SHIFTWORK

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1. Definition and Occurrence of Exposure

1.1 Definition of shiftwork

The International Labour Office (International Labour Organization, 1990a) defines working in shifts as "a method of organization of working time in which workers succeed one another at the workplace so that the establishment can operate longer than the hours of work of individual workers."

The European Council Directive 93/104 (1993) declares that "concerning certain aspects of the organisation of working time, shiftwork shall mean any method of organising work in shifts whereby workers succeed each other at the same work stations according to a certain pattern. Shiftworker shall mean any worker whose work schedule is part of shiftwork."

Besides these definitions, in the scientific literature, the term "shiftwork" has been widely used and generally includes any arrangement of daily working hours other than the standard daylight hours (7/8 am – 5/6 pm).

In most cases, shiftwork is synonymous of irregular, odd, flexible, variable, unusual, non-standard working hours.

1.2 Types of shiftwork

Several types of shiftwork exist and can be described as follows:

(*a*) permanent – people work regularly on one shift only, i.e. morning or afternoon or night; or rotating – people alternate more or less periodically on different shifts;

(*b*) continuous – all days of the week are covered; or discontinuous – interruption on weekends or on sundays;

(*c*) with or without night work – the working time can be extended to all or part of the night, and the number of nights worked per week/month/year can vary

considerably. Moreover, the definition of "period of night work" varies from country to country, i.e. in some countries it ranges from 8, 9 or 10 pm to 5, 6 or 7 am, and in many others from 11 or 12 pm to 5 or 6 am (See Table 1.1).

Table 1.1. Definitions of night work and night worker in some European countries

COUNTRY	NIGHT TIME/NIGHT WORK	NIGHT WORKER
AUSTRIA	Night work: period between 22:00 and 05:00	The workers who work at least 3 hours between 22:00 and 05:00 on at least 48 nights per year (EU-Nachtarbeits-Anpassungsgesetz 2002)
BELGIUM	Night work: a period, generally of 8 hours, between 20:00 and 06:00	Loi du 17/02/1997 et Loi du 04/12/1998: Act of 17 February 1997
FINLAND	Night work: Work carried out between 23:00 and 06:00	Night shift refers to a work shift with at least 3 hours of duty between 23:00 and 06:00 (Working Hours Act 605/1996)
FRANCE	Night time: a period between 22:00 and 05:00 Night work: whichever work period between midnight and 05:00	Any employee working usually at least 2 times per week for at least 3 hours over the period defined as night work (Loi 461/1998)
GERMANY	Night time: the time between 23:00 and 06:00 (in case of bakers between 22:00 and 05:00). Night work: all work which occupies more than 2 hours of night time	"Night workers" means workers who usually work nights on rotating shifts schedules, or work at night for not less than 48 days in a calendar year (Arbeitszeitgesetz 1994)
GREECE	Night time: a period of 8 hours which includes the period between 22:00 and 06:00	A worker who during night time works at least 3 hours of his/her daily working time or a worker who has to perform night work for at least 726 hours of his/her annual working time (Presidential Decree n. 88/1999)
IRELAND	Night time: period between midnight and 07:00	a) an employee who normally works at least 3 hours of his/ her daily working time during night time; b) an employee whose working hours during night time, in each year, equals or exceeds 50 per cent of the total number of hours worked during the year (Statutory Instruments n. 485/1998)

Table 1.1 (contd)

COUNTRY	NIGHT TIME/NIGHT WORK	NIGHT WORKER
ITALY	Night work: the activity carried out in a period of at least 7 consecutive hours comprising the interval between midnight and 05:00	a) any worker who during the night period carries out, as a normal course, at least 3 hours of his/her daily working time; b) any worker who during the night period, carries out part of his/her daily working time as defined by collective agreements; in default of collective agreements, any worker who works at night at least 80 working days per year (D.Lgs. 66/2003)
NETHERLANDS	Night work: work which covers all or part of the period from midnight to 06:00	
PORTUGAL	Night time: a period between 20:00 and 07:00	a) any worker who works at least 3 hours during the night period; b) any worker who during the night period, carries out part of its daily working time as defined by collective agreements (Decreto Lei 73/1998)
SPAIN	Night time: a period which includes the interval between 22:00 and 06:00	A worker who at night carries out at least 3 hours of his/her daily working time (Real Decreto Lei 1/1995)
SWEDEN	Hours between midnight and 05:00	A worker that works at least 3 hours of his/her daily work during night time, or a worker that most likely will work at least 38% of his/her annual work during the night (Working Hours Act 1982)
UK	Night time: a period lasting not less than 7 hours, and which includes the period between midnight and 05:00	A worker who, as a normal course, works at least 3 hours of his/her daily working time during night time, or who is likely, during night time, to work at least such proportion of his annual working time as may be specified for the purposes of these Regulations in a collective agreement or a workforce agreement (Statutory Instrument No.1833/1998).

Table compiled by the Working Group

The shift systems can also differ widely in relation to other organizational factors:

(*a*) length of shift cycle – a "cycle" includes all shifts and rest days lasting as long as the series of shifts restart from the same point; there can be short (6–9 days), intermediate (20–30 days), or long (up to 6 months or more) cycles.

(*b*) duration of shifts – in general, the length of a shift is 8 hours, but can range from 6 to 12 hours.

(*c*) number of workers/crews who alternate during the working day.

(*d*) start and finish time of the duty periods.

(*e*) speed of shift rotation – this depends on the number of consecutive days worked before changing shift. It can be fast (i.e. every 1, 2 or 3 days), intermediate (i.e. every week), or slow (i.e. every 15, 20 or 30 days). This factor has considerable influence on the number of consecutive night shifts and rest days.

(*f*) direction of shift rotation – it can be clockwise (i.e. morning/afternoon/night) or counter-clockwise (i.e. afternoon/morning/night) with consequent different duration of the intervals between shifts. Clockwise rotation is also referred to as "phase delay" or "forward rotation," and counter-clockwise rotation, "phase advance" or "backward rotation". They have a different impact on the adjustment of the circadian rhythm.

(*g*) number and position of rest days between shifts.

(*h*) regularity/irregularity of the shift schedules.

All of these factors can be combined in different ways depending on the demands specific to the occupation.

In the industrial sectors (i.e. mechanical and chemical), shiftwork is usually arranged in continuous three-shift systems. A similar number of crews/workers work both on day and night shifts, with regular shift schedules either on fast or slow rotating cycles, with fixed start and finishing times.

In the transport sector, schedules are often quite irregular, both in terms of number of consecutive shifts, shift rotation, start and finishing times, duration of the duty periods, location, and amount of rest days.

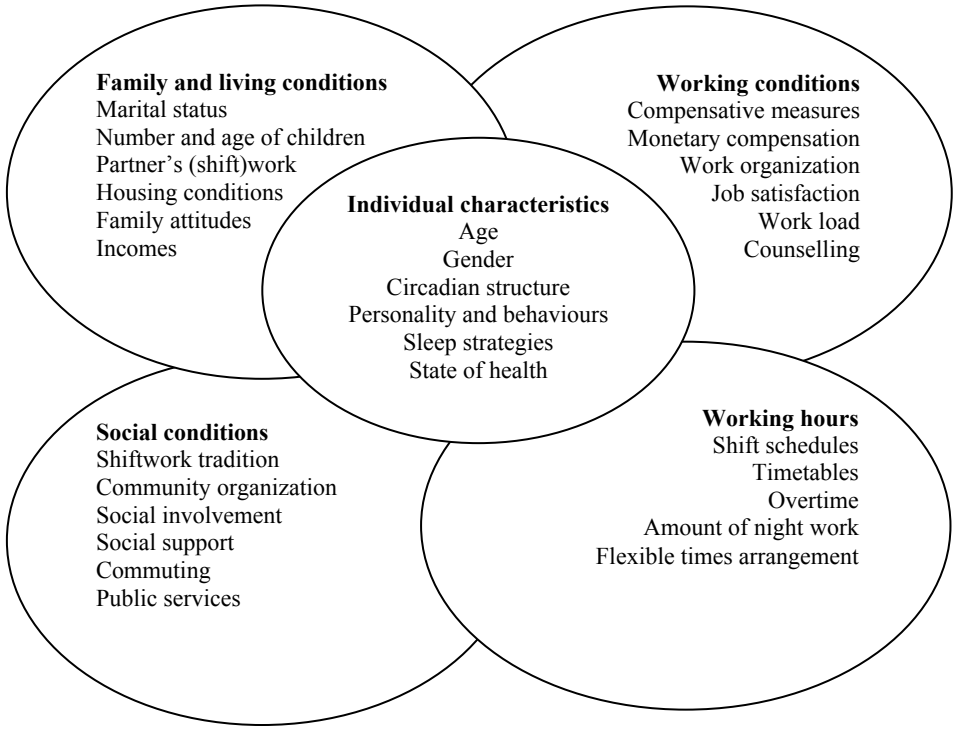
In the health-care sector, quite different shift schedules are operated with different rotation (clockwise or counter-clockwise), variable start and finishing time, and different amount of night shifts.

In the service sector, workers are commonly employed on split shifts, for example, very early morning and late afternoon shifts in road- and office-cleaning, merchandise delivery, or permanent night work (security guards).

In the leisure sector, work is mainly performed during the late afternoon and night hours, with a long duration of shifts.

Different shiftwork systems have potentially different impacts on the health of the workforce, disturbing the circadian rhythm, an essential biological function, in different ways, and also inducing sleep deprivation (see Section 4). In addition to shiftwork schedules, other factors can affect tolerance to shiftwork and night work such as individual characteristics, family situation, social conditions, and working conditions (Fig. 1.1; Costa *et al.*, 1989; Costa, 1996, 2003; Knauth, 1996; Knauth & Hornberger, 2003).

Figure 1.1. Factors that can affect tolerance to shiftwork and night work



(Costa, 2003)

1.3 Occurrence of shiftwork

Increasingly, shiftwork and night work are becoming more common in our so-called "24-hour" (or "24/7") society. Shiftwork and night work enable round-the-clock activities required for meeting technological needs (e.g. power plants, oil refinery, and steel industry), social services/utilities functions (e.g. hospitals, transports, police and security forces, firefighting, hotels, and telecommunications), productive and economic demands (e.g. textile, paper, food, mechanical, and chemical industry), and the needs of the leisure industry.

More than two and a half billion people are officially recognized as workers according to the most recent statistics of the International Labour Organization (International Labour Organization, 2006), two-thirds of which in the Asiatic continent. Reliable data on the numbers of workers employed in shiftwork is not easy to collect due to the lack of robust statistics in many countries, and/or differences in methods of data collection not always being comparable.

However, in Europe, the European Foundation for the Improvement of Living and Working Conditions has been carrying out periodical surveys on working conditions, including working hours, every 5 years since 1990. According to the third survey, carried out in 2000 in 15 European countries and involving 21703 workers, people who do normal or standard daytime work (that is those who do not work more than 40 hours per week, more than 10 hours per day, on shifts, at night, on sundays and/or saturdays, and part-time) represented only 24% of the whole population, 27% of employed workers, 8% of self-employed workers, with men and women sharing the same proportion (24%) (Costa *et al.*, 2004).

According to the results of the fourth survey carried out in 2005 (European Foundation, 2007), the weekly working hours among the 31 European countries examined ranged from an average of 34 hours in the Netherlands to 55 hours in Turkey, and from a minimum of 8 hours (as part-time work) to a maximum of 90 hours (as overtime work). Shiftwork, including night work, involved more than 17% of the total European Union (EU) working population (Table 1.2), with large variations among countries, and between old and new member States (from 6.4% to 30%). There were also quite large differences among EU countries when looking at evening (from 36% to 58%) and night work (from 18% to 24%) (Fig. 1.2). Evening and night work are mostly used in the hotel and restaurant industry, health care, and transport and communication sectors, usually employing an older workforce (Fig. 1.3). More generally, shiftwork in its different definitions is used by one-third of people working in the health-care sector and the hotel and restaurant industry, and in one fourth of cases in the manufacturing, transport, and communication sectors (Table 1.3). According to age and gender (Table 1.4), the average percentage of shiftwork including night work is quite similar in both men and women, with quite a high percentage of workers aged over 55 employed in night work (10.5%).

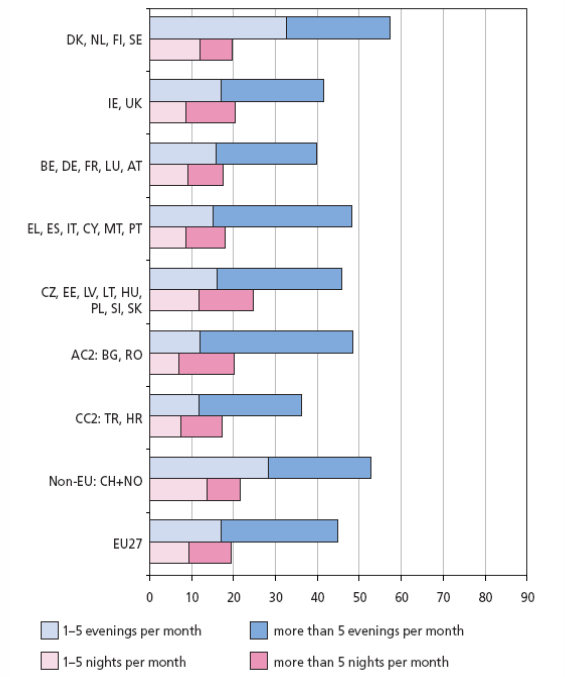
In the USA, according to the Bureau of Labor Statistics (US Bureau of Labor Statistics, 2005), in 2004, almost 15% of full-time salaried workers usually worked on alternate shifts. Men were more likely than women to work such shifts (16.7% and 12.4%, respectively). This was also true for the black population when compared to the caucasian, hispanic or latino, or asian populations, with shiftwork progressively decreasing with increasing age (Table 1.5). The prevalence of shiftwork was greatest among workers in the service industry (32.6%; Table 1.6), particularly the protective service industry (50.4%, includes police, firefighters and guards), food preparation and serving (49.4%), and those employed in production, transportation, and material-moving occupations (29%). The proportion of workers on alternate shifts was highest in the leisure and hospitality (45.8%), mining (31.5%), and transportation and utilities (27.8%) industries.

Table 1.2. Prevalence (%) of shiftwork that includes night work, by country in Europe in 2005 (4th EU Survey on working conditions)

Austria	13.2
Belgium	13.2
Bulgaria	21.0
Croatia*	33.5
Cyprus	11.8
Czech Republic	22.2
Denmark	9.3
Estonia	20.4
Finland	24.3
France	14.9
Germany	15.7
Greece	13.0
Hungary	20.7
Ireland	12.0
Italy	18.1
Latvia	21.9
Lithuania	19.4
Luxembourg	13.9
Malta	22.3
Netherlands	11.8
Norway	23.4
Poland	10.3
Romania	21.0
Slovakia	27.5
Slovenia	30.0
Spain	22.2
Sweden	16.0
Switzerland	12.9
Turkey*	6.4
United Kingdom	15.4
EU27	17.3
EU25	17.1
EU15	16.0
NMS	23.0

EU27: 25 EU Member States, plus the two countries that joined the European Union in 2007 – Bulgaria and Romania
EU25: 15 EU Member States. plus the 10 new Member States that joined in 2004
EU15: 15 EU Member States prior to enlargement in 2004
NMS: 10 New Member States that joined in 2004
* Two candidate countries for membership of the EU: Croatia and Turkey

Figure 1.2. Prevalence of evening and night work by group of country in Europe in 2005 (4th EU Survey on working conditions)



Country codes

EU15	15 EU Member States prior to enlargement in 2004		
NMS	10 new Member States that joined in 2004		
EU25	15 EU Member States, plus the 10 NMS		
EU27	25 EU Member States, plus the AC2		
AC2	Two countries that joined the European Union in 2007: Bulgaria and Romania		
CC2	Two candidate countries for membership of the EU: Croatia and Turkey		
AT	Austria	LU	Luxembourg
BE	Belgium	MT	Malta
BG	Bulgaria	NL	Netherlands
CY	Cyprus	PL	Poland
CZ	Czech Republic	PT	Portugal
DK	Denmark	RO	Romania
EE	Estonia	SK	Slovakia
FI	Finland	SI	Slovenia
FR	France	ES	Spain
DE	Germany	SE	Sweden
EL	Greece	UK	United Kingdom
HU	Hungary	HR	Croatia
IE	Ireland	NO	Norway
IT	Italy	CH	Switzerland
LV	Latvia	TR	Turkey
LT	Lithuania		

Country groups
Continental countries: AT, BE, DE, FR, LU
Ireland and the United Kingdom: IE, UK
Eastern European countries,: CZ, EE, HU, LT, LV, Pl, SI, SK
Southern European countries: CY, EL, ES, IT, MT, PT
Scandinavian countries and the Netherlands: DK, FI, NL, SE
Acceding countries: BG, RO
Candidate countries: HR, TR
EFTA (European Free Trade Association): CH, NO
Typology adapted from Esping-Andersen

Figure 1.3. Prevalence of evening and night work by work sector in Europe in 2005 (4th EU Survey on working conditions)

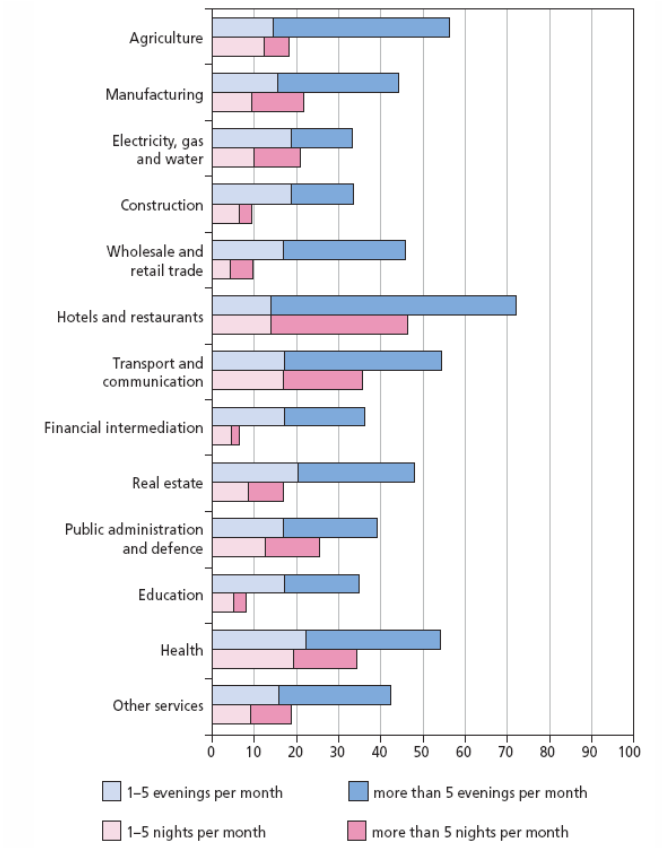


Table 1.3. Prevalence (%) of shiftwork that includes night work, by work activity in Europe in 2005 (4th EU Survey on working conditions)

Agriculture and fisheries	4.5
Armed forces	19.2
Clerks	13.4
Construction	5.3
Craft and related trades	17.6
Education	8
Electricity, gas and water supply	17.4
Elementary occupations	19.2
Financial intermediation	6.2
Health	35.5
Hotels and restaurants	29.9
Legislators, senior officials and managers	8.8
Manufacture and mining	25.8
Plant and machine operators and assemblers	34.5
Professionals	11.6
Public administration and defence	17.7
Real estate	9.5
Service, shop and market sales workers	26.9
Skilled agricultural and fishery workers	2.6
Technicians and associate professionals	14.3
Transport and communications	24.1
Wholesale and retail trade	16.3
Self-employed	5.7
Employee	19.8

Table 1.4. Prevalence (%) of shiftwork, including night work, by gender and age, in Europe in 2005 (4th EU Survey on working conditions)

Gender	Men	17.2
	Women	17.4
Age (years)	≤24	20.7
	25–39	19.1
	40–54	16.7
	≥55	10.5

Table 1.5. Percent distribution of shiftwork in full-time wage and salary workers by sex, race and ethnicity, in the USA in 2004 (US Bureau of Labor Statistics)

Total (>16 years)	14.8
Men	16.7
Women	12.4
White	13.7
Black or african american	20.8
Asian	15.7
Hispanic or latin ethnicity	16

Table 1.5 (contd)

20–24 years	22.3
25–34 years	15.2
35–44 years	14.1
45–54 years	12.8
55–64 years	12.5
≥65 years	10.3

Table 1.6 Percent distribution of shiftwork in full-time wage and salary workers, by occupation and industry, in the USA in 2004 (US Bureau of Labor Statistics)

Occupation	
Management professionals	8.7
Service occupations	36.1
Sales and office occupations	16.4
Natural resources, construction and maintenance	7.6
Production, transportation and material-moving occupations	26.4
Industry	
Private sector	15.4
Agriculture and related industries	9.5
Mining	31.5
Construction	2.8
Manufacturing	17.7
Wholesale and retail trade	22.0
Transportation and utilities	27.8
Information	15
Financial activities	7.0
Professionals and business services	9.4
Education and health services	12.8
Leisure and hospitality	45.8
Other services	13.0
Public sector	11.9
Federal government	14.7
State government	11.5
Local government	11.3

1.3.1 *Exposure assessment*

It is difficult to assess the effective "exposure" and the consequent "risk" of shiftwork with the common methods used (i.e. in toxicology) as the "dose" can widely differ not only in terms of quantitative load, i.e. in relation to the time spent in shiftwork, but mainly in terms of qualitative aspects, i.e. in relation to the interference that different shift

systems may have on biological and psychosocial functions, also taking into account several concurrent individual, social, and working factors.

The various combinations of these aspects can cause a different amount of stress and also different stress-related effects, thus making it difficult to compare groups without adjusting for the amount of "exposure", at least for the type of shift schedule and the years spent in shiftwork.

From a biological perspective, the occurrence and amount of night work is the most important factor to be considered. It is then possible to estimate roughly the effects (more or less severe) the different shift systems may have on health through interference on biological function, and on psychosocial issues.

Several methods have been proposed for assessing working time arrangements to evaluate their potential risk for health and well-being. The criteria most widely used are perturbation of the circadian rhythm, performance at work (ability to work efficiently), health, and social life (Wedderburn, 1994).

The "Rota Risk Profile Analysis," proposed by Jansen and Kroon (1995), describes several risk factors associated with with roster design, related to both physiological and psychosocial aspects, that must be considered. in particular: regularity of shift timetable, periodicity (i.e. the degree to which the "biological clock" is disturbed), shift load (i.e. the average length of shifts) and week load (i.e. the average length of the working week), opportunities for night rest (for sleeping between 11 pm and 7 am) and constancy in night rest (variation in the week), predictability of the shift cycles, opportunities and constancy for household and family tasks, opportunities and constancy for evening recreation (between 7 pm to 11 pm), opportunities and constancy for weekend recreation.

1.3.2 *Factors influencing shiftwork exposure and health*

Many health impairments associated with shiftwork have been reported. These include psychosomatic disorders of the gastrointestinal tract (colitis, gastroduodenitis, and peptic ulcer) and of the cardiovascular system (hypertension, ischaemic heart diseases), as well as metabolic disturbances, that are influenced by other time- and work-related factors and behaviours (Costa, 1996; Knutsson, 2003).

About 20% of all workers have to stop shiftwork altogether after a very brief period because of serious health problems, 10% do not complain about shiftwork during their whole working life, while the remaining 70% withstand shiftwork with different levels of intolerance that can become more or less manifest at different times and with different intensity in terms of discomforts, troubles or diseases (Waterhouse *et al.*, 1992).

1.3.3 *Some lifestyle factors that possibly modify the effects of exposure*

Some personal risk factors can act either as confounders or mediators, and/or modifiers, of the relation between shiftwork and health. Smoking and diet, generally considered as confounders in epidemiological studies, can also be intermediate factors of

the effects of shiftwork (i.e. for cardiovascular and gastrointestinal disorders). Many studies have reported that shiftworkers tend to smoke more (Bøggild and Knutsson, 1999; van Amelsvoort *et al.*, 2006) and/or increase their consumption of caffeinated or alcoholic drinks at night, as well as modify the composition and the caloric distribution of the different meals, i.e. by increasing carbohydrate intake at regular intervals (Reinberg *et al.*, 1979; Romon *et al.*, 1986; Lennernäs *et al.*, 1993). Metabolic disturbances have been found to be prevalent in shiftworkers (Knutsson *et al.*, 1990; Karlsson *et al.*, 2001). Of concern are mainly the risks for cardiovascular disease and obesity (Tenkanen *et al.*, 1998).

1.3.4 *Specificity of exposure to shiftwork for some particular occupations*

(a) Aircraft crew and transmeridian travel over time zones

Aircraft crews operating on long transmeridian flights have to cope with a shift in external time in addition to the shift of the working period. Therefore, the individual biological rhythms have to adjust to abnormal working hours in a changed environmental context. The short-term problems arising from these conflicts are similar to those of normal shiftwork, but are often aggravated by the fatigue due to the extended duty periods, and by a loss of the usual external time cues.

After a long transmeridian flight, the circadian system does not adjust immediately to the new local time, but requires several days in relation to the number of time zones crossed; the greater the number, the longer is the time required, considering that the human circadian system can adjust to no more than 60–90 minutes per day (Wegmann and Klein, 1985).

The adjustment is generally more rapid in westbound (about 1 day per hour of shift) than eastbound flights (about 1.5 day per hour of shift; Ariznavarreta *et al.*, 2002; Gander *et al.,* 1989; Suvanto *et al.,* 1990). In the first case, there is a progressive phase delay of the circadian rhythm in relation to the extended personal day, whereas in the latter there is a phase advance due to the compressed day (*directional asymmetry*). A complete readjustment after transition of six time zones was found to take 13 days and 10 days in eastward and westward flights, respectively (Wegmann and Klein, 1985).

In addition, crews are exposed to many other concurrent risk factors, such as cosmic radiation, electromagnetic fields, lighting, noise, acceleration, vibration, mental stress, fixed postures, and pressurization.

No statistics are currently available on the entire population employed in transmeridian flights, and consequently in related shiftwork, which is generally characterized by very irregular shift schedules. Only in the case of pilots and flight engineers, are there data that can provide a rough idea of the possible number of workers involved, considering that they generally account for about 20% of the total aircraft crew members.

The US Aircraft Owners and Pilots Association (IAOPA 2007) estimated that the civil aviation worldwide during 2004 consisted of approximately 370 000 aircraft and 1.3 million pilots flying some 39 million hours. On balance, roughly 600 000 pilots were employed in commercial air transportation worldwide (including cargo and charter).

The US Bureau of Labor Statistics (2007), reported that civilian aircraft pilots and flight engineers held about 107 000 jobs in the USA in 2006. About 79 000 worked as regular airline pilots, copilots, and flight engineers. The remainder were commercial pilots who worked as flight instructors at local airports or for large businesses that fly company cargo, and executives in their own airplanes or helicopters.

(b) Watchkeeping and driving

Ship's crew members engaged in long distance navigation work on continuous shiftwork, with some differences compare to land-based shiftworkers. For example, they can only take rest time in their place of work after the duty period, and usually have no rest days up until the end of the sea voyage is concluded. Moreover, they also have to cross several time zones (at different speed compared to flight crews), and their leisure time is limited both in terms of space and time. Several different shift systems are used. In merchant fleets, the personnel is generally divided into two or three crews working 12-hour or 8-hour shifts respectively, whereas on warships the crew work more frequently on the "4-hour watch" system, by dividing the 24-hour period into six 4-hour watches, and rotating on a "4-h on/8-h off" schedule, that allows one full night sleep in three. In general, in this shift schedule, the average amount of sleep is nearly the same as that of dayworkers, but the sleep is fragmented into two periods. A further system, the 6-hour on/6-hour off system is becoming more and more common on warships. However, high irregularity and variability of shift duration and rotation are quite frequent due to crew shortage, additional duties and unexpected situations, thus the amount of rest and sleep hours may vary considerably among days and subjects (Eriksen *et al.*, 2006).

The situation is similar for shiftworkers of offshore oil installations, who live in the same environment during both work and leisure time and stay away from home for several weeks, usually working in alternating 12-hour shift schedules (6:00–18:00, 18:00–6:00). In addition, in this occupational setting, the (mal)adjustment of the circadian rhythm may be more or less pronounced and depends on whether the fast or slow rotation is adopted, job characteristics (drilling, maintenance), and working organization (Barnes *et al.*, 1998).

Similar problems can be faced by long-haul truck and train drivers (i.e. coast-to-coast journeys, relay work), in which shiftwork, long working hours and time zone crossing interact in causing circadian disruption of the sleep/wake cycle and biological rhythms, as well as sleep deprivation, and overall fatigue (Jay *et al.*, 2006).

1.4 Biomarkers of circadian regulation and dysregulation

The production and release of nearly all hormones exhibits a circadian timing patterned on approximately a 24-hour cycle (Pandi-Perumal *et al.*, 2007). Agents that disrupt the circadian rhythm may therefore alter hormone levels.

At present, there is no known biomarker of exposure to shiftwork, which is thought to affect circadian regulation. In the past, core body temperature or blood cortisol levels have been used as markers of circadian regulation. However, given the importance of melatonin in the regulation of circadian rhythm, an indicator of melatonin levels is considered a preferable biomarker of circadian regulation and dysregulation, and has been more commonly used in recent studies on the effects of shiftwork in humans. Melatonin levels are comparatively robust in the presence of various external influences (Pandi-Perumal *et al.*, 2007). For example, excessive carbohydrate intake can significantly affect core body temperature and heart rate, whereas melatonin concentration remains relatively unaffected by this factor (Kräuchi *et al.*, 2002). Furthermore, the onset of melatonin production is largely unaffected by biochemical and physiological factors, which further suggests its greater reliability to measure circadian phase position (Lewy, 1999; Lewy *et al.*, 1999).

1.4.1 *Methods of measuring circulating melatonin*

(a) Melatonin in serum and plasma

Plasma melatonin, which has a very short biological half-life and is rapidly metabolized by the liver, reflects the amount of melatonin circulating at the point in time of the sample collection. Thus, measurement of melatonin in plasma at regular intervals (e.g. hourly) will map out a circadian rhythm, enabling identification of the onset of melatonin secretion, the duration of melatonin secretion, peak levels of circulating melatonin, and the time at which peak secretion occurred, and the total amount of melatonin secreted. Although such detailed information may be very useful in identifying the characteristics of the circadian rhythm in an individual, such measurement is possible only in a controlled setting (e.g. sleep laboratory), and is impractical in other applications such as an epidemiological study or other widespread population use.

(b) Melatonin in saliva

Melatonin can also be measured in saliva, using several different laboratory techniques. Salivary testing is a useful method for measuring melatonin in epidemiological studies, given that it is relatively non-invasive and generally acceptable to study participants. With proper training, study subjects can collect their own samples at home, to be later delivered to the laboratory for assay. Several researchers have found a high correlation between serum and salivary melatonin concentrations, and have concluded that salivary melatonin concentrations are reliable indices of serum melatonin

concentrations (Arendt *et al.*, 1985; Laakso *et al.*, 1990; Klante *et al.*, 1997; Davis *et al.*, 2001; Gooneratne *et al.*, 2003). However, Laakso *et al.* (1990) compared salivary and serum melatonin levels and found that saliva and serum measurements were not highly correlated in individuals with low serum melatonin levels, and that the proportion of melatonin found in saliva decreased with increasing serum melatonin levels. They concluded that melatonin concentrations measured in saliva do not always consistently reflect the absolute concentrations in blood. Gooneratne *et al.* (2003) reported similar results in that serum and saliva melatonin levels were less correlated in individuals with low serum melatonin levels. The primary drawback to measuring melatonin in saliva is that, similar to plasma and serum measurements, salivary melatonin reflects the amount of melatonin circulating in the body at a given time-point. To capture details of the rhythm of melatonin secretion, such as the time of onset, peak levels, and cumulative secretion, one has to collect the subject's saliva samples at regular intervals throughout the night.

(c) Melatonin in urine

Arendt *et al.* (1985) suggested that measurement of the primary metabolite of melatonin excreted in urine would allow the non-invasive study of pineal function, useful in a broad range of applications. If appropriately executed, measurement of melatonin in the urine reflects the cumulative amount of circulating melatonin corresponding to the time period between the prior urine void and the collection of the subsequent urine sample. Using this approach to quantify melatonin levels in urine is typically accomplished through the measurement of 6-hydroxymelatonin sulfate (aMT6s), the primary metabolite in urine, although some studies directly measure urinary melatonin. The principal methods for determination of urinary aMT6s include assay by either radioimmunoassay (RIA) or enzyme-linked immunosorbant assay (ELISA); commercial kits are available for both methods. Concentrations of aMT6s are often adjusted by urinary creatinine concentrations to account for differing urine output volume from one individual to the next, and for separate urine collections within individuals (Klante *et al.*, 1997).

The stability of such measurements further promotes the usefulness of this technique, since long-term levels of hormones are often of interest in diseases with long latency periods. Davis *et al.* (2001) evaluated nocturnal aMT6s levels in a group of women 20–74 years of age over 3 consecutive days, then repeated the measurement protocol 3–6 months later. Urinary aMT6s concentrations have been shown to be highly and significantly correlated on consecutive days, as well as between measurements sessions over long time period until 5-year time period in several studies (Levallois *et al.*, 2001; Travis *et al.*, 2003). Levallois *et al.* (2001) measured urinary aMT6s concentrations over 2 consecutive days and a found similarly high correlation.

(d) Comparison between blood and urinary melatonin levels

As melatonin is secreted primarily at night, studies have focused on nocturnal samples when evaluating the correlation between melatonin levels in blood and urine, and found a high degree of correlation between nocturnal measurements of urinary melatonin or urinary aMT6s, and plasma or serum melatonin. Graham *et al.* (1998) found a significant relationship between total nocturnal plasma melatonin and both urinary aMT6s corrected for creatinine and urinary melatonin. Combining the two urinary measures of aMT6s and melatonin accounted for 72% of the variance in total plasma melatonin. Furthermore, peak nocturnal levels of plasma melatonin were significantly related to morning levels of urinary melatonin and aMT6s. Cook *et al.* (2000) assessed the differences in melatonin levels between blood and urine samples collected in a laboratory-based setting with nocturnal urine samples collected in a field study, and found very high correlations ($P < 0.001$) between first morning void melatonin and creatinine-corrected aMT6s and both total nocturnal plasma melatonin output and peak nocturnal plasma melatonin.

Similarly high correlations have been found in studies that compared melatonin in plasma and serum with urinary melatonin and/or urinary aMT6s over a 24-hour period (Markey *et al.*, 1985; Baskett *et al.*, 1998). Bojkowski *et al.* (1987) found that total 24-hour urinary excretion of aMT6s was significantly correlated with the area under the curve of the respective profiles for plasma melatonin ($r = 0.75$), and plasma aMT6s ($r = 0.70$).

In conclusion, both urinary melatonin and urinary aMT6s are good indicators of melatonin secretion in blood with a significantly smaller variation for the former molecule (Pääkkönen *et al.*, 2006). Such measurements in urine samples would provide a suitable tool in epidemiological settings to study the modulation of the circadian rhythm in shiftworkers.

1.5 Regulations on shiftwork

Some international directives have been issued in the last decades addressing the need for a careful organization of shift and night work and the protection of shiftworkers' health: in particular, the International Labour Office (ILO) "Code of practice on working time" (1995) and Convention no. 171 (C171) on "Night work" (1990), and the European Directive no. 93/104/EC "concerning certain aspects of the organization of working time" (1993), which in European countries has been implemented through national legislation.

1.5.1 *ILO Night Work Convention and Recommendation*

(a) General population

The ILO C171 Night Work Convention (International Labour Organization, 1990a) refers only to *night work*, that is "all work which is performed during a period of not less than seven consecutive hours, including the interval from midnight to 5am," and *night worker*, who is "an employed person whose work requires performance of a substantial number of hours of night work which exceeds a specified limit, fixed by the competent authority. This convention applies to all employed persons except those employed in agriculture, stock raising, fishing, maritime transport and inland navigation."

In addition, the ILO R178 Night Work Recommendation (International Labour Organization, 1990b), supplementing the Night Work Convention C171, points out the following:

"Normal hours of work for night workers should not exceed eight in any 24-hour period in which they perform night work, except in the case of work which includes substantial periods of mere attendance or stand-by, in cases in which alternative working schedules give workers at least equivalent protection over different periods or in cases of exceptional circumstances recognized by collective agreements or failing that by the competent authority.

The normal hours of work of night workers should generally be less on average than and, in any case, not exceed on average those of workers performing the same work to the same requirements by day in the branch of activity or the undertaking concerned.

In occupations involving special hazards or heavy physical or mental strain, no overtime should be performed by night workers before or after a daily period of work which includes night work, except in cases of force majeure or of actual or imminent accident.

Where shift work involves night work: (a) in no case should two consecutive full-time shifts be performed, except in cases of force majeure or of actual or imminent accident; (b) a rest period of at least 11 hours between two shifts should be guaranteed as far as is possible."

(b) Women during pregnancy and around childbirth

At any point during pregnancy, once this is known, women night workers who so request should be assigned to day work, as far as is practical.

Measures shall be taken to ensure that an alternative to night work is available to women workers who would otherwise be called upon to perform such work: (a) before and after childbirth, for a period of at least 16 weeks of which at least 8 weeks shall be before the expected date of childbirth; (b) for additional periods in respect of which a medical certificate is produced stating that it is necessary for the health of the mother or child: (i) during pregnancy; (ii) during a specified time beyond the period after childbirth fixed pursuant to subparagraph (a) above, the length of which shall be determined by the

competent authority after consulting the most representative organizations of employers and workers. These measures may include transfer to day work where this is possible, the provision of social security benefits or an extension of maternity leave. During those periods, a woman worker shall not be dismissed or given notice of dismissal, except for justifiable reasons not connected with pregnancy or childbirth, and shall not lose the benefits regarding status, income, seniority and access to promotion which may attach to her regular night work position (ILO C171, 1990).

(c) Young people

With regard to young people, following the first Night Work of Young Persons (Industry) Convention (1919), the ILO Night Work of Young Persons (Industry) Convention (Revised) (1948), stated that: "young persons under eighteen years of age shall not be employed or work during the night in any public or private industrial undertaking (i.e. mines, quarries, manufactures, construction, transports, electrical-gas works, etc.). "Night" means a period of at least twelve consecutive hours. In the case of young persons under sixteen years of age, this period shall include the interval between ten o'clock in the evening and six o'clock in the morning. Moreover, in the case of young persons who have reached the age of sixteen years but are under the age of eighteen years, this period shall include an interval prescribed by the competent authority of at least seven consecutive hours falling between ten o'clock in the evening and seven o'clock in the morning. For purposes of apprenticeship or vocational training in specified industries or occupations which are required to be carried on continuously, the Convention stated that the competent authority may, after consultation with the employers' and workers' organizations concerned, authorise the employment in night work of young persons who have reached the age of sixteen years but are under the age of eighteen years."

(d) Seafarers

For specific groups of workers, the ILO Convention No. 180 "Concerning Seafarers' Hours of Work and the Manning of Ships" (1996) states limits on hours of work or rest, in particular: "a) maximum hours of work shall not exceed 14 hours in any 24-hour period, and 72 hours in any seven-day period; b) minimum hours of rest shall not be less than ten hours in any 24-hour period, and 77 hours in any seven-day period; c) hours of rest may be divided into no more than two periods, one of which shall be at least six hours in length, and the interval between consecutive periods of rest shall not exceed 14 hours. Moreover, no seafarer under 18 years of age shall work at night (which means a period of at least nine consecutive hours, including the interval from midnight to five a.m.)."

(e) Long-distance drivers

According to the US Bureau of Labor Statistics (2007) long-distance drivers may drive for 11 hours and work for up to 14 hours – including driving and non-driving duties – after having 10 hours off-duty. Moreover, they may not drive after having worked for

60 hours in the past 7 days or 70 hours in the past 8 days unless they have taken at least 34 consecutive hours off-duty.

(f) Airline pilots

According to the National Aeronautics and Space Administration guidelines (Dinges *et al.* 1996), for standard operations including day and night flying, the duty period for air pilots should not exceed 10 hours within a 24-hour period; in case of extended flight duty periods, the limit should be fixed at 12 hours, and accompanied by additional restrictions and compensatory off-duty periods. It is also recommended that in any 7-day period, there be no extended flight duty period that encroaches on any portion of the window of circadian low (i.e. period between 2–6 am for an individual's normal day–wake/night–sleep schedule).

Because of Federal Aviation Administration regulations, airline pilots flying large aircraft, cannot fly more than 100 hours a month or more than 1000 hours a year. Most airline pilots fly an average of 75 hours a month and work an additional 75 hours a month performing non-flying duties. To guard against pilot fatigue, which could result in unsafe flying conditions, the Federal Aviation Administration requires airlines to allow pilots at least 8 hours of uninterrupted rest in the 24 hours before finishing their flight duty.

Many countries in the world have national laws regulating night work according to ILO recommendations, whereas in many others this topic is regulated by means of collective or local agreements between parties (International Labour Organization, 1995).

1.5.2 *European Directive on Working Time*

(a) General population

In Europe, the EU Council Directive No 93/104/EC (European Council Directive, 1993) "concerning certain aspects of the organization of working time" (re-confirmed by EU Directive 2003/88/EC):

– defined "night time" as "any period of not less than seven hours, as defined by national law, and which must include in any case the period between midnight and 5 am"; and "night worker" as (a) any worker who, during night time, works at least three hours of his/her daily working time as a normal course, and (b) any worker who is likely during night time to work a certain proportion of his/her annual working time, as defined at the choice of the Member State concerned either by national legislation or by collective agreements. On the other hand, shift work means "any method of organising work in shifts whereby workers succeed each other at the same work stations according to a certain pattern, including a rotating pattern, and which may be continuous or discontinuous, entailing the need for workers to work at different times over a given period of days or weeks; consequently, "shift worker shall mean any worker whose work schedule is part of shift work."

– forced Member States to take the measures necessary to ensure that: normal hours of work for night workers do not exceed an average of 8 hours in any 24-hour period for normal work activities, but not more than 8 hours in any 24-hour period in case of work involving special hazards or heavy physical or mental strain; every worker is entitled to a minimum daily rest period of 11 consecutive hours per 24-hour period; where the working day is longer than 6 hours, every worker is entitled to a rest break; per each seven-day period, every worker is entitled to a minimum uninterrupted rest period of 24 hours plus the 11 hours daily rest; and it should preferably include Sunday; the average working time for each seven-day period, including overtime, does not exceed 48 hours; every worker is entitled to paid annual leave of at least four weeks in accordance with the conditions for entitlement to, and granting of, such leave laid down by national legislation and/or practice; the minimum period of paid annual leave may not be replaced by an allowance in lieu, except where the employment relationship is terminated.

Implementing such directive at national level, some European countries added the quantitative criterium of 80 night shifts worked per years as minimum level for establishing the compulsory periodical medical surveillance for night workers: this limit appears as a mere technical compromise among social parties (i.e. one third of the total working days), being not supported by any evidence based on the scientific literature.

There are also some differences among countries in the definition of both "night work" and "night worker" (see Table 1.7).

