Brain ependymal cilia

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by

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Abstract

Densely ciliated ependymal cells line the ventricular surface of the brain, cerebral aqueduct, and the central canal of the spinal cord and beat continuously at up to 40Hz.
Methodologies for the measurement and the containment of ependymal cilia were developed to allow their study in an *ex-vivo* setting. Three methods were used to measure ciliary beat frequency, the photomultiplier, photodiode and high speed imaging were established and compared.

The method used, to hold ciliated ependymal strips affected ependymal ciliary beat frequency. Tissue held in a microscope cover glass system, or under direct observation of a water immersion lens, showed a beat frequency of half that of cilia observed in an incubation chamber using an inverted microscope. The incubation system, using an inverted microscope, and high speed video system were chosen for the studies in this thesis.
The results obtained using this system may be summarised as follows:

- there was no change in ciliary length or beat frequency between ependyma from infant and adult Wistar rats. However, ependymal cilia beat twice as fast as respiratory cilia.
- a pH of less than 7 was associated with dramatic fall in ciliary beat frequency which was shown to be directly related to changes in intracellular pH.
- increasing viscosity decreased ciliary beat frequency, though cilia demonstrated a capability to adapt to an increasing viscous load.
- pentobarbitone only slowed cilia at levels which were incompatible with life. Halothane, a volatile anaesthetic, caused significant, but reversible ciliary slowing at concentrations which are used in a clinical setting.
- adrenergic agents and forskolin had no effect on ependymal ciliary function.
- ethanol and acetaldehyde had no effect on ependymal ciliary beat frequency.
- low concentrations of the toxin MAM, thought to be implicated in the development of Parkinsonism, caused ciliary slowing.

Chapter 1: Brain ependymal cilia : Literature Review

Abbreviations:

Ach	- acetylcholine
ALS	- amyotrophic lateral sclerosis
ANOVA	- analysis of variance
ATP	- adenosine triphosphate
BCECF	- 2',7'-bis(2'carboxy ethyl)'5(6)' carboxy fluorescein
BMAA	- beta-N-methylamino-L-alanine
BSA	- bovine serum albumin
CaM	- Ca^{2+}/calmodulin
cAMP	- cyclic adenosine 3'5' monophosphate
cGMP	- guanosine 3'5' monophosphate
CNS	- central nervous system
cP	- centipoise
CSF	- cerebrospinal fluid
DAG	- diacylglycerol
DIOD	- modified photodiode technique
DMSO	- dimethyl sulphoxide
DNA	- deoxyribose nucleic acid
EEG	- electro-encephalogram
G-proteins	- guanalyl nucleotide binding proteins
GABA	- gamma aminobutyric acid
GTP	- guanylyl triphosphate
HBS	- hepes buffered saline
HBSS	- HEPES buffered saline solution
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSV	- high speed video
Hz	- hertz
IBMX	- isobutly methyl xanthine
IL	- interleukin
iNOS	- inducible nitric oxide synthetase
IP3	- inositol 1,2,3, triphosphate
L-NAME	- N-nitro-L-arginine methylester
L-NMMA	- N-monomethyl-L-arginine
M	- Molar
MAC	- minimum alveolar concentration
MAM	- methylasoxymethanol
mRNA	- messenger ribonucleic acid
Ng	- nitro-L-arginine methyl ester
NMDA	- N-methyl-D-asparate
NO	- nitric oxide
NOS	- nitric oxide synthetase
P	- Probability
PDC	- Parkinsonism-dementia complex
PDE	- phosphodiesterase
pH	- log hydrogen ion concentration
pHi	- intracellular pH
pHo	- pH of extracellular buffer
PHOTO	- photomultiplier technique
PIP_2	- phosphatidylinositol diphosphate
PKA	- protein kinase A
PKC	- protein kinase C
PLC	- phospholipase C
Re	- Reynold's number
SD	- standard deviation
TFP	- trifluroperazine

TNF	- tumour necrosis factor
ug	- microgram
um	- micrometer

Chapter 1

Brain Ependymal cilia

Literature review

1.1 Introduction:

In lower vertebrates and invertebrates, ciliary transport is important in the feeding process and also in locomotion. In mammals this transport system aids the movement of gametes in the oviduct, sperm in the ductus efferentess of the testis and mucus and debris from the airways (Satir & Sleigh, 1990). Defective mucociliary transport is an important pathophysiological feature in several human respiratory diseases including cystic fibrosis, chronic bronchitis, and primary ciliary dyskinesia (Wanner, 1977). Although the role of mucociliary transport in tubal infertility has not been clearly established, there are well-documented cases of the association of immotile cilia syndrome and infertility (McComb et al, 1986). The role of ependymal cilia in the central nervous system, however, is less well understood. Although the precise role of ependymal cilia remains unclear it is unlikely that evolution would retain a layer of cells, with such a high metabolic demand, without reason.

This literature review describes the structure and function of cilia, much of which is relevant to subsequent chapters. Details of studies focusing on ependymal ciliary function are followed by a review of the ependymal cell layer which lines the ventricular system, aqueducts and central canal of the spinal cord. The chapter is completed by a brief review of cerebrospinal fluid.

1.2 Ciliary structure and function

The cilium is an extension of the cell surface, which houses a tubulin-based axoneme. The internal structure acts as a motorised unit creating the ciliary beat. The internal structure of a cilium consists of nine outer doublet microtubules and two single central pair microtubules, the 9+2 axoneme (Figure 1.a). These are constructed from heterodimers of alpha and beta-tubulin, arranged in protofilaments. The doublet microtubules consist of subfiber A comprised of 13

protofilaments on to which subfiber B (10-11 protofilaments) assembles. The midwall common to both subfibers, is composed of tektin, an intermediate filament like protein that resists detergent extraction (Linck et al, 1985: Hastie et al, 1992). Tektin appears to be very important to the structural ability of microtubules because the two subfibres separate in its absence.

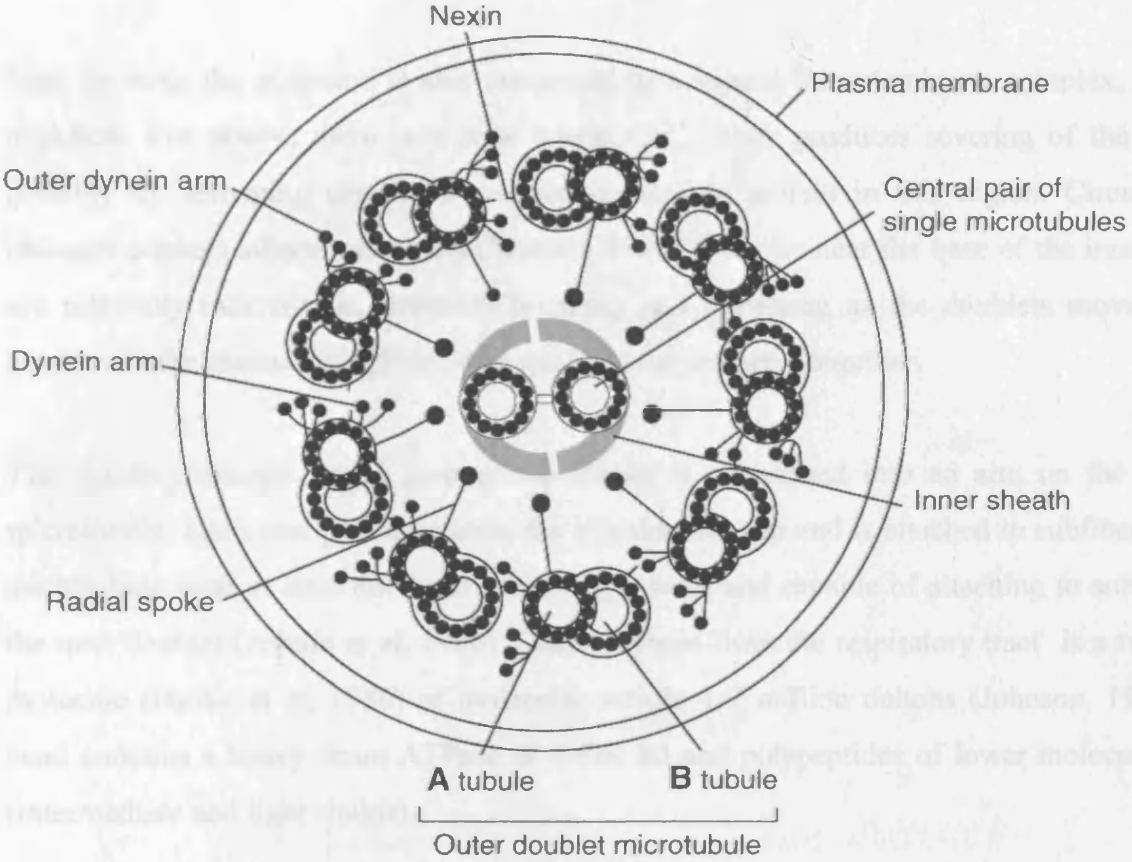


Figure 1a.
The arrangements of microtubules within a cilium.

In mammalian cells, assembly of the axoneme occurs in a fixed pattern at the cell surface above basal bodies (Dirksen, 1982). Axonemal assembly occurs directly on the basal body microtubules and complex doublets are polymerised. Axonemal doublets are polarised such that the fast polymerising (+) end is most distal to the basal body, which corresponds to the tip of the axoneme. Doublet stability relies on multiple interactions with other axonemal proteins (Luck, 1984). Mammalian cilia grow to 5-10 μm in length with human respiratory cilia being 6um. The doublet length is probably controlled by capping structures (Dentler, 1981) which is important as if the cilium is too long it may be less efficient (Afzelius et al, 1985). The axonemal components are arranged in repetitive units along the body of the cilium. Each unit along a

doublet microtubule is 96nm long. It consists of four outer dynein arms, three to four inner dynein arms, one spoke group (three radial spokes), and one pair of interdoublet links. At the tip of the cilium only the nine subfiber A's insert into a disc forming the cytoplasmic surface - the ciliary crown. The ciliary crown carries three to seven short claws (25 to 35 nm long), whose function in the respiratory tract is presumably to propel the mucus layer.

Near its base, the axoneme is also connected to a special transmembrane complex, the ciliary necklace. Just above, there is a zone where Ca^{2+} shock produces severing of the axoneme, possibly by activating centrin, a calcium contractile protein in this region. Circumferential linkages connect adjacent doublets (Warner, 1983). They lie near the base of the inner arm and are relatively inextensible, probably breaking and reforming as the doublets move past one another. In the absence of dynein, they can hold the axoneme together.

The dynein molecule which powers the cilium is compacted into an arm on the axonemal microtubule. Each arm projects across the interdoublet gap and is attached to subfiber A of one microtubule with at least one head projecting toward and capable of attaching to subfiber B of the next doublet (Avolio et al, 1986). Ciliary dynein from the respiratory tract is a two headed molecule (Hastie et al, 1986) of molecular weight 1-2 million daltons (Johnson, 1985). Each head contains a heavy chain ATPase of 4-500 kd and polypeptides of lower molecular weight (intermediate and light chains).

The dynein molecule moves the structure to which it is attached in an ATP-insensitive manner toward the base of the axoneme. The microtubule along which the dynein walks by its ATP-sensitive heads moves towards the tip of the axoneme. A single-head fragment of dynein is sufficient to give motility (Sale & Fox, 1988). Microtubule movement generated by dynein has been studied in sliding axonemes (Fox & Sale, 1987: Sale & Satir, 1977) or completely in vitro by using isolated dynein attached to glass and taxol-stabilised microtubules (Pasehal et al, 1987). In vitro sliding of microtubules in isolated, partially digested axonemes has been demonstrated directly for vertebrate sperm (Wolley & Brammall, 1987) and for mammalian, tracheal and oviduct cilia (Dirksen & Zeira, 1981).

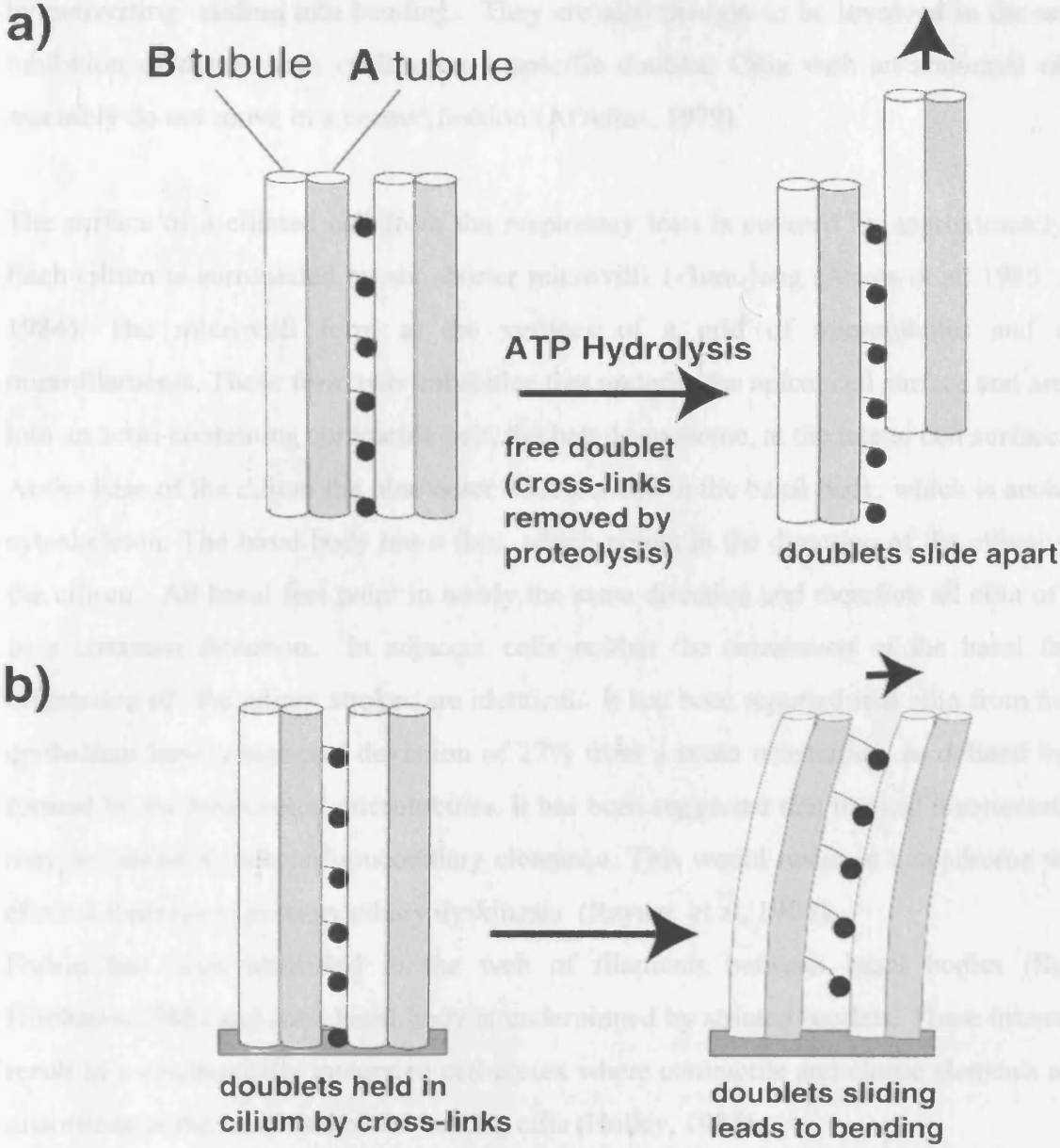


Figure 1.b

The bending of an axoneme. a) the sliding of outer microtubule doublets against each other causes the axoneme to elongate if the proteins that link the doublets together are removed by proteolysis. b) If the doublets are tied to each other at one end, the axoneme bends.

Paired links composed of nexin connect the nine outer doublet microtubules with each other. The radial spokes connect the doublet microtubules to the central pair of microtubules. They are a self-assembling, multipolypeptide structure (Luck, 1984) consisting of a cylindrical stalk and an expanded spoke head. Radial spokes limit microtubule sliding of active doublets

by converting sliding into bending. They are also thought to be involved in the activation or inhibition of dynein arm cycling on a specific doublet. Cilia with an abnormal radial spoke assembly do not move in a normal fashion (Afzelius, 1979).

The surface of a ciliated cell from the respiratory tract is covered by approximately 200 cilia. Each cilium is surrounded by six shorter microvilli 1-3um long (Arima et al, 1985: Reed et al, 1984). The microvilli form at the vertices of a grid of microtubules and actin-based microfilaments. These form two trabeculae that underlie the apical cell surface and are integrated into an actin-containing contractile belt, the belt desmosome, at the lateral cell surface.
At the base of the cilium the nine outer doublets end in the basal body, which is anchored to the cytoskeleton. The basal body has a foot, which points in the direction of the effective stroke of the cilium. All basal feet point in nearly the same direction and therefore all cilia of a cell beat in a common direction. In adjacent cells neither the orientation of the basal feet nor the orientation of the ciliary strokes are identical. It has been reported that cilia from human nasal epithelium have a standard deviation of 27% from a mean orientation, as defined by the plane formed by the two central microtubules. It has been suggested that marked disorientation of cilia may be linked to reduced mucociliary clearance. This would result in a syndrome with similar clinical findings to primary ciliary dyskinesia (Rayner et al, 1996).
Fodrin has been identified in the web of filaments between basal bodies (Kobayshi & Hirokawa,1988) and each basal body is underpinned by striated rootlets. These interconnections result in a mechanically integrated cell cortex where contractile and elastic elements act to resist distortions at the basal end of the beating cilia (Holley, 1984).

Brightman and Palay (1963) described, in detail, the transition from the cilium to the basal body of ependymal cilia in the brain of the rat. The basal body extends 0.3-0.4 microns into the cytoplasm. Its core is occupied by a clear matrix with a few coarse granules. In the deep portion of the basal body, its wall of nine peripheral doublets becomes a ring of nine triplet fibres. The subfibres of each triplet are aligned in a straight row and each row skewed so that subfibre A is closest to the centre of the basal body. Basal bodies of ependymal cilia are distinctive among mammalian cilia by their unusual filamentous appendages. Each basal body is fitted with a brush of fine filaments attached to the doublet or triplet fibres of the wall and extending into the

cytoplasm. The rootlet filaments are about 100A in diameter and about 0.4 microns long. The rootlets appear to arise in two main bundles. Those emerging from the lateral aspects of the

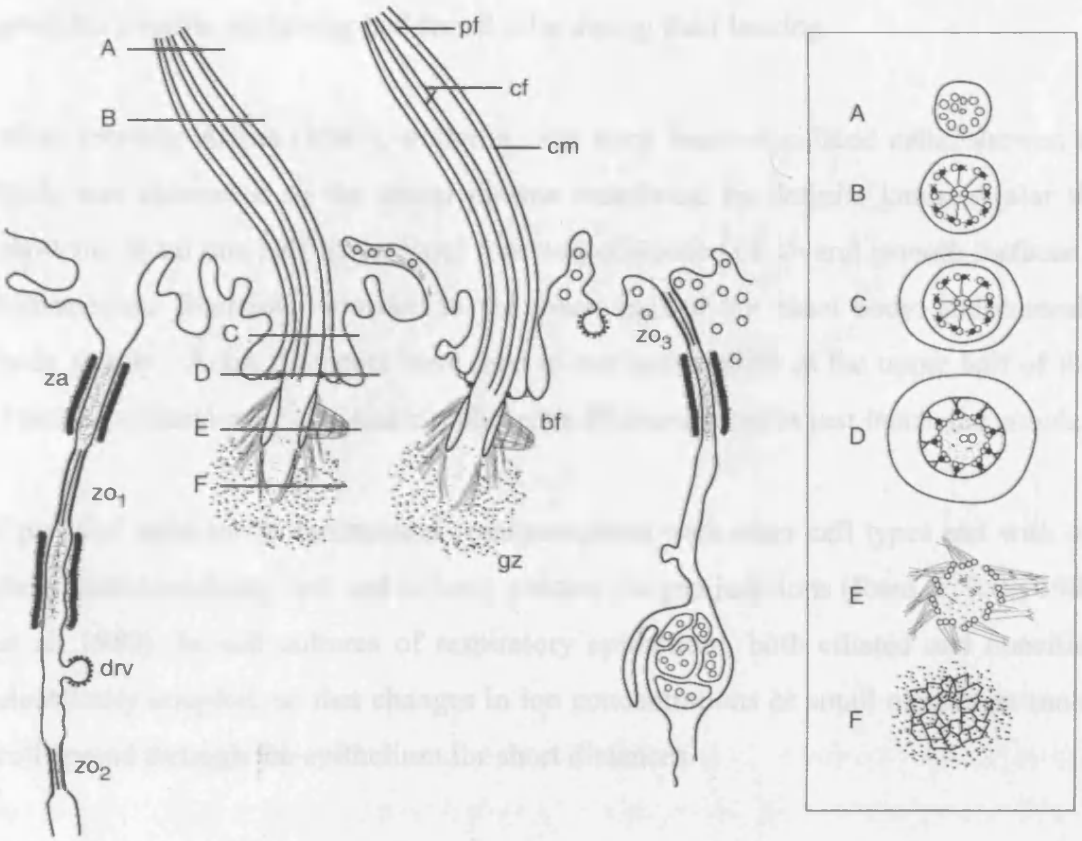


Figure 1.b.1
Diagram summarising the principal features of the lateral and apical surfaces of the ependymal cell of the rat. A ciliary complex including basal (D and E), rootlets (E and F), basal foot (bf), and granular zone (gz) is depicted in successive transverse planes designated by letters A - F. Densely rimmed vesicles (dvr) may be continuous with either the ventricular or the lateral cell membranes. A cluster of small neuronal processes occupies a "lacuna" or local distension of the intercellular space. CF = central fibres; CM = ciliary membrane; PF = peripheral subfibre; ZA = zonula adherens (Brightman & Palay, 1963).

basal body tended to diverge, while those leaving the proximal tip of the basal body converge below it. The basal foot consists of a short, conical collection of striated filaments attached to only one side of the basal body wall and extending into the cytoplasm at approximately 90 degrees to its longitudinal axis. There is a complex network between adjacent basal bodies which consists of microfilaments, microtubules, and intermediate filaments. The filamentous

networks are thought to play a functional as well as structural role during ciliary beating. The filament has networks, including the actin filaments, between basal bodies which have contractile ability. The relationship between basal bodies and the intermediate filament network provides a stable anchoring bed for all cilia during their beating.

More recently Arima (1985), studying cilia from tracheal ciliated cells, showed that the basal body was connected to the apical plasma membrane by definite laminae (alar sheets). They show the distal one half of the basal foot was composed of several smooth surfaced 12nm fibrils. Intermediate filaments extended to the lower half of the basal body, and enmeshed the basal body tightly. Actin filaments were seen to run horizontally at the upper half of the basal body. Tracheal ciliated cells also had circular actin filament bundles just inside the zonula adherens.

Epithelial cells are in mechanical communication with other cell types and with one another at their actin containing belt and in ionic contact via gap junctions (Reed & Satir, 1986: Sanderson et al, 1989). In cell cultures of respiratory epithelium, both ciliated and nonciliated cells are electrically coupled, so that changes in ion concentrations or small messenger molecules in one cell spread through the epithelium for short distances.

1.2.1 The ciliary beat cycle:

The ciliary beat cycle consists of a resting state, effective state and recovery state. During the recovery stroke the cilium swings almost 180 degrees backwards and close to the cell surface. It then fully extends and goes directly through its effective stroke in a plane perpendicular to the cell surface. After completion of the effective stroke, the cilium rests and then resumes its recovery and effective strokes (figure 1c).

1.2.2 The switch point hypothesis of ciliary motion:

The switch point hypothesis assumes that half of the doublets of the axoneme have active arms when the axoneme is moving in its effective stroke and that the other half has active arms during the return stroke (Satir, 1985). Activity then switches from one set of arms to another during a ciliary beat and back again at the beginning of the next beat.

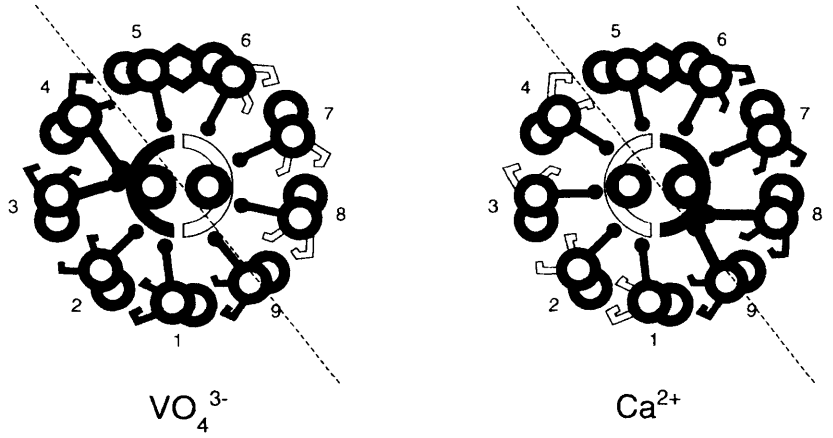


Figure 1.c: *Axonemal splitting in "hands down" versus "hands up" muscle gill lateral ciliary axonomes suggest that different halves of the axoneme have active arms. The figure shows predictions of activity in the axoneme arrested by the ions shown. The central pair splits from the active half axoneme (Satir and Matsuoka 1989).*

During ciliary beating, dynein arms form molecular bridges between subfibres A and B of adjacent outer doublet microtubules and, using energy from ATP hydrolysis, undergoes a conformational change that causes microtubules to slide relative to each other.

With an axoneme beating at 50 Hz, arms on subfiber A of a microtubule in the active half of the axoneme would switch off after 10 msec and would become non-cycling and refractory for 10 msec before resuming activity. During this refractory period, the doublet would move passively in the opposite direction. Spungin et al (1987) have produced negative stain images with two differently appearing arm distributions, one which may correspond to cycling arms and

one to the non-cycling state. Work on *Chlamydomonas* mutants suggests that the outer dynein arm is mainly responsible for regulation of ciliary beat frequency and inner dynein arms control the bend formation and beating form.

According to the switch point hypothesis one switch turns the arms of one set of doublets on and off, the other turns the complementary set on and off. When one switch is blocked, the cilia come to rest in a specific position, irrespective of where in the beat cycle the block is applied (Reed & Satir, 1986). If the second switch is blocked ciliary arrest occurs in a different position. Cilia can be moved from one arrest position to another without restarting beating by changing the blocking agent. Reactivation of hamster sperm flagella by local application of ATP generates a predictable pattern of bending, depending on initial position and consistent with activation of doublets 1- 4 or 6-9 (Yeung & Wolley, 1983). Sale (1986) has also shown that in sea urchin axonemes arrested in a specific position, ATP addition permits one subset of doublets, probably 5-7, to slide away from the remainder of the axoneme.

The opposite acting halves of the axoneme are determined by those doublets whose radial spokes interact with one central microtubule and its projections and not the other central microtubule (Satir, 1985). It is thought that the co-ordinated spoke-central sheath attachment in the active half of the axoneme converts sliding into bending. Mutations in spoke or central sheath proteins produce immotility but leave the sliding system intact. In metazoan cilia, the position of the central pair seems fixed (Fox & Sale, 1987). However, in protozoan axonemes, the central pair may rotate (Omoto & Whitman, 1981) either as a causal factor in switching of arm activity in the axoneme, or as a consequence of switching.

Cilia of swimming cells can drastically change their beat form so that the cell swims backwards (Lieberman et al, 1988) or turns toward a chemotactic stimulus (Brokaw, 1979). These changes may be explained by the switch point hypothesis if the timing of the switches controls the beat form of the axoneme. Where dynein arms are actively sliding for equal times in the two half-axonemes, the bends generated would be symmetrical. If timing was unequal, the principal bend would correspond to the longer on time, the reverse bend to the shorter (Satir, 1982).

It is likely that all arms along a particular doublet activate progressively with bend propagation. Arms on the doublets within a half axoneme may activate with a defined phase relationship with nine separate activation events rather than two. Axonemal bending may sometimes arise by mechanisms different from axonemal sliding (Eshel & Brokaw, 1987).

The switch point hypothesis does not specify the manner of bend propagation during beat. Studies, however, indicate that there are extensive feedback systems in the axoneme relating to bend generation and bend propagation (Gibbons, 1982: Sato et al, 1988).
The role of Ca^{2+}, calmodulin, cAMP, ATP and nitric oxide in ciliary activity are discussed in greater detail below.

1.3: Cellular control of ciliary movement:

The precise mechanisms of cellular control of ciliary beating have not been determined. However, research into changes in intracellular calcium, cyclic AMP and more recently nitric oxide (NO) has lead to greater understanding.

An increase in intracellular cAMP concentration increases ciliary beat frequency in mammalian respiratory epithelial cells. In human cells this increase is blocked by a kinase inhibitor H-7 (Tamaoki et al, 1989: Di Benedetto et al, 1991). Hamasaki et al (1989) showed that cAMP dependent protein kinase (PKA) phosphorylated specific axonemal targets both invitro and in permealised cells from *Paramecium.* In all species studied, increasing cAMP concentrations stimulates ciliary beat frequency through activation of PKA and the subsequent phosphorylation of ciliary targets. A dynein light chain, p29, was identified as one target. Phosphorylation of p29 is known to increase the velocity of microtubule gliding across dynein coated surfaces in vitro and to increase the swimming speed of *Paramecium* (Hamasaki et al, 1991).

However, a frequency regulating complex has been found between the radial spokes and inner dynein arms of Chlamydomonas flagella (Gardner et al, 1994). This decreases ciliary beat frequency in response to c-AMP dependent phosphorylation by an attached kinase (Howard et al, 1994). In addition protein kinase C (PKC) activation has been shown to decrease ciliary beat frequency in the respiratory cilia of mammals (Kobayashi et al, 1992).

Cyclic GMP, however, has been reported to increase ciliary beat frequency in cultured human respiratory epithelium following culture with a conditioning medium (Geary et al, 1995).

An increase in intracellular calcium concentration increases ciliary beat frequency while a decrease in intracellular calcium slows ciliary beating. It is still unclear how changes in intracellular calcium concentration lead to changes in ciliary beat frequency. It has been suggested that changes in intracellular calcium concentrations could act through calmodulin. There are reports that calmodulin inhibitors inhibit the effect of intracellular calcium on ciliary beat frequency. Di Benedetto and colleagues (1991), have shown that control of basal ciliary beat frequency is mediated through a calmodulin-sensitive system. Trifluroparazine (TFP) an inhibitor of calmodulin sensitive calcium protein kinases, decreased basal ciliary beat frequency and prevented ionophore stimulation of ciliary beat frequency.
Calmodulin could stimulate ciliary beat frequency through activation of a Ca^{++} / calmodulin kinase (CaM kinase) or by stimulation of a phosphatase with the dephosphorylation of a ciliary target. Both ciliary protein targets of CaM kinase (Chilcote & Johnson, 1990) and phosphatases, including calmodulin-dependent phosphatase, have been described in cilia (Tash et al, 1988).
Alternatively, calmodulin could mediate the effect of calcium directly or through a pathway independent of CaM kinase or phosphatase. Calmodulin in the hamster respiratory epithelium has been shown to be bound in a Ca^{++} dependent manner to microtubules, dynein arms, basal bodies and plasma membrane (Gordon et al,1982). Calmodulin may also activate an enzyme such as nitric oxide synthetase (NOS).

The following sections describe in more detail the effect of mechanical stimulation, ATP and nitric oxide on ciliary beat frequency.

1.3.1 Mechanism of the Mechanosensitive Response:

Ciliated cells are mechanosensitive. Stimulation of the apical surface of a single ciliated cell with a glass microprobe elevates the ciliary beat frequency of the stimulated and adjacent cells (Sanderson & Dirksen, 1989: Sanderson et al, 1988). The increase in beat frequency of each cell occurs after a lag-phase and is proportional to the distance from the stimulated cell. Stimulation of a nonciliated cell, adjacent to ciliated cells, also increases their beat frequency. A mechanical stimulus to either a ciliated or nonciliated cell induces an immediate increase in intra cellular

Ca^{2+} at the contact point which spreads throughout the cell. After approximately half a second, a wave of increasing intracellular Ca^{2+} occurs in adjacent cells travelling across up to 7 cells. The rise in intracellular Ca^{2+} always preceds an increase in ciliary beat frequency (Sanderson et al, 1990).

Ca^{2+} may enter the cell through stretch activated calcium channels (Kourie et al, 1990), on mechanical stimulation. However, under Ca^{2+} free conditions an increase in intracellular Ca^{2+} does not occur in the stimulated cell but does occur in adjacent cells. This implies that cellular propagation of Ca^{2+} waves involves release of Ca^{2+} from intracellular stores. The intracellular messenger inositol trisphosphate (IP_3) has been shown to release Ca^{2+} from intracellular stores in many cell types. The iontophoretic injection of IP_3 into rabbit tracheal cells, in the absence of extracellular Ca^{2+} , initiates the propagation of Ca^{2+} waves in rabbit tracheal cells.

Respiratory cells, in culture, have gap junctions and are electrically coupled (Sanderson et al, 1988). Sanderson and colleagues (1992) have proposed that IP_3 acts as both an intracellular and intercellular messenger in respiratory tract cells, moving through gap junctions increasing intracellular Ca^{2+} and ciliary beat frequency. This hypothesis requires that IP_3 is produced in the stimulated cell. One explanation of the initiation of a calcium wave under calcium free conditions may be through the production of IP_3.

The ciliary beat frequency of respiratory tract cells can also be elevated, in a dose-dependent manner, by isoproterenol, a beta-adrenergic agent (Sanderson & Dirksen, 1989). Increases in beat frequency induced by mechanical stimulation can be enhanced by isoproterenol. As beta-adrenergic agonists elevate intracellular cAMP, this supports the concept of at least two control mechanisms for the ciliary activity, namely, Ca^{2+} and cAMP.

Increases in temperature, intracellular Ca^{2+} induced by the ionophore ionomycin or cAMP induced by isoproterenol, increase ciliary beat frequency. This is achieved by decreasing the timing of the rest, recovery stroke and effective stroke of the beat cycle in a similar manner. This suggests that Ca^{2+} and cAMP regulate beat frequency in a similar manner by modifying the rate at which the axoneme can utilise ATP. Alternatively, Ca^{2+} or cAMP may regulate the availability of ATP (Lansley et al, 1992).

The cilia of other species often respond differently to Ca^{2+} and cAMP. Alteration of intracellular calcium either modifies the wave form of sperm or completely inhibits sperm mobility (Gibbons & Gibbons, 1980). The wave form of *Chlamyomonas* flagella may be modified to reverse its swimming direction (Hyams & Borisy, 1978). Cyclic AMP stimulates the activity of sperm (Tash & Means, 1983) but inhibits the invitro motility of Chlamydomonas (Hasegawa et al, 1987).

Cell models can be made permeable to allow small molecules such as Ca^{2+}, cAMP and ATP direct access to the ciliary interior. Saponin has been used to make ciliary membranes permeable which inactivates ciliary activity. The activity can be reactivated by addition of ATP. Reactivated, permeable, cells exhibit a lack of sensitivity to Ca^{2+} or cAMP. The lack of sensitivity to cAMP was seen at low concentrations of ATP. The modification of ciliary beat frequency by Ca^{2+} and cAMP in intact cells but not permeable cells, suggests that Ca^{2+} and cAMP interact with some intracellular component, rather that the axoneme, to induce ciliary beat frequency (Batts et al, 1992). Their relationship to NO and cGMP is discussed later.

1.3.2 Ciliary stimulation by Extracellular ATP:

Weiss et al (1992) studied the effect of exogenous ATP on the ciliary motility of the frog palate epithelium. The addition of micromolar amounts of ATP increased ciliary beat frequency. A similar effect was seen with exposure to adenosine triphosphate, a nonhydrolysable analog of ATP, confirming that ATP hydrolysis was not required. The enhancement of extracellular ATP on ciliary activity was dependent on the concentration of extracellular Ca^{2+} . A similar effect was seen when extracellular Ca^{2+} was replaced by Mg^{2+} . Potent inhibitors of voltage gated calcium channels had no effect on the stimulation of ciliary activity by ATP, over the concentrations where they are specific. Inhibition of K^+, calcium dependent channels by quinidine inhibited the effect of ATP. The authors suggested that exogenous ATP interacts with a membrane receptor in the presence of Ca^{2+}, mobilising intracellular calcium. The increase in the free intracellular calcium opens the calcium-activated K^+ channels, which then leads to an increase in membrane potential. Cilia respond by increasing their frequency (figure 1d).

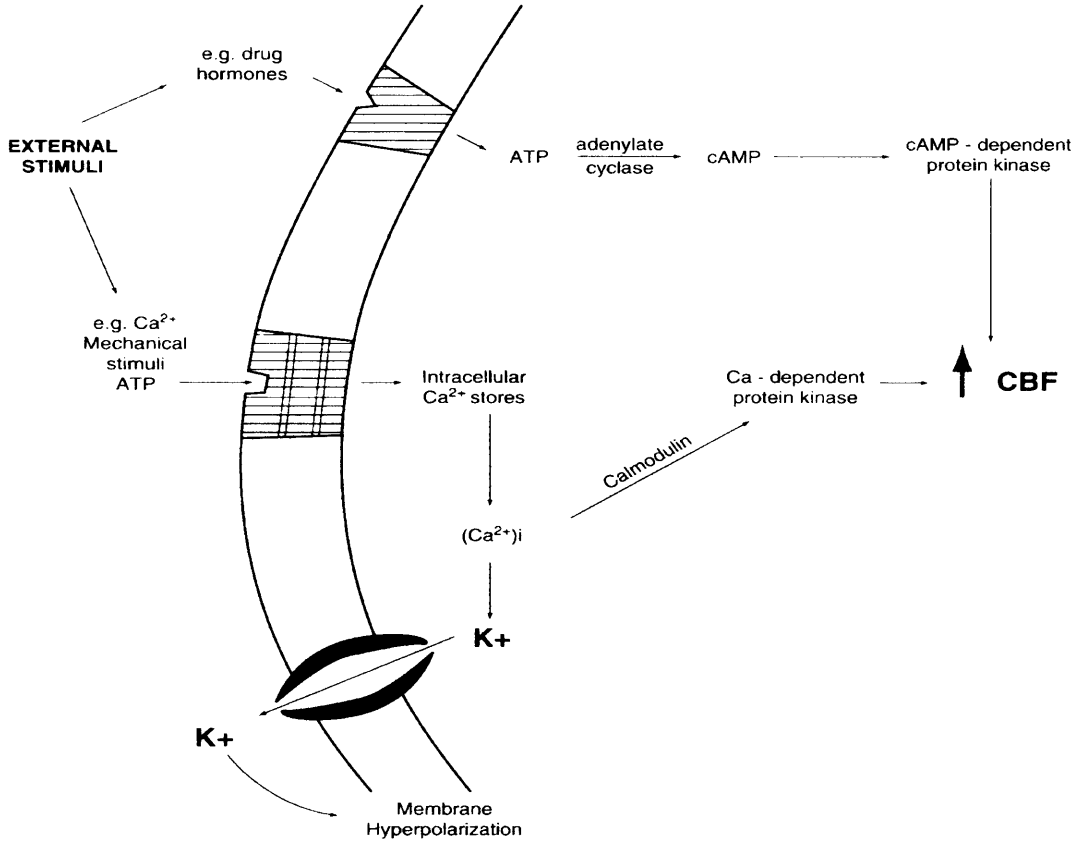


Figure 1.d *Summary of mechanisms proposed by Weiss et al (1992) to be important in the regulation of ciliary beat frequency.*

The results of Weiss et al (1992) are at variance with Tamaoki and colleagues (1989). The reason for this is unclear. Tamaoki et al (1989) found that in cultured rabbit tracheal epithelium, ciliary beat frequency was suppressed by adenosine and related substances. This was expected as a high affinity receptor for adenosine, the A_1-receptor, inhibits adenylate cyclase activity in tissues. In the presence of adenosine, the intracellular cAMP of the respiratory epithelium is decreased. The decrease in beat frequency and cAMP were reversed by phenyltheophylline, an adenosine receptor antagonist.

Simultaneous measurement of intracellular Ca^{2+} concentrations and ciliary beat frequency have been conducted by Komgreen and Priel (1994). They showed that ciliary beating and intracellular calcium concentrations do not correlate fully. Their results suggest that ATP triggers a plasma membrane receptor, releasing calcium from intracellular stores, stimulating ciliary beat frequency. An initial rapid rise in ciliary beat frequency over the first 2 seconds of stimulation was followed by a slower rise, lasting 20 seconds to the maximal value. The rise in intracellular Ca^{2+} concentration also showed a rapid rise over the first 2 seconds followed by a slower one lasting 10 seconds, which was twice as fast as the rise time of the ciliary beat frequency. After depletion of intracellular calcium stores with Thapsigargin, in a calcium free medium, extracellular ATP had no effect.

1.3.3 The role of nitric oxide (NO) in ciliary stimulation:

Ciliary beat frequency is increased in human and other species upon application of certain neurotransmitters and adrenergic or cholinergic drugs (Sanderson & Dirksen, 1989: Wong et al, 1988). Beta-adrenergic compounds, such as isoproterenol, increase beat frequency of mammalian respiratory cilia in vivo and in vitro. The effect is mediated through beta-adrenergic receptors as the beta blocker, propranalol blocks the response (Verdugo et al, 1980). Beta-adrenergic drugs will stimulate an increase in beat frequency when applied to either the ciliated surface or the basal surface of the tissue. However, stimulation was found to be more significant at the basal surface (Wong et al, 1988). Cholinergic drugs, however, may stimulate the ciliated surface slightly more effectively. The involvement of nitric oxide in these responses is discussed below.

Over recent years nitric oxide, a reactive gas, has been shown to function as a signalling molecule in endothelial and nerve cells and as a killer molecule by activated immune cells (Aagard, 1994). NO biosynthesis involves the hydroxylation of the nitrogen in the guanidino group of L-arginine. The reaction is catalysed by NO synthase (NOS). The process incorporates molecular oxygen into NO and citrulline. Several forms of NO synthase have been identified. The most important are the constitutive NOS (cNOS) present in the endothelium and neural tissue and the inducible enzyme (iNOS) formed in activated immune cells and vascular smooth cells. The constitutive forms synthesise small amounts of NO on demand. The synthesis of NO from L-arginine can be inhibited by analogues of L-arginine, such as N-monomethyl-L-arginine

(L-NMMA) and Ng-nitro-L-arginine methyl ester (L-NAME), which complete with L-arginine at the active site of NOS.

There is known to be local release of NO in the airways. Jain et al (1993) investigated the role of NO in ciliary movement. They demonstrated an NO dependent mechanism which upregulated ciliary beat frequency in bovine bronchial epithelial cells. NOS inhibitors were found to slow the increase in ciliary beat frequency induced by isoproterenol, bradykinin and substance P. These effects were reversed by L-arginine but not D-arginine. An NO donor, sodium nitroprusside, also reversed the effects of the NOS inhibitors. The NOS inhibitors used, L-NMMA and L-NAME induced ciliary slowing only when the ciliary beat frequency had been increased by agonists, suggesting resting ciliary beat frequency is not modulated to a significant extent by NO.

Other agents which increase ciliary beat frequency have been shown to be dependent on an NO/NOS signal transduction pathway. Sisson (1995), found ethanol, in concentrations found in the blood of social drinkers, stimulated ciliary beat frequency. The stimulation was dependent on NO. L-NMMA completely blocked the increase in ciliary frequency with ethanol which was restored by adding either L-arginine or sodium nitroprusside. The rapidity of the response suggested upregulation of the constitutive NO.

The conclusions of these studies are based on the premise that the NOS inhibitors were specific inhibitors of NOS. Tamaoki and colleagues (1995, 1996) found NO to be responsible for the increase in the respiratory ciliary beat frequency seen following incubation with the beta-2 stimulant, salbutamol, and with *Zizyphi Fructus* a constituent of an anti-asthmatic herbal remedy. They found stimulation to be blocked by incubation with L-NAME but not by D-NAME and that inhibition was reversed by L-arginine but not by D-arginine. In their experiments, NO, in the solution bathing the ciliated cells, was measured by detecting a current from a NO-selective electrode. A concentration dependent rise in NO was seen with increasing amounts of the stimulants studied.

Jain and colleagues (1995) found that inflammatory cells such as alveolar macrophages release factors that are capable of upregulating ciliary motility in airway epithelium. Antibody blocking

studies indicate that these effects are mediated through the release of TNF-alpha and /or IL-1beta. Complete blocking was seen when antibodies against TNF-alpha and IL-1beta were combined. Recombinant TNF-alpha or IL-1beta also upregulated ciliary motility. The latent increase in cytokine induced upregulation of motility appears to be due to new gene transcription and is reported to be inhibitable by steroids. It is consistent with the time delay seen during the induction of iNOS mRNA by cytokines. These results suggest the cytokine up regulation of ciliary beat frequency is NO- dependent as addition of NO inhibitors after stimulation with TNF-alpha or IL-1beta results in ciliary slowing. As with previous studies this inhibition was reversible by adding L-arginine or an NO donor.
Incubation of ciliated samples with a combination of isoproterenol and rTNF-alpha or rIL-1beta resulted in initial rapid stimulation lasting up to 2 hours followed by secondary stimulation when measured at 24 hours. Both episodes of stimulation could be blocked by NO inhibitors. The time dependence of the ciliary stimulation seen may be explained by activation of the constitutive NOS by isoproterenol and the new expression of iNOS by cytokines.

The parasympathetic nervous system is thought to play an important role in the airway defence system. One possible source of Ach in vivo is the extensive network of autonomic nerve fibres that innervate the airway (Latinen, 1985). These nerve fibres may provide neural control of the activity of cilia through the release of neurotransmitters. Although little is known about the effects of autonomic nerve stimulation on ciliary activity in mammalian airways, the strongest support of this concept comes from morphological evidence for direct synaptic contacts between intraepithelial nerve endings and ciliated cells in the frog palate. There is an increase in ciliary beat frequency on electrical stimulation of the palatine nerve to the explanted frog palate epithelium (Chu & Kennedy, 1994).

Methylcholine, a beta-methyl ester of acetylcholine, accelerates the mucociliary wave frequency, and this can be blocked by the non selective, muscarinic receptor antagonist, atropine (Hybbinette & Merke, 1982). Yang and colleagues (1997) have recently investigated signal transduction pathways in modulation of ciliary beat frequency by methacholine. They found stimulation of cilia by methacholine involves prostaglandin and NO second messengers and activation of a cGMP-dependent kinase. Figures 1.e and 1.f out line the pathways blocked by Yang and their proposed model of the action of methacholine in the stimulation of cilia. The stimulation was significantly inhibited when the cyclooxygenase pathway was blocked by

diclofenac, suggesting that the action of methacholine in stimulation of respiratory cilia requires endogenous prostaglandin generation.

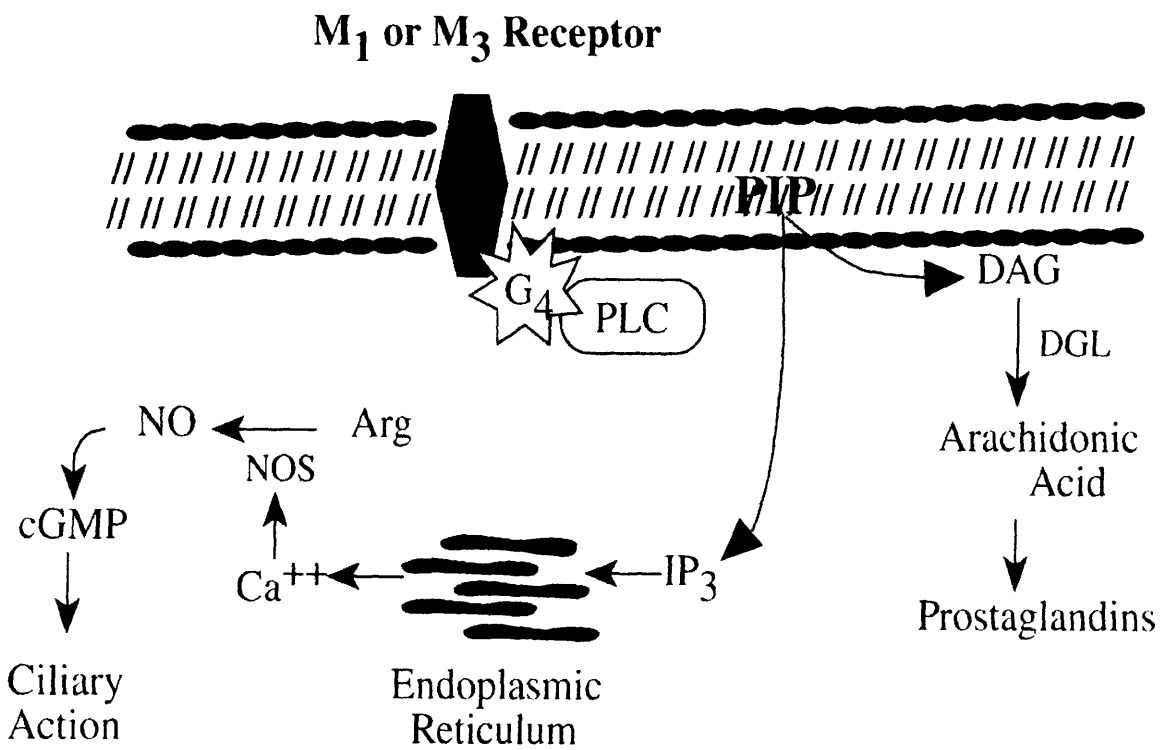


Figure 1.e:

Proposed signal transduction pathway for the stimulation of cilia by methacholine (Yang et al 1997). M1 and M3 muscarinic receptors are G protein-linked receptors (Gq) that are linked to phospholipase C (PLC). PLC hydrolyses phosphatidylinositol biphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,45-triphosphate (IP3). DAG can be degraded by diacylglycerol lipase (DGL) to arachidonic acid and further metabolised via cyclooxygenase pathway to prostaglandins. IP3 releases intracellular Ca^{2+} stores from endoplasmic reticulum. Increased intracellular Ca^{2+} can activate nitric oxide (NO) synthase (NOS) to produce NO from L-arginine (Arg). NO stimulates soluble guanylate cyclase to produce cyclic guanosine 3'5'-monophosphate (cGMP).

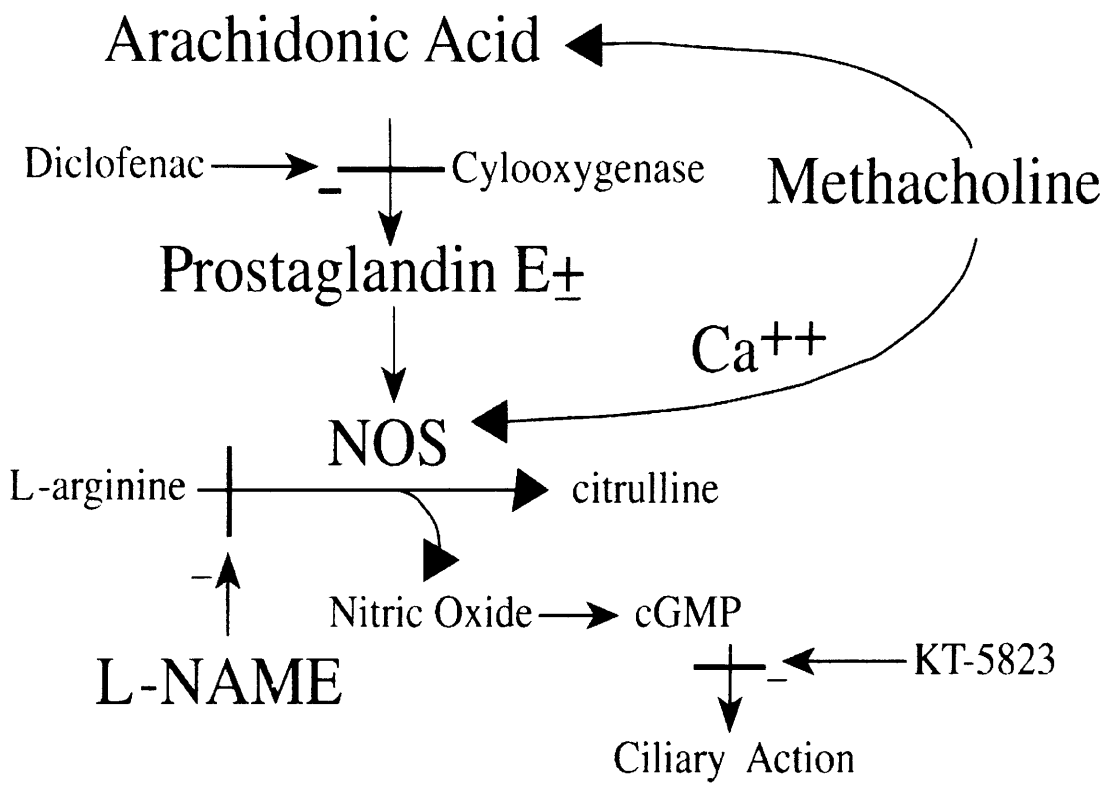


Figure 1.f

Diagram showing pathways Yang and colleagues (1997) blocked to study signal transduction pathways for methacholine ciliostimulation. Diclofenac is a cyclooxygenase inhibitor that blocks synthesis of prostaglandin from arachidonic acid. L-NAME competitively inhibits nitric oxide synthase (NOS) from L-arginine. KT-5823 inhibits cGMP-dependent protein kinase, and thus inhibits action of cGMP in the stimulation of cilia.

Prostaglandin synthesis may also be involved in the transduction of the muscarinic acetylcholine receptor signal. The cholinergic response mediated by the activation of M1 and M3 receptors is coupled to Gq proteins, which activate phosphatidylinositol turnover through the enzyme phospholipase C (Wess, 1993). In the same study stimulation of cilia by methacholine was significantly inhibited by both L-arginine free medium and the L-arginine analog, L-NAME, which block the endogenous production of NO. This inhibition was reversed by L-arginine.

Methacholine-induced ciliostimulation was also significantly inhibited by use of a cGMP kinase inhibitor (KT-5823).

Endogenous NO and cGMP synthesis are involved in the transduction of the muscarinic acetylcholine (Ach) receptor signal. Respiratory tissues contain predominantly M3 receptors and the muscarinic response mediated by the activation of M3 receptors is coupled to the Gq protein. Inositol triphosphate releases Ca^{2+} from intracellular stores. Increased intracellular Ca^{++} can activate NO synthase, the enzyme that synthesises NO from L-arginine (Sessa, 1994). Nitric oxide, in turn, activates soluble guanylate cyclase, resulting in the production of cGMP and activation of cGMP kinases.

Methacholine-induced ciliostimulation at a concentration of 10^{-6} mmol/l was significantly inhibited by the cAMP kinase inhibitor H-89. The methacholine effect on ciliary response at concentrations of 10^{-8} and 10^{-10} mmol/l was not significantly inhibited by H-89. At higher concentrations, methacholine could stimulate the cyclooxygenase pathway, producing prostaglandins. These endogenous prostaglandins could regulate ciliary beat frequency via stimulation of cAMP production. During the muscarinic response, activation of phosphatidylinositol turnover could result in diacylglycerol, phosphokinase C, inositol 1,4,5-triphosphate, arachidonic acid metabolises, Ca^{2+} , or NO. Any of these intermediates may have undetermined actions on cAMP production to stimulate ciliary beat frequency.

