes with 3.0 ml isopropanol, to precipitate the RNA. After centrifugation at 12,000 *g* 15 minutes at 4°C, the supernatant was discarded and the remaining RNA pellet was washed with 6.0 ml of 75% ethanol, vortexed and centrifuged at 7,500 *g* for ten minutes to remove salts and small inorganic material. Each pellet was resuspended with 200 µl of DEPC water. A 5 µl sample was diluted in sterile water (1:150) and the purity ratio was tested as previously described. Due to the variable and low purity ratios observed, each sample was cleaned by standard phenol-chloroform extraction and ethanol precipitation based on the same principles as the RNA extraction, until an acceptable purity was achieved.

4.4.2.2 Electrophoresis and RNA Blot Hybridisation

The total mRNA of each tissue sample was isolated on the basis of size by electrophoresis in agarose gel (containing 37% formaldehyde) using standard protocols. When a potential difference is applied across the gel, the mRNA moves toward the positive electrode since it carries negative charges on the phosphate groups making up the backbone. The distance of which the mRNA migrates through the gel is directly proportional to the size of the mRNA.

Volumes equivalent to 5 µg of rat brain mRNA and 20 µg of ovine hypothalamic and cortical mRNA, were evaporated under vacuum, and resuspended with 5.5 µl of diethyl pyrocabonate (DEPC) treated water for 30 minutes on ice. Prior to loading into the gel, each sample was denatured using 3.5 µl of 37% deionised formaldehyde and 10 µl of deionised formamide, heated for 5 minutes and then immediately iced. Two µl loading buffer (95% formamide, 0.09% xylene cyanol FF) containing 0.09% bromophenol blue, was added to visualise the samples and thus aid loading into the wells. The samples were loaded into the gel, and electrophoresised overnight at 22 V, room temperature (HE99X, Hoefer Scientific Instruments) while the electrophoresis buffer (0.625 mM EDTA, 0.5% MOPS, 0.2% Sodium Acetate Trihydrate) was continuously circulating.

The separated RNA was transferred from the gel onto Duralon UV™ membrane (Stratagen) using standard procedures (Ausubel *et al.* 1997), where the gel and membrane are sandwiched together between beds of weighted Whatmann paper, and RNA migration occurs towards the membrane due to simple capillary diffusion using electrophoresis buffer. Following transfer, the RNA was secured to the membrane by UV crosslinkage (UVC50, Hoefer Scientific Instruments) and stored in a self-sealed bag at -20°C until hybridisation. Blots could be stained with methylene blue (0.04% methylene blue, 5% acetic acid) to illustrate the total transfer and migration of RNA.

4.4.2.3 Oligolabelling of Prostaglandin D Synthase Probe Using [^{32}P]dCTP

The PGDS probe was labelled with [^{32}P]dCTP (Easytide, NEN Research Products, Dupont, USA) using a random priming kit (Pharmacia). In short, 50 µl of DNA was denatured by boiling for 3 minutes and then iced immediately. Fifty µl of PGDS probe, 10 µl of reagent mix (containing dATP, dGTP and ddTTP), 5 µl of [^{32}P]dCTP, 1 µl of polymerase Klenow fragment was made up to 49 µl with dd-H_2O and incubated for 60 minutes at 37°C. Fundamentally, using the DNA insert as a template, the polymerase enzyme synthesised the labelled probe by utilising nucleotides in the reagent mix and incorporating radioactive dCTP. The labelled oligonucleotide was separated from unincorporated [^{32}P]dCTP by

elution through a NICK™ column (Sephadex® G-50 Grade, Pharmacia) with 10 mM Tris-HCl (containing 1 mM EDTA, pH 7.5). One µl of labelled probe was taken before and after column purification procedure and was added to 5 ml of EcoScint to be counted. Incorporation above 50% was considered acceptable, and the labelled probe was used for hybridisation to the membrane within 7 days.

4.4.2.4 Hybridisation of Prostaglandin D Synthase mRNA and Visualisation by Phosphor-imaging

The labelled PGDS probe was hybridised to the PGDS mRNA transferred onto the membrane using conventional hybridisation techniques (Ausubel *et al.* 1997). The membrane was incubated in 15 ml of prehybrisation solution (0.75 M NaCl, 0.045 M $NaH_2PO_4.H_2O$, 6.25 mM EDTA, 50% formamide, 7% SDS, 0.1 mg of salmon sperm DNA, pH 7.4) for 3 hours at 42°C in a hybridisation oven (Xtron HI 2002, Bartelt Instruments, Australia). The labelled probe was denatured by boiling and subsequent cooling for 5 minutes respectively, then added to hybridisation buffer (same as prehybrisation solution) to achieve a final concentration of 2 x 10^6 cpm/ml. After 48 hour incubation at 42°C, the hybridisation solution was discarded and the unhybridised probe was rinsed off with low stringency washes using SSC (15 mM $NaCl_2$, 1.5 mM sodium citrate) containing 0.1% SDS solution for ten minutes at room temperature, then 2 x 0.5 hour washes at 35°C.

The probed membrane was then sealed in plastic bag and exposed to a pre-blanked storage phosphor-image screen (Molecular Dynamics, USA) for five days at room temperature. Upon exposure the hybridised radioactive probe excited adjacent phosphor molecules of the screen. Upon scanning of the phosphor-image screen by the phosphor-image scanner (Storm, Molecular Dynamics), the screen was exposed to red light, which causes the excited phosphor molecules to emit energy at predominately the intensity of blue wavelength 445 – 550 nm. The image was digitised and stored as a computer image in ImageQuaNT (Molecular Dynamics).

4.5 Results for RNA Blot Hybridisation

The rat PGDS probe was tested on 5 μg of total mRNA from the adult rat brain and 20 μg of total mRNA from the ovine hypothalamus and cortex from a 135 day gestation fetus (Fig. 4.11). Distinct expression of PGDS mRNA was detected in the rat brain sample at approximately 0.8 Kb, which is consistent with previous publications (Beuckmann *et al.* 1996; Garcia-Fernandez *et al.* 1993; Urade *et al.* 1989). No PGDS mRNA was detected in the sampled ovine brain tissue using the PGDS rat probe, despite increasing the amount of total mRNA load (10 to 20 μg) and using lower stringency washes (2 x 0.5 hours at 35°C).

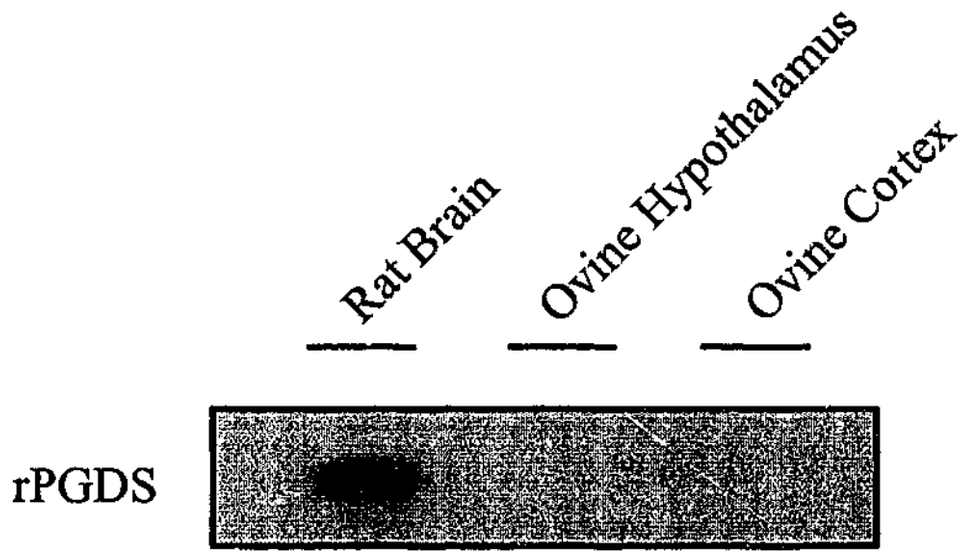


Figure 4.11
Expression of PGDS mRNA in 5μg of mRNA from adult rat brain and 20 μg of mRNA from ovine hypothalamus and cortex from a 135 days gestation fetus . The total RNA was blotted to a nylon membrane and hybridised with a rat PGDS cDNA probe corresponding to nucleotides 304 - 730. Expression of PGDS mRNA was restricted to the rat tissue and was not evident in the tested ovine tissue.

4.6 DISCUSSION

Based on amino acid sequence analysis it has been recently established that brain-type, glutathione-independent PGDS from the rat is structurally homologous to the human β-trace, a protein found in high concentrations in the CSF (Hoffmann *et al.* 1993). Therefore, it has been postulated that PGDS/β-trace in the CSF is enzymatically active, and could produce quantities of PGD_2 (Hayaishi 1997). Furthermore, it has been hypothesised that PGD_2 acts as a neuromodulator and stimulates the PGD_2-sensitive area of the ventro-basal forebrain (Watanabe *et al.* 1985) and the putative sleep centres of the anterior hypothalamus (Matsumura *et al.* 1994) to produce a global, soporific effect on the brain. In the ovine fetus, REM and NREM sleep, as defined by conventional parameters (e.g. ECoG, EMG, EOG) are virtually absent until approximately 120 days gestation, but by 130 days electrocortical activity has fully differentiated into REM and NREM-like activities accounting for ~95% of the time (Dawes *et al.* 1972); the remainder is occupied by brief (minutes) episodes thought to be arousal, or wakefulness (Szeto and Hinman 1985). We endeavoured to establish if PGD_2 and its synthesising enzyme PGDS play a role in initiating and maintaining REM/NREM sleep in the fetal sheep, as demonstrated in the adult rat (Hayaishi 1997).

In this study two antisera and a rat PGDS probe were employed to follow temporal changes in the expression of PGDS/β-trace protein and mRNA respectively in the fetal CNS in relation to the ontogenesis of prenatal sleep. The first antiserum, which was raised against the entire β-trace protein purified from human CSF, detected β-trace expression the both human and ovine CSF samples, but did not identify a protein in fetal brain samples. In comparison, the second antiserum used was raised against a 20 mer peptide fragment common to both the N-terminal of the rat-brain PGDS enzyme and human β-trace protein. It was expected that this antibody would recognise β-trace protein in the sheep brain and CSF, as the chosen sequence is highly conserved across species (Hoffmann *et al.* 1996b; Kuruvilla *et al.* 1991; Watanabe *et al.* 1994). Although the latter antiserum detected β-trace

in human CSF, it did not identify a PGDS/β-trace-like protein in ovine tissue samples. Furthermore, the rat PGDS probe corresponding to nucleotides 304 - 730 also did not hybridise with ovine PGDS mRNA. Hence, the amino acid sequence of PGDS in the sheep may vary considerably from that of the rat and human protein. These results suggest that phylogenic variations in amino acid sequences of PGDS/β-trace protein originating from different species and/or specific tissue are greater than suspected hitherto.

Using the antiserum raised against purified human CSF β-trace protein, it was shown that β-trace was barely detectable in fetal sheep CSF until 125 days gestation in sheep by immunoblot analysis. The functional significance of the delayed appearance of β-trace protein in CSF in late gestational age remains unclear. The absence of β-trace before approximately 125 days gestation may simply be due to the protein not being synthesised by the CP and secreted into the CSF until later ages. Alternatively the appearance of the protein in late gestation may reflect changes which occur in the permeability or clearance mechanisms of the CP or the BBB at this time. However, since the expression of β-trace greatly differs from the general trend of other major serum-derived CSF proteins, which are very high during the early stages (840 mg/100 ml, 30 days gestation) of development and then decline to twice the adult levels by late gestation (50 mg/ml, 125 days gestation; (Dziegielewska *et al.* 1980), the significant increase of β-trace at this certain stage of development may be indicative of the neuronal pocesses that develop at this particular time. Since the first appearance of β-trace in the fetal CSF coincides with the time when sleep states begin to differentiate and start to resemble adult-like sleep states, it suggests that there is an association between the two processes. However, whether β-trace and the onset of sleep is directly related requires further investigation.

Intriguingly, the antiserum that detected β-trace in ovine fetal CSF did not identify a β-trace-like protein in homogenates prepared from the brain or CP. Considering previously documented evidence describing the predominant expression of PGDS/β-trace in the brain and CP (Blodorn *et al.* 1996), the observed absence of β-trace expression in these tissues was unexpected. Several reasons can be given for this apparently anomalous result. Firstly,

taking into account that PGDS is synthesised prenatally in these tissues (as previously demonstrated in Chapter 3), it would be reasonable to assume that these findings suggests in the ovine fetus the PGDS enzyme in the brain (and CP) and the β-trace protein found in the CSF may not have the same amino acid sequence. However other factors, such as the immunogenicity of the PGDS in the ovine tissue, needs to be considered. Perhaps the enzyme was not detectable in CP and brain tissue for technical reasons. For example, the level of the protein may be low in the choroidal epithelium and the brain, due to immediate secretion of the protein into the CSF and brain parenchyma. Hence basal levels in the neural tissue and blood-CSF barrier may be low and below the sensitivity of either the antiserum or the detection system employed.