Table 1.7. Legislation on night work in 15 EU[a] countries

Country	Max. length of night work in hours	Legislation
AUSTRIA	–	Nachtschwerarbeitsgesetz nr. 354/1981 (rev. 1993)– "Night work": period of at least 6 hours between 22:00 and 06:00 for at least six nights a month. Additional breaks: 10 min paid break during the night shift. Additional vacations: 60 nightshifts per year, 2 work days, after 5 years on shift, 4 work days, after 15 years on shift, 6 work days. Health service, possibility of early retirement.
BELGIUM	**8**	Loi du 17/02/1997 et Loi du 04/12/1998: "Night time": a period, generally of 8 hours, between 20:00 and 06:00. "Night work": in principle, prohibited, but various derogations are possible.
DENMARK	–	The notions of night time and night worker have been defined generally in collective agreements**.**

Table 1.7 (contd)

Country	Max. length of night work in hours	Legislation
FINLAND	**–**	Working Hours Act 605/1996: "Night work": work of at least 3 hours between 23.00 and 06.00. An employer must notify the labour protection authorities of regular night work, when the said authorities so request.
FRANCE	**–**	Loi 461/1998: "Night time": period between 22:00 and 05:00 or whichever night work period between midnight and 05:00. "Night workers": any employee working usually at least 2 times per week at least 3 hours on the period defined as night work.
GERMANY	**8/10**	Arbeitszeitgesetz 1994: "Night time": a period which includes the time between 23.00 and 06.00, in the case of bakers between 22.00 and 05.00. "Night work": every kind of work which includes more than 2 hours of night time. The working time of a night worker and shiftworker shall not exceed 8 hours, or 10 hours if within a month or a 4-weeks period where the average working hours are 8 hours per day. The night workers are entitled to a health assessment before they take up the assignment and after that, every 3 years. After the age of 50, the time is reduced to 1 year. "Night worker": a worker who works at least 2 hours during night time. "Night workers" are those workers who usually work nights in rotating shifts system or works at night on not less than 48 days during a year. The working time of a night worker and shiftworker shall be laid out according to evidence based knowledge about human centred design of working hours from ergonomics.
GREECE	**8**	Presidential Decree no. 88/1999: "Night time": period of 8 hours which includes the period between 22:00 and 06:00. "Night worker": a worker who during night time works at least 3 hours of his daily working time or a worker who has to perform night work for at least 726 hours of his annual working time.
IRELAND	**9**	Statutory Instruments no. 485/1998: "Night time": period between midnight and 07.00. "Night worker": a) an employee who normally works at least 3 hours of his or her daily working time during night time; b) an employee whose working hours during night time, in each year, equals or exceeds 50 per cent of the total number of hours worked during the year.

Table 1.7 (contd)

Country	Max. length of night work in hours	Legislation
ITALY	–	D.Lgs. 66/2003: "Night work": the activity carried out in a period of at least 7 consecutive hours comprising the interval between midnight and 05.00 in the morning. "Night worker": a) any worker who during the night period carries out, in a not exceptional way, at least 3 hours of his daily working time; b) any worker who carries out, during the night, at least a part of his normal working hours. Night work does not have to be done obligatorily by: a) the working mother of a child under 3 years of age or, alternatively, by the cohabiting father; b) the worker who is the only entrusted parent of a cohabiting child of less than 12 years of age; c) the worker who takes care of a disabled subject. Women are forbidden to work from 24.00 to 06.00, from the assessment of state of pregnancy until the first year of age of the child. Thereafter their assignment to night work is on voluntary basis until the third year of age of the child.
LUXEMBOURG	–	There is no general legislation on night work or night worker.
NETHERLANDS	–	Wet van 23/11/1995: "Night work": work which covers all or part of the period from midnight to 06:00.
PORTUGAL	**8**	Decreto Lei 259/98: "Night time": a period between 20:00 and 07:00 L.73/98: "Night work": shall not exceed 8 hours. The night workers with risks shall not work more than 8 hours in a period of 24 hours. The employer ensures the worker the opportunity of a free health assessment before he takes up the assignment and during the period of work.
SPAIN	**8**	Real Decreto Lei 1/1995: "Night time": the period which includes the interval between 22.00 and 06.00. "Night work": shall not exceed the 8 hours in a work period of 15 days. The employer, who usually utilizes night work, has to inform the authority. "Night worker": the worker who at night carries out at least 3 hours of its daily working time".

Table 1.7 (contd)

Country	Max. length of night work in hours	Legislation
SWEDEN	–	Working Hours Act 1982: All employees shall be afforded free time for nightly rest. Such free time shall include the hours between midnight and 05:00. Exception could be made depending on the nature of the work. "Night worker": a worker that works at least 3 hours of his daily work during night time, or a worker that most likely will work at least 38% of his annual work during the night.
UK	**8**	Statutory Instruments.1833/1998: "Night time": a period the duration of which is not less than 7 hours, and which includes the period between midnight and 05:00. A nightworker's normal hours of work, in any reference period which is applicable in his case, shall not exceed an average of 8 hours for each 24 hours. "Night worker": a worker who, as a normal course, works at least 3 hours of his daily working time during night time, or who is likely, during night time, to work at least such proportion of his annual working time as may be specified for the purposes of these regulations in a collective agreement or a workforce agreement. An employer shall not assign an adult worker to work which is to be undertaken during periods such that the worker will become a night worker unless the employer has ensured that the worker will have the opportunity of a free health assessment before he takes up the assignment; or the worker had a health assessment before being assigned to work to be undertaken during such periods on an earlier occasion, and the employer has no reason to believe that that assessment is no longer valid.

[a] Council Directive 93/104/EC of 23 November 1993 concerning certain aspects of the organization of working time.
Compiled by the Working Group

(b) Women during pregnancy and around childbirth

For women, the EU Council Directive 92/85/EEC (European Council Directive, 1992), "on the introduction of measures to encourage improvements in the safety and health at work of pregnant workers and workers who have recently given birth or are breastfeeding," forced Member States to take the necessary measures to ensure that such workers are not obliged to perform night work during their pregnancy and for a period following childbirth which shall be determined by the national authority competent for safety and health. These measures must entail the possibility, in accordance with national

legislation and/or national practice, of transfer to daytime work, or leave from work or extension of maternity leave where such a transfer is not technically and/or objectively feasible.

In most legislations of European countries, women are prohibited to work at night from the assessment of state of pregnancy until the first year of age of the child. Thereafter, in many cases, assignment to night work is on voluntary basis until the third year of the child.

(c) Young people

For young people, the European Council Directive 94/33/EC (1994) on the protection of young people at work states that: "Member States shall adopt the measures necessary to prohibit work by children (less than 15 years of age) between 8 pm and 6 am (in case of cultural or similar activities allowed to children), and by adolescents (15–18 years of age) either between 10 pm and 6 am or between 11 pm and 7 am. For adolescents, there may be some exceptions in specific areas provided that they are supervised by an adult, but work between midnight and 4 am continues to be prohibited.

1.5.3 *Scientific guidelines*

The main indications for the design of better shift systems according to ergonomic criteria are (Knauth, 1996; Knauth and Hornberger, 2003; Wedderburn, 1994):

a) Quickly rotating shift systems are better than slowly rotating ones.

b) Clockwise rotation (morning/afternoon/night) is preferable to counter-clockwise (afternoon/morning/night).

c) Early starts for the morning shift should be avoided.

d) Prolonged work shifts (9–12 hour) should only be considered when the workload is suitable, there are adequate breaks, and the shift system is designed to minimize accumulation of fatigue and exposure to toxic substances.

e) Shift systems should be regular and able to guarantee as many free weekends as possible.

f) Permanent night work can be acceptable only for particular working situations which require a complete adjustment to night work to guarantee the highest levels of safety. Be aware that such complete adjustment requires people to maintain the inverted sleep/wake cycle also on rest days and to avoid exposure to bright light after night shifts (i.e. wearing dark sun glasses while commuting home).

g) Adequate time off between shifts should be allowed to compensate for fatigue and sleep as quickly as possible (i.e. two shifts in the same day must be avoided), and rest days should come preferably after the night duty period to allow prompt recovery from sleep deficit and an easier return to the normal sleep/wake cycle.

h) Some flexibility in working times is desirable to give the workers the possibility of combining better work duties with family and social life.

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2. Studies of Cancer in Humans

2.1 Introduction

Airline personnel flying over time zones are exposed to frequent disruptions of circadian rhythm, which has similarities with exposure to shiftwork. There are studies reporting cancer risk in about ten cohorts of airline cabin crew and a similar number of studies in cockpit personnel. The cabin crew cohorts support the strong evidence of significantly increased risk of breast cancer incidence found in most independent studies. Higher diagnostic activity (screening during annual health controls) may explain part of the excess when comparing with national population rates, and it should not confound internal comparisons within differently exposed subcohorts of cabin crew. Unfortunately, the studies published so far do not demonstrate precise dose–response evaluations according to the frequency of disruptions of circadian rhythm, for which the best proxy has been duration of work as flight attendant. In most studies, the excess is observed at around 10 years after first employment, and increases weakly with increasing duration. Differences in reproductive factors explain only a small fraction of the excess, while risk attributable to radiation may explain a quarter of the excess. It is unclear whether the substantial neutron component of cosmic radiation (25–50% of the effective dose but less than 5% of the absorbed dose) increases the proportion of risk attributable to radiation – this exposure can only be studied in flight crew personnel – but it is likely that there is a major part of the excess risk in breast that must be attributable to factors others than the factors listed above. Disruptions of circadian rhythm and related hormonal effects have been repeatedly mentioned as possible causal factors, and there are no data to exclude this possibility.

Prostate cancer incidence rates from the airline pilot cohorts are above the national reference levels. This excess has decreased over decades and is likely to be related to the prostate-specific antigen tests, common among pilots much earlier they became so in the general population. In the most recent follow-up reports, the SIRs among pilots have been only slightly increased. Only one study that combined cohorts of all pilots from five Nordic countries, with detailed individual level flight histories, was able to study the independent role of the long-haul flights over time zones in an internal analysis. A significant trend in risk for prostate cancer with increasing number of long-haul flights was observed, though there were only eight cases in the highest exposure category. Hence, the evidence related to the role of circadian rhythm disruptions in causing prostate cancer is weak.

2.2 Shiftwork

2.2.1 *Breast cancer*

Eight studies reported relative risk estimates for histologically confirmed breast cancer for female night shiftworkers, with vastly differing definitions of shiftwork in each study. The characteristics of these studies are presented in Tables 2.1–2.3. Two were prospective cohort studies (Schernhammer *et al.*, 2001; Schernhammer & Hankinson, 2005), one was a nationwide census-based cohort study (Schwartzbaum *et al.*, 2007), three were nested case–control studies (Tynes *et al.*, 1996; Hansen, 2001a; Lie *et al.*, 2006), and two were retrospective case–control studies (Davis *et al.*, 2001; O'Leary *et al.*, 2006). All eligible studies included caucasian women; only one study (O'Leary *et al.*, 2006) included a small proportion of Latino and African-American women (less than 10%). The majority of women studied were postmenopausal.

(a) Prospective cohort studies (Table 2.1)

The two prospective cohort studies of night shiftwork and breast cancer risk used data from the Nurses' Health Study cohorts (NHS and NHS II) (Schernhammer *et al.*, 2001; Schernhammer *et al.*, 2006). The NHS began in 1976, when 121 701 registered nurses 30–55 years of age and living in 11 large US states were enrolled and completed a questionnaire comprising items about their health status, medical history, and known or suspected risk factors for cancer. Since baseline, questionnaires have been mailed biannually with the exception of lifetime history of night work in years, which was only assessed once (in 1988). Follow-up data are available for more than 90% of the ongoing cohort. In 1988, the study participants were asked how many years in total they had worked rotating night shifts with at least three nights per month, in addition to days or evenings in that month. The second cohort, NHS II, was designed in a very similar fashion. It started in 1989, when 116 671 registered female nurses (no overlap with NHS) 25–42 years of age, and from 14 US states were enrolled. Since 1989, they have completed biennial questionnaires that include items about their health status risk factors for chronic disease. Response rates to questionnaires are at 90%. In NHS II, the 1989 baseline questionnaire included detailed questions on total months during which study participants had worked on rotating night shifts for at least three nights per month in addition to days or evenings in that month. This information was updated in 1991, 1993, 1997, and 2001. Questions were asked regarding both rotating night shifts and permanent night shifts for 6 months or more in this cohort.

In the NHS, Schernhammer *et al.* (2001) followed a total of 78 562 women who answered the 1988 question on night work and were cancer-free at baseline over 10 years (1988–1998): of these women, 2441 incident breast cancer cases were documented during that time. The relative risks (RRs) for breast cancer associated with rotating night work compared to women who reported never having worked rotating night shifts, after

Table 2.1. Cohort studies of night shiftwork and breast cancer

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/deaths	OR or RR (extreme group versus referent)	Adjustment for potential confounders	Comments
Schernhammer *et al.* (2001) USA Nurses' Health Study (NHS)	Prospective cohort study of 121 701 registered nurses from 11 large states, established in 1976; follow-up from 1988–1998	Self-reported life time years on rotating night shifts, one-timed assessment in 1988; rotating night shifts were defined as "at least 3 nights per month, in addition to evenings and afternoons in that month"	Breast cancer	*Years of rotating night work* Never 1–15 15–29 ≥30 *P* for trend	 925 1324 134 58	 1.0 (ref) 1.08 (0.99–1.18) 1.08 (0.90–1.30) 1.36 (1.0–1.78) 0.02	Age, age at menarche, parity, age at first birth, weight change, BMI, family history of breast cancer, benign breast disease, oral contraceptive use, age at menopause, alcohol consumption, use of postmenopausal hormones, menopausal status, height	

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/deaths	OR or RR (extreme group versus referent)	Adjustment for potential confounders	Comments
Schernhammer *et al.* (2006) USA Nurses' Health Study II (NHS II)	Prospective cohort study of 116 087 registered nurses from 14 states, established in 1989; follow-up from 1989–2001	Self-reported life time years on rotating night shifts, one-timed assessment in 1989; biannual update; rotating night shifts were defined as "at least 3 nights per month, in addition to evenings and afternoons in that month"	Breast cancer	*Years of rotating night work* Never 1–9 10–19 20+ *P* for trend	 441 816 80 15	 1.0 0.98 (0.87–1.10) 0.91 (0.72–1.16) 1.79 (1.06–3.01) 0.65	Age, age at menarche, parity, age at first birth, BMI, family history of breast cancer, benign breast disease, alcohol consumption, oral contraceptive use, smoking status, menopausal status, age at menopause, physical activity, postmenopausal hormone use	

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/deaths	OR or RR (extreme group versus referent)	Adjustment for potential confounders	Comments
Schwartzbaum *et al.* (2007) Sweden Register-based – all female residents of Sweden in the work force at census in 1960 and 1970	Register-based retrospective cohort study; 1 148 661 female workers; follow-up 1971–1989; 70 breast cancer cases among 3057 women with night work (40%)	Usual occupation & work hours (three-shift schedules and others) to define occupations with a large proportion of workers with night work; from in-person interview from annual survey of living conditions (1977–1981) among 55 323 randomly invited Swedes (84% participated)	Breast cancer	Shiftwork in 1970 Shiftwork in both 1960 & 1970	70 28	0.94 (0.74–1.18) 0.97 (0.67–1.40)	Age, socioeconomic status, occupational position (employed manager, other employee, self-employed with employees, self-employed without employees), county of residence (marital status and urbanization not important)	Shiftwork defined as occupations with at least 40% of the workers either reporting that they worked rotating shifts with 3 possible shifts or had work hours during the night ≥1 day before interview

controlling for known breast-cancer risk factors, were as follows: for 1–14 years, 1.08 (95% CI: 0.99–1.18); for 15–29 years, 1.08 (95% CI: 0.90–1.30); and for 30 or more years, 1.36 (95% CI: 1.04–1.78). The risk increased with increasing numbers of years in shiftwork (P for trend = 0.02). [The main strengths of this study are the prospective assessment of night work information and a wide range of potential confounding factors in a well defined occupation cohort of nurses, as well as the high follow-up rate (> 90%). Limitations of this study are its one-time assessment of night work and the inclusion of permanent night workers as well as those who worked < 3 nights per month among the unexposed reference group, which may have skewed the results towards the null].

Similarly, in 115 022 predominantly premenopausal women in the NHS II, Schernhammer *et al.* (2006) found an elevated breast cancer risk of 1.79 (95% CI: 1.06–3.01; $P = 0.65$) among women who worked 20 or more years of rotating night shiftwork compared with women who reported never having worked rotating night shifts, with 1352 incident breast cancer cases accruing over 12 years of follow-up (1989–2001). [The main strengths of this study are the prospective and updated assessment of rotating night work history and a wide range of potential confounding factors in a well defined occupational cohort of nurses, as well as the high follow-up rate (90%). Limitations are the inclusion of those who worked < 3 nights per month among the unexposed reference group, and the relatively small number of women ($n = 15$ women) in the category with longer durations of night work].

Schwartzbaum *et al.* (2007) found no increase in risk in female breast cancer from their definition of night work, based on 28 observed breast cancers versus 28.91 expected, diagnosed during 1971–1989. The design is a retrospective registry-based ecological cohort study comprising all 1 148 661 Swedish women that were active in the workforce according to both 1960 and 1970 census reports. Workers were followed up for breast cancer morbidity by linkage to the Swedish Cancer Registry. Information on occupation was derived from the censuses, which included each worker's industry and socioeconomic status. The annual surveys of living conditions (conducted during 1977–1981) among 46 438 randomly selected Swedish subjects who participated in a personal interview were used for assessing night work. Questions were asked regarding the usual occupation, work hours, and when they had started and ended working each day during the week preceding the interview. Shiftworkers were then defined as those who reported that their workplace had a rotating schedule with three or more possible shifts per day or had work hours during the night (any hour between 01:00 and 04:00) at least one day during the week preceding the interview. They classified as shiftworkers people working in job titles and industry combinations (from the censuses) with at least 40% shiftwork (as defined above). The reference group in their analyses comprised people in occupation–industry combinations in which less than 30% stated that they were shiftworkers. In analyses using 1970 census information for the definition of exposure, no increase in risk was reported among women with an occupation that was classified as shiftwork. Sub-analyses in this paper (which comprised all men and women working in Sweden) also considered 70% of shiftworkers as definition for occupation classification but due to

small sample size, this was not done for the women. [The weaknesses of this study include the implausibly small proportion of women working night shifts (only 0.3% worked in occupations with at least 40% shiftworkers working at least 20 hours per week), inadequate control for confounding, and that the three most common occupations that fell into their shiftwork classification were rather unusual (crane and hoist operators, delivery women in paper and paper-products manufacturing, printing and publishing industries, and midwives)].

(b) Nested case-control studies (Table 2.2)

Tynes *et al.* (1996) conducted a case–control study nested within a population-based cohort study of 2619 female Norwegian radio and telegraph operators working at sea and certified to work between 1920–1980, and followed up during 1961–1991. In total, 50 breast cancer cases were identified by linkage to the National Norwegian Cancer Registry, and each case was matched to four to seven disease-free controls from the cohort. For cases and controls, job histories on ships were collected and shiftwork as well as travel through time zones were classified for each ship mentioned in the job histories to define shiftwork. Shiftwork constituted frequent presence in the radio room both at night and during the day. After controlling for duration of employment, the SIR for breast cancer in this cohort was 1.5 (95% CI: 1.1–2.0). In the nested case–control study, there appeared to be an increased risk of breast cancer in women ≥ 50 years of age with increasing cumulative exposure to shiftwork, compared to no shiftwork (low exposure 0–3.1 years, adjusted for duration of employment, RR, 3.2, 95% CI: 0.6–17.3; high exposure 3.1–20.7 years, adjusted for duration of employment, RR, 4.3, 95% CI: 0.7–26.0; *P* for trend = 0.13). [The strength of this study is the use of internal controls, whereas the main limitation is its lack of control for confounding by breast cancer risk factors].

Hansen (2001a) conducted a population-based case–control study nested within the cohort of all female employees in Denmark established from the nationwide pension fund data, including information on all employments held since 1964. In total, 7035 women with incident breast cancer were identified by individual linkage to the files of the nationwide Danish Cancer Registry. Control subjects free of breast cancer were randomly drawn from the pension fund files and matched on year of birth and sex. The individual employment histories for cases and controls were reconstructed using files of the nationwide pension fund. Night work definition was based on information obtained from a nationwide interview-based survey on living and working environment conditions in 1976 among 2603 women. Trades in which at least 60% of female responders worked at night were considered to have a predominant night time schedule, whereas responders working in most trades with less than 40% reported night time schedules were regarded as day workers. The RR of breast cancer was 1.5 (95% CI: 1.3–1.7; 434 cases) among women who worked at least half a year at least 5 years before diagnosis in such trades, after controlling for age, social class, age at birth of first child, age at birth of last child, and number of children. For the subgroup of women with more than 6 years predominantly working at night, the RR was 1.7 (95% CI: 1.3–1.7; 117 cases). In further

Table 2.2. Nested case–control studies of night shiftwork and breast cancer

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	OR or RR (extreme group versus referent)	Adjustment for potential confounders	Comments
Tynes *et.al.* (1996) Norway Telecom cohort	Cohort of 2619 female radio and telegraph operators at sea, certified between 1920–1980; follow-up from 1961–1991. The nested case–control component comprised 50 cancer registry-identified cases and 4–7 matched (year of birth) controls	Collected detailed job histories from Norwegian seamen registry; "Work at night with exposure to artificial light." From cases and controls, detailed information on job histories on ship as well as shiftwork and travel through time zones was collected, classified by "ship"	*Shiftwork in women age < 50* None <3.1 yrs. >3.1 yrs *P* for trend *Aged 50+* None <3.1 yrs. >3.1 yrs *P* for trend	 12 5 12 3 6 12	 1.0 (ref) 0.3 (0.1–1.2) 0.9 (0.3–2.9) 0.97 1.0 (ref) 3.2 (0.6–17.3) 4.3 (0.7–26.0) 0.13	Age, duration of employment, parity, and age at first birth	

Table 2.2 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	OR or RR (extreme group versus referent)	Adjustment for potential confounders	Comments
Hansen (2001a,b) Denmark Linkage of Nationwide registries	Nested case–controls study; 7565 cancer-registry-derived women with breast cancer, 1:1 matched controls (year of birth and sex), follow-up 1964–1999	Individual employment histories were obtained from files of national pension fund	All night work combined in trades with >60% night work	434	1.5 (1.3–1.7)	Age, social class, age at birth of first child, age at birth of last child, number of children	Considered as night workers if employed ≥0.5 year in ≥1 trade in which ≥60% of the female responders had night time schedules Trades: beverage manufacture, land transport, catering, air transport
			Employed >6 years	117	1.7 (1.3–1.7)		
			Nurses	–	1.3 (1.1–1.4)		

Table 2.2 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/deaths	OR or RR (extreme group versus referent)	Adjustment for potential confounders	Comments
Lie *et al.* (2006) Norway Cohort of Norwegian nurses	Case–control study [537 cancer-registry-identified cases and 1:4 matched (year of birth) controls] nested within the 44 835 nurses-comprising cohort of Norwegian nurses; cases occurred between 1960–1982	Total work history reconstructed from occupational information for nurses from Norwegian Board of Health's registry, censuses 1960, 1970, & 1980	*Years night work* 0 1–14 15–29 30+ *P* for trend	 50 362 101 24	 1.0 (ref) 0.95 (0.67–1.33) 1.29 (0.82–2.02) 2.21 (1.10–4.45) 0.01	Total employment time as a nurse & parity; matched by birth year	

sub-analyses, the RR for nurses was also evaluated, a group in which 41% were considered having predominant night work (Hansen, 2001b), and a significantly increased risk of breast cancer was found (RR, 1.3; 95% CI: 1.1–1.4). [The strength of this study is its high number of incident cases and the apparent lack of selection and information bias due to use of routine data; its limitations include the crude exposure assessment with potential for non-differential misclassification as well as incomplete adjustment for confounding, in particular alcohol drinking.]

Lie *et al.* (2006) conducted a nested case–control study within a cohort of 44 835 Norwegian nurses based on information from the registry of the Norwegian Board on Health,established in 1949. In total, 537 breast cancer cases diagnosed during 1960–1982 were identified by linkage with the files of the nationwide cancer registry. Four age-matched controls were selected at random from the cohort, using incidence density sampling. Reconstruction of total work history was based on the nurses' registry (self-report of work history; until 1960 yearly updates, thereafter sporadically) and census information (1960, 1970, and 1980), accumulating from first year of employment until termination of the last employment. Based on experience, it was assumed that nurses employed at infirmaries worked nights (with the exception of managerial jobs, teaching, physiotherapy, and outpatients departments), whereas it was assumed that work sites other than infirmaries involved day work only. The authors found an association between duration of night work and breast cancer risk (P for trend = 0.01). The RR associated with > 30 years of night work was 2.21 (95% CI: 1.10–4.45), after adjustment for total employment time as a nurse and parity. [The main strength of this study is its high number of cases and the internal comparison, whereas limitations of this study are a lack of complete control for confounding as well as the potential for exposure misclassification, which is likely to be non-differential.]

(c) Case–control studies (Table 2.3)

Davis *et al.* (2001) conducted a case–control study of 813 women with breast cancer aged 20–75 years and 793 controls free from breast cancer. Cases were identified by the Cancer Surveillance System of Seattle, Washington, USA and controls were identified by random-digit dialling, frequency-matched on age (75% participation rate for controls). In-person interviews were performed from 1992–1995 to collect information about sleeping habits and light-at-night exposure during the 10 years before diagnosis as well as lifetime occupational history. The authors defined night work as at least one "graveyard" shift per week in the 10 years before diagnosis. "Graveyard" shiftwork was described as "beginning work after 19:00 and leaving work before 09:00". The RR of breast cancer was 1.6 (95% CI: 1.0–2.5) among women who had ever worked "graveyard" shifts. The RR of breast cancer was 1.06 for each hour increase per week of "graveyard" shift work (P = 0.03), after controlling for parity, family history of breast cancer, oral contraceptive use, as well as recent discontinued use of hormone replacement therapy. [The strengths of this study include its attempt to accurately define shiftwork assessment. One of the main

Table 2.3. Case–control studies of night shiftwork and breast cancer

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/deaths	OR or RR (extreme group versus referent)	Adjustment for potential confounders	Comments
Davis *et al.* (2001) Washington, USA	Cancer register based case–control study; case ascertainment (*n*=813) between 1992–1995, 793 matched (5-year age groups) controls identified by random-digit dialling	Information on sleeping habits, light exposure, lifetime occupational history obtained from in-person interview, considered as night workers if ≥1 graveyard shift/wk (8 hrs) in 10 years before diagnosis.	Breast cancer	*Years worked ≥3 nights/wk* None <1 1–3 3–4.6 4.7+ *P* for trend	 682 19 20 9 33	 1.0 (ref) 1.2 (0.6–2.3) 1.4 (0.7–2.8) 0.6 (0.3–1.5) 2.3 (1.2–4.2) 0.01	Parity, family history of breast caner (mother or sister), oral contraceptive use (ever), and recent (<5 years) discontinued use of hormone replacement therapy	Graveyard shift work defined as "beginning work after 7:00 pm and leaving work before 9:00 am."
O'Leary *et al.* (2006) Long Island, New York, USA Electromagnetic Fields and Breast Cancer on Long Island Study (EBCLIS)	Case–control study with 576 registry-identified cases and 585 1:1 matched (age in 5-year age groups) population-based controls; cases occurred between 1996–1997	Occupational history since age 16 & residential light-at-night exposures (sleep hours; frequency of turning on lights during night; length of time light was on) from in-person interview	Breast cancer	Any evening or overnight shiftwork Any evening shiftwork only Any overnight shiftwork only	174 148 26	1.04 (0.79–1.38) 1.21 (0.90–1.64) 0.55 (0.32–0.94)	Age (matched by 5-year age groups); parity; education; family history of breast cancer; history of benign breast disease	

limitations is the retrospective assessment of shiftwork with a modest potential for recall bias].

O'Leary *et al.*, (2006) conducted a case–control study in Long Island, New York, USA – the Electromagnetic Fields and Breast Cancer on Long Island Study (EBCLIS). They did not observe an association between night work and breast cancer risk (any evening or overnight shiftwork versus none, OR, 1.04; 95% CI: 0.79–1.38; only overnight shiftwork, OR, 0.55; 95% CI: 0.32–0.94). This study was built into another population-based case–control study among residents of Nassau and Suffolk counties. Cases were recorded during 1996–1997. Controls were frequency-matched by age and came from two different sources: 1) controls less than 65 years old were identified by random-digit dialling; 2) controls of age 65 and above were selected from the Health Care Financing Administration rosters. To evaluate the effects of electromagnetic frequency, women from within this case–control study were selected according to their degree of residential stability (EBCLIS component). EBCLIS comprised 576 breast cancer cases and 585 matched (1:1) population-based controls. In-person interviews were held to gather information on occupational history since the age 16 years as well as residential light-at-night exposures (sleep hours; frequency of turning on lights during night; length of time light was on). Shiftwork was defined as 'ever' working in at least one job during the past 15 years that included evening shifts (could start in the afternoon and end as late as 02:00), overnight shifts (could start as early as 19:00 and continue until the following morning), and various combinations thereof. The reference group comprised women who reported never having had a job involving shiftwork. Results were adjusted for age (matched by 5-year age groups), parity, education, family history of breast cancer, and history of benign breast disease. [An extreme and unlikely high proportion of controls (36.9%) and cases (35.7%) reported any 'evening or overnight shiftwork'; other limitations were the retrospective assessment of exposures and that the control selection was conducted from two different sources, introducing additional potential for bias].

(d) Meta-analysis

Megdal *et al.* (2005) conducted a meta-analysis that summarized six of the eight studies on night work (excluding the two most recent studies that gave negative results) and breast cancer, and found an increased risk for breast cancer (RR, 1.51; 95% CI: 1.36–1.68).

(e) Studies of biomarkers for night work (urinary melatonin) and breast cancer risk (Table 2.4)

Melatonin, the main biomarker for circadian dysregulation, can be measured in the urine by 6-sulphatoxymelatonin (aMT6s), the major metabolite of melatonin.

Skene *et al.* (1990) compared mean urinary aMT6s levels (measured by RIA) collected from 24-hour urine samples from British women attending a breast cancer screening clinic before biopsy and 160 normal female residents of Guernsey, the United Kingdom.

Table 2.4. Studies of biomarkers for light exposure (urinary melatonin) and breast cancer risk

Reference, study	Country or cohort and time period under observation	Source of information for exposure (i.e., light-at-night exposure)	Definition of biomarker for light exposure	Exposure category	No. of exposed cases	OR or RR (extreme group versus referent)	Adjustment for potential cofounders	Comment
Travis *et al*., (2004)	Nested case-control study; 5093 female residents of Guernsey (UK) recruited into a cohort of hormones and breast cancer (Guernsey III). Questionnaire at baseline (between 1977–1985);127 incident breast cancer cases that occurred before November 1, 2001 (mean follow-up ~12.6 yrs); 353 controls subjects; (1:2 matched for age, recruitment date, menopausal status, day of menstrual cycle/years postmenopausal); premenopausal: 77 cases, 214 controls postmenopausal: 50 cases, 139 controls	24-hour urine sample collected within ~16 days of recruitment	aMT6s by RIA; adjusted for urinary creatinine; tertiles of melatonin concentrations	aMT6s ng/mg creatinine High ≥18.32 ≥12.98	 46 26 20	*Overall* 0.99 (0.58–1.70) *Premenopausal* 0.99 (0.45–2.17) *Postmenopausal* 1.09 (0.46–2.60)	Matching factors (age ; date of recruitment, menopausal status, day of menstrual cycle that urine was collected or number of years post-menopausal); age, BMI, medication use thought to influence melatonin production, family history of breast cancer, parity and age at first birth, age at menarche, oral contraceptive use, season of urine collection, stage of menstrual cycle	

Table 2.4 (contd)

Reference, study	Country or cohort and time period under observation	Source of information for exposure (i.e., light-at-night exposure)	Definition of biomarker for light exposure	Exposure category	No. of exposed cases	OR or RR (extreme group versus referent)	Adjustment for potential cofounders	Comment
Schern-hammer & Hankinson (2005)	Nested case–control study; USA, Nurses Health Study II, prospective cohort study; 147 incident invasive breast cancer cases that occurred between 1996–1999 and 2001 and ≥91 matched (1:2) controls	First morning urine collection	aMT6s by ELISA; adjusted for urinary creatinine; quartiles of melatonin concentration	Urinary aMT6s ng/mg creatinine	23	*Overall* 0.59 (0.4–1.00) *P* for trend 0.13	Matching factors (year of birth, menopausal status at urine collection, month, year, time of day & luteal day of menstrual cycle at urine collection, fasting status at urine collection, ethnicity); age at menarche, parity, age at first birth, BMI, family history of breast cancer, benign breast disease, alcohol consumption, antidepressant use	Sub-analyses excluding night workers provided similar results; ~24% of the sample were postmenopausal

Mean levels for aMT6s excretion were 7.8±1.1 μg/24 hours in 14 benign cases, and 4.1±0.9 μg /24 hours in ten malignant cases. However, the lower aMT6s levels in the malignant cases compared to benign cases was entirely due to the difference in age between the two groups.

Only two studies have been published to date evaluating an association between melatonin, the main biomarker for the circadian rhythm, and risk of breast cancer.

Travis *et al.* (2004) conducted a case–control study nested within 5093 female residents of Guernsey, the United Kingdom, which were recruited into a cohort for the study of hormones in relation to breast cancer (Guernsey III). Overall, 127 incident breast cancer cases occurred before November 2001 (mean follow-up ~12.6 years). A total of 353 control subjects were matched 1:2 to these cases. There were 77 premenopausal cases (214 controls), and 50 postmenopausal cases (139 controls). A questionnaire was distributed at baseline between 1977–1985. Urine samples (24-hour) were collected within ~16 days of recruitment, and aMT6s concentrations were measured by RIA, and adjusted for urinary creatinine. Tertiles of melatonin concentrations were created, and the overall RR was 0.99 (95% CI: 0.58–1.70, comparing highest versus lowest tertile).

Schernhammer & Hankinson (2005) also conducted a nested case–control study of similar size and with primarily premenopausal women in the USA, and nested within the NHS II, a prospective cohort study of registered nurses only. A total of 147 incident invasive breast cancer cases that occurred in this cohort were enrolled into the nested case–control study during 1996–1999 and in 2001, and 291 controls were matched (1:2) to these cases; approximately 24% of participants were postmenopausal. During 1996–1999, a first morning urine sample was collected from roughly a third of the NHS II cohort. Concentrations of aMT6s were measured by ELISA, and adjusted for urinary creatinine. Quartiles of aMT6s level were created. Overall, there was a reduction in breast cancer risk among those in the highest quartile of melatonin (RR, 0.59; 95% CI: 0.4–1.00). Sub-analyses excluding night workers provided similar results.

2.2.2 *Prostate cancer*

(a) Cohort studies (Table 2.5)

A total of 14 052 men from 21 areas in Japan, 40–65 years old at baseline in 1988–1990, were extracted as a subcohort of the Japan collaborative cohort study for evaluation of cancer risk (Kubo *et al.*, 2006). A self-administered questionnaire was used to gather information at baseline on exposures related to lifestyle and work. At baseline, study participants were asked which form of work had they primarily been engaged in: daytime, fixed night or rotating shiftwork. In total, 31 cases of prostate cancer were documented from cancer registries during follow-up, based on 111 974 person–years (mean 8.0 years) from baseline until the end of 1997. Multivariate adjusted relative risks based on Cox proportional hazards models of fixed night shifts and rotating shifts were 2.3 (95% CI: 0.6–9.2; three cases), and 3.0 (95% CI: 1.2–7.7; seven cases), respectively, compared with

Table 2.5. Cohort studies

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Kubo *et al.* (2006), Japan, Japan Collaborative Cohort	Prospective cohort of 14 052 males, aged 40–65 years old enrolled from 45 areas in Japan between 1988–1990. Information on prostate cancer was obtained from cancer registries	Self-administered questionnaires at baseline included information on type of work schedule	Prostate	Day time work Fixed night Rotating shift	21 3 7	1.0 (ref) 2.3 (0.6–9.2) 3.0 (1.2–2.7)	Age, study area, family history of prostate cancer, BMI, smoking, alcohol drinking, job type, physical activity at work, workplace, perceived stress, educational level and marriage status	
Schernhammer *et al*. (2003) USA, American Nurses Health Study	Prospective cohort of 78 586 American nurses with a baseline question on rotating night work in 1988. Follow-up for colorectal cancer was through 1998	Self-reported from postal questionnaires: Rotating night shift was defined as "at least 3 nights per months, in addition to evenings and afternoons in that month"	Colorectal	No rotating night shifts 1–14 years ≥15 years *P* for trend 0.04	229 303 70	1.00 (ref) 1.00 (0.84-1.19) 1.35 (1.03-1.77)	Tobacco smoking, BMI, physical activity, aspirin use, colorectal cancer in relatives, endoscopy use, consumption of red meat, alcohol consumption, total calorie intake, postmenopausal hormones, menopausal status, height	No major differences in risk were seen for right or left colon, or colon and rectum separated
			Colon	No rotating night shifts 1–14 years ≥15 years *P* for trend 0.26	137 169 41	1.00 (ref) 0.93 (0.74–1.17) 1.32 (0.93–1.87)		
			Rectum	No rotating night shifts 1–14 years ≥15 years *P* for trend 0.15	41 48 14	1.00 (ref) 0.86 (0.56–1.30) 1.51 (0.82–2.81)		

Table 2.5 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Viswanathan *et al*. (2007), USA, American Nurses Health Study	Prospective cohort of 53 487 American nurses with a baseline question on rotating night work in 1988. Follow-up for endometrial cancer to 2004	Self-reported from postal questionnaires: Rotating night shift was defined as "≥3 nights/month, in addition to evenings & afternoons in that month"	Endometrial	No rotating night shifts 1–9 yrs 10–19 yrs ≥20 yrs	210 224 43 38	1.00 (ref) 0.89 (0.74–1.08) 1.06 (0.76–1.49) 1.47 (1.03–2.10) *P* for trend 0.04	Age, age at menarche, age at menopause, parity, BMI, duration of oral contraceptive, postmenopausal hormones, hypertension, diabetes, and pack–years of tobacco smoking	When stratifying for obesity, women with BMI>30 and having at least 20 years with rotating shifts had a more than 2-fold significant increased risk (*P* for trend 0.003)

Table 2.5 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Taylor & Pocock, (1972), England and Wales	Retrospective cohort study of 8603 manual workers all born before 1920 from 10 companies in England & Wales continuously employed for ≥10 years followed-up for death during 1956–1968.	Detailed job information since 1946, including work hours and types of shifts was obtained from payrolls kept at the companies	All cancers	Day	201	[1.02 (0.88–1.17)]	Age, calendar time	
				Shift	219	[1.16 (1.02–1.32)]		
				Ex-shift*	29	[1.12 (0.75–1.61)]		
				[Shift/Day]	[219/201]	[1.14 (0.94–1.38]		
			Lung	Day	95	[1.09 (0.80–1.33)]		
				Shift	94	[1.11 (0.90–1.36)]		
				Ex-shift	13	[1.15 (0.60–1.97)]		
			Stomach	Day	33	[1.24 (0.85–1.74)]		
				Shift	36	[1.43 (1.00–1.98)]		
				Ex-shift	4	[1.14 (0.31–2.93)]		
			Bladder	Day	4	[0.56 (0.15–1.44)]		
				Shift	7	[1.06 (0.42–2.19)]		
				Ex-shift	2	[4.00 (0.48–14.4)]		
			Leukaemia	Day	6	[1.54 (0.57–3.35)]		
				Shift	2	[0.54 (0.07–1.95)]		
				Ex-shift	2	[4.00 (0.48–14.4)]		

Table 2.5 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Schwartzbaum, (2007), Sweden	Ecological cohort study including male and female members of the working Swedish population participating in censuses in both 1960 and 1970, including information on usual job and industry, and followed-up for cancer in the cancer registry during 1971–1989	Job–exposure matrix based on surveys from 1977 to 1981; exposed defined as working in industries (≥20 hours/week) where at least 40% had work schedules with three or more possible shifts per day or work hours during the night at least one day per week	All cancers (29 for men and 18 for women)	Men Women	3799 103	1.01 (0.98–1.05) 1.00 (0.82–1.21)	Age, socioeconomic status, occupational position, county of residence	Significantly increased relative risks observed for kidney, skin, and other and unspecified cancers (men), whereas none of the female cancers were significantly different from unity. [Misclassification of shiftwork status is an invalidating problem]

BMI, body mass index
Ex-shift, men who did not qualify as shiftworkers but had done > 6 months on shiftwork & subsequently transferred to day work. They came under observation when they had completed 10 yrs' employment & the first 6 months of day work following their period of shiftwork. They remained under observation until the end of 1968 or until they had done a further 6 months of shiftwork.

predominantly day workers. [The major limitation of the study is the lack of statistical power, short follow-up for prostate cancer and a limited measure for shiftwork.]

(b) Case-control studies (Table 2.6)

A case–control study based on a cancer registry among residents of north-eastern Ontario, Canada, included 760 cases of prostate cancer, 45–85 years of age, and diagnosed during 1995–1998 (Conlon *et al.*, 2007). Cases were frequency-matched by age to 1632 male controls. A comprehensive mailed questionnaire was designed to gather information on exposures to lifestyle factors, and on each job held for one or more years, including information on usual work time (daytime shift, evening/night shift, rotating shift or other). The adjusted OR for 'ever' having worked rotating shifts on a full-time basis was 1.19 (95% CI: 1.00–1.42, 369 cases). Analyses of the duration in years of full-time rotating shifts (P for trend = 0.05) and age working the first full-time rotating shift (P for trend = 0.03) showed significant trends, but years since first full-time shifts did not show a significant trend (P for trend = 0.16). [The Working Group noted that the proportion of cases and controls classified with rotating shiftwork seemed unrealistically high and there was a lack of statistical power.]

2.2.3 *Colorectal cancer* (Table 2.5)

A prospective cohort study based on the American Nurses Health study including 78 586 nurses at baseline in 1988 was used for evaluating the association between colorectal cancer risk and rotating night work (Schernhammer *et al.*, 2003). Nurses completed a comprehensive questionnaire, including a question on how many years in total they had worked rotating night shifts at least three nights per month in addition to working days or evenings in that month. Based on 758 903 person–years during 1988–1998, a total of 602 cases of colorectal cancers were recorded. Cox proportional hazard models were used to estimate relative risks adjusted for potential confounders (tobacco smoking, body mass index, physical activity, aspirin use, colorectal cancer in relatives, endoscopy use, consumption of red meat, alcohol consumption, total caloric intake, postmenopausal hormones, menopausal status, and height). Compared with nurses who had never worked night shifts for at least three days per month, nurses who worked such shifts for 1–14 years and for at least 15 years had multivariate-adjusted RRs of 1.00 (95% CI: 0.84–1.19) and 1.35 (95% CI: 1.03–1.77), respectively. RRs adjusted for age only were similar and were reported as 1.00 (95% CI: 0.84–1.18) and 1.44 (95% CI: 1.10–1.89), respectively. Results for distinct sites such as right and left colon, combined colon, and rectum only marginally changed the results for the combined colorectal results. [Misclassification due to the relative crude definition of night shiftwork was likely to have resulted in bias towards the null.]

Table 2.6. Shiftwork - other sites than breast cancer. Case–control studies

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	No. of exposed cases	Exposure categories	Relative risk (95% CI)*	Adjustment for potential confounders
Conlon *et al*. (2007), Ontario, Canada, 1995–98	Prostate	760 cancer-registry-identified cases, aged 45–84 years and diagnosed during 1995–1998	1632 controls frequency-matched on age and from the same residence	Postal questionnaire (25 pages)	369	Rotating shiftwork	1.19 (1.00–1.42)	Age, family history of prostate cancer
						Years of shiftwork		
					115	≤7	1.44 (1.10–1.87)	
					87	7.1–22.0	1.14 (0.86–1.52)	
					81	22.1–34.0	0.93 (0.70–1.23)	
					86	>34.0	1.30 (0.97–1.74)	
						P for trend	0.05	

2.2.4 *Endometrial cancer* (Table 2.5)

Another prospective study based on the American Nurses Health study cohort included 53 487 women with an intact uterus who answered a question on rotating night work in 1988 (Viswanathan *et al.*, 2007). They were followed-up for endometrial cancer up to mid-2004, resulting in 515 cases (720 698 person–years). The RR was 1.47 (95% CI: 1.03–2.10) for nurses with 20 or more years of rotating shiftwork. When stratifying by body mass index, the RR was 2.09 (95% CI: 1.24–3.52) in the subgroup of nurses with a body mass index >30 kg/m^2 and at least 20 years of rotating shiftwork. In contrast, there was no difference in calorie consumption across night work categories. A significant trend ($P = 0.003$) of increasing relative risk was seen with increasing duration of rotating shiftwork in the group classified as obese. No significantly increased risk was observed in the group with a body mass index <30. The RRs were adjusted for potential confounders (age, age at menarche, age at menopause, parity, body mass index, duration of oral contraceptive use and postmenopausal hormones, hypertension, diabetes, and pack–years of tobacco smoking). [Misclassification due to the relative crude definition of night shiftwork was likely to have resulted in bias towards the null.]