Salathe and colleagues (1997) found Ach transiently increased intracellular Ca^{2+} and ciliary beat frequency in single tracheal epithelial cells in culture. The increase in intracellular calcium concentration was mainly from internal stores with a small delayed contribution from Ca^{2+} influx which was mediated via M3 receptors. They argue that the small increase in beat frequency of 2.1Hz seen (28%) is biologically relevant. Based on the work of Seybold et al (1990) a 16% increase in ciliary beat frequency in response to Ach was correlated with a 56% increase in mucociliary transport velocity in isolated whole tracheas. Interestingly, after depletion of intracellular Ca^{++} stores by thapsigargin, acetylcholine caused a rapid, transient decrease in both ciliary beat frequency and intracellular calcium concentrations. Thus acetylcholine can simultaneously activate pathways with opposite effects on intracellular calcium in the same cell.

1.4 Control of body asymmetry:

A commonly held hypothesis is that sidedness of asymmetric structures would be determined in a random fashion if it were not for superimposed genetic control. If this were true then situs inversus would have an incidence of 50%. This is the case in a mutant strain of mice in which parents homozygous for the autosomal recessive gene produce offspring with a 50% incidence of situs inversus (Bruckner et al, 1989). Based on the findings that 50% of patients with Kartagener's syndrome have situs inversus, Afzelius (1979) suggested that unimpaired ciliary function and cell movement of embryonic epithelial tissue play important roles in the development of body asymmetry. The reversed asymmetry is limited to the viscera, with the proportion of left handed persons being 8% which is close to that in the normal population of 5-7%.

More recently it has been shown that nitrous oxide (N_20) exposure both in vivo (Fruinaga et al 1990) and invitro (Fruinaga & Baden, 1989), during early organogenesis produces a high incidence of situs inversus in rats. Fruinaga and Baden (1991), subsequently found that administration of phenylephrine, an alpha-1 adrenergic agonist, to rat embryos caused a dose dependent increase of situs inversus with a maximum incidence of 25%. This effect could be blocked by co-administration of prazosin, an alpha-1 adrenergic antagonist. This strongly indicates that receptor mediated stimulation of the alpha-1 adrenergic pathway is involved in the control of normal body symmetry. The exact mechanism by which this occurs is yet to be determined.

1.5 Fluid movement by cilia:

Brain ependymal cilia move cerebrospinal fluid in a manner similar to the movement of water by other ciliated organisms. A zone of water surrounding a cilium is dragged along as the cilium moves. In the absence of significant inertial effects, the motion of the water stops as soon as the cilium stops moving. The propulsive effect of the cilium on water is approximately twice as high when the motion of the cilium is perpendicular to its long axis as when its motion is parallel to the long axis (Holwill & Satir, 1987). Water tends to adhere to the cell membrane opposing the tendency of the moving cilium to propel the fluid near the cell surface while fluid is much easier to move in the region of the ciliary tip (Sly, 1989).

During a typical beat cycle, the cilium moves through a large angle in an effective stroke, moving fairly quickly and perpendicular to its long axis. Water moves in the direction of the effective stroke because a larger volume of water is carried in the perpendicular stroke. Less movement is achieved during the recovery stroke as the cilium bends and moves backwards close to the cell surface. The water the cilium passes through during its recovery stroke is more adherent to the cell surface.

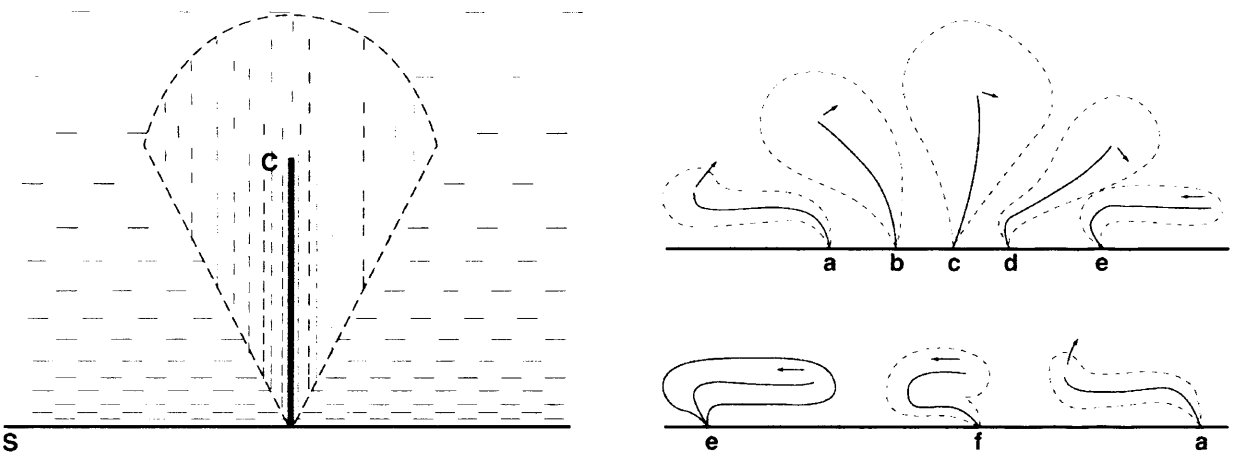


Figure 1g: *Extent of the zone of entrained water carried to the right in the cilium during the power stroke is much larger than that carried to the left during the recovery stroke (Blake & Sleigh, 1974).*

The propulsion of water by cilia is a low Reynolds number phenomenon, where viscous forces are more important than inertial forces. The Reynolds number (Re) for a cilium can be defined by:

$$Re = \frac{\text{fluid density} \times w\,Lr}{\text{fluid viscosity}}$$

where w = angular frequency, L = ciliary length, and r = ciliary radius. The Reynolds number for a cilium is low because the linear dimensions of cilia are so small.

The rate of propulsion of water by a cilium depends on ciliary length and beat frequency. The force generated by a cilium is related to the number of active dynein arms and to ciliary length. The 'stiffness' of a cilium depends upon passive mechanical properties of the axoneme, as well

as on active dynein arm attachment (Holwill & Satir, 1987). Cilia beat in close co-ordination in metachronal waves where each cilium provides some mutual assistance to the motion of neighbouring cilia. The importance of metachronism to water propulsion is that at any instant there are adjacent cilia involved in different stages of their effective stroke; each cilium does not accelerate water from rest during its effective stroke, but adds impetus to water already being moved by adjacent cilia. A continuous flow may be maintained which approaches the ciliary tip speed. The strength of the viscous-mechanical interaction between cilia depends on their positions relative to the beat direction and their separation relative to their length (Sleigh, 1984).

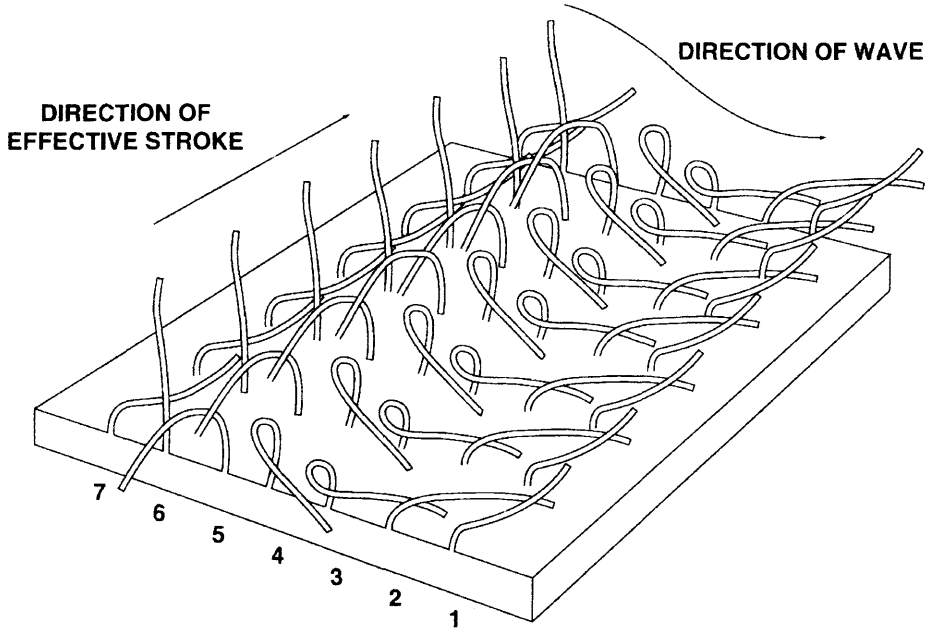


Figure 1.h: *Beating cilia on the surface of a cell interact so that in one direction, usually perpendicular to the direction of the effective stroke, they are out of phase. The result is a metachronal, or out of phase, wave that appears to move over the mat of cilia. In this diagram the cilia in Row 1 are at the end of the planar effective stroke. Those in Rows 2, 3 and 4 are in successive stages of the curling return stroke. Those in Row 5 have ended the return stroke and are beginning the effective stroke, which is ended in Row 7 (Satir, 1974).*

Interactions between adjacent cilia in the plane of the effective stroke tend to result in synchrony of beating, whereas interactions perpendicular to this plane produce metachrony. Metachronism

is a property of hydrodynamic coupling between closely packed, synchronously beating axonemes. Metachronal waves move in the direction towards which the cilium swings sideways in the recovery stroke. Metachronism can be reconstituted in single cells (Lieberman, 1988) or in respiratory epithelial cells (Weiver & Hard, 1985) after detergent treatment when membraneless axonemes are reactivated by Mg^{2+} -ATP. The wave length of a metachronal wave has been measured to be between 5 and 9 um. The wave propagates clockwise at an angle of approximately 54 to 125 degrees to the direction of the effective stroke. The mechanism by which metachronal waves are co-ordinated are not fully understood. Simple mechanical stimulation of other cilia by the cilium as it swings back from its resting state into the recovery stroke may play a part.

Because the propulsion of water by a cilium is only a local viscous phenomenon, only a shallow zone of water some two cilium lengths deep is transported across the ciliated surface (Blake & Sleigh, 1974); the total volume transported is therefore small, unless the surface is extensive, with many ciliary tracts in parallel, as on the ependymal surface.

1.6 Ependymal ciliary movement:

Purkinje (1836) was the first scientist to observe the movement of cilia on the walls of cerebral ventricles in full term foetuses, and he found that the cilia were long, pointed and vibrated. Chu et at (1942) observed the movement of dark particles, presumably melanin, in the cerebral ventricles of living *Anuran* larvae and concluded that this movement was due to local currents in the cerebro spinal fluid generated by the motion of the cilia of the ependymal cells lining the ventricles. He also observed the "swirling" of fluid by ciliary currents in the exposed ventricular surfaces of excised rat brains.

Konno and Shiotani (1956), in a study of isolated slices from walls of the lateral ventricles of dogs, reported that red blood corpuscles moved towards the foramen of Monro in the lateral ventricles of 2 - 8 week old dogs. Surprisingly they did not find ciliary activity in dogs older than two months.

Worthington and Cathcart (1963 & 1966) examined 200 samples of the ependymal lining of the ventricles of nine adult human brains obtained 2.5 - 6 hours post mortem. Cilia were found in

numerous places in each of the four ventricular cavities and were always present in any given area selected for examination. In several of the brains, cilia which were still beating were found at one or more sites. In the two best specimens of the series cilia, which were still beating in a rapid and uniform motion, were found in at least ten separate areas in each brain. The two brains in which ciliary motion was uniform, widespread, and rapid were from cases of sudden, accidental death in individuals without known disease. They were examined 2.5 - 3 hours after death. The ependymal cilia from a patient with hepatic cirrhosis showed no movement when examined 3.5 hours after death. Their findings suggested strongly that the ependyma of the adult human brain was completely ciliated. They rarely found any part of the ventricular system to lack cilia.

Cathcart and Worthing (1964) described the direction of the flow due to ependymal ciliary movement in the rat brain. The data they presented demonstrated conclusively that ciliary movements and ciliary currents are capable of circulating cells from the ventricular cavities towards the lateral apertures of Luschka and medial aperture of Magendie of the fourth ventricle at a very rapid rate, and that large amounts of cellular debris are cleared from ependymal surfaces of blind patches quickly. The direction taken by the ciliary currents in their specimens was the shortest distance to the next narrow opening in the system which is consistent with a constant turnover of fluid in all parts of the ventricles. In this system, even momentary stagnation in small pockets such as the supra optic recess, cannot occur. Milhorat (1975) proposed that the strong ciliary induced currents near the ventricular walls were important for intraventricular CSF circulation, but others have attributed only a minor role to the cilia. Current thoughts on the mechanism of CSF propulsion are dealt with later in this chapter.

Utilising the scanning electronmicroscopy Yamadori and Nara (1979 & 1983)observed directions of ciliary beat in adult mice on the walls of the whole brain ventricular system, including the central canal. They found the pattern of direction of cilial beat was the same in each mouse studied. The pattern of direction of the cilia was in the direction of anticipated CSF flow in all cases. It was postulated that ciliary movement must be important in the directional movement of CSF at the brain surface.

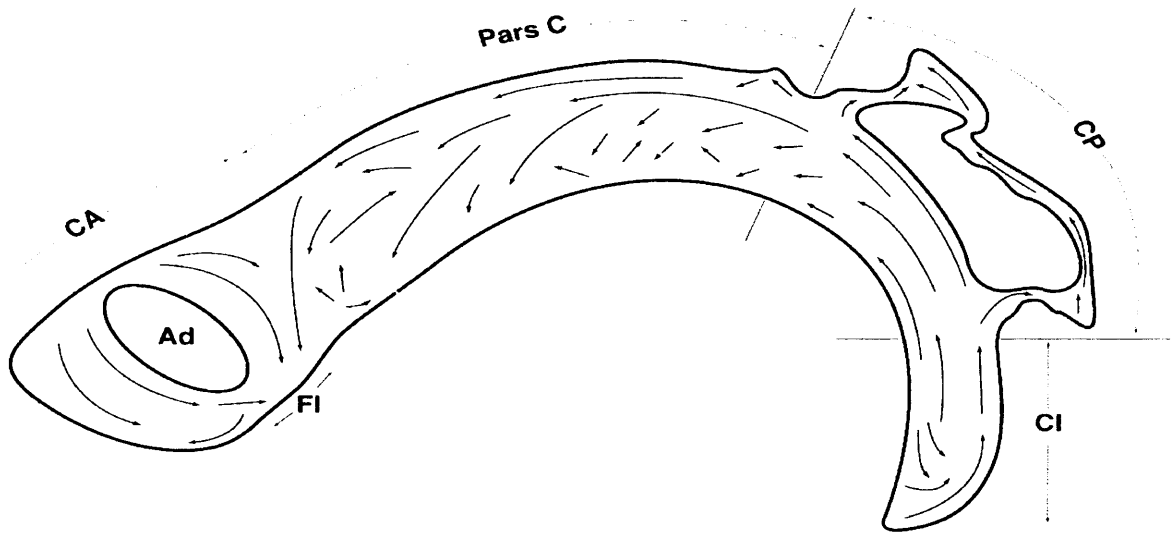


Figure 1.i

Schematic representation of the directions of ciliary beat on the wall of the lateral ventricle. Key: CA = anterior horn. Pars C = central part. CP = posterior horn. CI = inferior horn. Ad = wall adhesion. FI = interventricular foramen.

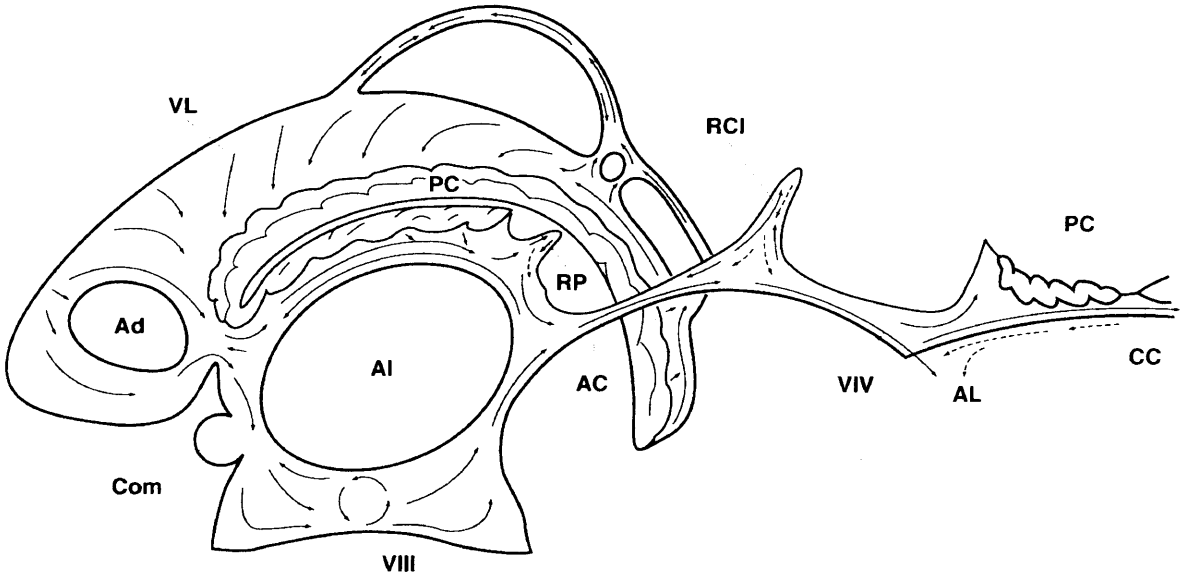
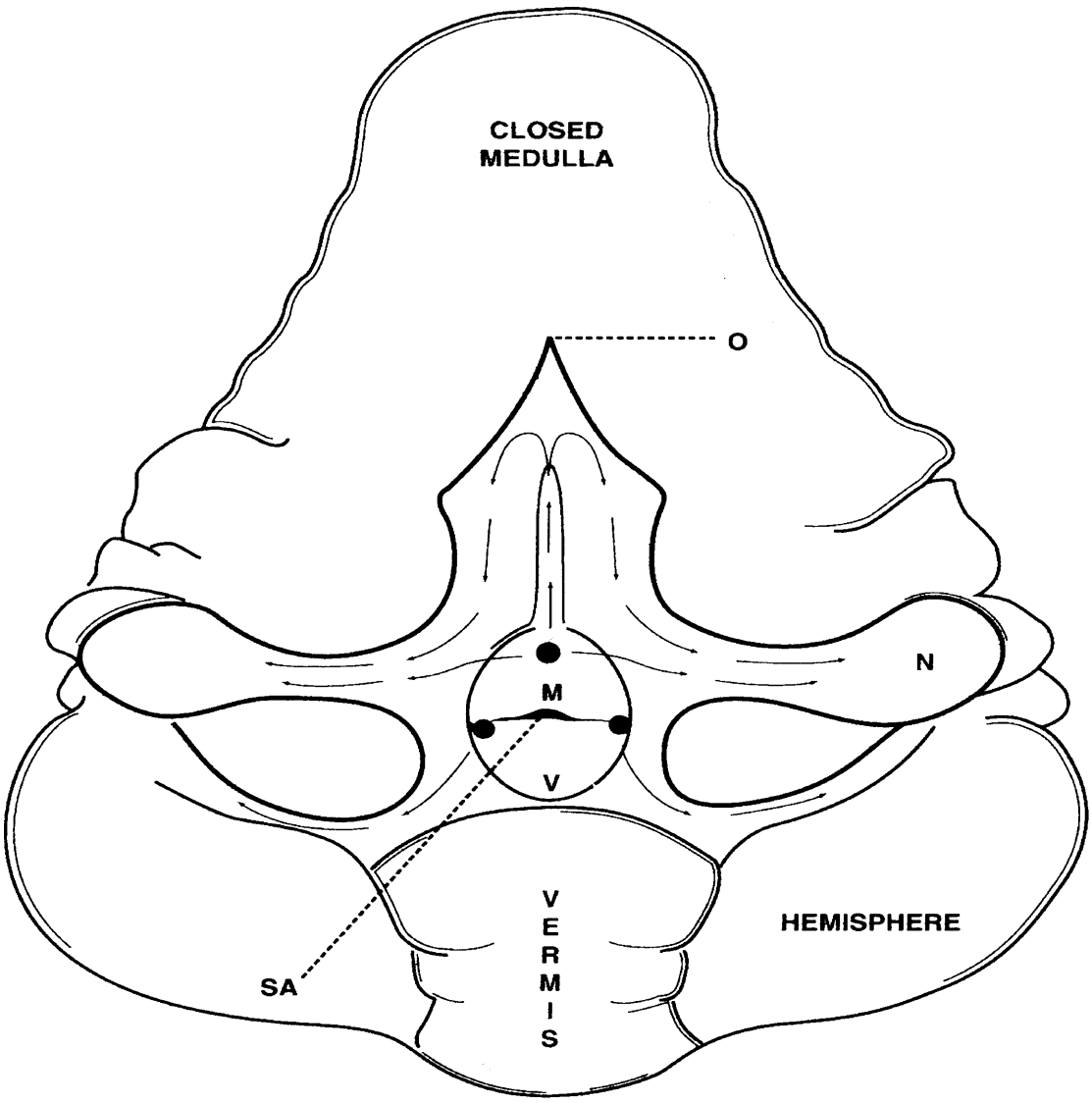


Figure 1.j

Schematic representation of the inferred CSF flow in the brain ventricles of a mouse. Broken lines indicate flow in the lateral part.

Key: VL = lateral ventricle. VIII = third ventricle. VIV = fourth ventricle. Com = anterior commisure. AI = interthalamic adhesion. RP = pineal recess. AC = cerebral aqueduct. RCI = recess of inferior colliculi. PC = choroid plexus. C = central canal. Al = lateral aperture. (Yamadori & Nara, 1979).



Fourth Ventricle

Figure 1.k

Exposed floor and roof of the fourth ventricle:

Arrows show the directions of the ciliary currents. The cerebellum has been retracted. The arrows in the lowest part of the diagram closest to the cerebella hemispheres show ciliary currents along the roof of the pathway of the cerebro spinal fluid towards the lateral apertures. The remaining arrows show the direction of ciliary currents on the floor of the ventricle (Cathcart & Worthington, 1964).

Key: O = Obex; N = eighth nerve; M = median eminence; V = anterial medullary vellum; SA = opening of cerebral aqueduct of Sylvius.

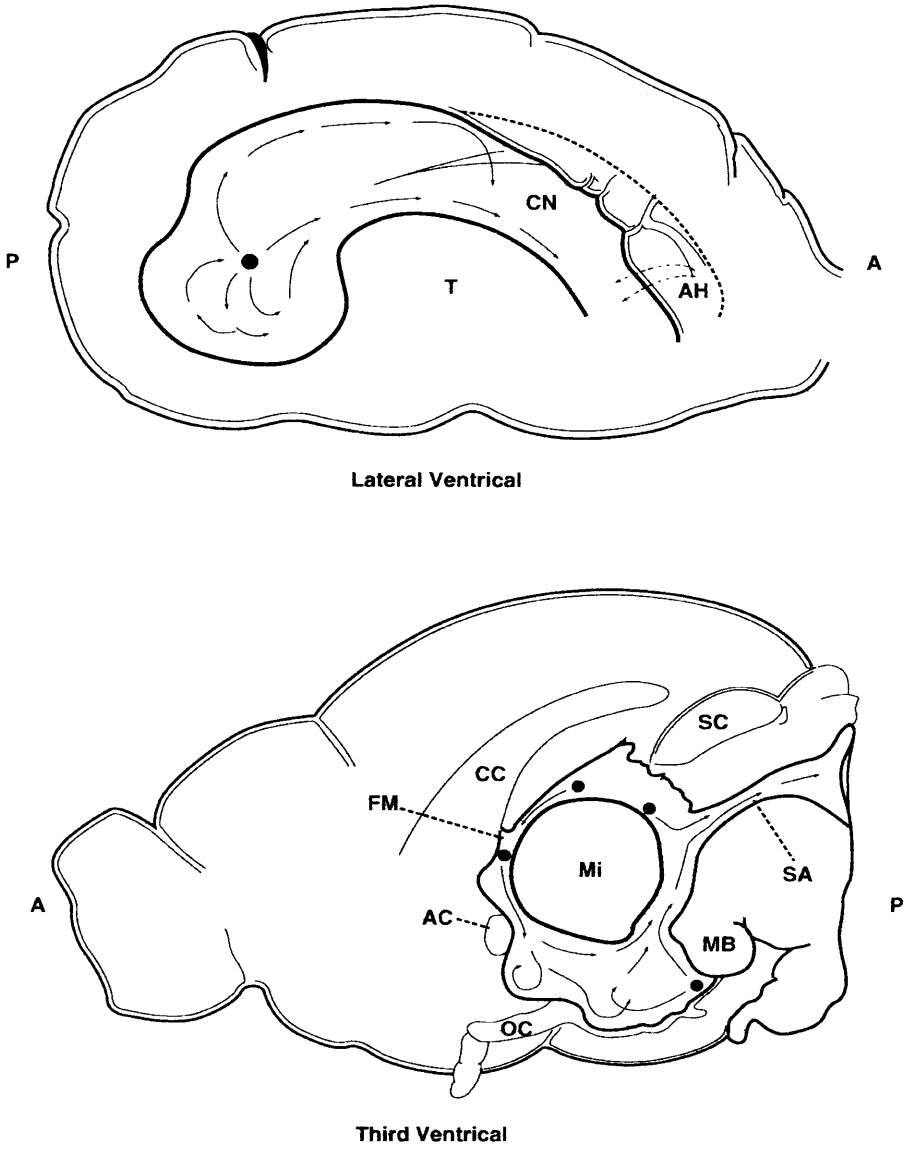


Figure 1.1

The top diagram shows an exposed lateral ventricle. The medial side of one hemisphere was sliced in a plane parallel to the lateral ventricle. This removed the medial wall of the ventricle and the foramen of Monro.

The lower image is of the exposed medial wall of the third ventricle. A mid-sagital slice between the hemispheres has been made and the brain stem and cerebellum removed by a coronal slice through the mid-brain. Direction of ciliary currents is shown by arrows.

The direction of CSF currents are shown by the arrows (Cathcart & Worthington, 1964). Key: P = posterior lobe. A = anterior lobe. CN = caudate nucleus. AH = anterior horn. T = Thalamus. SC = superior colliculus. CC = corpus callosum. AC = anterior commisure. MI = massa intermedia. OC = optic chiasma. MB = mammillary body. SA = aqueduct of Sylvius. FM = foramen of Monro.

1.6.1 Control of ependymal ciliary movement:

There are few studies relating to the movement or control of ependymal cilia Initial studies on the movement of brain cilia were conducted on rat and frog ependymal cilia. These are outlined below.

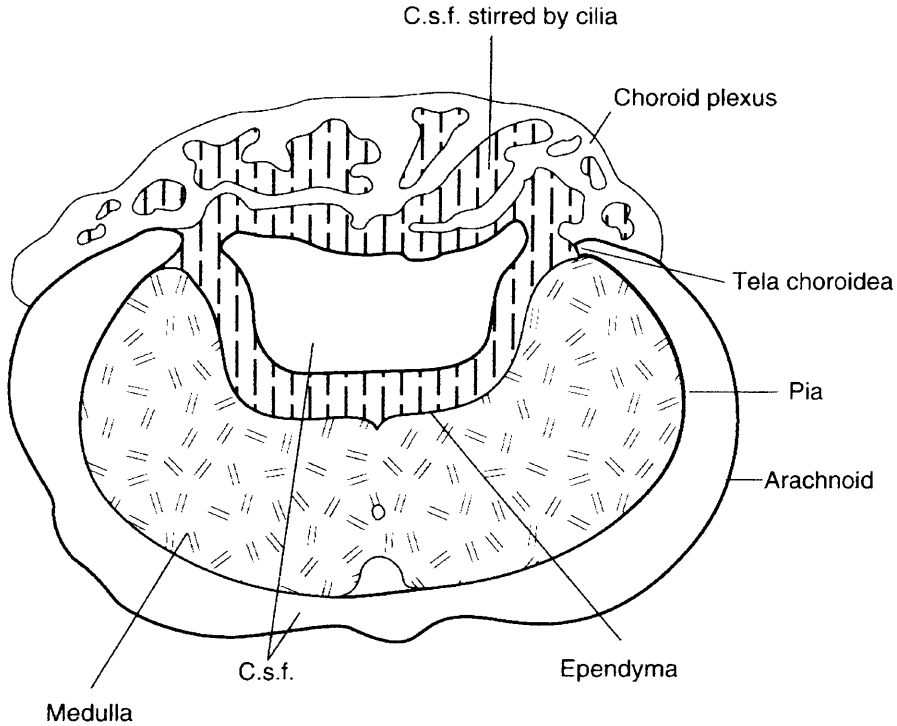


Figure 1.m

A cross-section of the 4th ventricle in a frog with associated meninges, neural tissue and choroid plexus.Lined areas represent those volumes of cerebrospinal fluid stirred by ependymal cilia. Nelson and Wright (1974) estimated that greater than 75% of the CSF within the ventricles of the frog is mixed as a direct result of ciliary activity.

Nelson and Wright (1974) studied the ciliary activity in the frog brain and found about 30 - 40 cilia emerged from the central region of each cell, and individual cilia were 20 microns in length and 0.25 microns in diameter. Conducting experiments at room temperature, cilia from the choroid plexus beat at rates of between 8 to 16 Hz while ependymal cilia of the lateral ventricle beat more quickly at 21Hz.

The authors also looked at the distance from the ependymal surface to where movement of surrounding CSF stopped. By inhibition of ciliary activity using DNP 2, 4-dinitrophenol or Ni^{2+} the thickness of the unstirred layer of CSF increased by 100μm, suggesting that cilia could

effectively stir the CSF within 100um from the surface of the cell. In a separate experiment cilia beating on the ependymal surface generated circular currents of the red blood cells as far as 200 microns away from the surface. From the dimensions of the frog brain it was estimated that greater than 75% of the CSF within the ventricles is mixed as a direct result of ciliary activity.

The effect of ions on ciliary movement was explored, though the precise methodologies are not clear. A lyotrophic series was determined for the ability of cations to depress ciliary beat frequency. Interestingly, anions had little effect (Singer and Goodman, 1966: Nelson and Wright, 1974). This was thought to be due to the net fixed negative charge on proteins at physiological pH. Divalent cations such as calcium chloride or magnesium chloride helped to maintain ciliary activity.

Early studies found an effect of pH on ciliary function. A pH of 6.0 caused ciliary stasis within 10 minutes (Singer and Goodman, 1966) while Nelson and Wright (1974) showed stasis caused by a pH of 4.4 was reversible following reperfusion with saline. In contrast Nakamura and Soto (1993) found that no effect on the flow of latex particles produced by cilia was seen with changes in pH between 6.0 and 8.0. The latter authors also found no effect of pressure changes of up to 100mmHg on ciliary movement.

Nelson and Wright (1974) found that the addition of adenosine triphosphatase (ATP) at a concentration of 1mM increased the beat frequency by 68 $\pm$ 11% over the baseline level, while cyclic adenosine 3, 5-monophosphate (cyclic AMP) increased the beat frequency by 61%. The time course of the response was over a period of 25 minutes with cyclic AMP whereas the same response was seen within 5 minutes when ATP was used. Theophylline, a known inhibitor of phosphodiesterase activity was significantly less effective than either ATP or cyclic AMP in its ability to stimulate ciliary activity. At concentrations of 1 and 5 mM the frequency increased by a maximum of 30%. Interestingly caffeine had no effect.
Metabolic inhibitors such as 2, 4-dinitrophenol (DNP), a potent uncoupler of oxidative phosphorylation irreversibly stopped ciliary activity within 7 minutes. Iodoacetic acid (IAA), which inhibits anaerobic glycolysis, also stopped ciliary activity over a 30 minute period. Cyanide also blocked ciliary activity. The ciliostatic effect of both iodoacetic acid and cyanide were irreversible. The inhibitory effects of DNP, IAA and cyanide on intact choroidal cilia are

readily explained on the basis that these agents block the production of ATP from glucose via glycolytic metabolism. Roth and colleagues (1985) studied ciliary beat frequency from various parts of the brain ventricular system and tracheal rings from hamsters. Using a photo electric system they measured frequencies of between 27 and 31 Hz in the lateral ventricle, aqueduct and fourth ventricle. Ciliary beat frequency was found to be less, at 20 Hz, in the third ventricle. Cilia from the trachea beat more slowly at 13.7Hz. The authors also found that bovine and cervical mucus reversibly inhibited ciliary beat frequency in the brain but not the trachea. Surprisingly, in contrast to respiratory cilia, neither glass microspheres (50-75um) or charcoal particles were transported by ependymal cilia, in their model, despite active ciliary movement.

Nakamura & Sato (1993) found difficulty in measuring ependymal ciliary beat frequency. As an indirect method they studied the effects of various conditions and drugs on the movement of 1um diameter latex beads by ependymal cilia. Metavanadate, a powerful inhibitor of dynein, caused a dose dependent decrease in flow of the latex particles. At lower concentrations this was partially reversed by addition of epinephrine. Various agents such as, dinitrophenol, nickel, Ethylene glycol, tetra acetic acid and colchicine, also suppressed the flow of latex beads.

1.7 Hydrocephalus and ependymal cilia:

Ependyma in humans with hydrocephalus may be normal (Bannister & Mundy, 1979) stretched, torn (Del Bigio et al, 1985: Weller & Shulman, 1972) or totally destroyed with only clusters of cells persisting in the gliotic wall of the ventricle. Ependymal damage is dependent on the severity of ventriculomegaly. In young and adult animals stretching and disruption of the ependyma begins as early as 12 hours after CSF obstruction (Clark & Milhorat, 1970: Diggs et al, 1986: Page, 1975). As the ventricle dilates the cuboidal and columnar ependymal cells flatten to increase their surface area (Del Bigio & Bruni, 1988: Page 1979) However, the rate at which ependyma can stretch is limited and only animals with slowly expanding ventricles are likely to retain an intact ependymal lining (Collins, 1979). Damage is minimal over the caudate nucleus. However, loss of cilia and microvilli and disruption of the epithelium occurs in the most severely affected regions which overlay the white matter, especially along the roof and dorsolateral angle of the lateral ventricles (Page, 1975) or over the septum pellucidum (Del Bigio & Bruni, 1988). Macrophages then appear on the ependymal surface presumably to remove debris (Go et al, 1976).

It is believed, by many authors, that ependymal cells can not regenerate following hydrocephalus-induced injury (Collins, 1979: Page, 1983: Weller et al, 1978). Others have suggested that germinal cells give rise to ependymal cells (Clark & Millhorat, 1970). Del Bigio & Bruni, (1988) have shown that existing ependymal cells proliferate at a greater rate following the induction of hydrocephalus. However, the small increase in mitotic activity is insufficient to maintain coverage of the enlarged ventricular surface. Whether the absence of an ependymal layer has an adverse effect on brain function is not known.

In rats with hereditary hydrocephalus, Linberg et al (1977) noted that cilia were shortened, fewer in number, and clumped or matted. Using scanning microscopy Bannister and Mundy (1979) studied ependymal surfaces of the ventricles of two congenitally hydrocephalic Hy-3 mice in comparison to that of an infant with hydrocephalus complicated by meningocele and Chiari malformation. In the basal regions of the lateral ventricles of the Hy-3 mice, the ependymal walls have normal cilia, but the upper ventricular walls were found to be devoid of cilia, whereas no change in the ependymal cilia of the lateral ventricle was noted. They concluded that the changes in the ventricular wall and the coarse ependymal cilia seen could be attributed to thinning of the ventricular wall resulting from increased intracranial pressure due to hydrocephalus, and was not the cause, but rather the result, of hydrocephalus.

Bryan (1983) studied mice homozygous for a recessive, pleiotropic, mutation of hydrocephalic-polydactyly. He found the male mouse was affected by post-natal hydrocephalus and complete sterility and that the female mouse had reduced reproductive performance. It was suggested that the fertility problems and development of hydrocephalus could have arisen as consequences of defective ciliary axonemes of the oviduct and the ependyma.

Only a small number of human patients with primary ciliary dyskinesia who also have hydrocephalus have been reported to date. The reason is probably due to the low incidence of primary ciliary dyskinesia, to the lack of the recognition that primary ciliary dyskinesia may be associated with mild hydrocephalus, and to insufficient medical examination. Afzelius (1979) postulated defective cilia were a primary cause of hydrocephalus and high intraventricular pressure its consequence. He examined the brains of seven patients with primary ciliary dyskinesia by CT scan. In three of these patients, the ventricular system and sulci were slightly enlarged.

Greenstone et al (1984) reported a 12 year old boy with primary ciliary dyskinesia and bronchiectasis who had developed hydrocephalus in the neonatal period. Jabourian et al (1986) described a 15 year old girl with Kartagener's syndrome who developed persistent headaches and was found to have communicating hydrocephalus with evidence of impaired CSF circulation at the level of the tentorium. There was symptomatic improvement following insertion of a ventriculo peritoneal shunt.

De Santi et al (1990) reported a particular form of primary ciliary dyskinesia, ciliary aplasia, in a girl with bronchiectasis who developed hydrocephalus in the neonatal period. A CT scan of her head showed triventricular hydrocephalus caused by aqueductal stenosis. Again, symptoms were relieved by the insertion of a ventriculo peritoneal shunt. These reports lend support to the fact that abnormal movement of ependymal cilia may be intimately related to development of the hydrocephalus seen in these cases.

Koto and colleagues (1987:1987) reported that WIC-Hyd rats found in the Wistar-Imamichi strain rat breeding colony were affected by a high incidence of congenital hydrocephalus. A difference in the severity of hydrocephalus between males and females was noted. Approximately 35% of the females developed slowly progressive and/or arrested hydrocephalus. Despite this they grew up to maturity and became capable of reproduction and seldom died of hydrocephalus. On the other hand, approximately 34% of the males were affected by rapidly progressively hydrocephalus which was clinically apparent as early as one week after birth. All of the males with hydrocephalus died of raised intracranial pressure due to triventricular hydrocephalus caused by secondary stenosis of the aqueduct of Sylvius within one month of birth. This data suggests that the hydrocephalus seen in the WIC-Hyd rats was communicating, inheritable and X-linked. Approximately half of the males with hydrocephalus were found to have total situs inversus viscera, while neither female rats nor those without hydrocephalus developed such visceral abnormalities.

Shimizu and Koto (1992) went on to study the ultrastructure and movement of cilia in the ependyma of the lateral, third and fourth ventricles, and the aqueduct of Sylvius, and in the tracheal walls. Scanning electron microscopy revealed that a marked decrease in the length and number of cilia in the ependymal and tracheal walls occurred in the affected male WIC-Hyd rats.

Interestingly this was noted prior to the development of ventricular enlargement. A moderate decrease in length and number of cilia was also seen among the normal ciliary tufts in affected female rats who developed mild hydrocephalus. Transmission electronmicroscopy revealed abnormal axonemal structures such as a lack of dynein arms and displacement of microtubules. These ultrastructural abnormalities were more common in affected male rats than in affected female rats. All cilia in affected male rats before and after development of hydrocephalus were immotile. A variety of movement disorders such as immobile, rotatory, and vibratory cilia were observed in addition to normally beating cilia in affected female rats who did not develop severe hydrocephalus. The hydrocephalus developing in affected male and female WIC-Hyd rats appears to be secondary to a motility disorder of ependymal cilia which is part of their primary ciliary dyskinesia. This study strongly suggests that dysfunction of ependymal ciliary movement may contribute to the development of hydrocephalus, in WIC-Hyd rats.

Nakamura and Sato (1993) developed a method for quantitatively analysing ciliary movement in the ependymal wall of the aqueduct in rats. This was achieved by cutting an axial slice of the mid-brain containing ependymal wall and observing a movement of 1 micron diameter latex particles. Ciliary movement of the culture fluid laden with latex particles was recorded on video. Aqueductal ciliary movement in congenitally hydrocephalic HTX rats, congenitally hydrocephalic WIC-Hyd, and other normal rats was evaluated. The results suggest that in congenitally hydrocephalic WIC-Hyd rats the degree of hydrocephalus related strongly to the degree of ciliary dyskinesia, but in congenitally hydrocephalic HTX rats it did not.
To support the hypothesis that ciliary dyskinesia is primarily responsible for hydrocephalus occurring in the WIC-Hyd rats Nakamura and Soto performed an in vivo experiment. Metavanadate, an inhibitor of ciliary movement, was infused into the third ventricle of normal Spraugue-Dawley rats for one week. All of the rats developed hydrocephalus

1.8.1 Modification of ependymal cilia:

Perraud and colleagues (1988) have described the presence of a brain lectin, cerebellar soluble lectin (CLS), on the surface of ependymal cilia. This lectin has a great affinity for oligomannosidic glycans. It is thought that CLS is produced by subependymal astrocytic cells. The membranes of ependymal cells appear to possess glycoprotein ligands for the lectin which explain the specific adhesion of CLS to the surface of cilia. Localisation of this adhesive

molecule on cilia of ependymal cells suggests that it may play a role in trapping foreign cells, micro-organisms or debris in normal or pathological situations.

1.8.2 Ependymal formation and repair in the foetal brain:

Foetal diseases that might affect the ependyma include cytomegalovirus, toxoplasmosis, herpes, periventricular leukomalacia and intraventricular haemorrhage. Periventricular leukomalacia, a complication of prematurity, is followed by extensive gliosis and often by widespread ependymal rosette and demirosette formation in the periventricular region.

The foetal ependyma differentiates regionally from the neuroepithelium, beginning with the floor plate of the neural tube at 4 weeks gestation and covering the last parts of the lateral ventricles as late as 22 weeks gestation (Dooling et al, 1977: Sanart, 1992). Mitotic activity of the undifferentiated foetal neuroepithelium is mainly at the ventricular surface and ceases as soon as ependymal cells differentiate (Smart, 1992). As the ependyma differentiates, the junctions between individual cells become less tight. This results in the ependyma becoming less of a barrier to the passage of proteins and other molecules (Mollgard et al, 1987).

The primitive neuroepithelium in the chick embryo is able to seal surgical incisions, restoring epithelial integrity. The repair is secondary to increased proliferation of neuroepithelial cells which then differentiate as new ependymal cells. Damage to young embryos heals faster and more consistently than that to older embryos (Clark & Scothorne et al, 1990: Lawson & England, 1992).

The foetal ependyma is a pseudostratified columnar epithelium. These cells already have microvilli and cilia at their apical surface. The ependyma not only shows morphological and structural abnormalities in many cerebral malformations but also exhibits metabolic aberrations that may contribute to the pathogenesis of these dysplasias. In lissencephaly the ependyma continues to strongly express S-100 protein, usually only expressed by foetal ependyma, in children up to 7 years.

The foetal ependyma plays an important role in the ontogeny of the CNS, participating in such developmental processes as the arrest of mitotic activity of the neuroepithelium and the guidance of axonal growth cones (Sanart, 1992).

1.8.3 Later ependymal repair:

The human brain ependyma does not appear to regenerate at any age, after birth, following damage. Mitoses are not seen in regions of ependymal damage in the brains of infants or adults regardless of the presence or absence of subependymal gliosis. In the foetus, once neuroepithelial cells begin differentiating into ependymal cells, they do not divide further. Indeed, mitotic activity in ependymal cells is taken as evidence of neoplasia.

Preliminary studies of monoclonal antibodies (MIB) against recombinant parts of the Ki-67 proliferating cell nuclear antigen (Cattoretti et al, 1992) also show no evidence of mitotic cycling phases in differentiated ependyma even in foetal life or in injured ependyma at any age. Intermediate filament proteins such as vimentin, glial fibrillary acidic protein (GFAP) or cyto-keratins, or secretary protein molecules such as S-100, which are strongly expressed by immature foetal ependymal cells are not expressed by mature ependymal cells in areas of damage.

Experimental hydrocephalus may be induced in animals by intracisternal injection of silicone or kaolin. The ependyma becomes discontinuous and the gaps are filled with glial processes. The subventricular glial cells do not become new ependymal cells (Page et al, 1979: Collins et at, 1990) and remaining ependymal cells do not proliferate. Subependymal cells are mobilised within hours after ependymal injury and mature astrocytes proliferate in about 6 days (Collins et al, 1992).

The transplantation of foetal cerebral cortical homografts into the spinal cord of adult rats is associated with mitotic proliferation of cells that become a new generation of ependymal cells to line the cysts within the graft (Bernstein et al, 1986). The origin of these cells is not from pre-existing ependymal cells, however.

1.8.4 Ependymal regeneration in the central canal of the spinal cord.

Regeneration of the ependyma can occur in non mammalian vertebrates. When the tail of a salamander is severed, ependymal cells proliferate to form a new central canal initiating growth of a new tail. Ependymal cells form tunnels which ensheathe and guide the regenerating axons

(Egar et al, 1972: Singer et al,1979). Ependymal regeneration is also seen in fish and reptiles (Alvarez et al, 1987: Anderson et al, 1986). Adrian and Walker (1962) observed thymidine-labelled ependymal cells in response to penetrating lesions of the central canal of the mouse spinal cord. Following spinal cord transection in the rat, ependymal cells proliferate and form basal processes that also attempt to envelop sprouting axons, but gliosis interferes with effective reconstruction (Mathews et al, 1979).

In man it has been postulated that proliferation of ependyma and astrocytes may represent the mechanism for normal occlusion of the central canal of the spinal cord (Kasantikul et al, 1979).

1.8.5 Microvilli and Glycocalyx

The apical surface of ependymal cells are covered by microvilli. A glycocalyx coating is seen on electron microscopy. Binding of ruthenium red and of lectins indicate the presence of sialic acid, poly-N-lactosamine, and D-galactose on ependymal microvilli in many species (Acarin et al, 1994; Adam et al, 1993; Damjanov and Black, 1987; Korte and Rosenbluth, 1982). Enzymatic removal of sialic acid exposes N-acetyl-D-galactosamine residues on adult human ependyma (Kuratsu et al, 1990) which is normally unmasked on human and rat ependyma during foetal development (Kaneko et al, 1991; Momoi et al, 1986). An intercellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM-1) are expressed on the microvilli of ependymal cells and this expression is increased following infection (Deckert-Schluter et al, 1994). Their role may be to mediate adhesion of inflammatory cells, including supraependymal macrophages, to the ependymal surface.

1.8.6 Ependymal cell communication

Ependymal junctions are specialised to complement the presence or absence of blood-brain barrier in adjacent capillaries. Tight junctions are only found between ependymal cells where capillaries lack tight junctions including those covering the choroid plexus. Ependymal cells are usually bound together at their apical surface by zonula adherens type junctions (Brightman and Reese, 1969). The ependyma allows slow access of CSF proteins or exogenous tracers (such as ferritin and horseradish peroxidase) to the brain extracellular space (Aird, 1984; Brightman, 1965; Fossan et al, 1985). Permeability of the ependymal membrane to proteins can be altered. An endogenous lectin present at the junctions of rat ependyma can bind mannose-containing

glycoproteins inducing the opening of ependymal junctions (Kuchler et al, 1994; Perraud et al, 1988).

Freeze-fracture studies reveal abundant gap junctions. Gap junctions allow transfer of tracer substances and electrical current between ependymal cells. There are differences in this regard between species as well as between ependymal cells cultured from several brain regions, with ependymocytes from circumventricular organs (CVOs) resembling primitive neurones in some aspects including less extensive dye coupling (Bouille et al., 1991; Connors and Ransom, 1987). Electrical coupling of ependymal cells may help to integrate ependymal cell function through intercellular communication (Mugnaini, 1986).

1.8.7 Specialised areas of ependyma

The ependymal surface has distinct areas which are devoid of cilia. These are known as circumventricular organs. The cells over these areas form tight junctions although the blood capillaries supplying the circumventricular organs are fenestrated. Circumventricular organs include the median eminence of the hypothalamus, the neurohypothesis, the area postrema, the pineal body and the subfornical organ. These are located around the third ventricle apart from the area postrema which is situated next to the fourth ventricle. The medial eminence, neurohypothesis and pineal body are involved in neuroendocrine regulation but the function of the other circumventricular organs is largely unknown.

Tanycytes are specialised non-ciliated ependymal cells located on the floor of the third ventricle. They form an anatomical connection between the CSF and the hypophysial-portal vasculature. The tanycytes may play a role in the regulation of the activity of the adenohypophysis. Hypophysiotropic hormones released from the hypothalamus diffuse into the CSF occupying the third ventricle and are transported to the portal circulation by the tanycytes. These hormones are then transported to the adenohypophysis where they effect a response (Flament-Durand, 1978; Bruni et al, 1985).

1.8.8 The choroid plexus:

Choroid plexus is found in the walls of the lateral ventricles and the roofs of the third and fourth ventricles. It consists of folds of dense blood capillaries and connective tissue covered by

ependyma. The capillary endothelium is fenestrated whilst the ependymal cells form tight junctions.

Other cellular elements have been observed on the ventricular surface. Stalks with dilated bulb-like ends project into the ventricular lumen from the ependymal cells. Their origin and function are unknown but may be cilia with cytoplasmic masses attached to them. The bulbs may contain mitochondrial groups or have a secretory function.

1.8.9 Relationship to Supraependymal Axons:

Axons arising in the rapine nuclei of the midbrain extend to most parts of the ventricular system (Ugrumov et al, 1985), lying on the surface of ependymal cells (Richards et al, 1980). Varicosities with serotonin and gamma amino butyric acid (GABA)-containing vesicles are widespread on the ependymal surface. Hamster ependymal cells show immunoreactivity for kynurenine, a metabolite of serotonin, suggesting that they may take up and process serotonin from these axons (Keith and Brownfield, 1985). Although there is evidence that serotonin activates chloride channels in choroid plexus cells (Garner et al, 1993), effects on ependymal cell function have not been reported. Synapse-like formations between serotonergic endings and ependymal cells have been observed on the subcommissural organ and in the cerebral aqueduct (Didier-Bazes et al, 1993, Meller and Dennis, 1993).

1.8.10 Reactive Protein Expression:

Ependymal cells increase their expression of certain protective proteins following injury or exposure to blood products. Haem oxygenase (heat shock protein 32), is present in normal rat brain at low levels, but following heat shock the mRNA coding for it increases dramatically in ependymal cells and less so in neurones (Ewing et al, 1992). This enzyme acts by converting haem into bile pigments which are potent antioxidants. Another antioxidant, superoxide dismutase, has been detected in rat and human ependymal cells (Mori et al, 1993; Zhang et al, 1994).

Cytokines may also be upregulated in ependymal cells following various stimuli. Infection and physical injury to rodent brains induces tumour necrosis factor alpha, interleukin 1-alpha, and transforming growth factor in ependymal cells. (Diaz-Ruiz et al, 1993; Logan et al, 1994; Tarlow et al, 1993: Tchelingerian et al, 1993). Administration of interferon gamma or systemic infection

with *Toxoplasma gondii* or interferon gamma induces the expression of MHC Class I and II antigens on ependymal cells of rats (Deckert-Schluter et al, 1994; Steiniger & van der Meide, 1988; Vass & Lassmann, 1990). Resting ependymal cells in the rat express mRNA for interleukin-6 and interleukin-6 receptor (Schobitz et al, 1993).

1.8.11 Movement of Water Across Ependyma:

Little is known about ion and water transport mechanisms in ependymal cells (Barres et al, 1989; Cardy and Fifth, 1993; Nielson et al, 1993; Zalc et al, 1984). It has been suggested that the ependyma may be important in regulating movement of water between the extracellular compartment and the CSF (Pollay and Curl, 1967). Vasopressin concentration in CSF is increased when intracranial pressure is raised and it can increase ependymal permeability to water (Chen et al, 1993; Rosenberg et al, 1986; Sorensen, 1986). The probable sources of vasopressin are circumventricular organs or vasopressinergic axons which terminate in the ependymal layer (Dubois-Dauphin et al, 1990; Lepetit et al, 1993).

1.8.12 Secretory Function:

The secretory product of ventricle-lining cells in embryonic chicks includes a chondroitin sulphate proteoglycan postulated to maintain expansion of the developing neural tube (Gato et al., 1993). Ependymal cells in adult humans show immunoreactivity for granulophysin, a membrane protein associated with exocytosis (Hatskelzon et al., 1993), and ependymal cells from mature rats express mRNA for the secretory proteins chromogranin B and secretogranin (Gee et al., 1993). The most obvious ependymal secretion is a proteinaceous material from the cells of the subcommissural organ in many species (Johnson and Gross, 1993).

1.8.13 Proteins Which Bind Metals:

Some heavy metals ultimately enter the CSF (Zhang et al, 1991). Metallothionein, a protein that binds copper, zinc, and some heavy metals, is present in the ependyma of rodents and humans (Blaauwgeers et al., 1993; Suzuki et al, 1994). Histochemical studies have shown that zinc, copper, iron, and the iron binding/transfer protein transferrin are present in ependymal cells (Benkovic & Connor, 1993; Connor and Benkovic, 1992; Moos and Mollgard, 1993). Ependymal uptake of reactive metal ions, such as iron and copper ions, from CSF may protect the brain parenchyma from oxidative damage by oxygen free radicals (Halliwell, 1992).

1.8.14 Phagocytosis and Degradative Enzymes:

Proteins can be ingested by ependymal cells by pinocytosis and degraded (Graff et al, 1993). Pinocytotic vesicles can incorporate serum proteins, ferritin particles, dyes, fluorescent tracers, and latex beads (Booz & Wiesen, 1976; Brightman, 1965; Broadwell & Sofroniew, 1993).

Rat ependymal cells contain glutamine synthetase which is involved in the metabolism of glutamate released by neurones (Akimoto et al, 1993; Graff et al, 1993). Ependymal cells also possess receptors for some drug classes (Benavides et al., 1983) and several enzymes known to metabolise drugs in the liver are also active in both the circumventricular organs and ependymal cells (Abramovitz et al, 1988: Ghersi-Egea et al, 1994; Senjo et al, 1986).

1.8.15 Regulation of Neuroactive Peptides:

A variety of transmitter substances and neuroactive peptides are transported in the CSF to their site of action (Fishman, 1992). Numerous enzymes which can degrade neuroactive peptides are produced by mammalian ependymal cells. It is postulated that inactivation of peptides by the ependyma may protect the brain from re-entry of these substances which could affect neuronal function (Bach-y-Rita, 1993; Begley and Chain, 1992).

Ependymal cells possess receptors for oxytocin and adenosine (Jones-Humble & Morgan, 1994; van Leeuwen et al, 1985) and possibly beta-endorphin (Bjelke and Fuxe, 1993).

Some peptidases present in rat ependymal cells are implicated in the synthesis or activation of neuropeptides that circulate in CSF. Angiotensin converting enzyme is active in ependymal cells (Bourne et al, 1989; Chai et al, 1987; Defendini et al, 1983). This enzyme is involved in the production of angiotensin II from angiotensinogen. Angiotensinogen may come from a subpopulation of astrocytes (Stornetta et al, 1988). An angiotensin II receptor involved in peripheral water balance has been detected on rat brain ependymal cells (Gehlert et al, 1991), suggesting that the angiotensin system may be involved in ependymal water regulation in the brain.