Similar results have been described in a previous study, where β-trace was also not detected in CP samples of the human neonate using an antiserum raised against two β-trace peptides isolated from the human CSF β-trace sequence (SVSVVETDYD, APEAQVSVQPNFQQD Harrington *et al.* 1993). Taking into account that the CP is considered to be the major source of the β-trace protein in adult CSF (Urade *et al.* 1985b), these results are unexpected and yet to be explained. Localisation of mRNA in ovine tissues will be required to establish whether the CP is a significant source of β-trace in the fetal sheep.

We successfully raised an antiserum (anti-P1) against the 20-mer peptide fragment common in the N-terminal of the human β-trace protein and rat brain PGDS. This anti-P1 antiserum identified β-trace protein from human CSF at the expected molecular weight of 27 kDa and on this basis was assumed to be specific for PGDS/β-trace protein. However, this antibody identified a protein of a molecular weight of 34 kDa in sheep and rat brain samples. Further validation procedures (firstly, pre-adsorbing the antiserum with the peptide; secondly affinity column purification), led to the conclusion that the 34 kDa protein was not likely to be PGDS/β-trace protein. Hence, the anti-P1 antiserum failed to detect the β-trace protein in ovine tissue and CSF, suggesting the N-terminal sequence of the human β-trace amino acid sequence may be markedly different to that in the sheep. This result is consistent with the observation that the rat PGDS probe was unable to hybridise with ovine PGDS mRNA.

Interestingly the 34 kDa protein that was identified by the anti-P1 antiserum was specific to the brain of both the sheep and rat, and was not present in other tissue such as the ovine fetal muscle and liver. The significance of the unknown brain-type protein is unclear. For future studies the protein sequence could be determined by isolation and sequencing techniques, and possibly further identified by searching complimentary amino acid sequences in the GenBank databases.

Elucidating our findings, recent work has confirmed that human and sheep β-trace proteins do in fact have different primary structures, especially within the region from which the synthesised peptide fragment was selected. Upon commencement of this experimental work in July 1996, the amino acid sequence of the ovine β-trace or PGDS had not yet been determined. Therefore, we followed conventional procedures by selecting a specific 20-mer peptide fragment on the basis of the high homogeneity exhibited between three animal species (human, rat and mouse). In April 1999, the amino acid protein deduced from the mRNA sequence of PGDS was characterised from the reproductive tract of the ram (unpublished, obtained from Fouchecourt *et al.* 1999). From the sequences (Fig. 4.12) it can be seen that the N-terminal of the human (APEAQVSVQPNFQQDKFLG) and ovine (APTEAALQPNFEEDKFLGR) sequence are divergent in terms of homology, exhibiting an identity ratio of only 40%. Thus, the antiserum produced from the synthetic peptide was likely to be species-specific and not likely specific for the ovine protein.

The production of any antiserum is essentially an exercise in probability. Rather than immunising a rabbit with a single peptide fragment, several previously cloned β-trace proteins purified from a cross-section of species could be used as reactive immunogens, thus increasing the likelihood of producing an antiserum immunogenic with a variety of species, including the sheep. We originally decided against this strategy, as it can magnify the risk of producing a highly non-specific antiserum. In comparison, the primary benefit of raising an antiserum against a desired peptide fragment is the production of antiserum with high specificity. In the present case, the produced antiserum was in fact species-specific, and precluded identification of ovine β-trace protein.

human MATHHTLWMGLVLLGLLGGLQAAPEAQVSVQPNFQQDKFLGRWFSAGLASNSSW*L
ovine ATPNRPWMGLLLLGVLGVLQTPAPTEAALQPNFEEDKFLGRWFTSGLASNSSWFL

REKKAALSMCKSVVAPATDGGLNLTSTFLRKNQCETRTMLLQPAGSLGSYSYRSPH
*EKRKVLSMCKSEVAPAADGGLNVTSTFLRKDQCETRTLLLRPAGPPGCYSYTSPH

WGSTYSVSVVETDYDQYALLYSQGSKGPGEDFRMATLYSRTQTPRAELKEKFTAFC
WSSTHEVSVAETDYETYALLYTESVRGPGPDSLMATLYSRTQTPRAEVKEKFTTFA

KAQGFTEDTIVFLPQTDKCMTEQ
RSLGFTEEGIVFLPKTDKCMEVRT

Figure 4.12
Comparison of the amino acid sequence of human β-trace derived from cerebrospinal fluid (above; Hoffman *et al.* 1993) and prostaglandin D synthase characterised from the reproductive tract of the ram (below; Fouchecourt *et al.* 1999). Common amino acids shared between the two sequences are marked in blue, whereas different amino acids are individually defined in black. The sequence demarcated in yellow describes the peptide fragment (P1) synthesised and used as an immunogen to produce anti-P1 antiserum. Asterisks indicates where amino acids are missing from the peptide chain compared corresponding protein.

Although the anti-P1 antiserum was raised against a peptide fragment that did include 13-mer of the rat PGDS sequence, it is not surprising that the antiserum failed to recognise PGDS in the rat brain homogenate. Presumably, this may be a result of small amounts of PGDS present in the rat tissue, or alternatively, the low affinity of the antiserum. The decrease in sensitivity of the antiserum to rat PGDS may be due to the polyclonal antiserum having few antibodies raised against epitopes specific to the rat sequence. Upon introduction of the foreign antigen, the peptide fragment is non-specifically phagocytised and partially degraded by macrophages. The degraded fragments (epitopes) are presented to the cell surface of the macrophages, which in turn attract binding T cells and lead to the subsequent proliferation and differentiation of polyclonal antibodies. Therefore during an immune response a large number of antibodies are produced with each specifically binding to individual epitopes (Harlow and Lane 1988). Technically, the sensitivity of the antiserum is dependent upon the fragments presented on the cell surface of the macrophage. Since the antigen also included epitopes that were only present in the human sequence, it is more than likely that the proportion of antibodies which specifically bind to epitopes derived from the rat sequence is relatively less. This would reduce the anti-P1 antiserum sensitivity to rat PGDS protein.

From this study it is difficult to definitely conclude that the development of fetal sleep is *causally* linked to the changes in expression of β-trace, although a strong temporal association appears to exist. The physiological properties and function of β-trace protein in CSF is yet to be defined. Since being identified as a member of the lipocalin superfamily, on the basis of conserved tertiary structure (Nagata *et al.* 1991), the β-trace protein has been originally thought to be involved in the transportation of small lipophilic molecules in aqueous media. In the adult β-trace is constituitively expressed in specific blood-tissue barriers including leptomeninges (Urade *et al.* 1993), CP (Blodorn *et al.* 1996), pigment epithelial cells of the retina (Beuckmann *et al.* 1996), and in the testes (Tokugawa *et al.* 1998). Thus, a potential role for this lipocalin protein in the maturation of these barriers includes the binding and transportation of essential nutrients. The appearance of β-trace protein on the CSF from about 120 days gestation may be related to the onset of secretory

functions in the CP, and perhaps also by the epithelial cells of the cerebral microvasculature, at this time. This raises the interesting possibility that β-trace/PGDS is also secreted into the brain parenchyma from this time, and heightens the need to identify the regions of mRNA expression in the fetal brain.

Whether β-trace has a general homeostatic role in providing a stable internal environment for the central nervous system, or has a specific role in transporting hydrophobic molecules across the blood brain barrier in late gestation remains to be elucidated. This thesis adds to the substantial evidence that implicates β-trace as the PGD_2 synthesising enzyme PGDS, which considered a neuromodulator of adult sleep. However, the role of β-trace as a sleep substance during fetal development requires further investigation.

Chapter 5

General Discussion

The exact mechanisms that underlie each behavioural state of sleep and wakefulness are not entirely understood. Several substances have been implicated in the maintenance of these physiological processes. More specifically, it has been postulated that somnolence and vigilance may be reciprocally regulated by two PGs, namely PGD_2 and PGE_2 (Hayaishi and Matsumura 1995). Although PGD_2 and PGE_2 are prostanoids derived from the same arachidonic acid precursor (Smith and Marnett 1991), studies have shown that they promote opposing effects on behavioural states in the adult. It is believed that endogenously synthesised PGD_2 exerts a soporific effect by acting at the sleep centres in the anterior hypothalamus, whereas PGE_2 induces arousal by acting at the wake centres of the posterior hypothalamus (Hayaishi 1988).

Recently PGDS, the synthetic enzyme responsible for the production of PGD_2, was found to be structurally (Hoffmann *et al.* 1993) and functionally (Watanabe *et al.* 1994) homologous to β-trace, a major protein constituent of CSF. The physiological significance of the homology between the enzyme and the protein is unclear. However, based on the assumption that PGDS and β-trace may be functionally the same protein *in vivo*, it has been hypothesised that PGDS/β-trace produces large amounts of PGD_2 in the CSF, which subsequently acts as a 'neurohormone' at the sleep centres of the VLPO area to globally facilitate the induction of sleep (Urade and Hayaishi 1999).

The behavioural states of sleep and wakefulness are complex phenomena that develop prenatally in precocial animals, such as humans (Roffwarg *et al.* 1966) and sheep (Dawes *et*

al. 1972). In the ovine fetus, ECoG activity first appears at 90 days gestation but the cardinal signs of sleep begin to develop at 110 – 120 days gestation to become completely differentiated into distinct NREM and REM sleep states by 130 days gestation (Clewlow *et al.* 1983). The exact cellular mechanisms that govern the ontogenesis of sleep during fetal development are not completely understood. This thesis set forth to investigate the role of PGD_2 and the synthesising enzyme PGDS in the development of fetal sleep and arousal. The first chapter illustrated the effect of inhibiting PGD_2 production using $SeCl_4$, an inhibitor of PGDS, on the sleep and aroused behavioural states in the late gestation ovine fetus. The following chapter determined whether PGD_2 and PGE_2 were synthesised in the hypothalamus, and to determine the enzymatic capacities of PGDS and PGES during the period of development that precedes and follows the appearance of fetal sleep. The final chapter followed the temporal expression of the PGDS/β-trace protein in the CSF throughout the time that sleeps develops *in utero*.

From the $SeCl_4$ and PGD_2 infusion studies, it was concluded that PGD_2 does have a role in the maintenance of the sleep-wake behavioural states in the late gestation fetus. The results showed that specific inhibition of PGDS by i.c.v infusion of $SeCl_4$ caused a significant dose-dependent increase in the incidence of a set of behavioural activities that have been used to identify arousal in the fetus. There are two possible mechanisms by which $SeCl_4$ promoted vigilance. Firstly, $SeCl_4$ may reduce the amount of PGD_2 acting on the sleep centres of the VLPO area; or secondly, inhibition of PGDS may cause a redirection of the arachidonic cascade and increase the production of PGE_2. Therefore it is conceivable that elevation of hypothalamic PGE_2 may act at the vigilance centres of the TMN of the posterior hypothalamus to increase arousal in the fetus, as it does in the adult (Onoe *et al.* 1992), although fetal studies in this area are some what lacking (see below). Conversely, i.c.v administration of doses of PGD_2 equal to and greater than 100 pmole/10 µl/min induced a decrease in the incidence of arousal. Furthermore, infusion of PGD_2 immediately following administration of $SeCl_4$ abolished the $SeCl_4$-induced increase in arousal, reducing it to below control levels. Together, these results further suggest that PGD_2 is a 'neurohormone' that regulates sleep-wake behaviours in the fetus, as proposed for the adult.

From these studies, it appears that both administration of exogenous PGD_2 and inhibition of endogenous PGD_2 could decrease and increase behavioural arousal in the fetus respectively. Building upon these conclusions, the proposed role of PGD_2 in the maintenance of sleep-wake behaviours during fetal life was further explored by investigating the synthetic capacity of hypothalamic homogenates to produce PGD_2 and PGE_2 during fetal development. It was found that fetal hypothalamic homogenates were capable of synthesising both PGD_2 and PGE_2 and that there were considerable concentrations of both PGs present in the hypothalamus from 90 days gestation. However, the synthetic capacity of PGDS and concentrations of PGD_2 in the hypothalamus did not change in a distinct way with respect to the onset of sleep-wake cycles during fetal development. These results do not totally negate the role of PGD_2 in the ontogenesis of sleep, since other mechanisms such as changes in PGD_2 receptor binding have yet not been explored. Interestingly, the enzymatic capacity of PGES increased in late gestation. This elevation in PGE_2 production may be indicative of the increased incidence of aroused behaviour during late gestation (Szeto and Hinman 1985). However, whether there is a causal link between the maximal conversion of PGE_2 and the incidence of arousal requires further elucidation. Infusion of PGE_2 into the fetal sheep has not been shown to increase arousal or alter the EEG pattern. Rather PGE_2 has been shown to decrease breathing activity (Kitterman *et al.* 1983), and to increase ACTH secretion (Morimoto *et al.* 1989), but a role for this prostanoid in sleep-wake activities is otherwise lacking. Therefore, the increased hypothalamic PGE_2 production at this stage may also be potentially related to other physiological processes governed by prostaglandins in the hypothalamus, such as release of ACTH (Brooks *et al.* 1992).