2.2.5 *Other cancers* (Table 2.5)

A cohort of 8603 male full-time manual workers from England and Wales were followed-up during 1956–1968 for cause-specific mortality, including all neoplasms, cancer of lung, stomach, bladder, and leukaemia (Taylor & Pocock 1972). Study subjects were from ten different companies in which they were employed on 1 January 1956. They were born before 1920 and had all been continuously employed at the same company for at least ten years during 1946–1968. Detailed information on all jobs held since 1946 was obtained from company payrolls, including information on working hours, and types of shifts. Based on this information, each worker was allocated into one of three groups: day worker ($n = 3860$), shiftworker ($n = 4188$), and ex-shiftworker ($n = 555$). The criteria for being classified as either a day worker or a shiftworker were that the worker had completed at least 10 years of either work since 1946, with a maximum of 6 months interruption during that period. The term shiftwork covered six types of working hours other than regular day work. The start of follow-up was initiated as soon as each worker met the 10 years of duration of employment criterion. At the end of follow-up, on 31 December 1968, it was possible to trace all but 22 men (0.25%). Information on date and cause of death for the 1578 men who died during the follow-up period was obtained from death certificates. Expected numbers of cause-specific deaths were calculated from the mortality experience of men in England and Wales in 5 year age-groups and calendar time groups. Observed versus expected numbers for all-cause mortality were not significantly different in any of three groups of day workers, shift- and ex-shiftworkers (736/756.4; 722/711; 120/100.9). For the all-neoplasms group, the shiftworkers experienced a significantly higher than expected all-cancer mortality than the general

population [SMR, 1.16; 95% CI: 1.02–1.32; 219 observed cases]. For the small group of ex-shiftworkers, 29 deaths were observed versus 25.9 expected [SMR, 1.12; 95% CI: 0.75–1.61], and in the group of day workers, 201 deaths versus 197.1 were observed during follow-up [SMR, 1.02; 95% CI: 0.88–1.17]. For death from cancers of the lung, stomach, bladder, and leukaemia, observed versus expected numbers were, respectively, 94/84.4, 36/25.2, 7/6.6, and 2/3.7 among shiftworkers. Similar patterns were seen in day workers and in ex-shiftworkers. [This study was based on a survivor population with 10 years or more experience of shiftwork which may have underestimated a true increased risk if less than 10 years of shiftwork increased the mortality.]

A census-based ecological cohort study from Sweden included all members of the Swedish population (≥ 20 hours/week) in both 1960 and 1970 (Schwartzbaum *et al.*, 2007). The censuses included individual information about social status and industry but not about work schedules. Therefore, a job–exposure matrix was established for assessment of shiftwork. It relied on a sample of the Swedish population ($n = 46\ 438$) collected during 1977–1981, which included information on usual occupation and work schedules. Shiftwork was defined as a schedule with three or more possible shifts per day or work hours during the night for at least one day during the week preceding the interview. About 3% of the men and less than 0.3% of the women participating in the censuses were classified as having done shiftwork, defined by working in industries in 1960 and 1970 where at least 40% of the participants from the survey had reported such a work schedule. Follow-up for cancer in the Swedish Cancer registry was from 1971–1989, and SIRs were calculated on the basis of person–years of follow-up and national rates of specific cancers taken from the Swedish Cancer registry. The SIRs for cancer among men were all close to unity during the 19 years of follow-up, except for kidney (1.14; 95% CI: 1.00–1.31), skin (1.20; 95% CI: 1.02–1.41), and other and unspecified cancers (1.27; 95% CI: 1.07–1.50). For the subgroup of men participating in the 1970 census only, the SIR for thyroid cancer was elevated (1.35; 95% CI: 1.02–1.79). Results changed minimally when the shiftwork status was based only on the 1970 census or other attempts to change the exposure definition. [The major limitation of this study was an unavoidable potential for misclassification of exposure resulting in null results, and to some extent, uncontrolled confounding. Cohort members were followed-up to 1989, although follow-up through 2006 had been possible].

2.3 Aircraft crew

Cancer risk of airline personnel has been studied since the 1990s in about ten countries. The main reason to study these cohorts has been exposure to cosmic radiation, and sometimes passive smoking or electromagnetic fields. Shiftwork as causal factor has not been explicitly mentioned, but in the latest studies, there has be discussion on the potential role of frequent disruptions of circadian rhythm. An alteration in melatonin metabolism decreasing the oncostatic function of this hormone has been hypothesized to be a potential biological mechanism. [It has been questioned whether flight attendants should be considered as shiftworkers.]

For most cabin crew, annual exposure to radiation ranges from 1–6 mSv, compared with approximately 2.4 mSv annually from background radiation. Cosmic radiation includes a substantial neutron component (25–50% of effective dose but less than 5% of absorbed dose). Because flight personnel are the only source of human data on the health effects of exposure to neutron radiation, it is hard to estimate how a large excess risk would be expected due to cosmic radiation. This further makes it difficult to judge how much of the observed excess could be for other risk factors such as shiftwork.

The number of flights over several time zones is used as a proxy of frequency of circadian rhythm disruptions. This number correlates with the dose of cosmic radiation, and therefore estimates of cancer risk in cumulative dose categories can also be interpreted to roughly reflect frequency of circadian rhythm disruptions. On the other hand, separation of the independent roles of these two factors is possible only in large studies with precise information on flight histories. Only one study, combining information on all pilots from the five Nordic cancer registries has been able to make this distinction to a certain extent. In general, the detailed flight histories of airline pilots are known quite well; while for cabin crew, normally only the beginning and end of employment is known. In the airline companies where the principle has been that all cabin crew members fly all routes, an approximation of the radiation dose and numbers of long flights over time zones for each person can be made based on his/her own annual numbers of flight hours, and the flight profile of the company.

All studies published on aircraft crew have been included in this evaluation, irrespective of whether they mention shiftwork or not. Only observations related to breast cancer and prostate cancer have been included in this review, because they are the only ones which have been considered to be associated with shiftwork. The observations related to breast cancer come from cabin crew personnel and those related to prostate cancer mainly from cockpit personnel, because almost all airline pilots are male, and the majority of the cabin crew, female.

In addition to the breast and prostate cancer findings presented below in detail, there is a consistent pattern of increased incidence of skin melanoma and basal cell carcinoma of the skin that are likely to be related to the more frequent sunbathing and sunburns among flight personnel in previous decades. Male cabin crew have also been shown to have a significantly increased risk of Kaposi sarcoma in most studies that included this cancer category. The risk of leukaemia, one of the main target sites in studies on effects of radiation, has been shown to be non-elevated in most studies.

2.3.1 *Breast cancer* (Table 2.7)

(a) Cohort studies

Pukkala *et al.* (1995) collected a cohort of 1577 all-female flight attendants who had ever worked for Finnish airline companies (first employment starting in the 1930s). This cohort was followed-up for cancer incidence during 1967–1992. The SIR for breast cancer was 1.87 (95% CI: 1.15–2.23, 20 cases), and the SIR was highest 15–19 years after

Table 2.7. Cohort studies of flight personnel and breast cancer

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Pukkala *et al*. (1995), Finland	1577 female cabin attendants who worked for Finnish airline companies; from files of Finnair Flight Company; follow-up for cancer incidence from date of recruitment as cabin crew worker (or January 1967 if later) to emigration, death, or December 1992	Calendar period, length of employment	Breast	Any Employment ≥2 years	20 NG	**SIR** 1.87 (1.15–2.23) 2.0 (1.2–3.2)	Age	Control for parity on group level (cohort vs. reference population): parity cannot explain the difference
Lynge (1996), Denmark	915 female airline cabin attendants in Denmark, follow-up for cancer incidence from 1970–1996	Cross-sectional census occupation 1970	Breast	Any	14	**SIR** 1.61 (0.90–2.70)	Age	
Wartenberg & Stapleton (1998), USA	287 retired flight attendants from one US airline; follow-up for cancer incidence		Breast	Any	7	**SIR** 2.0 (1.0–4.3)	Age	

Table 2.7 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Haldorsen *et al*. (2001), Norway	3105 female and 588 male airline cabin attendants with licences issued 1950–1994; follow-up for cancer incidence from 1953 to 1996 (≤38 years)	Total length of employment; length of employment before 26 years of age	Breast	Any Employment ≥15 years	38 5	**SIR** 1.1 (0.8–1.5) 1.0 (0.3–3.0)	Age, number children, age at first birth	
Rafnsson *et al*. (2001), Iceland	1532 cabin attendants, from Icelandic Cabin Crew Association and two airline companies; follow-up for cancer incidence 1955–1997	Year of employment, hired before or in/after 1971	Breast	Any	26	**SIR** 1.50 (1.00–2.10)	Age	Control for parity on group level (cohort vs. reference population): parity cannot explain the difference
Blettner *et al*. (2002), Germany	16 014 female cabin attendants who had been employed by two German airlines in 1953 or later; mortality follow-up through 1997		Breast	Any	19	**SMR** 1.28 (0.72–2.20)	Age	

Table 2.7 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Reynolds *et al*. (2002), California, USA	44 021 members of the Association of Flight Attendants in California; follow-up for cancer incidence 1988–1995	Route – international/domestic, length of service, age at entry	Breast			**SIR**	Age	
				Any	60	1.42 (1.09–1.83)		
				International	31	1.79 (1.21–2.54)		
				Employment ≥15 years	49	1.57 (1.16–2.08)		
				Starting age <25 years	41	1.72 (1.23–2.34)		
Linnersjö *et al*. (2003), Sweden	2324 women from Swedish Scandinavian Airline System employed 1957–1994; follow-up 1961–1996	High altitude, long distance flight hours	Breast			**SIR**	Age	Control for parity on group level (cohort vs. reference population): parity cannot explain the difference
				Any	33	1.30 (0.85–1.74)		
						Odds ratio		
				>5000 block hours in high altitude, long distance flights	5	3.27 (0.54–19.7)		
Zeeb *et al*. (2003), European countries	Cabin crew working 1961–1997 in Denmark, Finland, Germany, Greece, Iceland, Italy, Norway and Sweden, a total of 33 063 females and 11 079 male; follow-up for mortality up to 1997		Breast			**SMR**	Age	Overlap with the national incidence studies; breast cancer in women
				Ever	174	1.11 (0.82–1.48)		
				>0–<10	33	1.12 (0.75- 1.63)		
				10–<20 years	19	1.27 (0.74–2.07		
				≥20 years	7	0.80 (0.32–1.77)		

NG, not given

first employment (SIR, 3.4, 95% CI: 1.5–6.8), and slightly increased with increasing duration of employment. [The flight attendants were more likely than the general population to have multiple reproductive risk factors for breast cancer but these differences were insufficient to explain the magnitude of excesses observed.]

This observation was followed by other observations published in letters in the same journal. Lynge (1996) reported SIRs obtained from a routine tabulation of occupation-specific cancer risks in Denmark. In a 17-year follow-up, the SIR of breast cancer among the 915 women who had reported their occupation in 1970 as a flight attendant was 1.61 (95% CI: 0.90–2.70) when compared to average Danish women. Wartenberg and Stapleton (1998) also reported an increased breast cancer incidence in a small cohort of retired flight attendants from a US airline (SIR, 2.0; 95% CI: 1.0–4.3). This risk seems not to depend on the number of flights, and they suggest that exposure to dicophane (DDT), an organochlorine pesticide used to rid airplanes of insects during 1950–1970, may be a risk factor for breast cancer.

In 2001, two other Nordic studies were published with a setting similar to the earlier Finnish study. Haldorsen *et al.* (2001) studied a Norwegian cohort of 3144 female flight attendants and observed 38 cases of breast cancer. The SIR was 1.1 (95% CI: 0.8–1.5), and did not increase with increasing length of employment. The authors also had access to the dates of births of the children of the flight attendants for every woman born since 1934. The risk remained largely unchanged after controlling for age at first birth and parity.

Rafnsson *et al.* (2001) published a cohort study of 1532 cabin attendants from the Icelandic Cabin Crew Association and two airline companies and followed-up for cancer incidence during 1955–1997. The risk of breast cancer was significantly increased (SIR, 1.6, 95% CI: 1.0–2.1, lagged 15 years; SIR, 1.5, 95% CI: 1.0–2.1). Those hired in 1971 or later had the heaviest exposure to cosmic radiation at a young age, and had a signicantly increased risk of breast cancer (SIR, 4.1; 95% CI: 1.7–8.5). The information on reproductive factors among the cabin attendants and the Icelandic female population, obtained from the register of the Genetical Committee of the University of Iceland, provided an opportunity to evaluate the possible confounding due to reproductive factors on the risk of breast cancer in the present study in a similar way as has been recommended when evaluating confounding due to cigarette smoking in occupational studies. Predictive values were calculated on the basis of reproductive factors among the cabin attendants and the population. The risk of breast cancer was 1.0 for parous versus nulliparous, 1.0 for the number of children, and 1.1 for the age at birth of first child.

Rafnsson *et al.* (2003) also published a case–control study nested in the cohort of Icelandic cabin crew personnel. An increased risk of breast cancer was related to length of employment before 1971, the period before jet aircrafts were taken into operation. The authors concluded that the exposure related to the increased risk of breast cancer was solely confined to the period before 1971, because a long lag time may be required for inducing breast cancer, see Table 2.8. [Their result was compatible with the view that corresponds to a long induction period between ionizing radiation exposure and development of breast cancer.]

Table 2.8. Nested case–control studies of airline crew and cancer

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Rafnsson *et al.* (2003), Iceland	Breast	35 histologically confirmed cases, including 4 in situ cancers	140 age-matched flight attendants		≥5 versus <5 years work during:			Age at first delivery, parity	
					pre-jet era (<1971)	29	5.24 (1.58–17.4)		
					jet era (≥1971)	43	0.82 (0.34–1.97)		
Kojo *et al.* (2005), Finland	Breast	27 breast cancer cases diagnosed in 1975–2000 among cabin attendants (response proportion of 60%)	517 non-case cabin attendants (response proportion of 52%)	Self-administered questionnaire	Cumulative dose (per 10 mSv).	NG	0.93 (0.68–1.27)	Cumulative radiation dose, number of fertile years, parity, family history of breast cancer, alcohol consumption	To assess possible selection bias OR was also calculated for all the subjects in the cabin attendant cohort (44 breast cancer cases and 921 non-cases)
					Disruption of menstrual cycle sometimes or often	NG	0.56 (0.12–2.61)		
					Disruption of sleep rhythm sometimes or often	NG	1.52 (0.49–4.74)		

NG, not given

Blettner *et al.* (2002) studied cancer mortality among 16 014 female and 4537 male cabin attendants who had been employed by two German airlines in 1953 or later. The SMR for breast cancer was 1.28 (95% CI: 0.72–2.20; 19 observed deaths). The SMR did not increase with duration of employment.

In the largest of the studies, Reynolds *et al.* (2002) linked a group of 44 021 female members of the Association of Flight Attendants in California with the California Cancer Registry. They had to use a computer programme to conduct automated probabilistic record linkage, and to make several assumptions for estimations in the person–year accumulation which may have caused some inaccuracy in the results. During the follow-up, 60 cases of invasive and 12 cases of in-situ breast cancer were recorded during 1988–1995. The SIR for invasive breast cancer across all ethnicities was 1.42 (95% CI: 1.09–1.83) while the incidence of in-situ tumours did not differ significantly from what may have been expected compared to rates from the non-Hispanic Caucasian population or from the population of all races. Invasive breast cancer appeared to be significantly elevated (SIR, 1.79; 95% CI: 1.21–2.54) in flight attendants who were assigned to international routes compared with the general Californian reference population rates.

Linnersjö *et al.* (2003) observed 33 cases of breast cancer during 1961–1996 among the 2324 women employed at the Swedish Scandinavian Airline System. The SIR of breast cancer was 1.30 (95% CI: 0.85–1.74) when compared to the general population, and did not increase with increasing duration of employment. A case–control analysis nested within the cohort gave ORs for cancer incidence. For cabin crew with at least 5000 block hours in high altitude or long-distance flight hours compared with cabin attendants without this experience, the OR was 3.27 (95% CI: 0.54 –19.7).

Zeeb *et al.* (2003) reported the combined results from cabin crew cohorts from eight European countries employed during 1921–1997 in Denmark, Finland, Germany, Greece, Iceland, Italy, Norway, and Sweden. During follow-up of 485 831 person–years for women (until 1997), 174 breast cancer deaths were reported (SMR, 1.11; 95% CI: 0.82–1.48).

Kojo *et al.* (2005) reported on a nested case–control study of breast cancer among Finnish cabin crew attendants. The adjusted ORs were 0.93 (95% CI: 0.68–1.27) for cumulative dose per 10mSV, 0.56 (95% CI: 0.12–2.61) for disruption of menstrual cycle (sometimes or often), and 1.52 (95% CI: 0.49–4.74) for disruption of sleep rhythm (sometimes or often), see Table 2.8.

2.3.2 *Prostate cancer* (Table 2.9)

Band *et al.* (1990, 1996) reported cancer incidence and mortality in similar studies among pilots of two Canadian airline companies. Both of them demonstrated an increased incidence from prostate cancer (SIR, 1.54; SMR, 1.87), but only one study demonstrated slightly elevated mortality from prostate cancer (SMR, 1.52; 90% CI: 0.71–2.85; seven deaths) with no dependence on duration of the employment (Band *et al.*, 1996). The authors concluded that detection bias could be a likely explanation, as throughout their

Table 2.9. Cohort studies of flight personnel and prostate cancer

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Band *et al*. (1990), Canada	913 male pilots employed for 1 year or more by Canadian Pacific Airlines since 1950; cause of death and cancer incidence information up to October 31, 1988 ascertained through the divisions of vital statistics and the cancer registries of the Canadian provinces		Any	6	**SIR** 1.54 [0.56–3.35]	Age	
Band *et al*. (1996), Canada	2680 male pilots of Air Canada working 1year or more since 1950; cancer incidence and mortality up to 1992.		Any	34	**SIR** 1.87 [1.30–262]	Age	

Table 2.9 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Irvine & Davies (1992)	446 deaths among serving and retired British Airways (BA) pilots 1966–1989; deaths were ascertained from pension and registry listings, death registries, newspaper obituary listings	BA Personnel and pension records	Pilots	10	**PMR** 2.12 (1.02–3.89)	Age	
				10	**PCMR** 1.54 (0.74–2.83)		
Irvine & Davies (1999), England & Wales UK	6209 male pilots and 1153 male flight engineers employed since 1939 and for at least 1 year 1950–1992; followed for mortality	Years as flight deck crew, long/shorthaul	Pilots	15	**SMR** 1.11 (0.62–1.84)	Age	
			Flight Engineers	3	0.92 (0.19–2.69)		
			Longhaul vs shorthaul	4	**RR** 2.47 (0.83–7.65)		
Gundestrup & Storm (1999), Denmark	3790 male and 87 female pilots from 1921 and up, followed for cancer incidence 1943–1995	Flight hours	Jet	3	**SIR** 0.8 (0.2–2.2)		
			Non-jet	3	0.8 (0.2–2.2)		

Table 2.9 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Haldorsen *et al*. (2000), Norway	3815 authorized male pilots 1946–1994; follow-up for cancer incidence up to 1996	Block hours, estimated dose			**SIR**		Smoking among current pilots compared with reference population (slightly lower proportion among pilots)
			Any	25	1.0 (0.7–1.5)		
			≥10 000 block hours	14	1.1 (0.6–1.9)		
			≥20 mSv	6	1.8 (0.7–4.0)		
Rafnsson *et al*. (2000), Iceland	458 male pilots 1937–1985; follow-up for cancer incidence 1955–1997	Block hours, estimated dose, crossing time zones (yes/no)			**SIR**		
			Any	5	1.28 (0.41–2.98)		
			International flights	4	1.41 (0.38–3.61)		

Table 2.9 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Hammar *et al*. (2002), Sweden	1490 aircraft pilots and 2808 military pilots and navigators in the Swedish Air Force employed during 1957–1994; follow-up for cancer incidence 1961–1996	Numbers of block hours, high-altitude flights and long-distance flights			**SIR**		
			Civil	18	1.24 (0.74–1.97)		
			Military	49	1.17 (0.84–1.49)		
Pukkala *et al*. (2002), Denmark, Finland, Iceland, Norway, Sweden	10 032 male airline pilots employed 1921–1996; follow-up for cancer incidence through 1997	Employment duration, cumulative block hours (by aircraft type), estimated dose			**SIR**		The relative risk of prostate cancer increased with increasing number of flight hours in long distance aircraft (*P* for trend, 0.01)
			Any	64	1.21 (0.93–1.54)		
					RR		
			>10 000 hours long-haul (as compared with <5000 hours), ages 60+	8	3.88 (1.26–11.9)		

Table 2.9 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Zeeb *et al*. (2002), Germany	6061 male pilots 1960–1997; mortality follow-up through 1997	Employment durations, cumulative block hours, estimated dose	Any	8	**SMR** 1.26 (0.5–2.59)	Age	
Zeeb *et al*. (2003), European countries	Cabin crew working 1961–1997 in Denmark, Finland, Germany, Greece, Iceland, Italy, Norway and Sweden, a total of 33 063 females and 11 079 male; follow-up for mortality up to 1997			5	1.09 (0.35–2.68)		

Table 2.9 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustement for potential confounders	Comments
Blettner *et al*. (2003), European countries	Cockpit crew working 1921–1997 in Denmark, Finland, Germany, Great Britain, Greece, Iceland, Italy, Norway and Sweden, a total of 27 797 persons; follow-up for mortality		Any	54	**SMR** 0.94 (0.71–1.26)		Overlap with the national incidence studies

career, pilots underwent yearly physical examinations, including a digital rectal examination.

Irvine and Davies (1992, 1999) studied cancer mortality in British Airways pilots using the proportional mortality ratio (PMR) method (1992), and later (1999) based on SMRs. In the PMR study, there were ten deaths due to prostate cancer. The PMR was 2.12 (95% CI: 1.02–3.89) if the reference was all-cause mortality (excluding aircraft accident), and 1.54 (95% CI: 0.74–2.83) if the reference was all-cancer mortality. [It was however evident from other studies that both overall mortality and all-cancer mortality among airline pilots is markedly below the population mortality rates. In the British Airways pilots (Irvine & Davies, 1999), the SMR was 0.61 for all causes and 0.64 for all cancers, and therefore the PMRs in Irvine & Davies (1992) did not indicate excess prostate cancer mortality among pilots compared to the average population]. In the SMR study (Irvine & Davies, 1999), there were 15 prostate cancer deaths among British Airways pilots (SMR, 1.11; 95% CI: 0.62–1.84), and three deaths among flight engineers often travelling in cockpit (SMR, 0.92; 95% CI: 0.19–2.69). In the internal analysis, the age-adjusted RR between persons flying long-haul versus mainly short-haul (European) flights was 2.47 (95% CI: 0.83–7.65). Flight engineers were assumed to operate in long-haul operations.

Gundestrup & Storm (1999) studied cancer incidence among 3790 male and 87 female commercial Danish cockpit crew members, with records starting from 1921. They were followed for cancer mortality during 1943–1995. Three prostate cancer deaths were observed among both jet pilots and non-jet pilots versus 3.5–4.0 expected.

Haldorsen *et al.* (2000) published SIRs from a Norwegian cohort of 3815 authorised male pilots employed during 1946–1994. During the follow-up from 1953–1996, 25 cases of prostate cancer were observed (SIR, 1.0; 95% CI: 0.7–1.5); six were in the category of exposed to ≥ 20 mSv (SIR, 1.8; 95% CI: 0.7–4.0).

Rafnsson *et al.* (2000) studied a cohort of 458 Icelandic male pilots employed during 1937–1985, followed-up for cancer incidence from 1955–1997. There were only four cases of prostate cancer among pilots who had flown international flights (SIR, 1.41; 95% CI: 0.38–3.61), and therefore no dose–response analyses were performed for this cancer site.

The study by Hammar *et al.* (2002) reported cancer incidence both among civil and military pilots in Sweden. The incidence was about 20% above the national level in both categories, and did not vary with increasing number of block hours, high-altitude flights or long-distance flights.

Zeeb *et al.* (2002) analysed mortality data of 6061 German male pilots who had worked during 1953–1997. A total of eight deaths from prostate cancer were observed (SMR, 1.26; 95% CI: 0.53–2.59).

Zeeb *et al.* (2003) reported the combined results from cabin crew cohorts from eight European countries employed during 1921–1997 in Denmark, Finland, Germany, Greece, Iceland, Italy, Norway, and Sweden. During follow-up of 170 634 person–years for men (until 1997), five prostate cancer deaths were reported (SMR, 1.09; 95% CI: 0.35–2.68).

Blettner *et al.* (2003) combined mortality data from cockpit crew cohorts from nine European countries working during 1921–1997 in Denmark, Finland, Germany, Great Britain, Greece, Iceland, Italy, Norway and Sweden. During the approximately 28 000 person–years of follow-up, 54 prostate cancer deaths were reported (SMR, 0.94; 95% CI: 0.71–1.26).

Pukkala *et al.* (2002) conducted an analysis among cohorts of airline pilots from Denmark, Finland, Iceland, Norway, and Sweden. When compared to the national studies described above, the length of follow-up was extended. Unpublished Finnish data were added to this analysis. There were 10 032 male airline pilots employed during 1921–1996 who were followed-up for cancer incidence up until 1997. A total of 64 cases of prostate cancer were reported (SIR, 1.21; 95% CI: 0.93–1.54). The RR of prostate cancer increased with increasing number of flight hours on long-haul flights (P for trend = 0.01): the RR was 3.88 (95% CI: 1.26–11.9) for a duration of 10 000 hours long-haul compared with a duration < 5000 hours.

2.3.3 *Meta-analyses*

Ballard *et al.* (2000) published a meta-analysis of cancer incidence and mortality among flight personnel. The combined relative risk (meta-RR) based on a fixed effect model for breast cancer was 1.89 (95% CI: 1.40–2.56) overall, or 1.35 (95% CI: 1.00–1.85) if corrected for socio-economic status (based on two studies). The respective meta-RRs for prostate cancer incidence were 1.82 (95% CI: 1.31–2.52) overall and 1.65 (95% CI: 1.19–2.29) if corrected for socio-economic status, also based on two studies.

Seven observational studies among female air cabin crew (Pukkala *et al.*, 1995; Rafnsson *et al.*, 2001; Reynolds *et al.*, 2002; Wartenberg & Stapleton, 1998; Haldorsen *et al.*, 2001; Lynge, 1996) were considered in the review by Megdal *et al.* (2005) (studies are listed in Table 2.7). With only one exception (Haldorsen *et al.* 2001), these studies uniformly indicated an increased risk of breast cancer (meta-SIR, 1.44; 95% CI: 1.26–1.65; fixed effects model). All seven studies are incidence studies with the general population as the referent group. [The original rationale for these studies had been that the occupational exposure to cosmic radiation caused an anticipated excess cancer risk. In most studies, the excess starts about 10 years after first employment and increases weakly with increasing duration. Differences in reproductive factors can explain only a small fraction of the excess, and the risk attributable to (non-neutron) radiation, a similar fraction of the excess. Hence, it is likely that there is a major part of the excess risk in breast that must be attributable to factors others than reproductive factors and radiation]. It was reasoned subsequently that the observed increase in breast cancer risk could have been due as well to a melatonin deficiency resulting from work-associated light exposure at night (Mawson, 1998).

Buja *et al.* (2006) published a similar meta-analysis on cancer risk among female flight attendants, based on the same seven studies. They obtained a meta-SIR of 1.40 for

breast carcinoma (95% posterior interval, 1.19–1.65). The only other significant excess was in the incidence of melanoma (meta-SIR, 2.15; 95% CI: 1.56–2.88).

No publication bias was detected by any of the tests used in any of these two meta-analyses.

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3. Studies of Cancer in Experimental Animals

3.1 Modification of carcinogenesis by alteration of light/dark environment and central circadian pacemaker function

Introduction

The regular alternation of light and darkness over 24 hours synchronizes the endogenous circadian timing system of mammals through multiple pathways that involve (*a*) the elicitation of multiple gene transcription responses to glutamate and pituitary adenylcyclase activating peptide, the neuromediators that convey light messages to the suprachiasmatic nuclei (SCN) in the hypothalamus (Harmar *et al.*, 2002); the SCN are the main circadian pacemakers that coordinate the rhythmic organization of biological functions over 24 hours (Hastings *et al.*, 2003); and (*b*) the elicitation of multiple hormonal responses, including melatonin and corticosterone, in response to light. These neuroendocrine or endocrine effects seem to require SCN mediation and their extent depends upon the endogenous circadian time of exposure (Ishida *et al.*, 2005; Schibler & Brown, 2005); glucocorticoids, including corticosterone, can reset both the molecular clock and downstream clock-controlled genes in peripheral tissues, such as liver (Balsalobre *et al.*, 2000). In experimental systems, light exposure plays a key role in the resetting of the circadian timing system, that can involve, but does not require, melatonin signalling (Skene *et al.*, 1999).

The relevance of environmental light-dark schedules for cancer development or growth has been studied (see Table 3.1) with regard to the respective effects of (*a*) circadian time of carcinogen exposure in rodents kept in 24-hour light-dark regimens; (*b*) constant exposure to light or constant darkness; (*c*) experimental jet lag or other alterations of photoperiodic regimens; and (*d*) experimental mutations of clock genes. The intrication between carcinogen effects and circadian disruption was obvious in most reports. Conversely, circadian disruption, through experimental jet lag exposure, as well as clock genes mutations or SCN ablation, also leads to malignant processes taking place.

The influence of circadian time on carcinogen or promoter exposure and malignant growth was studied in intact mice or rats kept in a regular alternation of 12 hours of light and 12 hours of darkness (LD12:12) before exposure to carcinogens. Modifications to the regular alteration of light and darkness over 24 hours can disrupt both the circadian timing system at one or several levels of its hierarchical organization and the clock-controlled rhythms in mammals. The multifaceted changes that can occur range from clear whole organism response such as significant modifications in rest–activity rhythms and inability to rhythmically control core body temperature, to more subtle changes such as altered

Table 3.1. Summary of significant positive studies for each type of model and protocol: effect of light exposure during biological darkness and the circadian disruption on cancer incidence and growth

Experimental focus	Study type				
	No other exposure	Chemical initiation/ promotion models	Chemical transplacental carcinogenesis models	Tumour cell or graft transplantation studies	Total
Alterations in light exposures[a]	2/3[b]	5/6	1/1	10/10	18/20
SCN lesions[c]	–	–	–	1/1	1/1
Chronic experimental jetlag	–	–	–	2/2[d]	2/2
Pinealectomy-induced melatonin suppression	–	2/8	–	11/13	13/21
Direct effect of physiological concentration of melatonin on tumorigenesis	–	–	–	5/5	5/5
Clock gene mutations	1/1	1/2[e]	–	–	2/3
Circadian timing of carcinogen administration	–	4/4	–	–	4/4
Total	3/4	12/20	1/1	29/31	45/56

[a] Continuous bright light at night, dim light at night, intermittent or pulsed light at night
[b] The one negative study in this category was designed to be negative through the use of an inbred mouse with a genetic predisposition to retinal degeneration and was part of a study with one of the positive findings that had inadequate reporting.
[c] Electrolytic ablation of the superchiasmic nuclei
[d] Both of these studies were performed in the same laboratory with an experimental model that has not yet been used by other groups for cancer studies.
[e] These two studies used a radiation exposure in knockout animals as the cancer-initiating agent rather than a chemical.

activation of rhythmic signal–transduction pathways in multiple cell types in the body (Hastings *et al.* 2003: Levi & Schibler 2007). The rhythms that exist in the body in such diverse systems such as melatonin release, immune surveillance or cellular proliferation/apoptosis/DNA repair can be altered in different ways by different environmental exposures in different species (Deprés-Brummer *et al.*, 1995; 1997; Fu & Lee, 2003; Filipski *et al.*, 2005). The wide range in effects observed resulting from disruptions of the regular light-dark environment can make interpretation of the reported carcinogenic findings difficult. The rhythm alterations that most often result from chronic changes in the light-dark environment can either persist permanently or be restored through system feedback and adjustment over time. Finally, advancing light onset, as occurs with jet lag, has its own novel impacts on the circadian clock system and the clock-controlled rhythms at all three levels of the circadian timing system: hypothalamic pacemaker, circadian physiology, and molecular clocks (Reddy *et al.*, 2002; Nagano *et al.*, 2003; Filipski *et al.*, 2005). In essence, the circadian disruption that can occur through alterations in the light-dark environment is systematic and can lead to complex phenotypical changes that can only truly be understood in the context of the state of the entire circadian timing system and the downstream pathways it controls.

3.1.1 *Chronic alteration of light-dark environment*

Groups of 50 2-month old CBA mice, 50% females, were kept under a standard 300 lux light-dark regimen (LD12:12) or a constant 2500 lux light regimen until their natural death. All gross tumours and all tissues and organs with suspected tumour development were examined microscopically. [The Working Group noted that the microscopic examination of all relevant tissues in animals was not done but only done for cases where tumour development was suspected. This may have led to missing microscopic tumours.] No body weight difference between the groups was seen even though there was a significant 30% reduction in food consumption in the constant light group at 6 ($P < 0.01$), 8 ($P < 0.001$), 12 ($P < 0.02$) and 16 ($P < 0.02$) months. No cataracts were seen in either group. There were no significant changes in length of the estrous cycle, although mice in the constant light group were more likely to have irregular cycles at 3 ($P < 0.05$), 6 ($P < 0.001$) and 12 ($P < 0.002$) months. The total number of animals with malignant tumours was significantly increased in the constant light group (35% versus 10% of tumour-bearing mice; $P < 0.001$) as was the incidence of lung adenocarcinomas (7/50 (14%) versus 1/50 (2%); $P < 0.05$) and malignant lymphomas and leukaemia combined (6/50 (12%) versus 0/50; $P < 0.02$). There was also a marginal increase in hepatocellular carcinomas (4/50 (8%) versus 0/50; $P = 0.058$) (Anisimov *et al.*, 2004).

3.1.2 *Role of circadian time on two-stage models of carcinogenesis*

(a) Two-stage skin cancer carcinogenesis mouse model

(i) *N*-Nitroso-*N*-methylurea

Six groups of 77 to 105 hairless mice (hr/hr Oslo strain), 60–90 days of age, were kept in LD12:12 (06:30 to 18:30) and painted with *N*-nitroso-*N*-methylurea (NMU, 0.2 mg) once at 08:00, 12:00, 20:00, and 24:00 or once a week for 3 weeks at 08:00 and 20:00. There were equal numbers of male and female mice in each group. The mice were examined for appearance of skin papillomas once a week for 18 months. No difference was found between animals painted once at 05:30 or at 17:30 hours after light onset. The number of animals having tumours from the remaining four groups were 5/95 (5%) in the group painted once at 01:30, 6/77 (8%) in the group painted three times at 01:30, 10/104 (10%) in the group painted once at 13:30 and 13/96 (14%) in the group painted three times at 13:30. The difference between tumour incidence for the two single painting groups (01:30 and 13:30) were not significantly different ($P = 0.105$), the difference in the groups painted three times was marginal ($P = 0.054$). When the 01:30 groups were combined and compared to the combined 13:30 groups, the difference was significant ($P = 0.0137$). The authors noted that the highest incidence of papillomas was achieved when exposure to NMU occurred at a time corresponding to the lowest DNA synthesis rate when relatively large numbers of late G1 cells accumulated [no data presented] (Clausen *et al.*, 1984).

A total of 670 hairless mice (hr/hr Oslo strain, 50% females) kept in LD12:12 with light from 07:30 to 19:30 were exposed to a single topical application of two doses of NMU dissolved in 100 µL reagent-grade acetone. Mice (n = 351) were exposed in groups to a single application of 1 mg NMU at either 00:00, 04:00, 08:00, 12:00, 16:00 or 20:00. Also, several mice (n = 287) were exposed in three groups to a single application of 2 mg NMU at 08:00, 12:00 or 20:00. An additional group of 32 mice were also treated with 10 mg NMU to study the dose–response relationship. The development of all types of skin tumours was observed and the results presented as tumour rates (percentage of tumour-bearing animals in relation to the number of animals alive, appearance of the first tumour related to time), and tumour yields (the cumulative occurrence of all skin tumours standardized for comparison of groups of 32 mice related to time). Most animals were examined once a week for 54 weeks, but those to which 10 mg NMU was applied were observed for only 34 weeks. A circadian variation in tumour production after a single application of 1 mg NMU was demonstrated with a high tumour incidence after application in the period from 16:00 to 00.00, and a lower incidence between 04:00 and 12:00. When 2 mg NMU was applied, there was definitely a low tumour incidence after application at 04:00 compared to the two other times. There was a good and almost straight-lined dose–response relationship after the application of 1, 2, and 10 mg NMU. [Publication unavailable to the Working Group] (Iversen & Iversen, 1995).

(ii) *7,12-dimethylbenz[α]anthracene/12-O-tetradecanoylphorbol-13-acetate*

Groups of 25 female CD1 mice, 8–10 weeks of age, were kept in LD12:12, with light onset at 06:00 and were administered a single dose of 9,10-dimethyl-1,2-benzanthracene (DMBA, 2.5 μg/mouse). Starting 2 weeks later, the back of the mice were painted twice a week for 12 weeks with tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (1.9 μg). The application of 12-*O*-tetradecanoylphorbol-13-acetate was tested at: 05:00, 11:00, 17:00 (during the light span), and 23:00 (5 hours after the onset of darkness). The experiment was terminated at Week 15 following the first 12-*O*-tetradecanoylphorbol-13-acetate administration. In the group painted at 23:00 as compared to that exposed at 17:00, there were nearly twice as many mice with tumours within Weeks 8–11, and the weekly average number of tumours per mouse was nearly twice as high within Weeks 9–15 (Wille, 2003). [The Working Group noted that the tumour type was never clearly given but was assumed to be skin papilloma or carcinoma.]

(b) Intestinal carcinogenesis models in rats

(i) *1,2-dimethylhydrazine*

Male non-inbred rats from a local strain kept in LD12:12 were injected subcutaneously with five weekly doses of 21 mg/kg body weight 1,2-dimethylhydrazine 1 hour after light onset (10:00; $n = 23$) or 13 hours after light onset (22:00; $n = 24$). Injections at 13 hours after light onset were followed by a significant decrease in the incidence (from 91 to 75%; $P < 0.05$) and mean size of tumours (from 27.2 to 15.5 mm^2; $P < 0.05$) 6 months after the first injection; there were also relatively fewer large tumours (Dubina *et al.* 2002).

(ii) *Azoxymethane*

The effect of the time of administration of azoxymethane during the day to alter the yield of foci (recognized as precancerous lesions) in the colon was evaluated in six groups of 14 male Fischer 344 rats, 7 weeks of age. Azoxymethane was subcutaneously administered in a 15 mg/kg body weight saline solution on Days 7 and 14 of the experiment. Foci of aberrant crypts were evaluated with microscopy 28 days after the first azoxymethane dose in whole mounts of colons stained with methylene blue. In each of the three groups of rats, azoxymethane induced twice as many foci when administered between 8.40 and 11.00 than in the three other groups that were administered azoxymethane between 14.45 and 17.55 (205.7 ± 16.0 versus 110.2 ± 12.9; ANOVA, $P < 0.01$). [No indication as to the light-dark schedule in the animal rooms was given by the authors] (Pereira *et al.*, 1994).

Groups of 30 female mice from two inbred substrains of C3H mice, 100 days of age, were exposed to constant light [intensity not given] or LD12:12 (control) over 400 days. In substrain C3H-A, lifetime exposure to chronic light extended estrus by approximately 24 hours, and resulted in a significant increase in the incidence of and mortality from

mammary tumours [data only given in the form of a graph with no statistical details; 50% versus 0% and 70% versus 20% mammary tumour incidence at 151–200 and 201–250 days of age, respectively]. In substrain C3H-HeJ [this substrain has an inherited autosomal recessive retinal degeneration and has been well characterized in circadian behavioural paradigms], lifetime exposure to chronic light resulted in permanent estrus [onset time not given], a delayed onset of mammary tumour development, a reduced number of tumours, and an increased longevity [data only given in the form of a graph with no statistical details] (Jöchle, 1963; Joechle 1964).

3.1.3 *Constant light, light during darkness and constant darkness on experimental cancer*

(a) DMBA on mammary cancers in female rats

A group of 28 female non-inbred virgin rats was injected intravenously with 1.5 mg DMBA in saline emulsion six times once every 10 days. Four weeks after the last injection of the carcinogen, animals were divided into two equal groups. Females of the first group were exposed to constant electric light [300-W electric bulb], while the animals of the second group were kept under conditions of natural light. Animals of the constant light group had an increased multiplicity of mammary gland tumours (3.1 versus 1.7). Altogether, tumours developed in 12/14 rats (86%) in the first group and in 10/14 rats (71%) in the second group. In a second experiment, exposure to constant light that started 7 weeks before DMBA treatment resulted in mammary gland tumours in 5/17 rats (29%) while 6/15 females (40%) animals kept under natural light developed tumours (Khaetski, 1965).

Female Sprague-Dawley rats were exposed to LD12:12 or constant illumination starting on the 42nd day of life. At 50 days, the animals received 30 mg DMBA intragastrically. Eight months later, mammary tumours were found in 15/26 rats (58%) in LD12:12, with 16/36 (44%) tumours being adenocarcinomas. Conversely, 20/21 rats (95%) on constant light displayed mammary tumours, however, 53/57 (93%) tumours were fibroadenomas and only 4/57 (7%) were adenocarcinomas. Constant light reduced ($P < 0.001$) the weights of both the ovaries and pineal glands (Hamilton, 1969). [No information was given on the time of DMBA administration, sampling times or circadian effects of constant light.]

Pregnant female Holtzman rats, 10–12 days of age, were exposed to LD10:14 or to constant light, with light intensity of 150 lux, and their pups were subsequently exposed to the same regimen. The female offsprings were fed with 100 mg/kg DMBA intragastrically on Days 55 to 60. This procedure led to 20% mortality. Animals were palpated once weekly. Over the 180 days that followed DMBA delivery, 60% of the 25 rats in LD10:14 and 94% of the 47 rats in constant light developed tumours ($P < 0.02$). Of these tumours, 60% were adenocarcinomas in the LD10:14 group as compared to 95% in the constant light group ($P < 0.001$). The latency period of tumour appearance was also

significantly shorter in the constant light group ($P < 0.001$). Constant light exposure was associated with a 3-fold increase in plasma prolactin and a 5-fold increase in DNA synthetic activity in the mammary gland. Minor changes in plasma estradiol were found between the LD10:14 and constant light groups (Shah *et al.*, 1984; Kothari *et al.*, 1982, 1984; Mhatre *et al.*, 1984). [No information was given on the time of DMBA administration, sampling times or circadian effects of constant light, three factors that can help in the interpretation of these clearcut differences in carcinogenesis.]

A total of 100 female Sprague-Dawley rats were divided by weight into two groups of 50 animals. Starting at Day 26, one group was exposed to constant light, and the second group was exposed to LD8:16. Both groups received an 8 mg intragastric dose of DMBA at 52 days of age. At 13 weeks post-DMBA administration, there were significantly fewer mammary tumours in the constant light group compared with the LD8:16 group: the respective number of tumour-bearing rats were 8/50 (16%) vs 19/50 rats (38%) ($P < 0.05$), with an average mean number of tumours per rat of 1.1 and 2.6, respectively. The protective effect of constant light on tumorigenesis was ascribed to a substantial acceleration of mammary-gland development, past the temporal window of DMBA susceptibility in virgin animals. This was supported by the observation that 29/50 rats (58%) on constant light had lactating mammary nodules at necropsy compared to 0/50 in LD8:16 (Anderson *et al.*, 2000).