1.8.16 Teratogen induced hydrocephalus:

Rats exposed to 6-amino-nicotinamide in utero have been shown to develop hydrocephalus at term. Within 24 hours of exposure, the ependymal cells are flattened and lack cilia and microvilli. Recovery of the ependyma was not seen by term (Chamberlain, 1972). Rats exposed to ethylnitrosourea or ethylenethiourea in utero exhibit ventricular dilatation and areas of ependymal necrosis and denudition (Khera & Tryphonas, 1977: Koyama et al, 1972). Wide spread denudition of the ependyma with formation of subependymal glial nodules and ependymal rosettes were observed in new-born rats with zinc deficiency (Warkany & Adeloye, 1976). In foetal rats telliurium induces hydrocephalus (Duckett, 1971). Ventricular dilatation and ependymal flattening and subependymal oedema occurs within 1 to 3 days of injection. By day 5 there is more extensive ependymal flattening, loss of cilia, severe aqueductal stenosis and ventricular dilatation.

1.8.17 Ependymal damage by irradiation:

The severity of damage to the developing ependyma of the mouse depends on the stage of the developing brain when it is irradiated. Heinzman and colleagues (1978) found the ependyma did not regenerate following severe damage on gestational day 10 or later. Harkenson and von Mecklenburg (1981) studied the ependymal cells of patients irradiated for glioblastoma multiforme. Radiation therapy had a deleterious effect on ependymal cells that was more pronounced with larger doses of irradiation. The irradiated ependyma did not regenerate.

1.8.19 Viral infections of the ependymal layer:

Viral ependymitis in both humans and experimental animals leads to destruction of the ependyma and hydrocephalus with or without aqueductal stenosis. This occurs with a striking absence of focal inflammatory or astroglial reaction. Viral ependymitis is probably the most frequent cause of acquired aqueductal stenosis in humans.

The direct invasion of ependymal cells by viruses is well documented in both humans and experimental animals (Johnson & Johnson,1972: Johnson,1972: Wolinsky et al,1974: Takano et al,1993). Mumps ependymitis occurs in humans and is one of the important causes of acquired aqueductal stenosis (Timmons & Johnson,1970: Spartaro et al,1976: Thompson, 1979: Baumann

et al, 1982: Rotilio et al,1985). Johnson and Johnson (1968) described the occurrence of mumps viral ependymitis without an inflammatory response.

Within five days of intracerebral inoculation of mumps virus into suckling hamsters, antigen is detected in all ependymal cells and the cilia almost disappear. (Wolinsky et al,1974: Takano et al,1993). Histologically there is no inflammation of the ependyma. A perivascular infiltrate of lymphocytes and polymorphonuclear leukocytes and small haemorrhages (Johnson & Johnson, 1972) are seen. Only occasional mononuclear cells are seen in the subependymal zone. Necrosis and loss of ependymal cells from the ventricular surfaces and from the cerebral aqueduct occur. The paucity of ependymal reaction is striking (Johnson & Johnson, 1972). By 14 days, microglial nodules form in regions of previous small perivascular haemorrhages. Subventricular gliosis is minimal or absent, even in regions devoid of surface ependyma. Strands of eosinophilic fibrinous material cross the aqueduct. In the chronic phase, the aqueductal stenosis or occlusion is not accompanied by inflammatory cells, by extensive gliosis or by evidence of tissue reaction. Clusters and rosettes of residual ependymal cells are demonstrated, particularly in the posterior part of the aqueduct, and form ductules or blind diverticuli resembling "forking" of the aqueduct. In "non-neuroadapted" mumps viral infection, damage is limited to ependymal and choroid plexus epithelia. "Neuroadapted" strains of mumps virus infect neurones as well (Wolinsky et al, 1974).

In human children, aqueductal stenosis may appear clinically and radiographically as early as 3 weeks after mumps parotitis but is usually delayed by months (Spartaro et al, 1976: Thompson, 1979: Rotilio et al, 1985). Aqueductal stenosis may also follow influenza and parainfluenza type 2 infections.

In reovirus infection, virons aggregate at the apex of the ependymal cell in microtubular structures near the bases of cilia (Nielsen & Baringer, 1972). Degeneration of viral-infected ependymal cells occurs without ependymal inflammation or reactive gliosis. Experimental infection of hamsters and mice with respiratory syncytial virus causes hydrocephalus without aqueductal stenosis (Lagrace-Simard & Desconteaux, 1982).

Ependymitis occurring with human respiratory syncytial virus and murine hepatitis infections may cause hydrocephalus without obstruction of CSF pathway (Lagrace-Simard & Desconteaux,

1982: Tardieu et al, 1982). The resulting hydrocephalus is probably due to impaired absorption of ventricular CSF.

1.8.20 Inflammation:

Infection is the most frequent cause of inflammation involving the ependyma.
Ependymitis may become a complication of meningitis at any age. Neonatal patients, especially preterm infants, are at a particularly high risk because of their immature immune system. Ependymitis often is the cause of persistent meningitis that does not resolve clinically during treatment. Tumour necrosis factor-alpha, a cytokine which appears in CSF soon after endotoxin challenge, is demonstrated in epithelial cells of the ependyma and choroid plexuses in the guinea pig (Tarlow et al, 1993). Together with other cytokines such as Il-1 it may be important in the inflammatory response to bacterial infection.

Ependymitis due to bacterial infection of the ventricular fluid evokes an initial inflammatory response of polymorphonuclear leukocytes. Several days later lymphocytes may predominate. Intraventricular pus may obstruct CSF pathways, particularly the cerebral aqueduct, causing obstructive hydrocephalus. Fungal infections may result in meningitis and ependymitis (Sanart, 1995).

Ependymitis results in necrosis of ependymal cells and denudation of multiple areas of the ventricular surface. Ischaemia, acidosis, toxic products of infectious organisms and direct invasion of ependymal cells contribute to cellular loss. The role of macrophage activity, lymphocytic or polymorphonuclear cell infiltrates, microglial mobilisation and other natural responses to tissue damage are incompletely understood. Cerebrospinal fluid obtained by lumbar puncture or ventricular drainage rarely contains cells recognised as ependymal. Clusters of ependymal or choroid plexus cells may, however, exfoliate into the ventricular fluid in hydrocephalic infants (Wilkins et al, 1974).

The hydrocephalus that complicates ependymitis of bacterial, fungal or viral origin cannot be explained exclusively by obstruction. Ventricular dilatation begins well before evidence of blockage to CSF flow appears and may be due to impaired function of the ependyma as a transporter of fluid and ions.

1.9 The CSF Pathways:

This section briefly describes the formation, flow and composition of cerebrospinal fluid.

CSF circulates through the ventricular system, central canal of the spinal cord and the subarachnoid space. In each cerebral hemisphere there is a large space, the lateral ventricle, which is made up of an anterior horn, the body, a posterior horn and an inferior horn. The third ventricle is a cavity in the diencephalon. The lateral ventricle communicates with the third ventricle via the interventricular foramen of Monro. The aqueduct of Sylvius, a midbrain structure, connects the third ventricle to the fourth ventricle. The cavity of the pons forms the superior aspect of the fourth ventricle whilst its inferior aspect is the medullary cavity. It is roofed by the cerebellum and has a diamond-shaped floor. It has three openings to allow CSF to escape into the subarachnoid space - a single median foramen of Magendie and two lateral foramina of Luschka. The meninges that cover the brain and spinal cord consist of an outer layer, the dura mater, a middle layer, the arachnoid mater, and an inner layer, the pia mater. The subarachnoid space is the fluid-filled gap between the arachnoid and pia mater.

1.9.1 Formation of CSF:

Classically, the choroid plexuses are the main source of CSF formation, contributing to over 70% of the CSF (Bonadio, 1992). The remainder is formed in extrachoroidal sites such as the ependyma, the pia mater and the brain and spinal cord parenchyma. Such extrachoroidal sites are thought to account for a relatively small proportion of CSF but some investigators have claimed that they may be responsible for over 50% of CSF production (Greitz, 1993).

1.9.1.1 Choroidal secretion: The CSF is an ultrafiltrate of plasma across the fenestrated capillaries of the choroid plexus coupled with transport across the ependymal cells. Transport of sodium ions, the major osmotically active cation, is accomplished by the action of the sodium-potassium ion exchange pump along with passive diffusion of water. Glucose and other solutes enter the CSF via either similar ion pumps or facilitated diffusion. Diffusion gradients also determine the entry of serum proteins and the exchange of carbon dioxide.

1.9.1.2 Rate of CSF formation: In the adult human, total CSF volume ranges from 52 - 160 ml (Lyons and Meyer, 1990). It is produced at a rate of 500ml / 24 hours. The CSF, as a whole,

is renewed 4 or 5 times daily (Bonadio, 1992). The rate of formation depends upon the hydrostatic pressure of the blood and the activity of the sodium-potassium ion exchange pump.

1.9.2 Absorption Of CSF: The majority of CSF is thought to be absorbed by the arachnoid villi. These are microscopic finger like projections of the arachnoid mater, located on the convexities of the brain, that project into the aural sinuses. They have a valve-like action and when the CSF pressure exceeds venous pressure the valve 'opens' and the CSF enters the aural sinuses.

Alternative mechanisms have been suggested. CSF maybe absorbed into the bloodstream via the extracellular and paravascular spaces of the CSF (Greitz, 1993). The fenestrated capillaries supply the circumventricular organs which may also provide an additional route (Hashimoto, 1992). It is noteworthy that some mammals, including the rat, do not possess arachnoid granulations.

1.9.3 Composition:

Pressure: Normal CSF pressure in man is 5-15mmHg / 65-195mm H_2O. This is in equilibrium with capillary pressure. Variations in pressure occur for a number of reasons. Alterations in blood pressure, particularly in venous pressure, affect CSF pressure. An increase in venous pressure increases the volume of blood in the veins and dural sinuses increasing the pressure of the CSF. During respiration CSF pressure decreases in inspiration and increases in expiration due to changes in intracranial venous pressure (Lyons and Meyer, 1990). Coughing, sneezing and changes in posture affect CSF pressure. Acidosis causes a decrease in arteriolar resistance which increases cerebral blood flow and capillary pressure which consequently raises CSF pressure. Alkalosis is vasoconstrictive thereby decreasing the blood flow and CSF pressure.

1.9.4 Circulation: CSF flows from the lateral ventricle, through the third ventricle, the aqueduct and the fourth ventricle and finally leaves by the foramina of Magendie and Luschka into the subarachnoid space. It flows over the brainstem and the surfaces of the cerebral hemispheres. The CSF also circulates to the lower reaches of the spinal cord and a small proportion circulates through the central canal of the cord.

1.9.4 Flow dynamics: A number of factors are responsible for the flow dynamics of CSF. Bulk flow, due to the absorption of CSF at the villi produce forces to propel the fluid. Arterial pulsations of the choroid plexus have been shown to help drive the CSF from the ventricles into the subarachnoid sinuses. The motion of the brain within the cranial cavity may also help CSF flow. The role of ependymal cilia in CSF flow is rarely discussed in detailed reviews.

1.9.5 Function of the CSF: Its primary function has been thought to be physical. It provides buoyancy, reducing the effective weight of the brain from 1500g to 50g. The CSF acts as a protective buffer against acute changes in blood pressure and from impact. It is an important pathway for chemical substances and nutrients to reach the intracellular spaces of the brain, and because there are no lymphatics within the CSF, it returns the waste products to the venous circulation. The composition of the CSF is maintained within narrow limits and preserves a stable environment for the brain.

Table 1.1:
Concentrations of ions (mEq/kg H_2O) in plasma, plasma filtrate, choroid plexus fluid, and cisterna magna fluid of the cat (Ames et al, 1964)

	Cl	Na	K	Ca	Mg
Plasma	132	163	4.4*	2.62	1.35
Plasma ultrafiltrate	136	151	3.3	1.83	0.95
Choroid plexus fluid	138	158	3.28	1.67	1.47
Cisterna magna fluid	144	158	2.69	1.50	1.33

* Value for K^+ in plasma considered too high because of white cells etc. being present

Table 1.2:
Concentrations of various solutes (mEq/kg H_2O) in plasma and CSF of the rat (Manthei et al, 1973; Feise et al, 1976)

Substance	Plasma	CSF	R_{CSF}
Na	148	152	1.03
K	5.3	3.36	0.64
Ca	6.14	2.22	0.36
Mg	1.44	1.77	1.23
Glucose	7.19	5.38	0.75
Pyruvate	0.174	0.184	1.04
Lactate	0.713	2.08	2.92

Chapter 2

The measurement of ependymal ciliary beat frequency: a comparison of photodiode, photomultiplier and high speed video methods.

2.1 Summary:

A number of methods have been developed to measure the beat frequency of cilia. There has been no direct comparison of the most commonly used methods used to measure ciliary beat frequency, the high speed video, regarded as the gold standard, the photodiode and the photomultiplier. In this study we have measured brain ependymal ciliary beat frequency using each method at different temperatures.

Ciliated strips of ependyma attached to slices from the brain of Wistar rats were incubated at 30°C and observed using a x50 water immersion lens. Ciliary beat frequency was measured using each of the three techniques. Readings were repeated after 30 minutes incubation at 37°C. The mean (95% confidence intervals) beat frequencies determined by the high speed video, photomultiplier and photodiode at 30°C were 27.7(26.6-28.8), 25.5(24.4-26.6) and 20.8(20.4-21.3) Hz respectively. The mean (95% confidence intervals) beat frequencies determined by the high speed video, photomultiplier and photodiode at 37°C were 36.4(34-39.5), 38.4(36.8-39.9) and 18.8(16.9-20.5)Hz. The photodiode method greatly underestimates ciliary beat frequency compared to the high speed video. The limits of agreement were narrowest for the photomultiplier method at a temperature of 30°C.

The photodiode technique should not be used to measure ependymal ciliary beat frequency at the temperatures studied. Beat frequency measurements obtained from the high speed video and photomultiplier techniques can not be used interchangeably.

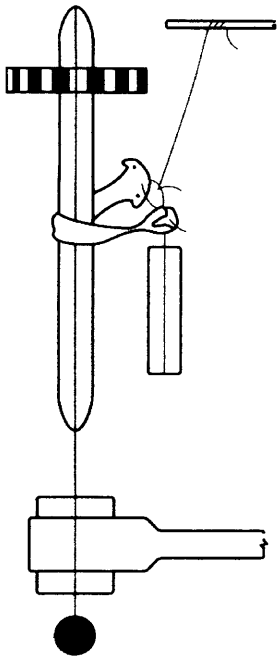
Intra subject and inter subject variability in the measurement of ciliary beat frequency using the high speed video was low. The intra and between observer reliability was 3.8% and 1% respectively.

2.2 Background:

During the last century a variety of methods have been developed to measure ciliary function.
One of the first objective techniques used to estimate ciliary beat frequency was the stroboscopic method. This technique involves flashing a light at known frequencies until a frequency is reached at which cilia appeared stationary (Grey, 1930; Ballenger et al, 1963; Bernstein et al, 1990). The frequency of the flashing light at this point relates to the ciliary beat frequency. Over recent years the stroboscopic method has been criticised by a number of authors, and is no longer in current use. Ballenger and colleagues (1966) found the method imprecise and experienced difficulty calculating the frequency of cilia beating slowly. Cilia in adjacent fields of view beat at different frequencies, varying by as much as 3Hz, and samples obtained were often several layers thick making it difficult to visualise individual cilia. Dalhamn (1970) also reported problems with the reproducibility and variability of results of the stroboscopic technique, particularly at high beat frequencies. In contrast to Ballenger (1966) his only success was in measuring the beat frequency of cilia from molluscs which beat at low frequencies.

Another early, ingenious, method was the cilioscribe. This consisted of a ciliated strip of epithelium from the frog palate which was able to rotate a glass rod, recording the trace on a rotating drum (figure 2.1).
When long strips of ciliated epithelium are obtained occasional strips roll up into a sphere which is rotated by the vigorous beating of cilia. The time to complete a certain number of revolutions was taken as an index of ciliary activity by Corsenn (1958) and Ballenger (1960). Obviously ciliary beat frequency can not be determined using this method.

Figure 2.1:



This figure shows a device called the cilioscribe invented by Inchley (1921). The glass tube 10cm long and 5 - 8mm in diameter, is tapered down and sealed at one end. At the other end is a strip of frog's ciliated membrane which is looped around the tube, with the ciliated surface in contact with it. The glass spindle slowly rotates due to ciliary activity. Near its upper end the spindle carries a disc of cork, about 3cm in diameter, graduated at the circumference to make rotation obvious.

An auditory method was developed by Bleeker (1971) which involved approximating the ciliary beat frequency to a sound generated at a known frequency. The ciliary beat frequency could be found when the beating cilia were in synchrony with the sound.

These methods have given way to measurements using a photodiode, a photomultiplier, cinematography and more recently high speed video.

2.2.1 The photodiode:

The photodiode method involves displaying the images of moving cilia, captured by a high resolution video camera, on a monitor. A photodiode cell is positioned on the monitor over the image of the moving cilia. The variation in light intensity resulting from ciliary movement is detected by the photodiode and a signal generated which may be displayed on an oscilloscope (Teictahl, 1986) (figure 2,2) or processed by computer to give a power spectrum analysis (Gyi, 1994) (figure 2.3).

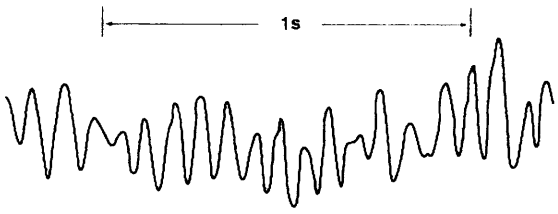


Figure 2.2:

Typical recording of beating cilia from the oscilloscope. Each beat should correspond to one cycle of a ciliary beat. The ciliary beat frequency of this epithelial area is 14Hz.

This method is simple to perform and provides real time analysis of ciliary beat frequency. Video recordings may be made at the same time to allow simple motion analysis.

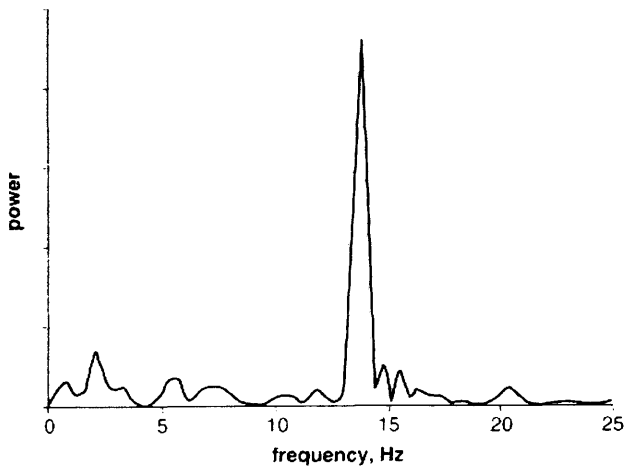


Figure 2.3:

Power spectrum of an area of beating cilia. The peak shows a ciliary beat frequency of 14Hz for this area.

Disadvantages of the method include interference from vibration of the sample at high

magnifications. Video recorders relay 25 images per second to the monitor, therefore, theoretically, problems may be encountered measuring cilia which beat at high frequencies.

2.2.2 The photomultiplier:

The photometric technique, initially described by Dahlman (1962), is currently the most popular method for estimating ciliary beat frequency (Ingels et al 1992: Rutland & Cole, 1980). Variations in the perpendicular light beam of the microscope, caused by beating cilia, are detected by a photosensitive cell. Voltage signals generated may then be displayed on an oscilloscope or a power spectrum analysis performed allowing frequency to be obtained. The use of power spectrum analysis, with this method, gives a more objective measurement of frequency. Photomultipliers are expensive and are also subject to vibration artefact. The photomultiplier system does not allow analysis of single cilia nor the precise beat pattern to be determined. A spectrum of frequencies is obtained and precise relationship of the frequencies and the ciliary beat cycle it is not always clear (Sanderson & Dirksen, 1985). The exact method also varies between authors particularly the size of the aperture used for the photomultiplier. The aperture is the area through which light passes through the sample to allow readings to be made. Dahlman suggests a small aperture of 2.5 microns as larger apertures may encompass more frequencies and out of phase metachronal activity (Sanderson & Dirksen, 1985).

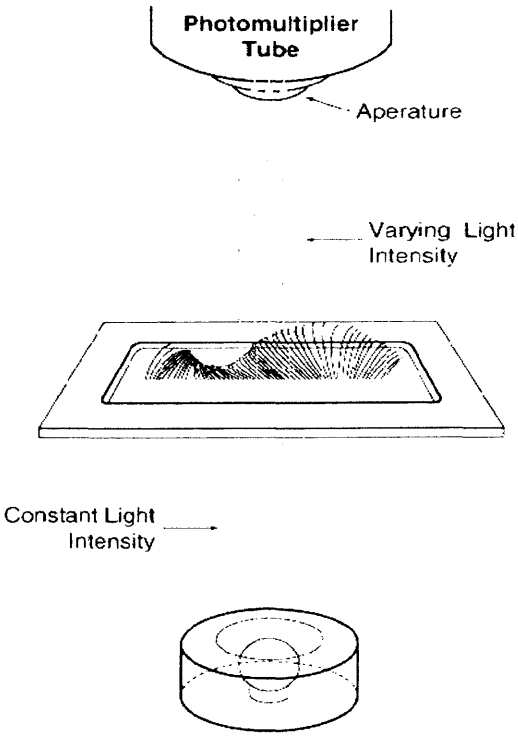


Figure 2.4:

Principle of the photomultiplier technique. Light at constant intensity is directed through the specimen. Due to the beating action of the cilia, light passes through the specimen in varying intensities. Variations in light intensity are transduced to voltage impulses by the photomultiplier Dahlman (1962).

The reflected light technique also makes use of a photomultiplier. A beam of light is directed at a ciliated surface and the reflected light detected by the photomultiplier giving an index of ciliary activity (Mercke et al, 1974). The origin of the surface reflections is not always clear but they may represent the muco ciliary wave frequency (Sanderson & Dirksen, 1985).

More recently this technique has been modified by the use of a laser light-scattering spectroscopy technique (Lee & Verdugo, 1976). A safe low power laser source is used to emit a laser beam, of approximately 15um diameter directly onto the specimen. The reflected laser light spectrum is analysed for fluctuations in the back scattered light intensity resulting from moving cilia. The fluctuations in back scattered light are detected by a photomultiplier and the frequency determined by a power spectrum analysis of the signal. The major disadvantages of the technique are that it is expensive, as is the case for all techniques measuring reflected light, and it is subject to error due to light scattered as a result of the slightest vibration in the fluid bathing the specimen.

2.2.3 High speed cinematography and video:

Cinephotographic techniques have been regarded as the gold standard for the measurement of ciliary beat frequency (Grey, 1930). These methods allow cilia to be recorded at high frame rates, up to 500/second, and then analysed by replaying in slow motion which allows the frequency to be determined and the precise beat pattern of a cilium to be seen. Although recognised to be the most accurate method (Dahlman, 1970; Ballenger et al, 1966; Greenstone et al, 1986: Baba & Hiramoto, 1970) it was technically difficult and real time analysis was not possible due to film processing. Over recent years high speed digital video cameras have been developed, allowing recordings to be made at frame rates up to 45,000 per second. High speed digital video cameras provide a simple and accurate method for determining ciliary beat frequency and for storing recordings to tape (Rautiainen et al, 1992). The beat pattern of individual cilia may be seen and analysed in slow motion following recording.

There have been no direct comparisons of the use of the photodiode, photomultiplier and high speed video techniques to measure the beat frequency of any ciliated system. The aim of this

study was to compare the measurement of ependymal ciliary beat frequency made by these three methods.

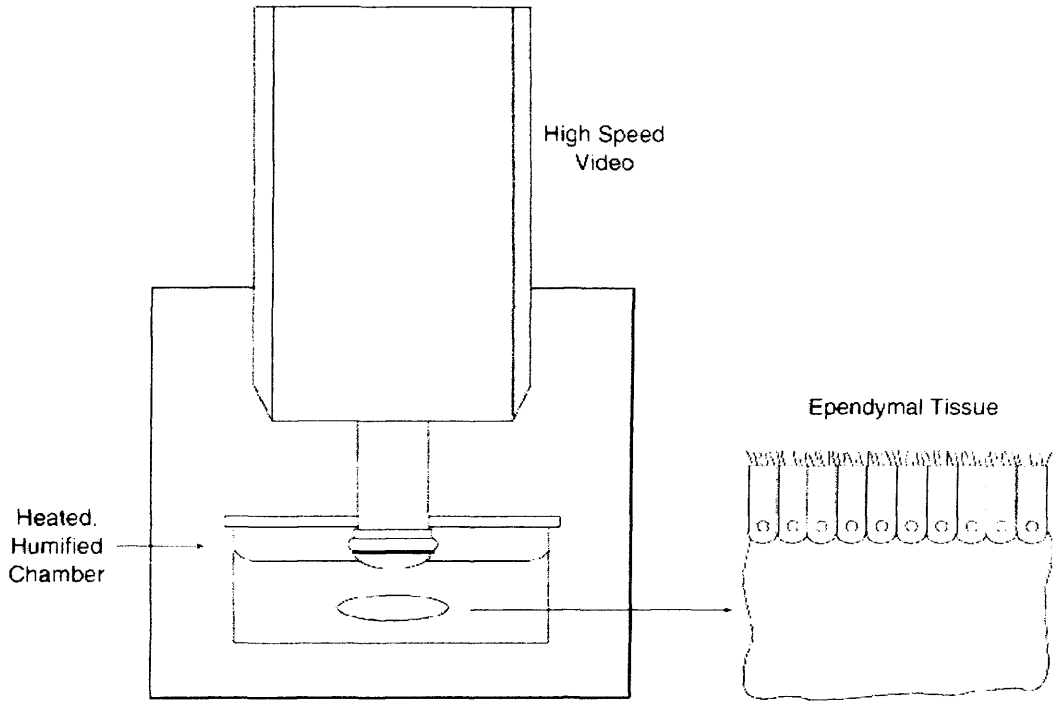


Figure 2.5:

Diagram of the well system and heated humidified chamber used to incubate brain slices.

2.3 Methods:

2.3.1 Preparation of brain ependymal tissue: Wistar rats (9-15 days of age) were sacrificed by cervical dislocation. The brain was immediately removed and stored in medium 199 (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) at 4°C until use. The fourth ventricle was chosen as the most reliable site for the presence of cilia. The brainstem and cerebellum were separated from the cerebral hemispheres and blood vessels and meninges removed.

To decrease trauma to the fragile ependyma caused by manual slicing, a vibratome was used to cut brain slices. The brainstem was mounted, aqueduct side down, on the cutting stage of a Vibroslice (Model 752: Campden instruments Ltd) using superglue as an adhesive. The bath surrounding the brain stem was filled with medium 199 and cooled to 4°C using a Peltier driven cooling device. Transverse slices of brain, 150um thick, were cut and stored in medium 199 at

4°C (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) until use. Ependyma from the fourth ventricle of 10-15 day old rats was used. For each piece of tissue associated with the fourth ventricle approximately 2-3 ciliated brain slices with intact ependymal edges were obtained. Ciliated ependyma which had obviously been disrupted or damaged was not used.
For the experiment, brain slices were mounted in a well containing 4 ml of medium 199 with Earle's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml). The well was placed in a purpose built environmental chamber which was thermostatically controlled to keep the fluid surrounding the ependymal sample at either 30°C or 37°C. The chamber was humidified to 75-80% to prevent evaporation from the well during the study period (see chapter 3 for details).

Three techniques were used to measure ciliary beat frequency: the high speed video; photomultiplier; and photodiode. These are described in greater detail below. The study involved incubation of the ependymal samples at 30°C for 30 minutes followed by initial measurements, using each of the three techniques. Samples were then incubated at 37°C for a further 30 minutes and readings repeated, again using the three methods. The order in which the various methods of measurement were made was varied from experiment to experiment to help exclude any bias due to their order of use.

Ciliary movement was observed using a x100 water immersion lens. The maximum time the tissue was in focus for was between 30-45 seconds for each reading.

In total 28 separate experiments on different ependymal strips were conducted at both 30°C and 37°C. The photodiode was not available for use in all of the experiments (n=16 at 37°C & n= 23 at 37°C) due to technical problems.
The following section describes the methods used to measure ciliary beat frequency.

2.3.2 High speed video: Beating ciliated strips were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 400 frames per second using a shutter speed of 1 in 2,000. The camera allowed video sequences to be downloaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study, ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips

in excess of 100um were studied. Using this method it is possible to observe the precise movement of individual cilia during their beat cycle.

2.3.3 Photodiode measurements: Video images of the beating ependymal cilia were relayed from a S-VHS video camera (Panasonic VW15) to a high resolution monitor. The photodiode, mounted in a pen like system, was held over the beating cilia on the monitor. Voltage signals were generated as cilia moved past the photodiode sensor and were fed via an oscilloscope to a power spectrum analysis program (ANADAT, Montreal, Canada) to determine ciliary beat frequency. The frequency of cilia was measured at 4 separate places along the ciliated edge allowing a mean value of ciliary beat frequency to be obtained.

2.3.4 Photomultiplier measurements: The aperture allowing light to reach the photomultiplier was adjusted to 2 um^2 and positioned over an area of beating cilia. Voltage signals generated were then displayed on an oscilloscope and relayed to the power spectrum analysis program (ANADAT, Montreal, Canada) to determine ciliary beat frequency.
The frequency of cilia was measured at 4 separate places over the ciliated edge allowing a mean value of ciliary beat frequency to be obtained.

2.3.5 Inter and intra observer variability using the high speed video system:
Following the results of the comparison between methods the high speed video system was selected as the method of choice for experiments conducted in this thesis.
The following study was conducted to establish inter and intra observer variability in measurements made using this technique.
Ten ciliated edges from different rats were incubated at 37°C for at least 30 minutes and a high speed video recording made. This was stored on video tape at a reduced frame rate for later analysis.
An observer measured beat frequency by determining the time taken for individual cilia to complete 5, 10 and 15 beat cycles. The edge from which readings were made was divided into quadrants and the beat frequency of an individual cilia from each quadrant made. Thus the effect of counting different beat cycles, using different ciliated edges and taking measurements from different places along a given edge could be assessed. Measurements were made again by the same observer 1 month later.

To compare the variation in beat frequency measurement between observers a second observer, independently, measured beat frequency from the same 10 edges. Frequency was measured by determining the time taken for individual cilia to complete 10 beat cycles. As with the first observer the edge was divided into quadrants and a reading taken from each quadrant.

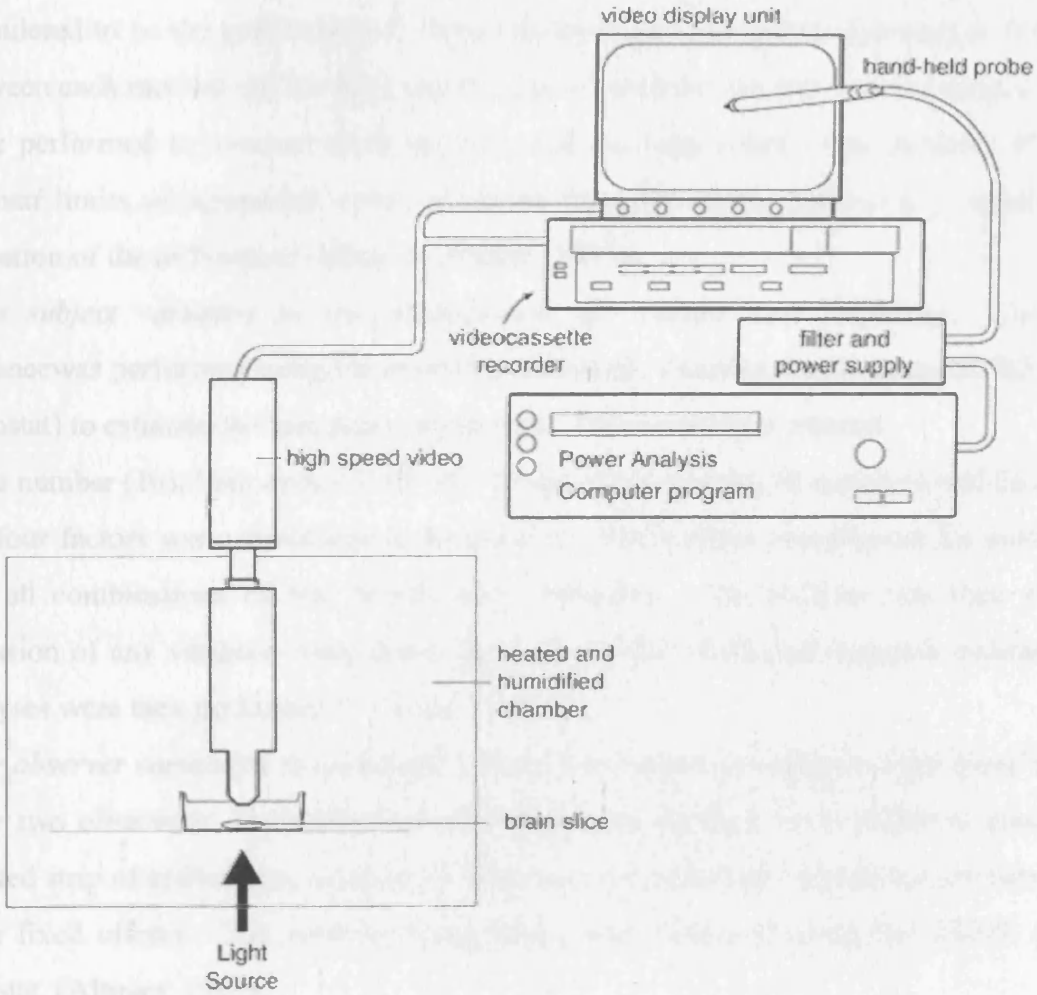


Figure 2.6: *Optical system and ciliary beat frequency analysis equipment. The image of the beating cilia is displayed on a high resolution monitor. Variations in light intensity caused by the beating cilia are detected by a photodiode probe whose amplified and filtered output is displayed on a recorder.*

2.4 Statistics:

A two way analysis of variance was performed with factors:

Temperature: 37°C or 30°C

Method: HSV, Photo, Diode.

As the difference between the methods varies with temperature, the limits of agreement were calculated for each temperature and each method separately. The high speed video method is considered to be the gold standard. Therefore the mean and standard deviation of the difference between each method and the high speed video at each temperature was calculated. Paired t tests were performed to compare each method with the high speed video method. The Bland and Altman limits of agreement were calculated from the mean difference $\pm$ twice the standard deviation of the differences (Bland & Altman, 1993).

Intra subject variation in the measurement of ciliary beat frequency: An analysis of variancewas performed using the restricted maximum likelihood estimation (RELM) procedure (Genstat) to estimate the variance components. There were four factors:

Edge number (10); Beat cycle (5, 10, 15); Quadrant (4 places); Occasion (1 and 2).

All four factors were considered to be random. The various components for each main effect and all combinations of two factors were estimated. The analysis was then repeated after omission of any variance components from the model which had negative estimates. Separate analyses were then performed 5, 10 and 15 cycles.

Inter observer variability in ependymal ciliary beat frequency using the high speed video: There were two observers. Each observer performed four readings at 10 different places along the ciliated strip of epithelium. Analysis of variance was performed considering the two observers to have fixed effects. The variants components were estimated using the REML procedure in Genstat (Altman, 1991).

2.5 Results:

The mean (95% confidence intervals) beat frequencies determined by the high speed video, photomultiplier and photodiode at 30°C were 27.7(26.6-28.8), 25.5(24.4-26.6) and 20.8(20.4-21.3) Hz respectively. At 37°C differences between the methods were greater. The mean (95% confidence intervals) beat frequencies determined by the high speed video, photomultiplier and photodiode were 36.4(34-39.5), 38.4(36.8-39.9) and 18.8(16.9-20.5)Hz respectively. The

individual results are shown in figure 1 where results from the high speed video are compared with those from the photodiode and photomultiplier.

There were highly significant effects of temperature ($P<0.001$), method ($P<0.001$) and a highly significant interaction between method and temperature ($P<0.001$). That is the difference between the methods differs between the two temperatures. The analysis of variance was repeated after omitting the photodiode method. There was still a significant interaction between temperature and method ($P=0.003$).

As the difference between the methods varies with temperature, the limits of agreement for each temperature and each method were calculated separately. These are shown in table 1 and graphically in figures 2.8a-d.

Table 2.1: *Mean (95% confidence intervals) readings of ependymal ciliary beat frequency using three different methods of measurement at two different temperatures.*

	Frequency in Hz (95% confidence levels)		
Temp	HSV	Photo Multiplier	Photo Diode
30°C	27.7 (26.6-28.8)	25.5 (24.4-26.6)	20.8 (20.4-21.3)
37°C	36.4 (34-39.5)	38.4 (36.8-39.9)	18.8 (16.9-20.5)

The photodiode method underestimates ciliary beat frequency greatly compared with the high speed video. The limits of agreement were narrowest for the photomultiplier method at a temperature of 30 °C.

Table 2.2: *Limits of agreement for comparison to high speed video method.*

Temperature °C	Method	Difference mean	SD	Limits of agreement
30	Photomultiplier	2.17	2.60	-3.03 to 7.37
37		-2.00	4.15	-10.30 to 6.29
30	Photodiode	6.58	2.96	0.65 to 12.51
37		18.34	4.94	8.46 to 28.21

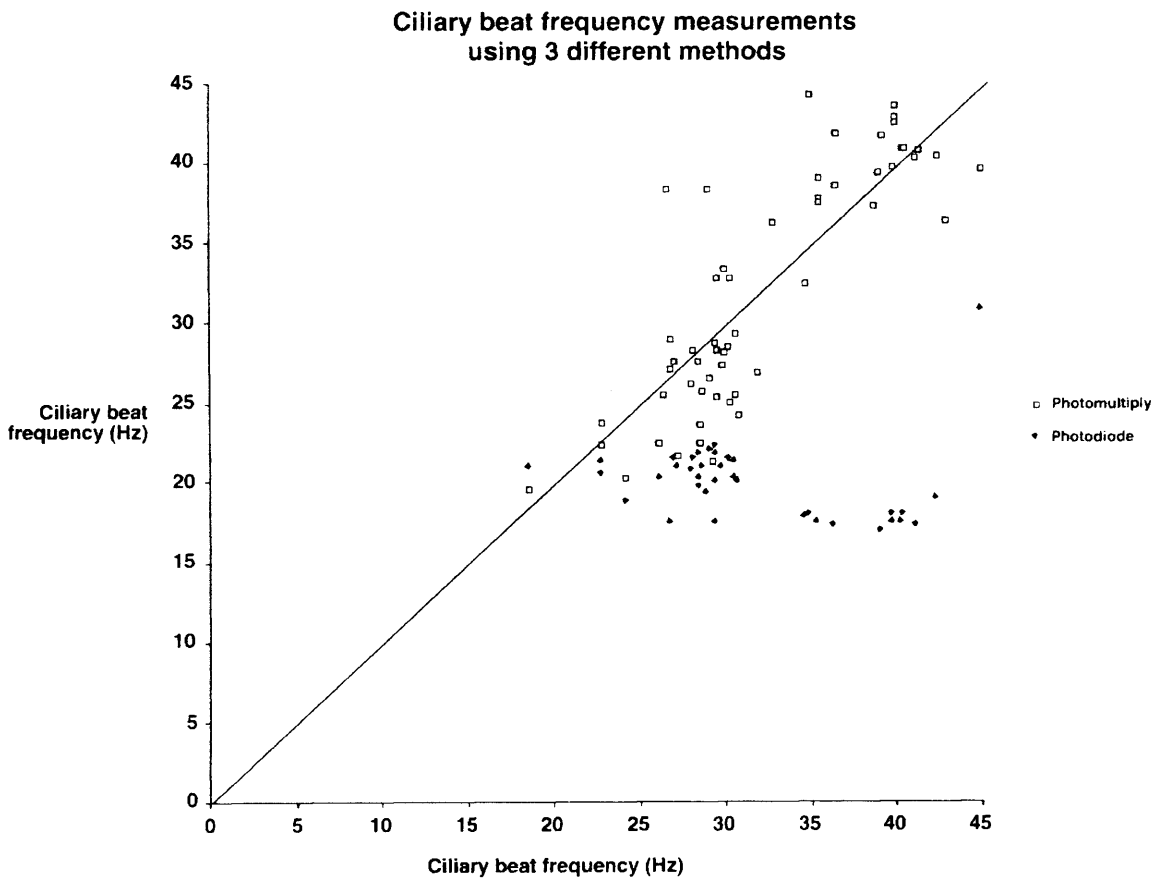


Figure 2.7: *Readings taken using the high speed video (x-axis) plotted against readings from the photomultiplier and photodiode(y-axis).*

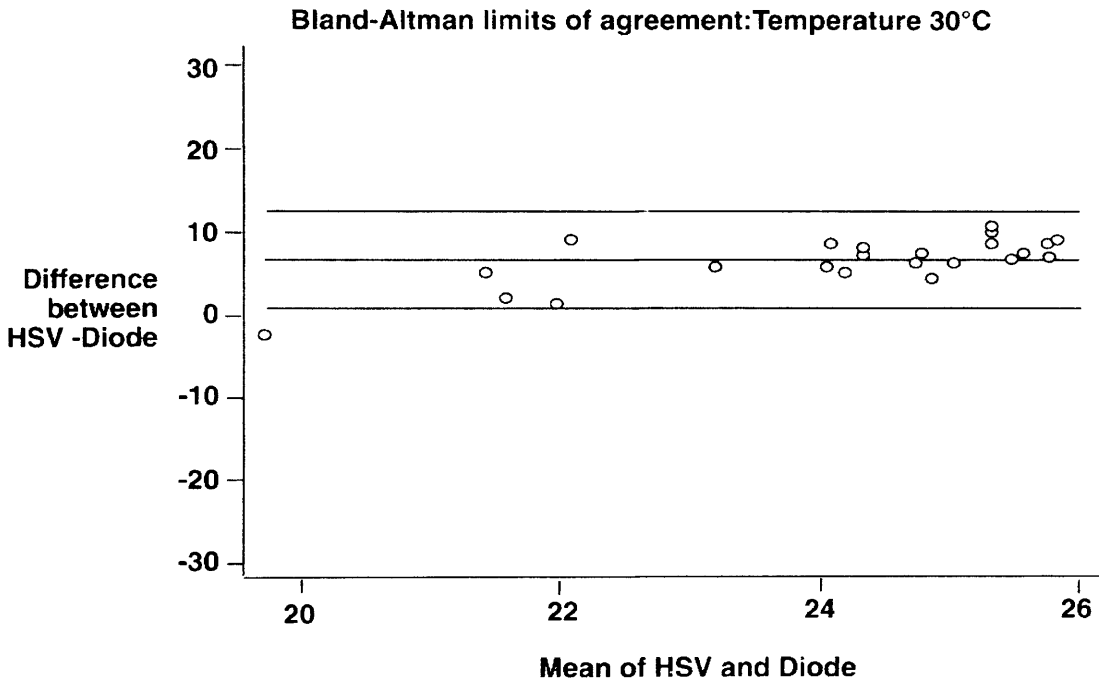


Figure 2.8a:

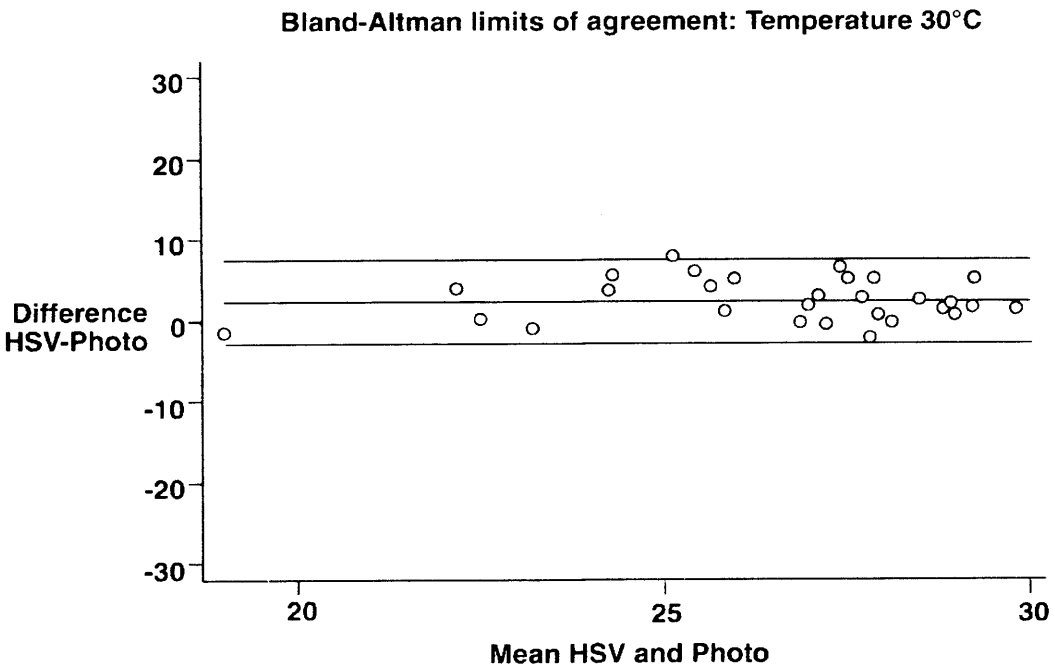


Figure 2.8b:

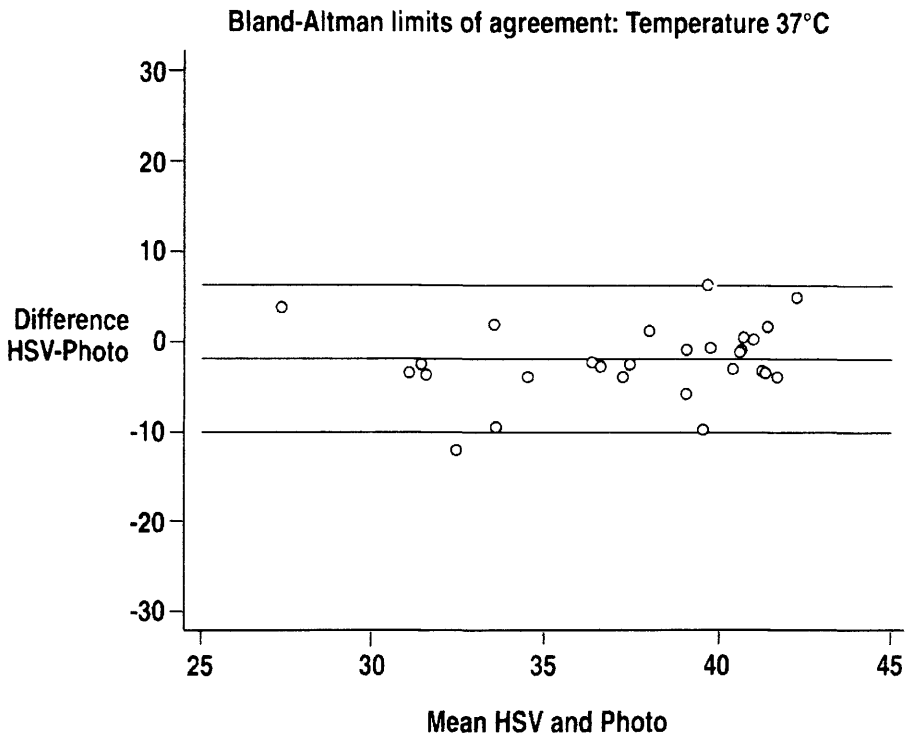


Figure 2.8c:

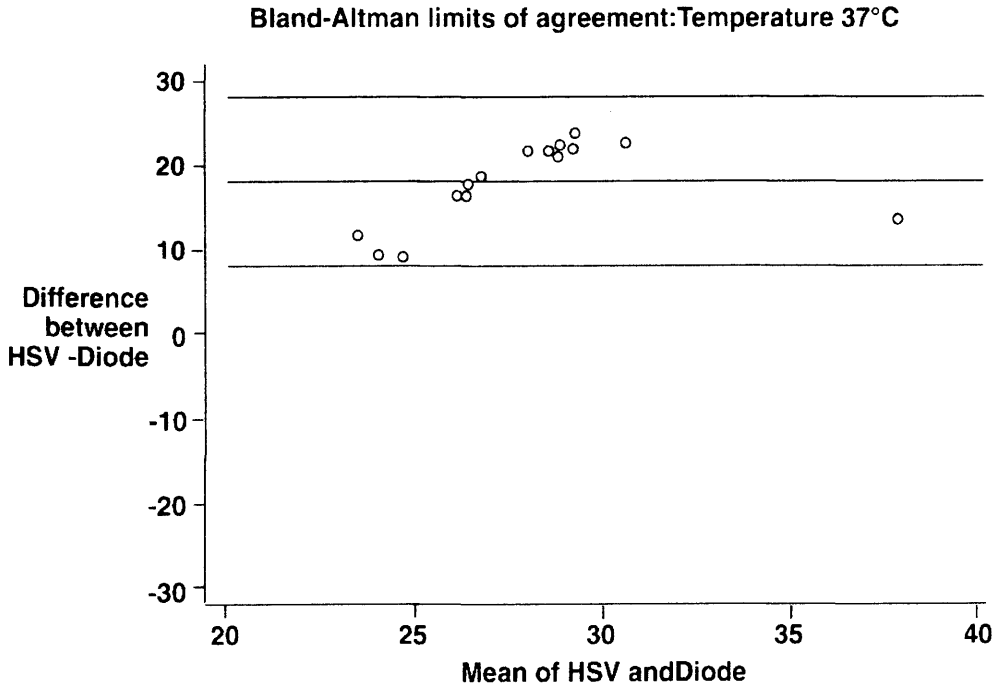


Figure 2.8d:

Intra subject variability in ependymal ciliary beat frequency using the high speed video: The variance components were remarkably similar even though the number of full cycles measured increased from 5 to 15. This implies that there is little advantage to be gained from increasing the number of cycles measured. The variants components were estimated and are summarised in the table below.

Table 2.3:

Component	**Variance component**	**Percentage of total**
Between edge	21.7	56%
edge quadrant	11.7	30.5%
beat cycle	0.1	1%
edge quadrant cycle	2.5	5.3%

Inter observer variability in ependymal ciliary beat frequency using the high speed video: Analysis of variance was performed considering the two observers to have fixed effects. This showed significant differences between the means of the two observers (p=0.02).
The variants components were estimated and are summarised in the table below.

Table 2.4:

Component	**Variance component**	**Percentage of total**
Between observers	1.49	3.8%
Between positions	25.4	65.5%
Between times	11.88	30.6%
Total	38.77	

Although there is a significant difference between the two observers, the between observer component of variance is only 3.8 per cent of the total variation (figure2.e).

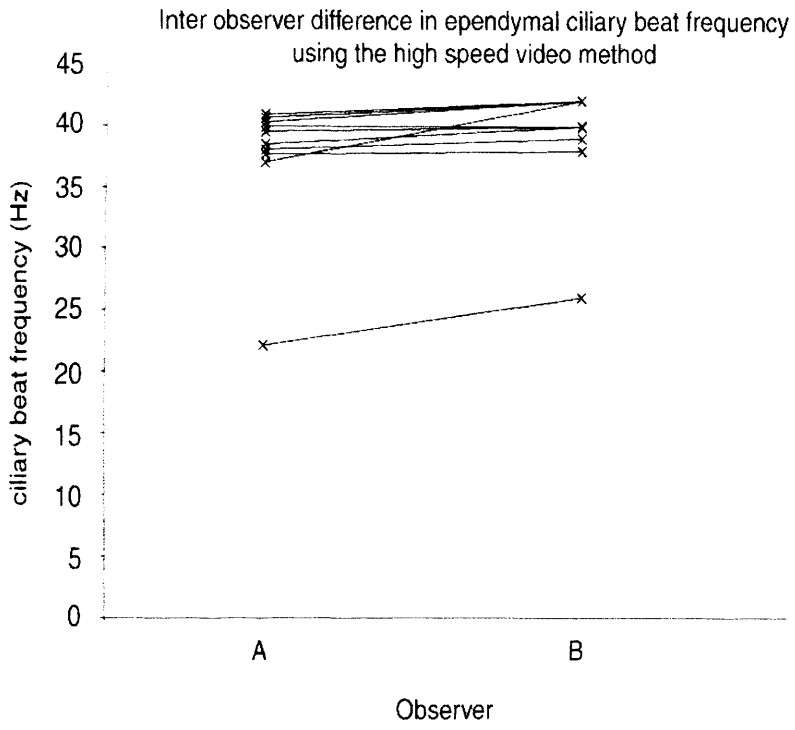


Figure 2.e:

2.6 Discussion:

This is the first study to directly compare the most commonly used methods of estimating ciliary beat frequency. The high speed video technique is held as the gold standard for the measurement of ciliary beat frequency. A major advantage of the high speed video system is the ability to obtain images at a rate of 400 per second and to make a permanent recording of the ciliary movement at a reduced frame rate. This allows frequency, amplitude, wave form, orientation, and synchrony of ciliary beat to be determined at a later time. Ciliary beat frequency may also be determined quickly during an experiment, movement of cilia reviewed and the integrity of the ciliated strip to be assessed.

Our results show that the photodiode method is unsuitable for the measurement of cilia beating at high frequencies. There is broad agreement between the ciliary beat frequency measured by the photomultiplier technique and by high speed video. The limits of agreement are narrowest at a temperature of 30°C, though still large. In addition the mean measurements made by the

photomultiplier at a temperature of 30°C are lower than those made by the high speed video whereas the reverse is true at a temperature of 37°C.

The photodiode technique involves the relay of images from a standard video camera to a monitor at a frame rate of 25/second. This explains the very poor agreement between ciliary beat frequency measured by the photodiode technique and the high speed video camera. If cilia are beating faster than 25 frames per second not all of the cycles are detected by the photodiode. The problems encountered with the photodiode may be shared by the analogue contrast enhancement technique which also relies on manipulations of standard video recordings. These techniques may still be suitable for recording the frequency of respiratory cilia which beat at less than 20 Hz. However, pharmacological stimulation which results in an increase of ciliary frequency may not be detected.

Although the limits of agreement between photomultiplier readings and high speed video recordings are in a similar range, the readings are different and the techniques can not be used interchangeably.

The photodiode and photomultiplier techniques are subject to vibration artefact. Readings made directly from the trace of an oscilloscope may include artefacts which may be impossible to differentiate from the true signal. The use of power spectrum analysis gives a more objective measurement of frequency with this method. A spectrum of frequency is obtained which helps to separate out artefactual results. However, it is not always clear which spectrum relates to the ciliary beat cycle (Sanderson & Dirksen, 1985). Two closely associated peaks of similar height are sometimes encountered making it difficult to know which to choose.
The precise method by which the photomultiplier is used varies between authors, particularly regarding the size of the aperture used. While Dahlman suggests a small aperture of 2.5 microns^2 other authors have used large apertures. It is likely that larger apertures may encompass areas where cilia are beating at different frequencies. One of the major draw backs of the photomultiplier method is that it does not allow analysis of single cilia nor the precise beat pattern to be determined.

The intra subject variation in measurement of ciliary beat frequency from slow motion video tapes was very low. The inter subject variation only accounted for 3.8% of the variability of the system. These results suggest recordings made may be studied at a later date by a trained observer to facilitate ongoing studies and to check previous results.

In summary the photodiode technique can not measure ependymal ciliary beat frequency. It follows that experiments using techniques involving standard video cameras can not be relied upon to detect stimulation of ciliary beat frequency. The photomultiplier may be used to detect differences in beat frequency at higher frequencies but results still vary considerably from those obtained using a high speed video system. Results from the two systems can not be directly compared.