The final study attempted to follow the temporal expression of PGDS/β-trace protein and mRNA in the hypothalamus, CP and CSF during fetal development, using immunoblot and RNA hybridisation methods respectively. Two antibodies were used to detect PGDS/β-trace: the first antibody had been raised by another laboratory against the entire β-trace protein derived from human CSF, and the second was raised by myself in rabbits against a

peptide sequence common in the N-terminal of β-trace from human CSF and PGDS of the rat brain (P1).

The antibody raised against the entire sequence of human β-trace detected β-trace protein in both human and ovine CSF samples; the results obtained using this particular antibody showed that β-trace was absent in ovine CSF from 90 days gestation. The physiological significance of the expression of β-trace from approximately 120 days gestation is unclear and may merely reflect the maturation of mechanisms that synthesise and then secrete β-trace from the choroid epithelium. However, since the expression of the β-trace protein in the fetus is markedly different from the general trend of other CSF proteins, which is maximal in early gestation and declines to adult levels throughout fetal development (Dziegielewska *et al.* 1980), it is probable that the relatively late production of β-trace has some functional significance. The findings show that the appearance of β-trace occurred at the same stage of gestation when the onset of NREM/REM sleep-like patterns emerges, and hence an association between the protein and the ontogenesis of fetal sleep may exist. However, whether this relationship is causal or coincidental requires further elucidation. Alternatively, the expression of the PGDS gene has been shown to be influenced by the thyroid hormone (Garcia-Fernandez *et al.* 1997), and the maturation of the hypothalamo-pituitary-thyroid axis from ~100 days gestation in sheep may be one of the systems that determines the appearance of β-trace in CSF at this time. To further explore β-trace's role during fetal development, it would be interesting to follow the cellular expression of β-trace at a number of sites at this time (e.g. retina, CP, leptomeninges, brain and testes), to ascertain whether the increase in β-trace CSF occurs because of a change in a specific secretory process at the blood/tissue barriers, or whether there is a change in the transcription and translation of the mRNA. Alternatively, knockout mice, which do not express the β-trace gene, would be a useful model in which to observe if any changes in behaviour that may manifest as a result of β-trace deficiency.

Although the antibody raised against human β-trace detected β-trace protein in ovine CSF, it was not immunoreactive with β-trace-like proteins in ovine brain tissue. A possible

explanation is that, in the sheep at least, β-trace in the CSF has a different amino acid sequence to PGDS/β-trace in the brain tissue. However, other technical factors such as antigen immunoreactivity may also be responsible for the lack of PGDS protein detection by the antiserum. Although the two proteins are structurally homologous (Hoffmann *et al.* 1993), whether they both function identically or are independent proteins that simply share a similar ancestry remains obscure. There is much evidence available describing the similar functional characteristics of each purified protein. Recent studies have demonstrated that β-trace purified from human CSF exhibits the same enzymatic properties as rat brain-type glutathione-independent PGDS *in vitro* (Watanabe *et al.* 1994). PGDS purified from the rat brain can also bind to retinoids like other lipocalins, such as retinol binding protein and β-lactoglobulin, with binding affinities sufficient for transportation (Tanaka *et al.* 1997). These *in vitro* studies performed using isolated β-trace and PGDS describe the potential role these proteins may have *in vivo*. However, whether β-trace in CSF is enzymatically active *in vivo* and contributes to the PGD_2-induced facilitation of somnolence by volume transmission requires further elucidation.

Definitive results were not obtained using the antibody raised against the synthetic P1 peptide. The present immunoblot and RNA hybridisation analysis suggested that there would be low homology between the amino acid and nucleic acid sequence of the human/rat and the ovine proteins, a result confirmed by a recent publication of the ovine β-trace sequence on the GenBank database (Fouchecourt *et al.* 1999). The significance of the 34 kDa protein that was identified in the fetal brain by this P1 peptide antibody is unclear. The identity of the protein could be established if further purification and sequences techniques were employed. However, this avenue of enquiry was not pursued, as the relevance of the protein to the specific objectives of this thesis appeared to be minimal.

From this thesis, it is concluded that PGD_2 influences the behavioural sleep-wake status of the fetus, presumably by acting on the putative sleep-centres of the VLPO area as it does in the adult. Since i.c.v administration of PGD_2 decreased the incidence of arousal experienced by the fetus and the appearance of β-trace in the CSF coincides with the

establishment of NREM/REM sleep, it is conceivable that PGDS/β-trace may produce PGD_2, which circulates throughout the ventricular system and percolates into the brain to globally induce sleep. The study also showed that hypothalamic homogenates have the enzymatic capacity to produce endogenous PGD_2. Therefore the brain tissue itself may synthesis endogenous PGD_2 that exerts a soporific effect by acting on the VLPO area sleep-centres. These results are consistent with previous studies in the adult that demonstrates administration of PGD_2 into the BF not only increased the incidence of sleep, but also increases FOS expression in the VLPO area (Scammell *et al.* 1998). Although it is apparent that PGDS and PGD_2 can influence arousal-like activity, it is more than likely that other systems also govern sleep and exclude wakefulness in the fetus, which are 'switched off' relatively quickly at birth. The PGD_2/PGDS system should therefore be seen as one modulatory substance of sleep that works in parallel with others to reduce CNS excitability and minimise the periods of wakefulness in the fetus. These include the interaction of neurosteroids with the $GABA_A$ receptor (Crossley *et al.* 2000), adenosine-dependent mechanisms in the brainstem (Karimi *et al.* 1996) and the opioid systems (Szeto *et al.* 1988). All of these substance may act synergistically with PGD_2 to decrease various parameters of fetal behaviour such as FBM, heart rate, blood pressure and ECoG, and induce potent sedative effects.

References

Abdel-Halim, M. S., Hamberg, M., Sjoquist, B. and Anggard, E. (1977) Identification of prostaglandin D_2 as a major prostaglandin in homogenates of rat brain. *Prostaglandins* **14**(4): 633-643.

Adelizzi, R. A. (1999) COX-1 and COX-2 in health and disease. *Journal of the American Osteopathic Association* **99**(11 Suppl): S7-12.

Adey, W. R., Bors, E. and R.W., P. (1968) EEG sleep patterns after high cervical lesions in man. *Archives in Neurology* **19**: 377-383.

Adamson, S. L., Engelberts, D. and Whiteley, K. J. (1997) Low dose indomethacin via fetal vein or cerebral ventricle stimulates breathing movements in fetal sheep. *Canadian Journal of Physiology and Pharmacology* **75**: 135-143.

Akarsu, E. S., Mamuk, S. and Comert, A. (1998) Inhibition of pentylenetetrazol-induced seizures in rats by prostaglandin D_2. *Epilepsy Research* **30**(1): 63 - 68.

Aldred, A. R., Brack, C. M. and Schreiber, G. (1995) The cerebral expression of plasma protein genes in different species. *Comparative Biochemistry & Physiology. Part B, Biochemistry & Molecular Biology* **111**(1): 1-15.

Arthur, J. R., Morrice, P. C., Nicol, F., Beddows, S. E., Boyd, R., Hayes, J. D. and Beckett, G. J. (1987) The effects of selenium and copper deficiencies on glutathione S-transferase and glutathione peroxidase in rat liver. *Biochemical Journal* **248**(2): 539-544.

Arthur, J. R., Nicol, F., Grant, E. and Beckett, G. J. (1991) The effects of selenium deficiency on hepatic type-I iodothyronine deiodinase and protein disulphide-isomerase assessed by activity measurements and affinity labelling. *Biochemical Journal* **274**(Pt 1): 297-300.

Asanuma, C. and Porter, L. L. (1990) Light and electron microscopic evidence for a GABAergic projection from the caudal basal forebrain to the thalamic reticular nucleus in rats. *Journal of Comparative Neurology* **302**(1): 159-172.

Aston-Jones, G. and Bloom, F. E. (1981) Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. *Journal of Neuroscience* **1**(8): 876-886.

Aston-Jones, G. (1985) The Locus Ceorulus: Behavioural functions of the locus ceoruleus derived from cellular attributes. *Physiological Psychology* **13**(3): 118-126.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seldman, J. G., Smtih, J. A. and Struhl, K. (1997) *Current Protocols in Molecular Biology*. USA, Wiley.

Balansky, R. M. and Argirova, R. M. (1981) Sodium selenite inhibition of the reproduction of some oncogenic RNA-viruses. *Experientia* **37**(11): 1194-1195.

Balzamo, E., Bradley, R. J., Bradley, D. M., Pegram, G. V. and Rhodes, J. M. (1972) Sleep ontogeny in the chimpanzee: from birth to two months. *Electroencephalography & Clinical Neurophysiology* **33**(1): 41-46.

Beckett, G. J., Russell, A., Nicol, F., Sahu, P., Wolf, C. R. and Arthur, J. R. (1992) Effect of selenium deficiency on hepatic type I 5-iodothyronine deiodinase activity and hepatic thyroid hormone levels in the rat. *Biochemical Journal* **282**(Pt 2): 483-486.

Behne, D., Hilmert, H., Scheid, S., Gessner, H. and Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. *Biochimica et Biophysica Acta* **966**(1): 12-21.

Beuckmann, C. T., Gordon, W. C., Kanaoka, Y., Eguchi, N., Marcheselli, V. L., Gerashchenko, D. Y., Urade, Y., Hayaishi, O. and Bazan, N. G. (1996) Lipocalin-type prostaglandin D synthase (β-trace) is located in pigment epithelial cells of rat retina and accumulates within interphotoreceptor matrix. *Journal of Neuroscience* **16**(19): 6119-6124.

Bissonnette, J. M., Hohimer, A. R. and Richardson, B. S. (1981) Ventriculocisternal cerebrospinal perfusion in unanesthesized fetal lambs. *Journal of Applied Physiology* **50**(4): 880 - 883.

Blodorn, B., Mader, M., Urade, Y., Hayaishi, O., Felgenhauer, K. and Bruck, W. (1996) Choroid plexus: the major site of mRNA expression for the β-trace protein (prostaglandin D synthase) in human brain. *Neuroscience Letters* **209**(2): 117-120.

Borbely, A. A. and Tobler, I. (1989) Endogenous sleep-promoting substances and sleep regulation. *Physiological Reviews* **69**(2): 605-670.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Brodal, P. (1992) *The Central Nervous System*. London, Oxford University Press.

Brooks, A. N., Currie, I. S., Gibson, F. and Thomas, G. B. (1992) Neuroendocrine regulation of sheep fetuses. *Journal of Reproduction & Fertility - Supplement* **45**: 69-84.

Broughton, R., de Koninck, J., Gagnon, P., Dunham, W. and Stampi, C. (1990) Sleep-wake biorhythms and extended sleep in man. *Sleep and Biological Rhythms*. Montplaiser and Godbout.

Cavallaro, V., Thompson, P. and Hearn, M. (1998) Solid phase synthesis of cyclic peptides: model studies involving i-(i+4) side chain-to-side chain cyclisation. *Journal of Peptide Science* **4**(5): 335-345.

Chiabrando, C., Noseda, A., Novella Castagnoli, M., Salmona, M. and Fanelli, R. (1984) Characterisation of arachidonic acid metabolic profiles in animal tissues by high-resolution gas chromatography-mass spectrometry. *Biochimica et Biophysica Acta* **794**(2): 292-297.

Clausen, J. (1961) Proteins in normal CSF not found in serum. *Proceedings of the Society Experimental Biology Medicine* **107**: 170-172.

Clausen, J. (1991) Uptake and distribution in rat brain of organic and inorganic selenium. *Biological Trace Element Research* **28**(1): 39-45.

Clewlow, F., Dawes, G. S., Johnston, B. M. and Walker, D. W. (1983) Changes in fetal breathing, electrocortical and muscle activity in unanaesthetised fetal lambs with age. *Journal of Physiology* **341**: 463-476.

Coenen, A. M. (1998) Neuronal phenomena associated with vigilance and consciousness: from cellular mechanisms to electroencephalographic patterns. *Consciousness & Cognition* **7**(1): 42-53.

Coons, S. and Guilleminault, C. (1984) Development of consolidated sleep and wakeful periods in relation to the day/night cycle in infancy. *Developmental Medicine & Child Neurology* **26**(2): 169-176.