The effects of four light–dark schedules on the growth of established mammary carcinomas was investigated using 64 female Sprague Dawley rats, exposed to a single intragastric dose of 20 mg/kg DMBA at 55 days of age. When the number of rats with palpated mammary tumours of size 1-cm in diameter was sufficient, the rats were randomized into four groups of 16 animals each. These groups were subjected to LD12:12, constant light (300 lux), LD12:12 (300 lux) with a 30-minute exposure to light near mid-dark, or exposed to low light intensity (0.21 lux) rather than real darkness during the 12-hour dark span. Tumour growth was fastest in the rats exposed to dim light during the dark phase, and slowest in those remaining in LD12:12 ($P < 0.01$). Tumours also grew faster ($P < 0.05$) in the rats exposed to light at night for 30 minutes and in those exposed to constant light, when compared to the LD12:12 group. The onset of accelerated tumour growth occurred at Week 4 for the dim-light-at-night group, at Week 6 for the light-pulsed-at-night group, and at Week 10 for the constant light group. On Weeks 11 and 12, the mean tumour surface of the three experimental groups with altered light–dark schedules were similar and differed significantly from the control LD12:12 group ($P < 0.05$). A significant decrease in night time urinary excretion of aMT6s was observed in all experimental groups. The usual light/dark difference in aMT6s excretion was eliminated in the dim-light-at-night and constant light groups but not in the light-pulsed-at-night group. A significant 5-fold increase in serum estradiol was observed in the dim-light-at-night group only (Cos *et al.*, 2006).

(b) NMU

The effects of altered endogenous night time melatonin concentrations on mammary tumour production were investigated in an NMU-induced breast cancer model in female Fischer 344 (F344)/N rats, as were the effects of suppressed serum melatonin concentrations on the incidence and progression of NMU-induced breast cancer. In-vivo studies were used to assess serum melatonin concentrations and the incidence of NMU-induced tumours after 1 day, 2, and 10 weeks of nightly administration of short-duration intermittent light exposure at night. Five 1-minute exposures to incandescent light every 2 hours after the start of the dark phase of the light–dark cycle decreased the magnitude of the nocturnal rise of serum melatonin concentrations in rats by approximately 65%. After 2 weeks of nightly intermittent light exposures, the peak night time serum melatonin concentrations decreased by approximately 35% on average. The amelioration continued and, at 10 weeks, peak night time serum melatonin concentrations had decreased further, by approximately 25%. Because peak endogenous night time serum melatonin values could be moderately suppressed for at least 10 weeks, a 26-week NMU mammary tumour experiment was conducted. Serum melatonin concentrations and incidence, multiplicity, and weight of NMU-induced mammary tumours were assessed. No effect on the development of mammary tumours in an NMU-induced tumour model in rats occurred when endogenous night time serum melatonin concentrations were moderately suppressed by short-duration intermittent light exposures at night. At necropsy, there were no alterations in mammary tumour incidence (28/40 NMU controls (70%), 28/40 NMU + light (70%)), multiplicity (2.18 tumours/tumour-bearing NMU control, 1.89 NMU + light), or average tumour weight (1.20 g NMU control, 1.19 g NMU + light). Tumour burden had no effect on the serum melatonin cycle. At 26 weeks, however, animals exposed to intermittent light at night exhibited an approximately 3-fold higher serum melatonin concentrations when compared to controls (Travlos *et al.*, 2001).

(c) Diethylnitrosamine on liver carcinogenesis in male rats

A total of 65 male Wistar rats were administered diethylnitrosamine (10 mg/kg/day p.o. in drinking-water) for 6 weeks and were randomized into three groups. Rats received either diethylnitrosamine only ($n = 20$), phenobarbital ($n = 22$, 30 mg/rat/day p.o. in drinking-water) for 4 weeks as a promoting agent or were exposed to constant light ($n = 23$). All three groups received diethylnitrosamine in LD12:12 for the initial 21 days and in constant light from Days 22 to 43. This procedure was chosen to suppress the main circadian physiology outputs expected to occur in the continuous light group by Day 77, simultaneously with the maximum phenobarbital promoting effect expected to occur in the phenobarbital group. In the constant light group, there was a 4-fold drop in 24-hour mean urinary aMT6s excretion when compared to the other groups (P from ANOVA < 0.001). The aMT6s rhythms were suppressed in constant light, but remained similar in the other groups. Laparotomy was then performed and macroscopic nodules were counted and measured in each of the four liver lobes, using a 1-mm scale, without knowing the

allocated group of the animal. The proportion of rats with macroscopic nodules was 72% (LD12:12 group), 89% (phenobarbital group), and 95% (constant light group) (P from $\chi^2 = 0.10$). Both the frequency of the lesions and the size of the largest lesion differed significantly among the three groups ($P < 0.05$). Nodules were more numerous and larger in the constant light group and in the phenobarbital group when compared to the LD12:12 group (P from $\chi^2 < 0.05$). Similarly, there were more rats with large tumours (≥ 3 mm) in the constant light group and in the phenobarbital group when compared to the LD12:12 group ($P < 0.05$). All the rats died with hepatocellular carcinomas, with a median survival of 5 months – this was similar in all three groups. (Van den Heiligenberg *et al.*, 1999). [Light-induced circadian clock suppression exerted a promoting effect similar to that caused by phenobarbital in this model. Reduced body weight through manipulation of feeding schedules has usually been associated with decreased tumour growth. Animals on constant light had increased incidence and volume of tumours and gained the least weight.]

(d) Transplanted Glasgow osteosarcoma in mice

Three groups of 10 male B6D2F1 mice were randomly assigned to remain in LD12:12 or exposed to constant darkness or to constant light throughout the whole study duration (5 weeks). Environmental conditions were confirmed to maintain circadian coordination in mice, with a period of 23.8 hours for the constant darkness group, and 26.5 hours for the constant light group. After 3 weeks, all the mice were inoculated subcutaneously with 3x3-mm fragments of mouse Glasgow osteosarcoma in each flank. Tumour size was measured three times weekly for 2 weeks. Exposure to constant darkness or to constant light had no significant effect on tumour growth (ANOVA: $P = 0.8$) nor survival (log-rank: $P = 0.66$) when compared to mice kept in LD12:12 (Filipski *et al.*, 2004). [The Working Group noted that in B6D2F1 mice, constant light and constant darkness did not suppress circadian outputs or melatonin secretion, explaining why the study was negative.]

(e) Tissue-isolated Morris rat hepatoma 7288CTC

Young adult male Buffalo rats (BUF/BUF/Ncr), 5 weeks of age, were allowed to acclimatize to a photoperiod of LD12:12 for 1 week, and were provided with standard laboratory chow and water *ad libitum*. Rats were randomly assigned to three different light exposure groups ($n = 12$ rats/group): LD12:12 (1st group), LD12:12 with a dark phase contaminated with dim light coming from an indirect light leak of 0.2 lux (2nd Group) through the door of the final animal group of constant bright light of 810 lux (3rd Group). Half of the animals were exposed to these lighting conditions for 4 weeks after which blood samples were collected at 4-hour intervals over a 24-hour period. The remaining animals were exposed to these lighting conditions for 2 weeks before their subcutaneous implantation of a single 3 mm cube of MT_1/MT_2 melatonin-receptor positive Morris rat hepatoma 7288CTC in a tissue-isolated manner ($n = 6$ rats/group).

Tumour growth (estimated tumour weights calculated from serial measurements of tumour size) was monitored throughout the course of exposure to these different lighting conditions for up to 3 additional weeks following tumour implantation. At the end of each tumour growth period in each light exposure group, tumour linoleic acid uptake and 13-hydroxyoctadecadienoic acid (13-HODE) release were measured via arteriovenous difference measurements; tumours were removed for analysis of linoleic acid content. Compared with the 1st group in which the nocturnal circadian increase in plasma melatonin and linoleic acid levels were intact, the mean tumour growth rate was increased over 2-fold ($P < 0.05$) and tumour latency decreased by 6 days [no statistical analysis peformed] in the 3rd group in which the nocturnal rise in the levels of both plasma melatonin and linoleic acid were extinguished. Plasma melatonin levels were consistently low while plasma linoleic acid levels remained the same throughout the 24-hour day in the 3rd group. Tumour linoleic acid uptake was increased 2-fold while 13-HODE release was augmented over 4-fold when compared to 1st group ($P < 0.05$). In the 2nd group, the mean tumour growth rate was increased nearly 2-fold ($P < 0.05$) and tumour latency decreased by 2 days [no statistics performed] when compared with the 1st group; the plasma profile of low plasma melatonin levels was similar to the 3rd group while the nocturnal circadian rise in plasma linoleic acid levels was preserved, as per the 1st group. Tumour linoleic acid uptake was increased 2-fold while 13-HODE release was augmented 3-fold when compared to the 1st group ($P < 0.05$). Under all three lighting conditions the total consumption of food throughout the day was virtually identical (Dauchy *et al.*, 1997).

In a follow-up confirmatory study of essentially identical design as that described above, virtually the same results were obtained on the growth, linoleic acid uptake and 13-HODE release in tissue-isolated rat hepatoma 7288CTC. The only substantial difference between the two studies was that the source of dim light exposure during the dark phase was from direct illumination (0.2 lux) provided by a mounted fluorescent lighting fixture placed directly in front of the animal cages rather than from an indirect, contaminating light leak in the previous study (Dauchy *et al.*, 1997).

In a light intensity dose–response study, adult male Buffalo rats were acclimatized to an LD12:12 photoperiod and then randomly assigned to one of six different light intensity exposures during the dark phase of an LD12:12 photoperiod in specially constructed light exposure chambers: total darkness, 0.02, 0.05, 0.06, 0.08 and 345 $\mu W/cm^2$; $n = 6$ rats per exposure. Indirect white, polychromatic fluorescent light reflected off of the walls of the chamber were used rather than direct light from the fluorescent tubes. Rats were exposed to these different lighting conditions for 2 weeks before the implantation of tissue-isolated Morris rat hepatoma (MT_1/MT_2 melatonin-receptor positive) 7288CTC. Following tumour implantation, rats were maintained on their respective light exposure regimen until the end of their respective tumour growth periods. Following 2 weeks of exposure, nocturnal serum levels of melatonin were suppressed in a dose–response manner until full suppression was reached at the highest light intensity. There was a marked dose-related increase in tumour growth rates, [^{3}H]thymidine incorporation into DNA, and DNA

content relative to the control animals exposed to the dark phase over a period of up to 3 weeks following tumour implantation ($P < 0.05$). Similary, tumour linoleic acid uptake, 13-HODE release, cyclic adenosine monophosphate (cAMP) levels ($P < 0.05$), extracellular signal-regulated kinase kinase (MEK) and extracellular signal-related kinase (ERK1/2) activation [no statistical analysis] were markedly increased as the light intensity during the dark phase increased. No significant dose–response effects of light were observed on the serum levels of corticosterone (Blask *et al.*, 2005).

(f) Tissue-isolated MCF-7 human breast cancer xenografts in female nude rats

Adult female inbred nude rats maintained on an LD12:12 photoperiod were implanted with estrogen/progesterone-receptor positive (ER+/PgR+) and MT_1/MT_2 melatonin-receptor positive MCF-7 human breast cancer xenografts in a tissue-isolated manner. Tumour growth was monitored for 40 days (estimated tumour weight of 2.5 g) at which time a subgroup of tumour-bearing rats ($n = 3$) was tranferred to constant bright fluorescent white light (300 lux) while the remaining rats ($n = 4$) were maintained on an LD12:12 photoperiod for the duration of the tumour growth period. Serum melatonin levels were measured in parallel groups of rats during the mid-day and mid-dark phases of an LD12:12 photoperiod, and during the subjective dark phase during constant bright light exposure ($n = 6$/group) after 5 weeks of being maintained under these conditions. In the group of tumour-bearing rats switched from LD12:12 to constant light, which induced a complete suppression of nocturnal circulating melatonin levels, the tumour growth rate increased ($P < 0.05$) 7-fold when compared to the control group maintained on LD12:12, and exhibited a robust nocturnal melatonin rise in the blood ($P < 0.05$). Tumour linoleic acid uptake increased 2-fold whereas 13-HODE production increased over 5-fold in the group exposed to constant bright light relative to the LD12:12 controls ($P < 0.05$) (Blask *et al.*, 2003).

Identical in design to the above study, a light intensity dose–response study was performed in adult female nude rats, implanted with tissue-isolated ER–/PgR– and MT_1 melatonin receptor positive MCF-7 human breast cancer xenografts (Blask *et al.*, 2003). Two weeks of exposure of rats to increasing intensities of white light before tumour implantation resulted in nocturnal serum levels of melatonin that were suppressed in a dose–response manner until full suppression was reached at the highest light intensity. Continued exposure of these tumour-bearing rats to the same conditions of increasing light intensities over a period of up to 5 weeks following tumour implantation resulted in a marked dose-related increase in tumour growth rates, [^{3}H]thymidine incorporation into DNA, and DNA content relative to the control animals exposed to the dark phase ($P < 0.05$). Similarly, tumour linoleic acid uptake, 13-HODE release and cAMP levels were significantly increased ($P < 0.05$) whereas extracellular MEK and extracellular ERK1/2 activation were markedly increased [no statistical analysis]. No significant dose–response effects of light were observed on the serum levels of either estradiol or corticosterone (Blask *et al.*, 2005).

3.1.4 *Effect of experimental chronic jet lag on cancer in the mouse*

Two groups of 16 male B6D2F1 mice each were assigned randomly to remain in standard lighting (LD12:12) or to be exposed to experimental chronic jet lag (through serial 8-hour advances of LD12:12 cycles every 2 days). This schedule was considered as being the most disruptive on the nest-activity circadian rhythm among schedules tested in previous experiments. The locomotor activity and body temperature of the mice were monitored with a radiotransmitter. Ten days after the start of light–dark cycle advances, animals in both groups were inoculated subcutaneously with a 3 × 3-mm fragment of transplantable mouse Glasgow osteosarcoma. Three mice in each group served as non-tumour-bearing controls. Survival was checked daily and tumour size measured with a caliper three times a week. Tumour weight was computed as (length × width^2)/2. Fifteen days after tumour inoculation, mice were sacrificed following exposure to constant darkness for up to 48 hours at four different circadian times, i.e. 0, 6, 12 or 18 hours after light onset. Tumour and liver samples were taken to measure the circadian expression of the clock genes mPer2 and mReverbα with an RNase protection assay (Filipski *et al.*, 2004). This experiment was replicated using 12 control mice in LD12:12 and 14 animals subjected to experimental chronic jet lag. Tumour size was measured daily or every alternate day. Tumours progressed significantly faster in animals undergoing "jet lag" when compared to those kept in LD12:12 in both experiments (ANOVA: $P < 0.001$ and $P = 0.002$, respectively). Both experiments indicated that chronic jet lag accelerated tumour growth predominantly between the 8th and the 11th day following tumour inoculation. In the first experiment, mean tumour weight (± SEM) on Day 11, i.e. before the death of the first animal, was 1330 ± 151 mg in experimental jet lag mice, and 647 ± 56 mg in controls (*t*-test: $P = 0.001$). In the replicated experiment, it was 1376 ± 131 mg, and 847 ± 107 mg in experimental jet lag and control mice, respectively (*t*-test: $P = 0.005$). The survival curves further differed with statistical significance as a function of lighting schedule with poorest survival seen in the experimental jet lag group, in each experiment considered separately (log-rank: $P = 0.013$ and $P = 0.0025$), or pooled ($P < 0.0001$) (Filipski *et al.* 2004).

The same protocol was applied to 13 male B6D2F1 mice on LD12:12 and to 14 mice undergoing experimental chronic jet lag for the previous 10 days. On Day 12, mean tumour weight was 1317 mg in the LD12:12 group and 1997 mg in the experimental chronic jet lag group ($P = 0.04$). Experimental chronic jet lag suppressed the circadian rhythms in mRNA transcription of the clock genes *Rev-erbα* and *Bmal1*, while dampening and phase-shifting that in *Per2* in the liver of male B6D2F1 mice. This resulted in a significant derepression of c-Myc that became profoundly rhythmic, while P53 was repressed. Both effects favour genomic instability and cellular proliferation [two effects that could favour liver carcinogenesis]. In the tumours of mice kept in LD12:12, the mRNA expression patterns of the clock genes was suppressed for *Rev-erbα* and markedly damped for *Per2* and *Bmal1*, when compared with liver. Experimental chronic jet lag suppressed these mRNA rhythms in tumours (Filipski *et al.*, 2005).

3.1.5 *Endogenous circadian disruption on experimental cancer*

(a) SCN ablation in mice

The SCN of male B6D2F1 mice were destroyed by bilateral electrolytic lesions, and body activity and body temperature were recorded with a radiotransmitter implanted into the peritoneal cavity. Mice were inoculated subcutaneously with 3 × 3-mm fragments of mouse Glasgow osteosarcoma tumours ($n = 16$ with SCN lesions, $n = 12$ sham-operated) or pancreatic adenocarcinoma tumours ($n = 13$ with SCN lesions, $n = 13$ sham-operated) to determine the effects of altered circadian rhythms on tumour progression. Complete SCN destruction was ascertained postmortem. Both types of tumours grew two to three times faster in mice with SCN lesions when compared to sham-operated mice ($P < 0.001$). The survival of mice with SCN lesions was significantly shorter compared with that of sham-operated mice (log-rank $P = 0.0062$). The 24-hour rest–activity cycle was ablated and the daily rhythms of serum corticosterone level and lymphocyte count were markedly altered in 75 additional mice with complete SCN destruction when compared to 64 sham-operated mice ($P < 0.001$). Thus, disruption of circadian rhythms in mice was associated with an accelerated growth of malignant tumours of two types, suggesting that the host circadian clock may play an important role in the endogenous control of tumour progression (Filipski *et al.*, 2002).

(b) Clock gene mutations in mice

Fu *et al.* (2002) observed that knock-out mice without expression of mPer2 (mPer2$^{m/m}$ mice) displayed salivary gland hyperplasia in both males and females and teratomas, predominantly of the epidermis in males at 6 months, with no other apparent pathological defect. By the age of 12 months, all mPer2$^{m/m}$ mice showed salivary gland hyperplasia, and all male mPer2$^{m/m}$ mice developed teratomas around the genital areas. In addition, 30% of the 34 mPer2$^{m/m}$ mice in the study died before the age of 16 months and 15% of the mPer2$^{m/m}$ mice died of lymphoma, an event that was not observed before the age of 20 months in the 40 wild-type mice studied ($P < 0.001$).

To examine further the role of *mPer2* in suppressing neoplastic growth, wild-type and mPer2$^{m/m}$ mice at 8 weeks of age were challenged with a single dose of whole-body radiation of 4 Gy 10 hours after light onset, and were monitored for illness and survival. The mPer2$^{m/m}$ mice were more sensitive to gamma radiation, as indicated by premature hair graying and hair loss, and an increased rate of tumour formation. Hair graying was observed in 50% of mutant mice at 12 weeks after irradiation, a difference that held up also at 22 weeks after irradiation. The irradiated mPer2$^{m/m}$ mice also showed an earlier onset of hyperplastic growth. At 7 months after irradiation, teratomas were observed in all irradiated male mPer2$^{m/m}$ mice, but not in any irradiated wild-type mice. At 16 months after irradiation, 71% of the mPer2$^{m/m}$ mice had developed malignant lymphomas, with a first case discovered at 5 months. Malignant lymphomas were found in multiple organs – liver, lung, spleen, heart, intestine, salivary glands, etc. Conversely, only 5% of the *wt*

mice displayed malignant lymphomas 16 months after irradiation. [The core circadian genes are induced by gamma radiation in wild-type mice but not in *mPer2* mutant mice. Temporal expression of genes involved in cell cycle regulation such as cyclin D1, cyclin A, *Mdm-2*, and *Gadd45*α, is deregulated in *mPer2* mutant mice. In particular, the transcription of *c-myc* is controlled directly by circadian regulators and is deregulated in the *mPer2* mutant. In this study, *c-myc* transcription in liver was derepressed and became rhythmic in $mPer2^{m/m}$ mice as compared to *wt* animals.] In the $mPer2^{m/m}$ mice, P53 was repressed when compared to *wt*. These studies suggested that the *mPer2* gene functions in tumour suppression through regulating DNA-damage-responsive pathways (Fu *et al.*, 2002).

Gauger and Sancar (2005) studied $Cry1^{-/-}/Cry2^{-/-}$ mice and fibroblasts derived from these mice for radiation-induced cancer and killing, as well as DNA-damage checkpoints and killing, respectively. They administered a single dose of 4 Gy to 24 *wt* C57/Bl6 and to 27 double mutant mice kept in LD12:12, 10 hours after light onset. No difference in survival was found between the two groups over the 90 weeks following radiation exposure and no overt lymphoma or other tumour was found in these mutant mice. Similarly, the $Cry1^{-/-}/Cry2^{-/-}$ mutant fibroblasts were indistinguishable from the wild-type controls with respect to their sensitivity to ionizing radiation and UV radiation and ionizing-radiation-induced DNA damage checkpoint response. According to the authors, their data suggest that disruption of the circadian clock in itself did not compromise mammalian DNA repair and DNA damage checkpoints and did not predispose mice to spontaneous and ionizing-radiation-induced cancers (Gauger & Sancar, 2005).

[A single timepoint of radiation exposure was tested here. No demonstration was offered in this study that $Cry1^{-/-}/Cry2^{-/-}$ mice had an ablated circadian clock. The in-vivo part of this study was performed in mice kept in LD12:12, an environmental condition that dampens, yet does not disrupt, 24-hour physiology in Cry1/Cry2 double mutants, while constant darkness exposure does (Nagashima *et al.*, 2005). The Working Group noted that recent data show that Crys do not seem to be required for normal circadian clock function in mouse fibroblasts (Fan *et al.*, 2007).]

3.1.6 *Transplacental carcinogenesis*

Three groups of 24 pregnant Wistar rats were exposed to different light–dark regimens consisting of either LD12:12 (control), constant darkness, or constant light from Day 1 of pregnancy. On the 18^{th} to 19^{th} day of pregnancy, the dams were injected with a single intravenous dose of 80 mg/kg *N*-nitrosoethylurea, a chemical known to cause tumours of the peripheral nervous system and kidney when administered under these conditions. Dams from the continuous darkness group were subjected to approximately 10 minutes of light during injection. All pups were kept with their dams during the lactating period (1 month) where they remained in the dams' initial light–dark regimen. Following lactation, pups were removed from the dams, housed in groups of 5–7 animals separated by sex and kept under the 12:12 light–dark cycle until their natural death. [The

rats were kept in this regimen since their conception until one month of age.] Full necropsies were performed on all animals with all suspected tumours and all tissues suspected for tumour growth examined microscopically. [The Working Group noted that the microscopic examination of all relevant tissues in animals was not systematically done and only for cases where tumour development was suspected. This may have led to mining microscopic tumours.] The incidence of any tumour, tumours of the peripheral nervous system, and tumours of the kidney in the perinatal constant light group were 2.6, 2.5 and 8.5 times higher than the LD12:12 controls, respectively. As for tumour-bearing animals, the incidences were: males (29/34 (85%) vs 16/61 (26%); $P < 0.01$) and females (38/54 (70%) versus 21/66 (32%); $P < 0.01$). Similar results were seen for the peripheral nervous tissue (males 21/34 (62%) versus 12/61 (20%); $P < 0.01$ and females 29/54 (54%) versus 17/66 (26%); $P < 0.01$) and for the kidney tumours (males 7/34 (21%) versus 1/61 (1.6%); $P < 0.01$ and females 5/54 (9%) versus 1/66 (1.5%); $P > 0.05$). All kidney tumours were classified as mesenchymal tumours with approximately equal localization to the left and right lobes. The nervous system tumours were equally distributed between benign (fascicular neurinoma, reticular neurinoma) and malignant (neuroblastoma, other malignant) [Even though this information was available, the Working Group was unable to separate malignant from benign tumours due to tumour multiplicity within animals in the same group]. The group exposed to perinatal constant darkness demonstrated a significant drop in the number of tumour-bearing animals for males (5/40 (12%); $P < 0.01$) and females (5/44 (11%); $P < 0.01$) and for both sexes combined ($P < 0.01$). For peripheral nervous system tumours (males 4/40 (10%); $P > 0.05$ and females 3/44 (7%); $P < 0.05$) the significant drop in tumour incidence applied only to females or both sexes combined. Kidney tumours (males 1/40 (2.5%); $P > 0.05$ and females 4/44 (9%); $P > 0.05$) showed no change from control in the perinatal constant darkness group. Finally, the mean survival of tumour-bearing rats [detected at necropsy] was significantly reduced in the constant light group ($P < 0.01$ for nervous system tumours, $P < 0.05$ for kidney tumours) for both tumour types and significantly extended ($P < 0.05$ for both tumour types) in the perinatal constant darkness group for both tumour types when combining both sexes (Beniashvili *et al.*, 2001).

3.2 Effects of pinealectomy and nocturnal physiological melatonin levels on the development and/or growth of chemically induced or transplantable experimental tumours in animals

Introduction

Pinealectomy consists in the surgical removal of the pineal gland from the brain. It is the only means of eliminating the nocturnal melatonin signal emanating from the pineal gland without also affecting the central circadian pacemaker in the SCN of the hypothalamus. Pinealectomy has been employed as one means of determining whether

the specific suppression of the physiological nocturnal melatonin signal leads to the enhancement of cancer development and/or growth in experimental animal models of tumorigenesis (see Table 3.1). At the same time, this procedure indirectly addresses whether the physiological nocturnal melatonin signal from the pineal gland is inhibitory to the process of tumorigenesis in experimental animal models. However, it is important to note that unidentified, non-melatonin compounds (i.e. small peptides) that possess anticancer activity both *in vivo* and *in vitro* have been isolated from the pineal gland (Bartsch *et al.*, 1992). Therefore, the mere removal of the pineal gland in the absence of physiological melatonin replacement would not unequivocally prove that only melatonin is responsible for the antineoplastic effects of the pineal or that the promotion of tumorigenesis by pinealectomy is exclusively due to the elimination of the nocturnal physiological melatonin signal. However, in view of the fact that these putative oncostatic substances have never been structurally identified, measured in the blood or other extracellular fluids or determined to be mediators of pineal/circadian physiology, their role in the pineal regulation of tumorigenesis will not be considered. In all of the studies that follow, the melatonin levels were not measured. Other sources of melatonin have been identified in rodents, including the Harderian gland and the intestine. Their relative contribution to the physiological rhythm in circulating melatonin levels are still poorly understood. [However, the Working Group felt confident that, in these studies, pinealectomy resulted in marked reduction in melatonin levels.]

3.2.1 *Undifferentiated neoplasms (Yoshida and Ehrlich tumour)*

Neonatal pinealectomy 24 hours after birth was evaluated on the growth and metastatic spread of Yoshida solid tumour cells transplanted intramuscularly into Sprague-Dawley rats [sex unspecified] 10–12 weeks following pinealectomy. Survival time was decreased in pinealectomized rats ($n = 10$) over intact control rats ($P < 0.001$) ($n = 4$). There was no difference in tumour weight between the pinealectomized and control groups. The prevalence of tumour metastases to the pancreas was markedly increased whereas metastatic foci were much less in liver [no statistical analysis] (Lapin, 1974).

The growth and mitotic index of another undifferentiated tumour, Ehrlich tumour, intraperitoneally or subcutaneously injected into six groups of Swiss inbred mice (25 g) that were either pinealectomized or sham-operated or not operated (controls) were evaluated. [The number of animals per group was not precisely provided but was assumed to be 17–18. Because of the high mortality, the Working Group had concerns regarding the adequacy of the sample size.] Pinealectomized animals were found to have more intraperitoneal ascite tumours ($P < 0.05$) with a greater mitotic index ($P < 0.01$). Solid subcutaneous tumour weight did not change, although there was an increase in the solid tumour mitotic index ($P < 0.001$) (Billitteri & Bindoni, 1969).

3.2.2 *Sarcoma*

As early as the 1940s it was demonstrated that pinealectomy could stimulate the growth of transplantable sarcomas in rats (Nakatani *et al.*, 1940; Katuguiri, 1943).

Thirty years later, the effects of pinealectomy were demonstrated 7 weeks following the subcutaneous injection of previously pinealectomized Holtzman rats [sex unspecified] with fibrosarcoma cells derived from rats treated with methylcholanthrene. Mean tumour volume in these animals was over 2-fold greater in pinealectomized rats than that in the intact control rats, and nearly 2-fold greater than in sham-operated rats. The prevalence of lymph node metastases was 2.5-fold ($P < 0.05$) [$P = 0.013$; pinealectomized versus sham-operated] greater in the pinealectomized group than in the combined control groups while the number of animals with lung metastases was virtually the same among all treatment groups (Barone *et al.*, 1972). The comparison of mean tumour volume in pinealectomized rats with that in the combined controls showed a significant increase ($P < 0.01$). [The Working Group questioned whether to use combined intact and sham group was reasonable.]

In contrast, exposure of Wistar rats [sex unspecified] to the polyoma virus failed to induce neosarcoma in neonatally pinealectomized or intact control animals (Wrba *et al.*, 1975). [The lack of details and of an effect in the controls make an interpretation of this study problematic.]

3.2.3 *Hepatocarcinoma*

Pinealectomy has been reported to inhibit the development of chemically induced hepatocarcinomas in rats (Lacassagne *et al.*, 1969).

More recently, the effects of pinealectomy versus sham-pinealectomy were examined to evaluate the growth of transplantable tissue-isolated Morris rat hepatoma (7288CTC) in male Buffalo rats over a 2-week period. The tumour growth rate in animals that were pinealectomized ($n = 8$) one week before tumour implantation was 2-fold ($P < 0.05$) greater than the tumour growth rate in sham-pinealectomized controls ($n = 8$) over a 3-week period, and latency to tumour onset was reduced by 50% ($P < 0.05$). The tumour uptake of linoleic acid and production of its metabolite 13-HODE, the mitogenic signal upon which hepatoma 7288CTC is dependent, were markedly increased in pinealectomized rats versus their sham-pinealectomized counterparts ($P < 0.05$) (Blask *et al.*, 1999).

3.2.4 *Ovarian and small bowel adenocarcinoma*

Previously pinealectomized ($n = 12$), sham-pinealectomized ($n = 10$) or intact ($n = 10$) hamsters [sex and strain not specified] were inoculated subcutaneously with ovarian tumour cells [type not specified]. The interval of time between the surgical procedures and tumour cell inoculation was not specified. Tumour growth evaluated over

a 30-day period following tumour inoculation revealed that tumour volume was about 5-fold greater in pinealectomized animals versus sham-pinealectomized; tumour volumes in sham-operated and intact hamsters were virtually equivalent [no *P* values shown]. No significant differences were observed in the growth of small bowel adenocarcinomas in the same pinealectomized versus sham-operated animals 2 weeks following tumour cell inoculation (Das Gupta, 1968). [The Working Group cannot clearly interpret the results of this study due to lack of details.]

3.2.5 *Walker 256 carcinosarcoma*

The effects of pinealectomy have been determined on the growth and spread of transplantable Walker 256 carcinosarcomas (carcinomatous variant) in male Sprague-Dawley rats. Young adult rats (40–50 g; 13 rats per group) were either pinealectomized, sham-pinealectomized or left intact 2 weeks before being injected into the thigh muscle with a homogenate of Walker carcinoma. Tumour size was measured every 2 days until the occurrence of spontaneous death from the tumour. The survival time of pinealectomized rats was significantly decreased by 14.6% compared to sham-pinealectomized rats ($P < 0.02$). Tumour size was significantly increased in pinealectomized animals by 43% versus sham-pinealectomized animals ($P < 0.01$). There were a greater number of rats with lung or lymph node metastases in the pinealectomized group than in either the sham-pinealectomized or intact groups, although the statistical significance of these differences were not determined (Rodin, 1963) [A Fisher's exact test performed by the Working Group revealed that the prevalence of nodal, but not lung, metastases in pinealectomized rats was significantly higher than in the sham-operated group ($P < 0.04$).]

In another confirmatory study in young inbred male Holtzman rats (40 – 60 g) (Barone & Das Gupta, 1970), it was demonstrated that the mean tumour volume in pinealectomized rats ($n = 27$) 24 days following subcutaneous injection of a cell suspension of Walker 256 carcinoma was 42% greater than in sham-operated animals ($n = 24$) ($P < 0.01$); pinealectomy and sham-pinealectomy were carried out 5 weeks before tumour cell injection. There was also a greater number of pinealectomized animals ($n = 39$) with metastatic lesions localized to the lungs as well as axillary and mediastinal lymph nodes than sham-pinealectomized rats ($n = 35$); however, no statistical analysis was performed [A Fisher's exact test revealed that these differences were statistically different for axillary nodes ($P < 0.02$), mediastinal nodes ($P < 0.001$), and lung ($P < 0.001$).]

3.2.6 *Melanoma*

The effects of pinealectomy were evaluated on the growth and metastatic spread of transplantable hamster melanoma cells – Melanotic Melanoma No. 1 (MM1) – in adult male and female Syrian hamsters. Animals were inoculated with a melanoma cell

suspension 5 weeks following pinealectomy or sham-operation; an additional control group was left intact. Tumour volume was measured every week for 5 weeks following tumour cell inoculation. At the end of the first 2 weeks following tumour cell inoculation, tumour volume was 10-fold higher in the pinealectomized group ($n = 10$) versus the sham-pinealectomized animals ($n = 10$) ($P < 0.001$). The overall tumour growth rate over the subsequent 3 weeks in the pinealectomized group was 3-fold higher than in the sham-pinealectomized group ($P < 0.001$); no significant differences were observed between the sham-operated and intact controls. There was also a higher frequency of metastasic foci in the lungs, liver, kidneys, spleen and axillary lymph nodes in the pinealectomized group when compared to sham-operated animals ($P < 0.001$) through 21 days; no significant differences were observed between the sham-operated and intact controls (Das Gupta & Terz, 1967).

In a follow-up study, melanoma growth was examined in young adult male Syrian hamsters, 4–6 weeks of age, that were either pinealectomized or sham-pinealectomized. One week following pinealectomy or sham surgery, the animals were injected subcutaneously with a tumour suspension of MM1 hamster melanoma cells derived from solid tumour tissue, and tumour weights were evaluated 3 and 6 weeks following tumour injection. After 3 weeks, mean tumour weight in the pinealectomized animals ($n = 12$) was 2.3-fold higher ($P < 0.05$) than in sham-operated animals ($n = 11$), and after 6 weeks it was 1.6-fold higher (pinealectomized, $n = 13$; sham-operated, $n = 12$; $P < 0.05$); tumour weight was not significantly different between sham-operated and intact animals either after 3 or 6 weeks ($n = 10$–11) (El-Domeiri & Das Gupta, 1973).

In a later study by this group, the effects of pinealectomy versus sham-pinealectomy were evaluated on the growth of MM1 hamster melanoma growth in young adult male Syrian hamsters, 5–6 weeks of age, under the conditions of a long photoperiod (LD14:10) or short photoperiod (LD6:18). Animals were maintained on either long or short days for 2 weeks before pinealectomy or sham surgery and continued on these photoperiods thereafter. One week following surgery, animals were injected subcutaneously with a suspension of cells derived from MM1 hamster melanoma. Under long days, the tumour growth rate in the pinealectomized hamsters ($n = 16$) was higher than in sham-operated animals ($n = 11$) over 38 days as determined by serial measurement of tumour volumes (no statistical comparison); tumour latency was identical in both groups. The final mean tumour weight in pinealectomized hamsters was 37% higher than in sham-operated controls ($P < 0.01$). In contrast, under short days, the tumour growth rate was lower in pinealectomized hamsters ($n = 8$) than in sham-operated controls ($n = 9$) over 51 days (no statistical comparison); tumour latency was significantly longer in pinealectomized versus sham-operated animals ($P < 0.05$). The final mean tumour weight in pinealectomized animals was nearly 50% lower than in sham controls ($P < 0.01$) (Stanberry *et al.*, 1983).

In a study by another group in a carcinogen-induced model of melanoma, the effects of pinealectomy versus sham-pinealectomy were examined on the development of melanomas induced by the intragastric administration of DMBA in male and female Syrian hamsters 2 days after pinealectomy or sham surgery. Thirteen months following

DMBA administration, the number of melanomas (> 1 mm and < 5 mm) in pinealectomized male animals ($n = 26$) was 74% higher than in sham-operated animals ($n = 45$) ($P < 0.001$), and 44% higher in pinealectomized females ($n = 11$) than in sham-operated females ($n = 20$) ($P < 0.001$). No significant differences were observed in tumour number in pinealectomized versus sham-operated males or females for tumours > 5 mm although tumour number tended to be smaller in the pinealectomized groups than in the sham-operated groups. The effects of pinealectomy on mean tumour size or incidence was not determined in this study (Aubert *et al.*, 1970). [No direct comparisons were done to compare direct measures of tumour size between groups.]

3.2.7 *Prostate carcinoma*

Only one study has examined the effects of pinealectomy on the growth of a fast-growing, androgen-independent transplantable rat Dunning R3327 prostate cancer in adult male Copenhagen-Fischer F_1 rats. Tumours were transplanted subcutaneously into pinealectomized ($n = 11$) or sham-operated rats ($n = 10$) [timing of surgery and tumour implantation in relationship to surgery were not specified]; no differences in growth rates were observed over a 75-day period following tumour transplantation (Toma *et al.*, 1987).

3.2.8 *Uterine carcinoma (Guerin malignant epithelioma)*

DMBA

Guerin malignant spontaneous epitheliomas of the Wistar rat uterus were transplanted into adult male Wistar rats that were either pinealectomized ($n = 7$) or left intact ($n = 17$). Three days after the pinealectomy, all rats were transplanted subcutaneously with Guerin epitheliomas. Mean life span in tumour-bearing pinealectomized animals was significantly reduced by 14 days when compared to intact controls ($P < 0.001$); the mean mitotic activity of tumours in pinealectomized rats was moderately (15%) but significantly higher than in intact controls ($P < 0.05$) 35–45 days after tumour transplantation (Lewiński *et al.*, 1993).

3.2.9 *Mammary carcinoma*

The effects of neonatal pinealectomy (24 hours after birth) on the development of mammary tumours induced by the intragastric administration of DMBA in adult female Wistar rats were evaluated against intact animals. Both groups of animals received a total of three intragastric DMBA treatments separated by 10-day intervals 3–3.5 months after pinealectomy, and were treated with saline following the first DMBA treatment. The incidence and final prevalence of DMBA-induced mammary tumours was the same in both pinealectomized ($n = 12$) and intact animals ($n = 12$) over the 400-day period following the first DMBA administration (Lapin, 1978).

In a subsequent study, the effects of pinealectomy versus sham-pinealectomy in animals on an LD12:12 light dark cycle were examined on DMBA-induced mammary tumorigenesis in young adult female Sprague-Dawley rats (58 days of age). Pinealectomy and sham surgery were performed 2 days before the administration of DMBA. Latency to onset and incidence of mammary tumours in pinealectomized rats ($n = 17$) versus sham-operated animals ($n = 20$) were not statistically different during the 140 days of tumorigenesis (Aubert *et al.*, 1980).

Pinealectomized young adult (50 days of age) female Sprague-Dawley rats administered a single low dose of DMBA (7 mg) 30 days following surgery showed a 4-fold higher incidence of mammary tumours in the pinealectomized group relative to the sham-operated controls 240 days following DMBA treatment ($P < 0.002$). When pinealectomized and sham-pinealectomized rats were administered a higher dose of DMBA (10 mg), tumour development in pinealectomized rats ($n = 30$) was 2-fold higher than in the sham-pinealectomized group ($n = 30$, $P < 0.03$) (Tamarkin *et al.*, 1981).

In a series of publications from one study that addressed the effects of neonatal (2 days of age) pinealectomy or sham surgery on DMBA-induced (Day 55) mammary tumorigenesis in adult female Holtzman rats maintained on a short photoperiod (LD10:14 light–dark cycle) from birth, no significant differences were found in the final prevalence of mammary tumours, mammary tumour number, mean latency to tumour onset, [^{3}H]thymidine incorporation into DNA in mammary tissue or the number of terminal end or alveolar buds in pinealectomized rats ($n = 23$) versus sham-operated rats ($n = 15$). The total duration of tumour-monitoring was 180 days after DMBA administration. No tumour incidence curves were presented in either of these studies so that the rates of tumour development could be statistically compared (Kothari *et al.*, 1984; Shah *et al.*, 1984).

In a subsequent study, it was similarly demonstrated that the incidence, final (55 days of age) prevalence, and number of DMBA-induced mammary tumours in adult female Holtzman rats that undergone pinealectomy neonatally (2 days of age) were not significantly different at the end of the tumorigenic period (30 weeks post-DMBA) between neonatally pinealectomized ($n = 20$) and intact animals ($n = 20$) maintained on a short photoperiod (LD10:14). However, 80% tumour prevalence in pinealectomized animals was achieved 12 weeks following DMBA treatment when compared to intact control animals that had a 10% prevalence at 12 weeks and a maximal tumour prevalence that was not apparent until 24 weeks following DMBA administration (70% tumour prevalence). [It was clear that under short photoperiod conditions, mammary tumours in pinealectomized animals developed at a rate that was substantially faster than in intact animals; however, these investigators did not statistically analyse the tumour incidence curves presented in their report.] (Subramanian and Kothari, 1991).

In another carcinogen-induced mammary tumour model, pinealectomized (3 days before the first NMU injection) adult female Sprague-Dawley rats ($n = 11$) maintained on an LD12:12 light–dark cycle and treated with the carcinogen NMU, on Day 50 and Day 57, exhibited a trend for an overall increase in the incidence and number of mammary

tumours over intact animals ($n = 14$) for the period encompassing 19 weeks following NMU injection; however, this increase was not statistically significant. Similarly, no significant difference was observed in tumour latency in pinealectomized rats versus intact controls (Blask *et al.*, 1991).

In a subsequent study using adult female Fischer 344/N rats maintained on an LD12:12 photoperiod, animals were pinealectomized ($n = 40$) at 4 weeks of age or left intact ($n = 40$) and given NMU intraperitoneally (50 days of age). Tumour development was documented over a 26-week period following NMU administration. There were no significant differences in tumour incidence, final prevalence, number, size or latency between the pinealectomized and intact groups even though the circadian melatonin rhythm was fully expressed in the intact animals and completely extinguished in the pinealectomized rats during the first half of the study. However, by the end of the study, a nocturnal melatonin signal was present in pinealectomized rats (Travlos *et al.*, 2001). [This latter result was difficult to explain; one possibility was that an extrapineal source of circulating melatonin (i.e. the gut) may have compensated for the loss of the pineal gland.]

3.3 Effects of physiological melatonin administration on experimental tumour growth activity in animals

Introduction

Most of the studies have demonstrated an oncostatic action of melatonin on tumour development and growth in experimental animal models of cancer. However, these studies have been performed using pharmacological doses of melatonin. The nocturnal, physiological blood concentrations of melatonin *in vivo* are inhibitory to tumorigenesis and have been inferred from studies employing pinealectomy as a technique for specifically eliminating the nocturnal melatonin signal and observing a stimulation of tumour development and growth (see section 3.2 above). Only a handful of recent studies (see Table 3.1) have directly investigated the role of physiological, nocturnal concentrations of melatonin on experimental cancer growth *in vivo*.