Chapter 3

The effect of methods used to observe brain ependymal cilia on the measurement of ciliary beat frequency

3.1 Summary:

A variety of techniques were developed and evaluated to establish the most appropriate experimental system for further work.

Methods were developed for:

- the preparation of ciliated ependymal tissue which was suitable for observation:
- for the containment of ciliated ependymal tissue to allow microscopy.

Although simple brushing of the respiratory epithelium results in excellent specimens of ciliated tissue for analysis, brushing of the ependymal lining of rat ventricles did not produce ciliated tissue of sufficient quality to study. Manual cutting of brain tissue slices provided tissue with occasional ciliated edges. The use of a vibratome to cut brain slices, in the region of the 4th ventricle, produced intact ciliated ependymal strips.

The following methods were used for containment of ciliated brain slices:

Microscope slide chamber: Brain slices were mounted in medium 199 within a well created from a microscope slide and coverslips. This method allows respiratory cilia to be monitored for several hours. However, ependymal ciliary beat frequency was rarely maintained for more than thirty minutes before slowing occurred (initial mean(SD) beat frequency = 17(3.6)Hz).

Perfusion chamber: To facilitate studies on the effect of various pharmacological stimuli on ependymal ciliary beat frequency a perfusion system was developed. The system developed allowed perfusion of up to three different solutions, at 37°C, with simultaneous ciliary beat frequency measurement. Using the perfusion system, ciliary beat frequency remained constant over a number of hours (mean (SD) beat frequency = 19.9(3.7)Hz).

Incubation chamber + water immersion lens: An incubation system was developed in which ciliated ependymal tissue was observed, using a water immersion lens. Brain slices were placed in a 4ml volume of cell culture medium maintained at 37°C. Using this method ependymal cilia

beat at approximately twice the frequency of samples studied using the perfusion system or slide and cover slip method (mean (SD) initial beat frequency = 36.5(3.6) Hz).

However, if the water immersion lens, used in the incubation system, was left in focus for over 30 seconds the ciliary beat frequency began to decline until cilia became stationary. Stasis usually occurred within 30 minutes. However, repeated, intermittent readings where the lens was in focus for 30 seconds or less caused no decrease in ciliary beat frequency over a 4 hour period.

Incubation chamber + inverted microscope: An inverted microscope was used to observe ependymal cilia in the incubation chamber. Ciliary beat frequency remained high and was maintained for several hours. The system allowed continual measurements to be made. The ciliary beat frequency was again approximately twice (35.2 (3.3) Hz) that seen when the slide and cover slip method or perfusion methods were used.

3.2 Introduction:

A variety of methods have been used to mount ciliated specimens, taken from the respiratory tract, to allow microscopic analysis. The static mount or hanging drop technique allows ciliated specimens to be suspended in cell culture medium between a glass slide and a cover slip. The cover slip is supported away from the glass slide, to allow cilia to move in a small chamber, by silicon grease (Rutland and Cole, 1981), a ring (Bleeker, 1971), or by use of a slide with a well in it (O'Callaghan, 1991). A similar preparation can be made by supporting the cover slip at either end with the walls built from cover slips. The specimen is placed between the walls in a droplet of cell culture medium and a cover slip applied (figure 3.a).

The alternative has been to place the specimen in a perfusion chamber. Several have been developed, including the rose chamber (Ballenger, 1966) and the Dvorak-Stotler (1971) chamber. Both employ a sealed transparent unit through which a medium may be perfused after heating. Such systems allow for the control of temperature and the effects of evaporation. It is of interest that no direct comparisons of the effect of these different methods on ciliary beat frequency have been performed.

Despite the ease at which ciliated respiratory tissue is obtained and observed, it took a considerable time to find a suitable preparation of ciliated ependyma which allowed reproducible readings of beat frequency to be made. Other investigators have experienced difficulties which may be responsible for the dearth of research into ependymal ciliary function. Indeed, problems with measurement of ependymal ciliary beat frequency forced Nakamura and Sato (1993) to use an indirect method to measure ependymal ciliary activity.

A brief description of the methods used to obtain ciliated samples is followed by details of the effects of different environments on ciliary beat frequency.

3.3 Methods:

3.3.1 Preparation of ciliated brain ependymal samples:

3.3.1.1 Brushings of ciliated ependyma: The brains of (9-15 day old) Wistar rats were removed following sacrifice. Dissection took place immediately after sacrifice and exposed surfaces of the lateral and fourth ventricles were brushed lightly using a bronchoscopy brush. While this is the most successful method of obtaining ciliated respiratory tissue, ependymal cilia were only seen on two occasions out of the 22 rats studied. Only isolated small clumps of cilia were observed.

Scanning electron microscopy was performed, to confirm the ventricular surfaces were ciliated. A representative scan showing the dense ciliary covering of the ependyma is shown in figure 3.a.

Following these experiments an attempt was made to preserve an edge of ciliated ependymal tissue by cutting thin slices of brain adjacent to the lateral and 4th ventricles, using a scalpel. Of the various slices studied from the lateral and fourth ventricle of 8 rats, cilia were seen to beat on 4 brain slices, three of which were from the fourth ventricle.

Figure 3.a: *Scanning electron microscopic picture of the floor of the fourth ventricle of a 15 day old Wistar rat. Details of the preparation of this sample for electronmicroscopy are given in Chapter 4.*

3.3.1.2 Preparation of brain ependymal slices using a vibratome: To decrease trauma to the fragile ependyma caused by manual slicing, a vibratome was used to cut brain slices. Ependymal samples from the fourth ventricle of 10-15 day old rats were chosen for the majority of studies in this thesis. The ventricular system of the rat is shown diagramatically in figure 3.b. Rats were sacrificed by cervical dislocation. The brain was immediately removed and stored in 4 ml of medium 199 with Earl's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) at 4°C until use. The brainstem and cerebellum were separated from the cerebral hemispheres and blood vessels and meninges removed by careful dissection.

The brainstem was mounted, aqueduct side down, on the cutting stage of a Vibroslice (Model 752: Campden instrument's Ltd) using superglue as an adhesive (figure3.c). The bath surrounding the brain stem was filled with medium 199 and cooled to 4°C using a Peltier driven cooling device. Transverse slices of brain, 150um thick, were cut and stored at 4°C. All experiments were conducted on the same day. For each piece of tissue associated with the fourth ventricle two to four ciliated brain slices with intact ependymal edges were obtained. Ciliated ependyma which had obviously been disrupted or damaged was not used.

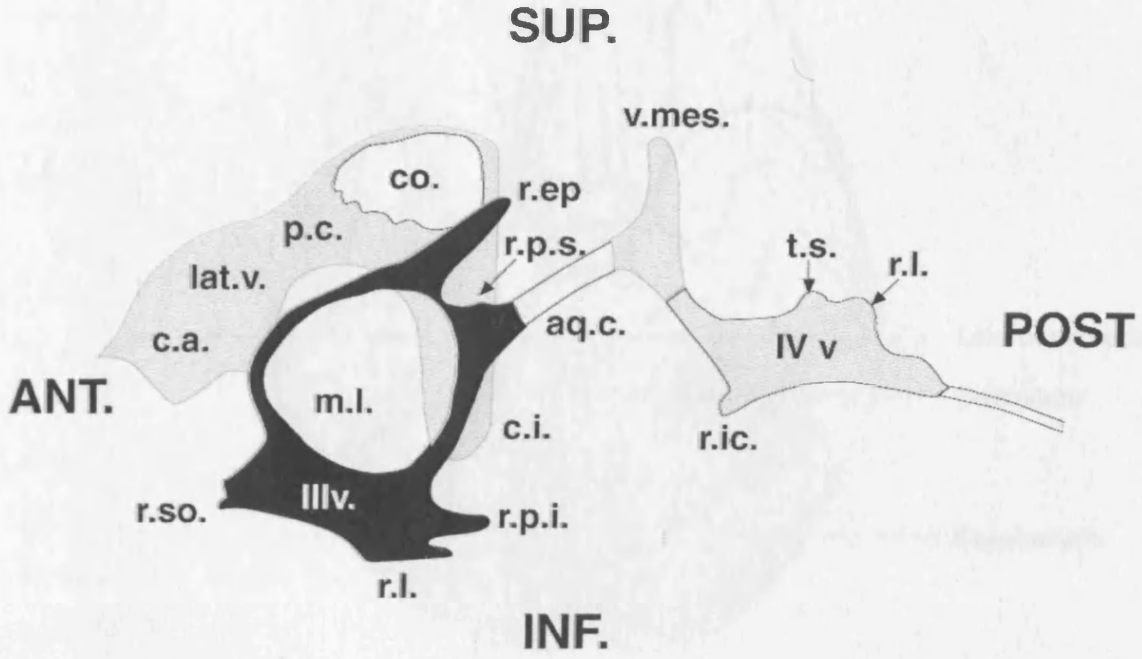


Figure 3.b:

Ventricular system of the rat.; Black, IIIrd ventricle; white, cerebral aqueduct; ant., anterior; aq.c., aqueductus cerebri; c.a., cornu anterius; c.c., canalis centralis; c.i., cornu inferius; ch., chiasma opticum; co., coarctatio ventriculi; f.i.v., foramen interventriculare; inf., inferior; lat., lateral; lat. v., ventriculus lateralis; m.i., massa intermedia; p.c. pars centralis; post., posterior; r.ep., recessus epiphysialis; r.i., recessus infundibularis. r.ic., recessus incertus; r.l., recessus lateralis; r.p.i. recessus posterior inferior; r.p.m., recessus posterior medius; r.p.s., recessus posterior superior; r.so., recessus supraopticus; r.s., recessus superior; sup., superior; v.bul.olf., ventriculus bulbi olfactorii; v.mes., ventriculus mesencephali; III v., ventriculus tertius; IV v., ventriculus quartus (Westergaard, 1969).

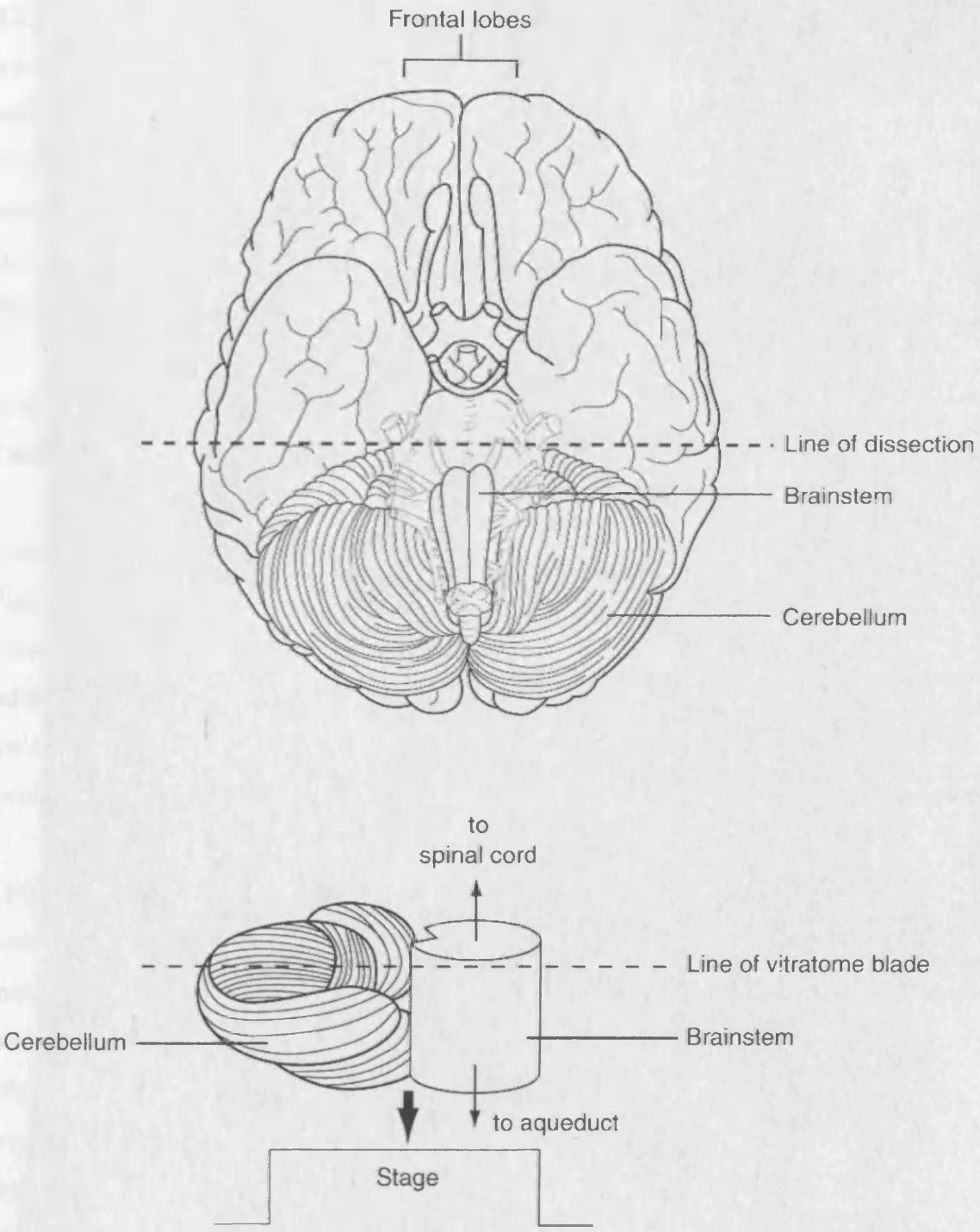


Figure 3.c

These figures show the line of initial dissection of the brain and the orientation of the tissue prepared for vibratome slicing.

3.3.2 Measurement of ciliary beat frequency: All measurements of ependymal ciliary beat frequency for this study were made using a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) recording at a rate of 400 frames per second. The camera allowed video sequences to be downloaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study, ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100um were studied.

3.3.3 The effect of the immediate environment of ciliated brain slices on the measurement of ciliary beat frequency:

During the course of this thesis I developed or adapted a number of techniques to maintain ciliated strips of ependyma in a fluid medium to allow repeated estimation of ciliary beat frequency. It became clear that the method used to maintain ependymal cilia in a fluid medium had a major effect on ciliary beat frequency. The methods used are described below.
The aim of this study was to determine the effect of the various techniques of holding ciliated ependyma in a fluid medium on beat frequency measurements.

3.3.3.1 The hanging drop technique: Measurements of respiratory ciliary beat frequency are commonly made on small strips of ciliated nasal epithelium obtained from nasal brushings. An open sided chamber was made, the sides of which were made of cover slips (400μm thick) mounted on a microscope slide(figure 3.b). The samples were placed with in a droplet of cell culture medium on the microscope slide between the two. A cover slip is then placed over the drop and the ciliated tissue held in the chamber. The same technique was used to mount ciliated brain slices. The slide containing the brain slice was mounted on a heated microscope stage which maintained the fluid content of the slide at 37°C. The temperature of the microscope slide was measured continually using a digital thermometer with its probe attached to the microscope slide in the vicinity of the ciliated ependyma.
Initially the cilia were observed continually via a x100 interference contrast oil immersion lens and readings taken at regular intervals. There was rapid ciliary slowing with persistent direct contact of the microscope lens. The study was, therefore, repeated with the microscope lens in contact and focused on the tissue for the time taken to perform a reading.

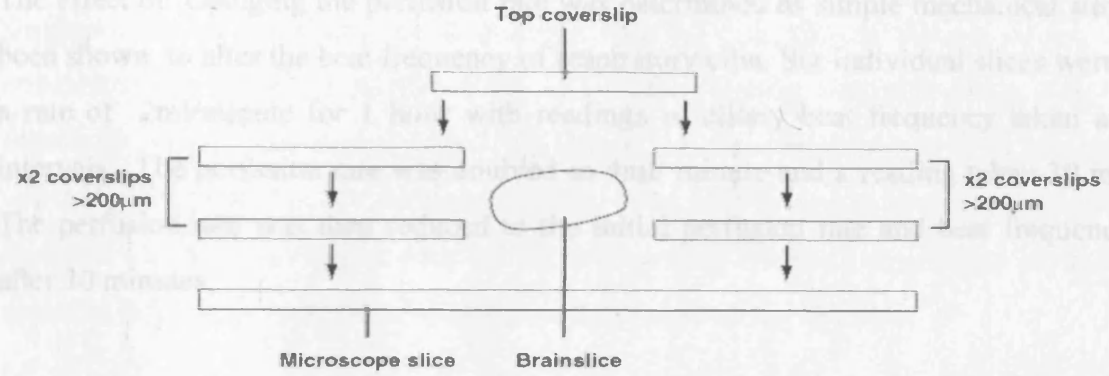


Figure 3.d: *Microscope slide, cover slip chamber.*

This was less than 30 seconds. Using this method readings were made 5 minutes after the temperature had stabilised at 37°C, again after 15 minutes and then at 30 minute intervals for up to two and a quarter hours.

3.3.3.2 The perfusion system: To facilitate studies on the effect of various pharmacological stimuli on ependymal ciliary beat frequency a perfusion system was developed. This was based on a system described by Forsythe (1991) which allows simultaneous perfusion with up to 3 solutions, at 37°C, whilst the tissue is continually observed by a x50, water immersion, microscope lens (figure 3.e). The high speed video camera was attached to the microscope to allow readings to be taken.

Experiments were undertaken to determine the time taken for a study solution to washout the existing medium and achieve a constant concentration within the chamber (volume 5ml). A solution of salbutamol was chosen as the perfusate as experiments on its effects on ependymal tissue were planned.

The perfusate was heated to 37°C to simulate experimental conditions and the peristaltic pump set to deliver a flow of 2ml/min to the perfusion chamber. Medium 199 was perfused for 15 minutes and then exchanged for a solution of salbutamol (25μg/ml). Samples (0.1ml) were collected from the area where the beating cilia are observed and in the region where the solution was being sucked via a needle to a waste container. Samples were taken at 1 minute intervals for 16 minutes. Salbutamol levels were determined by HPLC. Details of the assay are given in Appendix 1 .

The effect of changing the perfusion rate was determined as simple mechanical stimulation has been shown to alter the beat frequency of respiratory cilia. Six individual slices were perfused at a rate of 2ml/minute for 1 hour with readings of ciliary beat frequency taken at 30 minute intervals. The perfusion rate was doubled to 4ml/ minute and a reading taken 30 minutes later. The perfusion rate was then reduced to the initial perfusion rate and beat frequency measured after 30 minutes.

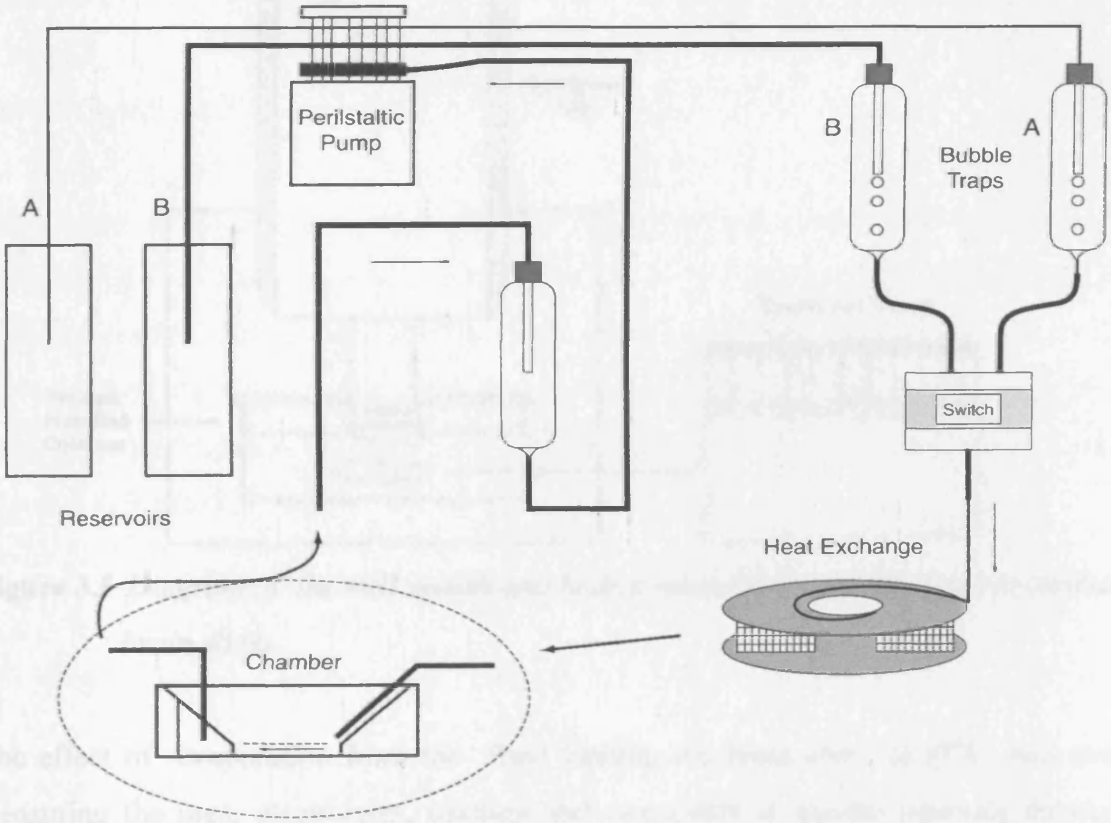


Figure 3.e: *The Forsythe perfusion chamber.*

3.3.3.3 Incubation chamber: An incubation system was developed to allow ciliated brain slices to be observed at a temperature of 37^0C for prolonged periods. It consisted of a large perspex chamber containing the microscope with the exception of the eye pieces, video camera attachment and light source (figure 3.f). A heating system was built using a coil from a hair dryer and a feed back control system developed such that a probe within the incubation system controlled the preset temperature at a constant level. The temperature of the fluid bathing ciliated samples was continually recorded by a thermocouple attached to a digital thermometer. The chamber holding the brain slice was filled with 4ml of fluid medium.

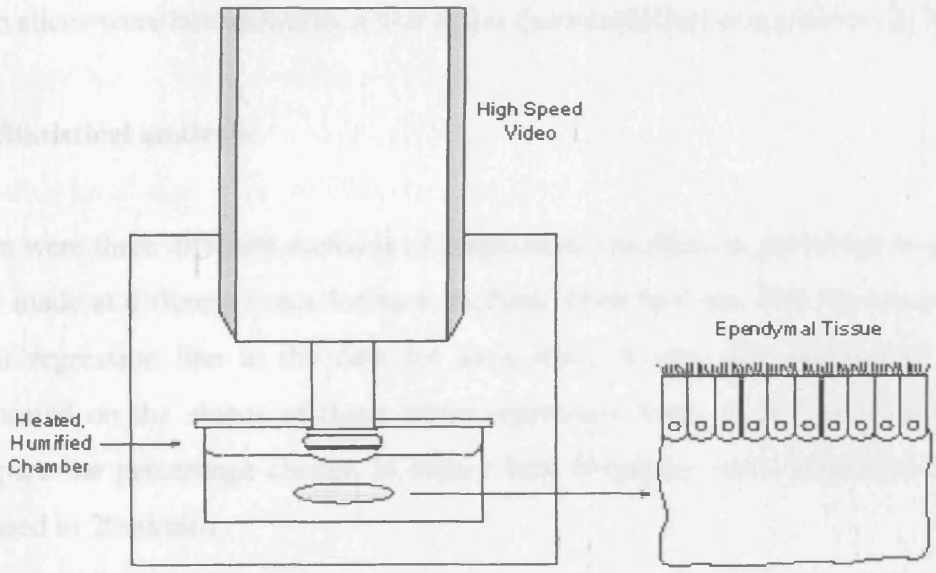


Figure 3.f *Diagram of the well system and heated humidified chamber used to incubate brain slices.*

The effect of evaporation from the fluid bathing the brain slice, at 37^0C, was evaluated by measuring the urea, electrolytes, calcium and osmolality at regular intervals during a 4 hour period. In an attempt to prevent evaporation a humidifier was used to introduce heated water vapour into the incubation chamber to give a humidity reading of 80%. To determine if this reduced evaporative losses, measurements of urea, electrolytes, calcium and osmolality were measured after a 4 hour period with the bathing fluid maintained at 37^0C.

A water immersion x50 lens was used to image ependymal cilia. As with the hanging drop technique, leaving the lens focused on the ependymal edge caused ciliary slowing with in a very

short period of time. Because of rapid ciliary slowing with persistent direct contact of the microscope lens the study was repeated with the microscope lens only focused on the tissue for the time taken to perform a reading which was less than 30 seconds.
Using this method readings were made 5 minutes after the temperature has stabilised at 37°C, again after 15 minutes and then at 30 minute intervals for two and a quarter hours.

In an attempt to achieve an experimental set up whereby cilia could be observed continually for long periods of time a second incubation system was constructed around a Lietz inverted microscope. With this system cilia were viewed through the base of the chamber holding the brain slice using a X50 objective. No contact occurred between the microscope and brain slice. Brian slices were held down by a fine nylon thread attached to a platinum C ring.

3.4 Statistical analysis:

There were three different methods of preparation: incubation, perfusion or slide. Measurements were made at different times for each method. Therefore, the data was summarised by fitting a linear regression line to the data for each slice. A one way analysis of variance was then performed on the slopes of these linear regression lines. A Wilcoxon test was performed to compare the percentage change in ciliary beat frequency when measured at a flow of 10 as opposed to 20ml/min.

3.5 Results:

The results from each slice studied with each method are shown graphically in figure 3e-3h.
There were striking differences in beat frequency when measurements were made using different methods. Mean (SD) ciliary beat frequency measured using the incubation system (36.5(3.6) Hz) was significantly (P=0.0001) greater than measurements obtained using the perfusion (19.9(3.7)Hz) or microscope slide method (17(3.6)Hz).

Ciliary stasis occurred within minutes using the microscope slide and the incubation methods when the microscope was kept continually in focus. However, use of the perfusion system,

where the sample was kept under continual focus, or use of the incubation system with an inverted microscope (35.2 (3.3) Hz) were not associated with a decrease in ciliary beat frequency over a three hour period.

To determine the effect of incubation time on ciliary beat frequency, when the different methods were used, a one way analysis of variance was performed to compare the mean slopes for the three methods (microscope slide: incubation chamber using the water immersion lens: and perfusion system). For the first two methods the lens was only kept in focus for sufficient time to perform a reading, whereas for the perfusion method cilia were continually observed. Bartlett's test of variance homogeneity was not significant (P=0.68). There were significant differences between the mean slopes in the 3 groups (P=0.03). Every pair of group mean slopes were compared and the Bonferroni procedure used to adjust the P values to take account of the multiple comparisons. The mean slope in the microscope slide group was significantly lower than the mean slope in the incubation group and perfusion group (P=0.04).

Using the perfusion chamber it was found that increasing the flow of fluid through the chamber from 2 to 4ml per minute caused a 17% (range -2 to 31%) increase in ciliary beat frequency (P=0.01).

Figures 3.e - h:

The effect of different experimental systems on ependymal ciliary beat frequency. Each point represents the mean of 4 readings of ciliary beat frequency from an ependymal edge.

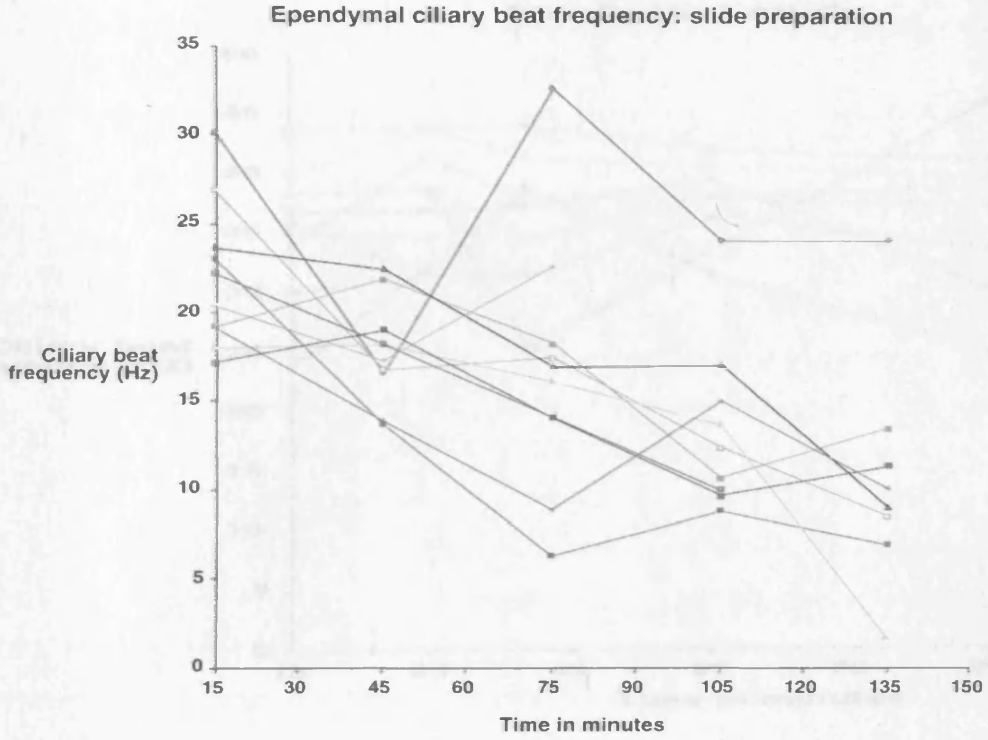


Figure 3.e:

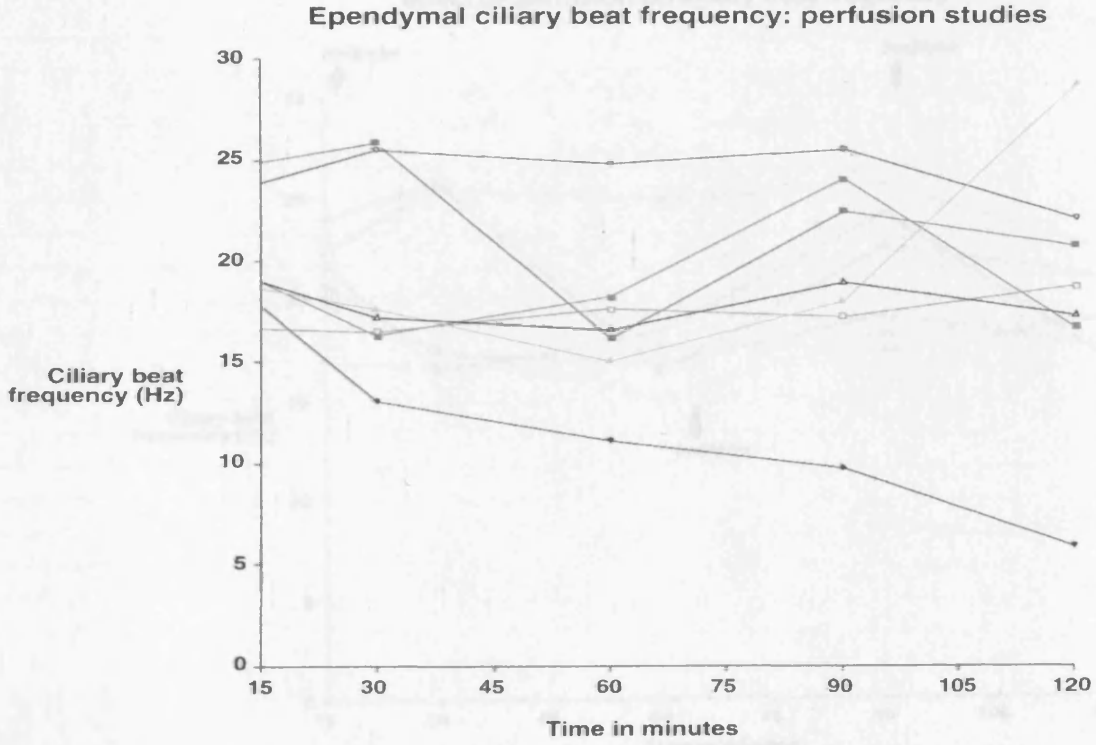


Figure 3.f:

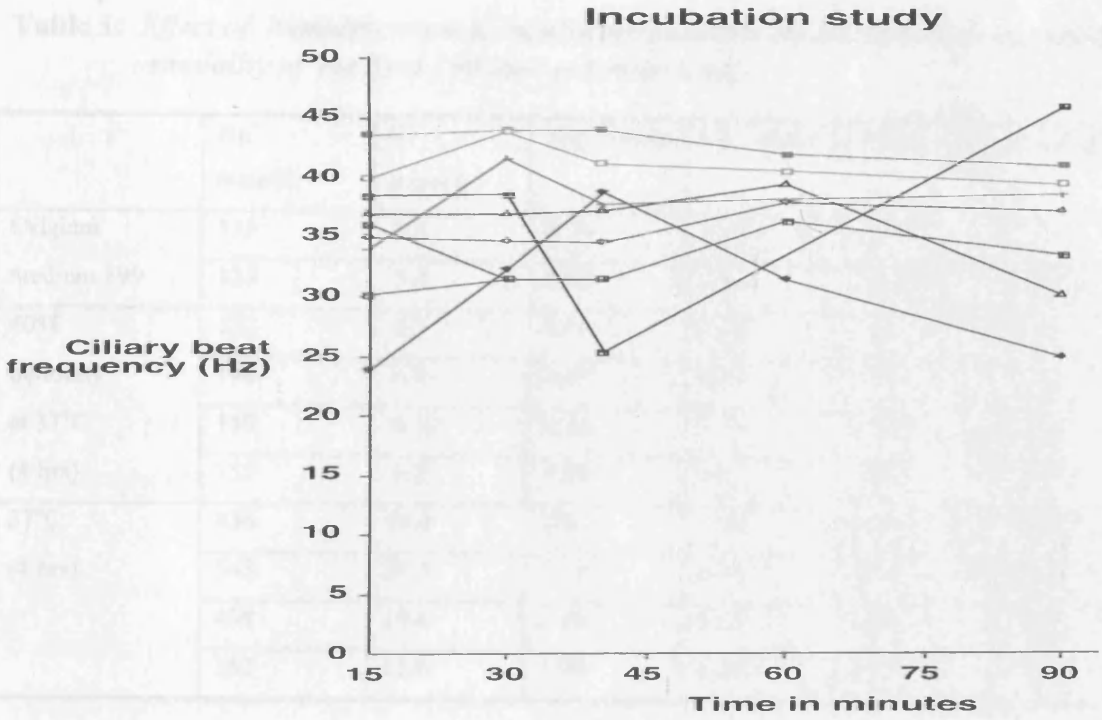


Figure 3.g

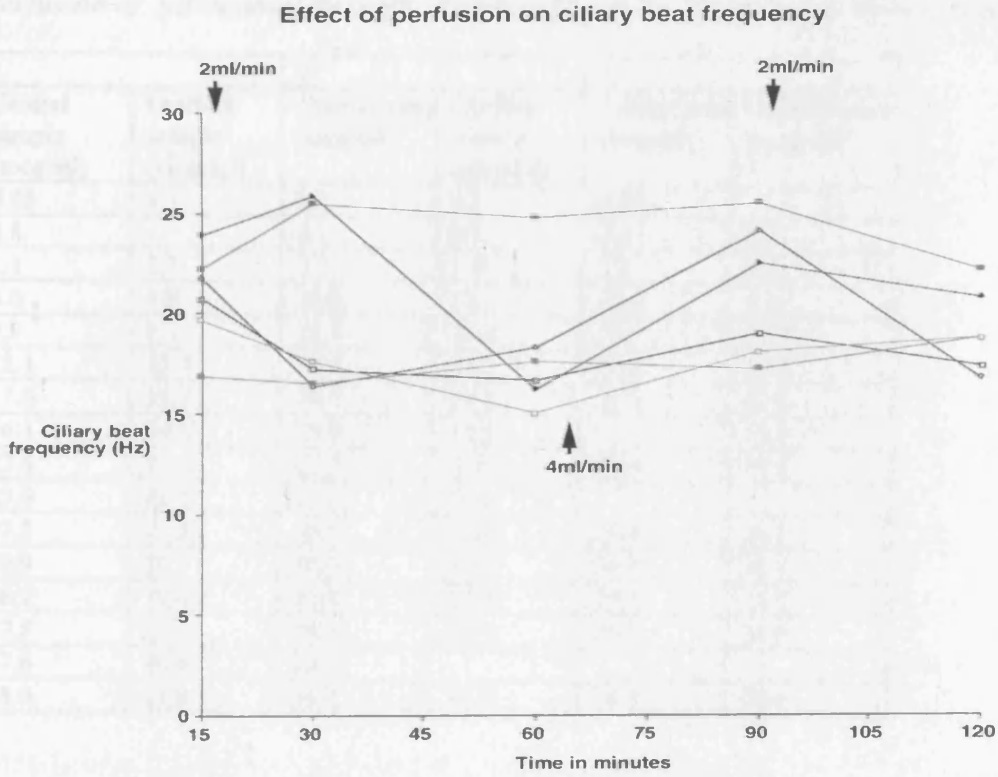


Figure 3.h:

Table 1: *Effect of humidification of incubation chamber on electrolyte concentration and osmolality of medium 199 over a 4 hour study.*

	Na^+ mmol/l	K^+ mmol/l	Mg^{++} mmol/l	Ca^{++} mmol/l	Glucose mm	Osmolality mos
Original	133	5.3	0.76	1.83	5.7	281
medium 199	133	5.4	0.69	1.84	5.8	278
80%	152	6.2	0.87	2.18	6.7	319
humidity	148	6.0	0.85	2.09	6.1	312
at 37°C	149	6.12	0.83	2.14	5.7	316
(4 hrs)	152	6.2	0.89	2.2	6.6	319
37°C	436	19.4	2.6	5.68	19.6	944
(4 hrs)	543	24.5	3.10	6.14	24.1	1169
	438	19.6	2.19	5.65	19.5	948
	352	15.6	1.90	4.89	15.9	772

Table 2: *Perfusion of salbutamol through the chamber of the Forsythe perfusion chamber.*

	1		2		3	
Time (mins)	Central sample (mcg/ml)	Outflow sample (mcg/ml)	Central samp (mcg/ml)	Outflow sample (mcg/ml)	Central sam (mcg/ml)	Outflow samp (mcg/ml)
1	0.05	1.1	0.2	0.1	0.08	
2	0.8	-	0.1	0.3	0.2	0.4
3	1.1	1.7	3.7	1.0	3.3	1.8
4	6.0	4.8	16.0	7.2	10.9	7.4
5	9.9	9.1	21.3	14.4	19.1	15.7
6	12.1	12.4	22.6	29.5	22.4	19.8
7	11.0	13.2	26.7	28.1	24.7	29.3
8	16.1	14.7	25.9	24.5		23.8
9	22.6	21.1	23.5	25.5	26.3	17.2
10	23.0	23.1	27.2	26.1		
11	27.2	25.5	26.7	25.6	26.4	26.1
12	26.9	26.1	26.0	25.9	28.6	26.1
13	26.7	30.6	25.8	27.2	25.3	25.1
14	27.8	25.5	26.4	26.9	26.5	25.0
15	27.6	26.4	26.5	-		
16	28.0	25.0	36.3	27.3	26.3	25.9

Incubation of ependymal slices at 37°C, without added humidification, resulted in marked evaporation. Table 1 shows the effects of evaporation on the electrolytes, glucose and

osmolality of the bathing solution. Addition of humidified air (80%) markedly reduced evaporation over a 4 hour study period. The osmolality increased to a maximum of 319 mosmol/K, compared to 543 mosmol/K, without humidification.

Perfusion of the perfusion system chamber with salbutamol resulted in a stable concentration of perfusate with in 11 minutes (see table 2).

3.6 Discussion:

The method by which cilia were incubated in cell culture fluid had a marked effect on measurements of ciliary beat frequency.
Ciliary stasis occurred within minutes using the microscope slide method when the microscope was kept in focus. This suggests that either cellular damage may have occurred due to a direct pressure effect or ciliary slowing was secondary to release of toxins from the brain slice.
When intermittent readings were made with the microscope slide in contact for less than 30 seconds, ciliary slowing occurred gradually over the 60 minute study period. Again this may have been due to a pressure effect or the slower release of toxic agents from the surrounding neuronal tissue of the brain slice.

A perfusion system was established in an attempt to overcome problems of ciliary slowing and to allow the effects of various pharmacological perfusates on ependymal ciliary beat frequency to be determined. Cilia studied in this system continued to beat at a constant rate for the 3 hour study period despite the constant pressure of the microscope lens. The frequency of ciliary beat was similar to the initial readings of beat frequency obtained using the microscope slide method. It is possible that the pressure exerted on the brain slice by the microscope lens of the perfusion system was not as great as that exerted by the microscope slide system. At this stage, however, we thought it more likely that ependymal ciliary function was being preserved because toxic substances were being continually washed away from the ciliated surface.
The importance of a pressure effect from the microscope lens however, was confirmed when ependymal cilia were observed continuously under a water immersion lens in a 4ml incubation well. Ciliary beat frequency decreased rapidly over a few minutes. However, when

measurements were made intermittently with the microscope in focus for less than 30 seconds ciliary beat frequency remained constant during the three hour study period. This suggested that release of a toxic material from a brain slice or cellular damage due to prolonged pressure may have been responsible for ciliary slowing.

The importance of pressure in producing ciliary slowing was emphasised when an inverted microscope was used to view brain slices in the incubation chamber. Using this system there is no pressure exerted by the microscope lens. The volume of the chamber was identical to that used for the water immersion lens experiment. No decrease in ciliary beat frequency occurred during a three hour period despite continuous observation.

It is possible that a toxic substance is released or that intracellular damage is caused by the pressure of the microscope lens which results in ciliary slowing. The presence of a toxic substance is given support by the fact that cilia beat at a constant frequency in the perfusion system, over a 3 hour period, despite the continual pressure of the focused microscope lens. The perfusion system would continually wash away toxic substances generated from the ependymal cells or the neuronal tissue of the brain slice.

Equally difficult to explain is the very large difference in ciliary beat frequency measurements depending on the method used. The microscope slide method and the perfusion method resulted in beat frequencies which were, at their fastest, half of those when the incubation system was used. Mechanical stimulation has been shown to increase ciliary beat frequency and appeared to be a possible explanation. However, this does not explain the presence of similarly high ciliary beat frequencies when measurements were made using an inverted microscope.Using this method there was no external stimulation. In support of these findings Roth and colleagues (1985), using an inverted microscope, found ependymal cilia beat considerably faster than respiratory cilia.

It is possible that the immediate surrounding of the ependymal cilia may behave differently depending on their local environment. For example, under the pressure of a lens or microscope slide it is conceivable that viscous or surface acting forces may exert an effect making it physically more difficult for the cilia to beat quickly.

Based on these experiments we chose the humidified incubation system and inverted microscope for the studies described in this thesis.

Chapter 4

Respiratory and Brain ependymal ciliary function

4.1 Summary:

Respiratory cilia from newborn humans beat slightly faster than those from adults. Our initial observations showed ependymal cilia were beating at rates far in excess of those recorded in the literature for respiratory cilia.

The aims of this study were:

- to compare beat frequencies of tracheal and ependymal cilia;
- to compare the beat frequencies of ependymal cilia from infant and adult rats;
- to determine the length of respiratory and ependymal cilia of infant and adult rats.

We used our ex-vivo model which allows ependymal ciliary beat frequency to be measured using a high speed video system. The beat frequencies of cilia, incubated at 37°C, were measured after an incubation period of 30 minutes.

Ependymal cilia beat at a similar frequency in 10-15 day old rats (mean 38.8Hz: 95% confidence intervals 37.1 - 40.6) as in adult animals (mean 40.7Hz: 95% confidence intervals 38.5 - 42.9). Respiratory cilia from adult animals, however, beat (mean 20.9Hz: 95% confidence intervals 14-27) at a significantly (P=0.003) lower frequency than ependymal cilia.

Ependymal cilia (mean length (SD): 8.19 (0.3)μm) measured by scanning electron microscopy were significantly (P=0.001) longer than respiratory cilia (mean length (SD): 5.53(0.6)μm) from the trachea of 9-15 day old rats. Cilia did not grow in length between 9-15 day old and adulthood. Adult respiratory and ependymal ciliary length (mean(SD)) were 5.62(0.5)μm and 8.12 (0.2)μm respectively.

4.2 Introduction:

As part of our initial investigations we were keen to know whether ependymal ciliary beat frequency and length changed from infancy to adulthood. It was also important to determine whether data from numerous experiments on respiratory cilia could be directly extrapolated to effects on ependymal cilia.

Ependymal cilia are water propelling as opposed to the mucus propelling cilia of the respiratory tract and may have different characteristics. Indeed, one previous study has suggested ependymal cilia beat more quickly than respiratory cilia (Roth et al, 1985).

The aim of this study was to determine whether ependymal cilia from very young, 10-15 day old, rats beat at the same frequency as those from adults and whether respiratory cilia beat at the same frequency as brain cilia. The secondary aim was to determine the relative lengths of ependymal and respiratory cilia.

4.3 Methods:

4.3.1 Sample preparation: The brains of Wistar rats (9-17 days of age) were dissected following sacrifice. Brain slices were prepared from the floor of the 4th ventricle of Wistar rats (9-17 days of age) immediately after sacrifice and mounted in a well containing 4 ml of medium 199 with Earl's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml). Ciliary movement was observed at a magnification of X50 using an inverted microscope. Samples were enclosed in a purpose designed environmental chamber which maintained the solution at 37°C, and the surrounding air at a humidity of 80% to minimise evaporation. Tracheal rings of 1mm thickness were prepared by careful dissection from eight of the eleven adult rats whose ependymal ciliary frequency was measured. Tracheal rings were mounted and observed in a similar fashion to ependymal strips.

4.3.2 Measurement of ciliary beat frequency: Beating cilia on ependymal strips were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 400 frames per second. The camera allowed video sequences to be down loaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each measurement time ciliary beat frequency was made from 4 different areas along each ependymal or tracheal edge.
Both respiratory and brain ciliary measurements were made after 30 minutes incubation at 37°C.

Thirty two ependymal edges were studied from a total of 11 adult rats. Four readings of ciliary beat frequency were made on each edge (total number of readings 128). Eight of these rats also had successful measurement of tracheal ciliary beat frequency (total number of readings 29). Twenty five 10-15 day old rats had brain ciliary measurements (total number of readings 100).

4.3.3 Measurement of ciliary length:
Ependymal ciliary length of the adult and 10-15 day old rats was measured from the video screen which had been calibrated using a graticule slide. In total the lengths of 34 cilia from 6 adults and 27 cilia from 7 rats 10 - 14 days old were measured.

We were keen to determine if respiratory cilia from the rat were the same length as ependymal cilia. Great difficulty was encountered in finding respiratory cilia to measure from the trachea of 9-15 day old rats using light microscopy and measurements from the video screen. Scanning electron microscopy was therefore used to obtain images from which measurement of tracheal cilia could be made. To allow comparrison using similar methods, measurements of the length of ependymal cilia from 9-14 day old rats were also made using images provided by scanning electron microscopy.

4.3.4 Scanning electron microscopy measurement of cilial length:
The brain and tracheal samples were fixed in 2.5% phosphate buffered gluteraldehyde and rinsed in fresh buffer prior to being post fixed in 1% osmium tetroxide (OsO_4). The rinsed samples were then dehydrated through graded ethanol and infiltrated and immersed in hexamethyldislazane (HMDS). The HMDS was then allowed to evaporate, reproducing the effect of critical point drying , allowing the tissue to dry to air thus avoiding phase boundary

damage. HMDS was chosen because critical point drying involves high rates of flow of liquid CO_2 which would damage the thin and fragile brain samples. The dried samples were fixed to aluminium stubs and sputter coated with gold prior to examination in the scanning electron microscope.

Ten fields from different cells bearing mature cilia were selected at random. Ten cilia from each field were measured using a computerised image analysis system. The brain and trachea from 5 different 9-15 day old and 4 adult (4-6 months) rats were studied. The system was calibrated in microns by means of the scanning electron microscopes internal standard and the calibration bar marked upon each image.

Representative electron micrographs of respiratory and ependymal tissue are shown in figures 4.a - d.

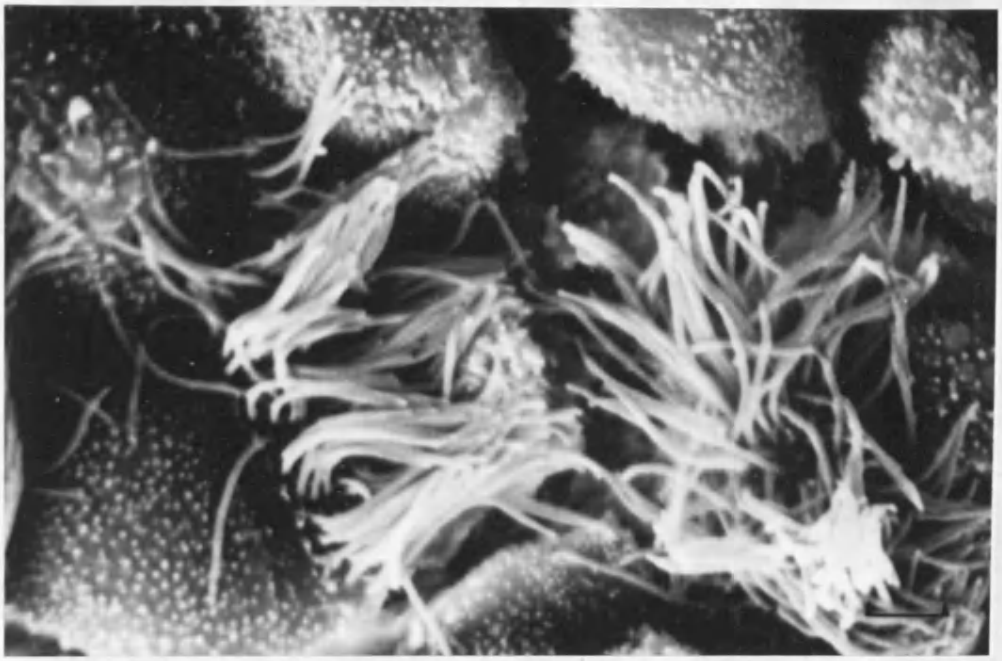


Figure 4.a: *Scanning electronmicrograph of cilia lining the trachea of a 12 day old Wistar rat. The 1cm bar on the photograph is equivalent to a length of 1.6μm*

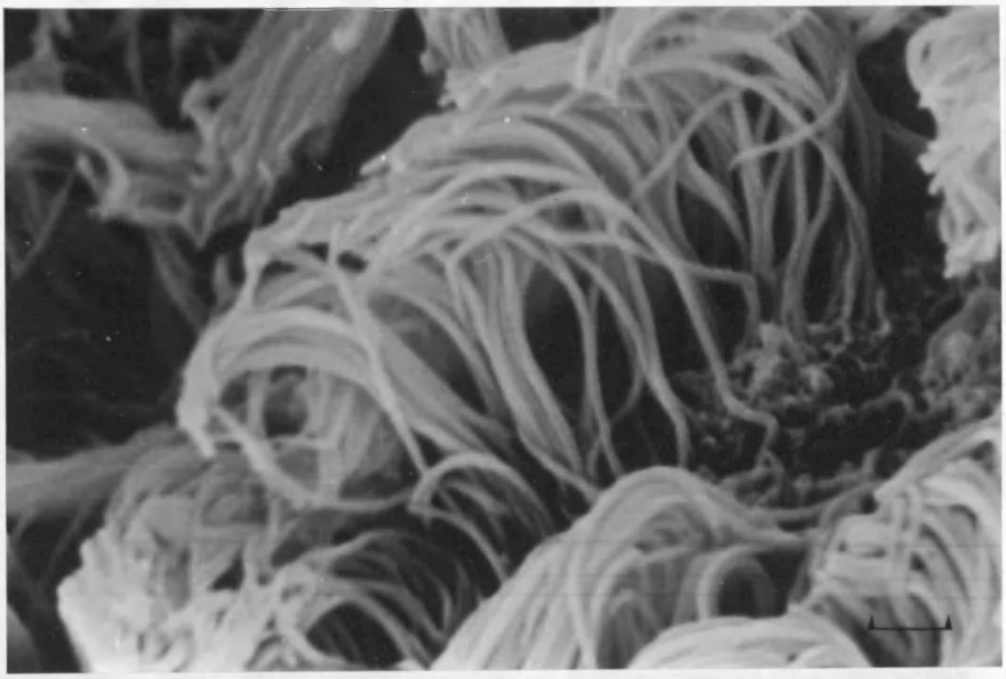


Figure 4.b: *Scanning electronmicrograph of the ciliated ependyma lining the floor of the 4th ventricle of a 12 day old Wistar rat. The 1cm bar on the photograph is equivalent to a length of 1.28μm.*

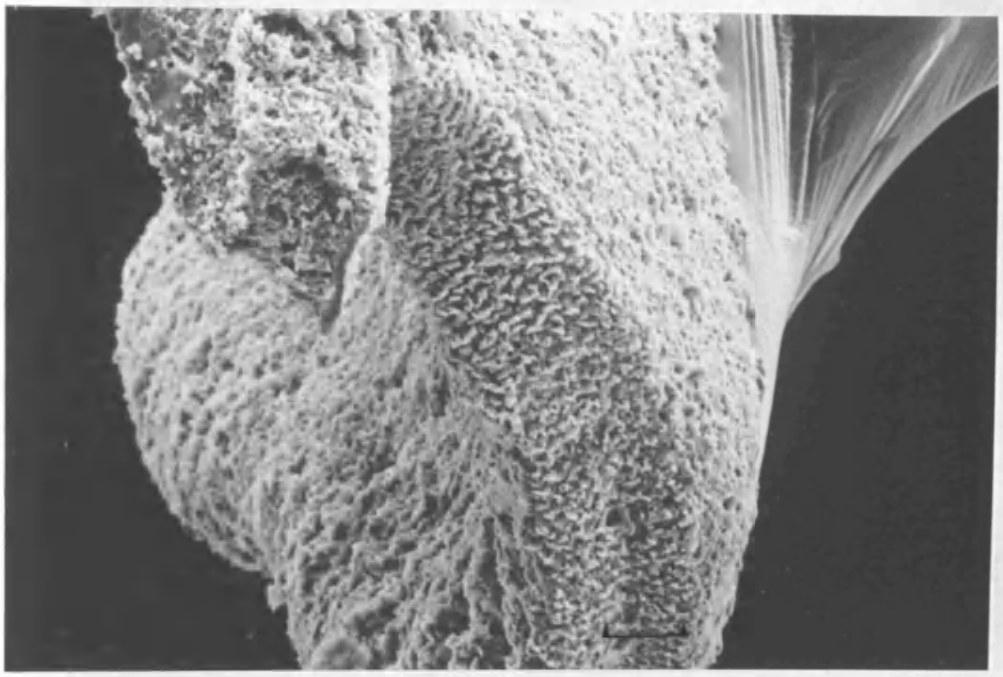


Figure 4.c: *This is a scanning electronmicrograph showing a densely ciliated ependymal edge. The edge curves round and has been fractured revealing the thin ependymal layer. This edge is representative of the ciliated edges from which beat frequency readings were made. The 1cm bar on the photograph is equivalent to a length of 50μm.*

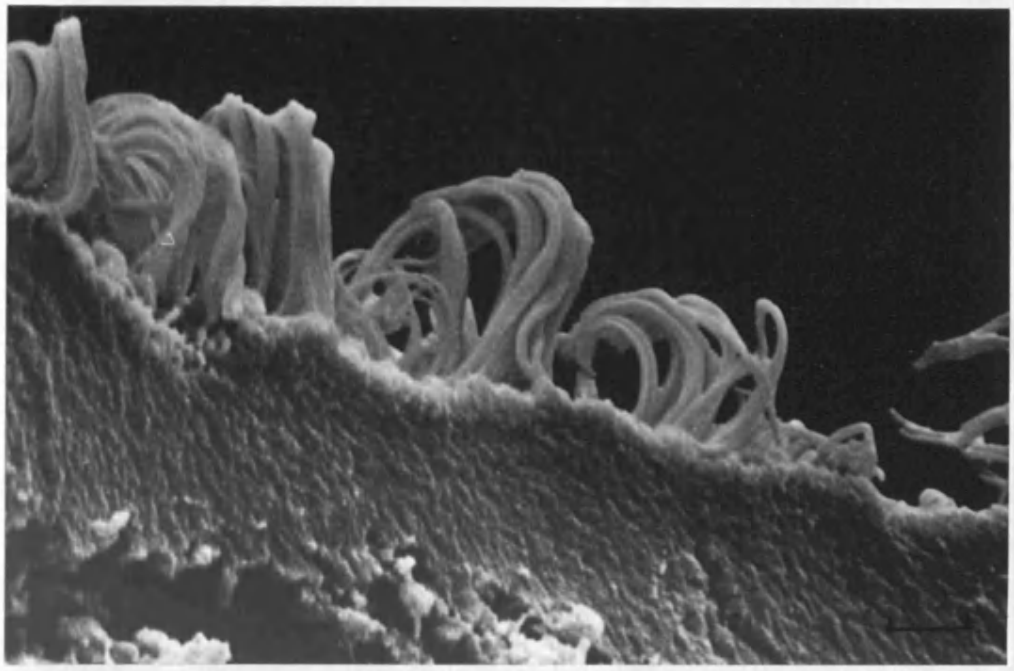


Figure 4.b: *Scanning electronmicrograph of the ciliated ependyma from the floor of the 4th ventricle of a 12 day old Wistar rat. The 1cm bar on the photograph is equivalent to a length of 2.9μm. The ciliated ependymal layer is clearly seen. The ependymal layer is one cell thick.*

4.4 Results:

The mean ciliary beat frequencies of the brain ependymal edges and tracheal respiratory samples are shown graphically in figure 1.