Crossley, K. J., Nicol, M. B., Hirst, J. J., Walker, D. W. and Thorburn, G. D. (1997) Suppression of arousal by progesterone in fetal sheep. *Reproduction, Fertility, & Development* **9**(8): 767-773.

Crossley, K. J., Walker, D. W., Beart, P. M. and Hirst, J. J. (2000) Characterisation of $GABA_A$ receptors in fetal, neonatal and adult ovine brain: region and age related changes and the effects of allopregnanolone. *Neuropharmacology* **39**(9): 1514-1522.

Davenne, D., Fregnac, Y., Imbert, M. and Adrien, J. (1989) Lesion of the PGO pathways in the kitten. II. Impairment of physiological and morphological maturation of the lateral geniculate nucleus. *Brain Research* **485**(2): 267-277.

Davson, H., Welch, K. and Segal, M. B. (1987) *The Physiology and Pathophysiology of the Cerebrospinal Fluid.* London, Churchill Livingstone.

Dawes, G. S., Fox, H. E., Leduc, B. M., Liggins, G. C. and Richards, R. T. (1972) Respiratory movements and rapid eye movement sleep in the foetal lamb. *Journal of Physiology* **220**(1): 119-143.

Dawes, G. S. (1984) The central control of fetal breathing and skeletal muscle movements. *Journal of Physiology* **346**: 1-18.

Dawson, D. and Encel, N. (1993) Melatonin and sleep in humans. *Journal of Pineal Research* **15**(1): 1-12.

de Roquefeuil, G., Djakovic, M. and Montagner, H. (1993) New data on the ontogeny of the child's sleep-wake rhythm. *Chronobiology International* **10**(1): 43-53.

Detari, L. and Vanderwolf, C. H. (1987) Activity of identified cortically projecting and other basal forebrain neurones during large slow waves and cortical activation in anaesthetized rats. *Brain Research* **437**(1): 1-8.

Dolling, M. and Seamark, R. F. (1979) Progestagen metabolites in fetal sheep plasma: the effect of fetal nephrectomy. *Journal of Developmental Physiology* **1**(5): 399-413.

Doolittle, R. F. (1989) Similar amino acid sequences revisited. *Trends in Biochemical Sciences* **14**(7): 244-245.

Dziegielewska, K. M., Evans, C. A., Malinowska, D. H., Mollgard, K., Reynolds, J. M., Reynolds, M. L. and Saunders, N. R. (1979) Studies of the development of brain barrier systems to lipid insoluble molecules in fetal sheep. *Journal of Physiology* **292**: 207-231.

Dziegielewska, K. M., Evans, C. A., Fossan, G., Lorscheider, F. L., Malinowska, D. H., Mollgard, K., Reynolds, M. L., Saunders, N. R. and Wilkinson, S. (1980) Proteins in cerebrospinal fluid and plasma of fetal sheep during development. *Journal of Physiology* **300**: 441-455.

Eguchi, Y., Eguchi, N., Oda, H., Seiki, K., Kijima, Y., Matsu-ura, Y., Urade, Y. and Hayaishi, O. (1997) Expression of lipocalin-type prostaglandin D synthase (β-trace) in human heart and its accumulation in the coronary circulation of angina patients. *Proceedings of the National Academy of Sciences of the United States of America* **94**(26): 14689-14694.

Evans, C. A., Reynolds, J. M., Reynolds, M. L., Saunders, N. R. and Segal, M. B. (1974) The development of a blood-brain barrier mechanism in foetal sheep. *Journal of Physiology* **238**(2): 371-386.

Everson, C. A. (1995) Functional consequences of sustained sleep deprivation in the rat. *Behavioural Brain Research* **69**(1-2): 43-54.

Fields, G. B. and Noble, R. L. (1990) Solid phase peptide synthesis utilising 9-fluorenylmethoxycarbonyl amino acids. *International Journal of Peptide & Protein Research* **35**(3): 161-214.

Fisher, R. S., Buchwald, N. A., Hull, C. D. and Levine, M. S. (1988) GABAergic basal forebrain neurons project to the neocortex: the localization of glutamic acid decarboxylase and choline acetyltransferase in feline corticopetal neurons. *Journal of Comparative Neurology* **272**(4): 489-502.

Forstermann, U., Heldt, R. and Hertting, G. (1983) Effects of intracerebroventricular administration of prostaglandin D_2 on behaviour, blood pressure and body temperature as compared to prostaglandins E_2 and $F_{2\alpha}$. *Psychopharmacology* **80**(4): 365-370.

Forstermann, U. and Neufang, B. (1983) Elimination from the circulation of cats of 6-keto-prostaglandin E_1 compared with prostaglandins E_2 and I_2. *Journal of Pharmacy & Pharmacology* **35**(11): 724-728.

Fossan, G., Cavanagh, M. E., Evans, C. A., Malinowska, D. H., Mollgard, K., Reynolds, M. L. and Saunders, N. R. (1985) CSF-brain permeability in the immature sheep fetus: a CSF-brain barrier. *Brain Research* **350**(1-2): 113-124.

Fouchecourt, S., Dacheux, F. and Dacheux, J. L. (1999) GenBank, National Centre for Biotechnology Information. *www.ncbi.nlm.nih.gov*.

Fowden, A. L., Harding, R., Ralph, M. M. and Thorburn, G. D. (1987) The nutritional regulation of plasma prostaglandin E concentrations in the fetus and pregnant ewe during late gestation. *Journal of Physiology* **394**: 1-12.

Freund, S. (1900) The interpretation of dreams. *Complete Physiological Works*. Strachey, T. London, Hogenth Press **4&5**.

Freund, T. F. and Gulyas, A. I. (1991) GABAergic interneurons containing calbindin D28K or somatostatin are major targets of GABAergic basal forebrain afferents in the rat neocortex. *Journal of Comparative Neurology* **314**(1): 187-199.

Friedman, L. and Jones, B. E. (1984) Computer graphics analysis of sleep-wakefulness state changes after pontine lesions. *Brain Research Bulletin* **13**(1): 53-68.

Fuxe, K. and Agnati, L. A. (1991) Two principal modes of electrochemical communication in the brain: volume versus wiring transmission. *Volume Transmission in*

the Brain: Novel Mechanisms for Neural Transmission. Fuxe, K. and Agnati, L. A. New York, Raven Press.

Gallopin, T., Fort, P., Eggermann, E., Cauli, B., Luppi, P.-H., Rossier, J., Audinat, E., Muhlethaler, M. and Serafin, M. (2000) Indentification of sleep promoting neurons in vitro. *Nature* **404**: 992-995.

Garcia-Arraras, J. E. and Pappenheimer, J. R. (1983) Site of action of sleep-inducing muramyl peptide isolated from human urine: microinjection studies in rabbit brains. *Journal of Neurophysiology* **49**(2): 528-533.

Garcia-Fernandez, L. F., Iniguez, M. A., Rodriguez-Pena, A., Munoz, A. and Bernal, J. (1993) Brain-specific prostaglandin D_2 synthetase mRNA is dependent on thyroid hormone during rat brain development. *Biochemical & Biophysical Research Communications* **196**(1): 396-401.

Garcia-Fernandez, L. F., Rausell, E., Urade, Y., Hayaishi, O., Bernal, J. and Munoz, A. (1997) Hypothyroidism alters the expression of prostaglandin D_2 synthase/β trace in specific areas of the developing rat brain. *European Journal of Neuroscience* **9**(8): 1566-1573.

Garcia-Fernandez, L. F., Urade, Y., Hayaishi, O., Bernal, J. and Munoz, A. (1998) Identification of a thyroid hormone response element in the promoter region of the rat lipocalin-type prostaglandin D synthase (β-trace) gene. *Brain Research. Molecular Brain Research* **55**(2): 321-330.

Gerashchenko, D. Y., Beuckmann, C. T., Marcheselli, V. L., Gordon, W. C., Kanaoka, Y., Eguchi, N., Urade, Y., Hayaishi, O. and Bazan, N. G. (1998) Localisation of lipocalin-type prostaglandin D synthase (β-trace) in iris, ciliary body, and eye fluids. *Investigative Ophthalmology & Visual Science* **39**(1): 198-203.

Gerena, R. L., Irikura, D., Urade, Y., Eguchi, N., Chapman, D. A. and Killian, G. J. (1998) Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. *Biology of Reproduction* **58**(3): 826-833.

Gerozissis, K., De Saint Hilaire, Z., Orosco, M., Rouch, C. and Nicolaidis, S. (1995) Changes in hypothalamic prostaglandin E_2 may predict the occurrence of sleep or wakefulness as assessed by parallel EEG and microdialysis in the rat. *Brain Research* **689**(2): 239-244.

Gerozissis, K., de Saint-Hilaire, Z., Python, A., Rouch, C., Orosco, M. and Nicolaidis, S. (1998) Microdialysis and EEG in rats reveal cortical PGE_2 changes during sleep and wakefulness. *Neuroreport* **9**(7): 1327-1330.

Gessa, G. L., Pani, L., Fadda, P. and Fratta, W. (1995) Sleep deprivation in the rat: an animal model of mania. *European Neuropsychopharmacology* **5**(Suppl): 89-93.

Giacomelli, S., Leone, M. G., Grima, J., Silvestrini, B. and Cheng, C. Y. (1996) Astrocytes synthesise and secrete prostaglandin D synthetase in vitro. *Biochimica et Biophysica Acta* **1310**(3): 269-276.

Gladyshev, V. N., Khangulov, S. V. and Stadtman, T. C. (1994) Nicotinic acid hydroxylase from Clostridium barkeri: electron paramagnetic resonance studies show that selenium is coordinated with molybdenum in the catalytically active selenium-dependent enzyme. *Proceedings of the National Academy of Sciences of the United States of America* **91**(1): 232-236.

Godovac-Zimmermann, J. (1988) The structural motif of β-lactoglobulin and retinol-binding protein: a basic framework for binding and transport of small hydrophobic molecules? *Trends in Biochemical Sciences* **13**(2): 64-66.

Gottesmann, C. (1999) Neurophysiological support of consciousness during waking and sleep. *Progress in Neurobiology* **59**(5): 469-508.

Graf, M. V., Kastin, A. J. and Fischman, A. J. (1985) The natural occurrence and some extra-sleep effects of DSIP. *Endogenous sleep substances and sleep regulation.* Inoue, S. and Borbely, A. A. Tokyo, Japnese Science Society Press.

Gritti, I., Mainville, L. and Jones, B. E. (1993) Codistribution of GABA- with acetylcholine-synthesizing neurons in the basal forebrain of the rat. *Journal of Comparative Neurology* **329**(4): 438-457.

Harlow, E. and Lane, D. (1988) *Antibodies, A Laboratory Manual.* New York, Cold Springs Habour Laboratory Publication.

Harrington, M. G., Aebersold, R., Martin, B. M., Merril, C. R. and Hood, L. (1993) Identification of a brain-specific human cerebrospinal fluid glycoprotein, β-trace protein. *Applied & Theoretical Electrophoresis* **3**(5): 229-234.

Hayaishi, O. (1988) Sleep-wake regulation by prostaglandins D_2 and E_2. *Journal of Biological Chemistry* **263**(29): 14593-14596.

Hayaishi, O. (1991) Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D_2 and E_2. *FASEB Journal* **5**(11): 2575-2581.

Hayaishi, O., Matsumura, H. and Urade, Y. (1993) Prostaglandin D synthase is the key enzyme in the promotion of physiological sleep. *Journal of Lipid Mediators* **6**(1-3): 429-431.

Hayaishi, O. and Matsumura, H. (1995) Prostaglandins and sleep. *Advances in Neuroimmunology* **5**(2): 211-216.

Hayaishi, O. (1997) Prostaglandin D synthase, β-trace and sleep. *Advances in Experimental Medicine & Biology* **433**: 347-350.

Hirst, J. J., Egodagamade, K. C. and Walker, D. W. (2000) Effect of neuroactive steriod infused into the cerebral ventricles of fetal sheep in utero using small infusion volumes. *Journal of Neuroscience Methods* **97**: 37-44.

Hobson, J. A. (1999) Sleep and dreaming. *Fundamental Neuroscience*. Zigmond, M. J., Academic Press: 1207-1227.

Hoffmann, A., Conradt, H. S., Gross, G., Nimtz, M., Lottspeich, F. and Wurster, U. (1993) Purification and chemical characterisation of β-trace protein from human cerebrospinal fluid: its identification as prostaglandin D synthase. *Journal of Neurochemistry* **61**(2): 451-456.

Hoffmann, A., Nimtz, M., Wurster, U. and Conradt, H. S. (1994) Carbohydrate structures of β-trace protein from human cerebrospinal fluid: evidence for "brain-type" N-glycosylation. *Journal of Neurochemistry* **63**(6): 2185-2196.