3.3.1 *Rat hepatoma*

In one study, 3-mm^3 of tissue-isolated Morris rat hepatomas (7288CTC) were sutured to the tip of a vascular stalk formed from the superficial epigastric artery and vein of groups of 5–9 male Buffalo rats. When these tumours reached approximately 5 g, the carotid artery and tumour vein were either cannulated or perfused *in situ*. Perfusion experiments used rat whole blood harvested during the early light phase just a few hours following light onset when endogenous melatonin levels were low. Perfusion studies using a high physiological concentration of melatonin (1 nM) for 2.5 hours reversibly

($P < 0.05$) blocked the uptake of linoleic acid, production of 13-HODE, and significantly decreased ($P < 0.05$) the incorporation of [^{3}H]thymidine into DNA. [These findings were strengthened by studies using several melatonin receptor antagonists that completely reversed the effects of the melatonin.] In the cannulated animals measured every 4 hours for 24 hours, tumour linoleic acid uptake and metabolism to 13-HODE were temporally correlated ($P < 0.05$) with the circadian rhythm and a significant difference was demonstrated between peak dark and peak light phase values ($P < 0.05$). Finally, pinealectomized rats hosting tumour tissue and given either daily subcutaneous injections of melatonin or provided oral melatonin (200 μg) demonstrated a significant delay in the latency to tumour palpability when compared to appropriate sham controls ($P < 0.05$) (Blask *et al.*, 1999).

In a subsequent dose–response study using the same experimental protocol, increasing concentrations of exogenous melatonin were administered to perfused in-situ tissue-isolated Morris rat hepatomas hosted in Buffalo rats, 4–5 weeks of age, using whole blood harvested from pinealectomized Sprague-Dawley donor rats. Final whole blood concentrations of melatonin reproduced those levels characteristic of the ascending limb of the nocturnal, endogenous melatonin surge. A significant ($P < 0.05$) dose-dependent suppression of tumour linoleic acid uptake and 13-HODE production occurred following melatonin perfusion. Similarly, a dose-dependent suppression of tumour-DNA content ($P < 0.05$) and [^{3}H]thymidine incorporation into DNA ($P < 0.05$) was seen in response to melatonin along this concentration range as well. The inhibitory effects of melatonin on tumour linoleic acid metabolism and cellular replication activity saturated at the highest physiological concentration of 1 nM. Additionally, tumour uptake and retention of melatonin itself, as a function of supply, ranged from 20 to 45% across all concentrations tested. In the same study, melatonin was added to a semi-purified 5% corn oil diet so that pineal gland intact animals ingested, primarily during the dark phase, either 50 ng, 500 ng or 5 μg/day of additional dietary melatonin to produce physiological, nocturnal concentrations of melatonin that added to the endogenous nocturnal surge. When animals began receiving melatonin in their diet 2 weeks before tumour implantation and continuously thereafter, tumour growth as well as linoleic acid uptake and metabolism to 13-HODE were significantly inhibited ($P < 0.05$) in a dose-dependent manner (Blask *et al.*, 2004).

3.3.2 *Human cancer xenograft*

Adult male Buffalo rats were implanted with 7288CTC hepatoma cells as described above (positive control), and adult female nude rats were implanted with tissue-isolated steroid receptor negative (SR–, no estrogen or progesterone receptor expression) or steroid-receptor positive (SR+) human breast cancer xenografts. The SR+ xenografts when perfused with Sprague-Dawley rat donor whole blood to which was added 1 nmol/L of synthetic melatonin showed significant reduction in [^{3}H]thymidine incorporation ($P < 0.05$) and in camp levels ($P < 0.05$). This reduction was completely

eliminated by coperfusion with 13-HODE. Although not shown, similar findings were reported for the SR– xenographs. The authors further investigated these models using differing levels of light at night to control melatonin levels. Using six animals per group and six different light intensities resulted in significant changes ($P < 0.05$) in Phos, ERK1/2, linoleic acid uptake, 13-HODE, c-AMP, [^{3}H]thymidine incorporation and tumour onset in both the 7288CTC model and the SR– model. Similarly, tissue-isolated SR–, MT_1 melatonin-receptor positive MCF-7 human breast cancer xenografts were perfused *in situ* for 1 hour with human whole blood from premenopausal females collected in daytime, night time and after exposure to bright light at night. There was a significant reduction of [^{3}H]thymidine incorporation (63% to 73%) between samples perfused at night time and daytime ($P < 0.05$) which was eliminated in experiments using blood from volunteers exposed to bright light at night. Other markers as noted above (linoleic acid uptake, 13-HODE, cAMP) behaved as expected. Finally, to determine if this was entirely driven by melatonin, 500 pmol/L of melatonin was added to blood from donors exposed to bright light at night for 90 minutes. The results were identical to what was seen for the night time blood sample experiments and these results could easily be blocked by using MT_1/MT_2 antagonists. Using a new perfusion system that minimized delivery time, it was subsequently demonstrated that perfusion of tissue-isolated estrogen-receptor negative MCF-7 human breast cancer xenografts *in situ* with melatonin-containing (1 nM final concentration) daytime-collected rat donor blood completely suppressed ($P < 0.05$) linoleic acid uptake and 13-HODE formation within 5 minutes of melatonin reaching the tumour, indicating that the melatonin suppression of tumour linoleic acid metabolism is extremely rapid (Dauchy *et al.*, 2006).

Using the same basic protocol used above, tissue-isolated FaDu human squamous-cell cancer xenografts (grade II human hypopharyngeal squamous cell carcinoma) were implanted into male athymic nude rats. Cancer xenografts were perfused *in situ* for 2 hours with daytime-collected male adult Buffalo rat donor whole blood to which melatonin was added at a final concentration of 1 nM. This perfusion resulted in a total blockade of linoleic acid uptake ($P < 0.05$) and 13-HODE formation ($P < 0.05$) as well as a significant ($P < 0.05$) 76% suppression of cAMP levels and a 50% inhibition ($P < 0.05$) of [^{3}H]thymidine incorporation into DNA and DNA content when compared to vehicle-containing daytime-collected control whole blood (Dauchy *et al.*, 2007).

3.4 References

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4. Mechanistic and Other Relevant Data

4.1 The pineal gland and melatonin

Introduction

There is considerable interest in the role of the pineal gland in the development and growth of malignant tumours and in the ability of melatonin, its main secretory product, to act as an oncostatic agent.

Melatonin is a messenger of time in the mammalian organism which transmits the information of environmental light and darkness obtained from the eye through the hypothalamus to all tissues of the body. It interacts with the mechanisms that form the mammalian time structure, and has to be understood in its relation to the organism's biological clock.

Melatonin has anti-proliferative effects on human cancer cells cultured *in vitro*. These oncostatic effects have been observed at physiological concentrations, and include reduction of cell-cycle progression by increasing the expression of the tumour-suppressor gene *TP53*, and inhibition of DNA synthesis. In addition, melatonin reduces the invasive and metastatic properties of human cancer cells *in vitro*, and increases intercellular communication between these cells. There is evidence from animal models that melatonin inhibits or reduces the induction of DNA damage by free radicals. Pinealectomized rats showed a higher level of DNA damage in response to treatment with a carcinogen than did rats with intact pineal glands. Melatonin also upregulates anti-oxidant enzyme systems.

4.1.1 *The pineal gland and its innervations*

The mammalian pineal is a secretory organ with specialized glandular cells, the pinealocytes, interstitial glial cells, and perivascular macrophages. The principal innervation is sympathetic and arises from the superior cervical ganglion. In addition, parasympathetic, commissural and peptidergic innervation are present. The sympathetic fibres contain norepinephrine and neuropeptide Y as neurotransmitters. The parasympathetic fibres contain vasoactive intestinal peptide and peptide histidine isoleucine. Neurons from the trigeminal ganglion reach the pineal gland containing substance P, calcitonin-gene-related peptide, and pituitary adenyl-cyclase-related activating peptide. Through the pineal stalk, nerve fibres originating in the brain and containing a variety of neurotransmitters innervate the pineal. In addition to its principal noradrenergic innervation, numerous receptors have been found in the pinealocyte cell

membrane, which are able to bind numerous neurotransmitters and influence the pinealocyte (for a review, see Møller & Baeres, 2002).

The secretory products of the pineal consist of melatonin which plays a major role in plant, animal, and human physiology, and several peptides the action of which is less well characterized.

4.1.2 *Melatonin and its production*

Melatonin (*N*-acetyl-5-methoxytryptamine) was first isolated by Lerner *et al.* (1958) from bovine pineal glands. Tryptophan is taken up from the blood stream and transformed to melatonin in four successive intracellular steps which are catalysed by tryptophan hydroxylase (EC1.14.16.4), aromatic amino acid decarboxylase (EC 4.1.1.28), arylalkylamine-*N*-acetyltransferase (EC 2.3.1.87), and hydroxyindole-*O*-methyltransferase (EC 2.1.1.4) (Axelrod & Weissbach, 1960; Lovenberg *et al.*, 1967). Arylalkylamine-*N*-acetyltransferase is thought to be the rate-limiting enzyme in the process of melatonin synthesis (Klein, 2007).

Melatonin is present in bacteria, in eukaryotic unicells, in numerous plants, vegetables, fruits, seeds, rice, wheat, and medicinal herbs, and diverse species of invertebrates (Hardeland & Poeggeler, 2003).

4.1.3 *Extrapineal production of melatonin*

The main source of circulating melatonin in mammals is the pineal gland. However, many extrapineal mammalian tissues and organs have the enzymatic mechanism to produce melatonin (Carrillo-Vico *et al.*, 2004, 2005).

The apparently large quantity of melatonin produced in tissues other than the pineal gland does not appear to contribute substantially to the circadian-rhythm-related plasma melatonin concentration as suppression of pineal function induced by surgical pinealectomy or constant light exposure markedly diminishes the circulating melatonin concentration (e.g. in Syrian hamsters) (Vaughan & Reiter 1986) and eliminates the nocturnal plasma melatonin concentration surge.

4.1.4 *Pineal production of melatonin*

In contrast, melatonin production in the pineal region is, in all mammalian species, periodic with high values during the dark phase irrespective of the activity or rest span of the species studied. In darkness, a marked 7–150 fold increase of arylalkylamine-*N*-acetyltransferase activity has been measured in the pineal region. The rhythm of production is endogenously generated by the activity of the suprachiasmatic nucleus (SCN) in the hypothalamus by a closed loop negative feedback of clock-gene expression. The rhythm is synchronized primarily by the environmental light–dark cycle. With irregular schedules of light exposure, it may be altered in its timing and in the duration of melatonin production. The pineal melatonin rhythm is driven by the circadian clock in the

hypothalamus through a multisynaptic pathway which consists of ganglion cells in the retina containing non-vision-dependent photoreceptors (Lucas *et al.*, 1999; Freedman *et al.*, 1999). Retinal ganglion cells containing the pigment melanopsin are thought to be the photoreceptors for the photic entrainment of circadian rhythms (Berson *et al.*, 2002). The photic information is transmitted to the central pacemaker in the SCN via the retino-hypothalamic tract.

The neural pathway from the SCN to the pineal region passes through pre-autonomic neurons of the paraventricular nucleus of the hypothalamus through the upper part of the spinal cord where synaptic connections are made with sympathetic preganglionic neurons relating to the superior sympathetic ganglia of the sympathetic chain (Møller & Baeres 2002). From there, postganglionic noradrenergic sympathetic neurons extend to the pineal gland. Norepinephrine is the neurotransmitter stimulating melatonin release in the pineal gland. It is released during the daily dark span in response to stimulating signals from the SCN.

Once formed, melatonin is not stored within the pineal gland but diffuses out into the capillary blood and cerebrospinal fluid (Tricoire *et al.*, 2003). The half-life of melatonin is bi-exponential with a first distribution half-life of 2 minutes and a second of 20 minutes or longer (Claustrat *et al.*, 2005). Melatonin released into the cerebrospinal fluid via the pineal recess reaches in the third ventricle concentrations up to 20–30 times higher than in the blood but decreases in concentration with increasing distance from the pineal region, suggesting cerebral tissue uptake (Tricoire *et al.*, 2003).

4.1.5 *Metabolism of melatonin*

Circulating melatonin is metabolized mainly in the liver which clears over 90% of the circulating melatonin. Melatonin is first hydroxylated and then conjugated as sulfate and excreted as 6-sulfatoxymelatonin (aMT6s) and in a small amount as glucuronide (Arendt, 1995; Claustrat *et al.*, 2005). Urinary and salivary aMT6s excretion closely parallels the plasma aMT6s profile. Melatonin passes into saliva in a low concentration which in its relative amount and timing corresponds also to the plasma profile. Urinary aMT6s and salivary melatonin lend themselves to the non-invasive study of melatonin secretion and its timing (Nowak *et al.*, 1987; Arendt, 1995).

Melatonin can also be metabolized non-enzymatically in cells and extracellularly by free radicals and other oxidants. Owing to its lipophilic character, it diffuses through cell membranes easily and can exert not only receptor-dependent but also receptor-independent actions (Claustrat *et al.*, 2005).

Once released into the blood, melatonin is primarily bound to albumin (70%) (Cardinali *et al.*, 1972), α-1-acid glycoprotein (Morin *et al.*, 1997), and haemoglobin (Gilad & Zisapel, 1995). Changes in circulating melatonin levels may be due in part to changes in the concentrations of one or more of these binding proteins. This could influence the availability of melatonin to various target tissues and its bioactivity in these tissues (Di *et al.*, 1998).

4.1.6 *The circadian rhythm of melatonin in plasma in humans*

The plasma melatonin rhythm in humans develops between the 2nd and 3rd month of life with peak melatonin concentrations found in prepubertal children (Waldhauser *et al.*, 1988). In the elderly, the nocturnal melatonin concentrations decrease at the end of the 5th decade and beyond to levels between 20–80% of levels found in young adults (Touitou *et al.*, 1984; Ferrari *et al.*, 1995; Magri *et al.*, 1997). This drop was not found in all studies probably due to different lifestyle, state of health, sampling techniques, etc (Kennaway *et al.*, 1999; Zeitzer *et al.*, 1999). The circadian melatonin profile varies considerably between clinically healthy human subjects, with some having no detectable melatonin concentrations during daytime or night time (Arendt, 1985). On the other hand, in healthy individuals, the timing, amplitude, and even the shape of the melatonin profile can be highly reproducible and characteristic for a given person (Arendt, 1988; Klerman *et al.*, 2002). Individual living habits like 'morningness' and 'eveningness' are expressed in differences in the phase of the circadian melatonin profile (Duffy *et al.*, 1999; Gibertini *et al.*, 1999). No consistent gender difference has been found in regard to melatonin concentrations, the melatonin profile, or its suppression by light (Arendt, 1985).

4.1.7 *Light and regulation of pineal melatonin production in humans and animals*

The light–dark cycle is the main entraining agent ("zeitgeber," synchronizer) of the regulating system of pineal melatonin secretion. Light suppresses melatonin secretion in humans (Lewy *et al.*, 1980) in an intensity-related manner (Bojkowski *et al.*, 1987a; McIntyre *et al.*, 1989). The endogenous rhythm governing melatonin synthesis and release is entrained to the daily dark span in different mammalian systems irrespective of the diurnal or nocturnal activity of the species. In diurnally active human subjects, high values of melatonin concentrations are released during the night. Light, in addition to acting as entraining agent of the circadian clock, will act as a masking agent when the subject is exposed to light during the habitual dark span. The photoreceptor system involved in clock regulation is distinct from the pathways associated with image formation. The sensitivity of the circadian system to light entrainment does not depend upon rod and cone photoreceptor integrity, and/or the loss of visual function (Foster *et al.*, 1991). Eye loss in mammals, including humans, confirms that photoentrainment originates within the eye (Haus *et al.*, 1967; Lockley *et al.*, 1997; Foster, 1998). The daily alteration between light and dark entrains the endogenous circadian clock in the hypothalamus to the astronomical day length (24 hours). The innate period of the hypothalamic clock tends to be slightly longer than 24 hours. Absence of this input due to loss of the eye leads to a tendency of the organism to "free-run" from the 24-hour environment following the non-24-hour endogenous period of the hypothalamic clock (Haus *et al.*, 1967; Lockley *et al.*, 1997; Skene *et al.*, 1999).

The photoreception acting as synchronizer upon the SCN is based upon a population of about 1% of retinal ganglion cells, which are photosensitive and respond to light directly (Sekaran *et al.*, 2003). These photoreceptors contain a photopigment based on an

opsin/vitamin A complex with peak sensitivity in the blue part of the spectrum, near 480 nm. In rodents as well as in humans this agent is more than likely OPN4 or melanopsin (Brainard *et al.*, 2001, Thapan *et al.*, 2001; Ruby *et al.*, 2002; Hattar *et al.*, 2003;).

The exploration of the human photoreceptors and the related circadian time organization requires the appropriate use and measurement of light stimuli (Foster *et al.*, 2007). The establishment of action spectra has been helpful in associating photopigments with the responses of photobiological systems. An action spectrum for spectral sensitivity of suppression of nocturnal melatonin concentration was identified by Thapan *et al.* (2001) using monochromatic light exposure for 30 minutes in clinically healthy subjects. The light pulse was administered at circadian time 16–18 hours at numerous wavelengths (λmax range, 424–548 nm), and a wide range of irradiance (0.7–65.0 μW/cm2). At each wavelength, suppression of plasma melatonin increased with increasing irradiance. The action spectrum revealed a peak in sensitivity at a λmax of 459 nm, which fitted best with the rhodopsin wavelength profile. A comparable action spectrum for wavelength was obtained in humans of both genders in night time melatonin suppression tests (over a wavelength range from 420–600 nm) with 446–477 nm as the most potent region for regulating melatonin secretion (Brainard *et al.*, 2001). The suppression of nocturnal melatonin concentrations by 1-hour light exposure of 200 or 500 lux was equal in both men and women, and proportional to the light intensity. Also, the levels and amplitude of the circadian rhythm in melatonin were not significantly different (Nathan *et al.*, 2000). In quantifying the biological response to light, the suppression of melatonin in a constant routine protocol showed a close relation to subjective alertness, slow eye movement, and theta-α activity (detected by electroencephalography, Cajochen *et al.*, 2000). These studies showed that light intensities as found in usual room-light illumination (90–180 lux) already have alerting and melatonin-suppressing effects (Bojkowski *et al.*, 1987a; Cajochen *et al.*, 2000; Zeitzer *et al.*, 2000). In clinically healthy subjects, maximal alertness in response to light exposure was found at very short wavelengths (420, 440 and 470 nm) of the visible spectrum (Revell *et al.*, 2006).

Human photosensitivity measured by melatonin suppression depends in part also on prior light exposure of the subjects. It increases after prior exposure of the subjects to dim light, indicating an adaptation of the photoreception or photoresponse to the recent photic history (Owen & Arendt, 1992; Hébert *et al.*, 2002; Smith *et al.*, 2004).

The blocking of the biologically most active short-wavelength light by the use of goggles that excluded all wavelengths of less than 530 nm prevented the suppression of the nocturnal salivary melatonin concentrations by 800 lux light intensity (Kayumov *et al.*, 2005). All subjects (11 men and eight women, 24.7 ± 4.6 years of age) preserved their melatonin levels in filtered light similar to their dim-light secretion profile, while unfiltered bright light drastically suppressed melatonin production. Normalization of the nocturnal melatonin production by elimination of short-wavelength light apparently did not impair the measures of performance, subjective sleepiness, or alertness. Relatively good colour recognition was maintained and visual light transmittance with the filters used was approximately 73% (Kayumov *et al.*, 2005).

Bright light exposure was able to phase shift and reset the circadian phase (Broadway *et al.*, 1987; Czeisler *et al.*, 1986). Also, much lesser light intensity like normal room light, (approximately 180 lux) produced a phase shift (Boivin *et al.*, 1996), and even very dim light (20 lux) was able to synchronize the circadian system in a subject following a regular sleep, wakefulness and meal schedule (Klerman *et al.*, 1997). In these studies with very low light intensity, the time of the daily dark span with sleep of the subjects may have reinforced the synchronizing effect of light. In a study carried without these time cues (scheduled sleep, darkness, activities), 14 days exposure of subjects to a schedule of light dark (LD) 12h:12h with 200 lux:< 8 lux were unable to maintain the initial circadian phase position (Middleton *et al.*, 2002).

Circadian phase shift after exposure to monochromatic short-wavelength light (with two peaks at 436 and 456 nm) in a 4-hour pulse mode (8 lux, 28 $\mu W/cm^2$) after habitual wake time led to a phase shift of the human melatonin profile comparable to an exposure to white light (12000 lux, 4300 $\mu W/cm^2$), in spite of the white light pulse containing 185-fold more photons than the short-wavelength light (Warman *et al.*, 2003).

Similarly, the circadian phase resetting of the free running plasma melatonin rhythm in clinically healthy subjects after a 6.5-hour exposure to monochromatic light at 460 nm induced a 2-fold greater circadian phase delay than the same time of exposure to 555 nm monochromatic light of equal photon density (Lockley *et al.*, 2003).

The sensitivity of the circadian pacemaker varies according to the resetting effect of retinal light exposure depending upon the circadian phase at which the light exposure occurs (Honma *et al.*, 1987; Dawson *et al.*, 1993; Van Cauter *et al.*, 1993). Using pre- and post-stimulus constant routines in dim light (approximately 2–7 1ux) with maintained wakefulness in a semi-recumbent posture, Khalsa *et al.* (2003) described a phase–response curve to a bright light exposure stimulus consisting of 6.7 hours first of a 6-minute fixed gaze exposure to 10000 lux followed by 5000–9000 lux for the remainder of the time span. Plasma melatonin was used to describe the phase of the onset, offset and midpoint of the melatonin profile. The resultant phase–response curve of the midpoint of the melatonin rhythm (with a peak-to-trough amplitude of 5 hours) showed phase delays when the light stimulus was centred before the critical phase of the core body temperature minimum, and phase advances when the stimulus was centred after the critical phase. No phase shift occurred when the stimulus was centred at the critical phase (the body core temperature minimum).

4.1.8 *Photoperiod and seasonal variations*

In addition to information on onset and offset of the daily photoperiod, melatonin provides information on day length. The duration of the melatonin secretion in animals and humans varies with the length of the dark span. The longer the dark span in the laboratory or the night in nature, the longer the time of melatonin synthesis and secretion, irrespective of whether the dark span is the time of activity in nocturnal rodents or of rest in diurnally active species, including humans (Arendt, 1995). Most mammals use the

changes in the length of the daily light and dark period to detect a change in seasons, and to regulate seasonal behaviour and/or synchronize circannual rhythms (Tamarkin *et al.*, 1985; Goldman, 2001). Seasonal variation in reproduction is directly controlled by the relative length of the light–dark span (Lincoln, 2002).

Under laboratory conditions imitating the winter season (short photoperiods), a longer sleep phase (recorded by electroencephalography) and a longer duration of nocturnal melatonin secretion was observed in human subjects (Wehr 1991, 2001). However, in the modern urban electrified environment, these changes are masked and not always detectable. In general, investigators found no seasonal change in duration of melatonin secretion at low- or mid-latitudes (Illnerová *et al.*, 1985; Bojkowski & Arendt 1988; Matthews *et al.*, 1991). In contrast, seasonal change with longer duration of melatonin secretion in winter was found at subpolar and polar high latitudes with marked changes in photoperiod and luminosity (Beck-Friis *et al.*, 1984; Martikainen *et al.*, 1985; Kauppila *et al.*, 1987; Makkison and Arendt 1991; Levine *et al.*, 1994), with higher daytime melatonin concentrations reported (Rönnberg *et al.*, 1990). Also, when people spent more time outdoors in the summer, even in temperate climate (mid-latitude), seasonal changes in secretion of melatonin and of cortisol were found (Vondrasová *et al.*, 1997). Kauppila *et al.*, (1987) suggested that these elevated melatonin concentrations may be associated with diminished reproductive function. A photoperiodic influence on human fertility was observed, resulting in increased fertility in spring, but appeared to be modified by different lifestyles (Wehr, 2001).

4.1.9 *Melatonin in relation to the circadian system*

It is well established that ocular light exposure in humans can affect hormonal secretion, either acutely as a direct response to the presence or absence of retinal light exposure, or indirectly as a result of the influence of light on circadian mechanisms. Indeed, light is the most powerful circadian synchronizer in humans (Czeisler & Wright, 1999), and can exert a profound effect on the phase and amplitude of the human circadian pacemaker (Czeisler & Klerman, 1999). Of particular interest in the context of melatonin as a biomarker is the effect of light on the pineal function in humans: nocturnal illumination of sufficient intensity completely suppresses melatonin production (Lewy *et al.*, 1980; Bojkowski *et al.*, 1987a); there is considerable individual variability in sensitivity to light at night (McIntyre *et al.*, 1990; Hébert *et al.*, 2002; Herljevic *et al.*, 2005); there appears to be a dose–response to light at night in that the brighter the light, the greater the reduction in nocturnal circulating melatonin (Bojkowski *et al.*, 1987a; McIntyre *et al.*, 1989; Zeitzer *et al.,* 2000); bright light shifts the phase of melatonin rhythm, with morning hours being associated with phase advance and evening hours with phase delays (Duffy & Wright, 2005); and light quality during the day affects night time melatonin production (Wehr *et al.*, 1995; Wehr, 1996; Lewy *et al.*, 1987; McIntyre *et al.*, 1990; Boivin *et al.*, 1996), as well as the human circadian pacemaker (Czeisler *et al.*, 1986).

Because melatonin is the best marker of internal clock timing and is quantifiable in the urine via well proven and reliable techniques applicable to non-laboratory studies, it has become a powerful tool as a biomarker of circadian dysregulation.

(a) Laboratory-based studies of melatonin and exposure to light at night

Using sleep laboratory-based protocols, several studies have used melatonin measurements to determine phase advance and delays resulting from controlled exposure to light at night. Deacon & Arendt (1996), Eastman and Martin (1999), Burgess *et al.* (2002), and Revell & Eastman (2005) have used the 'nudging' technique to simulate circadian rhythm disturbance in a laboratory environment. Progressively changing the timing of bright light exposure day by day (nudging) leads to a synchronized shift of the circadian system to a desired new phase. This method has been used to prepare astronauts for space flights (Eastman *et al.*, 1995). Using bright light exposure for 9 hours on five consecutive days, the same authors reported that urinary aMT6s acrophase took at least 5 days post-treatment to return to normal baseline pattern (Deacon & Arendt, 1996). Van Cauter *et al.* (1994) exposed volunteers to 3 hours of bright light (5000 lux) during constant routine conditions following a 7-day entrainment period, measured plasma melatonin at 20-minute intervals, and reported rapid phase shifts within 24 hours of bright light exposure. Results after exposure for 6.5 hours to light of dim to moderate intensity early in the biological night (Zeitzer *et al.*, 2000) showed that even small changes in ordinary light exposure during the late evening hours can significantly affect both plasma melatonin concentrations and the entrained phase of the human circadian pacemaker (Zeitzer *et al.*, 2000). Roach *et al.* (2001) conducted a simulated night shiftwork study in which participants 'worked' during seven consecutive 8-hour shifts, and reported a mean phase delay of 5.5 hours by Night 7, using salivary melatonin measurements to detect the phase shift. A later study by the same team reported similar results using urinary aMT6s levels to assess phase delay (Roach *et al.*, 2005).

(b) Field studies assessing the effects of shiftwork on melatonin secretion

Several studies have used measurements of melatonin in the blood or urine to evaluate and describe the effects of shiftwork on the circadian rhythm. Touitou *et al.* (1990) found that fast-rotating shiftwork modifies peak values and rhythm amplitudes of serum melatonin.

A series of studies on offshore oilrigs using aMT6s as a marker of internal timing and of melatonin suppression have shown complete circadian adaptation in some, but not all offshore schedules. When night-shift adaptation occurs, subjects experience desynchrony and melatonin suppression (approx. 20%) on the subsequent day shift. Thus, in addition to concerns for the health of unadapted night-shift workers, one should consider the implications of adaptation for subsequent health effects (Midwinter & Arendt, 1991; Ross *et al.*, 1995; Gibbs *et al.*, 2002, 2007).

Using data from the Nurses Health Study II, Schernhammer *et al.* (2004) reported a significant inverse association between increasing number of nights worked within the 2 weeks preceding morning-void urine collection and urinary melatonin levels. Along similar lines, Hansen *et al.* (2006) reported lower 24-hour urinary concentrations of aMT6s in nurses working the night shift compared to nurses working the day shift; urinary concentrations of aMT6s were also lower during a day off for night-shift workers, relative to day-off levels in day-shift workers. Several authors have reported inter-individual variability in the response of the melatonin rhythm to night shift (e.g. Sack *et al.*, 1992, 1997; Dumont *et al.*, 2001;; Gibbs *et al.*, 2007). In a study conducted by Quera-Salva *et al.* (1996, 1997) rapid change in sleep time and melatonin acrophase was reported in some night-shift workers, but not others, suggesting that some people have a physiological ability to readily adapt to rotating shift schedules, and reported for the first time a corresponding rapid shift in melatonin secretion. Dumont *et al.* (2001) measured urinary aMT6s every 2 hours during a 24 hour period after three consecutive nights of work in another group of nurses. Using cosinor analysis to estimate phase position, they reported individual variability in adaptation to night shiftwork, with five participants showing a phase delay, three a phase advance, and the remaining 22 demonstrating no phase shift (i.e. the timing of their melatonin secretion was typical of a day-shift worker). In a study involving offshore oil workers employed in a 1-week alternating shift schedule (1 week of nights, 1 week of days), Gibbs *et al.* (2002, 2007) reported adaptation to the night-shift schedule via a delay of aMT6s at the end of the week of night shifts. In another study of offshore oil workers employed in a 2-week alternating shift schedule (2 weeks of days 06:00h-18:00h, 2 weeks of nights 18:00-06:00h), Barnes *et al.* (1998a) reported similar results, with the delay of aMT6s occurring during the first week of the night shift. They also conducted another study in which urine samples were collected every 2 to 3 hours throughout the wake period (subjective days) and one collection over the sleep period (over-sleep sample) from a group of offshore oil workers employed in a 1-week alternating shift schedule (1 week of days (12:00-00:00h), 1 week of nights (00:00-12:00h)), and reported differing adaptation to the night shift depending on season of the year, using measurement of urinary aMT6s to detect phase shift (Barnes *et al.*, 1998b). Prior to this, Midwinter and Arendt (1991) reported differing shifts in the acrophase of melatonin secretion depending on the season of the year, using urine samples collected over 48 hours during a week of night-shift work in a group of workers stationed in the Antarctic; they further reported a slower readaptation to the rhythm following night-shift work during the winter compared to the summer.

A study conducted by Burch *et al.* (2005) compared melatonin levels among workers on permanent day, swing, and night shifts. Urinary aMT6s was measured in post-work and post-sleep samples, and disrupted circadian melatonin production was evaluated using the sleep:work aMT6s ratio. They reported that night workers had altered melatonin levels, disrupted sleep, and elevated symptom prevalence. Subjects grouped by their sleep:work aMT6s ratio rather than shift had even greater symptom prevalence. Risks for two or more symptoms were 3.5 to 8 times greater among workers with sleep:work ratios

≤ 1 compared to those with ratios > 1 (Burch *et al.*, 2005). The sleep:work ratio may be an objective means to assess circadian disruption.

(c) Melatonin as an indicator of diurnal type

Several studies have used melatonin measurements to compare whether diurnal type (morning versus evening) is associated with cumulative nocturnal melatonin secretion and/or onset of melatonin secretion. Madokoro *et al.* (1997) reported pronounced inter-individual differences in plasma melatonin concentration measured before and 1 year after beginning shiftwork. They constructed a ratio of melatonin concentration measured at 6 am to total melatonin concentration measured during the night, and reported that a higher Morningness-Eveningness score (indicating morning type according to Horne & Ostberg, 1976) was correlated with this ratio. Gibertini *et al.* (1999) measured nocturnal melatonin levels in blood on an hourly basis, and assessed the circadian type of each participant; they found that the circadian type was strongly related to the melatonin acrophase but not to the amplitude or the time of year of assessment, and that morning types experienced a more rapid decline in melatonin levels after the peak relative to evening types. Similarly, Liu *et al.* (2000) reported that morning type was associated with an earlier melatonin acrophase. More recently, Griefahn *et al.* (2002) found that the onset of melatonin synthesis was 3 hours earlier in morning than in evening types, using hourly salivary melatonin measurements; they also reported melatonin measurements to be a better indicator of diurnal type than rectal temperature measurements. Diurnal preference may be related to the ability to cope with shiftwork. Importantly, Duffy *et al.* (1999) and Wright *et al.* (2005) have established the close relationship between individual 'free-running' periodicity, entrained phase (entraining in a normal environment) and diurnal preference (Horne-Ostberg score).

(d) Melatonin as chronobiotic agent

Exogenous melatonin given at the right biological time can synchronize (Lockley *et al.*, 2000; Sack *et al.*, 2000; Arendt, 2000) and phase-shift the circadian time organization (Arendt *et al.*, 1984; Arendt, 1985). Depending upon the stage of the circadian rhythms of a subject at a given clock hour, melatonin is able to shift circadian timing to both later and earlier times (Lewy *et al.*, 1992). Appropriate timing of treatment to delay or advance can be predicted from a phase–response curve in subjects whose body clock phase is known (Lewy *et al.*, 1998).

Low doses (0.3–10 mg) of melatonin given during the 'biological day', when endogenous melatonin levels are low, can induce sleepiness or sleep, and lower body temperature (Cagnacci *et al.*, 1992; Deacon & Arendt, 1995, Arendt, 1995; Brzezinski *et al.*, 2005). A single melatonin treatment (5 mg fast release) given late afternoon in controlled studies can advance the timing of the internal clock by up to about 1.5 hours (Deacon & Arendt, 1995).

Timed melatonin administration (0.5–5.0 mg) given at 24-hour intervals, usually at desired bedtime can fully entrain the free-running (non-24 hour) circadian rhythm of most blind subjects (Lockley *et al.*, 2000; Sack *et al.*, 2000; Arendt, 2005). By acting as a circadian coupling agent countering desynchrony among central and peripheral clocks, and optimizing phase with respect to external time cues, cellular and system processes may be optimized and defence systems augmented (Arendt, 2005), with a broad range of potential therapeutic applications to be explored, including in medical oncology (Lissoni *et al.*, 1994a,b, 2003; Bartsch & Bartsch, 2006).

4.1.10 *Melatonin receptors*

Melatonin displays pleiotropic physiological functions. Owing to its small lipophilic structure, it can freely enter cells and can exert an effect independent of the specific receptors found widely in human tissues (Morgan *et al.*, 1994; Morgan & Williams, 1996; Dubocovich & Markowska 2005). For example, in over 130 locations within the brain alone (Masson-Pévet *et al.*, 1994; Pévet *et al.*, 2006; Wu *et al.*, 2006) melatonin receptors are co-localized with vasopressin-, oxytocin- and corticotropin-releasing neurons. In mammalian, two subtypes of high affinity membrane receptors for melatonin have been cloned, the MT1 and MT2 subtypes (Reppert *et al.*, 1994, 1995). They belong to the super-family of G-protein-coupled receptors. The two types show a different action spectrum with large variability among species in their distribution. The receptors are responsible for the chronobiological effects of melatonin at the level of the SCN. In most mammalian systems, MT1 receptors modulate neuronal firing, arterial vasoconstriction, affect the cell proliferation in cancer and other cells, and regulate reproductive and metabolic functions. Activation of MT2 melatonin receptors phase-shift circadian rhythms of neuronal firing in the suprachiasmatic nucleus, inhibit dopamine release in the retina, induce vasodilation and inhibit leukocyte movement in arterial beds, and enhance immune responses (Dubocovich and Markowska 2005).

Endogenous pineal melatonin is believed to feed back onto the master clock in the SCN and regulate neuronal activity and circadian rhythmicity through activation of the specific MT1 and MT2 receptors (Gillette and Mitchell 2002). The response to receptor activation varies with the circadian stage of the cell systems involved. Neurons in the SCN are most sensitive to inhibition of neuronal firing by melatonin in diurnally, as well as nocturnally, active species at dusk suggesting changes in clock excitability, possibly as expression of an endogenous circadian rhythm (Stehle *et al.*, 1989). Melatonin phase-shifts circadian rhythms with two windows of sensitivity corresponding to the day–night (dusk) and night–day (dawn) transitions (Dubocovich *et al.*, 1998; McArthur *et al.*, 1997). The MT1 and MT2 receptors are targets for drug development with receptor agonists and antagonists, which are of interest for eliciting or blocking the wide variety of actions related to melatonin (Zlotos, 2005).

4.2 Proposed mechanisms for carcinogenicity of shiftwork and circadian disruption

Epidemiological studies on genetic polymorphisms in clock-related genes and phenotypes such as morning/evening preference and depressive symptoms, have shown a significant association between a single-nucleotide polymorphism in the *PER3* gene and diurnal preference. In a wider sense, the circadian clock may function as a tumour suppressor at the systemic, cellular, and molecular levels. Clock-controlled genes and related factors involved in cell-cycle control include c-*Myc*, *Mdm2*, *Tp53* and *Gadd45a*, as well as caspases, cyclins, and various transcription factors. In transgenic mice, a deletion in *Per2* results in a shorter circadian period, a higher susceptibility to radiation-induced tumours, and reduced apoptosis in thymocytes. Disruption of the circadian rhythm in mice is associated with an accelerated growth of malignant tumours.

4.2.1 *Melatonin and cancer*

(a) Oncostatic effects of melatonin

Thirty years ago, it was hypothesized that diminished pineal function may promote the development of human breast cancer (Cohen *et al.*, 1978). The primary argument was that increased pineal calcification, presumably leading to lowered melatonin production, was most strongly associated with increased breast cancer risk. Although this was the first reference to environmental lighting, which necessarily includes both sunlight and artificial light, as a potential source of one of several endocrine abnormalities that may underlie the development of breast cancer, light at night was not specifically postulated as an etiological factor. It was proposed incorrectly that altered visual stimulation, by blindness or darkness, would impair pineal melatonin production, thereby leading to unopposed estrogen secretion and increased breast cancer risk. It is now known that overall melatonin production is not compromised in blind individuals (Lockley *et al.*, 1997) and that breast cancer risk is actually diminished in blind women (Hahn, 1991; Verkasalo *et al.*, 1999). It was postulated by Stevens (1987) that light exposure at night may represent a unique risk factor for breast cancer in westernized societies via its ability to suppress nocturnal melatonin production by the pineal gland. This postulate, referred to as the 'melatonin hypothesis', was based on in-vivo studies demonstrating that melatonin inhibits, while pinealectomy or constant bright light stimulates, the development and growth of experimental breast cancer in rodents, and by in-vitro studies showing that the proliferation of estrogen receptor positive (ER+) human breast cancer cells was directly suppressed by nocturnal physiological levels of melatonin (Stevens, 2006).

Many studies using pharmacological concentrations of melatonin have demonstrated a direct antiproliferative and/or apoptotic effect on cancer cells (usually human cancer cell lines) *in vitro*. A substantial number of investigations have also shown that nocturnal physiological concentrations of melatonin exert direct oncostatic effects on cancer cell

proliferation as well. However, several studies have also demonstrated cytotoxic effects of pharmacological levels of melatonin on cancer cells *in vitro*. A large number of studies in experimental animal models of tumorigenesis have shown that properly timed (i.e. relative to the light–dark cycle) administration of pharmacological doses of melatonin can inhibit the development and/or growth of a wide variety of murine tumours (i.e. chemically induced, transplantable, spontaneous) and human cancer xenografts. The mechanisms by which these tumour inhibitory effects are exerted are not totally clear but in some cases may involve the inhibition of tumour linoleic acid uptake and metabolism as well as direct oncostatic, immune enhancing, and/or free-radical/antioxidant actions (Blask, 1993, 2001; Blask *et al.*, 2002, 2005a).

In most of these in-vitro studies, ER+ MCF-7 human breast cancer cells were cultured for several days in the presence or absence of melatonin, usually at a high physiological concentration of 1 nM. Depending on the study, the robustness of melatonin's oncostatic effects could be quite variable ranging from 80% to less than 20% inhibition of cell proliferation. In several investigations involving either human MCF-7 human breast cancer, neuroblastoma, uveal melanoma or murine colon carcinoma cells, the dose–response to melatonin exhibited a bell-shaped pattern with the most robust antiproliferative effects occurring in nocturnal physiological range (Blask *et al.*, 2002). In a small number of studies, no oncostatic effects of melatonin were reported in the physiological range on MCF-7 cells or on several other human cancer cell lines (human cervical carcinoma [Hela], osteosarcoma [MG-63] and lymphoblastoid [TK6]) while cytotoxic effects were observed at pharmacological levels (Panzer *et al.*, 1998; Baldwin & Barrett, 1998; Baldwin *et al.*, 1998). These discrepancies were most likely due to the use of different culture conditions as well as subclones of cells with lower sensitivity to melatonin (Bartsch & Bartsch, 1981; Bartsch *et al.*, 2000).

The oncostatic action of melatonin at physiological concentrations, particularly on ER+ MCF-7 human breast cancer cells *in vitro*, encompasses a variety of molecular and cellular mechanisms, some of which involve the inhibition of the mitogenic action of hormones and growth factors, most notably estradiol (E2), epidermal growth factor, and prolactin. Major effects of physiological concentrations of melatonin on the cellular biology and cell-cycle control of ER+ MCF-7 cells include a slowing of the progression of cells from the G0-G1 phase of the cell cycle to the S phase (DNA synthetic phase) with a resultant lengthening of the transit time through the cell cycle. Evidence indicates that this is accomplished by a melatonin-induced increase in the expression of the tumour suppressor gene *TP53* which in turn activates p21WAF1 protein expression leading to eventual cell-cycle arrest via inhibition of the ability of cyclin-dependent kinases to phosphorylate the retinoblastoma protein (Rb). Additionally, melatonin inhibits DNA synthesis in that reduced proportion of MCF-7 cells that progress to the S phase of the cell cycle (Sánchez-Barceló *et al.*, 2003). While evidence indicates that pharmacological concentrations of melatonin can induce apoptosis in cancer cells, there is no convincing experimental evidence that programmed cell death is activated at physiological levels of this indoleamine (Cos *et al.*, 2002).

In addition to its oncostatic effects, melatonin is able to reduce the invasive and metastatic properties of MCF-7 cells *in vitro*. This appears to be mediated, in part, by a melatonin-induced upregulation in the expression of cell surface proteins E-cadherin and β1-integrin (Cos *et al.*, 1998). Melatonin at physiological levels can increase gap-junction-mediated intercellular communication between MCF-7 cells in culture (Cos & Fernández, 2000) and cause alterations in the cytoskelelal arrangements of ER+ T-47D human breast cancer cells in culture (Matsui & Machado-Santelli, 1997).

Melatonin exerts a major role as an antiestrogen in ER+ human breast cancer cell proliferation by suppressing the activity of the estrogen growth response pathway (Hill *et al.*, 1992). In MCF-7 cells in particular, the molecular mechanisms of this antiestrogen action centre around the fact that melatonin downregulates the transcription of ERα, prevents estrogen-dependent transcriptional activation by destabilizing the E2-ER complex from binding to the estrogen-responsive element of DNA via antagonism of calmodulin interactions with ERα, and by blocking E2-induced upregulation of cyclin D1 expression (Molis *et al.*, 1994; Rato *et al.*, 1999; del Río *et al.*, 2004; Cini *et al.*, 2005). Melatonin does not bind to ERα nor does it interfere with the binding of E2 to the ligand-binding domain of ERα. These molecular events mediating melatonin's oncostatic actions through suppression of the activity of the estrogen-response pathway appear to involve the MT1 melatonin receptor suppression of cAMP production as well as calmodulin antagonism (Ram *et al.*, 2002; Kiefer *et al.*, 2002; Rato *et al.*, 1999).

(b) Melatonin, aromatase and telomerase

In-vitro and in-vivo studies have examined the influence of melatonin, either at nocturnal physiological circulating concentrations or pharmacological doses, on the local biosynthesis of estrogens from androgens via modulation of aromatase activity by ER+ MCF-7 human breast cancer cells (Cos *et al.*, 2005) or dimethylbenzanthracene (DMBA)-induced rat mammary cancers (Cos *et al.*, 2006), and the impact on cell proliferation and tumour growth. Melatonin downregulated aromatase expression at the transcriptional level in MCF-7 cells and reduced aromatase activity in both MCF-7 cells and DMBA-induced rat mammary tumours resulting in a diminished rate of tumour cell proliferation and growth. This anti-aromatase action of melatonin is mediated via the MT1 melatonin receptor (González *et al.*, 2007).