A paired t test was used to compare adult brain and respiratory ciliary beat frequency. The adult respiratory cilia had a significantly (P=0.003) lower mean ciliary beat frequency (mean 20.9 Hz: 95% confidence intervals 14-27) than adult ependymal cilia (mean 40.7Hz: 95% confidence intervals 38.5 - 42.9). A two sample t test was performed to compare adult and neonatal ependymal ciliary beat frequencies and cilial lengths. There was no significant difference between the ependymal ciliary beat frequencies (P=0.19) of adult (mean 40.7: 95% confidence intervals 38.5 - 42.9) and 10-14 day old rats (mean 38.8: 95% confidence intervals 37.1 - 40.6). There was no significant difference in the mean length of ependymal cilia (SD) from 10-14 day old rats (10.0 (0.8) μm) and those from adult rats (9.6 (0.8)μm) measured using the graticule system.

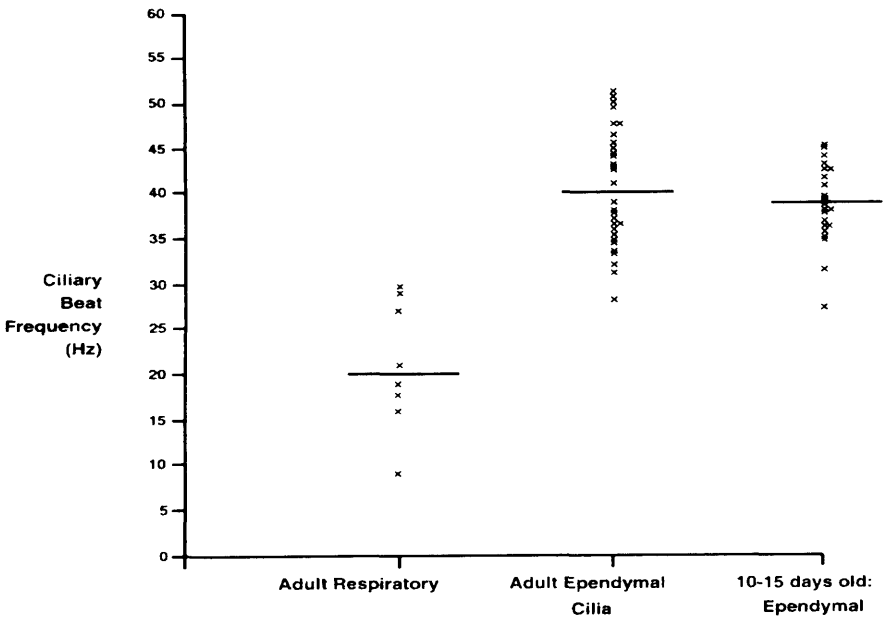


Figure 4.1:*Each data point on this graph represents the mean beat frequency of an individual ependymal or respiratory ciliated edge. Respiratory cilia beat considerably more slowly than ependymal cilia.*

Ependymal cilia (mean length (SD): 8.19 (0.3)μm), measured by scanning electron microscopy, were significantly (P=0.001) longer than respiratory cilia (mean length (SD): 5.53(0.6)μm) from the trachea of 9-15 day old rats. Respiratory and ependymal cilia did not increase in length with age. Mean (SD) adult respiratory and ependymal ciliary lengths were 5.6(0.5)μm and 8.12(0.2)μm respectively when measured using scanning electronmicroscopy.

4.5 Discussion:

We have shown that brain ependymal cilia from young rats beat at a similar frequency to those of adult rats and that no increase in cilial length occurred with growth to adulthood. However, respiratory cilia beat at a significantly slower rate than ependymal cilia confirming an earlier study by Roth et al (1985). Respiratory cilia for beat frequency analysis were taken from adult rats because of technical difficulties in the preparation of cilia from the trachea of young rats. Using scanning electron microscopy we subsequently demonstrated very poor ciliation of tracheas from 9-15 day old rats.

Measurements from the scanning electron micrographs revealed respiratory cilia to be significantly shorter than ependymal cilia. The length of respiratory and brain cilia did not change from infancy to adulthood. Initial measurements of ciliary length were made using light microscopy. Use of this method resulted in measurements of ciliary length greater than when measurements were made using scanning electronmicroscopy. The apparent enlargement of cilia when viewed by light microscopy may have been due to magnification by the coverslip and the fluid surrounding the cilia.

We have previously shown that respiratory cilia taken from human infants, within 2 days of birth, beat more quickly than those from healthy adults (O'Callaghan, 1991). Failure to find a similar difference between ependymal ciliary frequency between young and old rats may have been due to sampling at a later stage, 10 - 15 days after birth.

The mechanism whereby ependymal cilia beat at almost twice the frequency of respiratory cilia is being investigated. Caution must be observed when extrapolating results from experiments on respiratory cilia to ependymal cilia.

Chapter 5:

The effect of pH on brain ependymal ciliary function

5.1 Summary:

The pH of the cerebrospinal fluid is carefully maintained at a level just below that of the blood. Significant acidosis of the CSF is very strongly related to abnormal neurological function.

The aim of our study was to determine the effect of solutions of different pH on ependymal ciliary function.

We used our ex-vivo model using ependymal tissue from the fourth ventricle of Wistar rats, which allows ependymal ciliary beat frequency to be measured by high speed videophotography. Ependymal samples were incubated at 37°C in medium 199 at a pH of 7.4 for 30 minutes. The bathing solution was exchanged for one of study solutions which ranged from pH 6.0 to pH 8.0 and ciliary beat frequency measured at regular intervals. The effect of altering the external pH on intra cellular pH of ependymal cells was assessed by ratio measurements of the pH sensitive flurophore dye BCECF. To confirm if changes in ciliary activity were directly due to changes in intracellular pH, ciliary beat frequency was determined after incubation with a solution of pH 6.5. The intracellular pH was then rapidly increased by the addition of ammonium chloride whilst the extra cellular pH was maintained at 6.5.

Ependymal ciliary beat frequency at pH 7.4 did not change significantly during the experiment (mean (SD) initial beat frequency 33.7(3.4)Hz: beat frequency at 2 hours 36.6 (5)Hz). Bathing ependymal cilia in an acidic environment caused a gradual slowing of ciliary beat frequency ($p=0.01$). Cilia, beating at a frequency of 33(1.8) Hz, exposed to a pH of 6.0 became static within 15 minutes. Beat frequency returned towards normal (33.8(2)Hz) when the bathing solution was exchanged back to pH 7.4.

Intracellular pH was similar but slightly less than extracellular pH throughout the pH range studied. Rapid restoration of intracellular pH, by ammonium chloride, following incubation with a solution of pH 6.5 resulted in a rapid increase in ciliary beat frequency (from 21.2(4)Hz to 35.6(3.3)Hz).

We have shown acidic extracellular conditions cause depression of ependymal ciliary beat frequency which is directly related to parallel changes in intracellular pH.

5.2 Introduction:

Little information is available on the effect of important physiological parameters, such as pH, on ependymal ciliary function. While most studies of respiratory cilia show pH levels of less than 6.9 cause depression of ciliary function, the situation is less clear with regards to the effect of an acidic environment on ependymal cilia. Nakamura and Soto (1993), studying the rate of transport of red cells by rat ependyma as an indirect measure of ciliary function, found no change when the surrounding solution varied from pH 6 to pH 8.

The pH of cerebrospinal fluid is of importance as it appears to correlate with brain function.
The pH of the CSF in patients with certain types of acid base disorders, particularly subacute or chronic metabolic acidosis, tends to remain normal or near normal despite large shifts in the hydrogen ion concentration in arterial blood. There are advantages of keeping the CSF pH normal despite systemic acidosis. If the CSF and the brain extracellular space are in equilibrium, it would seem likely that pH shifts in the CSF may affect brain metabolism. This idea is supported by Posner and colleagues (1965) who found abnormal CSF pH and the degree of encephalopathy correlated closely. Severe acidosis measured in arterial blood is not usually associated with abnormal brain function as long as the CSF pH is maintained.
Respiratory acidosis, however, causes CSF pH to deviate from normal as much or more than arterial pH. Blood pH levels below 7.0 are never seen in awake patients with a respiratory acidosis but may be encountered in only mildly unwell patients with metabolic acidosis where CSF pH is likely to be normal.

The aim of our study was to investigate the effect of pH, on brain ciliary function and to determine whether change in ependymal ciliary beat frequency was linked to a change in the intracellular pH of the ependymal cells.

5.3 Methods:

5.3.1 Sample preparation: The brains of Wistar rats (9-15 days of age) were dissected following sacrifice. Following sacrifice brain slices were prepared immediately, using a vibratome. They were mounted in a well containing 4 ml of medium 199 with Earle salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) and kept on ice until the study began. The well was placed in a purpose built environmental chamber which was thermostatically controlled to keep the fluid surrounding the ependymal sample at 37°C. The chamber was humidified to 75-80% to prevent evaporation from the well during the study period. Ciliary movement was observed using a x50 lens.

5.3.2 Measurement of ciliary beat frequency: Beating ciliated edges were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 1000 frames per second. The camera allowed video sequences to be down loaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100um were studied.

5.3.3 Study design:

5.3.3.1 Effect of pH on ciliary beat frequency: Ciliated ependymal strips were incubated in medium 199 (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) in the environmental chamber for 30 minutes at 37°C. Base line readings were made and the surrounding cell culture fluid exchanged for one of the study solutions pre heated to 37°C. The study solutions comprised of medium 199 with a range of pH values of 6, 6.5, 6.8, 7.0, 7.4 and 8.4. Ciliary beat frequency was measured 15, 30 and 60 minutes after the exchange.

To determine whether ciliary slowing seen at low pH values was reversible, samples exposed to pH 6, which caused rapid ciliary stasis. The surrounding medium was changed to medium 199 at a pH of 7.4. Readings of ciliary beat frequency were repeated after one hour.

5.3.3.2 Measurement of intracellular pH (pHi): The aim of this experiment was to determine the relationship between extracellular and intra cellular pH for brain ependymal cells. Measurement of pHi has been described in detail in previous work (Ng et al, 1993). Ependymal edges were loaded with BCECF by incubation with the cell permeant ester BCECF-acetoxymethyl ester (10 umol/l in TC199 for 30 min at 37°C). The extracellular dye was then washed off and the edges left to de-esterify the ester for 30 min. The edges were then mounted on the heated stage (37°C) of a Nikon Diaphot inverted fluorescence microscope and baseline readings obtained in HEPES buffered saline (HBSS, composed of, in mM, NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5, HEPES 15, BSA 1 g/l, pH 7.4) after a period of equilibration of 15 min. Excitation wavelengths of the dual grating fluorometer (Deltascan, Photon Technology International Inc., South Brunswick, New Jersey, USA) were set at 500 and 439 nm (5 nm slit widths) and epifluorescence emission measured at 530 nm. The ependymal edges with beating cilia were visualised through a collimator and the fluorescence determined by a single photon counting photomultiplier. Ratio (500/439 nm) readings were obtained at a rate of 5 per second. The external solution was then changed to HBSS with pH adjusted to 6, 6.5, 6.8, 7.0, 7.4 and 8.0 in random order. When the 500/439 ratio (equivalent to pHi) had stabilised, mean readings were obtained. Calibrations were then performed using isotonic KCl buffer composed of, in mM, KCl 140, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5, HEPES 15, nigericin and monensin (5 uM each), with pH adjusted to values between 6 and 8. A pulse of NH_4Cl was used to determine intrinsic buffering capacity (30 mM at pH 6.5), substituting NH_4Cl for KCl so that isotonicity was maintained (1).

5.3.3.3 Rapid alteration of intracellular pH using ammonium chloride: Ammonium chloride is known to pass rapidly into cells increasing intracellular pH. The results of our intracellular pH measurements allowed calculation of the amount of ammonium chloride which, when added to ependymal cells, bathed in medium 199 at pH 6.5, would result in a rapid increase of intra cellular pH towards normal physiological values.

The amount of NH_4Cl needed to increase the intracellular pH from 6.5 to 7 whilst the external pH remained at 6.5 was estimated to be 30 mM. The mean (SD) buffering capacity of the ependymal edges, estimated, using the NH_4C1 prepulse method was 18.9 (1.1) mM/pH (n = 6). Buffering was calculated using the following formula:

$$\text{Intrinsic buffer capacity} = [\text{total } NH_4\,]\,(\,10^{pHo - pK})\,/\,(1 + 10^{pHo - pK})\,(pH_a - pH_b)$$

where pHo is the pH of the extracellular buffer, pH_b and pH_a are the pHi values before and after adding the NH_4Cl, and pK the dissociation constant for NH_3. This was confirmed in a series of experiments where ependymal edges were incubated in Medium 199 at pH 6.5 for 30 minutes. Intracellular pH was measured. This solution was then exchanged for medium 199 of pH 6.5 containing 30 mM of NH_4Cl and intracellular pH measured. Five minutes later the solution was exchanged for medium 199 at a pH of 6.5 without NH_4Cl and intracellular pH levels determined.

A separate set of experiments were then performed to determine the effect on ciliary beat frequency of rapidly increasing intracellular pH after ependymal cells had been incubated at a pH of 6.5 for 30 minutes. Ependymal strips were exposed to Hepes buffered Medium 199 at pH 7.4 for 30 minutes to allow base line readings of ciliary beat frequency to be taken. The bathing solution was then changed for one of pH 6.5 for 30 minutes and beat frequency measured. The solution surrounding the ependymal strip was exchanged for medium 199 at pH 6.5 containing 30mM of NH_4Cl. Ciliary beat frequency was measured within 2 minutes. Five minutes later the bathing solution was exchanged for medium 199 at a pH of 6.5 without NH_4Cl and ciliary beat frequency measured within 2 minutes.

5.4 Statistical analysis:

A split unit analysis of variance was performed with factors pH and time. The effect of pH was assessed relative to the between slice variation while the effect of time and the interaction between pH and time was assessed relative to the within slice variation. Contrasts testing for linear trends were included, with pH and with time.

For the study of the effects of rapid change in intracellular pH with time the means of the duplicate values were analysed. There were 4 times 30, 45, 47 and 52 minutes. An analysis of

variance with factors time and slice was performed. Contrasts were performed to compare the mean values at the different time points using the pooled estimate of the standard deviation from the analysis of variance

5.5 Results:

Incubation with acidic solutions caused a decrease in ciliary beat frequency (figure 1a - 1f). Statistically there was a highly significant interaction between pH and time ($P<0.001$). Incubation of cilia in medium 199 at a pH 6 caused ciliary stasis within 15 minutes. Stasis was confirmed by a second reading taken 15 minutes later. Returning the bathing solution to pH 7.4 restored ciliary beat frequency (figure 1a).
The effect of incubation at a pH of 6.5 was slightly less marked with areas of ciliary stasis noted within 1 hour. After 2 hours exposure to solutions of pH 6.8 and pH 7.0 ciliary beat frequency had decreased by 76% and 26% respectively.

The control samples (pH 7.4) retained their normal beat frequency throughout the experiment. No reduction in beat frequency was found at pH 8. When the analysis of variance was repeated including only the data for pH 7.4 and 8 no significant effect of a change in pH was seen ($P=0.45$). A significant interaction between pH and the linear trend with time ($P=0.96$) did not occur. There was a significant linear trend with time ($P=0.02$). This suggests that there was a slight increase in ciliary beat frequency, measured from brain slices exposed to pH7.4 and 8, over the study period.

As expected a decrease in extracellular pH was reflected by a decrease in intracellular pH. Levels of intracellular pH were lower than extracellular pH (Figure 2).

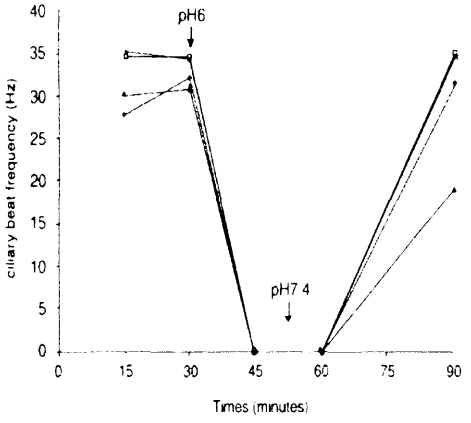
Exposure to an external pH of 6.5 for 30 minutes resulted in an intracellular pH of 6.6. Addition of 30mM of NH_4Cl in medium 199 at a pH of 6.5 resulted in an immediate and highly significant ($P<0.001$) increase of intracellular pH to pH7. Rewashing with medium 199 pH 6.5 at this stage decreased intracellular pH to pH 6.45.

The experiment was repeated on fresh ependymal strips to determine the effect of these manipulations on ciliary beat frequency. Figure 3 shows significant ($P<0.001$) slowing of ciliary beat frequency (from 34.9 (3.5) to 21.2(4.2)Hz) following exposure to pH 6.5 Hepes buffered medium 199. When washed with Hepes buffered medium 199 pH 6.5 with 30mM NH_4Cl a rapid rise in ciliary beat frequency was seen (mean (SD) from 21(4.2) to 35.6(3.3)Hz). Rapid washing of the ependymal strip with medium 199 at pH 6.5 without ammonium chloride resulted in very significant decrease in ciliary beat frequency (35.6(3.3) to 13.1(6.4)Hz) ($P<0.001$).

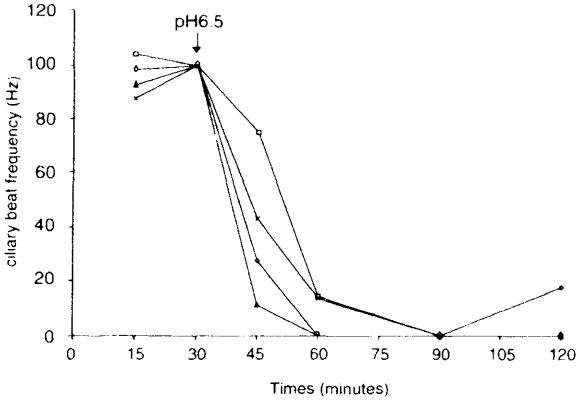
Figure 5.1 a - f.

Graphs 1a to 1f show the effect of incubation of ependymal cilia in bathing solutions of pH values ranging from 6.0 to 8.0. Cilia were incubated in medium 199, pH 7.4, for 30 minutes prior to exchange with one of the study solutions. Each line represents an experiment on a different brain slice. Each point on the line represents the mean of 4 readings along that edge. In experiment 1a the medium 199 at pH 6.0 was exchanged for medium 199 pH 7.4 15 minutes after total ciliary stasis had occurred. Recovery of ciliary beat frequency is seen when measured 30 minutes later.

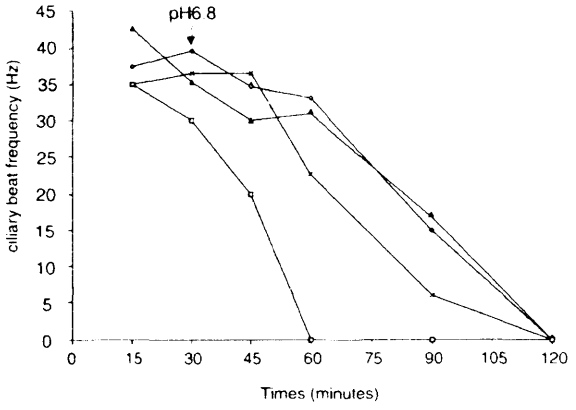
a)


pH6
pH7.4
ciliary beat frequency (Hz)
Times (minutes)

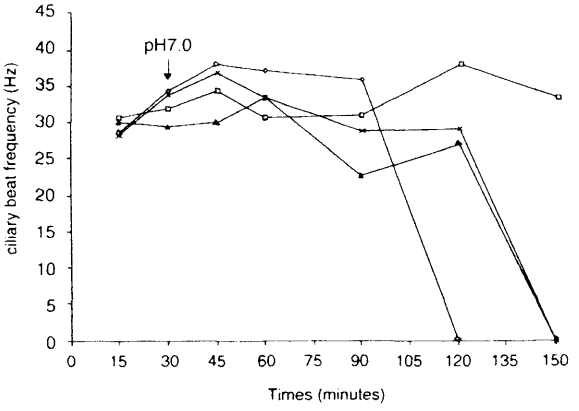
b)

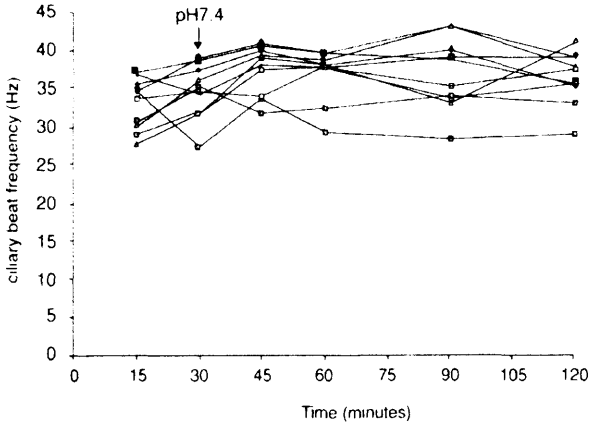

pH6.5
ciliary beat frequency (Hz)
Times (minutes)

c)

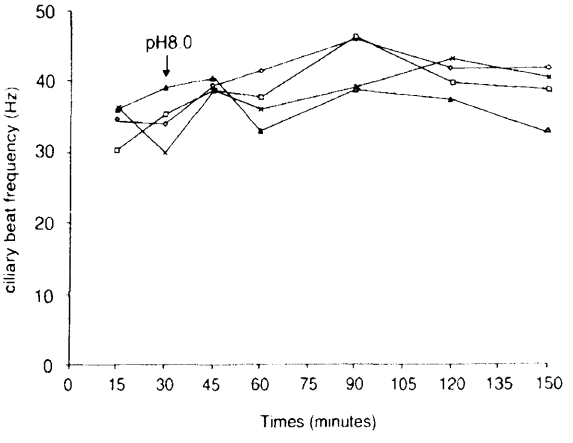

pH6.8
ciliary beat frequency (Hz)
Times (minutes)

d)


pH7.0
ciliary beat frequency (Hz)
Times (minutes)


pH7.4
ciliary beat frequency (Hz)
Time (minutes)

e)


pH8.0
ciliary beat frequency (Hz)
Times (minutes)

f)

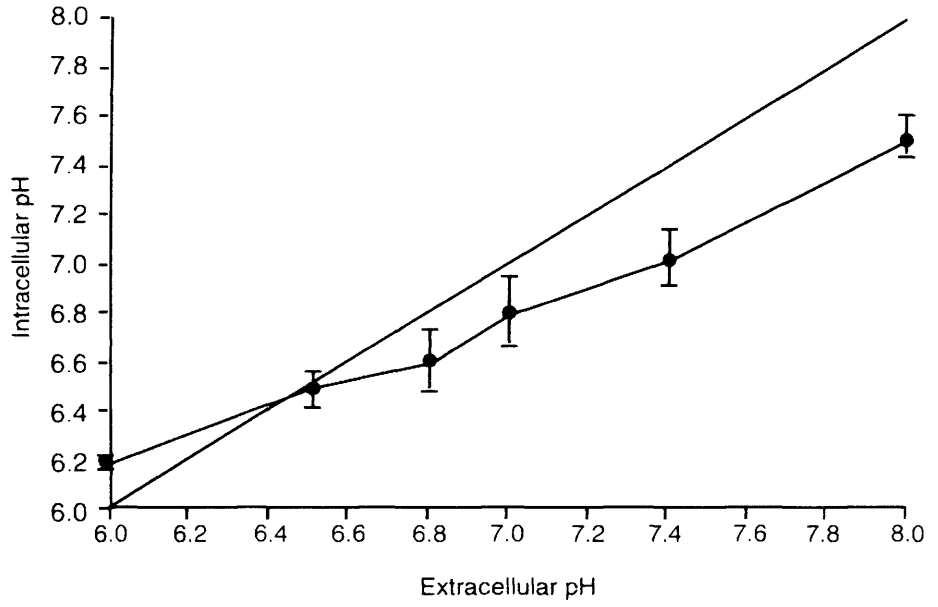


Figure 5.2.

A comparison between the extracellular pH and the intracellular pH of ependymal cells. Over a pH of 6.5 the intracellular pH is lower than that of the extracellular pH.

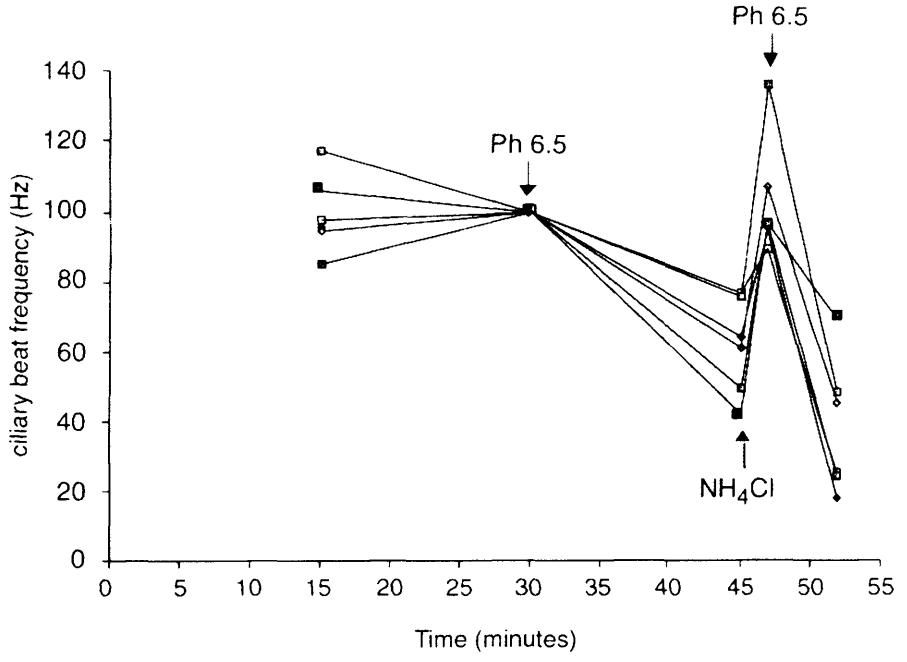


Figure 5.3.

Ciliary slowing was induced by incubation with medium 199, pH 6.5, for 30 minutes. The solution was exchanged for medium 199, at a pH of 6.5, plus ammonium chloride. The amount

of ammonium chloride added had been shown, in a separate study, to rapidly increase the intracellular pH of ependymal cells from 6.4 to 7. Ciliary beat frequency is seen to rise and then decreases again as the solution is exchanged for medium 199, pH 6.5, without ammonium chloride.

5.6 Discussion:

In this chapter we have measured brain ciliary beat frequency from the ependymal lining of the ventricular system of young rats. Their cilia beat continually at approximately 35 Hz and they are clearly sensitive to changes in pH at levels which may be encountered clinically. The decrease in ciliary beat frequency was related to changes in the intracellular pH of ependymal cells.

To establish that changing the extracellular pH resulted in change in intracellular pH, fluorescent measurements were obtained using a hydrogen ion sensitive dye. This confirmed that a decrease in extracellular pH resulted in a decrease in intracellular pH. As with other cell types the intracellular pH of ependymal cells was slightly lower than the external pH. Changes in ciliary beat frequency occurred gradually following exposure to an acidic environment which fits with a gradual decrease in measured intracellular pH.

To confirm that intracellular pH alone was the important factor in the change in ciliary beat frequency, studies were performed using ammonium chloride. Incubating the ependymal strips in an acidic (pH 6.5) solution caused gradual ciliary slowing. The pH of the surrounding media was exchanged for a solution of similar pH (pH6.5) containing sufficient ammonium chloride to increase intracellular pH rapidly towards normal. This resulted in a rapid rise in ciliary beat frequency, directly related to the rapid rise in intracellular pH. Washing ammonia out of the cells by replacing the solution with one of pH 6.5, without ammonium chloride, had the effect of rapidly decreasing the intracellular pH and ciliary beat frequency.

In other cells the majority of H^+ loading at low external pH in a physiological solution can not be ascribed to reduced acid exit. The predominant mechanism under such conditions is attributable to increased H^+ entry. The pathways for H^+ entry across cell membranes

previously suggested include H^+ transport on membrane fatty acids or water molecules, H^+ diffusion through protein (water filled) channels, molecular HCl diffusion across the lipid bilayer, and H^+ transport on membrane ion exchangers. At present there are 4 known exchangers, two of which are used for acid extrusion, the Na^+/H^+ antiporter, the Na^+ - dependent Cl-/HCO_3- exchanger and, for H^+ loading, the Na^+ - independent Cl^-/HCO_3- exchanger and Na+/HCO_3- cotransporter. The use of a Hepes buffer medium for the experiments removes bicarbonate from the extra cellular environment. Thus a proportion of intra cellular acidification in our experiments may have been due to the reduced activity of the Na^+ - dependent Cl/HCO_3- exchanger which acts as an acid extruder. The acid loading ability of the Cl^-/HCO_3- sodium independent acid loader may have been enhanced by the reduction in extra cellular bicarbonate.

Studies on the effect of hydrogen-ion concentration on ciliary activity have been reported in lower, sea water organisms whose cilia were shown to function at pH values as low as 3.5-6.2 (Sleigh, 1962). Irvani (1976), found that the activity of cilia from the bronchus of the rat began to decrease below pH 6.8 and that cilial stasis occurred at pH 5.2. In alkaline surroundings the cilia were active at pH 9.5. Holma et al (1977) studying bovine tracheas demonstrated normal cilial activity, in vitro, between pH 6.7 and 9.6. They noted cell membranes were destroyed below pH 6.7 with the appearance of small pores on cell surfaces. The cell cytoplasm discharged and left the cell membranes as empty shells. Cilia, however, kept their normal appearance down to the lowest pH investigated, pH 4. The most severe effect observed at low pH values (pH 6) was the total shedding of the mucosa, leading to a naked basement membrane. Examining human bronchial cilia Luk and Dulfano (1983) found optimal ciliary beat frequency was seen between pH 7-9, with a marked reductions outside these limits.

The effect of pH on ependymal cilia is dealt with very briefly in two early papers looking at various aspects of ependymal ciliary function. Singer and Goodman (1966) found a pH of 6.0 caused ciliary stasis within 10 minutes while Nelson and Wright (1974) showed stasis using a pH of 4.4 which was reversible following reperfusion with saline. The range of pH values studied was not mentioned in these studies. More recently Nakamura and Soto (1993) studying the rate of transport of red cells by a preparation of exposed ependyma, as an indirect

measure of ciliary function, found no effect of external pH changes in the range of 6.0 to 8.0. Their description of these experiments are very brief and it remains unclear why their results are so different from ours.

The lack of response to incubation in a solution of pH 8 confirms previous reports that cilia tolerate alkaline conditions better than acidic conditions. Gray (1926) found the gill cilia of the Mytilus would tolerate a pH value of 9.2. Interestingly the type of alkali used was essential to the result. Ammonium carbonate of 1% concentration caused immediate ciliostasis, while sodium bicarbonate of the same strength had no harmful effect. He also showed the effects to be reversible.

The pH of the CSF in normal subjects remains within narrow limits. As mentioned in the introduction the tendency for CSF pH to remain normal is most pronounced in subacute or chronic metabolic acidosis, less evident in metabolic and respiratory alkalosis and non existent in respiratory acidosis (Posner et al, 1965). The CSF pH almost entirely depends on its carbon dioxide-bicarbonate ratio and contains so little protein that its buffering capacity resembles a 20 millimolar bicarbonate solution. Under normal conditions the pH in cerebrospinal fluid is slightly lower than blood and the same has been observed in patients with chronic acid-base imbalance, especially in metabolic acidosis.

Under normal conditions the hydrogen ion concentration is higher in CSF than in blood. This suggests the existence of active mechanisms for the relative stability of hydrogen ion concentration. The blood brain barrier is permeable to CO_2 but not to HCO_3^-, which needs active transport. This explains the parallel changes in pH in both CSF and blood observed in respiratory acidosis or alkalosis, with CO_2 passing easily into or out of the CSF.
In cases of metabolic acidosis it has been suggested that the change in CSF pH is dependent upon the velocity at which the change in blood pH occurs. For example, in acute metabolic acidosis there is an almost immediate compensatory diminution in the CO_2 levels in blood, due to increased ventilation. CO_2 will move rapidly out of the CSF equilibrating with blood levels. Bicarbonate levels will not have changed during this short time. CSF pH will, therefore, turn alkaline since its absolute hydrogen ion concentration will be lower than in the blood.

Gesell and Hertzman (1926) found that in dogs breathing CO_2, the arterial blood, venous blood, and CSF became more acidic. When sodium bicarbonate was infused, both CO_2 and bicarbonate were increased in blood, but only CO_2 passed into the CSF. Consequently, blood pH rose but CSF pH fell. Ventilation reflected the pH change in CSF rather than in blood, so that the respiratory rate increased as CSF pH fell and decreased as CSF pH rose.

Davies et al (1973) studied the acid-base relationship between CSF and blood during acute metabolic acidosis caused by the infusion of acidic solutions into anaesthetised dogs whose arterial PCO_2 was kept constant by ventilation. They found that the increase in CSF hydrogen ion concentration was mainly due to an increase in the CSF PCO_2. The decrease in CSF HCO^{3-} concentration accounted for less than a third of the increase in CSF hydrogen ion concentration.

To explain their results they suggested that hydrogen ions move rapidly towards the negatively charged capillary wall while HCO_{3-} is repelled more slowly from the wall. The combination of H^+ and HCO_{3-} results in the transient formation of CO_2 near the capillary wall. The increased PCO_2 near the capillary wall may then act as a back pressure to the free diffusion of CO_2 out of the CSF thereby causing an increase in the difference in PCO_2 between CSF and blood. It is possible that that entry of CO_2 into the CSF is facilitated by rapid CSF flow adjacent to the ependymal surface, by ciliary activity, which may enhance the diffusion gradient down which CO_2 may pass.

Chapter 6

Viscous loading of brain ependymal cilia

6.1 Summary:

The effect of viscous loading, which may occur during certain diseases, on the function of ependymal cilia has not been investigated. Ependymal cilia, for example, may be exposed to cerebrospinal fluid of increasing viscosity during meningitis. The aim of this study was to determine the effect of changes in viscosity on the beat frequency of ependymal cilia.

Ciliated strips of ependyma were exposed to solutions of different viscosity ranging from 1-60cp. Beat frequency measurements were made using a high speed video camera (400 frames/second) over a 1 hour period. Ciliary amplitude was determined from slow motion digital video recordings.

The mean (SD) baseline ciliary beat frequency measured after 30 minutes incubation in medium 199 solution at 37°C was 34.9(2.9)Hz. Increased viscous loading was followed by a rapid decrease in frequency of ependymal cilia compared to base line readings ($P<0.001$). After 15 minutes of exposure to the increased viscous load ciliary beat frequency reached a new equilibrium value that remained fairly stable while the viscous load was maintained. Compared to base line measurements of ciliary beat frequency, viscous loading of 3.7cP caused a 16%, 10.4cP a 34% and 24cP a 70% decrease in beat frequency. Further viscous loading at levels up to 60cP resulted in no further suppression of ependymal ciliary beat frequency.
Solutions of 24cP and 40 cP had no effect on ciliary amplitude. A viscosity of 60cP caused a significant (30%: $p=0.001$) decrease in the amplitude of the ciliary beat.

6.2 Introduction:

The hydrodynamic load on respiratory cilia undergoes wide variations due to rheological changes in the mucus. Johnson and colleagues (1991) found that when rabbit tracheal cilia were exposed to high viscous loads, ciliary beat frequency decreased only slightly and the beat amplitude of cilia remained relatively constant. This autoregulatory response, in the face of increasing viscous loads, was considered to take place within the cell as the cultured monolayers of ciliated cells studied were devoid of nerve terminals.

Studying the effect of viscosity on the ciliary activity of the *Paramecium*, Machemer (1980) found ciliary beat frequency falls linearly with exponentially rising viscosity. Yoneda (1962), studying the single cilium of *Mytilus edulis* found a tendency of the force exerted by the cilium to increase with increasing viscosities, up to a level where ciliary movement ceased. It was estimated that in free motion a force of only one twentieth of maximum is developed. This was thought to suggest that some sort of 'intracellular machinery' analogous to that of muscle must be involved in ciliary movement. The role of increased intracellular calcium in such a response is discussed later. The results of studies on Peristomial cilia of *Stenor* resemble those of *Mytilus* (Sleigh, 1956). Despite an increase in viscosity of up to 3.5 times, the frequency of beat of the *Stentor* cilia maintained the level of 75% of its initial value with no change in amplitude. An equation for the force produced by a single cilium in free motion was derived. Expressing the force in terms of torque referred to the base of the cilium:

$$Torque = angular\ velocity \times viscosity \times (length\ of\ the\ cilium)^3 \times constant$$

Experiments in such water propelling cilia suggest that increase in viscosity resulted in increase in torque. The power, or rate of doing work, of a cilium was calculated from the equation:

$$Power = torque \times angular\ velocity$$

Thus power also increased with increasing viscosity.

CSF has a similar viscosity to water. However, ependymal cilia may encounter a slight increase in viscous loading with conditions such as Guillian Barre syndrome or a very significant increase in meningitis when gel formation may occur. Meningitis may be associated with decreased CSF flow and hydrocephalus.

The effect of increasing viscous loads on ependymal ciliary function has not been described. We were interested to determine whether the water propelling ependymal cilia from the rat brain behaved in a similar manner to other water propelling cilia. The aim of this study was, therefore, to determine the effect of changes in viscosity on the beat frequency and beat amplitude of ependymal cilia.

6.3 Methods:

6.3.1 Tissue preparation: The brains of Wistar rats (9-15 days of age) were dissected following sacrifice. Brain slices from the floor of the 4th ventricle of Wistar rats (10-15 days of age) were prepared immediately after sacrifice and mounted in a well containing 4 ml of medium 199 with Earl's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml). Samples were enclosed in a purpose designed environmental chamber which maintained the solution at 37°C, and the surrounding air at a humidity at 80% to minimise evaporation. The chamber was humidified to 75-80% to prevent evaporation from the well during the study period. Ciliary movement was observed using a x50 water immersion lens.

6.3.2 Measurement of ciliary beat frequency: Beating ciliated edges were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 400 frames per second. The camera allowed video sequences to be down loaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100μm were studied.

6.3.3 Viscosity measurement:

Viscosity was determined in accordance with the British Standards guidelines (1977) using a ubelhode viscometer. For the purposes of this British Standard the following definitions apply.

- *Viscosity* is internal friction in a fluid; viscous forces oppose the motion of one portion of a fluid relative to another. That is the property of a liquid to resist shear deformation increasingly with increasing rate of deformation.

- *Shear* is the orderly movement of layers of liquid relative to parallel adjacent layers. In a steady shear the rate of relative movement of layers is known as the *rate of shear* and the tangential force per unit area applied to the layers to sustain the motion is the *shear stress.* A liquid is said to exhibit Newtonian flow when the shear stress and the rate of shear are directly proportional; that is, at constant temperature and pressure the viscosity is independent of the rate of shear.
- *Dynamic viscosity, n.* The shear stress divided by the rate of shear for steady flow of the liquid.
- *Kinematic viscosity, v.* The dynamic viscosity divided by the density of the liquid, both measured at the same temperature.

6.3.3.1 Measurement of viscosity:

The time was measured for a reproducible volume of the liquid to flow through a capillary viscometer under an accurately reproducible head and at a closely controlled temperature. The kinematic viscosity was then calculated from the measured flow time and the calibration factor (C) of the viscometer. If the density is measured at the same temperature as applied to the determination of kinematic viscosity, the product of the two results is the dynamic viscosity at that temperature.

Four precepts for precise viscometry were observed; a clean instrument; a clean liquid constant temperature during the course of a measurement; and accurate measurements of temperature and flow time.

6.3.3.2 Apparatus:

Viscometers: Ubelode glass capillary viscometers were used.

Thermometer: a total immersion thermometer capable of temperature measurements around 37°C.

Thermostatic Bath: a liquid bath was used so that no portion of the sample was less than 20mm below the surface of the bath medium or less than 20mm above the bottom of the bath.

6.3.3.3 General procedure: The bath was maintained at the test temperature, 37°C, and the entire liquid column of the thermometer was immersed in water so that the top of the mercury column was visible just below (about 2mm) the surface of the medium. The thermometer was

mounted vertically by sighting its parallelism with a plumb line. The viscometer was charged with the sample, making sure to avoid trapping bubbles. Thirty minutes were allowed for the charged viscometer to reach the same temperature as the bath.

When temperature equilibrium was attained, tube M (figure 6.a) was closed and suction applied to capillary tube N until the liquid reached a level about 5mm above mark E. The liquid was held at this level by closing tube N. Tube M was opened so that the sample dropped away from the lower end. When the sample was clear of the capillary end and the lower end of tube M, tube N was opened. The flow time was the time taken for the bottom of the meniscus to pass from the top edge of mark E to the top edge of mark F. Thermometer readings were made at the beginning and end of the timed flow.

Measurements of flow time were repeated immediately, without emptying or recharging until two

successive flow times were found to agree within 0.2%.

6.3.3.4 Calculation and expression of results:

The kinematic viscosity was then calculated from the measured flow time and the calibration factor of the viscometer. The density was measured at the same temperature as the determination of kinematic viscosity allowing the product of the two, the dynamic viscosity at that temperature, to be calculated.

Kinematic viscosity (v). $v = C\ t$

where t is the mean flow time (in seconds); C is the factor for the viscometer, given in its calibration certificate.

The result is expressed in mm^2/s. The SI unit of kinematic viscosity is the metre squared per second (m^2/s)

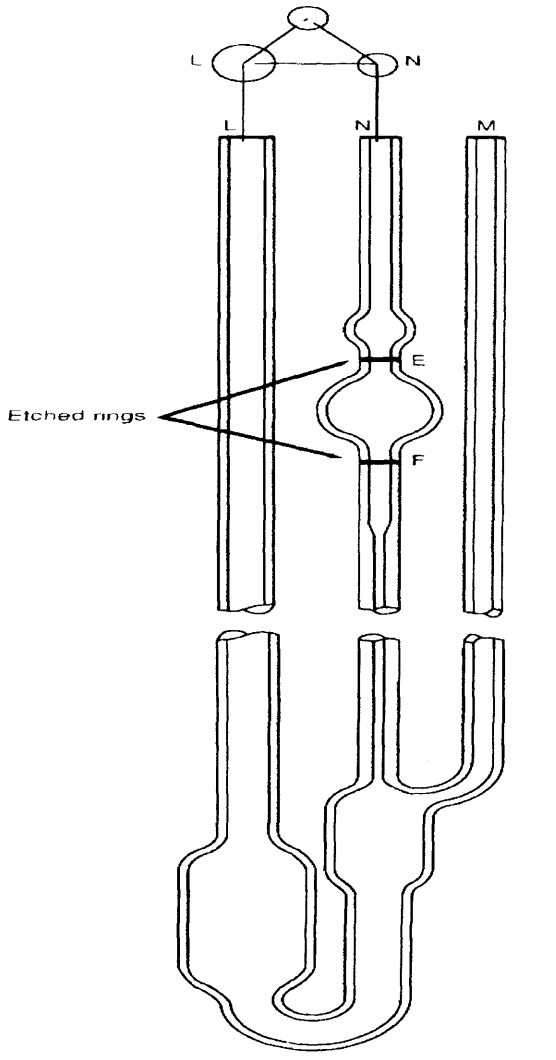
Dynamic viscosity (n). $n = V\ p$

where v is the kinematic viscosity (in mm^2/s), p is the density measured at the same temperature (in g/cm^3)

The SI unit of dynamic viscosity is the Pascal second (Pa s). The millipascal second (mPa s) is equal to the centipoise (cP). Viscosity in this study are reported in units of cP to allow easier reference to previous investigations into the effect of viscosity on cilial function.

Repeatability: Duplicate results by the operator using the same viscometer showed that their difference was less than 0.35% of their mean.

The type of direct-flow viscometers we used were Ubelohde, and have calibration constants of 0.004988, 0.02914 and 0.3172 mm^2/s^2.



Ubbelohde Suspended-Level Viscometer

Figure 6.a

6.3.4 Effect of viscous loading on ciliary beat frequency:

Ependymal strips were incubated for 15 minutes to allow the solution to reach 37°C and an initial reading made. A second reading was made 15 minutes later. The medium 199 bathing the ependyma was then exchanged with one of the experimental solutions. Those conducting the experiment were blinded to the nature of the solution. Solutions of the following viscosities

were used; 3.7cP, 10.4cP, 25.3cP, 40cP and 60cP. A solution of medium 199 (1cP) was used as a control. All solutions were preheated to 37°C prior to exchange. Readings of ciliary beat frequency were taken at 3, 15, 30, 45 and 60 minutes after exchange.

6.3.4.1 Effect of a low sodium high osmolality solution:

The higher concentrations of dextran resulted in a low measured sodium (95 mmol/l) and a high osmolality (375 mmol/kg). To determine whether a solution of low viscosity (1cP), high osmolality and low sodium (95mmol/l), also caused ciliary slowing the following experiment was conducted. A solution of artificial CSF (in mmol/l; NaCl 125, KCl 2.5, Glucose 10, Hepes 10, $MgCl_2$ 1, $CaCl_2$ 2) adjusted to a pH 7.3 was made.

NaCl almost completely dissociates in water. This means that 1M of NaCl will contribute 2 Osmoles towards the osmolality of a solution. However, sucrose, not being a salt, does not dissociate, and therefore, only contributes 1 Osmole per mole.

The amount of sucrose required to increase the osmolality up to 375, and to produce a solution with only 90mmol/l of Na was determined and the dynamic viscosity measured.

For example, a 45 mM decrease in NaCl (in aCSF), from 135 mmol/l to 90mmol/l will cause a 90 mosmol/kg decrease in osmolality (from approximately 300 to 210). Therefore, to increase the osmolality up to 375, whilst maintaining a low (90mmol/l) Na content, 165 mmol/l of sucrose was needed.

6.3.5 Effect of viscous loading on the range of cilial motion:

The effect of viscous load on the distance travelled by the tip of a single ependymal cilium during its power stroke was determined. Ciliated cells were observed sideways using a x50 water immersion lens. Brain slices were stored in medium 199 with Earl's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml), at 37° C for 30 minutes, and ciliary motion recorded at a frame rate of 400 per second. The bathing solution was then exchanged for a preheated (37°C) solution of either 24 cP(n=7), 40 cP (n=6), or 60 cP (n=8). Ciliary motion was recorded, again, after twenty minutes.

The position of the cilium (magnified 1500 times) was stored at intervals of 2.5 milliseconds. The distance travelled by the tip of the cilium was measured during its forward stroke. This was divided by the cilial length. This was to offset any differences in measurement due to the

increased refractive index of the more viscous solutions. This index was used to compare initial movement with that following fluid exchange with results expressed as a percentage of the cilium's movement in medium 199.

Table 1.

Measurements made in determining viscosity of solutions used.

% Dextran	Time (mins)	Temperature (°C) before reading	Temperature (°C) after reading	Density (g/cm^3)	Mean dynamic Viscosity
Artificial CSF (O%)	147.37	37	37		0.73cP
	146.03	37	37		
	145.3	37	37	1.0	
Low Na+ (0%)	2.43.52	37	37		0.83cP
	2.45.61	37	37	1.03	
	2.43.08	37	37		
5%	2.04.24	37.05	37.05		3.73cP
	2.05.83	37.05	37.05	1.03	
	2.09.52	37.05	37.05		
	2.04.32	37.05	37.05		
10%	5.56.47	37.05	37.05	1.03	10.43cP
	5.59.23	37.05	37.05	1.0	
	6.00.81	37.05	37.05		
15%	1.18.13	37.05	37.05	1.02	24.29cP
	1.18.15	37.05	37.05	1.02	
17.50%	1.54.58	37	37	1.04	40.3cP
	1.54.27	37	37		
20%	2.39.90	37	37		60cP
	2.38.17	37	37	1.00	

6.4 Statistics:

A split unit analysis of variance was performed with two factors, viscosity and time. The effect of viscosity was assessed relative to the between slice variability, while the effects of time and the interaction between viscosity and time were assessed relative to the within slice variability. To determine the effect of exchanging the control medium with an artificial CSF or with a low sodium CSF, the means of the duplicate values were analysed. Paired t tests were performed to compare artificial CSF (aCSF) with control and low sodium CSF with control. A two sample t test was used to compare the mean change from control for the two media.

To determine the effect of changing viscosity on beat amplitude a one way analysis of variance was used to compare the three viscosities (24, 40, and 60cP) studied. The Bonferroni procedure was used to compare every pair of means (Altman, 1991). For each viscosity a one sample T-test was used to compare results to readings obtained in medium 199.

6.5 Results:

The mean (SD) baseline ciliary beat frequency measured after 30 minutes incubation in medium 199 solution at 37°C was 34.9(2.9)Hz. Increased viscous loading was followed by a rapid decrease in frequency of ependymal cilia. After 15 minutes of exposure to the increased viscous load the beat frequency reached a new equilibrium value that remained fairly stable while the viscous load was maintained. For the full model there was a highly significant interaction between viscosity and time ($P<.001$).

The beat frequency decreased to 84% of base line readings with the addition of a solution with a viscosity of 3.7cP to 66% at 10.4cP and 30 % at 24cP. Further viscous loading at levels up to 60cP resulted in no further suppression of ependymal ciliary frequency. When the analysis was restricted to viscosities of 24cP or more there was no significant interaction between viscosity and time ($P=0.29$) and no significant effect of viscosity ($P=0.47$). The time course of the effect of viscous loading on the frequency of ependymal cilia is shown in figure 6.4a-f.

The effect of viscous load on ciliary beat amplitude after equilibration at 24cp, 40cP and 60cP compared to base line readings are shown in figure 6.2. The analysis of variance used to

compare the effect of the 3 viscosities (24, 40, and 60cP) on ciliary amplitude showed highly significant differences between the three means ($p<0.001$). The highest viscosity was significantly different from either of the other two viscosities. A one sample t test comparing the mean % amplitude with 100% was only significant for the 60cP solution.

There was no significance difference in ciliary beat frequency between aCSF and control (P=0.22) (figure 6.5) or between low sodium CSF and control (0.09) (figure 6.4). Exchange of medium 199 with artificial CSF or artificial CSF with a high osmolality (*388mosmol/l*) and low sodium concentration (91mmol/l) resulted in a 9.8% and 7% increase in ciliary beat frequency respectively.

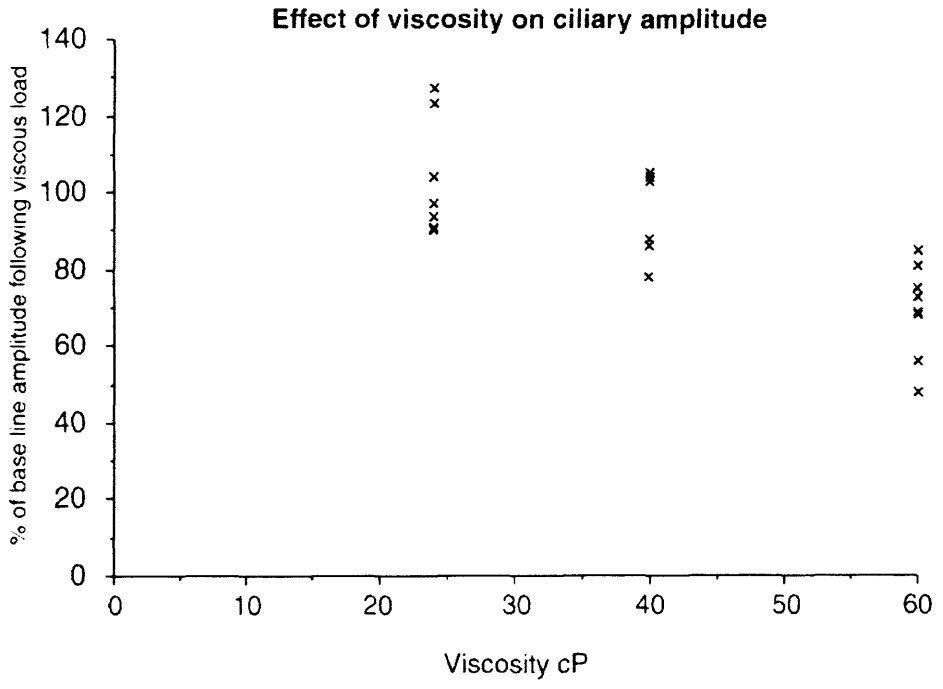


Figure 6.2:

The effect of viscous load on the distance travelled by the tip of an ependymal cilia during its power stroke. High speed video recordings were made at a frame rate of 400 per second. Ciliated cells were observed sideways using a x50 water immersion lens. The position of the cilium (magnified 1500 times) was stored at intervals of 2.5 milliseconds. For each ciliated sample the relative (%) change of distance travelled by the cilial tip is referenced to its own control measured at the end of a 30 minute incubation period in medium 199 (1cP).