Hoffmann, A., Bachner, D., Betat, N., Lauber, J. and Gross, G. (1996a) Developmental expression of murine β-trace in embryos and adult animals suggests a function in maturation and maintenance of blood-tissue barriers. *Developmental Dynamics* **207**(3): 332-343.

Hoffmann, A., Gath, U., Gross, G., Lauber, J., Getzlaff, R., Hellwig, S., Galla, H. J. and Conradt, H. S. (1996b) Constitutive secretion of β-trace protein by cultivated porcine choroid plexus epithelial cells: elucidation of its complete amino acid and cDNA sequences. *Journal of Cellular Physiology* **169**(2): 235-241.

Hoffmann, A., Nimtz, M. and Conradt, H. S. (1997) Molecular characterisation of β-trace protein in human serum and urine: a potential diagnostic marker for renal diseases. *Glycobiology* **7**(4): 499-506.

Hollingsworth, E. B. and Patrick, G. A. (1985) Involvement of the serotonergic system in the prolongation of pentobarbital sleeping time produced by prostaglandin D_2. *Pharmacology, Biochemistry & Behavior* **22**(3): 365-370.

Horner, R. L., Sanford, L. D., Annis, D., Pack, A. I. and Morrison, A. R. (1997) Serotonin at the laterodorsal tegmental nucleus suppresses rapid-eye-movement sleep in freely behaving rats. *Journal of Neuroscience* **17**(19): 7541-7552.

Huang, K., Lauridsen, E. and Clausen, J. (1994) Selenium-containing peroxidases of germinating barley. *Biological Trace Element Research* **46**(1-2): 173-182.

Ibuka, N., Inouye, S. I. and Kawamura, H. (1977) Analysis of sleep-wakefulness rhythms in male rats after suprachiasmatic nucleus lesions and ocular enucleation. *Brain Research* **122**(1): 33-47.

Igarashi, M., Nagata, A., Toh, H., Urade, Y. and Hayaishi, O. (1992) Structural organisation of the gene for prostaglandin D synthase in the rat brain. *Proceedings of the National Academy of Sciences of the United States of America* **89**(12): 5376-5380.

Inokuchi, A. and Oomura, Y. (1986) Effects of prostaglandin D_2 on the activity of hypothalamic neurons in the rat. *Japanese Journal of Physiology* **36**(3): 497-509.

Inoue, S., Honda, K., Komoda, Y., Uchizono, K., Ueno, R. and Hayaishi, O. (1984) Differential sleep-promoting effects of five sleep substances nocturnally infused in unrestrained rats. *Proceedings of the National Academy of Sciences of the United States of America* **81**(19): 6240-6244.

Ip, C. and Ganther, H. E. (1991) Combination of blocking agents and suppressing agents in cancer prevention. *Carcinogenesis* **12**(2): 365-367.

Islam, F., Urade, Y., Watanabe, Y. and Hayaishi, O. (1990) A particle concentration fluorescence immunoassay for prostaglandin D synthase in the rat central nervous system. *Archives of Biochemistry & Biophysics* **277**(2): 290-295.

Islam, F., Watanabe, Y., Morii, H. and Hayaishi, O. (1991) Inhibition of rat brain PGD synthase by inorganic selenocompounds. *Archives of Biochemistry & Biophysics* **289**(1): 161-166.

Jacobsen, M., Clausen, P. P., Jacobsen, G. K., Saunders, N. R. and Mollgard, K. (1982) Intracellular plasma proteins in human fetal choroid plexus during development. I. Developmental stages in relation to the number of epithelial cells which contain albumin in telencephalic, diencephalic and myelencephalic choroid plexus. *Brain Research* **255**(2): 239-250.

Jakobsson, P. J., Thoren, S., Morgenstern, R. and Samuelsson, B. (1999) Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proceedings of the National Academy of Sciences of the United States of America* **96**(13): 7220-7225.

Jones, B. E. and Yang, T. Z. (1985) The efferent projections from the reticular formation and the locus coeruleus studied by anterograde and retrograde axonal transport in the rat. *Journal of Comparative Neurology* **242**(1): 56-92.

Jones, B. E. and Beaudet, A. (1987) Distribution of acetylcholine and catecholamine neurons in the cat brainstem: a choline acetyltransferase and tyrosine hydroxylase immunohistochemical study. *Journal of Comparative Neurology* **261**(1): 15-32.

Jones, B. E. (1989) Basic mechanisms of REM sleep. *Sleep '88*. Horne, J. New York, Gustav Fischer Verlag, Struttgart: 163-167.

Jones, S. A., Adamson, S. L., Engelberts, D., Bishai, I., Norton, J. and Coceani, F. (1993) PGE_2 in the perinatal brain: local synthesis and transfer across the blood brain barrier. *Journal of Lipid Mediators* **6**(1-3): 487-492.

Jones, S. A., Adamson, S. L., Bishai, I., Engelberts, D., Norton, J. L. and Coceani, F. (1994) Prostaglandin E_2 in cerebrospinal fluid of fetal and newborn sheep: central versus peripheral source. *Biology of the Neonate* **66**(6): 339-351.

Jouvet, M. (1962) Recherches sur les structures nerveuses et les mechanismes responables des differentes phases du sommeil physiologique. *Archives of Italian Biology* **100**: 125-206.

Jouvet, M. (1999) Sleep and serotonin: an unfinished story. *Neuropsychopharmacology* **21**(2 Suppl): 24S-27S.

Jouvet-Mounier, D., Astic, L. and Lacote, D. (1970) Ontogenesis of the states of sleep in rat, cat, and guinea pig during the first postnatal month. *Developmental Psychobiology* **2**(4): 216-239.

Karimi, A., Ball, K. T. and Power, G. G. (1996) Exogenous infusion of adenosine depresses whole body O_2 use in fetal/neonatal sheep. *Journal of Applied Physiology* **81**(2): 541-547.

Karni, A., Tanne, D., Rubenstein, B. S., Askenasy, J. J. and Sagi, D. (1994) Dependence on REM sleep of overnight improvement of a perceptual skill [see comments]. *Science* **265**(5172): 679-682.

Katsuki, H. and Okuda, S. (1995) Arachidonic acid as a neurotoxic and neurotrophic substance. *Progress in Neurobiology* **46**(6): 607-636.

Kawaoi, A. and Tsuneda, M. (1985) Functional development and maturation of the rat thyroid gland in the foetal and newborn periods: an immunohistochemical study. *Acta Endocrinologica* **108**(4): 518-524.

Kayama, Y. and Koyama, Y. (1998) Brainstem neural mechanisms of sleep and wakefulness. *European Urology* **33**(Suppl 3): 12-15.

Kelly, R. W., Deam, S., Cameron, M. J. and Seamark, R. F. (1986) Measurement by radioimmunoassay of prostaglandins as their methyl oximes. *Prostaglandins Leukotrienes & Medicine* **24**(1): 1-14.

Kilunga Kubata, B., Eguchi, N., Urade, Y., Yamashita, K., Mitamura, T., Tai, K., Hayaishi, O. and Horii, T. (1998) Plasmodium falciparum produces prostaglandins that are pyrogenic, somnogenic, and immunosuppressive substances in humans. *Journal of Experimental Medicine* **188**(6): 1197-1202.

Kitterman, J. A., Liggins, G. C., Fewell, J. E. and Tooley, W. H. (1983) Inhibition of breathing movements in fetal sheep by prostaglandins. *Journal of Applied Physiology* **54**(3): 687-692.

Kormuller, A. E., Lux, H. D., Winkel, K. and Klee, M. (1961) Neurohumoral ausgelosate sclafzustande and tieren mit gekreutem kreislauf unter der Kontrolle von EEG-Ableitungen. *Natuwissenshaften* **14**: 503-505.

Korth, C. (1995) A co-evolutionary theory of sleep [published erratum appears in Med Hypotheses 1996 Jan;46(1):57]. *Medical Hypotheses* **45**(3): 304-310.

Koyama, Y. and Hayaishi, O. (1994) Modulation by prostaglandins of activity of sleep-related neurons in the preoptic/anterior hypothalamic areas in rats. *Brain Research Bulletin* **33**(4): 367-372.

Kropf, W. and Kuschinsky, K. (1991) Electroencephalographic correlates of the sedative effects of dopamine agonists presumably acting on autoreceptors. *Neuropharmacology* **30**(9): 953-960.

Krueger, J. M., Pappenheimer, J. R. and Karnovsky, M. L. (1982) Sleep-promoting effects of muramyl peptides. *Proceedings of the National Academy of Sciences of the United States of America* **79**(19): 6102-6106.

Krueger, J. M. (1985) Muramyl peptides and interleukin-1 as promoters of slow wave sleep. *Endogenous sleep substances and sleep regulation.* Inoue, S. and Borbely, A. A. Tokyo, Japanese Science Society Press.

Krueger, J. M. (1990) Somnogenic activity of immune response modifiers. *Trends in Pharmacological Sciences* **11**(3): 122-126.

Krueger, J. M., Obal, F., Opp, M., Cady, A. B., Johannsen, L., Toth, L. and Majde, J. (1990) Putative sleep neuromodulators. *Sleep and Biological Rhythms.* Montplaiser and Godbout. New York, Oxford Press.

Krueger, J. M., Kapas, L., Opp, M. R. and Obal, F., Jr. (1992) Prostaglandins E_2 and D_2 have little effect on rabbit sleep. *Physiology & Behaviour* **51**(3): 481-485.

Krunic, N., Adamson, S. L., Bishai, I. and Coceani, F. (1997) Prostaglandin uptake and catabolism by the choroid plexus during development in sheep. *Brain Research. Developmental Brain Research* **100**(1): 82-89.

Kuruvilla, A. P., Hochwald, G. M., Ghiso, J., Castano, E. M., Pizzolato, M. and Frangione, B. (1991) Isolation and amino terminal sequence of β-trace, a novel protein from human cerebrospinal fluid. *Brain Research* **565**(2): 337-340.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(259): 680-685.

Lands, W. E. (1989) Differences in n-3 and n-6 eicosanoid precursors. *Advances in Prostaglandin, Thromboxane, & Leukotriene Research* **19**: 602-605.

Lefauconner, J.-M. (1992) Transport of amino acids. *Physiology and Pharmacology of the Blood Brain Barrier*. Bradbury, M. W. B. Germany, Springer-Verlag: 117-142.

Lerner, R. A., Green, N., Alexander, H., Liu, F. T., Sutcliffe, J. G. and Shinnick, T. M. (1981) Chemically synthesised peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. *Proceedings of the National Academy of Sciences of the United States of America* **78**(6): 3403-3407.

Li, D. Y., Hardy, P., Abran, D., Martinez-Bermudez, A. K., Guerguerian, A. M., Bhattacharya, M., Almazan, G., Menezes, R., Peri, K. G., Varma, D. R. and Chemtob, S. (1997) Key role for cyclooxygenase-2 in PGE_2 and $PGF_{2\alpha}$ receptor regulation and cerebral blood flow of the newborn. *American Journal of Physiology* **273**(4 Pt 2): R1283-1290.

Lin, J. S., Sakai, K. and Jouvet, M. (1988) Evidence for histaminergic arousal mechanisms in the hypothalamus of cat. *Neuropharmacology* **27**(2): 111-122.

Lin, J. S., Sakai, K. and Jouvet, M. (1994) Hypothalamo-preoptic histaminergic projections in sleep-wake control in the cat. *European Journal of Neuroscience* **6**(4): 618-625.

Lin, J. S., Hou, Y., Sakai, K. and Jouvet, M. (1996) Histaminergic descending inputs to the mesopontine tegmentum and their role in the control of cortical activation and wakefulness in the cat. *Journal of Neuroscience* **16**(4): 1523-1537.

Lin, J.-S., Sakai, K., Vanni-Mercier, G. and Jouvet, M. (1989) A critical role of posterior hypothalamus in the mechanisms of wakefulness determined by microinjection of muscimol in freely moving cats. *Brain Research* **479**: 225-240.

Lindvall, M. and Owman, C. (1981) Autonomic nerves in the mammalian choroid plexus and their influence on the formation of cerebrospinal fluid. *Journal of Cerebral Blood Flow & Metabolism* **1**(3): 245-266.

Link, H. (1967) Immunoglobulin G and low molecular weight proteins in human cerebrospinal fluid. Chemical and immunological characterisation with special reference to multiple sclerosis. *Acta Neurologica Scandinavica* **43**(Suppl): 1-136.

Loeschcke, H. H. (1982) Central chemosensitivity and the reaction theory. *Journal of Physiology* **332**: 1-24.

Lu, J., Greco, M. A., Shiromani, P. and Saper, C. B. (2000) Effect of lesions of the ventrolateral preoptic nucleus on NREM and REM sleep. *Journal of Neuroscience* **20**(10): 3830-3842.

Mallick, B. N., China, G. S., Sundaram, K. R., Singh, B. and Kumar, V. M. (1983) Activity of preoptic neurons during synchronisation and desynchronisation. *Experimental Neurology* **81**(3): 586-597.