Melatonin at physiological nocturnal circulating levels and pharmacological concentrations inhibits both the expression and activity of telomerase in MCF-7 human breast cancer cells in culture, and in xenografts in female nude mice. Telomerase, an enzyme responsible for the elongation of telomeres at the ends of chromosomes, is activated in most human cancers. Melatonin appears to regulate telomerase mRNA expression via both membrane and nuclear melatonin-receptor-mediated mechanisms (Leon-Blanco *et al.*, 2003, 2004).

(c) Effects of melatonin on sex hormones

In animals, the effects of physiological levels of melatonin, either endogenously produced or exogenously administered, on reproductive hormones (pituitary gonadotrophins, prolactin, gonadal steroids) are well known, particularly in animal species that breed seasonally (Goldman, 1999, 2001). In these animals, melatonin can exert either inhibitory, stimulatory or modulatory effects on these hormones depending upon the species and situation.

In humans, the situation is more problematic. While low pharmacological doses of melatonin administered to human subjects over the course of several days to a few weeks had no effect on either pituitary or gonadal hormones (Arendt, 1985; Wright *et al.*, 1986; Luboshitzky *et al.*, 2000; Arendt, 1995), extremely high doses of melatonin produced a slight reduction in blood levels of pituitary gonadotrophic hormones (Voordouw *et al.*, 1992). As part of a contraceptive study, the administration of large oral doses of melatonin (300 mg) on a daily basis for 30 days to young adult women with normal menstrual cycles, caused significantly decreased mean circulating levels of luteinizing hormone (LH), estradiol and progesterone compared with non-treated controls, possibly through mechanisms dependent on (i.e. enhanced) or independent of steroid negative feedback (Voordouw *et al.*, 1992). On the other hand, much lower doses of oral melatonin (3 mg) enhanced LH and follicle-stimulating hormone (FSH) levels in response to a gonadotropin-releasing hormone (GnRH) challenge during the follicular but not luteal phase of the menstrual cycle (Cagnacci *et al.*, 1995a). Pharmacological levels of melatonin (2–5 mg) have been demonstrated to stimulate prolactin secretion into the blood following oral administration in adult men and women (Terzolo *et al.*, 1993; Kostoglou-Athanassiou *et al.*, 1998) whereas in another study, it had no effect at this dose range (Terzolo *et al.*, 1990).

(d) Melatonin, free radical scavenging and antioxidation

The role of free radicals in oncogenesis encompasses the initiation, promotion and progression stages of tumour development and growth. For example, the exposure of normal somatic cells to chemical carcinogens can generate free radicals that can cause DNA damage that, in turn, may lead to the initiation of cancer. DNA mutations caused by free radicals may become fixed as a consequence of a wave of clonal expansion due to the relative growth advantage that new mutations confer on cells. Additional genomic instability induced by faulty cell division or defective DNA-repair mechanisms may further increase the rate of tumorigenic mutations. Moreover, free radicals and other reactive oxygen species may provide additional stimulation of signal transduction mechanisms that may lead to enhancement of cell proliferative and survival mechanisms (Marte, 2004). While there is no question that melatonin at pharmacological concentrations has potent direct free radical scavenging effects, the role of physiological levels in free radical scavenging remains controversial (Reiter *et al.*, 2001). At

pharmacological levels, melatonin pretreatment substantially reduces the formation of DNA damage in liver tissue in rats caused by the chemical carcinogen safrole, implying that these levels of melatonin have the potential to prevent the initiation of hepatic carcinogenesisby reducing free radical-induced DNA damage. In pinealectomized rats, a further increase in DNA-adduct formation over pineal-intact controls was observed, indicating that the endogenous physiological melatonin signal confers a degree of partial protection against free-radical-induced nuclear DNA damage, and in doing so, may reduce the probability of cancer initiation (Reiter, 2001). Physiologically relevant melatonin levels have been reported to upregulate endogenous antioxidant enzyme systems such as glutathione (GSH) peroxidase, γ-glutamylcysteine-synthetase, the rate-limiting enzyme responsible for GSH synthesis as well as GSH levels, and superoxide dismutase (Blask *et al.*, 1997; Hardeland, 2005). In the case of the enzyme for GSH synthesis, this upregulation appears to be mediated via a mechanism mediated by the melatonin receptor (Hardeland, 2005). Not only do physiological concentrations of melatonin markedly elevate total GSH concentrations in MCF-7 human breast cancer cells, but adequate intracelluar levels of GSH appear to be an absolute requirement for the oncostatic action of melatonin in these cells *in vitro*. Furthermore, ER– HS578T human breast cancer cells that are ordinarily insensitive to the oncostatic action of physiological melatonin concentrations can be coerced into responding to melatonin by raising intracellular GSH levels (Blask *et al.*, 1997). Paradoxically, physiological levels of melatonin have been shown to actually enhance the production and release of reactive oxygen species into the incubation medium by human monocytes co-cultured with human cancer cell lines. This resulted in increased lethality to the cancer cells, indicating a beneficial pro-oxidant effect for melatonin (Morrey *et al.*, 1994).

4.2.2 *Circadian genes and cancer: possible mechanisms*

(a) Genetic determinants of circadian rhythms

Although the daily oscillation of physiological and behavioural processes in plants and animals were observed thousands of years ago, it wasn't until the 1960s that such oscillating rhythms were found to be regulated genetically (Pittendrigh, 1967). The first circadian gene, *Period*, was cloned from fruitflies in the mid-1980s (Bargiello *et al.*, 1984; Reddy *et al.*, 1984). Since then, rapid advances in the field of circadian biology have revealed that these clocks are operated by numerous gene products that function in interacting feedback loops in all species studied. Biological clocks provide organisms with a survival advantage, by organizing their behaviour and physiology around cyclic changes in the environment.

At the time of writing, nine core circadian genes have been identified: *Clock* (King *et al.*, 1997), casein kinase I epsilon (*CSNK1E*) (Takano *et al.*, 2000), cryptochrome 1 (*CRY1*), cryptochrome 2 (*CRY2*) (Hsu *et al.*, 1996), Period 1 (*PER1*), Period 2 (*PER2*), Period 3 (*PER3*) (Shearman *et al.*, 1997; Tei *et al.*, 1997), neuronal *PAS* domain protein 2

(*NPAS2)* (Reick *et al.*, 2001), and aryl hydrocarbon receptor nuclear translocator-like (*ARNTL*) (also referred to as brain and muscle Arnt-like protein-1, *BMAL1*) (Hogenesch *et al.*, 1998; Honma *et al.*, 1998). The three *PER* genes encode PER–ARNT–Single-minded protein (*PAS*)-domain proteins that function as surfaces allowing heterodimerization among different clock proteins. The *CLOCK* and *BMAL1* genes encode basic–helix–loop–helix (*bHLH*)-*PAS* transcription factors. *NPAS2* is a paralogue of the transcription factor *CLOCK*, which is a major player in the SCN. The human *CRY* genes encode proteins similar to plant blue-light receptors within class I photolyases. A common feature of these circadian genes is that the levels of mRNAs and proteins that they code for, with the exception of those coded for by *CLOCK* and *CSNK1E,* oscillate throughout a 24-hour period (Reppert & Weaver 2001).

(b) Circadian genes and clock control

The circadian system is divided into two parts, the central pacemaker and the peripheral clocks. The mammalian circadian clock contains three components: input pathways, the central pacemaker, and output pathways. The input pathways transmit information from environmental cues to the central pacemaker. The central pacemaker synchronizes with the environment to generate endogenous rhythms. The output pathways convert the instructions from the central pacemaker into daily oscillations in various physiological and behavioural processes (Hastings, 2000; Hastings & Herzog, 2004; Fu & Lee, 2003). The central pacemaker in mammals resides in the SCN of the anterior hypothalamus. The SCN is composed of multiple, single-cell circadian oscillators that can generate coordinated circadian outputs when synchronized (Welsh *et al.*, 1995; Liu *et al.*, 1997).

A model of transcription-translation feedback loops of circadian genes has been proposed to explain the molecular clockwork of the mammalian central pacemaker (Reppert & Weaver, 2001, 2002; Young & Kay, 2001). At the molecular level, the circadian clock is organized as positive and negative feedback loops based on transcription-translation. The positive components of the loops are CLOCK (or NPAS2) and BMAL1, which form a heterodimer that regulates the expression of genes containing E-box regulatory segments in their promoter regions (Reppert & Weaver 2001; Fu *et al.*, 2002). This heterodimer directly induces the genes coding for the *PERs* and *CRYs*, which constitute the major components of the negative feedback loop. An additional level of regulation within the positive feedback loop is provided by *REV-ERBα*, which controls cyclic *BMAL1* expression (Preitner *et al.*, 2002). The CLOCK–(or NPAS2–)BMAL1 complex also regulates the expression of multiple other clock-controlled genes, either directly or indirectly.

Similar interacting loops of core circadian gene products regulate circadian rhythms in peripheral tissues. The "peripheral clocks" are regulated by the SCN pacemaker, through both the autonomic nervous system and neuroendocrine systems (Bartness *et al.*, 2001; Kalsbeek & Buijs 2002). The rhythmic expression of core circadian genes is observed in most peripheral tissues (Zylka *et al.*, 1998), and can be induced in cultured

fibroblasts (Balsalobre *et al.*, 1998). Ablation of the SCN has been shown to abolish circadian gene oscillation in peripheral tissues as well (Balsalobre *et al.*, 1998; Sakamoto *et al.*, 1998). These findings suggest that the peripheral clocks are either driven or synchronized by the SCN pacemaker. Both the SCN central clock and peripheral tissue clocks regulate cell functions by affecting the expression of clock-controlled genes. Studies have indicated that 2–10% of all mammalian genes are clock-controlled genes (Le Minh *et al.*, 2001; Duffield *et al.*, 2002; Storch *et al.*, 2002).

Recent data also reveals that microRNAs play a key role as regulators of the circadian-timing process. *miR-219–1* is a clock-controlled gene that plays a role in regulating the length of the circadian rhythm. *miR-132* is light-inducible and modulates the phase-shifting capacity of light. Both microRNAs potentially regulate both clock periodicity and clock entrainment (Cheng *et al.*, 2007).

Time information from the central oscillator to the peripheral oscillators is transmitted by neural and humoral stimuli (Schibler *et al.*, 2003; Buijs & Kalsbeek, 2001), which are currently not well characterized but among which a secretory product of the pineal gland, melatonin, is thought to play a prominent role. The central oscillator in turn is kept in step ("synchronized") with the periodic surrounding by the light–dark alteration of the astronomical calendar day. The oscillators in the peripheral tissues are also subject to the entraining (synchronizing) effects of social routine, physical exercise, and food uptake. These secondary synchronizers determining the phase setting of many peripheral oscillators may compete with the lighting regimen, acting over the central oscillator in the SCN in a multifactorial way (Challet & Pévet, 2003). In some tissues, like the liver, the time of food uptake may become the dominant synchronizer and determine the staging of the metabolic rhythms in this organ (Stokkan *et al.*, 2001; Hara *et al.*, 2001). In daily life, circadian rhythms determine the rhythmically varying degree of cognitive functioning and physical strength and dexterity, resulting in predictable timing of best and worst work performance and efficiency. Optimal function of the human body requires a certain sequential and phase-related ordering of the many circadian rhythms extending from the molecular to organismic level.

(c) Circadian gene polymorphisms and circadian disturbance in humans

A limited number of epidemiological studies have examined genetic polymorphisms in clock-related genes and phenotypes such as morning/evening preference and depressive symptoms (Johansson *et al.,* 2003). The T3111C variation of the *CLOCK* gene was first reported by Katzenberg *et al.* (1998) to be associated with morning/evening preference as assessed by the Horne-Ostberg scale (Horne & Ostberg, 1976). A single nucleotide polymorphism located in the 3′ flanking region of the human *CLOCK* gene was demonstrated to be a predictor of diurnal preference in a population-based random sample of 410 normal adults. A smaller study of 105 subjects, however, did not confirm this association (Robilliard *et al.*, 2002). In addition, associations of *PER3* polymorphisms with delayed sleep phase syndrome or diurnal preference have recently been reported (Ebisawa *et al.*, 2001; Archer *et al.*, 2003; Johansson *et al.*, 2003). Archer

et al. (2003) used the Horne-Ostberg scale to examine a *PER3* length polymorphism in which the longer allele was associated with 'morningness' and the shorter allele with 'eveningness'. The shorter allele was also strongly related to delayed sleep phase syndrome, and they reported allele frequencies to be 68% for the shorter allele and 32% for the longer allele. A recent study found that the homozygous *PER3* longer allele (5/5) had a considerable effect on sleep structure and waking performance (Viola *et al.*, 2007). Rapid eye movement (REM) sleep was increased in *PER3* (5/5) compared to *PER3* (4/4) individuals. In addition, the decrement of cognitive performance in response to sleep loss was significantly greater in the *PER3* (5/5) individuals. By contrast, the circadian rhythms of melatonin, cortisol, and peripheral *PER3* mRNA expression were not affected. These findings show that this polymorphism in *PER3* predicts individual differences in the sleep-loss-induced decrement in performance, and the differential susceptibility may be mediated by its effects on sleep homeostasis. Johansson *et al.* (2003) examined a single nucleotide polymorphism in the *PER3* gene (*647 Val/Gly*) in a Swedish population and their results showed a significant association between this *PER3* genetic variation and diurnal preference.

(d) Circadian genes: potential tumour suppressors

A novel role of circadian genes in tumorigenesis comes from discoveries demonstrating that the circadian clock may function as a tumour suppressor at the systemic, cellular and molecular levels through its involvement in cell proliferation, apoptosis, cell cycle control, and DNA-damage response.

(i) *Regulation of cell cycle and apoptosis*

The cell cycle is regulated by an internal circadian clock. In cells of peripheral tissues, the circadian clock controls cell proliferation and apoptosis by regulating the expression of circadian-controlled genes. The circadian clock mechanism is directly involved in the regulation of cell division (Reddy *et al.*, 2005; Lee, 2005; Gery *et al.*, 2006). Studies have shown that about 7% of all clock-controlled genes identified in rodents regulate either cell proliferation or apoptosis (Kornmann *et al.,* 2001; Akhtar *et al.,* 2002; Duffield *et al.,* 2002; Panda *et al.,* 2002). These clock-controlled genes include c-*Myc* and *Mdm2*, the tumour-suppressor genes *Tp53* and *Gadd45a*, as well as genes that encode the caspases, cyclins, transcription factors, and ubiquitin-associated factors that are involved in regulating the cell cycle and apoptosis. In humans, the rhythmic expression of several cyclins and the tumour-suppressor p53 are also regulated by the circadian clock (Bjarnason *et al.*, 1999). Moreover, the expression patterns of these clock-controlled genes are synchronized with the circadian oscillation patterns of *PER1* and *BMAL1* expression in the same tissue (Bjarnason *et al.*, 2001). The *Per2* gene may also play an important role in tumour suppression by inducing apoptotic cell death by enhanced pre-apoptotic signalling and attenuated anti-apoptotic processes (Hua *et al.*, 2006).

(ii) *Modulation of cell proliferation*

In addition to controlling the expression of cell-cycle genes and tumour-suppressor genes at the transcriptional and post-transcriptional levels, the core circadian genes are also involved directly in modulating the intracellular signalling pathways that regulate cell proliferation. It has been shown that a core circadian regulator, *CSNK1E* also functions in promoting cell proliferation by stabilizing β-catenin (Lee *et al.*, 2001). β-Catenin can interact with transcription factors of the T-cell-specific transcription factor/lymphoid enhancer factor-1 family to regulate transcription (van de Wetering *et al.*, 2002), and promote tumorigenesis (Morin, 1999).

(iii) *Control of the cell-cycle checkpoint*

Whether the circadian clock and the cell-cycle clock are connected *in vivo* was subject to debate until a recent study of a mouse model demonstrated that apoptosis induced by γ-radiation is dependent on circadian time in both wild-type and *Per2*-mutant thymocytes (Fu *et al.*, 2002). Specifically, when irradiated at the early stage of the active phase or at the early stage of resting phase, *Per2*-mutant thymocytes show a G2/M-specific resistance to radiation-induced apoptosis. Therefore, the circadian clock not only regulates the expression of cell-cycle genes but could also be involved in controlling cell-cycle checkpoint function.

(iv) *Response to DNA damage*

Studies from animal models have also shown that the core circadian genes can respond directly to γ-radiation. However, disruption of the *Per2* gene stops the response of all core circadian genes to γ-radiation (Fu *et al.*, 2002), suggesting that the molecular clock itself can be modulated by genotoxic stress in peripheral tissues. The ability of circadian genes to mediate the DNA-damage response seems to be cell autonomous, since *Per2*-mutant thymocytes have been shown to attenuate p53-induction in response to γ-radiation *in vitro*. *Per2*-mutant mice also show altered cell division, increased sensitivity to ionizing radiation with impaired DNA repair, and development of malignancies (Fu *et al.*, 2002; Matsuo *et al.*, 2003). Besides γ-radiation, it has also been shown that the clock genes can respond to low levels of ultraviolet irradiation in cultured human keratinocytes (Kawara *et al.*, 2002). *Per1* also plays an important role in regulating growth and DNA-damage control, and it interacts with proteins in the cell-cycle pathway (Gery *et al.*, 2006).

The findings that circadian genes are involved in cancer-related biological pathways such as cell-cycle control and DNA repair support the assumption that disturbances in circadian oscillator functions may increase the risk of carcinogenesis in a variety of tissues. Animal experiments also suggest the genetic circadian oscillator system may be involved in carcinogenesis. These reports have justifiably raised widespread health concerns.

(e) Loss of circadian gene functions in tumorigenesis

In animal models, mice with disruptions in the core circadian gene *Per2* have recently been shown to display salivary-gland hyperplasia and develop spontaneous lymphoma (Fu *et al.*, 2002). It is likely that deregulation of multiple molecular pathways contribute to the cancer-prone phenotype of the *Per2*-mutant mice. Deregulation of the *Myc*-mediated growth-regulatory pathway is proposed to be one possible mechanism by which disruption of the circadian clock could promote tumour formation. Zheng *et al.* (1999) constructed a mouse in which there was a deletion mutation in *Per2*, resulting in a shorter circadian period (~22 hours). Fu *et al.* (2002) reported that this mouse was also more susceptible to radiation-induced tumours, and showed reduced apoptosis in thymocytes. This mouse showed temporal deregulation of *cyclin D1*, *cyclin A*, and c-*Myc*. Moreover, the disruption of circadian rhythms in mice is associated with accelerated growth of malignant tumours, suggesting that the host circadian clock may play an important role in endogenous control of tumour progression (Filipski *et al.*, 2002).

In humans, direct evidence has demonstrated an association between the loss of human PERIOD (hPER1 and hPER2 and hPER3) function and human breast and human endometrial cancer. One study showed that ~95% of breast tumours (53 out of 55 specimens) displayed no or deregulated levels of PER1, PER2 or PER3 proteins in the breast tumour cells when compared to the adjacent normal tissue (Chen *et al.*, 2005). In another study, it was also observed that the loss of PER1 protein was common in human endometrial carcinoma but not in the adjacent normal cells (Yeh *et al.*, 2005). Chen *et al.* (2005) further suggested that the loss of clock-gene expression was linked to DNA-methylation of clock-gene promoters rather than genetic mutations of the clock genes (see paragraph below). A recent study also showed a downregulation of PER1 in human breast and lung cancer tissue (Gery *et al.*, 2006). It was suggested that altered PER expression, resulting in the disruption of normal circadian clock control, may benefit the survival of cancer cells and promote carcinogenesis.

(f) Epigenetic alterations of circadian genes in cancer tissue

The expression level of a gene can be dramatically influenced by the methylation status of its promoter region, and alterations in global methylation patterns have been associated with cancer development. Similarly, changes in circadian gene methylation patterns have also been observed in cancer tissue. One study revealed disturbances in the expression of the three period genes in over 95% of breast cancer cells when compared to non-cancerous cells (Chen *et al.*, 2005). Promoter methylation of *PER1*, *PER2*, and/or *CRY1* was detected in one-third of endometrial cancers compared to one-fifth of non-cancerous endometrial tissues (Shih *et al.*, 2006). The expression levels of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2* and *BMAL1* were also significantly impaired for those having chronic myeloid leukaemia, and the promoter region of *PER3* was found to be methylated in all of these leukaemia patients (Yang *et al.*, 2006). Microarray analysis also identified *PER1* as being expressed at lower levels in non-small cell lung cancer tissue compared to

normal tissue, and artificially induced expression of *PER1* in non-small cell lung cancer cell lines resulted in a significant reduction in growth. It is believed that DNA hypermethylation and histone H3 acetylation are potential mechanisms for this silencing of *PER1* expression (Gery *et al.*, 2007).

(g) Evidence from genetic association studies in humans

The human *PER*3 gene was the first circadian gene to be linked to increased risk of breast cancer in a genetic association study (Zhu *et al.*, 2005). The *PER*3 gene, which is a central component of the clockwork mechanism, contains a polymorphic repeat region comprised of four or five copies of a 54 bp repetitive sequence in exon 18. This length variation results in the insertion/deletion of 18 amino acids and has been found to be associated with delayed sleep phase syndrome and diurnal preference (Ebisawa *et al.*, 2001; Archer *et al.*, 2003). A case–control study found that the variant genotypes (heterozygous + homozygous 5-repeat alleles) were associated with an increased risk of breast cancer among premenopausal women (OR, 1.70; 95% CI: 1.00–3.0) (Zhu *et al.*, 2005).

NPAS2, the largest circadian gene, is a member of the basic helix–loop–helix-PAS class of transcription factors. *NPAS2* forms heterodimers with *BMAL1*, which transcriptionally activates *PER* and *CRY* expression, which are required for maintaining biological rhythms in many organisms (Vitaterna *et al.*, 1994; Bunger *et al.*, 2000). The *NPAS2* gene is very conserved, with only one missense mutation (SNP database accession No. rs2305160, *Ala394Thr* located in alternative exon 22), listed in the NCBI SNP database. A recent breast cancer case–control study found that women with the heterozygous *Ala394Thr* genotype had a significantly reduced breast cancer risk compared to those with homozygous *Ala394Ala* (OR, 0.61; 95%CI: 0.46–0.81) (Zhu *et al.*, 2008). Furthermore, this protective role was more evident in premenopausal women (OR, 0.44; 95%CI: 0.27–0.77) than in postmenopausal women (OR, 0.65; 95% CI: 0.49–0.91). This is the first piece of evidence demonstrating an association between *NPAS2* and human breast cancer.

The same polymorphism (rs2305160, *Ala394Thr*) in the *NPAS2* gene has also been genotyped in another population-based case–control study of non-Hodgkin lymphoma (Zhu *et al.*, 2007). These results demonstrated that risk of non-Hodgkin lymphoma was significantly reduced among individuals with the heterozygous Ala/Thr genotype (OR, 0.69; 95%CI: 0.53–0.90), the homozygous variant *Thr/Thr* genotype (OR, 0.55; 95%CI: 0.36–0.85), and both variant *Thr* genotypes combined (*Ala/Thr* & *Thr/Thr*) (OR, 0.66; 95%CI: 0.51–0.85), when compared to those with the homozygous *Ala/Ala* genotype. Similar reduced risks were detected for B-cell lymphoma and its two major subtypes: B-cell chronic lymphocytic leukaemia/prolymphocytic leukaemia/small lymphocytic lymphoma, and follicular lymphoma. Previous studies have shown that disruption of circadian rhythm may cause disordered immune responses such as aberrant immune cell trafficking and abnormal cell proliferation cycles (Mormont & Lévi, 1997; Vgontzas & Chrousos, 2002). Given the established association between immune

dysregulation and non-Hodgkin lymphoma (Filipovich *et al.*, 1992), the observed role of the circadian genes in lymphomagenesis could be explained by their impacts on immune activity.

(h) Expression of circadian genes

Mammalian circadian oscillators were originally believed to exist only in neurons of the SCN (Ralph *et al.*, 1990). However, with the identification of the mammalian clock and clock-controlled genes, this view has changed dramatically. Circadian oscillators have been uncovered in both central and peripheral tissues, with the SCN presumed to coordinate cyclic gene expression in the periphery by neural and/or humoral signals (Balsalobre *et al.*, 2000; Kramer *et al.*, 2001; Le Minh *et al.*, 2001; McNamara *et al.*, 2001; Cheng *et al.*, 2002). Robust daily oscillations in gene expression could be detected in almost all investigated tissues (Schibler *et al.*, 2003). These daily cycles were believed to be the result of cyclic humoral or neuronal signalling from the SCN. However, the autonomy of peripheral oscillators is now under debate as peripheral tissues explanted and maintained in culture demonstrate continued oscillation of *Per2* for up to 20 days, and SCN lesioning does not abolish this circadian oscillation (Yoo *et al.*, 2004). Moreover, the mechanisms of regulation of peripheral clocks and indeed, their function, remain largely obscure. Furthermore, disturbances in the expression of the three *PER* genes (*PER*1, *PER*2, and *PER*3) have been detected in human breast cancer cells in comparison with nearby non-cancerous cells (Chen *et al.*, 2005). Because the circadian clock controls expression of cell-cycle-related genes, it was suggested that altered *PER* gene expression may result in the disruption of the control of the normal circadian clock, hence benefit the survival of cancer cells, and promote carcinogenesis.

(i) Light exposure and circadian gene expression

Light is the most powerful circadian synchronizer among all environmental cues (Lucas *et al.*, 2001; Wright & Czeisler, 2002). The molecular mechanisms involved in synchronization to light have been demonstrated in previous experiments. For example, both *Per1* and *Per2* could be induced in SCN tissue by light exposure in mice (Albrecht *et al.*, 1997). In the SCN of wild-type mice, light exposure also evoked a transient interaction between Protein Kinase C α and Per2 proteins that affects Per2 stability and nucleocytoplasmic distribution (Jakubcakova *et al.*, 2007). Using oral mucosa samples, a recent study showed that the induction of human *PER*2 expression was stimulated by exposing subjects to 2 hours of light in the evening (Cajochen *et al.*, 2006). The increase in *PER*2 expression relative to a non-light control condition was statistically significant after exposure to light at 460 nm (blue), but not after exposure to light at 550 nm (green). The authors concluded that the non-image-forming visual system is involved in human circadian gene expression (Cajochen *et al.*, 2006).

(j) The human time structure and its alteration by phase shift

The temporal organization of the human body has to be understood to appreciate the impact of night work and shiftwork upon worker health and well-being. The human body has not only a structure in space, as expressed by its gross and microscopic anatomy, but it has a structure in time, as expressed by rhythms of numerous frequencies superimposed upon linear trends associated with development and aging (Touitou & Haus, 1992). The rhythmic variations encountered vary in period from milliseconds, e.g. in individual nerve cells, to minutes or hours (ultradian rhythms) to 24 hours (circadian rhythms), and to longer periods, such as the menstrual cycle in women, and yearly cycles (circannual rhythms) in both men and women (Haus & Touitou, 1992; Hildebrandt *et al.*, 1998).

Rhythms of a person, synchronized to diurnal activity by the ambient light–dark cycle and social routine, must undergo phase readjustment when forced to adhere to a new activity–sleep schedule due, for example, to night work or geographic displacement across several time zones. The central and peripheral oscillators will follow the new schedule, not immediately however, but over a certain number of transient cycles, to adapt to the changed phase of the environmental synchronizer. During this time of adaptation, disruption of the usual sequence and biological order of the numerous rhythmic events takes place with some clock genes responding faster than others. The result is an internal phase desynchronization within the oscillator mechanism (Sakamoto & Ishida, 2000; Nagano *et al.*, 2003; Nakamura *et al.*, 2005). The circadian oscillators in the anterior region of the SCN undergo a faster time adaptation than those in the posterior portion (Nagano *et al.*, 2003; Nakamura *et al.*, 2005). During the re-adjustment period, desynchronization occurs within the oscillators as well as among different oscillatory tissues and brain regions that re-adjust their phases at different rates (Stokkan *et al.*, 2001; Abe *et al.*, 2002; Nagano *et al.*, 2003). Within the oscillators, after a shift in the light–dark regimen, there is a faster shift of *Per1* and *Per2* oscillator genes and a slower shift of *Cry1*, another component of the oscillator mechanism (Reddy *et al.*, 2002). In the molecular oscillator mechanism, as in the organism as a whole, there is a difference in response with different directions of the phase shift. Phase advances (earlier timing) of the lighting schedule lead to a more prolonged desynchronization within the SCN than do phase delays (Nakamura *et al.*, 2005). Also *Per1* and *Per2* genes have been found to behave differently during advancing and delaying phase shifts (Yan & Silver, 2002; Albrecht *et al.*, 1997). Moreover, the phase-shifting kinetics of circadian rhythms in transcriptional activity show region-specific differences (Nagano *et al.*, 2003; Nakamura *et al.*, 2005), with different tissues exhibiting different resetting behaviour than the SCN or behavioural rhythms (Abe *et al.*, 2002). The adaptation of the peripheral oscillators is independent – in part – of the hypothalamic control (Stokkan *et al.*, 2001). Thus, during the phase-resetting process, internal desynchronization is manifested within the individual oscillators and simultaneously also between central and peripheral oscillators. In the absence of hypothalamic control and synchronization, peripheral oscillators of diverse tissues cycle with different periods; thus, during the process of adaptation, they express

different phases and changed phase relations. The unique circadian phase and period values expressed by each tissue suggest that the quantitative properties of the circadian oscillators in each tissue are unique and tissue-specific (Yoo *et al.*, 2004) and/or may be the expression of different synchronizing mechanisms acting upon different tissue oscillators (Lakatua *et al.*, 1975, 1983, 1988). The overall effect of a phase shift of this nature is alteration at several hierarchical levels of the internal time organization during the transitional duration of adjustment. For example, the top physical efficiency that is typically observed in the afternoon becomes delayed into the night time. The propensity to sleep, which is the expression also of a circadian rhythm, may be high during the environmental time that requires alertness and efficiency, and it may be low during the time reserved for rest, resulting in insomnia and non-restorative sleep. During adaptation, this external and internal desynchronization of the human organism leads to a functional disturbance of the time organization ("dyschronism"), with loss in performance efficiency plus the expression of a set of symptoms, similar to those of jet lag (Hildebrandt *et al.*, 1974; Harris, 1977; Ribak *et al.*, 1983; Folkard & Akerstedt, 2004; Folkard & Lombardi, 2004).

In this context, it is important to understand that a circadian phase shift: (1) affects all metabolizing and proliferating cells in the organism; (2) leads to transient internal desynchronization on a molecular basis within the individual cellular oscillators; (3) results in desynchronization among the cellular oscillators in the SCN and peripheral tissues; (4) is not immediate but requires time (days) for complete adjustment, occurring over several transient cycles; and (5) varies by variable and function in the amount of time required for phase adaptation and, with regard to cell and tissue proliferation, may extend over several weeks' time.

A circadian phase shift exerts its effects upon molecular cell and tissue physiology and occurs over an extended period during which the time sequence of the biological rhythms of many variables is different from that found in day–night-adapted individuals, i.e. the circadian time organization which is thought to be linked to optimal function (Touitou & Haus, 1992, Winget *et al.*, 1992; Monk, 1992; Mormont & Waterhouse, 2002). Changes in the neuroendocrine web, controlling cell and tissue proliferation during the internally desynchronized span of phase adaptation, may permit or even promote growth of abnormal cell proliferation in target tissues that find themselves out of phase with their usual controlling influences.

(k) Summary

Exposure to artificial light during the night may disrupt circadian gene function in the SCN, which in turn may alter circadian-regulated biological pathways, such as cell-cycle regulation and DNA repair. The impact of artificial light on the circadian pacemaker might be modified by genetic variants of the core circadian genes, although such gene–environment interactions have yet to be explored. Given the roles of circadian genes in tumorigenesis, the light-mediated dysfunction of circadian genes may provide a possible

mechanism for the putative carcinogenic effect of light, which may or may not involve melatonin.

4.2.3 *Melatonin as part of the neuroendocrine immune axis*

Pineal melatonin plays an important part in the neuro-immune-endocrine web regulating mammalian immune defenses. Immune functions in different species of mammals, including man, show circadian and seasonal variations with enhancement during short days, which correlates with the prolonged duration of the daily secretion of melatonin (Nelson & Drazen 2000).

However, in addition to the circadian and seasonally periodic pineal melatonin, the recent observations on the synthesis of melatonin by immune-competent cells in different parts of the immune system suggest a role for locally produced melatonin in the regulation of the immune response. The presence of melatonin and the mechanisms for melatonin-synthesis in many peripheral tissues raises questions about the function of the peripherally produced and/or stored melatonin. There are questions about potential release of this peripheral melatonin into circulation and about its potential participation in circadian system regulation. The relationship of the peripherally formed melatonin to the circadian timekeeping system and its disruptions has not been widely explored, but will be of interest since the same immune-competent cells carry the circadian oscillator genes, and are subject to multifrequency time regulation.

(a) Observations in animals

(i) *Pinealectomy – surgical and functional*

Surgical and functional pinealectomy by continuous bright light exposure led to abnormal development of the immune organs of mammals and birds (Vaughan *et al.*, 1987; Janković *et al.*, 1994). Impairment of different aspects of the immune response after pinealectomy was reported in rats (Liebmann *et al.*, 1996; Molinero *et al.*, 2000; Beskonakli *et al.*, 2001), in mice (Maestroni *et al.*, 1986; del Gobbo *et al.*, 1989; Vermeulen *et al.*, 1993; Mocchegiani *et al.*, 1996;), other rodents (Haldar *et al.*, 2001), and birds (Rosołowska-Huszcz *et al.*, 1991; Moore *et al.*, 2002; Moore & Siopes, 2003). These defects in immune function could be reversed by the administration of melatonin.

In studies on male Wister rats, constant light which suppresses pineal function and melatonin production induced a 30% depression of the phagocytic ability of blood neutrophils throughout the whole 24-hour cycle without altering its circadian oscillations. It was deduced that the daily dark span serves as synchronizer, and the rhythmic melatonin secretion is involved in the maintenance of the level of phagocytosis and the timing of its circadian rhythm, but does not cause the circadian oscillation as such (Hriscu *et al.*, 2002).

Pharmacological inhibition of melatonin synthesis in mice by the β-receptor antagonist propanolol was shown to be associated with suppressed humoral and cellular

immunological responses (Liebmann *et al.*, 1996). Given before onset of the daily dark span, propanolol markedly decreased primary and secondary antibody formation in Balb/c mice injected with sheep red blood cells (Maestroni *et al.*, 1986; Maestroni & Conti, 1996).

(ii) *Relation to length of daily photoperiod*

The relationship between the photoperiod and various aspects of immune function is stronger with short day lengths (light spans) in diurnal as well as in nocturnal species (Nelson, 2004). There is a correlation between the elevated night time (dark span) melatonin concentrations with the number and response of immunocompetent cells in humans, and in several rodent species (Giordano *et al.*, 1993; Haldar *et al.*, 2001; Prendergast *et al.*, 2003).

(b) Melatonin receptors in immune-competent cells

(i) *Observations in animals*

Immune-competent cells, including splenocytes, lymphocytes and monocytes carry membrane-bound and nuclear receptors. The membrane-bound MT1 high affinity receptor is coupled to G-protein. The lower affinity MT2 receptor binding sites are not bound to G-protein and have a different pharmacological profile. The melatonin actions in the immune system are mainly mediated by the MT1 receptor. The nuclear receptors found belong to the retinoid-related (retinoid Z receptor/retinoid-related orphan receptors) superfamily of nuclear receptors (Dubocovich, 1995; Dubocovich & Markowska, 2005; Nosjean *et al.*, 2000).

(ii) *Observations in humans*

Specific membrane and nuclear receptors were found in peripheral blood lymphocytes and monocytes (Lopez-Gonzalez *et al.*, 1992; Pozo *et al.*, 2004). The Kd values of these receptors suggest that they can recognize physiological concentrations of melatonin in circulating blood at night and endogenous melatonin generated locally by the immune system (Carrillo-Vico *et al.*, 2005).

4.2.4 *The immunomodulatory response to exogenous melatonin*

(a) Observations in animals

Melatonin administration in animals leads to immuno-enhancement at several levels of the immune system ,and in several immune-system-related functions. These actions of melatonin are most pronounced when the animal's immune system is suppressed, e.g. by light exposure or by corticosteroid suppression (Haldar *et al.*, 2004).

Melatonin administration increased the proliferative capacity of mouse splenocytes (Demas & Nelson, 1998) and rat lymphocytes (Martins *et al.*, 1998), and led to an increase in tissue mass of thymus and spleen (Vaughan & Reiter, 1971; Vaughan *et al.*,

1987; Rai & Haldar, 2003). The enhancement by melatonin of mouse splenocytes in response to the T-cell mitogen concanavalin A was blocked by the administration of luzindole, a high-affinity melatonin receptor antagonist. Luzindole also reduced the ability of splenocytes to proliferate during the daily dark span (night) when endogenous melatonin concentrations are naturally high. This effect was not observed during daytime (light span) when melatonin concentrations are low (Drazen *et al.*, 2001). The authors suggested that melatonin enhancement of splenocyte proliferation was mediated directly by melatonin receptors on splenocytes and also that the circadian rhythm in splenocyte proliferation was mediated by splenic melatonin receptors (Drazen *et al.*, 2001). Melatonin also acts upon the non-specific immune response and leads to an increase in the number of natural killer (NK) cells and monocytes in the bone marrow (Currier *et al.*, 2000), and enhances the antibody-dependent cellular cytotoxicity (Giordano *et al.*, 1993).

Melatonin *in vivo* modulates several cytokines active in immune responses via the regulation of their gene expression and production. Among those that are in mice in splenic and/or peritoneal macrophages: the production of tumour necrosis factor α (TNFα), interleukin-1 (IL-1), major histocompatibility complex-II (MHC-II), macrophage-colony stimulating factor (M-CSF), transforming growth factor β (TGFβ), interferon gamma (IFNγ) and IL-10 (Pioli *et al.*, 1993; Liu *et al.*, 2001; Raghavendra *et al.*, 2001a,b). In rats, melatonin increases the generation of thymosin-α1 via an increase in pro-thymosin-α gene expression (Molinero *et al.*, 2000). Interaction between the effects of melatonin upon the immune system with the opiatergic system have been suggested, as in some studies, naltrexone, a specific opioid antagonist, prevented the immune-stimulatory effects of melatonin. Similar effects were observed with the administration of β-endorphin and dynorphin (Maestroni *et al.*, 1988a, Maestroni, 2001).

(b) Observations in humans

In humans, exogenous melatonin acts upon NK-cell activity (Lissoni *et al.*, 1986) in a biphasic pattern. In diurnally active subjects, resting during the night, melatonin given in the afternoon (15:00) led to a stimulation of NK-cell activity during the first 4 hours, followed by a phase of apparent inhibition, suggesting an ultradian periodicity.

4.2.5 *Retinal/pineal/hypothalamic/pituitary/adrenal interaction*

The relation between the hypothalamic-pituitary-adrenal (Hth-Pit-Adr) axis and the retinal-hypothalamic-pineal (Ret-Hth-Pin) axis are characterized by variables that are rhythmic in several frequencies in each one of these organs (for a review, see Haus, 2007). The optimal function of the mammalian organism depends upon certain time relations between rhythmic variables. In animal experiments, the adrenocorticotropic hormone (ACTH) will elicit a strong adrenal response if given at certain stages of the circadian adrenal cycle in responsiveness when activation of the gland is expected. The response will be substantially less when it is given at other circadian stages both *in vivo* (Haus, 1964) and *in vitro* (Ungar & Halberg, 1962; Sánchez de la Peña, 1993). In this

regard, the extent of the response of the Hth-Pit-Adr axis to stress (e.g. handling of an animal or saline injection) may be out of phase with the activation of the adrenal gland directly by ACTH due to temporal differences in the cycles of responsiveness of the adrenal and of superimposed controls (Haus, 1964). The rhythmic interactions among hormonal stimuli, rhythmic receptor activity, and target organ response imply that a study of an endocrine and neuroendocrine interaction at a certain (single) time represents a snapshot characterizing only that certain time point. Furthermore, the interactions between the variables studied may be quite different quantitatively and even qualitatively a few hours earlier or later. This is the case in environmentally synchronized organisms and much more so during a phase shift of the organism when different variables have a different time course in phase adaptation (Haus & Halberg, 1969; Fève-Montange *et al.*, 1981). The rhythmicity in the variables studied without a chronobiological experimental design has led to many publications that are difficult to interpret or even contradictory. Studies without such an experimental design have to be qualified as valid only for that specific time and constellation of rhythms of the variables studied.

(a) Melatonin receptors in human central nervous system and pituitary

The MT1 melatonin receptor is widely distributed in the human hypothalamus and pituitary. In addition to the SCN, MT1 immunoreactivity was found in numerous sites including the paraventricular nucleus, periventricular nucleus, supraoptic nucleus, and others. The MT1 receptor was colocalized with some vasopressin neurons in the SCN, and with vasopressin and oxytocin neurons in the paraventricular nucleus, and with parvocellular corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus (Wu *et al.*, 2006).

The colocalization of MT1 and CRH suggest that melatonin might directly modulate the Hth-Pit-Adr axis in the paraventricular nucleus suggesting cross-modulation between the systems at the hypothalamic level, which may have implications for stress reactions and other conditions (Wu *et al.*, 2006).

In the pituitary, strong MT1 expression was observed in the *pars tuberalis* while only weak staining was found in the anterior and posterior pituitary (Wu *et al.*, 2006).

(b) Animal studies on pineal-adrenal interaction

Both the *N*-acetyltransferase activity in the pineal glandwhich is the rate limiting step in melatonin synthesis, as well as adrenal activity are under sympathetic control (Buijs *et al.*, 1999). The rat pineal gland expresses glucocorticoid receptors in a density comparable to the liver (Ballard *et al.*, 1974; Ferreira *et al.*, 2005). The receptors are functional and participate in glucocorticoid-induced effects upon the pineal, which are blocked by the high-affinity glucocorticoid receptor antagonist mifepristone (Gagne *et al.*, 1985). Melatonin binding sites have been identified in the adrenal in rats (Persengiev, 1992), suggesting the existence of a direct interaction between pineal melatonin and adrenal cortex steroid production (Persengiev & Kanchev, 1991).

Glucocorticoids *in vitro* were reported to decrease the norepinephrine-stimulated melatonin secretion in the rat pineal gland (Fève-Montange & Abou-Samra 1983), and to participate in lowering pineal *N*-acetyltransferase activity and melatonin production during stress (Joshi *et al.*, 1986).

In perifusion studies on adult rat pineal glands, corticosterone and dexamethasone, but not deoxycorticosterone, decreased melatonin production in pharmacological doses. Lower concentrations had no effect, regardless of the circadian stage (Zhao & Touitou, 1993). Torres-Farfan *et al.* (2003) studied MT1 melatonin receptors in the adrenal glands of the capuchin monkey, and through sampling at a single time point found inhibition of ACTH-stimulated cortisol production by melatonin. This effect was reversed by the MT1/MT2 antagonist luzindole.

In studying the interaction of the pineal gland with pituitary and adrenal glands *in vitro*, Sánchez de la Peña *et al.* (1983a,b) found in a chronobiological study design that the effect of aqueous pineal gland extracts and of melatonin upon production of corticosterone in the mouse adrenal gland did depend critically upon the circadian stage in which the adrenal glands were harvested. In LD12:12-synchronized B6D2F1 mice, pineal gland extracts harvested at one circadian stage and ACTH 1–17, (an analogue of the natural ACTH pituitary hormone), added *in vitro* to adrenal glands harvested at six different circadian stages showed that, depending upon the circadian stage, when the adrenal glands were obtained, the pineal gland extract or melatonin either stimulated or inhibited or had no effect on adrenal corticosterone production stimulated by ACTH 1–17 (Sánchez de la Peña *et al.*, 1983a, b). The effect of the pineal gland extract or melatonin did depend upon the circadian cycle of responsiveness of the target organ both quantitatively and in direction. The circadian-stage-dependent effect of the pineal gland on the adrenalgland may explain many of the controversial results reported in the literature.