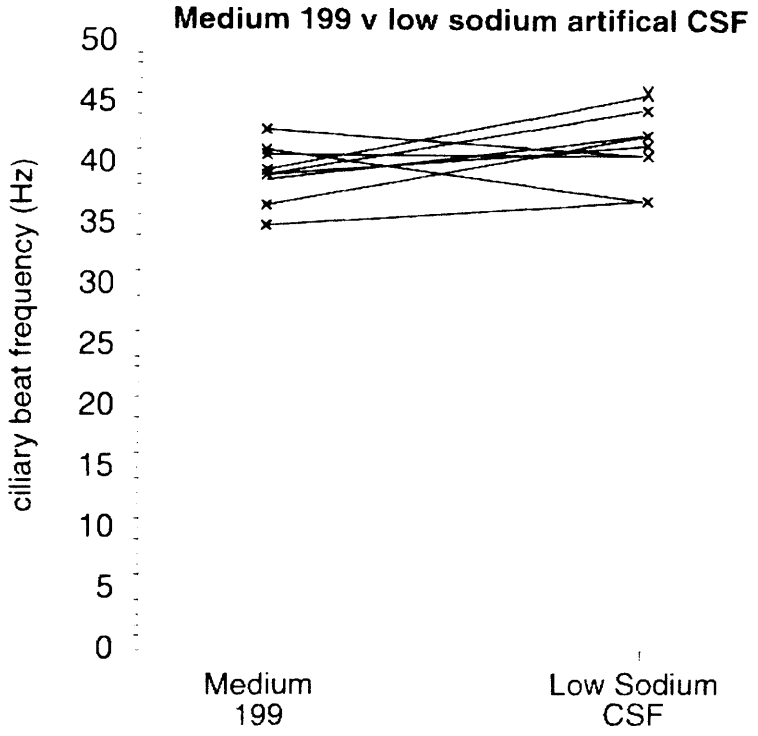


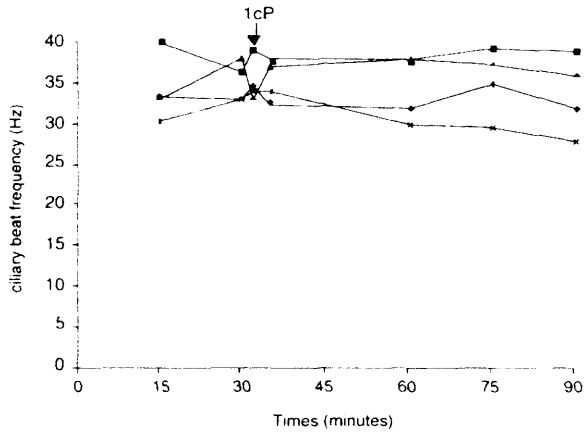
Figure 6.3:

Medium 199 and dextran at its highest concentration (20%) resulted in a viscous solution of high osmolality (380mosmol/l) and low sodium concentration (95mmol/l). This study was performed to determine the effect of a solution of low viscosity, high osmolality and low sodium on ependymal ciliary beat frequency. Ciliated samples were incubated in a control solution of medium 199 which was exchanged for either artificial CSF or artificial CSF with an osmolality of 380mosmol/l and a sodium concentration of 90mmol/l. The viscosity of these solutions was similar

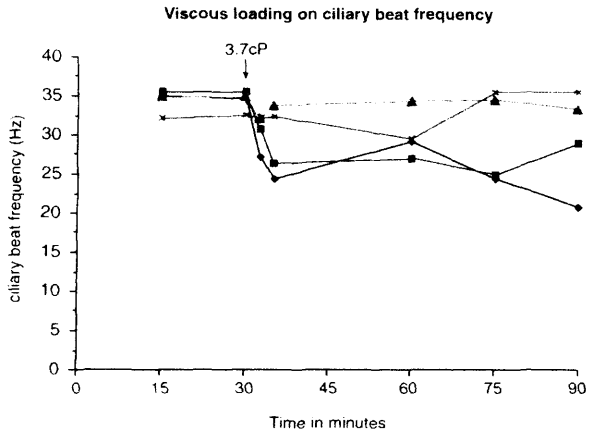
Figures 6.4a-f (next page):

Time course of the effect of viscous loading on the frequency of ciliary beat of ciliated ependymal cells. After a 30 minute control period in medium 199, the ciliated ependymal cells were exposed to solutions of medium 199 and dextran with different viscosities (1-60cP). Each line on a graph is represents an experiment on a single ependymal edge. Each point on the graph is the mean reading of four experiments on that edge.

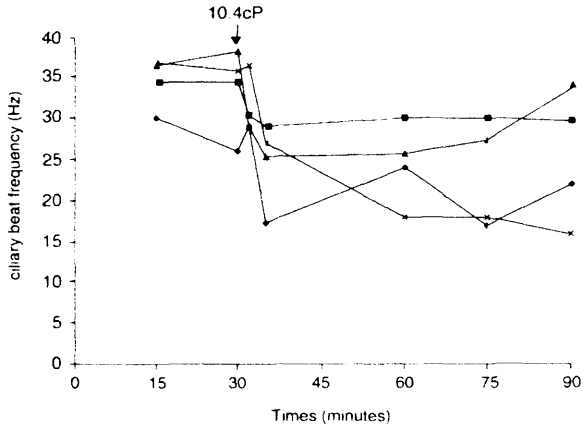
a)


1cP
ciliary beat frequency (Hz)
Times (minutes)

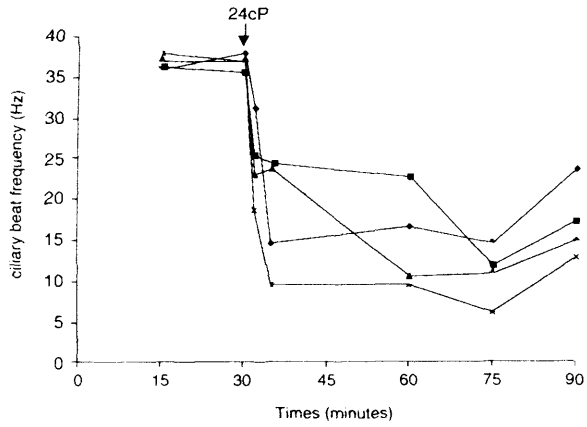
b)


Viscous loading on ciliary beat frequency
3.7cP
ciliary beat frequency (Hz)
Time in minutes

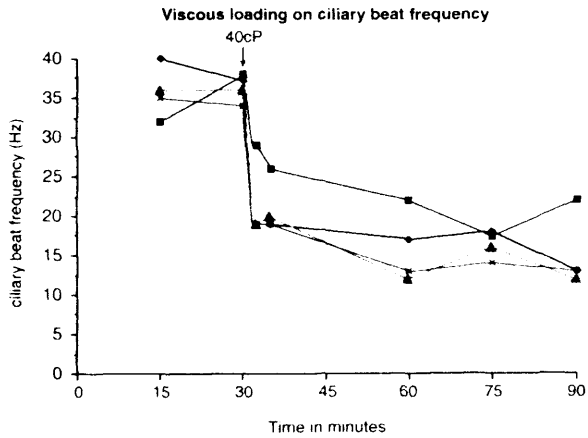
c)


10.4cP
ciliary beat frequency (Hz)
Times (minutes)

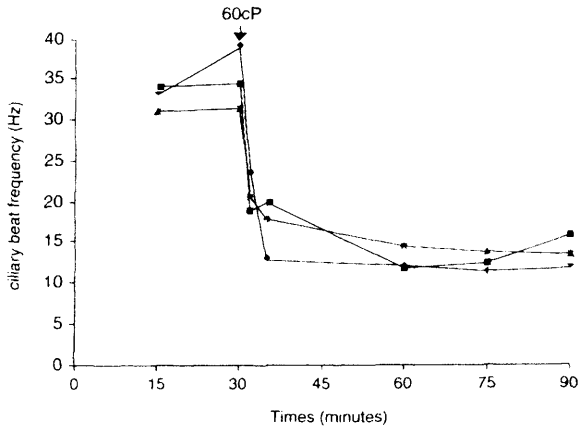
d)


24cP
ciliary beat frequency (Hz)
Times (minutes)

e)


Viscous loading on ciliary beat frequency
40cP
ciliary beat frequency (Hz)
Time in minutes

f)


60cP
ciliary beat frequency (Hz)
Times (minutes)

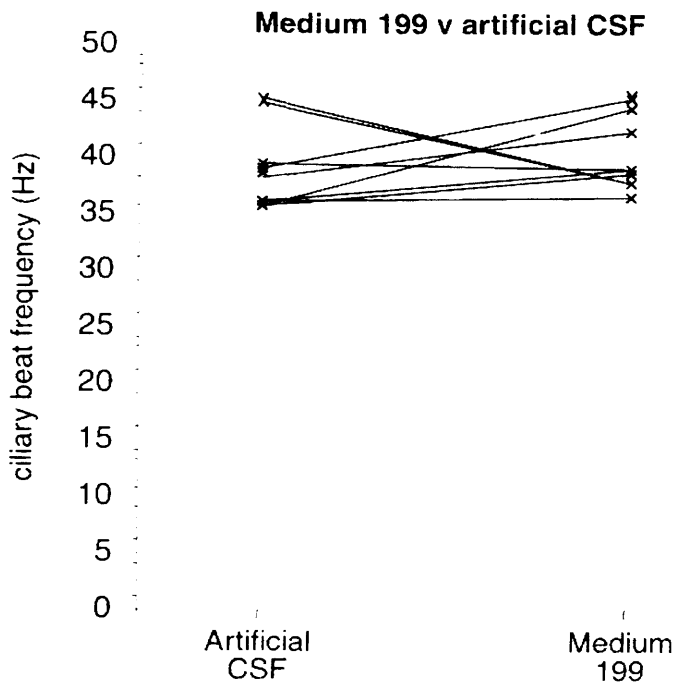


Figure 6.4:

This graph shows the effect of incubating ciliated samples in either medium 199 or in artificial CSF at 37°C.

6.6 Discussion:

We have shown that increasing the viscosity of fluid surrounding brain ependymal cilia results in a rapid decrease in ciliary beat frequency. Once this rapid decrease in beat frequency occurred cilia beat at their new, lower, frequency for the remainder of the study period. Viscous loading of 3.7 cP, 10.4 cP and 24cP caused a progressive decrease in ciliary beat frequency. However, further viscous loading up to 60cP did not depress beat frequency further.

At viscosities up to 40cP the distance moved by the cilial tip during its active stroke, the amplitude, did not change. However, a viscous loading of 60cP reduced the ciliary amplitude significantly. Ependymal cilia, therefore, show some ability to autoregulate their activity. This is

in agreement with studies by Yondea (1962) who estimated that a water propelling cilia in free motion produced a force of only on twentieth of its maximum potential.

Our results are not dissimilar to those of Machemer (1972). He studied the effect of increasing viscous loads on the water propelling cilia of *Paramecium*. The percentage decrease in beat frequency with viscous loading was similar to the decrease in frequency seen when similar viscous loads were applied to ependymal cilia.

Although water propelling cilia do appear to be able to adapt to increasing viscosity they do not appear to be as well adapted as mucous propelling respiratory cilia described by Johnson and colleagues (1991). They found that mucous propelling cilia of the respiratory tract from confluent clusters of cells or single cells showed autoregulatory properties when exposed to an increased viscous load. Despite the use of viscous loads (1-150cP), which were considerably higher than those used in our experiments, the beat amplitude and frequency exhibited only slight variations over viscosities ranging from 12-150cP. A recovery response was seen after approximately 30 minutes of exposure to viscosities less than 55cP. This ability to auto regulate activity is not secondary to an external nerve mediated reflex as cultured monolayers are devoid of nerve terminals. It is not immediately clear why ependymal cilia did not tolerate viscous loading as well as respiratory cilia. Cilial length may be a factor in that ependymal cilia are significantly longer than those from the respiratory tract.

Methodological differences may also have played a part in the results. In Chapter 3 it was demonstrated that the design of the environment within which ciliary samples were viewed may have a very significant effect on the beat frequency recorded. It is possible that the use of the Rose chamber by Johnson and colleagues reduced the relative decrease in beat frequency of cilia observed following exposure to viscous loading. The cilia observed by Machemer and in this study were in an open bath system.

It is unlikely, in our study, that the ciliary activity decreased the apparent viscosity of the medium 199-dextran solutions as such solutions have been shown to behave as Newtonian fluids when exposed to shear rates much higher than those produced by ciliary activity. The possibility of the lower sodium and higher osmolality of the high dextran solutions being responsible for the decrease in ciliary beat frequency is also unlikely as a solution of similar sodium concentration and osmolality but of low viscosity did not affect ciliary beat frequency.

The ability of cilia to respond to increasing viscous loading may be due to cellular reaction to changes in mechanical load or be due to inbuilt properties of the ciliary axoneme. Ciliated cells are mechanosensitive. Stimulation of the apical surface of a single ciliated cell with a glass microprobe elevates the ciliary beat frequency of the stimulated and adjacent cells (Sanderson & Dirksen, 1986: Sanderson et al, 1988). The increase in beat frequency of each cell occurs after a lag-phase and is proportional to the distance from the stimulated cell. Stimulation of a nonciliated cell, adjacent to ciliated cells, also increased their beat frequency. A mechanical stimulus to either a ciliated or nonciliated cell induces an immediate increase in intra cellular Ca^{2+} at the contact point which spreads throughout the cell. After approximately half a second, a wave of increasing intracellular Ca^{2+} occurs in adjacent cells travelling across up to 7 cells. The rise in intracellular Ca^{2+} always preceded an increase in ciliary beat frequency (Sanderson et al, 1990). The axoneme may also play a role as demembranated-ATP- reactivated axonemes of newt lung ciliated cells, under conditions in which calcium concentrations are kept constant, appear to autoregulate beat frequency under increased viscous loads (Hard et al, 1985)

The effect of various physiological variables on the ability of ependymal cilia to move CSF has received little attention. The movement of fluid by small structures such as cilia may be markedly affected by viscosity. The propulsion of water by cilia is a low Reynolds number phenomenon, where viscous forces are more important than inertial forces. The Reynolds number (Re) for a cilium can be defined by:

$$Re = \frac{\text{fluid density} \times w\,Lr}{\text{fluid viscosity}}$$

where w = angular frequency, L = ciliary length, and r = ciliary radius. The Reynolds number for a cilium is low because the linear dimensions of cilia are so small. A capture zone of water is dragged along around the cilium as it moves, and in the absence of any appreciable inertial effects, the motion of the fluid will stop as soon as the cilium stops moving.

The overall propulsive effect depends upon the arrangement of the cilia and their pattern of beating, as well as on ciliary length and beat frequency. Efficiency in transmitting force to the surrounding water during the effective stroke will be lost if the cilium is not stiff enough to remain reasonably straight. Such loss is reduced by co-operation between adjacent ependymal cilia, which beat in close co-ordination in metachronal waves where each cilium provides some mutual assistance to the motion of neighbouring cilia. Such co-operation will occur in our

model which comprises of many attached ciliated cells as the ependymal strips studies are approximately 150um in thickness and over 100um long.

The formation of metachronal waves is important in water propulsion. As mentioned cilia densely line the ependymal surface and their orientation facilitates the directional flow of the CSF towards and through the cerebral aqueduct and beyond. The importance of metachronism to CSF propulsion is that at any instant there are adjacent cilia involved in different stages of their effective stroke. Each cilium does not accelerate CSF from rest during its effective stroke, but adds impetus to water already being moved by adjacent cilia. A continuous flow can therefore be maintained at a level which approaches the ciliary tip speed, and the lack of inertial momentum is overcome by use of continuously overlapping cilia. Because the propulsion of water by a cilium is only a local viscous phenomenon, only a shallow zone of water some two cilium lengths deep is transported across the ciliated surface (Blake & Sleigh, 1974) and the total volume transported is therefore small. With an extensive covering of ependymal cilia the volume of CSF they transport may be considerably greater.

Chapter 7

The effect of Pentobarbital Sodium on ependymal ciliary function

7.1 Summary

The ex-vivo model, which allows measurement of brain ependymal ciliary beat frequency, was used to determine the effect of various concentrations of pentobarbitone on ependymal ciliary beat frequency. Using paired incubation chambers, ependymal cilia were exposed to pentobarbitone (25, 50 or 250μg/ml) or control cell culture medium over a 3 hour study period. Pentobarbitone at concentrations of 25 and 50μg/ml had no effect on ciliary beat frequency compared to controls (P= 0.6 and 0.4 respectively). Mean ciliary beat frequency (SD) after the 3 hour study was 34.1(3.4)Hz for pentobarbitone 25μg/ml and 33.5(3.8)Hz for control: at 50μg/ml mean readings were 44.1(6)Hz and 42(4.2)Hz for controls. A significant (P=0.002) decrease in ciliary beat frequency was seen following incubation with a pentobarbitone concentration of 250μg/ml (mean (SD) frequency, 24(8)Hz compared to controls, 38(9)Hz).

7.2 Introduction:

Many studies of CSF flow have been conducted in animals under pentobarbital anaesthesia (Marmarou et al, 1994). We were concerned that if this anaesthetic caused significant depression of ependymal ciliary function, the contribution of ependymal cilia to the flow of cerebrospinal fluid may not have been appreciated. Our concerns were based on evidence that a number of anaesthetic agents have been shown to cause significant slowing of respiratory ciliary beat frequency (O`Callaghan et al, 1994: Gyi et al, 1994: Raphael et al, 1996).

Prior to conducting studies on the role of ependymal cilia in the flow of cerebrospinal fluid it was essential to know whether the anaesthetic to be used, pentobarbitone, would interfere with ependymal ciliary function.

The aim of this study was to determine the effect of clinically relevant concentrations of pentobarbitone on ependymal ciliary beat frequency.

7.3 Methods:

7.3.1 Sample preparation: The brains of Wistar rats (9-15 days of age) were dissected following sacrifice. Brain slices from the floor of the 4th ventricle of Wistar rats (10-15 days of age) were prepared immediately after sacrifice and mounted in a well containing 4 ml of medium 199 with Earl's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) and kept on ice until the study began. For the experiment the well was placed in a purpose built environmental chamber which was thermostatically controlled to keep the fluid surrounding the ependymal sample at 37°C. The chamber was humidified to 75-80% to keep prevent evaporation from the well during the 3 hour study period. Ciliary movement was observed using a x50 water immersion lens.

7.3.2 Measurement of ciliary beat frequency: Beating ciliated strips were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 1000 frames per second. The camera allowed video sequences to be downloaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100um were studied.

7.3.4 Study design: Ciliated ependymal strips were incubated for 30 minutes in the environmental chamber at a temperature of 37°C. Base line readings were then made and the surrounding cell culture fluid exchanged for either pentobarbitone sodium or a control solution of medium 199. Readings were repeated at hourly intervals for 3 hours. Solutions were preheated to 37°C prior to fluid exchange. The effect on ciliary beat frequency of ependymal

strips was measured at the following concentrations of pentobarbitone: 25(n=10), 50(n=9) and 250μg/ml (n=8). For each experiment involving addition of pentobarbitone a control strip of ependyma was studied simultaneously.

7.4 Statistical analysis: The mean of the four replicate measurements of each ependymal slice at each time was analysed. An unbalanced analysis of variance (using the restricted maximum likelihood procedure: Genstat 5 software) was performed with three fixed factors:

a) pentobarbitone concentration - 25, 50, 250μg/ml:

b) controls for pentobarbitone experiment:

c) 0, 1, 2, 3 hours.

There were two random factors: between slice and within slice components of variance.

7.5 Results:

There were a total of 308 measurements of ciliary beat frequency from the ten paired samples exposed to pentobarbitone over a 3 hour period at a concentration of 25μg/ml. Two hundred and sixty eight measurements were made from the 8 paired samples exposed to 50μg/ml and a similar number of readings made at a concentration of 250μg/ml.

At a pentobarbitone concentration of 25μg/ml no significant (P=0.6) effects were found in ciliary beat frequency (mean (SD)after 3 hours = 34.1(3.4)Hz) compared with control samples (mean (SD) after 3 hours = 33.5(3.8)Hz). At a concentration of 50μg/ml (mean (SD) after 3 hours = 44.1(6)Hz) there was also no significant effect compared to control samples (mean(SD) after 3 hours = 42(4.2)Hz) (P=0.4). At a concentration of 250μg/ml, however, there was significant (P=0.002) slowing of ependymal ciliary beat frequency (mean (SD) after 3 hours = 24(8)Hz) compared to control samples (mean(SD after 3 hours = 38(9) Hz).

Results of ciliary beat frequency when exposed to pentobarbitone and control medium are shown in figure 7.2a - f..

Ciliary slowing at a pentobarbitone concentration of 250μg/ml was not associated with an abnormal ciliary beat pattern.

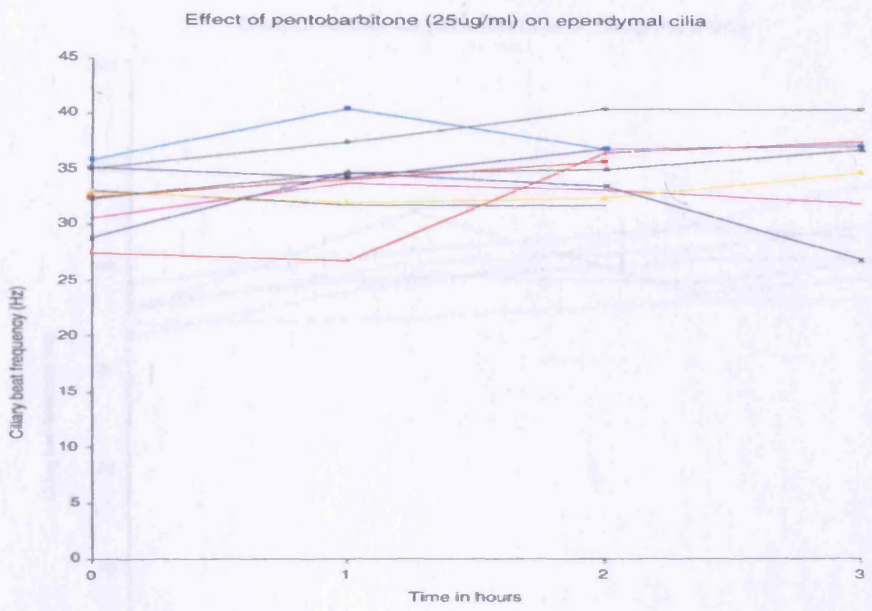


Figure 7.2a

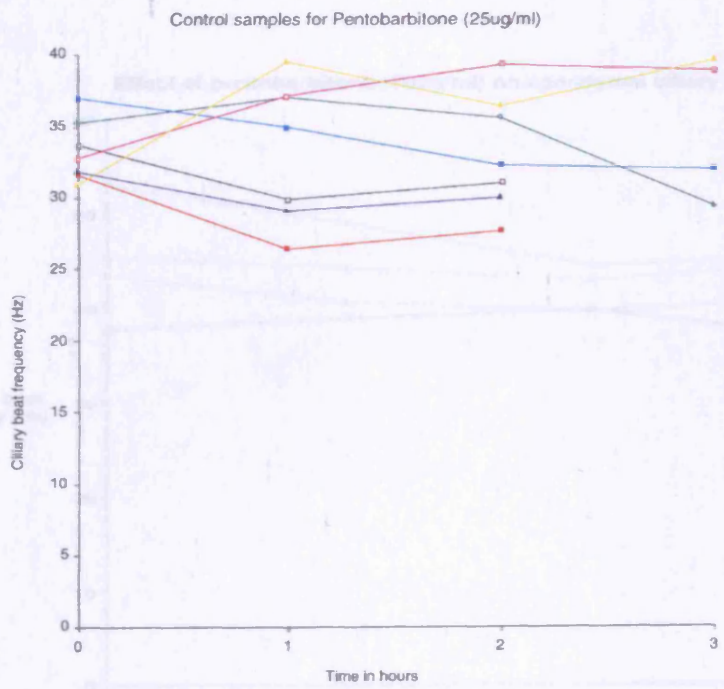


Figure7.2b

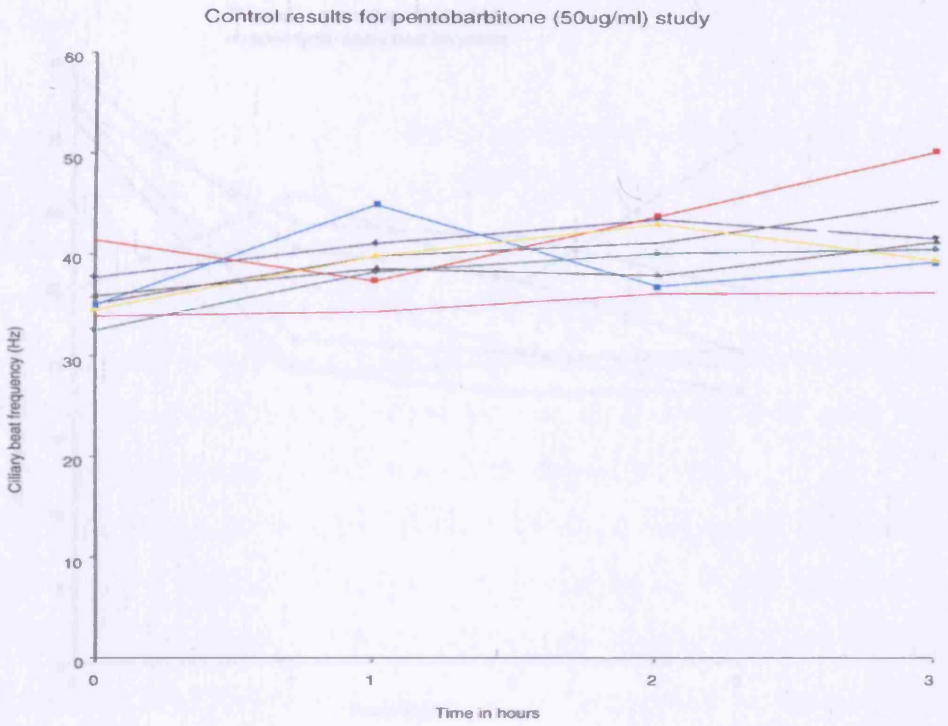


Figure 7.2c

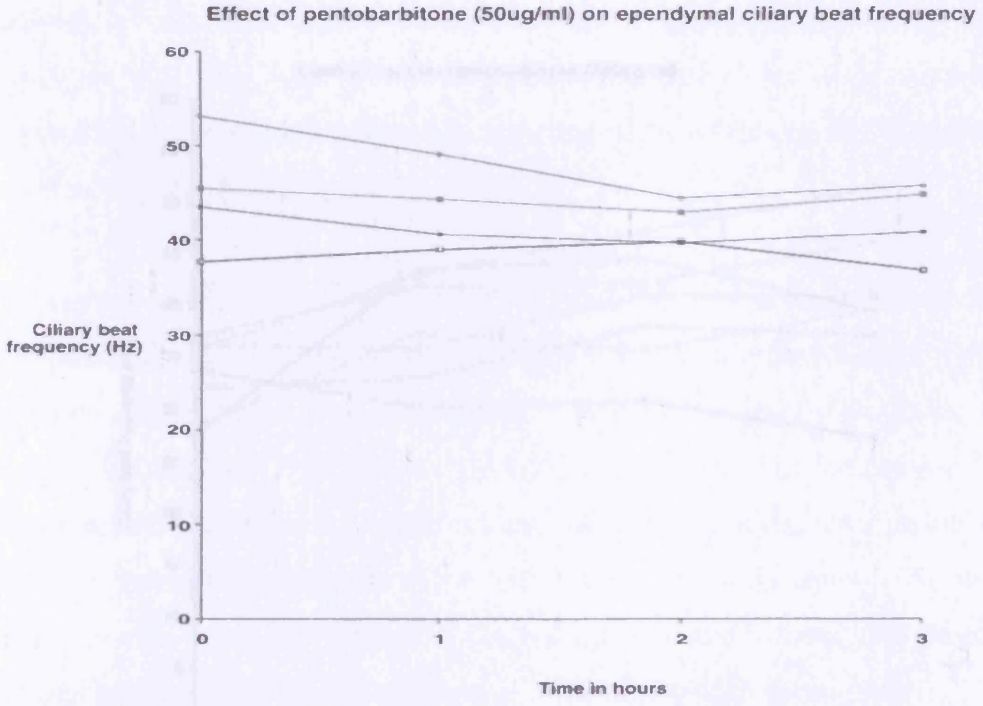


Figure 7.2d

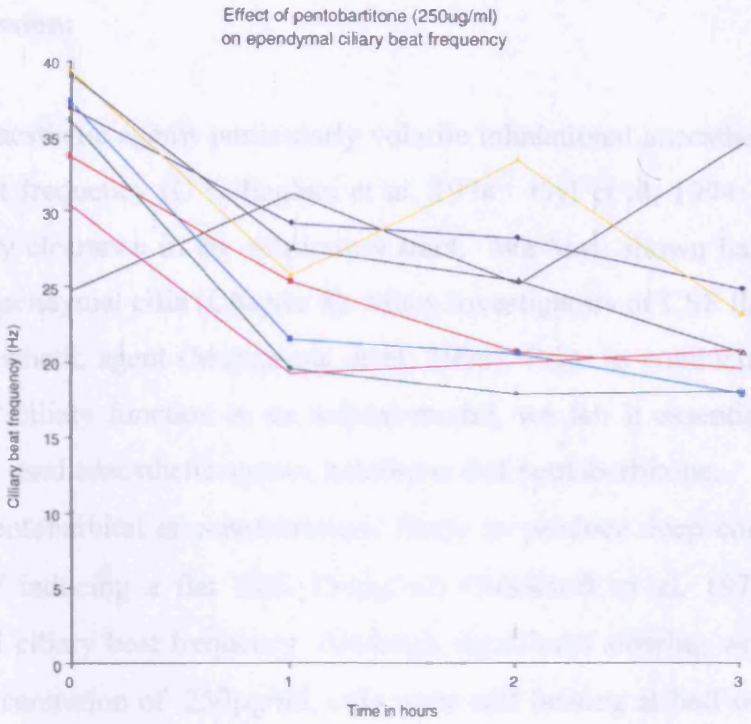


Figure 7.2e

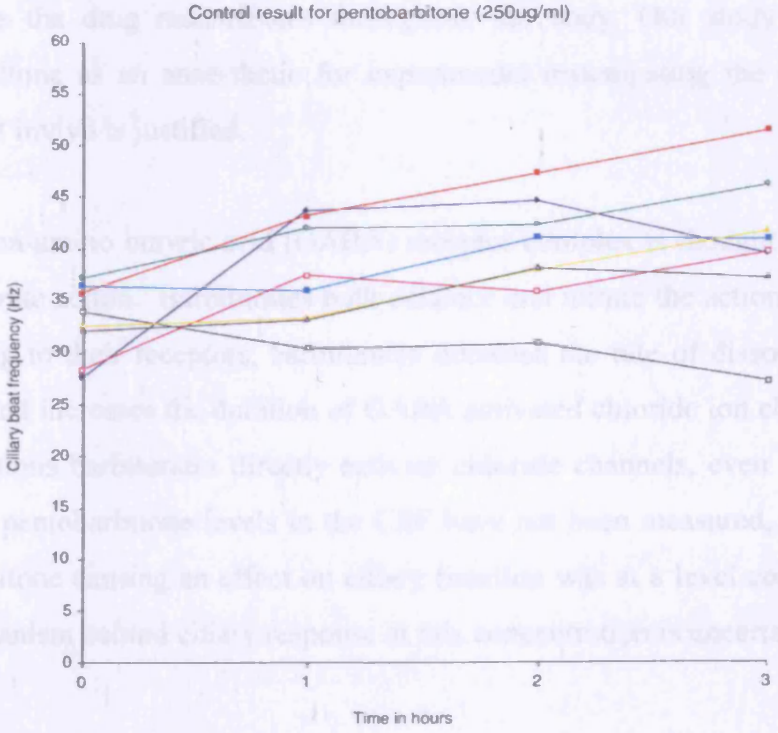


Figure7.2f

7.6 Discussion:

Certain anaesthetic agents particularly volatile inhalational anaesthetics may cause depression of ciliary beat frequency (O'Callaghan et al, 1994: Gyi et al, 1994: Raphael et al, 1996) and of mucociliary clearance in the respiratory tract. We have shown halothane to have a depressant effect on ependymal cilia (Chapter 8). Many investigators of CSF flow have used pentobarbitone as an anaesthetic agent (Marmarou et al, 1994). Prior to conducting investigations relating to ependymal ciliary function in an animal model, we felt it essential to study the effect of two commonly used anaesthetic agents, halothane and pentabarbitone.

Sodium pentobarbital at concentrations likely to produce deep coma (25μg/ml) and at levels capable of inducing a flat EEG (50μg/ml) (Rockhoff et al, 1979) had no effect on brain ependymal ciliary beat frequency. Although significant slowing was seen following incubation with a concentration of 250μg/ml, cilia were still beating at half of their initial rate and had a normal beat pattern. Highly perfused, relatively low volume tissues such as the brain equilabrate repidly with the high early concentrations of barbiturates, such as pentobarbitone, in the blood, resulting in induction of anaesthesia (Fragren & Avram, 1994). Drug levels then decrease quickly as the drug redistributes throughout the body. Our study suggests that the use of pentobarbitone as an anaesthetic for experiments investigating the effect of epedymal ciliary movement invivo is justified.

The gamma-amino butyric acid (GABA) receptor complex is thought to be the most likely sight of barbiturate action. Barbiturates both enhance and mimic the action of GABA (Olsen, 1988). By binding to their receptors, barbiturates decrease the rate of dissociation of GABA from its receptor and increases the duration of GABA activated chloride ion channel openings. At higher concentrations barbiturates directly activate chloride channels, even in the absence of GABA. Although pentobarbitone levels in the CSF have not been measured, the only concentration of pentobarbitone causing an effect on ciliary function was at a level considered lethal to humans. The mechanism behind ciliary response at this concentration is uncertain.

Chapter 8

The effect of Halothane on the beat frequency of brain ependymal cilia.

8.1 Summary:

Rapid removal of CSF adjacent to the brain surface is thought to facilitate the movement of toxins from the brain to the CSF for clearance. Anaesthesia with volatile anaesthetics is associated with central nervous system side effects. The aim of this study was to determine the effect of halothane on brain ependymal ciliary function.

Our ex-vivo model was used to measure ependymal ciliary beat frequency by high speed videophotography. The beat frequency of cilia, incubated at 37°C, were measured before and after exposure to various concentrations of halothane for 3 hours. Measurements were repeated after a washout period of one hour.

Exposure to halothane caused a significant reduction in ciliary beat frequency of 2% ($P=0.006$), 31% ($P<0.001$), and 32% ($P<0.001$) for halothane concentrations of 1.8%, 3.4% and 4.4%, respectively, compared to controls where beat frequency rose by 6%.

Following a one hour wash out period there was no significant difference between control samples and cilia that had been exposed to 1.8% ($P=0.5$) and 3.4% ($P=0.3$) halothane. The beat frequency of cilia exposed to 4.4% halothane had increased following the wash out period but cilia were still beating significantly more slowly than cilia from the control group ($P=<0.001$).

Halothane reversibly inhibits the rate at which ependymal cilia beat. It is unclear whether slowing of ependymal ciliary beat frequency is responsible for some of the secondary central nervous system effects of volatile anaesthetic agents.

8.2 Introduction:

The depressant effects of anaesthetic agents on the cilia of the ciliated protozoan, *Tetrahymena pyriformis*, were described by Nunn (1974). Inhalational anaesthetic agents have been shown to depress mucociliary transport in the respiratory tract invivo in animals and in man (Forbes, 1979; Lichtiger, 1975). Manawatu et al (1979) investigated the effects of halothane on ferret tracheal cilia by noting the presence or absence of ciliary activity at different sites and reported a reduction in the number of functioning cilia after prolonged exposure. Lee & Park (1980) found the activity of cilia from rabbit tracheal specimens was reduced by halothane and enflurane. Gyi et al, (1994) has shown human respiratory ciliary beat frequency may be reversibly inhibited by halothane and Raphael and colleagues (1996) found similar levels of ciliary depression following the exposure of respiratory cilia to isoflurane and enflurane.

No investigations have been performed on the effect of inhalational anaesthetics on brain ependymal ciliary beat frequency. Although ciliary structure is similar in different species, ciliary function and mechanisms of ciliary control are not uniform (Sleigh, 1966; Wanner, 1977) making it difficult to extrapolate results from tissue to tissue or from animal to animal.
We were keen to study the effect of halothane on ependymal ciliary function for two reasons. Firstly, it would provide further information on the central nervous effects of inhalational anaesthetics. The use of halothane is associated with a rise in intracranial pressure (Schettini, 1980).
Secondly, halothane is commonly used as an anaesthetic agent for animal experiments. Before using ependymal cilia, obtained following halothane anaesthesia, in physiological experiments it was essential to determine the effect of halothane on ependymal ciliary beat frequency.

The aim of this study was to investigate the effect of various concentrations of halothane on brain ependymal ciliary function and to determine whether any effect seen was reversible.

8.3 Methods:

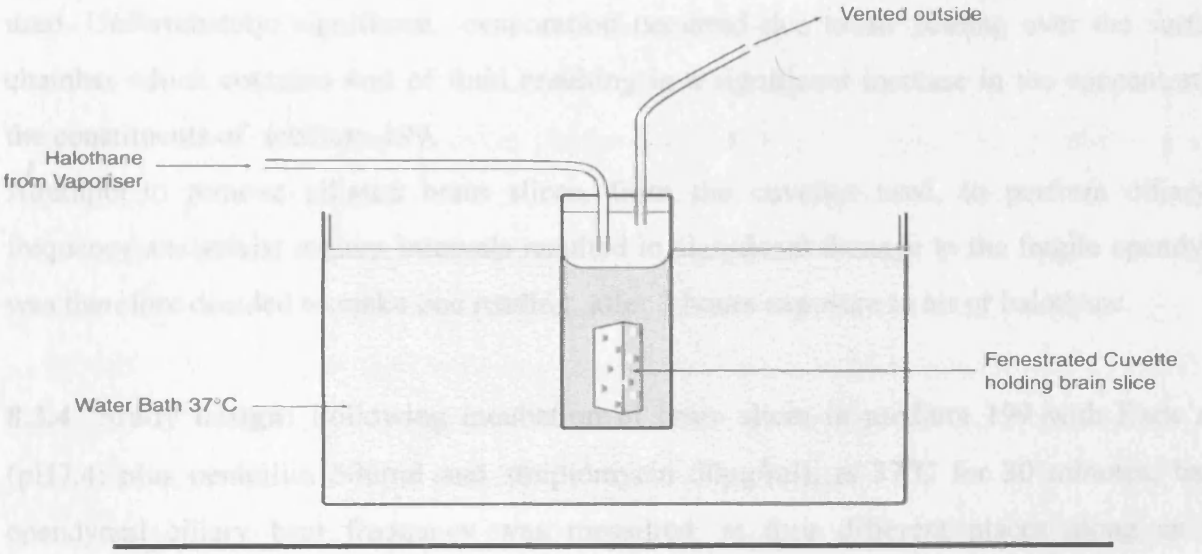
8.3.1 Sample preparation: The brains of Wistar rats (9-15 days of age) were dissected following sacrifice. Brain slices were prepared from the floor of the 4th ventricle of Wistar rats (10-15 days of age) immediately after sacrifice and mounted in a well containing 4 ml of medium 199 with Earle's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) and kept on ice until the study began. For the experiment the well was placed in a purpose built environmental chamber which was thermostatically controlled to keep the fluid surrounding the ependymal sample at 37°C. The chamber was humidified to 80% to prevent evaporation from the well during the 3 hour study period. Ciliary movement was observed using a x50 lens.

8.3.2 Measurement of ciliary beat frequency: Beating ciliated strips were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 400 frames per second. The camera allowed video sequences to be downloaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100um were studied.

8.3.3 Delivery of Halothane: A glass bottle was filled with 40ml of medium 199 with Earle's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml). Ciliated samples were placed in plastic cuvettes within the fluid. Multiple holes were cut in the cuvettes to allow free passage of medium 199 (figure 1). The use of cuvettes allowed the ciliated samples to be suspended in the same region of the bottles for all experiments. The bottle containing a ciliated sample was placed in a heating bath to maintain the temperature of the fluid medium at 37°C. The halothane vaporiser (Flurotec 3 plenum: Cyprane) was set to the required level and air passed through it at a flow of 1l/min (measured at the distal end of the tubing) taking halothane to the fluid medium surrounding the ciliated samples.

Figure 8.1

Experimental set up used to expose ciliated brain slices to halothane.



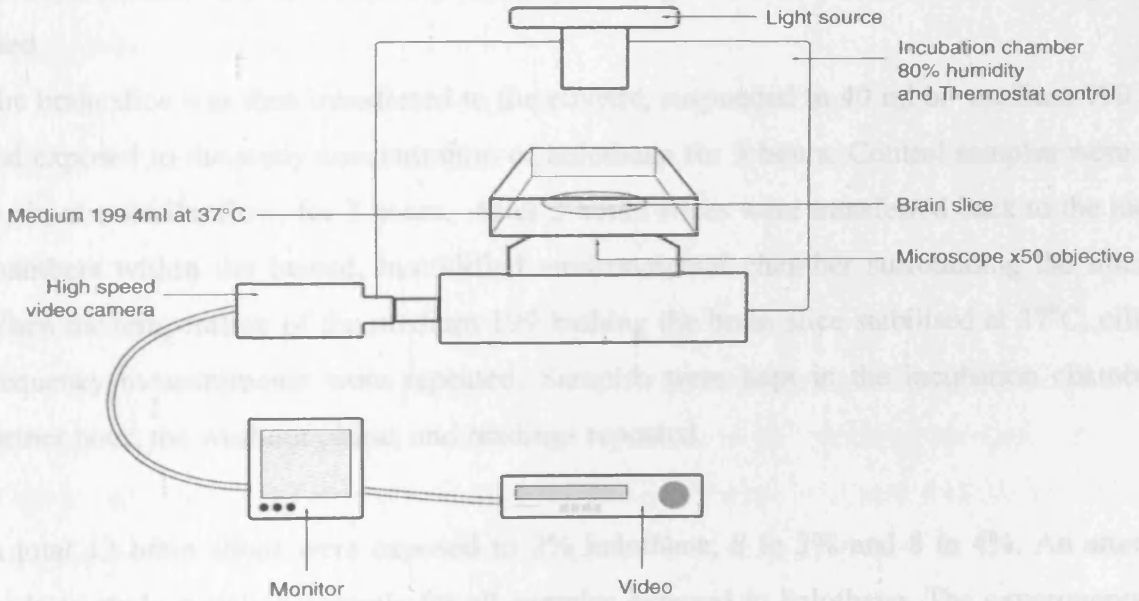


Figure 8.2 *System used to observe ependymal cilia and to measure beat frequency using the high speed video camera.*

The plastic tubing passed through the lid of the bottle containing medium 199 blowing halothane across the fluid surface. Gas exited the bottle via a separate tube and was vented outside.

Our experimental preparation did not allow frequent measurements to be made during halothane exposure. Attempts were made to measure ciliary beat frequency continually. An incubation system we have developed which allows continual observation of ciliary beat frequency was used. Unfortunately, significant evaporation occurred due to air passing over the surface of chamber which contains 4ml of fluid resulting in a significant increase in the concentration of the constituents of medium 199.

Attempts to remove ciliated brain slices, from the cuvettes used, to perform ciliary beat frequency analysis at regular intervals resulted in significant damage to the fragile ependyma. It was therefore decided to make one reading, after 3 hours exposure to air or halothane.

8.3.4 Study design: Following incubation of brain slices in medium 199 with Earle's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml), at 37°C for 30 minutes, baseline ependymal ciliary beat frequency was measured, at four different places along an intact ependymal strip. Disrupted ependymal strips or edges less than 100 microns in length were not used.

The brain slice was then transferred to the cuvette, suspended in 40 ml of medium 199 at 37°C, and exposed to the study concentration of halothane for 3 hours. Control samples were exposed to air, at a similar flow, for 3 hours. After 3 hours slices were transferred back to the incubation chambers within the heated, humidified environmental chamber surrounding the microscope. When the temperature of the medium 199 bathing the brain slice stabilised at 37°C, ciliary beat frequency measurements were repeated. Samples were kept in the incubation chamber for a further hour, the washout phase, and readings repeated.

In total 12 brain slices were exposed to 2% halothane, 8 to 3% and 8 to 4%. An attempt was made to study matched controls for all samples exposed to halothane. The experimental set up was identical for controls. Air rather than halothane was blown across the surface of the fluid medium. The total number of control brain slices was 11 for the 2% halothane experiment, 6 for the 3% concentration and 7 for the 4% concentration.

To determine the exposure of ciliated tissue to halothane a separate study was conducted to directly measure the equilibration time and levels of halothane in the plastic cuvettes. This was considered important as inhalational anaesthetic agents are volatile and adhere to plastics.

Equilibration times were determined for halothane in the fluid immediately adjacent to the brain slices by gas chromatographic analysis (table 1). Halothane at 3 different vaporiser settings was delivered to the bottle containing medium 199 and brain slices for 3 hours. An Flurotec type 3 vaporiser was used for all experiments. Aliquots of 200μl were taken, using a glass syringe, from the area next to where the brain slices were placed at 1, 2 and 3 hours after exposure to Halothane. A further aliquot was taken 1 hour after the vaporiser was switched off. The aliquots were mixed immediately with n-heptane (100μl) in glass vials on ice. The vials were sealed to prevent evaporation before analysis. The non-aqueous phase was injected onto a 30-m DB17 megabore column under the following conditions: injection temperature 100°C; oven temperature 90°C; flame ionisation detector at 100°C. The amount of anaesthetic in each sample was determined from the peak against a standard curve for the agent.

These samples were then analysed by gas chromatography (Perkins Elmer 8410) with a DB-17 column, using helium as carrier gas and detection by flame ionisation that had been standardised for halothane (Rutledge, 1963).

For halothane standard curves dilutions of liquid halothane were made with Heptane in glass ampoules. Measurements of halothane concentration were carried out in duplicate for each concentration studied.

8.4 Statistics:

An analysis of variance was performed using two factors: halothane concentration and time. The effect of halothane concentration was assessed relative to the variation between slices while the effect of time was assessed relative to the within slice variation. A series of contrasts were fitted. For halothane concentrations each concentration was compared with control. For time each possible pair of time points was compared. A separate analysis of variance for each of these combinations of contrasts was performed.

8.5 Results:

The system established to expose brain slices to halothane took up to 2 hours to reach a plateau level (table 1). The mean readings determined by gas chromatography when stable levels were reached after 120 minutes were, 1.8%, 3.4% and 4.4% halothane.

Stopping halothane after 180 minutes rapidly reduced the halothane concentration of the solution.

The effect of exposure to air controls and 1.8%, 3.4%, and 4.4% halothane and the effect of wash out period are shown in figures 8.3a-d.

There was a highly significant interaction between concentration and time ($P<0.001$).

Compared to controls significant suppression of ciliary beat frequency occurred after exposure to halothane concentrations of 1.8% (($P=0.006$), 3.4% ($P=<0.001$), and 4.4% ($P=<0.001$) for 3 hours. This corresponds to a mean increase of 6% in the ciliary beat frequency of controls compared to a 2% ,31% and 32% reduction in ciliary beat frequency following exposure to, respectively, 1.8%, 3.4% and 4.4% halothane.

Following a one hour wash out period there was no significant difference between control samples and cilia that had been exposed to 1.8% ($P=0.5$) and 3.4% ($P=0.3$) halothane. Cilia exposed to 4.4% halothane had increased in frequency following one hours wash out but were still beating significantly more slowly than cilia from the control group ($P=<0.001$).

Table 8.1: Percentage halothane in medium 199 bathing ciliated brain slices during the study period. Halothane was blown across the medium 199 containing the brain slices for 180 minutes. A further reading was taken after a 1 hour wash out period (240 minutes).

	time in minutes			
	60	**120**	**180**	**240**
% halothane: study 1	1.6	1.7	1.8	0.5
% halothane: study 2	2.8	3.5	3.3	0.3
% halothane: study 3	3.0	4.5	4.3	0.4

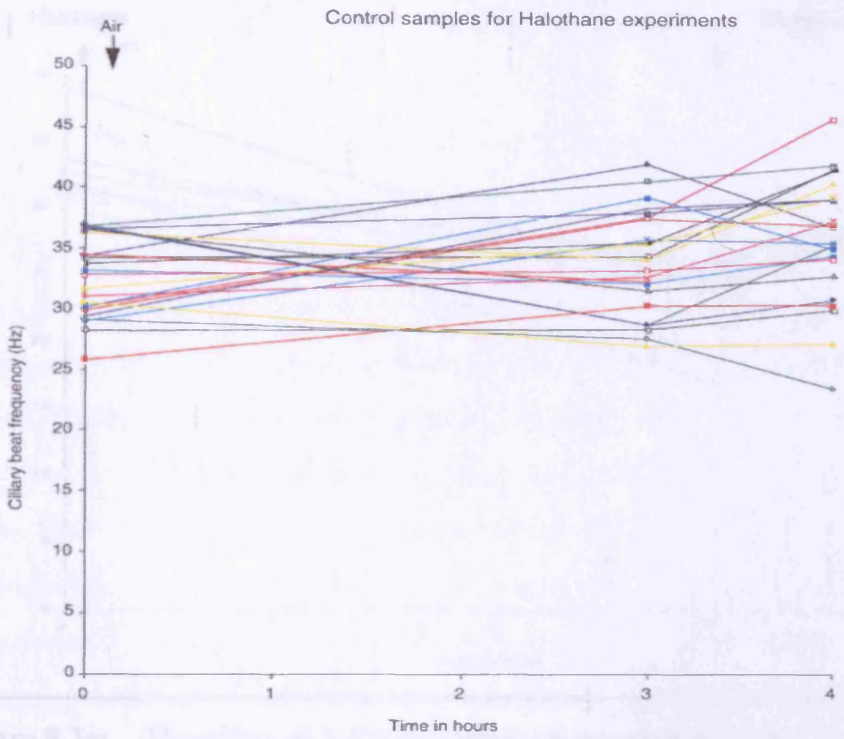


Figure 8.3a: Ciliary beat frequency of ependymal cilia exposed to air.

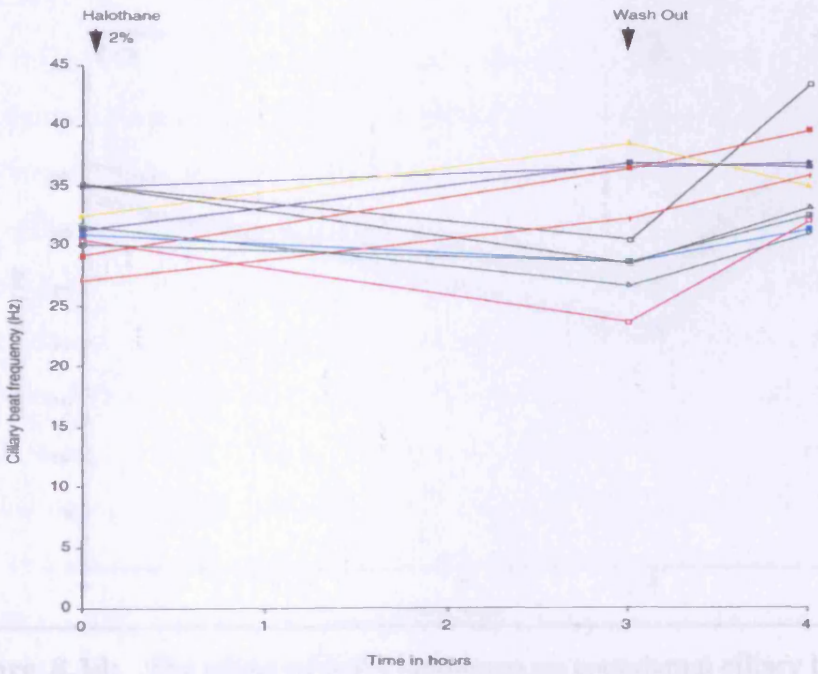


Figure 8.3.b: Effect of 1.8% halothane on ependymal ciliary beat frequency.

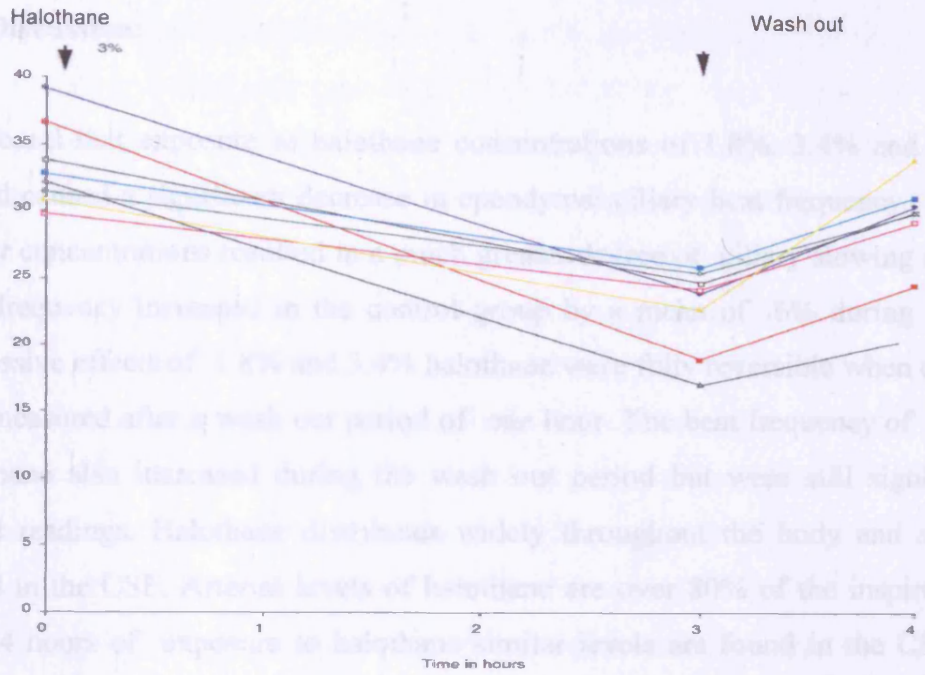


Figure 8.3c: The effect of 3.4% halothane on ependymal ciliary beat frequency

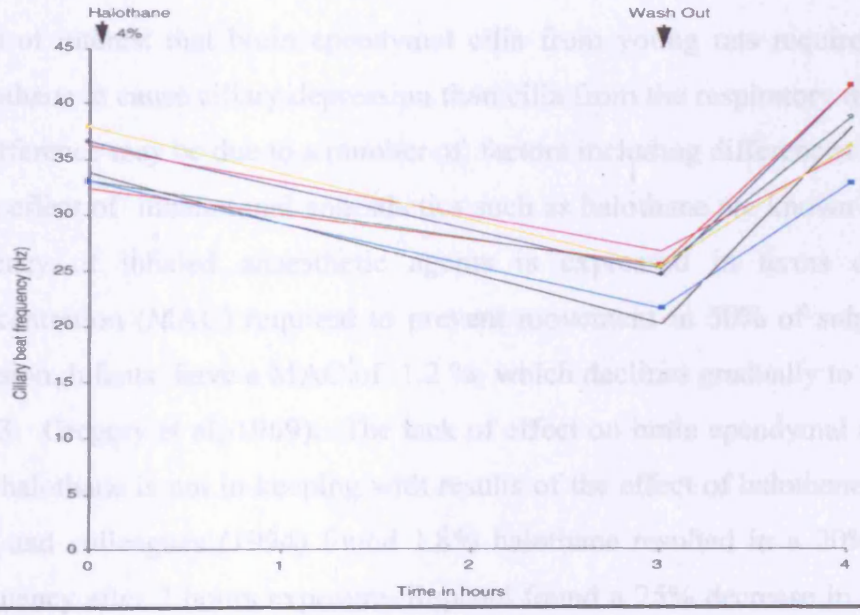


Figure 8.3d: The effect of 4.4% halothane on ependymal ciliary beat frequency.