Maquet, P., Degueldre, C., Delfiore, G., Aerts, J., Peters, J. M., Luxen, A. and Franck, G. (1997) Functional neuroanatomy of human slow wave sleep. *Journal of Neuroscience* **17**(8): 2807-2812.

Marks, G. A., Shaffery, J. P., Oksenberg, A., Speciale, S. G. and Roffwarg, H. P. (1995) A functional role for REM sleep in brain maturation. *Behavioural Brain Research* **69**(1-2): 1-11.

Marshall, P. J., Kulmacz, R. J. and Lands, W. E. (1987) Constraints on prostaglandin biosynthesis in tissues. *Journal of Biological Chemistry* **262**(8): 3510-3517.

Matsumura, H., Goh, Y., Ueno, R., Sakai, T. and Hayaishi, O. (1988) Awaking effect of PGE_2 microinjected into the preoptic area of rats. *Brain Research* **444**(2): 265-272.

Matsumura, H., Honda, K., Choi, W. S., Inoue, S., Sakai, T. and Hayaishi, O. (1989a) Evidence that brain prostaglandin E_2 is involved in physiological sleep-wake regulation in rats. *Proceedings of the National Academy of Sciences of the United States of America* **86**(14): 5666-5669.

Matsumura, H., Honda, K., Goh, Y., Ueno, R., Sakai, T., Inoue, S. and Hayaishi, O. (1989b) Awaking effect of prostaglandin E_2 in freely moving rats. *Brain Research* **481**(2): 242-249.

Matsumura, H., Takahata, R. and Hayaishi, O. (1991) Inhibition of sleep in rats by inorganic selenium compounds, inhibitors of prostaglandin D synthase. *Proceedings of the National Academy of Sciences of the United States of America* **88**(20): 9046-9050.

Matsumura, H., Nakajima, T., Osaka, T., Satoh, S., Kawase, K., Kubo, E., Kantha, S. S., Kasahara, K. and Hayaishi, O. (1994) Prostaglandin D_2-sensitive, sleep-promoting zone defined in the ventral surface of the rostral basal forebrain. *Proceedings of the National Academy of Sciences of the United States of America* **91**(25): 11998-12002.

Matsumura, H., Gerashchemko, D., Satoh, S., Nakajima, T., Kashara, K. and Hayaishi, O. (1995) Prostaglandin D_2 and nitric oxide: key factors indulging the regulation of slow waves sleep and paradoxial sleep in the rat brain. *Sleep Research* **24A**: 134.

Matsumura, K., Watanabe, Y., Onoe, H. and Hayaishi, O. (1990) High density of prostaglandin E_2 binding sites in the anterior wall of the 3rd ventricle: a possible site of its hyperthermic action. *Brain Research* **533**(1): 147-151.

McCormick, D. A. and Bal, T. (1997) Sleep and arousal: thalamocortical mechanisms. *Annual Review of Neuroscience* **20**: 185-215.

Mendlewicz, J., Linkowski, P., Kerkhofs, M., Leproult, R., Copinschi, G. and Van Cauter, E. (1999) Genetic control of 24-hour growth hormone secretion in man: a twin study. *Journal of Clinical Endocrinology & Metabolism* **84**(3): 856-862.

Merchant-Nancy, H., Vazquez, J., Aguilar-Roblero, R. and Drucker-Colin, R. (1992) c-fos proto-oncogene changes in relation to REM sleep duration. *Brain Research* **579**(2): 342-346.

Miles, L. E. and Dement, W. C. (1980) Sleep and aging. *Sleep* **3**(2): 1-220.

Mirmiran, M., Scholtens, J., van de Poll, N. E., Uylings, H. B., van der Gugten, J. and Boer, G. J. (1983) Effects of experimental suppression of active (REM) sleep during early development upon adult brain and behavior in the rat. *Brain Research* **283**(2-3): 277-286.

Mirmiran, M. (1995) The function of fetal/neonatal rapid eye movement sleep. *Behavioural Brain Research* **69**(1-2): 13-22.

Mollgard, K., Balslev, Y., Lauritzen, B. and Saunders, N. R. (1987) Cell junctions and membrane specialisations in the ventricular zone (germinal matrix) of the developing sheep brain: a CSF-brain barrier. *Journal of Neurocytology* **16**(4): 433-444.

Monti, J. M. (1993) Involvement of histamine in the control of the waking state. *Life Sciences* **53**(17): 1331-1338.

Moore, R. Y. (1990) The Circadian system and sleepwake behaviour. *Sleep and Biological Rhythms*. Montplaiser and Godbout. New York, Oxford Press.

Morimoto, A., Murakami, N., Nakamori, T., Sakata, Y. and Watanabe, T. (1989) Possible involvement of prostaglandin E in development of ACTH response in rats induced by human recombinant interleukin-1. *Journal of Physiology* **411**: 245-256.

Nagata, A., Suzuki, Y., Igarashi, M., Eguchi, N., Toh, H., Urade, Y. and Hayaishi, O. (1991) Human brain prostaglandin D synthase has been evolutionarily differentiated from lipophilic-ligand carrier proteins. *Proceedings of the National Academy of Sciences of the United States of America* **88**(9): 4020-4024.

Naito, K., Osama, H., Ueno, R., Hayaishi, O., Honda, K. and Inoue, S. (1988) Suppression of sleep by prostaglandin synthesis inhibitors in unrestrained rats. *Brain Research* **453**(1-2): 329-336.

Namima, M. and Okamoto, K. (1987) Modulatory action of prostaglandin D_2 on the release of ^{3}H-norepinephrine from rat cerebellar slices. *Japanese Journal of Pharmacology* **43**(2): 197-204.

Narumiya, S., Ogorochi, T., Nakao, K. and Hayaishi, O. (1982) Prostaglandin D_2 in rat brain, spinal cord and pituitary: basal level and regional distribution. *Life Sciences* **31**(19): 2093-2103.

Nauta, W. J. H. (1946) Hypothalamic regulation of sleep in rats: an experimental study. *Journal Neurophysiology* **9**: 285-316.

Newcomer, M. E., Jones, T. A., Aqvist, J., Sundelin, J., Eriksson, U., Rask, L. and Peterson, P. A. (1984) The three-dimensional structure of retinol-binding protein. *EMBO Journal* **3**(7): 1451-1454.

Nicholson, A. N., Pascoe, P. A. and Stone, B. M. (1985) Histaminergic systems and sleep. Studies in man with H_1 and H_2 antagonists. *Neuropharmacology* **24**(3): 245-250.

Nicol, M. B., Hirst, J. J., Walker, D. W. and Thorburn, G. D. (1997) Effect of alteration of maternal plasma progesterone concentration on fetal behavoural state during late gestation. *Journal of Endocrinology* **152**(379-386).

Nicol, M. B., Hirst, J. J. and Walker, D. W. (1999) Effect of the 5-α reductase inhibitor finasteride on fetal behavioural states. *Proceedings of the Australian Neuroscience Society* **10**: 83.

Nichols, N. R., Dokas, L., Ting, S. M., Kumar, S., de Vellis, J., Shors, T. J., Uenishi, N., Thompson, R. F. and Finch, C. E. (1996) Hippocampal responses to corticosterone and stress, one of which is the 35,000 M(r) protein, glycerol phosphate dehydrogenase. *Journal of Neuroendocrinology* **8**(11): 867-876.

Nilsson, C., Lindvall-Axelsson, M. and Owman, C. (1992) Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Research - Brain Research Reviews* **17**(2): 109-138.

Ogorochi, T., Narumiya, S., Mizuno, N., Yamashita, K., Miyazaki, H. and Hayaishi, O. (1984) Regional distribution of prostaglandins D_2, E_2, and $F_{2\alpha}$ and related enzymes in postmortem human brain. *Journal of Neurochemistry* **43**(1): 71-82.

Ogorochi, T., Ujihara, M. and Narumiya, S. (1987) Purification and properties of prostaglandin H-E isomerase from the cytosol of human brain: identification as anionic forms of glutathione S-transferase. *Journal of Neurochemistry* **48**(3): 900-909.

Ohe, Y., Ishikawa, K., Itoh, Z. and Tatemoto, K. (1996) Cultured leptomeningeal cells secrete cerebrospinal fluid proteins. *Journal of Neurochemistry* **67**(3): 964-971.

Okuda-Ashitaka, E., Sakamoto, K., Giles, H., Ito, S. and Hayaishi, O. (1993) Cyclic-AMP-dependent Ca^{2+} influx elicited by prostaglandin D_2 in freshly isolated nonchromaffin cells from bovine adrenal medulla. *Biochimica et Biophysica Acta* **1176**(1-2): 148-154.

Olson, D. M., Lye, S. J., Skinner, K. and Challis, J. R. (1984) Early changes in prostaglandin concentrations in ovine maternal and fetal plasma, amniotic fluid and from dispersed cells of intrauterine tissues before the onset of ACTH-induced pre-term labour. *Journal of Reproduction & Fertility* **71**(1): 45-55.

Olsson, J. E. and Link, H. (1973) Distribution of serum proteins and β-trace protein within the nervous system. *Journal of Neurochemistry* **20**(3): 837-846.

Olsson, J. E., Link, H. and Nosslin, B. (1973) Metabolic studies on ^{125}I-labelled β-trace protein, with special reference to synthesis within the central nervous system. *Journal of Neurochemistry* **21**(5): 1153-1159.

Olsson, J. E. and Nord, L. (1973) Immunochemical and immunofluorescence studies of β-trace protein in different species and organs, with special reference to the central nervous system. *Journal of Neurochemistry* **21**(3): 625-633.

Onoe, H., Ueno, R., Fujita, I., Nishino, H., Oomura, Y. and Hayaishi, O. (1988) Prostaglandin D_2, a cerebral sleep-inducing substance in monkeys. *Proceedings of the National Academy of Sciences of the United States of America* **85**(11): 4082-4086.

Onoe, H., Watanabe, Y., Ono, K., Koyama, Y. and Hayaishi, O. (1992) Prostaglandin E_2 exerts an awaking effect in the posterior hypothalamus at a site distinct from that mediating its febrile action in the anterior hypothalamus. *Journal of Neuroscience* **12**(7): 2715-2725.

Osaka, T. and Matsumura, H. (1994) Noradrenergic inputs to sleep-related neurons in the preoptic area from the locus coeruleus and the ventrolateral medulla in the rat. *Neuroscience Research* **19**(1): 39-50.

Osaka, T. and Hayaishi, O. (1995) Prostaglandin D_2 modulates sleep-related and noradrenaline-induced activity of preoptic and basal forebrain neurons in the rat. *Neuroscience Research* **23**(3): 257-268.

Osaka, T. and Matsumura, H. (1995) Noradrenaline inhibits preoptic sleep-active neurons through α 2-receptors in the rat. *Neuroscience Research* **21**(4): 323-330.

Pace-Asciak, C. R. and Rangaraj, G. (1978) Distribution of prostaglandin biosynthetic pathways in organs and tissues of the fetal lamb. *Biochimica et Biophysica Acta* **528**(3): 512-514.

Pandey, H. P., Ram, A., Matsumura, H. and Hayaishi, O. (1995a) Concentration of prostaglandin D_2 in cerebrospinal fluid exhibits a circadian alteration in conscious rats. *Biochemistry & Molecular Biology International* **37**(3): 431-437.

Pandey, H. P., Ram, A., Matsumura, H., Satoh, S. and Hayaishi, O. (1995b) Circadian variations of prostaglandins D_2, E_2, and $F_{2\alpha}$ in the cerebrospinal fluid of anesthetized rats. *Biochemical & Biophysical Research Communications* **213**(2): 625-629.

Pappenheimer, J. R., Miller, T. B. and Goodrich, C. A. (1967) Sleep-promoting effects of cerebrospinal fluid from sleep-deprived goats. *Proceedings of the National Academy of Sciences of the United States of America* **58**(2): 513-517.

Peitsch, M. C. and Boguski, M. S. (1991) The first lipocalin with enzymatic activity. *Trends in Biochemical Sciences* **16**(10): 363.

Pentreath, V. W., Rees, K., Owolabi, O. A., Philip, K. A. and Doua, F. (1990) The somnogenic T lymphocyte suppressor prostaglandin D_2 is selectively elevated in cerebrospinal fluid of advanced sleeping sickness patients. *Transactions of the Royal Society of Tropical Medicine & Hygiene* **84**(6): 795-799.

Pieron, H. (1913) *Le problem physiologique du sommeil.* Paris, Maason.

Pinzar, E., Kanaoka, K., Inui, T., Eguchi, N., Urade, Y. and Hayaishi, O. (2000) Prostaglandin D synthase gene is involved in the regulation of non-rapid eye movement sleep. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 4903 - 4907.