The adrenal androgen dehydroepiandrosterone sulfate (DHEA-S) stimulated melatonin production and secretion by 50–80% in perifused isoproterenol-stimulated rat pineal glands which had been removed during the light phase, while in pineals obtained during the dark span, only the highest doses of DHEA-S increased melatonin secretion, and by only 25% (San Martin & Touitou, 2000). No such effect was observed from dehydroepiandrosterone (DHEA) which is also secreted by the adrenal gland.

In in-vivo studies, a direct inter-relation between the pineal gland and the Hth-Pit-Adr axis could be shown in some models but not in others. DHEA-S, given as single injection in pharmacological doses (500 μg), induced a significant increase in nocturnal pineal gland melatonin content and an increase in *N*-acetyltransferase in Wistar rats, both young and old. DHEA-S or DHEA at lower doses (50 and 250 μg administered acutely) and at doses of 100 μg administered daily over 8 days had no effect (Djeridane & Touitou, 2004). In a strain of mice with the enzymatic mechanisms to produce DHEA, melatonin stimulated DHEA production in ex-vivo adrenal incubates at all stages of the circadian rhythm (Haus *et al.*, 1996). The administration of tryptophan in rats caused a marked rise

in plasma melatonin but had no effect upon corticosterone concentrations (Hajak *et al.*, 1997).

The acute and longer-lasting exposure of rats to stress led to a significant rise in adrenal corticosterone secretion but had no effect upon circulating melatonin levels (Hajak *et al.*, 1997). Some in-vivo models of stress which increase corticosterone secretion such as immobilization (Lynch *et al.*, 1977), forced swimming (Wu *et al.*, 1988) and insulin-induced hypoglycaemia (Tannenbaum *et al.*, 1987) did increase daytime levels of melatonin, and attenuate nocturnal light-pulse inhibition in melatonin synthesis (Funk & Amir, 1999).

In a model of chronic inflammation in rats exposed to Bacillus Calmette-Guérin (BCG), Ferreira *et al.* (2005) reported in Wistar rats kept on an LD12:12 lighting regimen and sampled at the early part of the light span (09:00–11:00) that corticosterone potentiated noradrenaline-induced melatonin and *N*-acetylserotonin production in pineal organ culture in a bell-shaped curve through the action of the glucocorticoid receptor. Glucocorticoids exerted a positive control on the secretion of melatonin by the pineal gland in animals undergoing a chronic inflammation process (Ferreira *et al.*, 2005).

When mice were exposed to competing synchronizers (e.g. light versus time-limited meal feeding), the circadian rhythm in corticosteroids tended to counteract the internal desynchronization between central and peripheral oscillators, and tended to stabilize the internal circadian time organization (Le Minh *et al.*, 2001).

In the more complex models, often both the Ret–Hth–Pin and the Hth–Pit–Adr axes react concomitantly. Inconsistencies and controversial results reported in the literature often may be due to the variable constellations seen in one- or two-point snapshots of two or more high-amplitude rhythmic systems.

(c) Human studies on pineal-adrenal interaction

Glucocorticoid secretion was not modified by either acute or chronic melatonin administration in close to physiological doses (Wright *et al.*, 1986; Waldhauser *et al.*, 1987). No correlation was found between the nocturnal urinary excretion of melatonin and cortisol, either among healthy subjects or among patients with panic disorder (with increased excretion of cortisol) or in insomnia patients (with a high incidence of low melatonin secretion) (Hajak *et al.*, 1997).

The circadian rhythms of cortisol and melatonin are related in their timing within the framework of the day–night-synchronized human time organization with activity during the day, and in free-running blind subjects (Skene *et al.*, 1999). The plasma melatonin concentration begins to rise (melatonin onset) when the cortisol concentration is at its lowest, and peaks when the cortisol concentration begins to rise, it then begins to drop (melatonin offset) when the cortisol concentration reaches its peak (Arendt, 1988; Rivest *et al.*, 1989). In case of a rapid phase shift in shiftwork or travel over time zones, melatonin rapidly follows the light–dark and sleep–wakefulness pattern while cortisol

phase-shifts only slowly over a large number of transient cycles (Fève-Montange *et al.*, 1981).

In clinically healthy men, in samples collected every 30 minutes over 24 hours, the ultradian rhythms of cortisol and melatonin followed ultradian periods of about 8 hours and 5.5 hours, respectively (Rivest *et al.*, 1989), suggesting an intrinsic difference in the mechanism controlling their secretion. Similarly, the cortisol and melatonin response to 24 hours of complete bed rest under dim light was also different.

Cagnacci *et al.* (1995b) administered a high pharmacological dose of melatonin (100 mg) or placebo at 08:00 on two consecutive days to a group of young women (22–32 year of age) in early follicular phase and a group of postmenopausal women (54–62 years of age), and observed gender and age differences in melatonin and cortisol blood concentrations over 48 hours. The postmenopausal group had higher cortisol concentrations than the young group during the daytime (especially at lunch time and early in the evening).

In pituitary- and adrenal-dependent Cushing syndrome with hypercortisolemia patients, the circadian rhythm of melatonin was abolished, and the nocturnal melatonin levels and the integrated 24-hour secretion were significantly lower than in controls (Werner *et al.*, 1981; Soszyński *et al.*, 1989).

In human subjects, melatonin concentrations were markedly reduced after the administration of a low dose (1 mg) of dexamethasone at 22:00 (Beck-Friis *et al.*, 1985). Similar results were reported by Demisch *et al.* (1988). However, a higher dose of dexamethasone (4 mg given during 1 day in a dosage of 1 mg orally at 08:00, 12:00, 18:00 and 00:00) had no significant effect on melatonin concentration (Beck-Friis *et al.*, 1983).

The inhibition of cortisol synthesis with the use of metyrapone resulted in an increase in melatonin urinary excretion (Brismar *et al.*, 1985). No significant difference in cortisol was found during a propanolol-induced decrease in melatonin (Beck-Friis *et al.*, 1983, 1984).

The Hth–Pit–Adr and the Ret–Hth–Pin axes are two major branches of the human time-keeping system and provide time information to peripheral tissues. The two axes behave differently during phase shift and phase adaptation. A direct interaction is suggested by the presence of functional receptors for melatonin in the central nervous system, the pituitary gland, and the adrenal gland. However, these interactions, if they are truly functional, may vary with the circadian stage and the responsiveness of the target tissues. At the time of writing, a consistent relationship has not yet been established and many of the studies reported suffer from a lack of a chronobiological study design, which may be required to obtain meaningful results.

4.2.6 *Melatonin and the neuroendocrine reproductive axis*

Melatonin, as the messenger of darkness in diurnally and nocturnally active species, plays a major role in directing the activity of the reproductive system. It provides input on

the length of the daily dark span, thus indicates the season to the species' neuroendocrine system in the parts of the northern and southern hemisphere where seasonal changes in luminosity occur.

The majority of mammals are seasonal breeders. The role of the pineal gland and of melatonin in controlling mammalian reproduction among seasonal breeders is now well established (e.g. Goldman, 2001). There are differences among different species in the mechanisms elicited by melatonin stimulus, e.g., in the hamster, the reproductive system is inhibited by short photoperiods with a prolonged melatonin signal leading to an anestrous effect in females, and testicular regression in males (Hoffman, 1973; Carter *et al.*, 1982). The anti-gonadal effect of the short photoperiod is prevented by pinealectomy (Reiter 1972, 1980) while gonadal inhibition can be achieved even during long days by the daily injection of melatonin (Stetson *et al.*, 1976). In species like the ewe, the mechanism is different with suppression of the estrous cycle during spring and summer with fertility resumed during autumn and winter (Goldman, 1999, 2001). In this species, there is a different response to the prolonged melatonin signal, leading to a release of gonadotropin (Bittman *et al.*, 1983). Interspecies differences have to be kept in mind if the results of animal experiments are to be applied to humans.

The melatonin effects on the reproductive system in humans and animals appear to take place at several locations, and appear to be receptor-mediated. Melatonin has been shown to exert its reproductive effects at the level of the central nervous system where the presence of melatonin receptors and responsive neurons has been widely demonstrated. Melatonin directly inhibits hypothalamic GnRH pulses (Bittman *et al.*, 1983), and suppresses the pituitary response to GnRH (Martin *et al.*, 1980). However, peripheral actions at the level of the gonads have also been reported. The ovary in experimental animals and humans takes up circulating [3H]melatonin more effectively than most other tissues (Wurtman *et al.*, 1964; Cohen *et al.*, 1978).

(a) Central mechanisms in animal studies

Melatonin exerts its regulatory effects on the reproductive axis predominantly through actions upon the central nervous system (e.g. Glass & Lynch 1981; Lawson *et al.*, 1992; Goldman, 2001). Melatonin binding sites have been demonstrated in numerous areas in the hypothalamus, which are involved in reproductive functions (Migaud *et al.*, 2005; Vaněcek, 1988; Weaver *et al.*, 1989) together with the *pars tuberalis* of the pituitary gland (Morgan, 2000). In sheep, the pre-mammillary hypothalamus was identified as the site where melatonin regulates seasonal reproduction (Malpaux *et al.*, 2001; Lincoln, 2002). In times of reproductive quiescence induced by short photoperiods or melatonin treatment, the pituitary gland remains responsive to exogenous GnRH (Robinson *et al.*, 1986) favouring a mechanism located in the hypothalamus rather than the pituitary gland. Animal experiments have shown that the action of melatonin may be mediated by way of regulating gonadotropin release through effects upon hypothalamic monoamines and GnRH (Martin & Sattler, 1982; Arendt, 1986; Reiter, 1991), and by action at the level of the pituitary gland through cAMP- and Ca2+-dependent mechanisms leading to inhibition

of the pituitary response to GnRH (Martin & Klein, 1976; Vanecek, 1995; Vanecek & Klein, 1995). Control of seasonal prolactin operates via the MT1 receptors of the *pars tuberalis* (Lincoln, 2002, 2006a, b; Lincoln *et al.*, 2003).

In the Syrian hamster, neurons expressing melatonin receptors in the dorsomedial nucleus of the hypothalamus are implicated in the regulation of gonadotropin secretion and gonadal activity (Maywood *et al.*, 1996). In LSH/SsLaK female hamsters, melatonin treatment given approximately one hour before light-off in an L14:D8 regimen decreased significantly the weight of the uterine and pituitary glands, FSH, LH, and prolactin (Lawson *et al.*, 1992). In ovariectomized virgin animals of the same strain, melatonin (25 μg/day subcutaneously) given at the same circadian phase reduced the number of cells expressing estrogen receptor immunoreactivity in the medial preoptic area (Lawson *et al.*, 1992).

At the level of the pituitary gland, melatonin acts in sheep and other photoperiodic animals via MT1 receptors in the *pars tuberalis* to control seasonal prolactin secretion (Morgan, 2000; Lincoln & Clarke, 1994; Hazlerigg *et al.*, 2001). In the *pars tuberalis*, it appears that circadian clock genes provide a molecular mechanism by which melatonin duration is decoded (Lincoln *et al.*, 2002; Lincoln, 2006a). The ovine *pars tuberalis* expresses the core clock genes with a 24-hour rhythm in mRNA levels distinct for each gene and different in timing and amplitude from the clock-gene profiles of the SCN (Lincoln *et al.*, 2002; Hazlerigg *et al.*, 2004). In the *pars tuberalis*, but not in the SCN, the timing of the clock gene rhythms is markedly modulated by photoperiod and manipulation of melatonin (Hazlerigg *et al.*, 2004; Johnston *et al.*, 2006). *Cry1* is controlled in sheep and also in rodents via the MT1 receptor (Hazlerigg *et al.*, 2004; von Gall *et al.*, 2005; Johnston *et al.*, 2005, 2006) with a low amplitude circadian rhythm remaining after melatonin suppression by exposing animals to constant light (Johnston *et al.*, 2006). Under constant light conditions, melatonin was effective at all times in activating *Cry1* expression, but suppressed RNA levels for the other clock genes measured (*Bmal1*, *Per1*, *Per2*, *Rev-erbα*) only at the times when endogenous gene expression was increased (Johnston *et al.*, 2006). A phase-dependence of melatonin action upon the stage of the endogenous rhythms at the level of the target organ may explain many controversial resultsMelatonin onset at dusk activates *Cry1* gene expression (the dusk oscillator) and melatonin offset at dawn activates *Per1* gene expression (the dawn oscillator), and the interval between these events corresponds to the night length, and thus varies with the seasons. The *Per/Cry* interval dictates the level of Per/Cry protein complexes in the *pars tuberalis* cell nucleus achieved during each circadian cycle, and governs the functional output of the *pars tuberalis* (Lincoln, 2006a, b).

(b) Interactions of estrogen with melatonin at the level of the target organs in animal studies

Estrogens stimulate the growth of ER+ breast cancer cells by stimulating the transcription of cell-cycle progression genes, and downregulating the expression of genes that block cell-cycle progression (Métivier *et al.*, 2003; Stossi *et al.*, 2006). Chromatin

remodelling mediated by the estrogen receptor α (Erα) has been suggested as constituting an essential part of mammary tumorigenesis (Sahar & Sassone-Corsi, 2007). Cyclin D1 stimulates mammary growth and in its overexpression leads to mammary tumorigenesis associated with Erα, and enhances its activity by antagonizing the repressor *BRCA1* (Wang *et al.*, 2005). Since cyclin D1 is under clock control (Fu *et al.*, 2002), a direct relation between cell proliferation and circadian regulation or dysregulation may play a role in mammary gland cell proliferation and carcinogenesis. Also *CLOCK* and other linked circadian regulators appear to play a role in cell-cycle regulation, and DNA repair. Actions of *CLOCK* in its enzymatic functions as enzyme histone acetyletransferase may be involved in chromatin remodelling in response to estrogens in a circadian manner (Sahar & Sassone-Corsi, 2007), and in the case of disrupted circadian rhythms, may lead to alterations in cell proliferation and cancer.

In addition to its central regulatory functions, melatonin has been shown to interfere with the proliferation of human breast cancer cells *in vitro* (Blask & Hill, 1986; Hill & Blask, 1988). The local inhibitory and anti-estrogenic effects of melatonin have been studied largely in human breast cancer cells of the ER+ and estrogen responsive MCF-7 cell line. Melatonin was shown to downregulate the ER expression in MCF-7 cells, and its anti-proliferative effects appeared to be mediated through the estrogen response pathway (Hill *et al.*, 1992). The melatonin anti-proliferation effect upon breast cancer cells is limited to ERα+ MCF-7 cells and is not found in ERα– (MDA-MB-231) breast cancer cells (Hill *et al.*, 1992). There is substantial literature supportive of an anti-proliferative action of melatonin in physiological concentrations corresponding to peak night time and daytime serum values found in humans, which directly inhibit the MCF-7 cell line *in vitro* (Blask & Hill, 1986; Hill *et al.*, 1992; Hill & Blask, 1988; Cos & Sánchez-Barceló, 1995). In a recent study, it was shown in a human breast cancer xenograft rodent model that melatonin-rich human blood obtained during night time reduced tumour proliferation while melatonin-depleted blood obtained during daytime or following exposure to bright polychromatic light at night enhanced human breast cancer xenograft proliferative activity (Blask *et al.*, 2005b).

With regard to mechanisms, melatonin suppresses both ERα protein and *ERα* RNA in a time- and dose-dependent manner (Molis *et al.*, 1994) but does not compete with E2 for binding to the ERα (Molis *et al.*, 1994). In MCF-7 cells, melatonin pretreatment significantly reduced E2-induced *ERα* transactivation and ERα estrogen-responsive-element-binding activity (Kiefer *et al.*, 2002). Melatonin also inhibited the E2-induced elevation of cAMP levels; melatonin, in this model, acting as biological modifier to affect ERα transcriptional activity (Kiefer *et al.*, 2002).

(c) Prenatal exposure to melatonin in animals

Throughout fetal development, expression of the melatonin receptor exhibits considerable plasticity. During early stages of development, melatonin receptors are transiently expressed in multiple neural and endocrine tissues (Davis, 1997). Expression of MT1 receptors is subject to developmental and circadian control, which may modulate

the physiological actions of melatonin. In studies with cloned regions of the ovine *MT1* promoter and studies of the rat promoter, Johnston *et al.* (2007) suggested a model in which the melatonin expression in the mammalian pituitary gland during development is determined by the changing balance between stimulating and inhibiting transcription factors. In these studies, the authors also suggested that the circadian variation in *MT1* gene expression does not depend upon the direct action of circadian clock genes (Johnston *et al.*, 2003a,b, 2006).

In rats, the maternal pineal gland and melatonin (which passes the placental barrier) are necessary for normal sexual maturation. Prenatal melatonin treatment was shown to produce delayed sexual maturation (Díaz López *et al.*, 2005), and hyperprolactinemia in 30-days-old offspring. Melatonin treatment during pregnancy was shown to influence the ontogeny of the hypothalamus–pituitary–gonadal (Hth–Pit–Gnd) axis that begins during intrauterine life, and leads to alterations in gonadotropin and prolactin secretion of both female and male rats during sexual development (Díaz López *et al.*, 2005). The feedback of E2 on LH secretion by the pituitary gland was altered in the female offspring exposed to melatonin in utero, resulting in precocious initiation of puberty. In the male offspring, both the LH and FSH feedback mechanism were delayed. Modification of the fetal endocrine environment caused by prenatal melatonin administration induced changes in the sensitivity of gonadotropin regulation and the prolactin feedback response to exogenous androgens indicative of a delayed sexual development of the male offspring (Díaz López *et al.*, 2005). Increased exposure to melatonin during intrauterine life resulted in an inhibitory effect on postnatal androgen biosynthesis (Díaz Rodríguez *et al.*, 1999). Both maternal pinealectomy and melatonin treatment led to alterations of oocyte development in the female offspring (Fernández *et al.*, 1995).

In the fetus of mother capuchin monkeys (90% of gestation), the MT1 receptor and the clock genes *Bmal1*, *Per2*, *Cry2* and *Clock* showed circadian changes in the SCN and adrenal gland, and a rhythm of DHEA-S concentration was found in plasma (Torres-Farfan *et al.*, 2006). Maternal melatonin suppression by a constant light exposure changed the expression of *BMAL1*, *Per2* and MT1 in the fetal SCN. These effects were reversed by maternal melatonin replacement. In contrast to the SCN, maternal melatonin suppression nor its replacement had an effect on the clock genes or MT1 expression in the fetal adrenal gland or the circadian rhythm of fetal plasma DHEA-S. The authors suggested that maternal melatonin is a zeitgeber (synchronizer) for the fetal SCN but probably not for the adrenal gland (Torres-Farfan *et al.*, 2006).

(d) Melatonin effects at the level of the ovary in human studies

The uptake of melatonin in animal and human ovarian tissue has been reported (Wurtman *et al.*, 1964; Cohen *et al.*, 1978). High levels of melatonin which undergo circadian and seasonal variations are foundin human pre-ovulatory follicular fluid (Yie *et al.*, 1995a; Rönnberg *et al.*, 1990). In human granulosa-luteal cells, melatonin binding sites have been detected (Yie *et al.*, 1995b), and a stimulation of progesterone production by melatonin has been shown (Brzezinski *et al.*, 1992; Webley & Luck, 1986). Several

forms of melatonin receptor genes are expressed in human granulosa-luteal cells (Niles *et al.*, 1999). Woo *et al.* (2001) identified the melatonin receptor subtypes MT1-R and MT2-R. Cloning and sequence analysis revealed that these receptors were identical to their brain counterparts. Treatment of these cells with melatonin significantly increased the *LH* receptor mRNA levels without any effect on the expression of the FSH receptor gene. After melatonin treatment, both GnRH and *GnRH* receptor mRNA were significantly decreased to 61% and 45% of control levels, respectively. In the same study, melatonin itself had no effect upon basal progesterone production, but enhanced human chorionic gonadotropin (hCG) stimulated progesterone production. There appeared to be a complex receptor-mediated direct melatonin action upon ovarian steroidogenesis involving the *LH* and *GnRH* receptor gene expression in the steroid-producing human granulosa-luteal cells. These peripheral actions of melatonin complement its central actions and can conceivably lead to an alteration of the gonadal steroid balance (Woo *et al.*, 2001).

(e) Melatonin during puberty in humans

Serum night time melatonin concentrations are high in children, and drop by 75% from childhood (1–5 years) to young adulthood (Waldhauser *et al.*, 1984; Waldhauser & Dietzel, 1985). The morning values are uniformly low without change over different ages. The night time melatonin concentration were negatively correlated with the Tanner Stages of sexual development. In contrast, the aMT6s excretion in children and adults was similar in per day amount (Tetsuo *et al.*, 1982; Bojkowski *et al.*, 1987b). It appears that the amount of melatonin secreted by the pineal gland from childhood to young adulthood remains about the same, but as it is distributed over a larger body volume, the serum concentration is lower.

(f) Melatonin during the menstrual cycle in humans

Conflicting results have been reported in studies of the circadian melatonin rhythm during the menstrual cycle. Some studies reported increased melatonin levels during the luteal phase (Wetterberg *et al.*, 1976; Arendt 1978, 1988; Webley & Leidenberger, 1986; Brun *et al.*, 1987) or no difference between the phases (Brzezinski *et al.*, 1988; Wright *et al.*, 2001).

Brzezinski *et al.* (1988) found no significant change of plasma melatonin during the normal menstrual cycle in 14 clinically healthy normally cycling women (± 36 years of age (range 19–34)) studied at 2-hour intervals over a 24-hour span during early follicular, periovulatory, and luteal phase of the menstrual cycle. Circadian phase, amplitude, and total amount of melatonin secreted were consistent among the three profiles.

Studying the relations of melatonin to FSH and LH in 79 healthy women of different ages, Fernández *et al.* (1990) found a significant correlation of melatonin with FSH and E_2 in menstruating women during the follicular phase, while during the luteal phase, a negative correlation was found between melatonin, progesterone, and E_2. During the perimenopausal period, there was no significant correlation between the serum hormone

concentrations. In menopause, as during the follicular phase, melatonin and FSH were negatively correlated.

Exposure to bright light at night appears to have some effects upon the regulation of the menstrual cycle (Dewan, 1967; Lin *et al.*, 1990). A response of menstrual cycle length to nocturnal light exposure (100 W bulb with 235 lux) has been reported in women with long and irregular menstrual cycles (Lin *et al.*, 1990). Nocturnal light may also have effects upon the menstrual cycle phase (Putilov *et al.*, 2002).

There is no direct evidence that either endogenous, nocturnal circulating levels of melatonin or the administration of exogenous doses of melatonin mimicking circulating physiological concentrations exert any influence on pituitary gonadotrophins, prolactin, or gonadal steroids in humans. However, exposure of normal menstrual cycling women to continuous light (500–800 lux measured at eye level) during the night suppressed nocturnal circulating melatonin and prolactin concentrations while elevating FSH concentrations; no clear-cut effects were observed on LH levels when compared to control subjects maintained in the dark (Miyauchi *et al.*, 1991). This same group later reported that the incidence of menstrual irregularities in a cohort of 766 women who worked in various occupations was highest in nurses (24.9%), factory workers (36.8%), and barmaids (40.0%) when compared to teachers (13.1%) and office personnel (14.9%); the incidence of menstrual irregularities were significantly higher in those working during the night versus the day. In a small subset of nurses, those working during the night ($n = 5$) had significantly lower blood concentrations of melatonin and prolactin (sampling at 22:00 and 02:00) versus nurses resting in their quarters ($n = 6$); however, no differences were observed in plasma LH or FSH levels (Miyauchi *et al.*, 1992). In a study of 53 healthy women exposed to light during the night, circulating melatonin levels during the night were suppressed while there was no point for point changes in matching mesures of circulating E2 levels regardless of whether women were in the follicular or luteal phases of the menstrual cycle. Furthermore, in women who chronically secrete low or high levels of melatonin during the night (area under the curve range) had similar E2 blood levels (Graham *et al.*, 2001). This was also true in nude female rats exposed to increasing intensities of white, fluorescent light during the dark phase (0, 0.02, 0.05, 0.06, 0.08 and 345 μW/cm2) of an LD12:12 regimen – a dose-dependent suppression of the nocturnal amplitude of blood melatonin levels was observed while circulating levels of E2 were unchanged (Blask *et al.*, 2005b)

Disruption of circadian rhythms is associated with disturbances in menstrual function. Female shiftworkers compared to non-shiftworkers are more likely to report menstrual irregularity, and longer menstrual cycles (Baker & Driver, 2007).

Menstrual cycle irregularities have been reported in female airplane crew members, which may be the result of frequently repeated phase shift, light exposure at times unusual for their circadian cycle, or other causes specific to air travel (Iglesias *et al.*, 1980). The frequent phase shift in airline personnel has also been reported to lead to cognitive deficits (Cho *et al.*, 2000) and even associated with organic changes in the temporal lobe area

(Cho, 2001). No observations on reproductive axis dysregulation were mentioned in these reports

(g) Seasonal variations of pineal-ovarian relations in humans

In human subjects, the availability and exposure to artificial illumination appears to decrease the seasonal differences in environmental daily light–dark span, and the associated changes in pineal–gonadal relation. However, in Northern countries with strong seasonal variation in luminosity, melatonin also seems to contribute to the seasonal control of reproductive function in humans. During the dark months of the year, the activity of the pituitary-ovarian axis (Ronkainen *et al.*, 1985) on the conception rate (Timonen *et al.*, 1964; Sandahl, 1978) is decreased.

Studying serum melatonin as the likely messenger of the length of the daily dark span, Kivelä *et al.* (1988) found that the serum melatonin concentrations on menstrual cycle Days 2 and 10 of 12 clinical healthy, diurnally active women were 27% and 49%, respectively, higher in the winter than in the summer. Night time serum LH levels at mid-cycle were 76% higher in the summer than in the winter. The high levels of melatonin in the winter may have had an inhibiting effect upon serum LH levels (Kivelä *et al.*, 1988).

Kauppila *et al.* (1987) found that the area-under-the-curve of 24-hour melatonin profiles obtained by 2-hourly serum sampling during the dark (winter) season in 11 normally cycling women were significantly larger than during the light season. The duration of the nocturnal melatonin pulse during the dark season was lengthened whereas the mean serum E2 concentration was significantly decreased at the time of ovulation and during the luteal phase of the cycle in spite of increased gonadotropin concentration, indicating lowered ovarian responsiveness. The concentration of free testosterone was also lower during the dark season.

(h) Aging of the pineal-gonadal axis in the rat

In middle-aged female rats (11-months-old) with irregular estrous cycles and lowered gonadotropin surge during proestrus (perimenopausal in human equivalent), melatonin enhanced the amount of LH, FSH, and prolactin released during the surge at the proestrus day and restored the afternoon preovulatory surge in LH, FSH, and prolactin to values equivalent to those found in young rats. E2 concentrations were markedly increased in the treated animals on the day of proestrus which preceded the FSH, LH, and prolactin surges in the afternoon (Díaz *et al.*, 1999). Melatonin administration in middle-aged female rats regulated the activity of the hypothalamo-pituitary unit, and particularly improved gonadotropin secretion in response to the luteinizing-hormone-releasing hormone (Díaz López *et al.*, 2005; Díaz *et al.*, 1999, 2000).

In acyclic 23–25-months-old rats, melatonin reduced the elevated LH and FSH concentrations and increased the prolactin concentration (Diaz *et al.*, 2000). The responsiveness of the pituitary to the luteinizing-hormone-releasing hormone *in vivo* was increased by melatonin treatment, which in aging animals restored the pituitary

responsiveness to levels similar to that seen in young rats (Díaz *et al.*, 1999; Fernández Alvarez *et al.*, 1999). This is in contrast with in-vitro findings in the neonatal pituitary gland (Martin & Klein, 1976). Therefore, the effect of melatonin changes with the age of the animals.

(i) Menopause in humans

Under controlled 'constant routine' conditions, there was no significant difference in the amplitude of the salivary melatonin circadian rhythm between healthy middle-aged premenopausal (age 42 ± 4 years) and postmenopausal (55 ± 2 years) women (Walters *et al.*, 2005). In this respect, this study agrees with the findings of Zeitzer *et al.* (1999), which showed no age-related difference in melatonin amplitude when subjects were studied under constant routine conditions (although the age difference of the groups in this study was small).

There was a significant advance of the timing of the melatonin acrophase in the postmenopausal compared to the premenopausal women (1.1 ± 0.5 hours versus 2.3 ± 0.3 hour clock time in decimals, respectively) (Walters *et al.*, 2005). This result is in agreement with the studies of Cagnacci *et al.* (1995b), Duffy *et al.* (2002) and Yoon *et al.* (2003) while other investigators reported a phase delay (Sharma *et al.*, 1989), or no change in timing (Youngstedt *et al.*, 2001).

The correlation between melatonin and LH and melatonin and FSH was negative in perimenopausal and menopausal women before treatment with melatonin. After 6 months of treatment with 3 mg of melatonin at bedtime, a significant decrease in plasma LH was found only in the younger women (43–49 years of age) and not in the older women (50–62 years of age). There was a significant decrease of FSH especially in women with low basal overnight melatonin levels. In the same study, the women treated with melatonin had a significant increase in concentrations of total thyroid hormones, triiodothyronine (T_3), and thyroxine (T_4) in comparison to the women treated with placebo, without concomitant changes in the thyroid-stimulating hormone (TSH) on single-time-point sampling (Bellipanni *et al.*, 2001).

(j) Melatonin in disorders of the human hypothalamic–pituitary–gonadal axis

(i) *Women athletes with functional hypothalamic amenorrhoea*

Women athletes have abnormalities of the hypothalamic–pituitary–ovarian (Hth–Pit–Ova) (Veldhuis *et al.*, 1985; Loucks *et al.*, 1989) and hypothalamic–pituitary–adrenal (Hth–Pit–Adr) axes (Loucks *et al.*, 1989; Ding *et al.*, 1988). The changes in the Hth–Pit–Ova axis resemble those of women with functional hypothalamic amenorrhoea who do not exercise (Berga *et al.*, 1989; Suh *et al.*, 1988) in whom the magnitude and duration of nocturnal melatonin secretion is increased. Elevated daytime plasma concentrations of melatonin were observed in cycling and in amenorrhoeic women athletes compared to sedentary women. In contrast, nocturnal melatonin concentrations in sedentary and

cycling athletic women were indistinguishable while the amenorrhoeic athletic women had a marked increase in nocturnal peak amplitude and delay in melatonin offset leading to a 2-fold amplification of the nocturnal melatonin secretion (Laughlin *et al.*, 1991). Neither opioidergic (naloxone) nor dopaminergic (metoclopramide) blockade changed melatonin secretion in any of the three groups. The mechanisms of the amenorrhoea in the athletes appeared to be similar to that of sedentary functionally amenorrhoeic women. These mechanisms seemed related to a common hypothalamic dysregulation rather than to athleticism which was accompanied by daytime elevated values of melatonin, and not by the characteristic elevation seen in the amenorrhoeic subjects during night time (Laughlin *et al.*, 1991).

(ii) *Functional hypothalamic amenorrhoea*

Plasma melatonin concentrations in seven women with functional hypothalamic amenorrhoea (aged 22–35 years, mean 28.4), measured in 2-hourly sampling over a 24-hour span (daytime and night time), were significantly higher than in concomitantly studied healthy controls observed during three stages of their menstrual cycle (Brzezinski *et al.* 1988). Similar results were reported by Berga *et al.* (1988) in seven women with the same condition sampled at 30-minute intervals over a 24-hour span. While the daytime melatonin levels were undetectable in both groups, the integrated night-time levels were three times greater in the amenorrhoeic women than in the matched controls. The rise was due both to an increased peak amplitude and an extended duration of melatonin secretion towards morning, in spite of a comparable light–dark regimen. In a further study by Okatani & Sagara (1994), 20 women with functional hypothalamic amenorrhoea had significantly higher nocturnal melatonin concentrations than 11 matched controls. Negative correlations between the cumulative melatonin concentration (between 20:00 and 08:00) and serum E_2, and between the peak serum melatonin values and serum E_2 were observed. Intravenous administration of conjugated estrogen (Premarin 20 mg) significantly suppressed nocturnal melatonin secretion.

Five women with endometriosis and a low estrogen state induced by a GnRH agonist treatment had an increase in nocturnal melatonin secretion which was similar to that of the women with hypothalamic amenorrhea. This observation suggests that melatonin does not alter gonadotropin responses in humans to GnRH (Weinberg *et al.*, 1980; Fideleff *et al.*, 1976).

(iii) *Hth–Pit–Gnd disorders in the male*

Hypogonadotropic hypogonadism and delayed puberty are based on GnRH deficiency. Both of these conditions in young males resulted in a marked increase in nocturnal melatonin concentrations and integrated nocturnal melatonin secretion values (area-under-the-curve) when compared to normal pubertal male controls (Luboshitzky *et al.*, 1995). There was no correlation between melatonin and LH or between melatonin and prolactin concentrations, suggesting that circulating sex steroids rather than LH modulate melatonin secretion in a reverse fashion (Luboshitzky *et al.*, 1995). This nocturnal

increase in melatonin secretion was corrected using a testosterone treatment resulting in the melatonin concentrations returning to normal levels (Luboshitzky *et al.*, 1996a). In a single case report of female delayed puberty treated with estradiol, a marked decrease of melatonin production was noted in the course of treatment (Arendt et al., 1989).

In contrast, untreated males with hypergonadotropic hypogonadism, due to untreated Klinefelter syndrome and sampled overnight, all had elevated FSH, LH, and E_2 concentrations. Klinefelter syndrome patients with low testosterone concentrations had significantly lower melatonin nocturnal concentrations and area-under-the-curve profiles when compared to Klinefelter syndrome patients with normal testosterone levels, and controls. No correlations between the melatonin concentration and LH, FSH, or E_2 levels were observed (Luboshitzky *et al.*, 1996b).

In male and female patients with central precocious puberty with elevated sex steroid levels, serum melatonin levels were lower than normal subjects (Waldhauser *et al.*, 1991, 1993) indicating that in general, in conditions in which sex hormones are lower with decreased or normal gonadotropin concentrations, melatonin was found to be elevated, and vice-versa. (Berga *et al.*, 1988; Brzezinski *et al.*, 1988; Laughlin *et al.*, 1991; Tortosa *et al.*, 1989; Okatani & Sagara 1994; Puig-Domingo *et al.*, 1992).

4.2.7 *Time organization in the normal and abnormal human breast*

(a) Periodicity in the normal human breast

Periodicities in the normal human breast were studied non-invasively by measuring the skin temperature above the mammary gland by ambulatory thermography monitoring. Thermography methods study both the metabolic activity of the gland and the blood flow in the overlying tissue. In some studies, the thermography readings over the breast were related to simultaneously monitored skin temperatures obtained at other sites or to oral temperature allowing to partly separate these two components, and obtain a corrected "breast-specific temperature" (Simpson *et al.*, 1989). Circadian (about 24-hour), circaceptan (about 7-day), and circamenstrual (about 28–32 day) rhythms were identified (Gautherie & Gros, 1977). The breast temperature in clinically healthy diurnally active women exhibited a circadian rhythm similar to that of the oral or the core temperature with a peak (acrophase) in the evening (Mansfield *et al.*, 1973; Gautherie & Gros, 1977). The circamenstrual rhythm in breast temperature included changes in the circadian rhythm parameters in mean amplitude, and a characteristic periovulatory rise with a peak occurring approximately 24 hours after ovulation (Phillips *et al.*, 1981; Wilson *et al.*, 1983). In large numbers of breast biopsies taken from women in different phases of the menstrual cycle, Anderson *et al.* (1982) found the highest incidence of epithelial mitoses to occur before the onset of the menstrual period.

(b) Time of initiation of breast cancer

The initiation of breast cancer may occur many years before the clinical manifestation of the tumour. In many instances, a breast cancer may begin to develop in the early premenopausal period (Simpson *et al.*, 1988). Findings concerning the influence of age at first pregnancy (MacMahon *et al.*, 1970) and the relation of the time of radiation exposure to the appearance of the tumour (Howe, 1984) support the concept that the predominant environmental initiation of breast cancer occurs in the premenopausal period during the reproductive lifespan. This is when the epithelium is proliferating and biologically vulnerable to carcinogenic agents interfering with the circadian periodicity of cell proliferation (Simpson *et al.*, 1988).

An increased cancer incidence in shiftworkers may be related both to initiation of the tumour and to events occurring during the period of promotion of the malignancy, until it becomes clinically manifest.

(c) Circadian time structure and risk to develop breast cancer

Gautherie and Gros (1980) reported on a large series of women who received routine breast examination, including a breast thermogram. Of these, 1245 were followed up for a period of 12 years because of a questionable abnormal thermal pattern. During the follow-up period, 501 of these developed breast cancer.

In a group of 106 women with apparently healthy breast and no family history of related medical conditions, but with an abnormal thermal pattern, 27.2% developed breast cancer. In a group of 31 women with family history for breast cancer and an abnormal thermogram, 11 women (35.8%) developed breast cancer when compared to only 3.9% of 486 women with a normal thermogram. The abnormal thermal pattern preceded the clinical diagnosis of cancer in the majority of cases by 4 to 5 years, but in some instances beyond 5 years, but usually less than 10 years (Gautherie, 1983; Amalric *et al.*, 1981).

Rhythm alterations in the circadian timing of 12 hormonal variables were reported in women with a high risk (epidemiologically determined) of developing breast cancer (Ticher *et al.*, 1996). A total of 24 clinically healthy, diurnally active non-obese American women of three age groups (adolescent 17 ± 2 yr, $n = 8$; young adult 33 ± 1 yr, $n = 10$; and postmenopausal 56 ± 7 yr, $n = 8$) were studied. The women were characterized as high risk ($n = 12$) or low risk ($n = 12$) of developing breast cancer according to the epidemiological index criteria. Risk assessment followed a scale based upon the epidemiological data presented by MacMahon *et al.* (1973), Farewell *et al.* (1977), and Choi *et al.* (1978), which are similar to another review of this topic (Gail *et al.*, 1989). No subjects with a family history of *BRCA1* or *BRCA2* mutations were included. A family history of first degree relatives with (sporadic) breast cancer was the primary distinction, as it applied to all age groups. No medications known to affect prolactin secretion, including oral contraceptives, were allowed 6 months before the start of the study until its completion.

Most subjects were sampled throughout a series of four 24-hour spans during a single year, once each season, and at a different menstrual stage. There were no seasonal differences in the incidence of the different stages of the menstrual cycle. The total number of time series analysed was 44 for the cohort of high-risk subjects (31 in regularly adult menstruating women and 14 in postmenopausal women) and 41 for the cohort of low-risk subjects (26 in regularly non-menopausal menstruating women and 15 in postmenopausal women). The number of time series analysed per season was as follows: $n = 21$ spring, $n = 20$ summer, $n = 20$ autumn, $n = 24$ winter. Blood for cortisol and prolactin was collected every 100 minutes over each 24-hour span, and blood for the other variables (aldosterone, cortisol, DHEA-S, E2, insulin, LH, 17-hydroxyprogesterone, TSH, thyroxine, and triiodothyronine) every 100 minutes. The data for each subject were analysed for each variable by the single cosinor test (Nelson *et al.*, 1979) yielding a calculated peak time (acrophase), an amplitude, and a circadian-rhythm-corrected mean value (MESOR). The differences and similarities between the high- and low-risk groups and the age groups in the dispersion of the set of acrophases and the ratio of amplitude over MESOR were analysed by a multiple Pearson correlation test and the resulting correlation matrix was used for cluster analysis. Two main profiles of acrophase dispersion were detected according to the level of breast cancer risk. The circadian time organization was similar in women with a high risk to develop breast cancer, irrespective of age, and different from the pattern in women with a low risk. In contrast, the amplitude/MESOR ratio was characteristic for the age group, and unrelated to breast cancer risk (Ticher *et al.*, 1996).

In the same study, Lewy *et al.* (2007) compared the distribution of circadian and ultradian (in the range of 4–18 hours) rhythms in low-risk and high-risk patients sampled during the four seasons for prolactin and cortisol. The high-risk and low-risk patients expressed different rhythmic output patterns in both variables, also suggesting that the genetic background as defined by the risk state to develop breast cancer was related to differences in the circadian time structure including the ability to change the subjects' predominant rhythm periods as a function of season. The low-risk patients exhibited a statistically significant change in the rhythm periods of both variables with a shift from the circadian to an ultradian rhythmicity as a function of the season while the high-risk patients did not.

Rhythm alteration in the menstrual temperature rhythm of patients at high risk to develop breast cancer was described by Simpson *et al.* (1989). The high-risk state in this study was defined by previous excision of an ipsilateral or contralateral breast tumour. While the basic breast-specific temperature in the women at usual risk of breast cancer showed the usual variation characteristic for the menstrual period with a sustained rise after ovulation and high values during the luteal phase, the high-risk patients had three temperature peaks separated by 7 and 6 days, respectively, the largest (first peak), preceding the salivary progesterone peak by about 6 days, the second and the third peaks appearing 2 days and 8 days after the salivery progesterone peak, respectively (progesterone peak appearing 8 days after the ovulation).

These data indicate significant changes in the circadian and menstrual (possibly adaptive) characteristics in the human time structure related to the risk state to develop breast cancer.

4.2.8 *Sleep deprivation – impact upon the neuroendocrine and immune system*

The most prevalent health problem for the night worker and shiftworker is the quantity and quality of sleep. The night worker, but also the early-morning shiftworker, sleeps about >2 hours less than the average day worker, and often with decreased sleep efficiency and sleep quality. Sleep deprivation impacts heavily upon the entire neuroendocrine-immune system complex regulating several biological functions, including cell proliferation, immune defence and adaptation, and defence to everyday stresses.

(a) Sleep deprivation and the immune system

The immune system in all its components is closely integrated in two-way communication and feedback loops with the nervous and the endocrine system, forming a web of biological regulation, which functions rhythmically in multiple frequencies. In the circadian frequency range, the immune system is subject to the central hypothalamic oscillator in the SCN with peripheral oscillators in immunocompetent cells and organs. It is kept in pace by neural as well as neuroendocrine and endocrine messengers and synchronizers. Some of the multifrequency periodic neuroendocrine variables (e.g. prolactin, melatonin) enhance immune reactions, while other variables (e.g. cortisol) keep them in check and control their intensity, or if these variables are overexpressed, they might suppress the immune response.

Immune reactions taking place in mammalian organisms (including humans) exert feedback effects upon the regulatory centres. The immunocompetent cells involved in an immune reaction produce humoral messengers that act on the neuroendocrine system signalling the occurrence of damage and/or of an ongoing immunodefence reaction. The feedback mechanism from the peripheral cells to the centre elicits a neuroendocrine response, which in turn regulates the peripheral cellular response to the stimulus encountered. While cortisol acts as an immunosuppressor, melatonin enhances IFNγ and IL-1 production (Maestroni *et al.*, 1986; Caroleo *et al.*, 1992; Colombo *et al.*, 1992; Morrey *et al.*, 1994), and antagonizes the immunosuppressive effect of cortisol (Maestroni *et al.*, 1988a, b). There is a bidirectional link between sleep and the immune system, in which cytokines such as IL-1, IL-2, interferon, and TNF induce sleep (Krueger & Obál, 1993).

In addition to cytokines, human peripheral leukocytes, e.g. infected by a virus or exposed to endotoxin will synthesize immunoreactive ACTH, and endorphins. The immunoreactive ACTH produced by the immunocompetent cells appears to be identical to pituitary ACTH, and acts upon the same receptors in the target tissues and shows a steroidogenic response in mice. The production of ACTH, both by pituitary cells and by

leukocytes in response to synthetic corticotropin-releasing factor (CRF), is suppressed by dexamethasone *in vitro* and *in vivo* suggesting that the production of ACTH and endorphins by leukocytes is controlled by the CRF (Smith *et al.*, 1986). CRF in itself has anti-inflammatory effects that are independent of the pituitary and adrenal glands (Kiang *et al.*, 1987; Gao *et al.*, 1991; Wei & Gao 1991; Serda & Wei 1992). CRF is produced in the hypothalamus in a cyclic fashion, and also in response to a wide variety of environmental stimuli as a stress response and in response to pro-inflammatory cytokines such as IL-1 (Anderson *et al.*, 1993; Rivier, 1993). In animal experiments, elimination of cyclicity by exogenous CRF leads to altered response patterns after challenge (e.g. by bacterial endotoxin) (Linthorst *et al.*, 1997).