8.6 Discussion:

We found that exposure to halothane concentrations of 1.8%, 3.4% and 4.4% over a 3 hour period caused a significant decrease in ependymal ciliary beat frequency. Exposure to the two higher concentrations resulted in a much greater degree of ciliary slowing of up to 35%. Ciliary beat frequency increased in the control group by a mean of 6% during the same time. The depressive effects of 1.8% and 3.4% halothane were fully reversible when ciliary beat frequency was measured after a wash out period of one hour. The beat frequency of cilia exposed to 4% halothane also increased during the wash out period but were still significantly slower than initial readings. Halothane distributes widely throughout the body and significant levels are found in the CSF. Arterial levels of halothane are over 80% of the inspired concentration and after 4 hours of exposure to halothane similar levels are found in the CSF (Stoelting, 1973). Following cessation of a halothane anaesthetic, CSF halothane decreases significantly more slowly than arterial halothane levels. It is likely, therefore, that halothane administered during anaesthesia may induce slowing of ependymal cilia and should not be used in studies which relate to the investigation of ependymal ciliary activity.

It is of interest that brain ependymal cilia from young rats required higher concentrations of halothane to cause ciliary depression than cilia from the respiratory tract of human adults. Such a difference may be due to a number of factors including differences in species, tissue or age.
The effect of inhalational anaesthetics such as halothane are known to be age dependent. The potency of inhaled anaesthetic agents is expressed in terms of the minimum alveolar concentration (MAC) required to prevent movement in 50% of subjects in response to a skin incision. Infants have a MAC of 1.2 % which declines gradually to 0.8 in adults (Lerman et al, 1983: Gregory et al, 1969). The lack of effect on brain ependymal cilia following exposure to 2% halothane is not in keeping with results of the effect of halothane on respiratory epithelium. Gyi and colleagues (1994) found 1.8% halothane resulted in a 20% reduction in ciliary beat frequency after 2 hours exposure. Raphael found a 25% decrease in frequency at one hour and 40% after two hours exposure to 2.3% halothane.
Significant variations in the depressive effect of inhalational anaesthetics has been found depending on the tissue preparation used. For example, Raphael et al (1996) found the depression of ciliary beat frequency using nasal turbinate preparations of 33%, 25% and 33%

with 3 MAC of halothane, enflurane and isoflurane respectively differed from that found with nasal brushings, where reductions of 28, 10 and 2% were seen. Our tissue is more similar to the turbinate preparation in that relatively intact strips of ciliated ependyma attached to neuronal tissue are used. Using rabbit trachea, Lee found reductions in ciliary beat frequency using 3MAC of halothane of 22%. (Lee, 1980). In a study of tracheal mucus transport in dogs using radioactive droplets and scintillation counters, Forbes found a mucus transport rate of 20% of controls at around 3 MAC of halothane and enflurane (Forbes, 1976 and 1977).
Forbes measured tantalum bronchographic clearance in dogs anaesthetised with halothane and found that 1.2 MAC of halothane administered for two hours delayed the clearance of tantalum for more than four hours after the termination of anaesthesia (Forbes, 1979). This may represent additional effects of the anaesthetics on the mucus itself. Alternatively, even relatively short periods of ciliary beat frequency depression which produce mucus stasis could alter the physical properties of the mucus and therefore impair mucus transport for a longer period.

The recovery characteristics following general anaesthesia are dependent on the physical properties of the anaesthetic agent. Halothane has a high lipid/water solubility coefficient and may take longer to diffuse out from the fat soluble tissues of the preparation at the higher concentration. Raphael and colleagues (1996) found the return to baseline values of ciliary beat frequency following exposure to 3 MAC of halothane, enflurane and isoflurane for one hour took one hour in the cases of enflurane and isoflurane and one and a half hours in the case of halothane.

Inhalational anaesthetics produce a decrease in the cerebral metabolic rate, which is maximal when the functional activity of the brain cell stops, that is, when the EEG is isoelectric. Hanson et al (1989) during halothane and isoflurane anaesthesia in rats, demonstrated a strong correlation between cerebral metabolic rate and cerebral flow within individual anatomical regions.

Neurosurgeons are reluctant to use volatile anaesthetics, such as halothane, in brain surgery even when intracranial pressure is normal. The fact that inhalational anaesthetics are cerebral vasodilators explains why they may increase intracranial pressure, especially when intracranial compliance is reduced. The greatest reduction in cerebro vascular resistance is produced by

halothane. Low concentrations of inhalational anaesthetics reduce the efficacy of auto regulation, which is finally abolished at high concentrations. During halothane anaesthesia in cats, auto regulation is lost at concentrations below 1 MAC (Todd and Drummond, 1984). Similar results were also found in experiments on baboons (Brussel et al, 1991). By hyperventilation it is possible to blunt, abolish, or even reverse the cerebral vaso dilation that occurs when volatile anaesthetics are administered. This only applies predictably to the normal brain and may not be so in the presence of intracranial pathology. For example, following a cryogenic brain injury in rabbits, intracranial pressure increased during both halothane and isofluorane anaesthesia, and the increase could not be prevented by hyperventilation (Ringaert et al, 1988).

Schettini (1980) following measurement of brain water and electrolyte concentration concluded that halothane also induces metabolic brain oedema. The movement of CSF by ependymal cilia is in the predicted direction of CSF flow and may serve to clear metabolites and toxins from the brain by improving the diffusion gradient between neuronal tissue and CSF. The relationship between such a role and metabolic brain oedema is speculative.

The cellular mechanisms responsible for the activity of halothane remain to be clarified. Halothane and isofluorane alter the tension of isolated cerebro vascular smooth muscle, a response that is further modulated by the CO_2 tension (Reinstrup et al 1994). The vaso dilatory action of halothane is thought by some to involve nitric oxide (NO) (Coenig et al 1993) but by others to be independent of it (Hart et al, 1992). Indeed, halothane reduces the vaso dilatory effect of NO, (Jing et al 1993) an effect that ought to attenuate vaso dilatation.
It is generally agreed, however, that halothane depletes intracellular (sarcoplasmic) Ca^{2+} stores (Yamamoto et al, 1993; Wheeler et al, 1994). Another interesting finding is that volatile anaesthetics produce hyperpolarisation in snail neurones (Yost et al, 1993)

The slowing of respiratory cilia by inhalational anaesthetics may lead to mucous retention predisposing to post operative chest infection. The clinical effects of acute slowing of brain ependymal cilia is unknown. It is yet to be determined whether slowing of ependymal ciliary beat frequency is responsible for some of the secondary central nervous system effects of these agents.

Chapter 9

The effect of adrenergic agonists on ependymal ciliary beat frequency

9.1 Summary:

A number of studies have demonstrated the stimulatory effect of beta agonists on respiratory cilia. This study was undertaken to determine the effect of beta adrenergic agents on ependymal ciliary function. The study was conducted in a blind fashion.

Ependymal slices were incubated, at 37°C, with a range of salbutamol concentrations (10^{-8} to 10^{-3}M) and beat frequency measured using high speed videophotography. In case ependymal cilia were beating at their maximal rate at 37°C the study was repeated at 30°C. The effect of a non selective beta sympathomimetic, isoprenaline, forskolin and forskolin with a phosphodiesterase inhibitor (IBMX) were also determined.

There was no significant change in ependymal ciliary beat frequency, compared to control, with any concentration of salbutamol (30°C, P=0.28: 37°C, P=0.57). No change in ciliary beat frequency was observed after incubation of ependymal cilia with isoprenaline (P=0.17) or forskolin (P=0.35). The combination of forskolin and IBMX caused ciliary slowing over the 3 hour study period (P=0.001).

Cilia incubated at 37°C (mean (SD) beat frequency = 36.8 (6.4) Hz) beat significantly (P = 0.001) more quickly than cilia beating at 30°C (mean (SD) beat frequency = 27.5 (2.9) Hz).

These results show that ependymal cilia do not respond to beta adrenergic stimulation.

9.2 Background:

Respiratory ciliary beat frequency has been shown to increase in human (Clarke et al, 1983: Konietzko et al, 1983) and animal (Blair & Woods, 1969: Iravani & Melville, 1976: Van As, 1974: Yanaura et al, 1979: Verdugo et al, 1980: Lopez-Vidriero et al, 1985: Hybbinette & Mercke, 1982) tissue following stimulation with adrenergic and cholinergic drugs. The increase in ciliary beat frequency is related to an increase in cAMP.

Cilia of other species often respond differently to stimulation by Ca^{2+} and cAMP. Alteration of intracellular calcium either modifies the wave form of sperm or completely inhibits sperm mobility (Gibbons & Gibbons, 1980). The wave form of *Chlamyomonas* flagella may be modified to reverse its swimming direction (Hyams & Borisy, 1978). Cyclic AMP stimulates the activity of sperm (Tash & Means, 1983) but inhibits the in-vitro motility of Chlamydomonas (Hasegawa et al, 1987).

The aim of this study was to determine the effect of beta-adrenergic stimulation on the beat frequency of ependymal cilia.

9.3 Methods:

9.3.1 Sample preparation: The brains of Wistar rats (9-15 days of age) were dissected following sacrifice. Brain slices from the floor of the 4th ventricle were prepared immediately after sacrifice and mounted in a well containing 4 ml of medium 199 with Earl's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) and kept on ice until the study began. For the experiment the well was placed in a purpose built environmental chamber which was thermostatically controlled to keep the fluid surrounding the ependymal sample at 37°C. The chamber was humidified to 75-80% to prevent evaporation from the well during the 3 hour study period. Ciliary movement was observed using an inverted Nikon microscope and a x50 lens.

9.3.2 Measurement of ciliary beat frequency: Beating ciliated strips were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 400 frames per second. The camera allowed video sequences to be downloaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study, ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100μm were studied.

9.3.3 Exposure to salbutamol: Ciliated ependymal strips were incubated, in medium 199, for 30 minutes at a temperature of 37°C. Base line readings of ciliary beat frequency were made and the surrounding cell culture fluid exchanged for one of the study concentrations of salbutamol, preheated to 37°C. Ciliary beat frequency was measured after 10, 30 and 60 minutes. Concentrations of salbutamol chosen were 10^{-3} (n= 6), 10^{-4} (n= 6), 10^{-5} (n= 6), 10^{-6} (n= 4), 10^{-7} (n=3) and 10^{-8} M(n= 3). Ten control edges were also studied.

Interim results showed little increase in beat frequency following incubation with any of the concentrations of salbutamol chosen. A number of other investigations into the effect of beta agonists on ciliary beat frequency were conducted at low, unphysiological, temperatures. In case ependymal cilia were beating at their maximal rate at 37°C the study was repeated at 30°C. This lower temperature was chosen as it may be encountered clinically by patients with severe head injuries who have their body temperature reduced in an attempt to decrease further brain damage.

Ciliated ependymal strips were incubated, in medium 199, for 30 minutes at 30°C. The cell culture fluid was exchanged for one of the study concentrations of salbutamol, preheated to 30°C.

Concentrations of salbutamol chosen were 10^{-3} (n=6), 10^{-4} (n= 6), 10^{-5} (n=6), 10^{-6} (n=6), and 10^{-7}M (n=6). Eight control edges were also studied.

9.3.4 Exposure to isoprenaline:

Ciliated ependymal slices were challenged with the non selective beta agonist isoprenaline, at 37°C, using a similar experimental design. Concentrations of isoprenaline chosen were 10 and 100μM. Ten control slices were also studied.

9.3.4 Exposure to forskolin and IBMX:

Forskolin has the ability to directly activate all AC isoforms independently of receptor-G protein interactions. It is one of the most potent stimulators of cAMP production known.

Ciliated ependymal strips were incubated, in medium 199, for 30 minutes in the environmental chamber at a temperature of 37°C. Base line readings of ciliary beat frequency were made and the surrounding cell culture fluid exchanged for one of the study concentrations of forskolin (0, 1 and 10μmol/ml) preheated to 37°C. Ciliary beat frequency was measured after 5, 30 and 60 minutes.

Separate ependymal slices were then exposed to a combination of forskolin (10μM) and IBMX (isobutly methyl xanthine) (500μM) or control. An identical experimental design was used as for the forskolin study. IBMX is a non selective phosphodiesterase inhibitor. Thus any increase in cAMP would be maintained, due to a lack of cAMP breakdown, resulting in maximum levels of intracellular cAMP.

9.4 Statistics:

A separate analysis of variance was performed for each temperature (30°C and 37°C). A split unit analysis of variance was performed on the concentration of salbutamol and time (30, 40, 60, 90 minutes). The effect of concentration was assessed relative to the between slice variation, while the effect of time and the interaction between concentration and time were assessed relative to the within slice variation. The effects of salbutamol concentration and time were divided into a linear trend with concentration and deviations from the linear trend.

For the isoprenaline study the slope of the regression line, for each slice, relating to frequency with time was calculated. A one way analysis of variance was performed on these slopes to compare the three isoprenaline concentrations (0, 10, 100 mmol/L). A split unit analysis of variance was also performed. The effect of isoprenaline concentration was assessed relative to the variation between slice while the effect of time and the interaction between concentration time was assessed relative to the variation within slices.

The effect of the concentration of forskolin on ciliary beat frequency was estimated by comparing the rate of change in frequency over time. The average slopes for each concentration of forskolin were compared using both parametric (one way analysis variance) and non-parametric (Kruskal Wallis) tests (Altman, 1991).

For the study of IBMX and forskolin combined, against control the slope of the regression line of frequency versus time was calculated. They were compared using an unpaired t-test and a Mann Whitney U-test.

9.5 Results:

Graphs of the results from each experiment are shown in figures 9.1.a-k.

At a temperature of 30°C there was no significant linear trends with concentration of salbutamol (P=0.28), no significant linear trend with time (P=0.52) and no significant interaction between these linear trends (P=0.21). The beat frequency of one slice decreased by half following incubation at 30^{oC} with 10^{-4}M salbutamol, presumably due to trauma. A similar decrease in frequency followed by recovery of beat frequency was observed for two slices incubated at 37°C with 10^{-3}M salbutamol. The reason for this is unclear.

At a temperature of 37°C there was no significant linear trend with concentration of salbutamol (P=0.57), a significant linear trend with time (P 0.02) and no significant interaction between these linear trends (P=0.26).

Cilia incubated at 37°C (mean (SD) beat frequency = 36.8 (6.4) Hz) beat significantly (P = 0.001) more quickly than cilia beating at 30°C (mean (SD) beat frequency = 27.5 (2.9) Hz).

No significant difference was shown between the mean slopes for the three concentrations used in the isoprenaline study (p=0.17). There was also no significant trend with isoprenaline concentration (p=0.2). There was a highly significant linear trend with time (p<0.001). There was no significant effect of isoprenaline (p=0.08) and no significantly different linear trends with time in the three isoprenaline groups (p=0.08).

The one-way analysis of variance suggested that the mean slope of the regression line of frequency versus time depended on the concentration of forskolin (p=0.05). There was also evidence that the association with concentration was linear (p=0.04).
Using the Kruskal Wallis test, which makes less assumptions about the distribution of slopes, results were similar to the analysis of variance. There was a statistically significant difference in the median slopes (p=0.03) and evidence of a linear trend with concentration (p=0.01). Thus, from both analyses, there is some evidence that increasing concentrations of forskolin linearly decrease the rate of change of ciliary beat frequency.

The combination of IBMX and forskolin caused a significant slowing of beat frequency with time. The mean slope in the control group was significantly different from that in the IBMX-forskolin group (p=0.0003). Using the Mann Whitney U-test test there was also evidence of the difference between the groups (p=0.02).

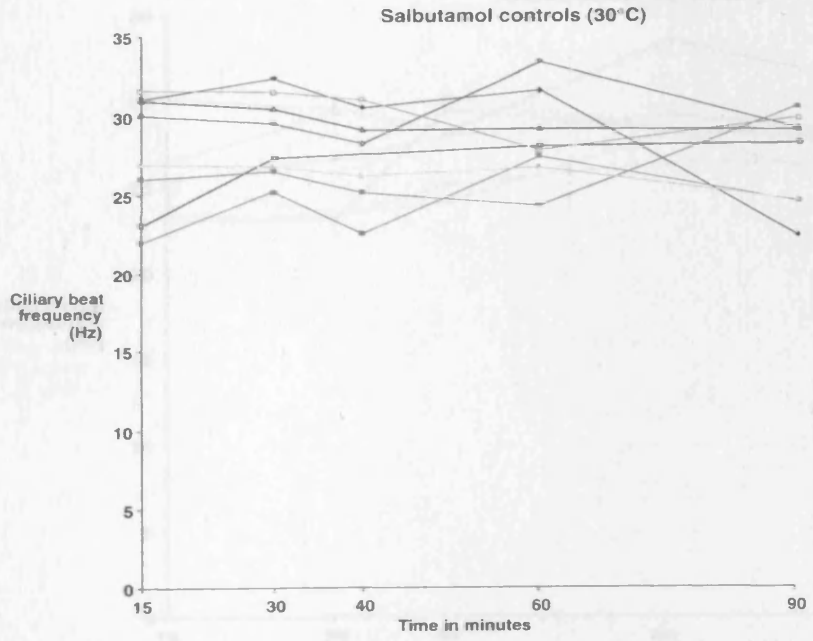
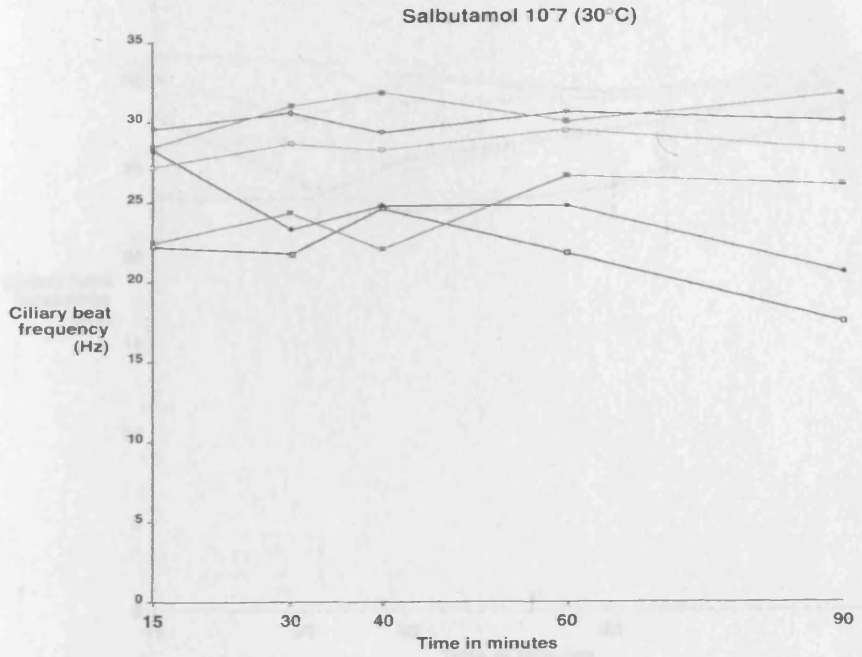


Figure 9.1.a:



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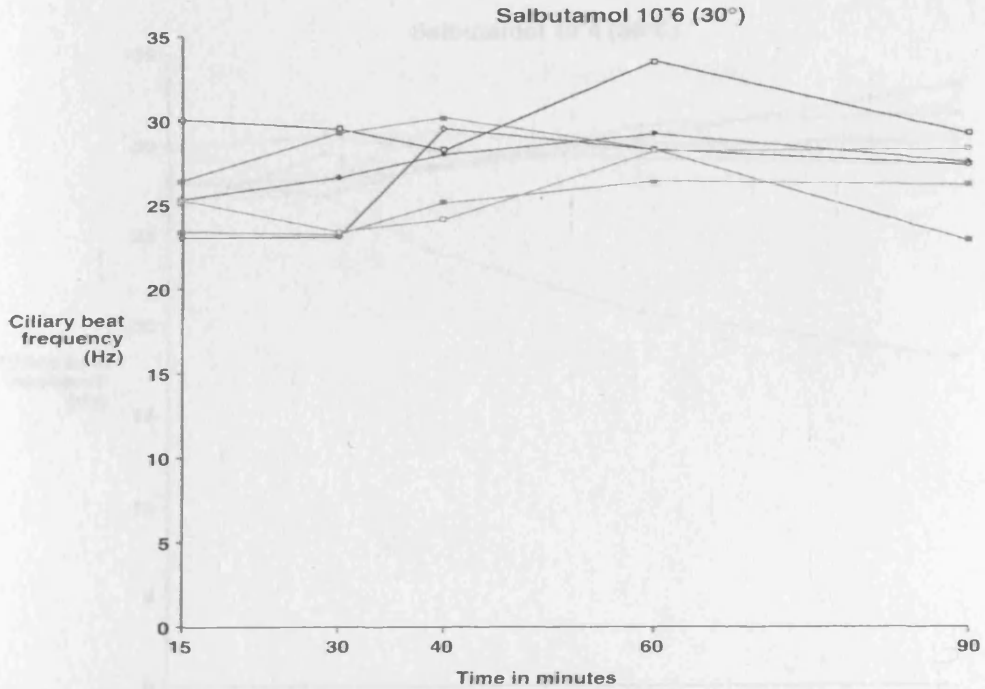

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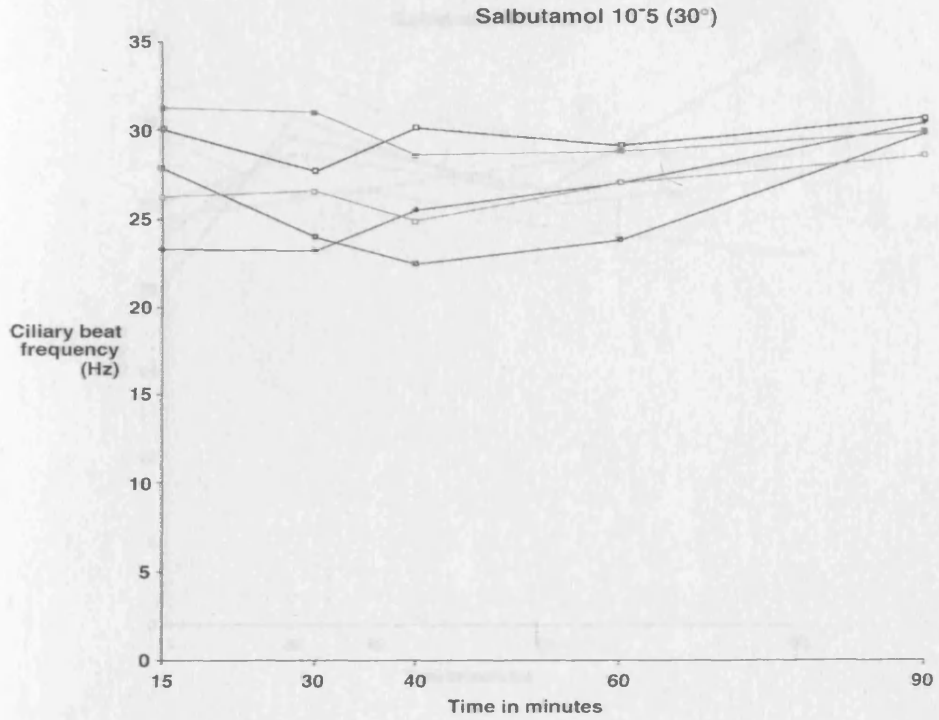

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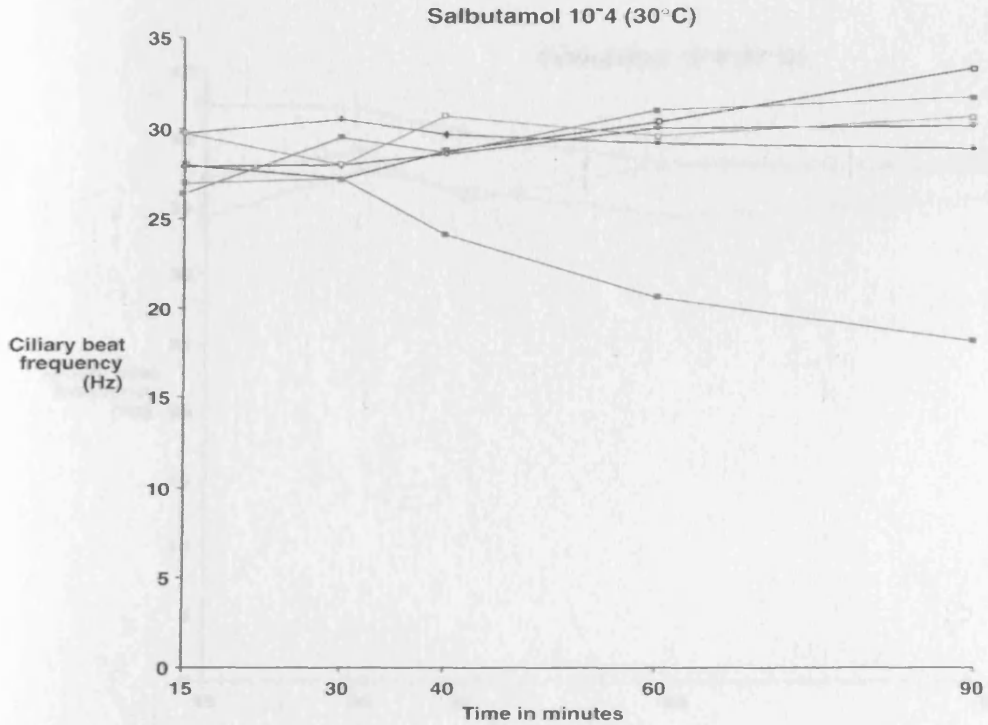

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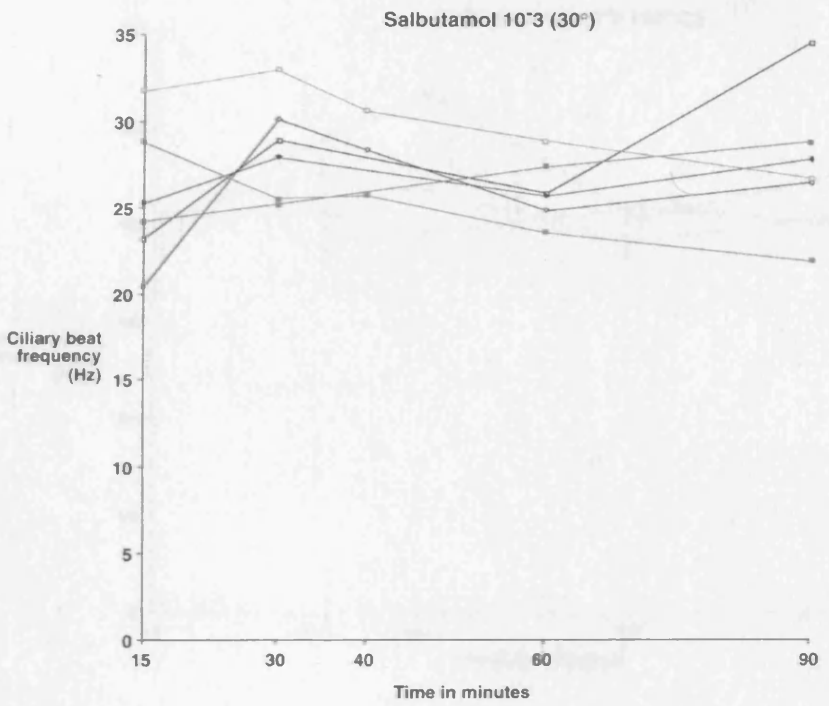

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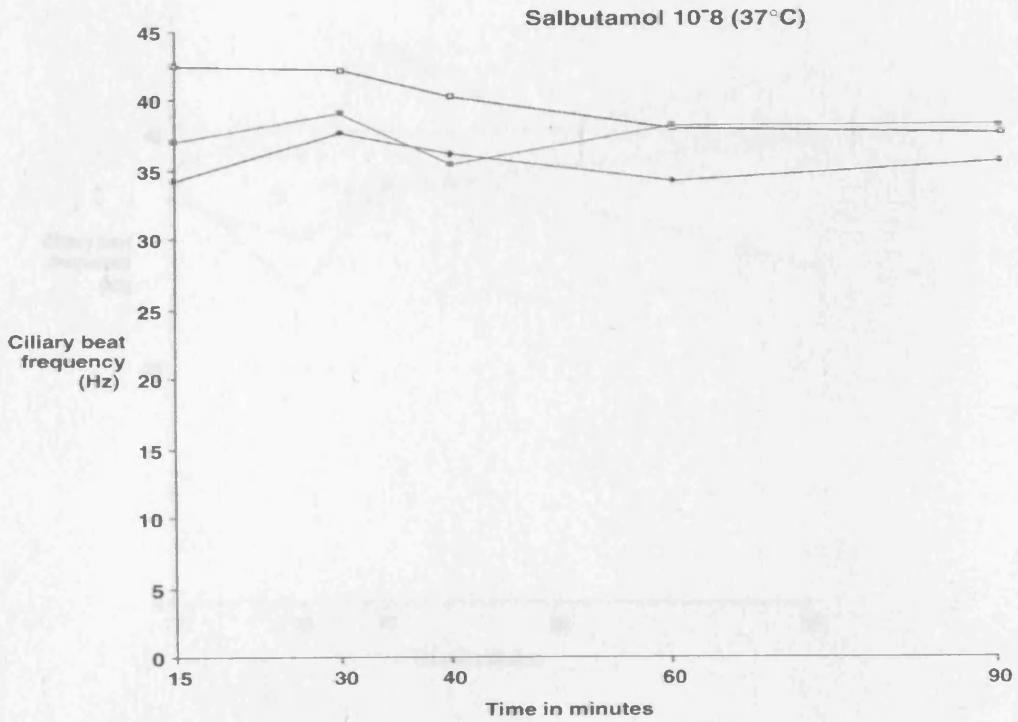

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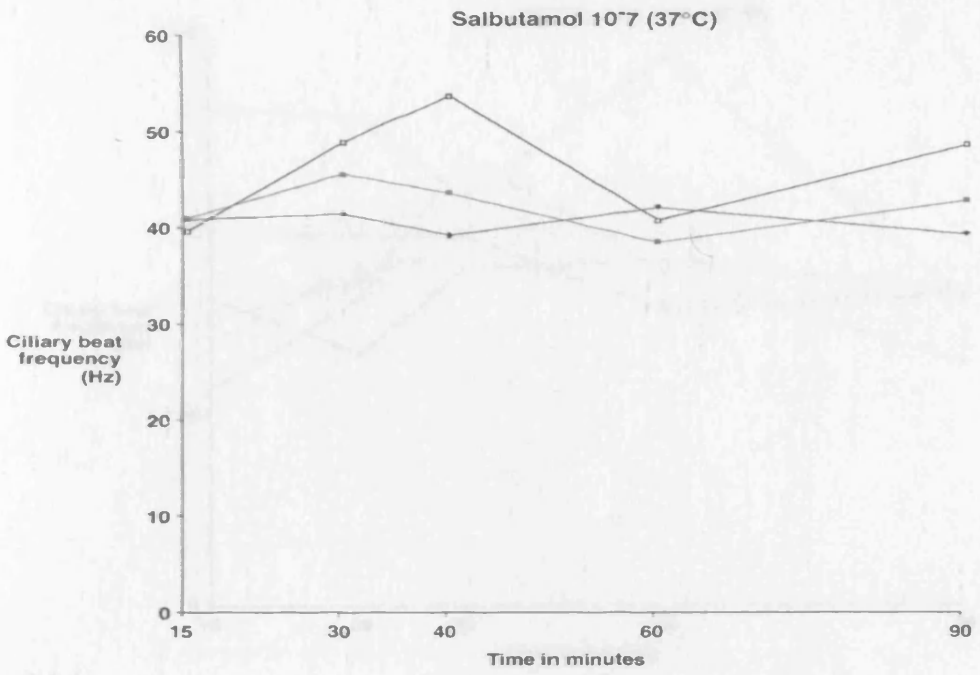

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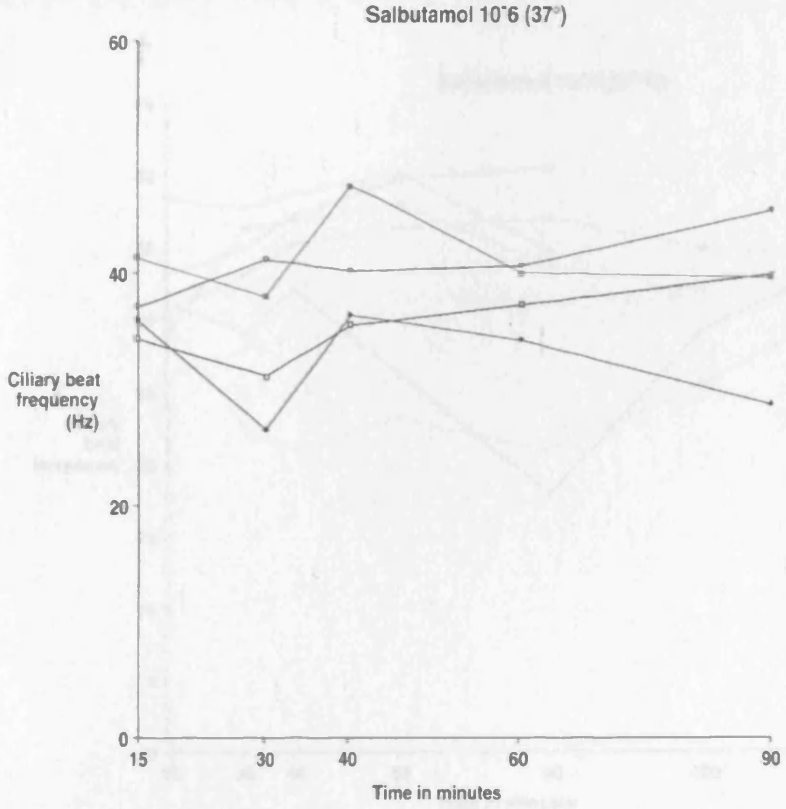

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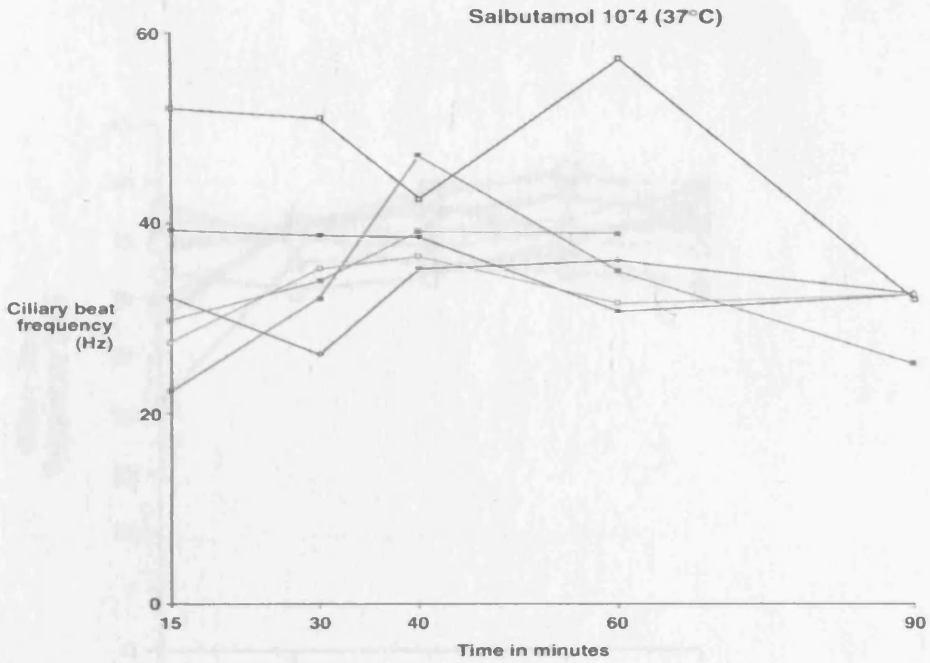

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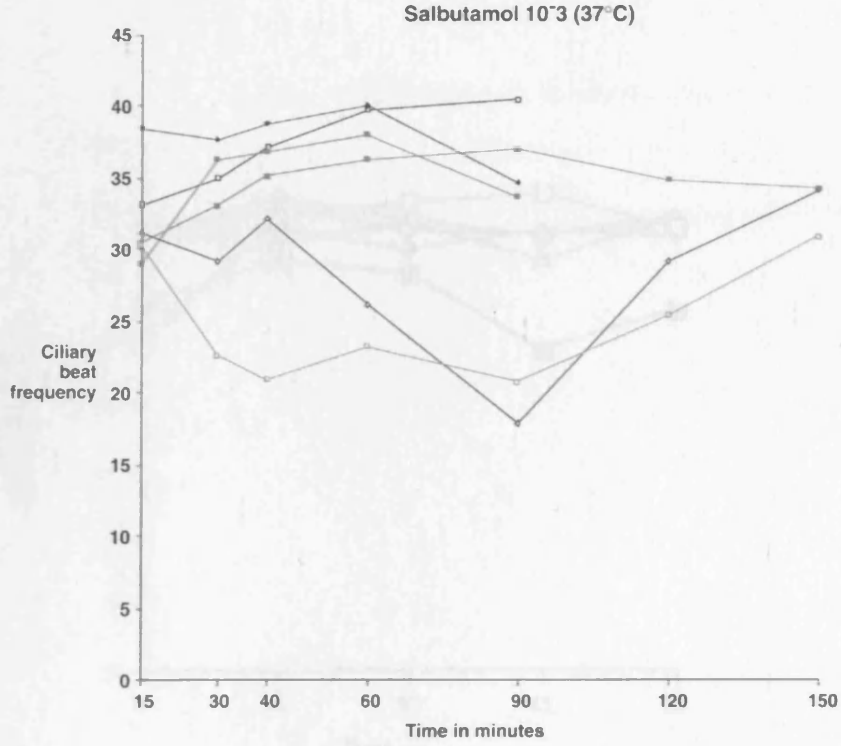

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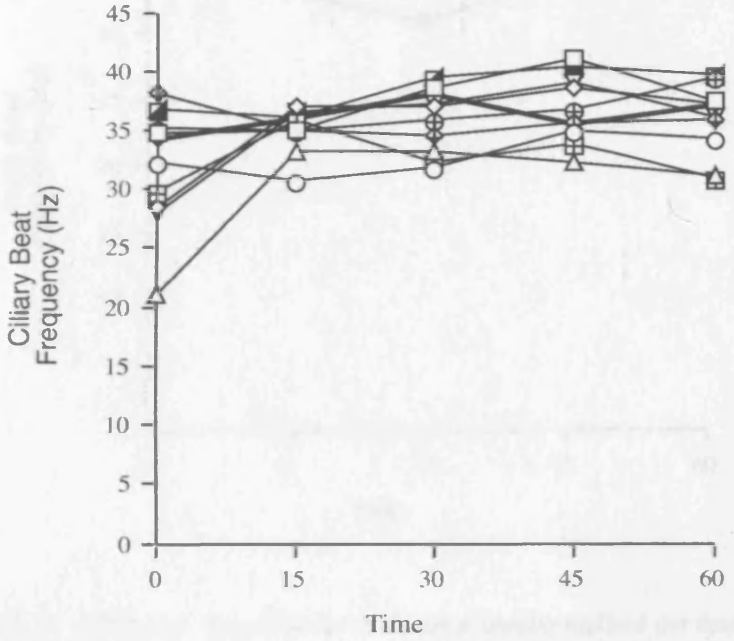

Figure 9.2.a *Control experiments run in parallel with isoprenaline experiments*

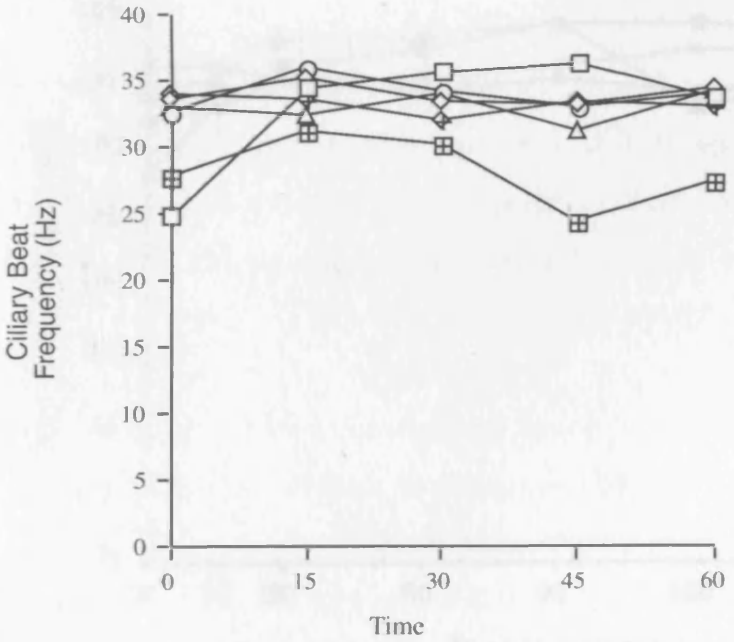

Figure 9.2.b *Effect of incubation in 10μmol isoprenaline on ependymal ciliary beat frequency.*

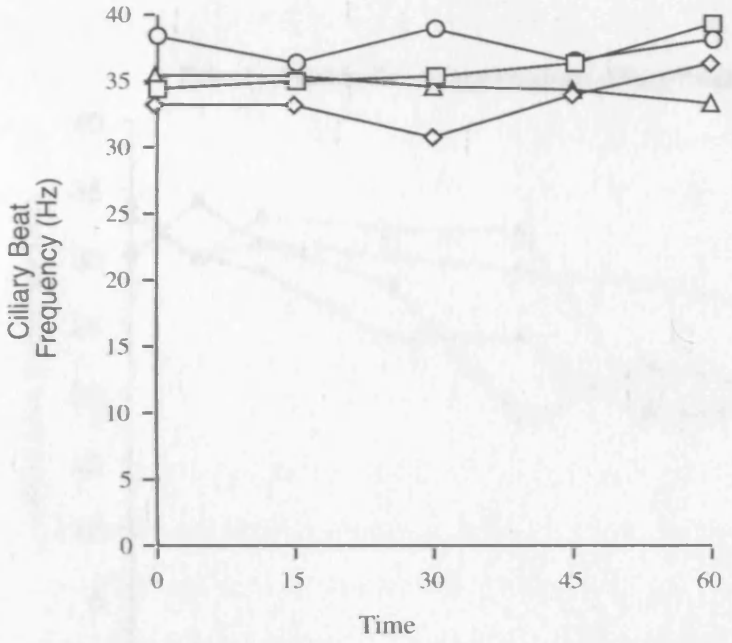

Figure 9.2.c *Effect of incubation in 1 μmol isoprenaline on ependymal ciliary beat frequency.*

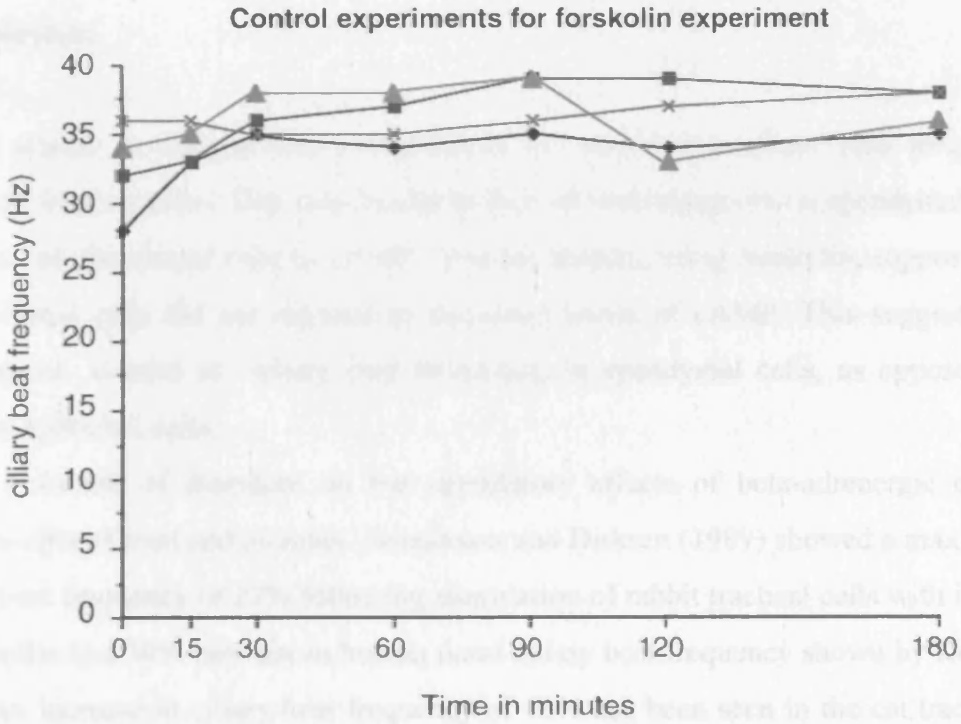

Figure 9.4.a

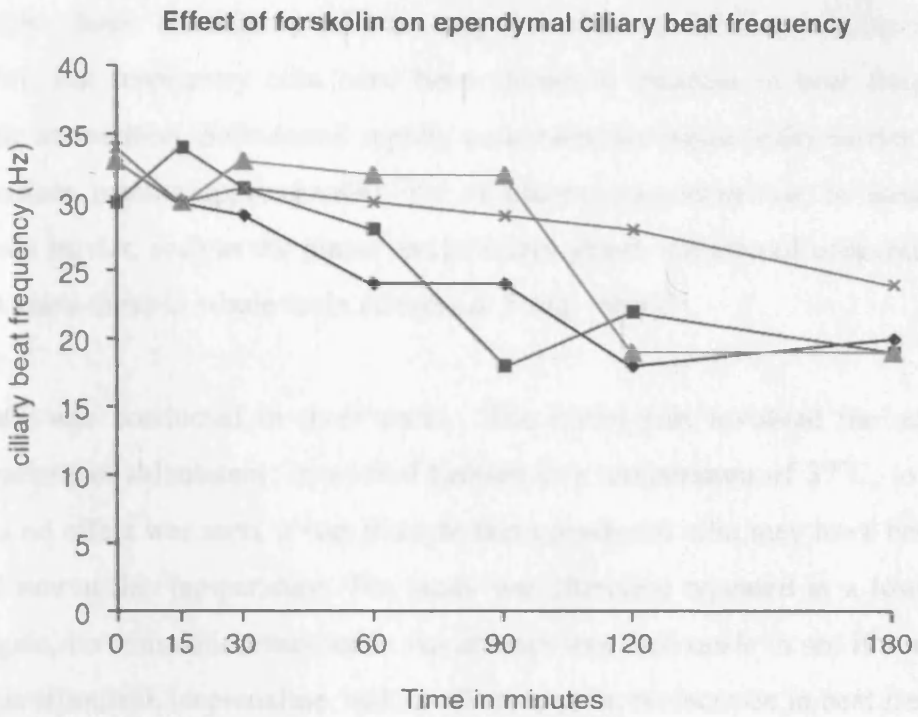

Figure 9.4.b

9.6 Conclusion:

We were unable to demonstrate a stimulation of ependymal ciliary beat frequency in the presence of beta agonists. This may be due to lack of beta receptors on ependymal cells or lack of response of ependymal cilia to cAMP. Further studies, using forskolin, support the latter in that ependymal cilia did not respond to increased levels of cAMP. This suggests a different mechanism of control of ciliary beat frequency, in ependymal cells, as opposed to ciliated respiratory epithelial cells.

There is a wealth of literature on the stimulatory effects of beta-adrenergic drugs on the respiratory cilia of man and animals. Sanderson and Dirksen (1989) showed a maximal increase in ciliary beat frequency of 27% following stimulation of rabbit tracheal cells with isoproterenol. This is similar to a 30% increase in human nasal ciliary beat frequency shown by Konietzko et al (1983). An increase in ciliary beat frequency of 16% has been seen in the cat trachea (Blair & Woods, 1969) and 22% in the rabbit's maxillary sinus (Hybbinette & Mercke, 1982). Indeed, previous studies on cultured respiratory tract ciliated cells and in rat tracheal explants, have

shown a maximum increase of 60% (Verdego et al, 1980) and 50% (Lopez-Vidriero et al, 1985) respectively. Such stimulatory effects may be blocked by the beta-adrenergic antagonist propanolol. Rat respiratory cilia have been shown to increase in beat frequency upon beta-adrenergic stimulation. Salbutamol rapidly penetrates the blood brain barrier of rats with brain concentrations reacing approximately 5% of plasma concentrations. In structures outside the blood brain barrier, such as the pineal and pituitary gland, salbutamol concentrations reach more than 100 times those in whole brain (Caccia & Fong, 1984).

This study was conducted in three parts. The initial part involved the addition of various concentrations of salbutamol, in a blind fashion at a temperature of 37°C, to the ciliated brain slice. As no effect was seen, it was thought that ependymal cilia may have been beating at their maximal rate at this temperature. The study was therefore repeated at a lower temperature of 30°C. Again, no stimulation was seen. An attempt was then made to see if a non-selective beta-adrenergic stimulant, isoprenaline, had an effect. Again, no increase in beat frequency was seen. Cellular communication relies on membrane mediated biochemical events involving membrane bound proteins. Adenyl cyclase (AC) is an enzyme used to relay extracellular signals into the cell. A chemical first messenger binds to a receptor which then transduces the signal across the plasma membrane via guanalyl nucleotide binding proteins (G-proteins). Activated G-proteins regulate the activity of AC to generate the second messenger, cAMP. The second messenger is then free to activate cAMP dependent protein kinase enzymes which are responsible for phosphorylation of target proteins. As mentioned in the literature review (Chapter 1) increased cAMP is associated with an increase in respiratory ciliary beat frequency. Beta agonists are thought to stimulate adenyl cyclase and the resultant increase in cAMP leads to increased ciliary beat frequency. Individual AC isoforms are regulated by a variety of molecules. The diterpene, forskolin was first discovered in the late seventies [Bhat et al, 1977], and soon after its effects (smooth muscle relaxation and positive ionotropic effects) were attributed to activation of AC [Metzger and Lindner, 1981]. Forskolin has the ability to directly activate all AC isoforms independently of receptor-G protein interactions. In an attempt to determine whether stimulating ependymal cells to produce cAMP produced a measurable increase in ciliary beat frequency, they were challenged with forskolin. No effect was seen, indeed the results showed a slight decrease in ciliary beat frequency with time. Ependymal cilia were then exposed to forskolin in the presence of IBMX, a phosphodiesterase inhibiting enzyme. Phosphodiesterase (PDE) is the

enzyme which is responsible for termination of the cAMP signal and this enzyme also exists as a family of structurally related isoforms [Beavo, 1996]. This combination is likely to result in maximal levels of intracellular cAMP. Again, no stimulatory activity was see. Indeed, beat frequency decreased over the study period. This decrease in frequency may be due to lack of available ATP or to toxicity related to increased levels of cAMP with related phosphorylation.

The ependymal cilia in our experiments are bound to underlying neuronal tissue. Wong and colleagues (1988) were able to show that stimulation of the mucosal surface as opposed to the serosal surface by beta-2 agonists caused a greater increase in ciliary beat frequency. However, changes seen on stimulation of either surface were very significant.

Our studies suggest that ependymal cilia attached to brain slices to not respond to adrenergic stimulation. They do not respond to increases in cAMP, suggesting a different mechanism of intracellular control of ependymal ciliary function compared to that seen in respiratory tissue. It is possible that brain ependymal cilia are programmed to beat at a given frequency and beat constantly as they are in a water filled environment.

The next approach would be to determine if beta-2 receptors are actually present on ependymal ciliated cells using autoradiography to detect the receptors. We are unable at present to measure intracellular cyclic AMP levels, but are in the process of establishing this assay to lend support to the lack of effect seen in our system.

Chapter 10

The effect of ethanol and acetaldehyde on brain ependymal ciliary beat frequency

10.1 Summary:

Ethanol has been shown to stimulate the beat frequency of respiratory cilia at concentrations encountered during social drinking. One of its metabolites, acetaldehyde, caused marked ciliary depression and it has been suggested that damage to cilia secondary to acetaldehyde exposure may be cumulative. While ciliated cells in the respiratory tract are regularly replaced, ciliated ependymal cells, which densely line the ventricular surface of the brain, cerebral aqueducts and central canal of the spinal cord, do not regenerate following damage.

The aim of this study was to determine the effect of ethanol and acetaldehyde on ependymal ciliary function. We used our ex-vivo model which allows ependymal ciliary beat frequency to be measured using a high speed video system. The beat frequencies of cilia, incubated at 37°C, were measured before and after exposure to a control solution of medium 199 and to various concentrations of ethanol and acetaldehyde.

Exposure of ependymal cilia to control, 0.1%, 0.5% and 1% ethanol solutions resulted in a 6.1, 6.8, 11 and 15 % increase, respectively, in the ciliary beat frequency from baseline values. A one way analysis of variance comparing the mean slopes for the 3 concentrations of ethanol and control showed no significant differences between the 4 groups ($P = 0.21$).

Exposure of ependymal cilia to control, 100μM and 250μM acetaldehyde solutions resulted in a 12%, 12.2% and 15% increase, respectively, in the ciliary beat frequency from baseline. A one way analysis of variance performed to compare the mean slopes in these 3 groups showed no significant differences ($P = 0.9$).

Exposure of brain ependymal cilia to the concentrations of ethanol likely to be encountered during episodes of heavy drinking and acetaldehyde at concentrations well above those encountered by man, did not have a significant effect on ependymal ciliary beat frequency.

10.2 Background:

Sisson and colleagues (1991) have shown marked slowing or complete ciliary stasis of bovine respiratory cilia on exposure to acetaldehyde, a metabolite of ethanol. We were particularly concerned at their suggestion that chronic ethanol exposure would be likely to result in cumulative dysfunction if the rate of adduct formation by acetaldehyde exceeded the ability of the cells to replace damaged proteins. Ciliated brain ependymal cells, unlike those from the respiratory tract are not replaced following damage. The implication being that exposure to high levels of ethanol may result in permanent damage to ependymal cilia.

Although extremely high concentrations of ethanol have been linked to slowing of respiratory cilia, Maurer and Liebman (1988) found concentrations of alcohol which may be achieved from social drinking had no detrimental effect on respiratory cilia. Indeed they reported stimulation of ciliary beating after exposure to low levels of ethanol. Sisson (1995) also found ethanol rapidly stimulated respiratory ciliary beat frequency at concentrations as low as 10μM and found suppression of ciliary beat frequency did not occur until levels exceeded 1,000μM.
To study the effects of ethanol and acetaldehyde on ependymal cilia we used our ex-vivo system which allows measurement of the beat frequency of cilia from the brains of Wistar rats. The aim of our study was to determine the effect of ethanol and its metabolite acetaldehyde on ependymal ciliary beat frequency.