Porterfield, S. P. and Hendrich, C. E. (1993) The role of thyroid hormones in prenatal and neonatal neurological development--current perspectives. *Endocrine Reviews* **14**(1): 94-106.

Powell, W. S. (1980) Rapid extraction of oxygenated metabolites of arachidonic acid from biological samples using octadecylsilyl silica. *Prostaglandins* **20**(5): 947-957.

Rage, F., Lee, B. J., Ma, Y. J. and Ojeda, S. R. (1997) Estradiol enhances prostaglandin E_2 receptor gene expression in luteinizing hormone-releasing hormone (LHRH) neurons and facilitates the LHRH response to PGE_2 by activating a glia-to-neuron signaling pathway. *Journal of Neuroscience* **17**(23): 9145-9156.

Ram, A., Pandey, H. P., Matsumura, H., Kasahara-Orita, K., Nakajima, T., Takahata, R., Satoh, S., Terao, A. and Hayaishi, O. (1997) CSF levels of prostaglandins, especially the level of prostaglandin D_2, are correlated with increasing propensity towards sleep in rats. *Brain Research* **751**(1): 81-89.

Rammsayer, T. H. (1997) Are there dissociable roles of the mesostriatal and mesolimbocortical dopamine systems on temporal information processing in humans? *Neuropsychobiology* **35**(1): 36-45.

Roffwarg, H. P., Muzio, J. N. and Dement, W. C. (1966) Ontogenetic development of human sleep-dream cycle. *Science* **152**: 604-619.

Ruckebusch, Y., Gaujoux, M. and Eghbali, B. (1977) Sleep cycles and kinesis in the foetal lamb. *Electroencephalography & Clinical Neurophysiology* **42**(2): 226-237.

Rueter, L. E., Fornal, C. A. and Jacobs, B. L. (1997) A critical review of 5-HT brain microdialysis and behavior. *Reviews in the Neurosciences* **8**(2): 117-137.

Sakai, K. (1985) Anatomical and physiological basis of paradoxial sleep. *Brain Mechanisms of Sleep.* McGinty, D., Drucker-Colin, R., Morrison, A. R. and Patmeggiani, P. L. New York, Raven Press.

Sallanon, M., Denoyer, M., Kitahama, K., Aubert, C., Gay, N. and Jouvet, M. (1989) Long-lasting insomnia induced by preoptic neuron lesions and its transient reversal by muscimol injection into the posterior hypothalamus in the cat. *Neuroscience* **32**(3): 669-683.

Salzarulo, P. and Fagioli, I. (1995) Sleep for development or development for waking?--some speculations from a human perspective. *Behavioural Brain Research* **69**(1-2): 23-27.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* New York, Cold Springs Laboratory Press.

Saunders, N. R. (1977) The blood brain barrier in the foetal and newborn lamb. *Annales de Recherches Veterinaires* **8**(4): 384-395.

Saunders, N. R., Habgood, M. D. and Dziegielewska, K. M. (1999a) Barrier mechanisms in the brain, II. Immature brain. *Clinical & Experimental Pharmacology & Physiology* **26**(2): 85-91.

Saunders, N. R., Habgood, M. D. and Dziegielewska, K. M. (1999b) Barrier mechanisms in the brain, I. Adult brain. *Clinical & Experimental Pharmacology & Physiology* **26**(1): 11-19.

Scammell, T., Gerashchenko, D., Urade, Y., Onoe, H., Saper, C. and Hayaishi, O. (1998) Activation of ventrolateral preoptic neurons by the somnogen prostaglandin D_2. *Proceedings of the National Academy of Sciences of the United States of America* **95**(13): 7754-7759.

Scammell, T. E., Elmquist, J. K., Griffin, J. D. and Saper, C. B. (1996) Ventromedial preoptic prostaglandin E_2 activates fever-producing autonomic pathways. *Journal of Neuroscience* **16**(19): 6246-6254.

Scheen, A. J., Byrne, M. M., Plat, L., Leproult, R. and Van Cauter, E. (1996) Relationships between sleep quality and glucose regulation in normal humans. *American Journal of Physiology* **271**(2 Pt 1): E261-270.

Scherschlicht, R. (1985) Role for GABA in the control of the sleep-wakefulness cycle. *Sleep: Neurotransmitters and Neuromodulators*. Wauquier, A., Monti, J. M., Gaillard, J. M. and Radulovacki, M. New York, Raven Press.

Schnedorf, J. G. and Ivy, A. G. (1939) An examination of hypnotoxin theory of sleep. *American Journal of Physiology* **125**: 491-505.

Schoenenberger, G. A. and Monnier, M. (1977) Characterisation of a δ-electroencephalogram (-sleep)-inducing peptide. *Proceedings of the National Academy of Sciences of the United States of America* **74**(3): 1282-1286.

Schror, K. (1997) Aspirin and platelets: the antiplatelet action of aspirin and its role in thrombosis treatment and prophylaxis. *Seminars in Thrombosis & Hemostasis* **23**(4): 349-356.

Schwarz, K. and Foltz, C. M. (1989) The Journal of Biological Chemistry, Volume 233, 1958: Factor 3 activity of selenium compounds [classical article]. *Nutrition Reviews* **47**(4): 108-110.

Scott, S. C., Inman, J. D. and Moss, I. R. (1990) Ontogeny of sleep/wake and cardiorespiratory behavior in unanesthetized piglets. *Respiration Physiology* **80**(1): 83-101.

Seregi, A., Keller, M. and Hertting, G. (1987) Are cerebral prostanoids of astroglial origin? Studies on the prostanoid forming system in developing rat brain and primary cultures of rat astrocytes. *Brain Research* **404**(1-2): 113-120.

Sherin, J. E., Shiromani, P. J., McCarley, R. W. and Saper, C. B. (1996) Activation of ventrolateral preoptic neurons during sleep. *Science* **271**(5246): 216-219.

Shimizu, A. and Himwich, H. E. (1968) The ontogeny of sleep in kittens and young rat bits. *Electroencephalography & Clinical Neurophysiology* **24**(4): 307-318.

Shimizu, T., Mizuno, N., Amano, T. and Hayaishi, O. (1979a) Prostaglandin D_2, a neuromodulator. *Proceedings of the National Academy of Sciences of the United States of America* **76**(12): 6231-6234.

Shimizu, T., Yamamoto, S. and Hayaishi, O. (1979b) Purification and properties of prostaglandin D synthetase from rat brain. *Journal of Biological Chemistry* **254**(12): 5222-5228.

Shimizu, T., Yamashita, A. and Hayaishi, O. (1982) Specific binding of prostaglandin D_2 to rat brain synaptic membrane. Occurrence, properties, and distribution. *Journal of Biological Chemistry* **257**(22): 13570-13575.

Shiromani, P. J., Armstrong, D. M., Bruce, G., Hersh, L. B., Groves, P. M. and Gillin, J. C. (1987) Relation of pontine choline acetyltransferase immunoreactive neurons with cells which increase discharge during REM sleep. *Brain Research Bulletin* **18**(3): 447-455.

Shiromani, P. J., Kilduff, T. S., Bloom, F. E. and McCarley, R. W. (1992) Cholinergically induced REM sleep triggers Fos-like immunoreactivity in dorsolateral pontine regions associated with REM sleep. *Brain Research* **580**(1-2): 351-357.

Shiromani, P. J., Magner, M., Winston, S. and Charness, M. E. (1995) Time course of phosphorylated CREB and Fos-like immunoreactivity in the hypothalamic supraoptic nucleus after salt loading. *Brain Research. Molecular Brain Research* **29**(1): 163-171.

Sibley, Y. D., Walker, A. M. and Maloney, J. E. (1982) The influence of behavioural state on the cardiovascular system of the newborn. *Journal of Developmental Physiology* **4**(2): 107-119.

Siegel, J. M. (1990) Mechanisms of sleep control. *Journal of Clinical Neurophysiology* **7**(1): 49-65.

Siren, A. L. (1982) Central cardiovascular and thermal effects of prostaglandin D_2 in rats. *Prostaglandins Leukotrienes & Medicine* **8**(4): 349-359.

Smieja, Z., Zakar, T., Walton, J. C. and Olson, D. M. (1993) Prostaglandin endoperoxide synthase kinetics in human amnion before and after labor at term and following preterm labor. *Placenta* **14**(2): 163-175.

Smith, W. L. and Marnett, L. J. (1991) Prostaglandin endoperoxide synthase: structure and catalysis. *Biochimica et Biophysica Acta* **1083**(1): 1-17.

Stadtman, T. C. (1983) New biologic functions--selenium-dependent nucleic acids and proteins. *Fundamental & Applied Toxicology* **3**(5): 420-423.

Stadtman, T. C. (1990) Selenium biochemistry. *Annual Review of Biochemistry* **59**: 111-127.

Staines, W. A., Daddona, P. E. and Nagy, J. I. (1987) The organization and hypothalamic projections of the tuberomammillary nucleus in the rat: an immunohistochemical study of adenosine deaminase-positive neurons and fibers. *Neuroscience* **23**(2): 571-596.

Steininger, T. L., Wainer, B. H., Blakely, R. D. and Rye, D. B. (1997) Serotonergic dorsal raphe nucleus projections to the cholinergic and noncholinergic neurons of the pedunculopontine tegmental region: a light and electron microscopic anterograde tracing and immunohistochemical study. *Journal of Comparative Neurology* **382**(3): 302-322.

Steriade, M. and Hobson, J. A. (1976) Neuronal activity during the sleep-wake cycle. *Progressive Neurobiology* **6**: 155.

Steriade, M. and McCarley, R. W. (1990) *Brainstem control of sleep and wakefulness.*, New York: Plenum Press.

Steriade, M. (1993) Central core modulation of spontaneous oscillations and sensory transmission in thalamocortical systems. *Current Opinion in Neurobiology* **3**(4): 619-625.

Sterman, M. B. and Clemente, C. D. (1962) Forebrain inhibitory mechanisms: Sleep patterns induced by basal forebrain stimulation. *Experimental Neurology* **6**: 103-117.

Suzuki, F., Hayashi, H. and Hayaishi, O. (1986) Transport of prostaglandin D_2 into brain. *Brain Research* **385**(2): 321-328.

Suzuki, F., Hayashi, H., Ito, S. and Hayaishi, O. (1987) Methyl ester of prostaglandin D_2 as a delivery system of prostaglandin D2 into brain. *Biochimica et Biophysica Acta* **917**(2): 224-230.

Szeto, H. H. (1983) Effects of narcotic drugs on fetal behavioural activity: acute methadone exposure. *American Journal of Obstetrics & Gynecology* **146**(2): 210 - 216.

Szeto, H. H. and Hinman, D. J. (1985) Prenatal development of sleep-wake patterns in sheep. *Sleep* **8**(4): 347-355.

Szeto, H. H., Zhu, Y. S., Umans, J. G., Dwyer, G., Clare, S. and Amione, J. (1988) Dual action of morphine on fetal breathing movements. *Journal of Pharmacology & Experimental Therapeutics* **245**(2): 537-542.

Szeto, H. H. (1992) Behavioral states and their ontogeny: animal studies. *Seminars in Perinatology* **16**(4): 211-216.

Szymusiak, R. and McGinty, D. (1986a) Sleep suppression following kainic acid-induced lesions of the basal forebrain. *Experimental Neurology* **94**(3): 598-614.

Szymusiak, R. and McGinty, D. (1986b) Sleep-related neuronal discharge in the basal forebrain of cats. *Brain Research* **370**(1): 82-92.

Szymusiak, R. and McGinty, D. (1989) Effects of basal forebrain stimulation on the waking discharge of neurons in the midbrain reticular formation of cats. *Brain Research* **498**(2): 355-359.

Szymusiak, R. (1995) Magnocellular nuclei of the basal forebrain: substrates of sleep and arousal regulation. *Sleep* **18**(6): 478-500.

Szymusiak, R., Alam, N., Steininger, T. L. and McGinty, D. (1998) Sleep-waking discharge patterns of ventrolateral preoptic/anterior hypothalamic neurons in rats. *Brain Research* **803**(1-2): 178-188.

Taishi, P., Chen, Z., Obal, F., Jr., Hansen, M. K., Zhang, J., Fang, J. and Krueger, J. M. (1998) Sleep-associated changes in interleukin-1β mRNA in the brain. *Journal of Interferon & Cytokine Research* **18**(9): 793-798.

Takahata, R., Matsumura, H., Kantha, S. S., Kubo, E., Kawase, K., Sakai, T. and Hayaishi, O. (1993) Intravenous administration of inorganic selenium compounds, inhibitors of prostaglandin D synthase, inhibits sleep in freely moving rats. *Brain Research* **623**(1): 65-71.

Takahata, R., Matsumura, H., Eguchi, N., Kantha, S. S., Satoh, S., Sakai, T., Kondo, N. and Hayaishi, O. (1996) Seasonal variation in levels of prostaglandins D_2, E_2 and $F_{2\alpha}$ in the brain of a mammalian hibernator, the Asian chipmunk. *Prostaglandins Leukotrienes & Essential Fatty Acids* **54**(1): 77-81.

Tanaka, T., Urade, Y., Kimura, H., Eguchi, N., Nishikawa, A. and Hayaishi, O. (1997) Lipocalin-type prostaglandin D synthase (β-trace) is a newly recognized type of retinoid transporter. *Journal of Biological Chemistry* **272**(25): 15789-15795.

Terao, A., Matsumura, H. and Saito, M. (1998) Interleukin-1 induces slow-wave sleep at the prostaglandin D_2-sensitive sleep-promoting zone in the rat brain. *Journal of Neuroscience* **18**(16): 6599-6607.

Thoman, E. B., Waite, S. P., Desantis, D. T. and Denenberg, V. H. (1979) Ontogeny of sleep and wake states in the rabbit. *Animal Behaviour* **27**(Pt 1): 95-106.

Tokugawa, Y., Kunishige, I., Kubota, Y., Shimoya, K., Nobunaga, T., Kimura, T., Saji, F., Murata, Y., Eguchi, N., Oda, H., Urade, Y. and Hayaishi, O. (1998) Lipocalin-type prostaglandin D synthase in human male reproductive organs and seminal plasma. *Biology of Reproduction* **58**(2): 600-607.

Tokumoto, H., Watanabe, K., Fukushima, D., Shimizu, T. and Hayaishi, O. (1982) An NADP-linked 15-hydroxyprostaglandin dehydrogenase specific for prostaglandin D_2 from swine brain. *Journal of Biological Chemistry* **257**(22): 13576-13580.

Tononi, G., Pompeiano, M. and Cirelli, C. (1994) The locus coeruleus and immediate-early genes in spontaneous and forced wakefulness. *Brain Research Bulletin* **35**(5-6): 589-596.

Toth, P., Lukacs, H., Hiatt, E. S., Reid, K. H., Iyer, V. and Rao, C. V. (1994) Administration of human chorionic gonadotropin affects sleep-wake phases and other associated behaviors in cycling female rats. *Brain Research* **654**(2): 181-190.

Tumani, H., Reiber, H., Nau, R., Prange, H. W., Kauffmann, K., Mader, M. and Felgenhauer, K. (1998) β-trace protein concentration in cerebrospinal fluid is decreased in patients with bacterial meningitis. *Neuroscience Letters* **242**(1): 5-8.

Turner, D. M., Ransom, R. W., Yang, J. S. and Olsen, R. W. (1989) Steroid anesthetics and naturally occurring analogs modulate the gamma-aminobutyric acid receptor complex at a site distinct from barbiturates. *Journal of Pharmacology & Experimental Therapeutics* **248**(3): 960-966.

Ueno, R., Ishikawa, Y., Nakayama, T. and Hayaishi, O. (1982a) Prostaglandin D_2 induces sleep when microinjected into the preoptic area of conscious rats. *Biochemical & Biophysical Research Communications* **109**(2): 576-582.

Ueno, R., Narumiya, S., Ogorochi, T., Nakayama, T., Ishikawa, Y. and Hayaishi, O. (1982b) Role of prostaglandin D_2 in the hypothermia of rats caused by bacterial lipopolysaccharide. *Proceedings of the National Academy of Sciences of the United States of America* **79**(19): 6093-6097.

Ueno, R., Honda, K., Inoue, S. and Hayaishi, O. (1983) Prostaglandin D_2, a cerebral sleep-inducing substance in rats. *Proceedings of the National Academy of Sciences of the United States of America* **80**(6): 1735-1737.

Ueno, R., Hayaishi, O., Osama, H., Honda, K., Inoue, S., Ishikawa, Y. and Nakayama, T. (1985a) Prostaglandin D_2 regulates physiological sleep. *Endogenous Sleep Substances and Sleep Regulation.* Inoue, S. B., A. Tokyo, Japanese Scientific Society Press: 193-201.

Ueno, R., Osama, H., Urade, Y. and Hayaishi, O. (1985b) Changes of enzymes involved in prostaglandin metabolism and prostaglandin binding proteins in rat brain during development and aging. *Journal of Neurochemistry* **45**(2): 483-489.

Urade, Y., Fujimoto, N. and Hayaishi, O. (1985a) Purification and characterisation of rat brain prostaglandin D synthetase. *Journal of Biological Chemistry* **260**(23): 12410-12415.

Urade, Y., Kaneko, T., Fujimoto, N., Watanabe, Y., Mizuno, N. and Hayaishi, O. (1985b) Purification, characterization, and immunohistochemistry of rat brain prostaglandin D synthetase. *Advances in Prostaglandin, Thromboxane, & Leukotriene Research* **15**: 549-551.

Urade, Y., Fujimoto, N., Kaneko, T., Konishi, A., Mizuno, N. and Hayaishi, O. (1987) Postnatal changes in the localization of prostaglandin D synthetase from neurons to oligodendrocytes in the rat brain. *Journal of Biological Chemistry* **262**(31): 15132-15136.

Urade, Y., Nagata, A., Suzuki, Y., Fujii, Y. and Hayaishi, O. (1989) Primary structure of rat brain prostaglandin D synthetase deduced from cDNA sequence. *Journal of Biological Chemistry* **264**(2): 1041-1045.

Urade, Y., Kitahama, K., Ohishi, H., Kaneko, T., Mizuno, N. and Hayaishi, O. (1993) Dominant expression of mRNA for prostaglandin D synthase in leptomeninges, choroid plexus, and oligodendrocytes of the adult rat brain. *Proceedings of the National Academy of Sciences of the United States of America* **90**(19): 9070-9074.

Urade, Y., Eguchi, N., Kantha, S. S., Kanaoka, Y., Beuckmann, C., Watanabe, K., Hayaishi, O., Kitahama, K., Ohishi, H., Kaneko, T., Mizuno, N., Mader, M. and Murphy, C. (1995a) Prostaglandin D synthase, the key enzyme of sleep regulation, is mainly localised in the leptomeninges and choroid plexus, and secreted into cerebrospinsl fluid. *Sleep Research* **24A**: 107.

Urade, Y., Tanaka, T., Eguchi, N., Kikuchi, M., Kimura, H., Toh, H. and Hayaishi, O. (1995b) Structural and functional significance of cysteine residues of glutathione-independent prostaglandin D synthase. Identification of Cys^{65} as an essential thiol. *Journal of Biological Chemistry* **270**(3): 1422-1428.

Urade, Y., Watanabe, K. and Hayaishi, O. (1995c) Prostaglandin D, E, and F synthases. *Journal of Lipid Mediators & Cell Signalling* **12**(2-3): 257-273.

Urade, Y., Hayaishi, O., Matsumura, H. and Watanabe, K. (1996) Molecular mechanism of sleep regulation by prostaglandin D_2. *Journal of Lipid Mediators & Cell Signalling* **14**(1-3): 71-82.

Urade, Y. and Hayaishi, O. (1999) Prostaglandin D_2 and sleep regulation. *Biochimica et Biophysica Acta* **1436**(3): 606-615.

Van Essen, D. C. (1997) A tension-based theory of morphogenesis and compact wiring in the central nervous system. *Nature* **385**(6614): 313-318.

Vanni-Mercier, G., Sakai, K. and Jouvet, M. (1984) [Specific neurons for wakefulness in the posterior hypothalamus in the cat]. *Comptes Rendus de l Academie des Sciences - Serie Iii, Sciences de la Vie* **298**(7): 195-200.

Vogel, G., Neill, D., Hagler, M. and Kors, D. (1990) A new animal model of endogenous depression: a summary of present findings. *Neuroscience & Biobehavioral Reviews* **14**(1): 85-91.

Von Economo, C. (1931) *Encephalitis Lethargica: Its sequelae and treatment.* London, Oxford University Press.

Walch, L., Labat, C., Gascard, J. P., de Montpreville, V., Brink, C. and Norel, X. (1999) Prostanoid receptors involved in the relaxation of human pulmonary vessels. *British Journal of Pharmacology* **126**(4): 859-866.

Walker, D. W. and Pratt, N. (1998) Effect of probenecid on breathing movements and cerebral clearance of prostaglandin E_2 in fetal sheep. *Journal of Physiology* **506**(Pt 1): 253-262.

Watanabe, K., Shimizu, T., Iguchi, S., Wakatsuka, H., Hayashi, M. and Hayaishi, O. (1980) An NADP-linked prostaglandin D dehydrogenase in swine brain. *Journal of Biological Chemistry* **255**(5): 1779-1782.

Watanabe, K., Urade, Y., Mader, M., Murphy, C. and Hayaishi, O. (1994) Identification of β-trace as prostaglandin D synthase. *Biochemical & Biophysical Research Communications* **203**(2): 1110-1116.

Watanabe, K., Kurihara, K., Tokunaga, Y. and Hayaishi, O. (1997) Two types of microsomal prostaglandin E synthase: glutathione-dependent and -independent prostaglandin E synthases. *Biochemical & Biophysical Research Communications* **235**(1): 148-152.

Watanabe, T., Taguchi, Y., Shiosaka, S., Tanaka, J., Kubota, H., Terano, Y., Tohyama, M. and Wada, H. (1984) Distribution of the histaminergic neuron system in the

central nervous system of rats; a fluorescent immunohistochemical analysis with histidine decarboxylase as a marker. *Brain Research* **295**(1): 13-25.

Watanabe, Y., Yamashita, A., Tokumoto, H. and Hayaishi, O. (1983) Localisation of prostaglandin D_2 binding protein and NADP-linked 15-hydroxyprostaglandin D_2 dehydrogenase in the Purkinje cells of miniature pig cerebellum. *Proceedings of the National Academy of Sciences of the United States of America* **80**(14): 4542-4545.

Watanabe, Y., Tokumoto, H., Yamashita, A., Narumiya, S., Mizuno, N. and Hayaishi, O. (1985) Specific bindings of prostaglandin D_2, E_2 and $F_{2\alpha}$ in postmortem human brain. *Brain Research* **342**(1): 110-116.

Watanabe, Y., Mori, K., Imamura, K., Takagi, S. F. and Hayaishi, O. (1986) Modulation by prostaglandin D_2 of mitral cell responses to odor stimulation in rabbit olfactory bulb. *Brain Research* **378**(2): 216-222.

Watanabe, Y., Hamada, K., Bommelaer-Bayt, M. C., Dray, F., Kaneko, T., Yumoto, N. and Hayaishi, O. (1989) Distinct localization of prostaglandin D_2, E_2 and $F_{2\alpha}$ binding sites in monkey brain. *Brain Research* **478**(1): 143-148.

Webster, H. H. and Jones, B. E. (1988) Neurotoxic lesions of the dorsolateral pontomesencephalic tegmentum-cholinergic cell area in the cat. II. Effects upon sleep-waking states. *Brain Research* **458**(2): 285-302.

Wever, R. A. (1975) The circadian multi-oscillator system in man. *International Journal of Chronobiology* **3**: 19-55.

White, D. M., Mikol, D. D., Espinosa, R., Weimer, B., Le Beau, M. M. and Stefansson, K. (1992) Structure and chromosomal localisation of the human gene for a brain form of prostaglandin D_2 synthase. *Journal of Biological Chemistry* **267**(32): 23202-23208.

White, D. M., Takeda, T., DeGroot, L. J., Stefansson, K. and Arnason, B. G. (1997) β-trace gene expression is regulated by a core promoter and a distal thyroid hormone response element. *Journal of Biological Chemistry* **272**(22): 14387-14393.

Yamaguchi, K., Hama, H. and Watanabe, K. (1997) Possible roles of prostaglandins in the anteroventral third ventricular region in the hyperosmolality-evoked vasopressin secretion of conscious rats. *Experimental Brain Research* **113**(2): 265-272.

Yamamoto, K., Miwa, T., Ueno, R. and Hayaishi, O. (1988) Muramyl dipeptide-elicited production of PGD_2 from astrocytes in culture. *Biochemical & Biophysical Research Communications* **156**(2): 882-888.

Yamashita, A., Watanabe, Y. and Hayaishi, O. (1983) Autoradiographic localisation of a binding protein(s) specific for prostaglandin D_2 in rat brain. *Proceedings of the National Academy of Sciences of the United States of America* **80**(19): 6114-6118.

Yamuy, J., Mancillas, J. R., Morales, F. R. and Chase, M. H. (1993) C-fos expression in the pons and medulla of the cat during carbachol-induced active sleep. *Journal of Neuroscience* **13**(6): 2703-2718.

Zahn, M., Mader, M., Schmidt, B., Bollensen, E. and Felgenhauer, K. (1993) Purification and N-terminal sequence of β-trace, a protein abundant in human cerebrospinal fluid. *Neuroscience Letters* **154**(1-2): 93-95.