10.3 Methods:

10.3.1 Sample preparation: The brains of Wistar rats (9-15 days of age) were dissected following sacrifice. Brain slices from the floor of the 4th ventricle were prepared immediately after sacrifice and mounted in a well containing 4 ml of medium 199 with Earl's salts (pH7.4: plus penicillin 50u/ml and streptomycin 50μg/ml) and kept on ice until the study began. For the experiment, the well was placed in a purpose built environmental chamber which was thermostatically controlled to keep the fluid surrounding the ependymal sample at 37°C. The

chamber was humidified to 75-80% to prevent evaporation from the well during the 3 hour study period. Ciliary movement was observed using an inverted Nikon microscope and a x50 lens.

10.3.2 Measurement of ciliary beat frequency: Beating ciliated strips were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 400 frames per second. The camera allowed video sequences to be downloaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study, ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100um were studied.

10.3.3 Exposure to ethanol: Ciliated ependymal strips were incubated, in medium 199, for 30 minutes in the environmental chamber at a temperature of 37°C. Base line readings of ciliary beat frequency were made and the surrounding cell culture fluid exchanged for one of the study concentrations of ethanol in medium 199 or medium 199 alone, preheated to 37°C. Ciliary beat frequency was measured after 15, 30 and 60 minutes. The concentrations of ethanol studied were 0.1% (n=5), 0.5% (n=8) and 1% (n=6). Matched controls in medium 199 were studied for each concentration of ethanol (controls n=21).

10.3.4 Exposure to acetaldehyde: The study protocol was similar to that used for exposure to ethanol. Base line measurements of ciliary beat frequency were made following incubation in medium 199 for 30 minutes at 37°C. Medium 199 surrounding the cells was exchanged for a known concentration of acetaldehyde (100μM or 250μM) in medium 199 or a control solution of medium 199, preheated to 37°C. Sisson and colleagues (1991) had shown marked ciliary slowing following exposure to similar concentrations of acetaldehyde, within minutes. Pilot studies were conducted, measuring ciliary beat frequency every few minutes. When no change in ependymal ciliary beat frequency occurred the study design was changed to one where ciliary beat frequency was measured after 30, 60, 90, 120, 150 and 180 minutes. The concentrations of acetaldehyde studied were 100μM (n=6) and 250μM (n=6) using medium 199 alone as a control solution (n=4).

10.4 Statistical analysis:

There were 3 different concentrations of ethanol (0.1, 0.5 and 1%) and a control studied. For each ciliated brain slice the slope of the regression line of frequency against time was calculated. A one way analysis of variance was performed to compare the mean slopes in the 4 groups. A linear trend was also tested for in the 4 group means.

The analysis was repeated after expressing the data as % change from the baseline value. For each slice the slope of the regression line of % change against time was calculated. A one way analysis of variance was performed to compare the mean slopes in the 4 groups. A linear trend was tested for in the 4 group means.

Similar statistical analysis was performed for the 2 concentrations of acetaldehyde (100 and 250 μM) and the control group studied.

10.5 Results:

The ciliary beat frequency from individual brain slices following exposure to ethanol or control solutions are shown in figure 10.1a-d.

There were 3 different concentrations of ethanol (0.1, 0.5 and 1%) and a control studied. The one way analysis of variance comparing the mean slopes for the 3 concentrations of ethanol and control studied showed no significant differences between the 4 groups (P = 0.21). The linear trend tested for in the 4 group means was not significant (P = 0.10).

Exposure of ependymal cilia to control, 0.1%, 0.5% and 1% ethanol solutions resulted in a 6.1, 6.8, 11 and 15 % increase, respectively, in the ciliary beat frequency from baseline values. To test whether analysing % change form base line made a difference to the interpretation of our results the slope of the regression line of % change against time was calculated for each slice. The one way analysis of variance comparing the mean slopes in the 4 groups showed no significant differences (P = 0. 1). The linear trend tested for in the 4 group means was also not significant (P = 0.5). The conclusions were the same as in the previous analysis.

The ciliary beat frequency from individual brain slices following exposure to acetaldehyde or control solutions are shown in figure 10.2a & b.

For the two concentrations of acetaldehyde (100 and 250 μM) and the control group studied the slope of the regression line of frequency against time was calculated for each slice. A one way analysis of variance performed to compare the mean slopes in these 3 groups showed no significant differences (P = 0.9). The linear trend tested for in the 3 group means was not significant (P = 0.8).

Exposure of ependymal cilia to control, 100μM and 250μM acetaldehyde solutions resulted in a 12%, 12.2% and 15% increase, respectively, in the ciliary beat frequency from baseline. Again the analysis was repeated after expressing the data as % change from the baseline value. The one way analysis of variance comparing the mean slopes in the 3 groups showed no significant differences (P = 0.7). The linear trend tested for in the 3 group means was not significant (P= 0.7).

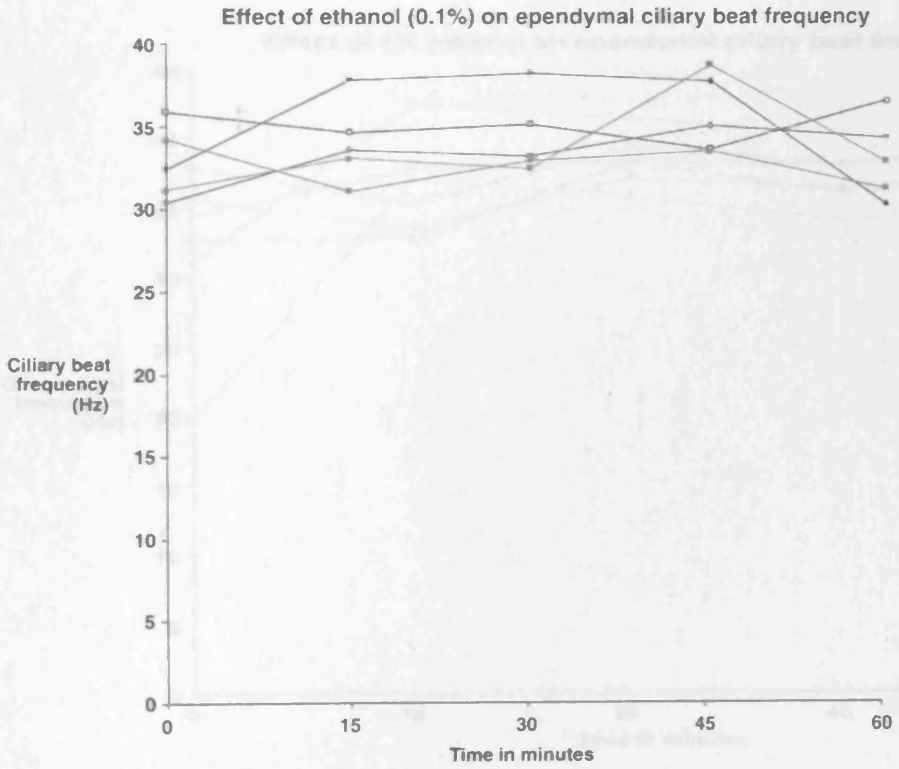

Figure 10.1a

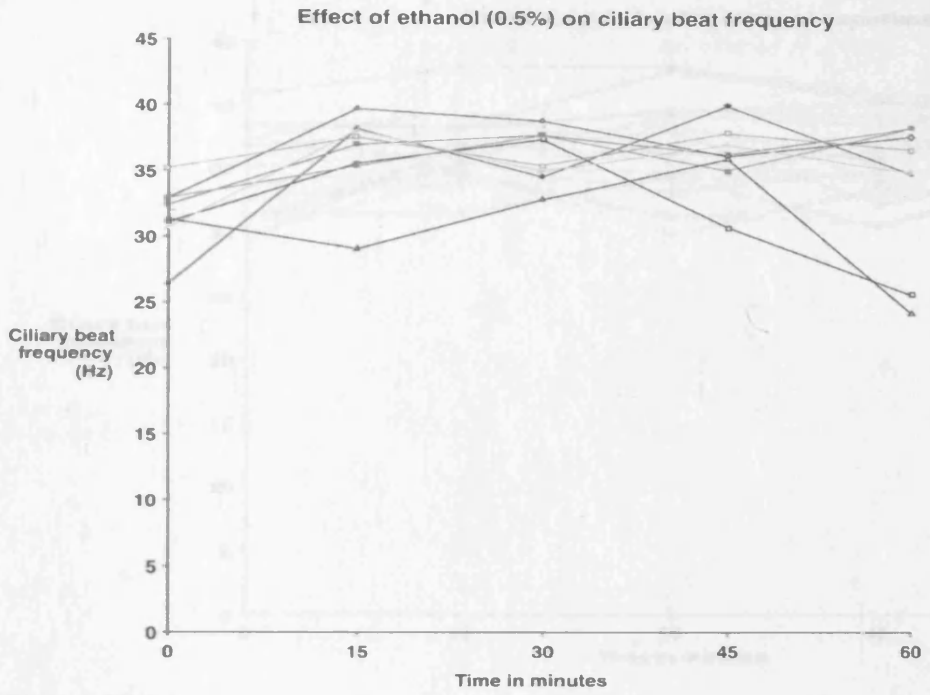

Figure 10.1b

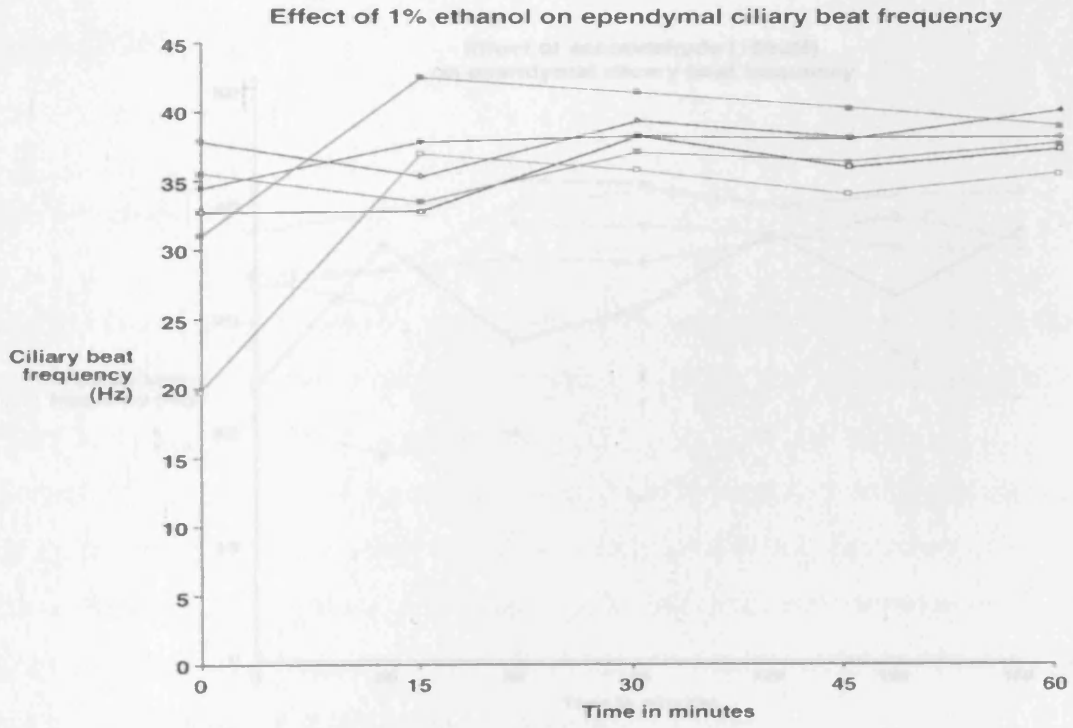

Figure 10.1c

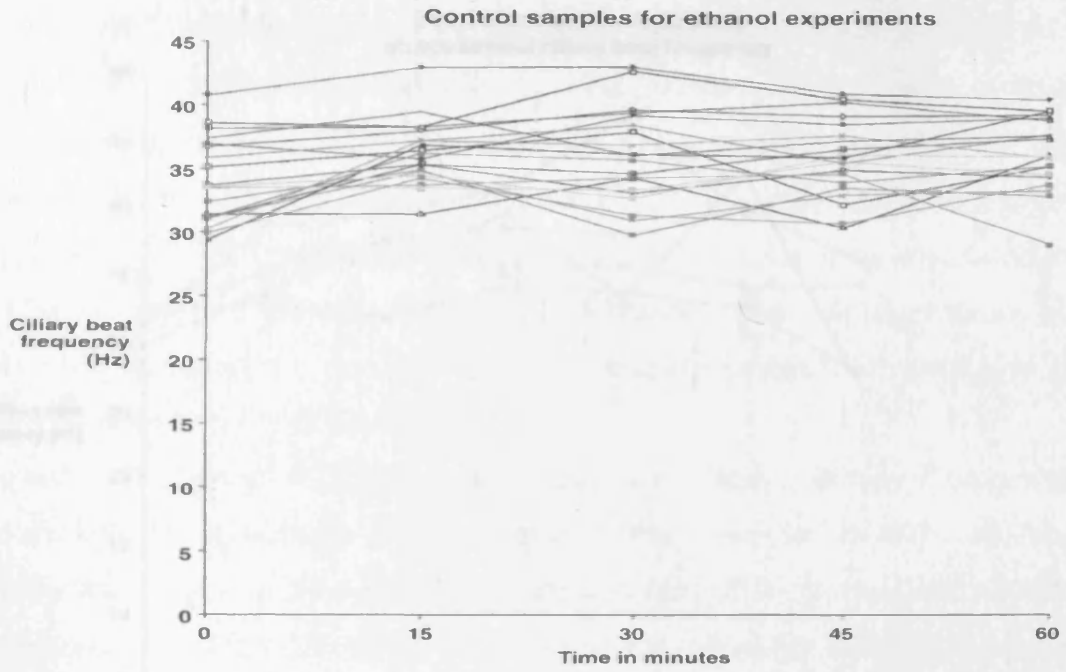

Figure 10.1d

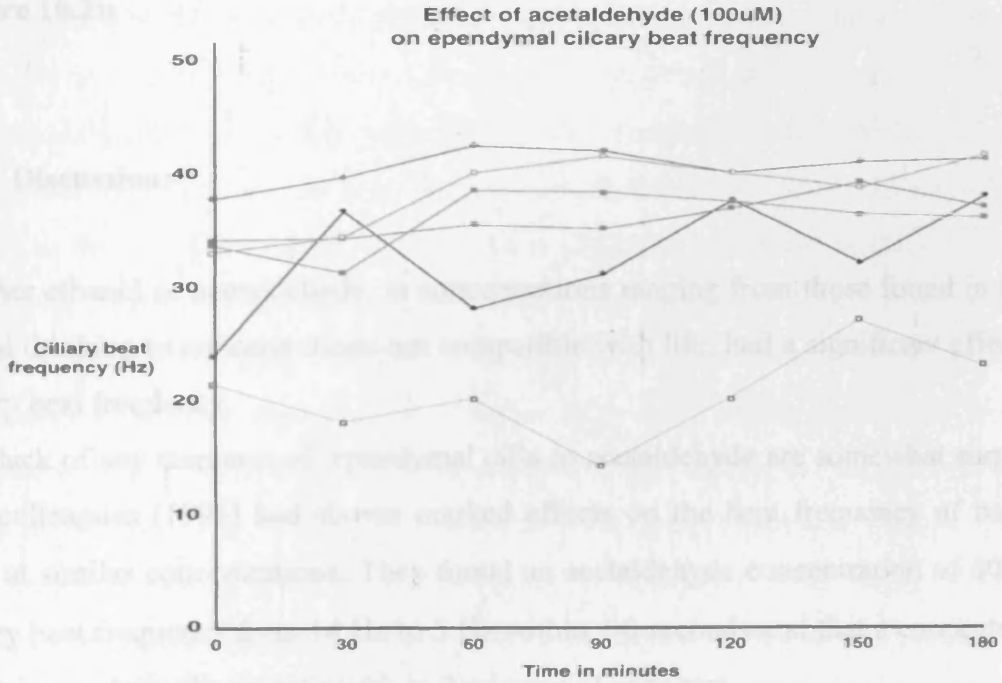

Figure 10.2a

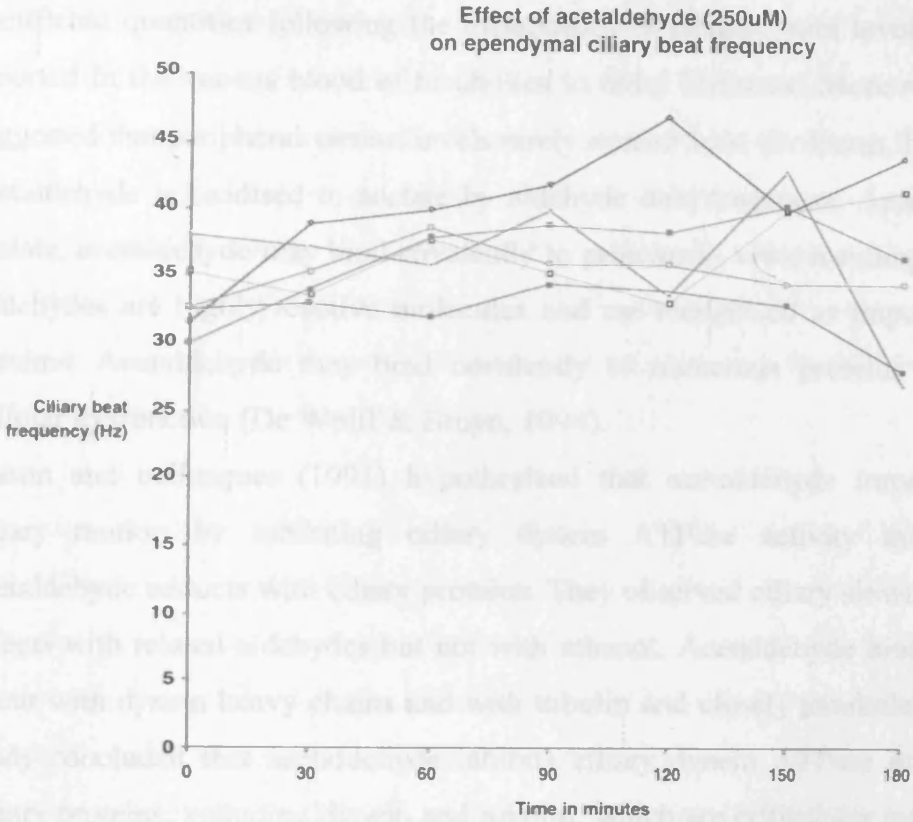

Figure 10.2b

10.6 Discussion:

Neither ethanol or acetaldehyde, at concentrations ranging from those found in the blood during social drinking to concentrations not compatible with life, had a significant effect on ependymal ciliary beat frequency.

The lack of any response of ependymal cilia to acetaldehyde are somewhat surprising as Sisson and colleagues (1991) had shown marked effects on the beat frequency of bovine respiratory cilia at similar concentrations. They found an acetaldehyde concentration of 60μM reduced the ciliary beat frequency from 14 Hz to 5 Hz within 30 seconds and that a concentration of 250μM caused complete ciliary stasis with in 2 minutes of exposure.

Over 90% of the ingested dose of ethanol is oxidised by different metabolic pathways, the most important being the alcohol dehydrogenase route. Alcohol dehydrogenase is a single dependent enzyme which oxidises ethanol to acetaldehyde. Acetaldehyde is produced in biologically

significant quantities following the metabolism of ethanol with levels as high as 50μM being reported in the venous blood of alcoholics in older literature. More recently investigators have suggested that peripheral venous levels rarely exceed 5μM (Eriksson, 1987). After its formation, acetaldehyde is oxidised to acetate by aldehyde dehydrogenase. Apart from being oxidised to acetate, acetaldehyde may bind covalently to proteins in vivo, resulting in acetaldehyde adducts. Aldehydes are highly reactive molecules and are recognised as important toxins in biological systems. Acetaldehyde may bind covalently to numerous proteins resulting in protein and cellular dysfunction (De Wolff & Bruyn, 1994).

Sisson and colleagues (1991) hypothesised that acetaldehyde impaired bronchial epithelial ciliary motion by inhibiting ciliary dynein ATPase activity through the formation of acetaldehyde adducts with ciliary proteins. They observed ciliary slowing and ATPase inhibitory effects with related aldehydes but not with ethanol. Acetaldehyde binding was demonstrated to occur with dynein heavy chains and with tubulin and closely paralleled ATPase inhibition. The study concluded that acetaldehyde inhibits ciliary dynein ATPase activity, and also binds to ciliary proteins, including dynein and tubulin, which are critical for motion. These results make the lack of effect of very high concentrations of acetaldehyde on rat ependymal cilia surprising given the similarity in structure and function of cilia from various sites and animals.

Our investigations involving acetaldehyde are particularly reassuring, as damaged ciliated ependymal cells are not replaced and functional abnormalities of ependymal cilia have been linked to the development of hydrocephalus. Shimizu and Koto (1992) found hydrocephalus, developing in adult male and female rats, was due to motility disorders of ependymal cilia. This was part of a generalised primary ciliary dyskinesia affecting these animals in that their respiratory cilia were also abnormal. Cases of primary ciliary dyskinesia in man have been reported in which idiopathic hydrocephalus was discovered in the neonatal period, suggesting a function of cilia in the embryology of the CSF pathways (Greenstone, 1984).

The human brain ependyma does not appear to regenerate at any age, after birth, following damage (Sanart, 1992). Mitoses are not seen in regions of ependymal damage in the brains of infants or adults regardless of the presence or absence of subependymal gliosis. In the foetus, once neuroepithelial cells begin differentiating into ependymal cells, they do not divide further. Indeed, mitotic activity in ependymal cells is taken as evidence of neoplasia.

Extremely concentrated solutions of 4% ethanol (Dalhamn, 1971) have been show to produce ciliary stasis. Maurer and Liebman (1988) studied the effects of concentrations of alcohol which may be achieved from social drinking, on ciliary beat frequency. Ciliary beat frequency was stimulated at ethanol concentrations ranging from 0.01% up to but not including 0.1%, unchanged at 0.5 and 1% and slowed at 2%. Although their results confirmed earlier studies of the inhibition of ciliary motility at very high ethanol levels, no acute impairment in ciliary function was seen at ethanol concentrations compatible with social drinking. A slight stimulation of ciliary beating at low levels of ethanol was noted.

The intoxicating and anaesthetic properties of alcohol have been attributed to their solubility in the hydrophobic inner part of the membrane's lipid bilayer (Wolf & Bruyn, 1994). More recently it has been argued that the transmitter gated ion channels are sensitive to alcohol at pharmacological concentrations (Wieght, 1992). This is in agreement with the reversibility of the effects of acute intoxication. Voltage clamping studies on cells in the presence of ethanol, at pharmacological concentrations, reveal an increase in intracellular calcium concentration. Increased intracellular calcium concentrations are linked to an increase in ciliary beat frequency.

Sisson (1995) also found ethanol caused a rapid stimulation of bovine respiratory cilia with concentrations as low as 10μM ethanol and that no detectable decreases in ciliary beat frequency was found until ethanol concentrations exceeded 1,000μM. A stereospecific nitric oxide synthetase inhibitor, N-monomethyl-L-arginine (L-NMMA) completely blocked ethanol induced stimulation of ciliary beat frequency. The frequency could be restored by adding either sodium nitroprusside which is a direct NO donor or L-arginine. These results indicate that ethanol, at clinically relevant concentrations, stimulated the release of nitric oxide by airway epithelium that upregulates ciliary motility.

Our studies showed a slight increase in ciliary beat frequency on exposure to ethanol but that this was not significantly different from control samples. The reason for the lack of stimulation of ependymal cilia is unclear, although both the tissue and animals used in out experiments were different. The ependymal cilia we studied were part of fresh long strips of ependyma attached to neuronal tissue as opposed to ciliated cell rich aggregates of bovine respiratory cilia prepared over night. The temperature at which studies were performed were also different. Our

experiments were conducted at a physiological temperature, 37°C, as opposed to a temperature of 24°C, chosen by Sisson. It is possible that ependymal cilia in our studies were beating at a near maximum rate making any response less obvious. Bovine cilia studied at such a low temperature are capable of increasing their beat frequency considerably which may have made them more likely to show a response.

In summary, exposure of brain ependymal cilia to the concentrations of ethanol, likely to be encountered during episodes of heavy drinking and acetaldehyde at concentrations well above those encountered by man, did not have a significant effect on ependymal ciliary function. These results are in contrast to studies on respiratory tissue, where ciliary stimulation was seen following exposure to ethanol and marked depression of ciliary activity seen following exposure to acetaldehyde.

Chapter 11

The effect of MAM on brain ependymal cilia

11.1 Summary:

Toxins released from cycads are thought to be responsible for the very high incidence of patients with amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia complex (ALS-PDC) on the South Pacific island of Guam. The two toxins thought most likely to cause toxic damage are B-N-mehthylamino-L-alanine (BMMA) and methylasoxymethanol (MAM). When BMMA from cycads is fed to Maques, a variety of monkey, corticoneuronal dysfunction and Parkinsonian features develop. Despite being extremely carcinogenic, MAM failed to produce any neurological disease. Recently MAM injected directly into the lateral ventricles of rats was shown to cause selective damage of the ependymal layer.

The aim of this study was to determine the effect of different concentrations of MAM on ependymal ciliary function.

Complete ciliary stasis occurred within 15 minutes when ependymal strips were exposed to 1% MAM and within 90 minutes when exposed to 0.5% MAM. A marked decrease in ciliary beat frequency was only seen in 2 of the 10 ependymal strips (P=0.19) exposed to 0.1% MAM. Ethyl acetate in medium 199, used as a control, and medium 199 had no effect on ciliary beat frequency over the 2 hour study period.

We have shown low concentrations of MAM cause ependymal ciliary stasis.

11.2 Introduction:

The seeds of many cycads are distinguished by a brilliant red or orange seed coat. There has been an association between the possible role of cycads in amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia complex (ALS-PDC) in the indigenous Chamorro population of Guam. ALS has its onset around the age of 35, and is characterised by wasting and weakness of skeletal muscles due to motor neurone degeneration at spinal or bulba level. ALS-PDC usually occurs at the age of forty and is characterised by dementia and extra pyramidal manifestations. Both forms have an insidious onset and lead to death within 4 - 6 years. Neuro fibrillary tangles of the same type as in Alzheimer's dementia are reported in the cells of the anterior spinal horn. Cell loss in the substantia nigra and the locus ceruleus is also observed in 'classical' Parkinson's disease, which suggests an association between the latter and ALS-PD (De Wolff & Bruyn, 1995).

ALS and ALS-PDC were not recognised as an epidemic in Guam until a series of epidemiological studies began at the end of World War 2. During difficult times, such as World War 2, cycad became an important source of carbohydrate in the diet (Kurland, 1993). A known toxic component of the cycad had to be removed by repeated soaking over several days. After soaking, the slices are dried and ground into flour which is used to prepare tortillas or cakes (Kisby et al, 1992). Uncertainty as to whether the toxin was totally removed raised suspicions that it could be the cause of ALS and ALS-PDC. (Kurland, 1993). The toxin, at that time was known as cycasin, is extremely hepatotoxic and carcinogenic. Interestingly, ruminants grazing on cycad leaves show ataxia of the hind quarters, muscle wasting, and long track degeneration.

Spencer and colleagues (1987) fed Macques, a breed of monkey, with a cycas amino acid, beta-N-methylamino-L-alanine. This is a low potency convulsant, had excitotoxic activity in the mouse brain and was considered to be the most likely cause of ALS and ALS-PDC. The animals developed cortico motor neural dysfunction, Parkinsonism features, and behavioural anomalies, with cromatolytic and degenerative changes of motor neurones, cerebral cortex and spinal cord. The sense of smell appears to be lost early in Parkinson's disease and Alzheimer's disease and in patients with PDC on Guam. Olfactory neurones are seriously depleted in cases

of PDC. This suggests that a portal of entry of a toxin may be through the nasal mucosa and the olfactory nerve. The possible role of cycad BMAA in ALS-PD is still questioned. Duncan et al (1990) analysed the actual amount of the amino acid present in flour processed according to Guam customs. It appeared that at least 85% of the total BMAA was removed. The contents of BMAA in the flour was only in the order of 0.005% by weight. However, the effect of chronic low level exposure to minute amounts may have a role.

The second class of compounds from cycads with neurotoxic potential is formed by the azoxyglycosides. The major components in cycad seed is cycasin: methylazoxymethanol-beta-D-glucoside. Cycasin is metabolised to methylazoxymethanol (MAM) by beta-glucosidase. MAM is a direct alkylating agent with toxic, carcinogenic and teratogenic properties (McMahon and Cunningham, 1991). Current research into a possible relationship between cycad consumption and ALS-PD is now mainly directed towards cycasin toxicity. Kisby et al (1992) were able to detect cycasin in cycad flour in an amount that could lead to human exposure to milligrams of cycasin per day. It has been suspected, however, that low levels of cycasin may silently alter the function of post mytotic neurones in a manner that precipitates a slowly progressive degeneration of cells (Spencer et al, 1991).

To determine if MAM had any effect upon the central nervous system it was injected directly into the lateral ventricles of rats (Unpublished results: personal communication from Professor Alan Seawright: MRC toxicology unit, Leicester). The investigators believed that MAM would have no neurotoxic effects and could be excluded as a possible cause of PCD. Surprisingly the rats, who were sacrificed after 3 weeks, showed evidence of mild ventricular dilatation. On microscopy the only abnormality seen was selective damage of the ependymal layer. The aim of our experiment was to determine the concentration of MAM required to cause stasis of ependymal cilia.

11.3 Methods:

11.3.1 Sample preparation: The brains of Wistar rats (9-15 days of age) were dissected following sacrifice. Strips of ciliated ependyma (100um thick and 300um long) from the floor of the 4th ventricle were prepared immediately after sacrifice and mounted in a well containing

4 ml of medium 199 with Earl's salts (pH7.4: plus penicillin and streptomycin) and kept on ice until the study began. For the experiment the well was placed in a purpose built environmental chamber which was thermostatically controlled to keep the fluid surrounding the ependymal sample at 37°C. The chamber was humidified to 75-80% to prevent evaporation from the well during the 3 hour study period. Ciliary movement was observed using a Nikon inverted microscope with a x50 lens.

11.3.2 Measurement of ciliary beat frequency: Beating ciliated strips were recorded by a high speed video camera (Kodak EktaPro Motion Analyser, Model 1012) at a rate of 400 frames per second. The camera allowed video sequences to be downloaded at reduced frame rates, allowing ciliary beat frequency to be determined directly by timing a given number of individual ciliary beat cycles. At each time point of the study ciliary beat frequency was measured at 4 different areas along each ependymal strip. Only intact, undisrupted, ciliated strips in excess of 100um were studied.

11.3.3 Study design: Ciliated ependymal strips were incubated for 30 minutes in the environmental chamber at a temperature of 37°C. Base line readings were then made and the surrounding cell culture fluid exchanged for either medium 199 (control; n=25), one of the study concentrations of MAM in medium 199 (0.1%, n=10: 0.5%, n=8: 1%, n = 5), and ethyl acetate in medium 199 (1%, n=7). Ethyl acetate, the solvent used to help dissolve MAM, in medium 199 acted as a second control. Readings were taken after 15 minutes for the highest concentration of MAM (1%) as rapid ciliary stasis was noted during pilot studies at this concentration. For all other concentrations readings were made at 30 minute intervals for up to 2 hours. All solutions were preheated to 37°C prior to fluid exchange.

11.4 Statistics:

There were 5 groups: 3 concentrations of MAM (0.1, 0.5 and 1%) in medium 199 and 2 control groups (medium 199 alone (0%), medium 199 and ethyl acetate (99) - the solvent used with MAM). Two statistical approaches were used. A survival analysis was used to compare survival times in the 5 groups. Log rank tests were performed which test for the equality of survivor functions. When all 5 groups were compared, there were highly significant

differences. Each group was then compared with each of the control groups. The log rank test can only be performed when there is at least one death in one of the groups to be compared. The output gives the observed number of deaths in each group and the expected number of deaths if the null hypothesis of the same survival experience in all groups were true.

For each slice the slope of the regression line of frequency against time was calculated. A one way analysis of variance was performed to compare the mean slopes in the 5 groups. Repeated values of zero were omitted from the same slice when the slopes were calculated.

11.5 Results:

The results of individual experiments on brain slices are shown in graphs 11.1a-e. Complete ciliary stasis occurred within 15 minutes when ependymal strips were exposed to 1% MAM and within 90 minutes when exposed to 0.5% MAM. A marked decrease in ciliary beat frequency was only seen in 2 of the 10 ependymal strips exposed to 0.1% MAM. Ethyl acetate in medium 199 and medium 199 alone had no effect on ciliary beat frequency over the 2 hour study period.

Two statistical approaches were used to analyse the results. When all 5 groups were compared, using the log rank test, there were highly significant differences. Each group was then compared with each of the control groups. All comparisons were significant.

The analysis of variance showed highly significant differences between the 5 group means ($P < 0.001$), but the assumption of equality of variances was violated. Therefore, the analysis was repeated after omitting the two highest concentrations of MAM. There were no significant differences between the 2 control groups and the lowest MAM concentration ($P=0.19$).

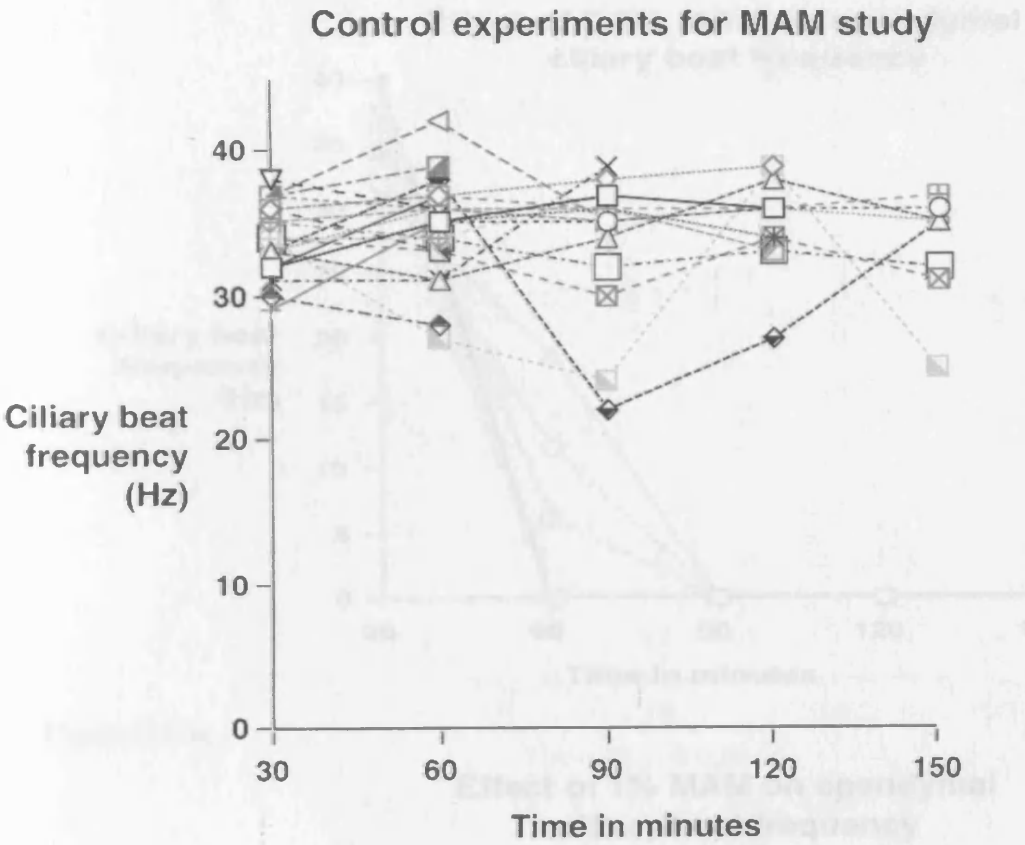

Figure 11.1a

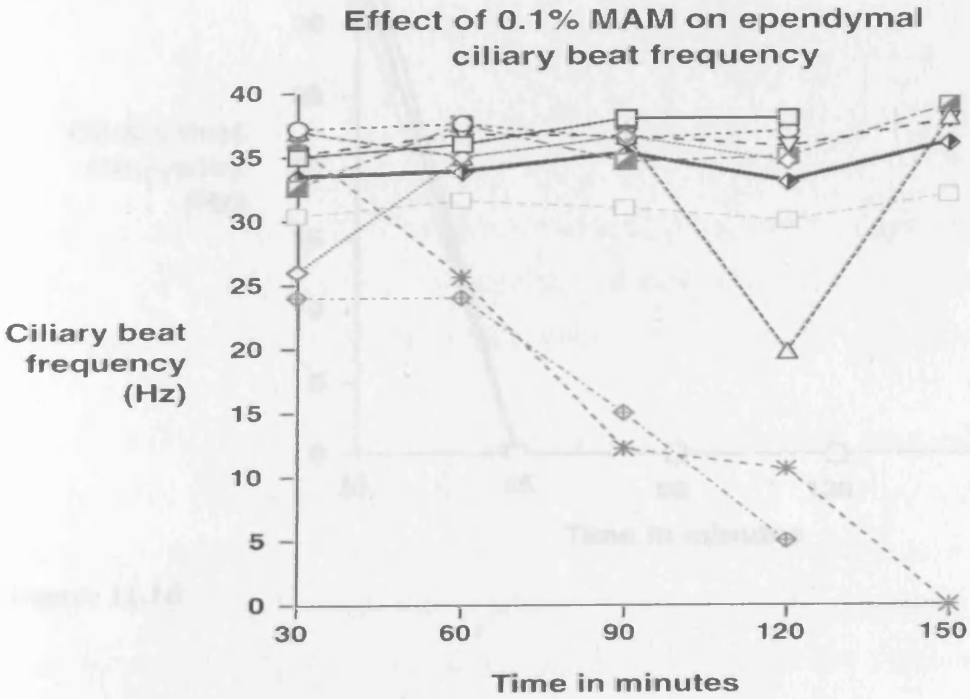

Figure 11.1b

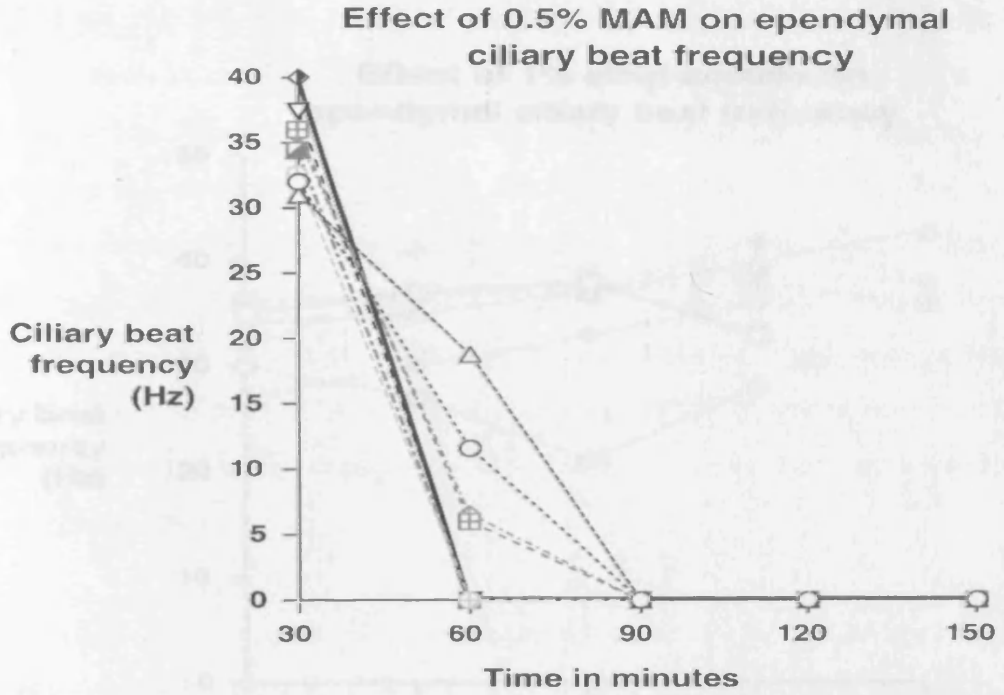

Figure 11.1c

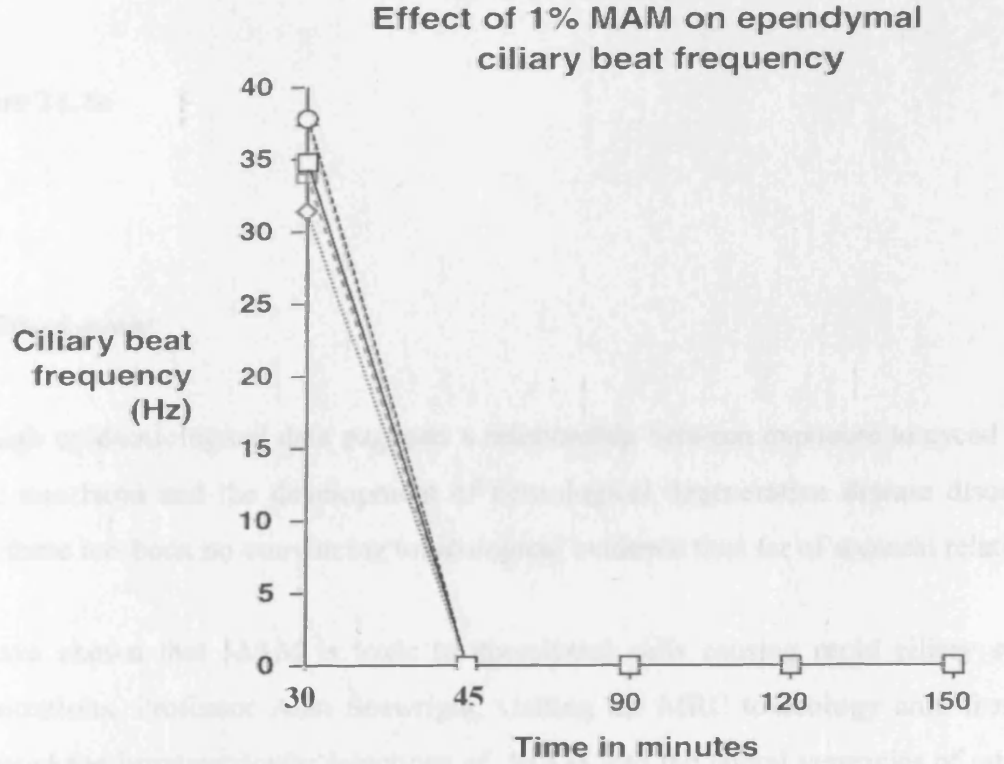

Figure 11.1d

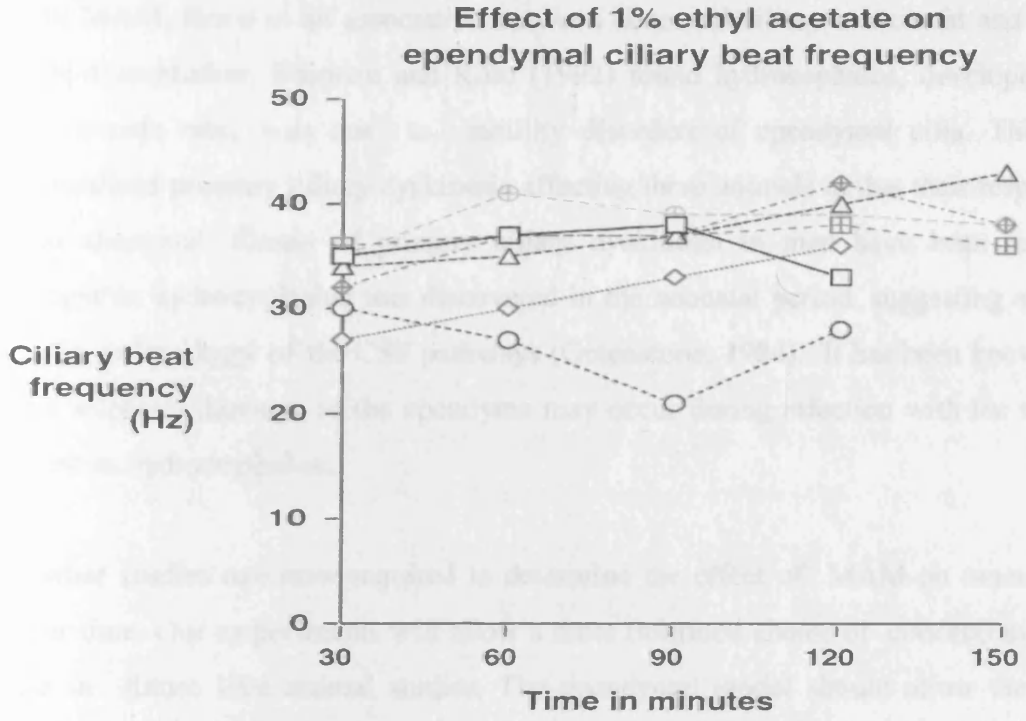

Figure 11.1e

11.6 Discussion:

Although epidemiological data suggests a relationship between exposure to cycad constituents on the one hand and the development of neurological degenerative disease disorders on the other, there has been no convincing toxicological evidence thus far of a causal relationship.

We have shown that MAM is toxic to ependymal cells causing rapid ciliary stasis at low concentrations. Professor Alan Seawright, visiting the MRC toxicology unit, from Brisbane, performed the intraventricular injections of MAM into the lateral ventricles of rats. When the rats were sacrificed, 3 weeks later, selective damage of the ependymal layer and slight ventricular enlargement was seen. The dose chosen for ventricular injection was large as no neurological damage was expected.

While it is not possible to be certain of the cause of ventricular dilatation in the rats injected with MAM, there is an association between abnormal ciliary movement and the development of hydrocephalus. Shimizu and Koto (1992) found hydrocephalus, developing in adult male and female rats, was due to motility disorders of ependymal cilia. This was part of a generalised primary ciliary dyskinesia affecting these animals in that their respiratory cilia were also abnormal. Cases of primary ciliary dyskinesia in man have been reported in which idiopathic hydrocephalus was discovered in the neonatal period, suggesting a function of cilia in the embryology of the CSF pathways (Greenstone, 1984). It has been known for some time that selective damage to the ependyma may occur during infection with the mumps virus and result in hydrocephalus.

Further studies are now required to determine the effect of MAM on neurological outcome over time. Our experiments will allow a more informed choice of concentrations of MAM to use in future live animal studies. The ependymal model should allow the mechanisms of damage of relevant toxins and the effect of repeated exposure to low levels of toxin to be investigated.

Chapter 12

Summary

Since the first description of brain ependymal ciliary movement by Purkinge in 1836, there has been a relative dearth of published literature in this area. While there have been a number of papers focusing on the scanning electronmicroscopic appearance of ependymal cilia in a variety of animals, ranging from rat to man (Brightman & Palay, 1963: Yamadori and Nara, 1979 & 1983: Bannister & Mundy, 1979), there have been very few that have concentrated on ependymal ciliary movement. Most investigators have relied on indirect measurement of ciliary activity using transport of particles via the cilia to indicate underlying movement (Chu et at,1942: Konno & Shiotani, 1956). We suspect that the lack of information on ependymal ciliary movement and function may be partly due to difficulty in sample preparation. Nakamura and Sato (1993) commented that measurement of ependymal ciliary beat frequency was unreliable and difficult to standardise.

A significant amount of the experimental work for the thesis involved development of a methodology for the preparation and containment of ciliated ependymal edges. These developments allowed reproducible measurements of ciliary beat frequency to be made over a prolonged period of time. Simple brushing techniques used to prepare strips of ciliated respiratory epithelium did not result in viable tissue. The use of a vibratome to cut cooled brain samples provided brain slices with an intact ciliated ependyma. The effect on ciliary beat frequency of the different methods used to contain ependymal slices was described in detail in Chapter 3. The experimental work described in the thesis was performed using an inverted microscope to observe the ependymal edge of brain slices mounted in a well containing cell culture fluid. The well was enclosed within a much larger incubation chamber which served to heat and humidify the surrounding air, keeping the tissue at 37°C and preventing evaporation of cell culture fluid. This circumvented the problem of the decrease in ciliary beat frequency seen following direct observation using the water immersion system or

brain slices contained within a microscope well with a cover slip on top. The system I developed allowed cilia to be observed continually for several hours.

The system allowed us to explore the effects of both pH and viscosity on ependymal ciliary function. Results of the pH experiments were similar to those observed with other ciliated tissue within the body, that is ciliary function was relatively preserved above pH 7 but rapidly decreased below this level. We were able to show this was directly due to a change in intracellular pH, which has not previously been documented.

Increases in viscosity caused a rapid initial decrease in ciliary beat frequency. Following this rapid decrease cilia beat at constant frequency. Increasing the viscous load from 20cP to 40cP did not result in a decrease in ciliary beat frequency or amplitude of the power stroke of the cilia. This suggests ependymal cells are capable of auto-regulating ciliary activity when exposed to increased viscous loading, which is similar to the autoregulatory properties of ciliated cells lining the respiratory tract (Johnson et al, 1991).

Ependymal cilia were found to be approximately 3μm longer than respiratory cilia and to beat at between 34-40Hz which is twice as fast as respiratory cilia.

The response of ependymal cilia to pharmacological stimuli also differed to that expected of respiratory cilia. Increased intracellular cAMP is associated with an increase in respiratory ciliary beat frequency. Beta-agonists, such as salbutamol, are thought to stimulate adenyl cyclase and the resultant increase in cAMP leads to increased ciliary beat frequency. However, ependymal cilia showed no response to salbutamol or isoprenaline. The diterpene, forskolin which has the ability to directly activate all AC isoforms, independently of receptor-G protein interactions, and increase intracellular cyclic AMP levels, did not alter ependymal ciliary beat frequency. The combination of forskolin and a phosphodiesterase inhibitor was used to obtain maximal intracellular concentrations of cyclic AMP. This combination caused ciliary slowing. Our results suggest that ependymal cilia do not respond to agents known to raise intracellular cyclic AMP. Further work is required to determine the cellular control of ependymal cilia, in particular the role of intracellular calcium concentration.

Of surprise was the lack of stimulation of ependymal cilia by ethanol. Ethanol has been shown to increase the beat frequency of respiratory cilia which is related to the production of nitric oxide (Sisson,1995). A metabolite of ethanol, acetaldehyde, has been shown to cause a concentration dependent slowing or stasis of bovine respiratory cilia (Sisson et al,1991). Ependymal cilia exposed to similar concentrations of acetaldehyde maintained their beat frequency. Lack of toxic damage to the ependyma by acetaldehyde is reassuring as dead ependymal cells are not replaced.

Certain anaesthetic agents have been shown to slow the beating of respiratory cilia. Prior to conducting investigations relating to ependymal ciliary function in an animal model, we felt it essential to study the effect of two commonly used anaesthetic agents, halothane and pentobarbitone. Highly perfused, relatively low volume tissues such as the brain equilibrate rapidly with the high early concentrations of barbiturates in the blood, resulting in induction of anaesthesia (Fragren & Avram, 1994). Drug levels then decrease quickly as the drug redistributes throughout the body. Pentobarbitone, even at very high levels caused no decrease in ciliary beat frequency throughout the study period. Its use in experiments investigating the effect of ependymal ciliary movement appears justified. Halothane, however, caused a decrease in ependymal ciliary beat frequency. The magnitude of decrease in ependymal ciliary beat frequency was less than that seen following exposure of adult human respiratory cilia to similar concentrations of halothane. It is well known that more inhalational anaesthetic is required for younger patients to achieve the same anaesthetic effect. Ependymal cilia of young animals were studied which may help to explain the difference in response. Halothane distributes widely throughout the body and significant levels are found in the CSF. Arterial levels of halothane are over 80% of the inspired concentration and after 4 hours of exposure to halothane similar levels are found in the CSF. Following cessation of a halothane anaesthetic, CSF halothane decreases significantly more slowly than arterial halothane levels (Stoelting, 1973). It is likely, therefore, that halothane administered during anaesthesia may induce slowing of ependymal cilia. It should not be used in studies which relate to the investigation of ependymal ciliary activity. Whether ependymal ciliary slowing contributes to the central nervous system effects of halothane anaesthesia remains unclear.

Finally, we used our ependymal model to look at the toxicological effect of MAM, thought to be implicated as a causal agent of the Parkinsonism-dementia complex. This work has allowed us to estimate the lowest dose of MAM which may be injected directly into the ventricular system and cause ependymal ciliary stasis. Follow-up work is planned to monitor Wistar rats injected with this toxin to determine its neurological effects.

Future research:

My aim is to continue exploring the physiology and cellular mechanisms of ependymal ciliary function. Since the thesis has been completed we have set up a cell culture method to allow us to study ciliated ependymal cells.

My second line of investigation is to use the ependymal model to study the effect of bacteria and their virulence factors.

A neglected aspect of studies on meningitis is consideration of damage to the ependyma. Ependymal cells line the walls of the ventricles and aqueducts in the brain and the central canal of the spinal cord forming a barrier between cerebrospinal fluid, which is infected in meningitis, and neuronal tissue.
Postulated roles for brain cilia include:

- continual directional movement of cerebrospinal fluid,
- maintenance of a diffusion gradient, between the CSF and brain tissue, facilitating the movement of toxins and metabolites to the CSF for clearance,
- keeping the surface of the brain clear of debris and preventing margination of bacteria during meningitis. Thus, damage to the ependyma may result in physiological damage to the brain.

Interestingly defective brain ciliary movement has been linked to the development of hydrocephalus, a recognised complication of meningitis.

Appendix 1:

Salbutamol HPLC:

Reagents:

Salbutamol - analytical working standard

Benzyl Bithenyl - recrystallised

Ammonium acetate solution (0.1% w/v)

Methanol

Water - purified

HPLC determination:

Chromatographic conditions:

Column:

10cm x 4.6mm internal diameter packed with 5um spherisorb ODS 1

Mobile phase:

800ml methanol + 300ml ammonium acetate solution filtered through a Hortman GF/A glass fibre filter

Flow rate: 2ml per minute

Temperature : 50°C

Detector: variable wave length UV set at 276nm

Sensitivity: 0.05 AUFS

Injection volume: 200ul

Measurement: electronic determination of peak area or height

Typical retention times: salbutamol 1.34 minutes: benzyl biphenyl 2.07 minutes

Calculations:

Relative weight response factor (RWR)

The RWR factor was calculated for each of the injections of the RWR solutions.

The mean value obtained from all the RWR injections was used in the subsequent calculations.

The relative standard deviation of the RWR values was determined.

$$R = \frac{S_2 \times W_1 \times P}{S_1 \times W_2 \times 100}$$

Where:

S_1 = Area (Height) of Standard peak in RWR solution

S_2 = Area (Height) of Internal Standard peak in RWR solution

W_1 = Weight of Standard (μg) in 5.0ml of Standard solution

W_2 = Weight of Internal Standard (μg) in 5.0ml of Internal Standard solution.

P = Percentage purity of the standard used

Deposition in Stage 1 and Stage 2 and on the actuator/Valve

Average Salbutamol content per sample was calculated as follows:

$$\text{sample salbutamol} = \frac{S_1 \times W_2 \times R}{S_2 \times 10}$$

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