The alternation of sleep and wakefulness is a fundamental part of the organization of circadian time and coordinates numerous neuroendocrine variables. Several immunologically active hormones and peptides influence the sleep–wake cycle of the brain and are involved in a bidirectional or multidirectional communication between neuroendocrine, immune, and central nervous system functions. Most of those possess circadian rhythmicity, and some are influenced by environmental factors acting as synchronizers or as masking agents. IL-1 and other cytokines play a regulatory role on the sleep–wake cycle (Opp *et al.*, 1992), and interact in this process with various immunologically active neuroendocrine substances (Krueger *et al.*, 1990a,b). In the physiological regulation of the sleep–wake rhythm, the natural sleep-promoting or wakefulness-promoting substances require a specific constellation of multiple variables that in part is determined by their circadian rhythms and in part by environmental and behavioural conditions (Moldofsky, 1994). Periods of predisposition to sleepiness are separated by periods of resistance to sleep (Lavie & Weller, 1989).

Sleep deprivation in experimental animals and in human subjects leads to an impairment in immune function which, if prolonged, will lead to the death of the animals and of the human subjects (fatal familial insomnia syndrome) (Everson, 1993; Portaluppi *et al.*, 1994). Sleep deprivation in rodents, even for a brief 7-hour period, leads to a downregulation in immune defence against viral infection, and after challenge, to significantly decreased antibody titres (Brown *et al.*, 1989a, b). With more prolonged sleep deprivation, Everson (1993) reported in rats a breakdown in immune defences with a systemic infection by pathogenic organisms, leading to the death of the animals.

Phase shifts, as they are encountered in shiftworkers or after transmeridian flights or rhythm disturbances under irregular work schedules, lead to internal desynchronization of immune-related circadian rhythms, and to the impairment of immune functions. In studies on shiftworkers, Nakano *et al.* (1982) reported lower proliferative responses in lymphocytes when compared to regular daytime workers. As shiftwork is usually accompanied by a certain degree of sleep deprivation, it is unclear whether the impairment of immune function in shiftworkers is a consequence of circadian desynchronization, of sleep deprivation, or of both. In night shiftworkers, a short daytime sleep isa consequence of circadian desynchronization as a result of the misalignment of

the circadian rhythm in sleep propensity of the worker with the time for sleep allowed by the work schedule.

(i) *Total sleep deprivation*

Unfortunately, many of the studies in this area have limited their sampling to single or very few time points per day, which, in view of the high amplitude circadian rhythms of immunocompetent cells and their responsiveness to stimulation, raises questions as to their interpretation since they often do not allow a distinction between an actual change in level of the variable studied or a shift in circadian phase (Haus, 1996; Haus & Smolensky, 1999). This problem is compounded by different sampling times used in different studies. In prolonged studies of sleep deprivation (e.g. 64 hours) with measurements of circulating immunocompetent cells limited to single time points at a given clock hour only, biphasic or other reaction patterns may be an expression of circadian phase alteration level changes or masking. These sampling limitations in much of the published literature are a likely explanation of the many contradictory results obtained by different investigators.

After 48 hours of sleep deprivation, with blood sampling at single time point at 08:00 before and after 24 and 48 hours of sleep deprivation, and 24 hours after recovery sleep, Ozturk *et al.* (1999) found a decrease in the proportion of NK cells during sleep deprivation with return to normal after recovery sleep. In contrast, Dinges *et al.* (1994) sampled blood daily at 22:00 in 20 healthy young adults of both genders over a 64-hour span of total sleep deprivation and at 22:00 in the pre-deprivation day and on the first recovery day. They reported during total sleep deprivation an increase in the number of circulating white blood cells, including granulocytes, monocytes, and NK cells as well as an increase in NK-cell activity, and increased response of lymphocytes to phytohaemagglutinin, a T-cell mitogen. On the basis of their observations, Dinges *et al.* (1994) assumed that an activation of these branches of the immune system occurred with 64 hours of total steep deprivation.

Seventy-seven hours of total sleep deprivation in eight clinically healthy women led to reduced phagocytosis by polymorphonuclear granulocytes, increased interferon production by lymphocytes, and increased the plasma cortisol concentration (Palmblad *et al.*, 1976). Sampling was limited to a single time point at 12:30, and after 28 and 76 hours of total sleep deprivation. The experimental design included a stressful surrounding during sleep deprivation with simulated battlefield environment.

Also using a single time point of measurement in 12 healthy young men after 48 hours of total sleep deprivation and in-vitro phytohaemagglutinin stimulation of their lymphocytes, Palmblad *et al.* (1979) reported a decreased lymphocyte blastogenesis.

These differences in the outcome of single-time point studies of variables with high amplitude circadian rhythms emphasize the need for studies at more than one circadian stage, or the use of a marker rhythm (Haus *et al.*, 1988) when studying conditions like shiftwork in which circadian phase alterations are to be expected.

(ii) *Partial sleep deprivation*

In contrast to prolonged total sleep deprivation, partial sleep deprivation with wakefulness either during the first part or the latter part of the night corresponds more closely to the situation encountered by the shiftworker.

Irwin and colleagues (1994) studied NK-cell activity in 23 healthy adult men (22–61 years of age) with sampling at a single clock hour (between 07:00 and 09:00) after one night of late night partial sleep deprivation, with no sleep from 03:00 to 07:00 following three baseline nights of regular sleep, and again after a recovery night of sleep. The late night sleep deprivation was associated with a decreased NK-cell activity in 18 of the 23 subjects with an average reduction in lytic activity of 28%.

In a similar study of early night of partial sleep deprivation with no sleep until 03:00, Irwin *et al.* (1996) found in 42 clinically healthy men after a single night by single-time point sampling at the same clock hour (between 07:00 and 09:00), a reduction of the natural immune response as expressed by a decrease in NK-cell activity, NK activity per number of NK cells, decrease in lymphokine-activated killer cell number and activity, and lymphokine-activated killer activity per number of lymphokine-activated killer precursors. IL-2 production stimulated by concanavalin-A was also suppressed. After one night of recovery, sleep NK-cell activity had returned to baseline levels while IL-2 production remained suppressed. These data indicate that even a modest disturbance of sleep produces a reduction in natural immune responses and T-cell cytokine production.

(iii) *Sleep deprivation and cytokine balance*

The immune system is organized in a two-branch model. The pro-inflammatory Type 1 T-helper1 (Th1) cytokines (IL-2IFNγ and IL-12) produced by immunocompetent peripheral blood mononuclear cells is counterbalanced by the anti-inflammatory group of Type 2 T-helper2 (Th2) cytokines (IL-4 and IL-10) (Lucey *et al.*, 1996). In diurnally active human subjects, the Type 1 immunodefence pattern predominates during night hours (Petrovsky, 2001; Dimitrov *et al.*, 2004a). The Type 1 cytokines support the cellular aspects of immune response and are moderated in their pro-inflammatory action by the Type 2 cytokines. Maintenance of this balance is essential since excessive production of one or the other type of cytokines leads to immune disturbances with either inflammation and tissue damage or with susceptibility to infection and allergy (Lucey *et al.*, 1996). The balance among the cytokine groups is maintained in the healthy organism by cross-inhibition and by superimposed neuroendocrine control (Romagnani, 1996), which in its time organization is directed by the SCN, and the related circadian clock mechanisms.

Sleep is an integral part of the circadian time structure and plays a vital role in the regulation of the immune system (Bryant *et al.*, 2004). There is a sleep-associated shift towards Type 1 cytokine activity in T-cells (Dimitrov *et al.*, 2004a). Sleep deprivation leads to alterations in the cytokine balance. A shift towards Type 2 activity has been reported for sleep deprivation in otherwise healthy subjects, in insomnia, under stress, and in the aged (Dimitrov *et al.*, 2004b; Sakami *et al.*, 2002–2003; Glaser *et al.*, 2001).

Elevation of sympathetic tone during the night also contributes to a reduction of cellular immunity, e.g. in psychological stress situations (Irwin *et al.*, 1990a; Irwin *et al.*, 1991).

Covering an entire 24-hour span with frequent sampling and sleep permitted from 23:00 to 07:00, and a second 24-hour span without sleep in the same subjects at least 4 weeks apart, Lange *et al.* (2006) studied by flow cytometry IL-12- and IL-10-producing monocytes, representing messengers of the Th1 and Th2 pattern, respectively. During sleep, there was an increase in the number of IL-12- producing monocytes and a concurrent decrease of IL-10-producing monocytes, leading to a circadian rhythm in these cells with a peak at 02:20 and 11:30, respectively. These apparently rhythmic temporal variations were absent during continuous wakefulness. Monocytes are a major contributor to pro-inflammatory cytokine production in the peripheral blood. Nocturnal sleep shifts monocyte cytokine production to Type 1 cytokines, which is regarded as a prerequisite for sleep-associated predominance of Th1-mediated adaptive immune defence (Lange *et al.*, 2006). The human monocytes are regarded as direct precursors of antigen-presenting cells, and can be directly assessed by flow cytometry in blood samples (Geissmann *et al.*, 2003). The study of Lange *et al.* (2006) shows a dependence of the cytokine rhythm on sleep and its apparent absence during continuous wakefulness. The circadian variation in monocyte-derived IL-12 and IL-10 production, and the respective Type 1/Type 2 cytokine balance, which are induced primarily by sleep, are vulnerable to sleep disturbances and sleep deprivation.

With regard to mechanisms, growth hormone and prolactin shift the Type 1/Type 2 balance towards Type 1, whereas cortisol and norepinephrine shift it towards Type 2 (Dimitrov *et al.*, 2004a, b; Elenkov & Chrousos, 2002). Both prolactin and growth hormone rhythms are altered during sleep deprivation (Lange *et al.*, 2006). There was a positive correlation between the prolactin level and IL-12+ monocyte numbers, and between norepinephrine and IL-10+ monocyte numbers, and a negative correlation between the cortisol level and IL-12+ monocyte numbers (Lange *et al.*, 2006; Petrovsky & Harrison, 1998). *In vitro*, studies of prolactin and cortisol effects support the assumption of a direct hormonal action upon IL-12+ monocytes (Petrovsky & Harrison, 1998; Visser *et al.*, 1998; Petrovsky, 2001; Elenkov & Chrousos, 2002; Lange *et al.*, 2006). A direct effect of growth hormone on the immunocompetent cells is less well documented (Elenkov & Chrousos, 2002; Lange *et al.*, 2006). Melatonin also stimulates Type 1 activity (Petrovsky, 2001), and nocturnal suppression of melatonin may counteract this shift.

Irwin *et al.* (2006) studied in 30 diurnally active healthy adult men (n = 17) and women (n = 13) the monocyte intracellular pro-inflammatory cytokine production across 3 days of baseline testing, and after 1 day of partial sleep deprivation with wakefulness from 23:00 to 03:00. Sampling occurred at 08:00, 12:00, 16:00, 20:00 and 23:00. In the morning after sleep loss, but not at the other times of sampling, the monocyte production of IL-6 and TNFα was significantly greater when compared to the same time (08:00) following uninterrupted sleep. Sleep loss apparently led to an activation of these pro-inflammatory cytokine genes with a more than 3-fold increase in transcription of *IL-6*

mRNA, and a 2-fold increase in *TNFα* mRNA. This change was the expression of a functional difference in the monocytes and did not relate to any difference in the numbers of cells. Global gene expression profiling in leukocyte total RNA by high density oligonucleotide assay in five subjects before and after sleep deprivation revealed a set of 22 genes that were significantly upregulated after partial sleep deprivation. These included the circadian clock gene *Per1*, several epidermal-growth-factor-related genes, and multiple inflammatory response genes. The complex ensemble of functional genomic alterations induced by sleep loss included multiple immediate early response genes, and signal transduction mediators. The remodelling of leukocyte gene expression by sleep and its alteration by sleep loss may point to molecular sites of action in the immune system, and also more generally in cellular physiology and pathology.

(b) Sleep deprivation and the neuroendocrine system

(i) *Prolactin and sleep*

Prolactin plasma concentrations show pulsatile episodic hormone secretion patterns superimposed upon ultradian rhythms as well as circadian oscillation. The prolactin 24-hour profile reflects both tonic and intermittent hormone release (Veldhuis *et al.*, 1992). The normal secretory pattern of prolactin consists of a series of daily pulses, occurring every 2–3 hours, which vary in amplitude. The bulk of the hormone is secreted during REM sleep. In diurnally active human subjects, REM sleep occurs predominantly during the latter half of the nightly sleep phase, so that the highest plasma prolactin concentrations usually occur late during the night (Sassin *et al.*, 1972, 1973). In men and non-pregnant and non-lactating women, REM sleep is the dominant organizer of prolactin secretion. It has been shown that, in turn, prolactin infusion increases REM activity in the electroencephalogram (Obál *et al.*, 1994; Roky *et al.*, 1995). In lactating women, the reflex elevation of prolactin and oxytocin by nipple stimulation during nursing becomes the predominant controller of circulating prolactin concentrations (Leake *et al.*, 1983).

Sleep onset is associated with an increase in prolactin secretion also during daytime naps, irrespective of the time of the day, but the amplitude of the prolactin rise during daytime sleep is usually less than during nocturnal sleep. Conversely, modest elevations in prolactin concentration may occur at the time of the usual sleep onset even when one remains awake. Thus, prolactin plasma concentrations appear to be regulated by a circadian rhythm and superimposed pulsatile secretions modulated by the sleep-wakefulness pattern, with maximal secretion when sleep and circadian rhythmicity are in phase (Spiegel *et al.*, 1994, 1999; Waldstreicher *et al.*, 1996). Shallow and fragmented sleep, prolonged awakening, and interrupted sleep patterns, as frequently seen in the elderly, are associated with a dampening of the nocturnal prolactin rise, decreased amplitude of the nocturnal prolactin pulses (van Coevorden *et al.*, 1991; Greenspan *et al.*, 1990), and decreased prolactin concentrations (Spiegel *et al.*, 1995).

Prolactine secretion in man is normally restrained by the action of dopamine, which is secreted from the hypothalamus. Prolactin is the only pituitary hormone that is secreted at

unrestrained high levels when completely isolated from any tropic influences of the hypothalamus. However, a variety of stimulatory prolactin secretagogues have been identified including steroids (estrogen), hypothalamic peptides, vasoactive intestinal peptide, and oxytocin, and growth factors such as epidermal growth factor, and fibroblast growth factor-2. Numerous medications used in everyday clinical practice elevate prolactin secretion, and this can mask physiological rhythmicity and occasionally may even lead to symptomatic hyperprolactinaemia. These agents include commonly used antidepressants, antiemetics, and narcotics, which antagonize dopamine action or elevate serotonin or endorphin bioactivity (Ben-Jonathan, 1994). Hypnotics like benzodiazepines (e.g. triazolam) and imidazopyridines (e.g. zolpidem) taken at bedtime (concordant with the tendency of the daily prolactin rise) may lead to substantial rises of serum prolactin concentrations into the range regarded as abnormal (Copinschi *et al.*, 1990, 1995). Melatonin itself acutely stimulates prolactin release in humans (Wright *et al.*, 1986; Waldhauser *et al.*, 1987). Endogenous estrogens play a role in the differential regulation of prolactin in relation to age and sex. Mean prolactin concentrations, pulse amplitude, and pulse frequency are all higher in normally cycling young women than in either postmenopausal women or in men (Katznelson *et al.*, 1998). Blunting of the nocturnal rise is not specific and is found also in other medical conditions, including breast cancer.

(ii) *Prolactin and the immune system*

The effects of prolactin in the human body are manyfold. Of importance is the regulatory role it plays on the immune system. Prolactin receptors are found on most immune-precursor and -effector cells in each of the major haematopoietic and lymphopoietic organs, such as the bone marrow, spleen, and thymus. However, the action of prolactin upon the immune system is complex, and depends upon the stage of both the circadian timing of the prolactin rhythm and its time relations to the circadian rhythms of immune-related functions in the target organs (Cincotta *et al.*, 1995).

In laboratory experiments, prolactin restores immune competence in hypophysectomized animals (Gala, 1991). Inhibition of prolactin secretion by bromocriptine results in immunosuppression (Hiestand *et al.*, 1986; Bernton *et al.*, 1988; Berczi, 1989). Prolactin antagonizes the immunosuppressive effects of glucocorticoids (Bernton *et al.*, 1992). While lowered prolactin concentration leads to immunodeficiency, and exogenous prolactin in short-term experiments produces immunoenhancement, persistently elevated prolactin levels, due to a variety of medical conditions, are associated with immunosuppression (Karmali *et al.*, 1974; Jungers *et al.*, 1982; Gerli *et al.*, 1987; Lavalle *et al.*, 1987; Nicoletti *et al.*, 1989; McMurray *et al.*, 1991; Vidaller *et al.*, 1986, 1992).

Some of the discordant results of investigations pertaining to the effects of prolactin on the immune system may be due to the pronounced circadian variation in its regulatory action (Cincotta *et al.*, 1995), and the marked time-dependent difference of immunocellular responses. In the male BALB/c mouse, the immunostimulatory activity of prolactin was restricted to only an 8-hour daily interval, from 4–12 hours after light on

in animals kept on an LD12:12 regimen. Prolactin administration outside this sensitive interval was occasionally associated with immunosuppressive effects both in the one-way mixed lymphocyte reaction and in the hapten-specific delayed-type hypersensitivity responses. Reducing endogenous levels of prolactin with bromocriptine inhibited immune functions only when the medication was administered during this daily interval of immunoregulatory sensitivity to the hormone (Cincotta *et al.*, 1995). This observation is similar to that of Bernton *et al.* (1992) who found that the effect of the prolactin inhibitor cysteamine (a dopamine β-hydroxylase inhibitor) on splenocyte mitogenic response was circadian-time-dependent.

A chronobiological explanation of the interaction of the rhythms in human prolactin secretion and in target cell responsiveness, however, has not been reported. It appears that in the immunoregulatory action of prolactin, the overall level of plasma prolactin is of less importance than the circadian rhythmicity of prolactin and that of the apparently circadian periodic responses of the immunocompetent target cell systems. The phase relation between the circadian rhythm in prolactin and that of the rhythms in immunocellular response may be the determining factor for the prolactin effect upon the immune system. Circadian rhythm disruption or phase shifts of either of these rhythms may be associated with immunological dysfunction, which may be of interest for shiftwork and transmeridian flights, and in the elderly, circadian rhythm and sleep disturbances.

Prolactin effects on human immune activity and immunological disorders, including lupus erythematosus and the postpartum exacerbation of rheumatoid arthritis, have been reported (Vidaller *et al.*, 1986; Gerli *et al.*, 1987; Lavalle *et al.*, 1987; Nicoletti *et al.*, 1989; Gala, 1991).

(iii) *Hypothalamic–pituitary–thyroid axis and sleep*

The hypothalamic–pituitary–thyroid (Hth–Pit–Thy) axis possesses an intricate time structure with rhythmic variations of multiple frequencies found at all levels of the system, from the hypothalamic neurons to the cells of the peripheral target tissues. The frequencies observed include pulsatile secretions and ultradian, circadian, and circannual rhythms. The time-dependent rhythmic (and non-rhythmic) variations of the Hth–Pit–Thy system interact with, and are modulated by, similar time-dependent variations of other neuroendocrine, metabolic, and immune functions.

The thyrotropin-releasing hormone is a tripeptide neurotransmitter that exerts multiple actions in the central nervous system and beyond (Metcalf & Jackson, 1989; Nicolau & Haus, 1992). It is produced also in peripheral tissues, including the immune system (Simard *et al.*, 1989). In addition to its capacity to stimulate the release of TSH from the anterior pituitary, it also stimulates prolactin.

TSH is secreted from the pituitary gland in a series of discrete pulses with an average pulse frequency of 9 pulses/24 hours (range 7–12) in normal men and women (Brabant *et al.*, 1990; Nicolau & Haus, 1992). These pulses are not equally distributed, but cluster during the evening and night hours when fusion of the pulses and an increase in amplitude leads to the nightly increase of TSH concentration, forming the circadian rhythm of this

hormone with a maximum in day–night-synchronized subjects occurring usually between 02:00 and 04:00 (Brabant *et al.*, 1990; Samuels *et al.*, 1990). The relatively high peak values of individual TSH pulses during sleep have to be kept in mind, as the values reached may be slightly above the usually accepted normal range.

The pulse pattern may be necessary for normal thyroid gland function due to the better response of the pituitary thyrotrops to intermittent rather than continuous thyrotropin-releasing hormone stimulation (Spencer *et al.*, 1980). Loss of the usual nocturnal variation in TSH and pulse amplitude may be sufficient to cause clinical hypothyroidism (Samuels *et al.*, 1990).

Sleep deprivation and sleep fragmentation result in a marked decrease in the mean 24-hour TSH secretion as well as a lowering of pulse amplitude also without change in peak frequency (Behrends *et al.*, 1998; Brabant *et al.*, 1990; Spiegel *et al.*, 1999).

(iv) *Growth hormone and sleep*

The 24-hour profile of growth hormone (GH) in adult subjects consists of stable low values interrupted by secretory pulses. There is a marked sexual dimorphism of the secretory pattern. In men, the highest pulse, amounting to about 70% of the secretory output per 24 hours, occurs shortly after sleep onset with the first phase of slow-wave sleep (Van Cauter *et al.*, 1998). In normally cycling women, there is a wider distribution of GH pulses throughout the day. The sleep-onset-associated pulse is still found in most women, but accounts only for a smaller fraction of the total 24-hour secretory product (Ho *et al.*, 1987). The linkage of a major GH pulse to sleep onset leads to an immediate shift in the circadian rhythm in GH with any change of the sleep–wake cycle, e.g. in shiftworkers and after transmeridian travel over several times zones. This linkage also leads to alterations of GH secretion in the case of sleep irregularities (Van Cauter *et al.*, 1998). The mechanism of this association is based on the hypothalamic relationship of the GH releasing hormone to areas of the brain involved in the regulation of sleep (Krueger & Obál, 1993). Inhibition of endogenous GH releasing hormone action by a specific antagonist or by immunoneutralization inhibits both sleep and GH secretion (Ocampo-Lim *et al.*, 1996). On the other hand, substances which promote sleep also lead to increases in nocturnal GH secretion (Gronfier *et al.*, 1996; Van Cauter *et al.*, 1997).

Total sleep deprivation with absence of recovery sleep leads to a markedly decreased growth hormone secretion (Van Cauter *et al.*, 1992; Weibel *et al.*, 1997). Recovery from total sleep deprivation irrespective of the time of day when recovery sleep occurs leads to a robust increase in GH secretion. When bedtime is acutely delayed by a few hours, nocturnal GH levels remain low as long as the subject is awake, and rebound as soon as sleep is initiated (Van Cauter *et al.*, 1998). Semichronic partial sleep deprivation more closely resembles the condition experienced by shiftworkers. Spiegel *et al.* (2000) studied 11 clinically healthy men after 6 nights of restricted bedtimes (01:00 to 05:00) and after 7 nights of extended bedtimes (21:00 to 09:00). After 1 week of sleep extension to 12 hours, the major GH peak occurred at the same time as the usual 8-hour sleep time after onset of sleep. After 1 week of bedtime reduced to 4 hours, the GH secretory rate

exhibited a biphasic pattern with a large pulse occurring during waking around the usual time of sleep onset on a standard 8-hour bedtime schedule, an expression of the circadian rhythm in GH secretion, followed by a second sleep-induced pulse after onset of the (shortened) sleep span. The state of subchronic partial sleep deprivation (sleep debt) was associated with a markedly different temporal association of GH secretion. The biphasic nature of the GH secretory pattern during sleep restriction resulted in a longer exposure of peripheral tissues to elevated GH concentrations (4 hour 12 minute ± 25 minute vs 3 hour 25 minute ± 33 minute during sleep extension). This biphasic pattern represents an adaptive process to the subchronic sleep deprivation as it was not found in studies with acute sleep deprivation (Van Cauter & Copinschi, 2000). The prolonged exposure of the peripheral tissues to GH may have played a role in the marked deterioration of glucose tolerance that was found in these subjects after 1 week of subchronic sleep restriction (Spiegel *et al.*, 1999). In relation to this study, the question can be raised if a curtailment of sleep by a phase advance due to earlier rising rather than delayed bedtime may avoid the biphasic secretion, and lead to a different adaptive response, possibly with less or different side-effects.

(v) *The Hth–Pit–Adr axis and sleep*

The corticotropic axis with the CRH, the ACTH of the pituitary, and cortisol from the adrenal cortex is, in addition to the direct effects upon multiple systems, a major messenger of time information in the circadian regulatory system. In addition, CRH is synthesized and produced in multiple peripheral tissues with likely involvement in the regulation of energy balance, metabolism, and immune response (Richard *et al.*, 2000; Baigent, 2001). CRH in the rat brain is produced in the arcuate and paraventricular nuclei of the hypothalamus (Sawchenko & Swanson, 1985), and receives time information from SCN neurons. As a neurotransmitter, CRH acts within the brain to elicit changes in neuroendocrine, autonomic and behavioural activity similar to those observed after stress. Centrally administered CRH induces suppression of NK-cell cytotoxicity (Irwin *et al.*, 1988), an action which appears to be mediated through sympathetic activation as it can be counteracted by adrenergic-receptor blockade (Irwin *et al.*, 1990b). Stress-induced suppression of NK activity appears to be mediated by CRH, and can be antagonized by the central immunoneutralization of CRH (Irwin *et al.*, 1990a). The immunoregulatory role of CRH is not associated with the activation of the pituitary-adrenal axis (Irwin *et al.*, 1990a).

The corticotropic axis receives time information through inputs from oscillator neurons in the SCN to the CRH-ergic neurons in the paraventricular and arcuate nucleus, which release CRH into the hypophyseal portal vein, in a periodic and pulsatile pattern leading to the characteristic periodic and pulsatile ACTH release which is followed by corresponding pulses of cortisol secretion. The clinically manifest activity of the axis reflects the interaction of cycles of hormone secretion and of responsiveness of the endocrine target organs (pituitary and adrenal), and of the corticoid responsive peripheral tissues to the stimulation.

The rhythmicity of the corticotropic axis is quite stable and not rapidly altered in its circadian peak by minor changes of the sleep–wakefulness pattern, light, and other environmental stimuli. The normal circadian rhythm of the hypothalamic-pituitary-adrenal axis that is regarded as the major transducer of stress, is primarily regulated by the circadian oscillator system, and is only minimally modulated by sleep. Sleep onset is associated with an acute inhibition of cortisol secretion (Born *et al.*, 1988; Weibel *et al.*, 1995). Awakening during the night, and especially in the morning, is followed by secretory cortisol pulses (Pruessner *et al.*, 1997; Späth-Schwalbe *et al.*, 1991). These changes are absent if a person is prevented from sleeping.

Chronic insomniacs with difficulty falling or staying asleep, with less than 6.5 hours sleep time and a sleep efficacy of less than 80%, exhibited a significantly higher 24-hour ACTH and cortisol secretion than a matched control population with greatest differences in plasma concetrations in the evening and during the first half of the night (Vgontzas *et al.*, 2001). The circadian rhythm in ACTH and cortisol as such was maintained.

In clinically healthy diurnally active young men, partial sleep deprivation (sleep from 04:00 to 08:00) or total sleep deprivation for one night led on the following day to an elevation of plasma cortisol concentration during the evening (18:00 to 23:00), and the onset of the daily quiescent period in plasma cortisol was delayed (Leproult *et al.*, 1997).

In a study of semichronic sleep deprivation in 11 young men whose sleep time was restricted to 4 hours per night (01:00 to 05:00) for 6 nights, Spiegel *et al.* (1999) found similar changes in the 24-hour profile of plasma cortisol in comparison to the circadian profile of the same subjects studied after a 6-day recovery period. The changes observed after semichronic sleep deprivation also consisted of a shortened quiescent period (537 ± 44 minute versus 634 ± 24 minute) due largely to a delay in its onset of nearly 1.5 hours and raised cortisol concentrations in the afternoon and early evening (Spiegel *et al.*, 1999).

Some studies of sleep loss did not find evidence of a stress reaction in urinary cortisol and catecholamine excretion (Kant *et al.*, 1984) or plasma cortisol (Akerstedt *et al.*, 1980; Davidson *et al.*, 1991; Follenius *et al.*, 1992; Lange *et al.*, 2006; Vgontzas *et al.*, 2004), which, in part, may be due to the relatively short time span in which a deviation from the usual cortisol concentrations can be recognized. These studies suggest that sleep loss does not constitute an acute stimulus for the Hth-Pit-Adr axis, i.e. a "stressor."

The Hth-Pit-Adr axis possesses powerful and far reaching immunoregulatory activity. CRH directing the characteristic rhythmicity of this system also inhibits endotoxin-stimulated production of IL-1 and IL-6 by human monocytes. ACTH suppresses IFNγ production by human lymphocytes (Johnson *et al.*, 1984).

The glucocorticoids exert an extensive and multifaceted immunoregulatory activity. They are powerful anti-inflammatory agents inhibiting inflammatory mediators including cytokines, phospholipid products, proteases, and oxygen metabolites. They downregulate cytokine expression of IL-1, Il-2, IL-3, IL-6, IL-4, IL-8, IFNγ, and TNFα (for a review, see Petrovsky, 2001). In contrast to the downregulation of cell-mediated immunity, glucocorticoids enhance immunoglobulin production (Cooper *et al.*, 1979) and also

induce the macrophage migration inhibitory factor, a pro-inflammatory cytokine which counteracts and moderates the anti-inflammatory effects of glucocorticoids (Calandra *et al.*, 1995), maintaining a balance between the pro-inflammatory and anti-inflammatory components of the system. By stimulating the production of IL-4, IL-10 and IL-13, glucocorticoids favour the Th2 mode of the immune system (Ramírez *et al.*, 1996). The changes in the immune system found in sleep deprivation and shiftwork may in part be related to the circadian rhythm alterations in the Hth–Pit–Adr system experienced during these conditions.

Sleep loss, similar to aging, may slow down the rate of recovery of the corticotropic axis response following a challenge, and may facilitate the development of central and peripheral disturbances associated with glucosteroid excess. Especially elevated cortisol concentrations at the time of the normal daily quiet period may, in the long run, result in undesirable side-effects, such as memory deficits, insulin resistance, and osteoporosis (Dallman *et al.*, 1993; McEwen, 1998; Dennison *et al.*, 1999; Plat *et al.*, 1999).

In circadian phase shift, as may be experienced in shiftwork or under the effect of competing synchronizers between the light-directed SCN and peripheral stimuli like the time of food uptake, the rhythmic reaction of glucocorticoids inhibits the uncoupling of peripheral circadian oscillators from the central pacemaker (Le Minh *et al.*, 2001). This may counteract the internal circadian desynchronization and favour maintenance of the circadian time organization, and, if a phase shift takes place, determine in part the time of phase adaptation.

(c) Sleep deprivation and metabolism

Numerous studies over the last decade have consistently reported, with cross-sectional as well as with prospective design, an inverse relation between the numbers of hours of sleep and body weight in both children and adults with some age and gender differences noted (Vioque *et al.*, 2000; Sekine *et al.*, 2002; Cournot *et al.*, 2004; Hasler *et al.*, 2004; Patel *et al.*, 2004; Taheri *et al.*, 2004; Reilly *et al.*, 2005). Obesity in shiftworkers has been associated with a short duration of sleep (van Amelsvoort *et al.*, 1999; Moreno *et al.*, 2006). The incidence and degree were related to the duration of the shiftwork. A significant increase in the waist:hips ratio was found in workers after 2–5 years' involvement in shiftwork, and in the body mass index after more than 5 years in shiftwork (van Amelsvoort *et al.*, 1999). A causal relationship between sleep restriction and weight gain is supported by metabolic studies.

Spiegel *et al.* (1999, 2004b) studied 24-hour hormone and metabolic profiles in 11 young adult men after 6 days of sleep deprivation (4 hours' bedtime, 01:00 to 05:00) and 1 week of recovery. After 6 days of sleep deprivation, the mean circadian leptin concentration and the circadian amplitude of leptin were decreased, and ghrelin concentrations were increased together with increased hunger and appetite (Spiegel *et al.*, 2004a, b). The leptin concentrations were similar to the values found after calorie restriction (Chin-Chance *et al.*, 2000) in spite of adequate calorie intake. Similar findings of short sleep duration associated with reduced leptin, elevated ghrelin sampled at a single

time point in the morning and increased body mass index was reported by Taheri *et al.* (2004). In animal experiments, a marked increase in food uptake was found in sleep-deprived rats (Rechtschaffen & Bergmann, 1995). It appears that sleep deprivation alters the regulation of leptin and ghrelin production, and, accordingly, the feedback on the energetic needs and appetite control to the brain, which may lead to an increase in food uptake and represent a risk factor for obesity.

Estimations of the sympathovagal balance derived from recordings of heart-rate variability were significantly higher during sleep restriction (Spiegel *et al.*, 1999). The higher sympathetic activity may be related to metabolic changes (e.g., insulin-resistance) and other metabolic and cardiovascular changes. In sleep deprivation and during sleep-debt conditions, an impairment of carbohydrate tolerance develops with a slower rate of glucose clearance, with a decrease in glucose effectiveness, and a lower acute insulin response to glucose (Spiegel *et al.*, 1999) leading to conditions found in natural aging (Kahn *et al.*, 1993), and close to findings in non-insulin-dependent diabetics (Bergman, 1989) or gestational diabetes (Catalano *et al.*, 1993). Decreased carbohydrate tolerance and increased sympathetic tone are risk factors for the development of insulin resistance, obesity, and hypertension (Reaven *et al.*, 1996), corresponding to the condition described as "metabolic syndrome." It appears likely that some endemic disorders of the modern society like diabetes and obesity are in part a consequence of chronic sleep deprivation (Sekine *et al.*, 2002; Taheri *et al.*, 2004; Cizza *et al.*, 2005; Gangwisch *et al.*, 2005; Taheri, 2006). This includes the increased incidence of obesity in shiftworkers (van Amelsvoort *et al.*, 1999; Gangwisch *et al.*, 2005; Moreno *et al.*, 2006), which again, may be related to an increased cancer risk in these workers.

The high intake of dietary fat at night by rotating shiftworkers (40% of total calories) (Lennernäs *et al.*, 1994) leads to marked postprandial increases in triacylglycerols and non-esterified fatty acids such as linoleic acid (Holmbäck *et al.*, 2002). Linoleic acid provides a robust stimulatory signal for cancer growth via its mutagenic metabolite, 13-hydroxyoctadecadienoic acid (13-HODE). Elevated physiological nocturnal melatonin levels in the blood of human premenopausal women have the capacity to inhibit the uptake of linoleic acid, and its metabolism to 13-HODE, and tumour proliferative activity in (estrogen receptor negative/progesterone receptor negative (ER–/PgR–) and ER+/PgR+ tissue in isolated breast cancer xenografts perfused *in situ.* Exposure of these subjects to bright white light at night suppresses melatonin production resulting in substantially increased linoleic acid uptake, 13-HODE formation, and tumour proliferative activity in human breast cancer xenografts perfused *in situ*, with this melatonin-depleted blood. These results suggest that nocturnal circadian melatonin levels in women may protect against the breast cancer growth-promoting effects of increased dietary linoleic acid levels ingested at night (Blask *et al.*, 2005b).

4.3 Mechanistic arguments

Melatonin has been shown to have antiproliferative effects on human cancer cells cultured *in vitro*. These oncostatic effects have been observed at physiological

concentrations, and include reduction of cell-cycle progression by increasing the expression of the tumour suppressor gene *TP53*, and inhibition of DNA synthesis. In addition, melatonin reduces the invasive and metastatic properties of human cancer cells *in vitro*, and increases intercellular communication between these cells. There is evidence from animal models that melatonin inhibits or reduces the induction of DNA damage by free radicals. Pinealectomized rats showed a higher level of DNA damage in response to treatment with a carcinogen than did pineal-intact rats. Melatonin has also been shown to upregulate anti-oxidant enzyme systems.

Epidemiological studies on genetic polymorphisms in clock-related genes and phenotypes such as morning/evening preference and depressive symptoms, have shown a significant association between a single-nucleotide polymorphism in the *PER2* gene and diurnal preference. In a wider sense, the circadian clock may function as a tumour suppressor at the systemic, cellular, and molecular levels. Clock-controlled genes involved in cell-cycle control include *c-MYC*, *MDM*2, *TP*53 and *GADD45*a, as well as caspases, cyclins, and various transcription factors. In transgenic mice, a deletion in *Per2* results in a shorter circadian period, a higher susceptibility to radiation-induced tumours, and reduced apoptosis in thymocytes. The disruption of the circadian rhythm in mice is associated with the accelerated growth of malignant tumours.

Functional loss of the *Period* genes has been observed in various human tumours, and is probably based on epigenetic changes, i.e. the modulation of the methylation pattern in the promoter region. The loss of the clock protein function and the aberrant methylation of *PER1*, *PER2*, *PER3*, *CRY1* and *CRY2* promoters has been found in tumours of the breast, endometrium, lung, and in leukaemia. Artificially induced expression of *PER1* in non-small lung cancer cells *in vitro* results in a significant reduction in growth.

The human circadian gene *PER3* is linked to breast cancer risk. A polymorphic repeat region in this gene results in a PER3 protein of different length, which is associated with delayed sleep-phase syndrome, and diurnal preference. The variant genotypes are associated with an increased breast cancer risk in premenopausal women.

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5. Summary of Data Reported

5.1 Exposure data

Several types of shift systems exist, described according to several main characteristics: permanent or rotating, continuous (all days of the week are covered) or discontinuous (interruption at the weekend or on Sunday), or with or without night work. Other important organizational factors that may have an impact on health are: length of the shift cycle, duration of shifts, number of alternating workers/crews, start and finish time of the duty periods, speed and direction (clockwise or anticlockwise) of shift rotation, number and position of rest days and regularity/irregularity of the shift schedules.

The amount of night work in any shift period is the most important factor to be considered in the disruption of biological functions. The amount of sleep of the shiftworker decreases both in terms of quantity and quality, both on night shifts (due to circadian and environmental reasons) and on early-morning shifts.

Shiftwork, that includes night work, is estimated to involve about 15–20% of the total working population, although reliable and comparable statistics are not available in most countries. In Europe, large differences have been recorded among countries (from 6.4 to 30%), and between self-employed (5.7%) and employees (19.8%); in the USA, the average prevalence of shiftwork that includes night work is 14.8% (16.7% in men and 12.4% in women). Shiftwork is most prevalent among workers in the health care, transportation, communication, leisure and hospitality sectors (above 30%), and in the service, mining and industrial manufacturing sectors (20–30%). The prevalence of shiftwork is more common in work schedules of younger workers but decreases with the age of the workers, from more than 20% in the youngest decades of life to approximately 10% after 55 years of age.

At the time of writing, there is no known biomarker of exposure for shiftwork. However, because of the importance of melatonin in the relation to the activity in the circadian rhythm, levels of melatonin could be useful biomarkers of circadian disruption. Melatonin can be measured in the blood, saliva, or urine. The measurement of melatonin concentration in plasma at regular intervals (e.g. hourly) can identify the onset, offset and duration of melatonin secretion, the time at which peak secretion occurred and the total amount of melatonin secreted. Salivary melatonin concentration is a good alternative measure as it is highly correlated with serum concentrations. The primary urinary metabolite of melatonin, 6-sulfatoxymelatonin, may also be a useful biomarker.

The disruptive effects of night work on the biological functions and social life have been recognized by some regulators both at the national and international levels, i.e. the International Labour Organization, the European Union, and the Federal Aviation Administration.

5.2 Human carcinogenicity data

Female breast cancer

Eight studies from various geographic regions have been designed to assess the relationship between breast cancer and shiftwork that involves night work. Six of these eight studies, including two prospective cohort studies in nurses, have consistently pointed towards a modestly increased risk of breast cancer among long-term employees who performed night shiftwork, defined in different ways. Most studies reported this increased risk after controlling for potential confounders. Two of the eight studies, one of which appeared to be hampered by important limitations in design, were not supportive of an association between shiftwork and breast cancer. There were a relatively limited number of studies (most focused on a single profession, i.e. nurses), some potential for confounding by unknown risk factors, and inconsistent and inaccurate exposure assessments of shiftwork, which may have biased the results towards the null.

Another occupational group of shiftworkers is flight cabin crew personnel, who also experience circadian disruption due to the crossing of time zones. The incidence of breast cancer has been studied in eight cohorts of female flight attendants, all but one consistently reported an increased risk for breast cancer which was greater after a longer duration of employment. Limitations of these studies included the potential for detection bias among female cabin crew due to a higher prevalence of breast cancer screening in this occupational group, proxy measures of exposure used in dose–response relationships, and potential confounding by reproductive factors and cosmic radiation.

The Working Group concluded that the evidence for an association with breast cancer and shiftwork that involves night work was consistent in the studies that were specifically designed to address this question. The studies of cabin crews provided additional support.

Other cancers

Few studies have investigated the association between shiftwork and cancers at other organ sites. Increased risks of cancers of the prostate, colon, and endometrium have been reported. The earliest studies of airline pilots also showed a markedly elevated incidence of prostate cancer compared with national reference levels, but limitations of these studies included the potential for detection bias due to a higher prevelance of screening for prostate cancer in this occupational group.

5.3 Animal carcinogenicity data

Animal models have been used extensively to test the impact of the circadian system (central circadian pacemaker in the suprachiasmatic nuclei and the pineal gland/melatonin-generating system) and its disruption (i.e. phase shifts, light during the dark period, melatonin suppression) on tumour development and growth at all stages of oncogenesis.

Two studies examined the impact of continuous high-intensity light versus low-intensity light on tumour development in mice. One study demonstrated clear increases in the incidence of lung adenocarcinomas, leukaemias and lymphomas combined. The second study showed an increase in the incidence of and mortality from mammary tumours in one substrain that had normal vision, and no increase in a substrain of the same strain that had retinal degeneration due to genetic predisposition. A third study showed no effects.

All of the remaining experimental studies used initiation–promotion protocols or tumour growth models following the transplantation of syngeneic tumour fragments, cells, or human cancer xenografts. The species used in these studies included both sexes of rats, mice and hamsters, all of which yielded positive results in at least one study. The types of rodent model systems studied included mammary adenocarcinoma/fibroadenoma, cancers of the peripheral nervous system and kidney, hepatocarcinoma, pancreatic adenocarcinoma, colon adenocarcinoma, prostate adenocarcinoma, squamous-cell carcinoma and fibrosarcoma, osteosarcoma and carcinosarcoma, melanoma, neuroblastoma, and undifferentiated neoplasms.

The model systems used to study the role of the central circadian function and its disruption on cancer development and/or growth encompassed the exposure of animals to chronic alterations in the light–dark environment (i.e. constant bright light, constant darkness, altered light–dark schedules, intermittent light during darkness, single light pulse during darkness). Other model systems used more focused experimental manipulations that included phase-shifting central circadian activity only (i.e. exposure to experimental chronic jet lag), suppression or ablation of the nocturnal circadian melatonin signal (i.e. pinealectomy or exposure to dim light during darkness), ablation of the central circadian activity and of melatonin production (i.e. induction of lesions in suprachiasmatic nuclei), clock gene mutations (i.e. mPer2 knockouts) and the impact of carcinogen administration at different circadian times on tumorigenesis. A specialized model system evaluated the acute proliferative activity of tissue-isolated melatonin-receptor-positive murine or human tumours perfused *in situ* with different physiological levels of melatonin from natural diurnal blood changes and artificial manipulation.

The major patterns of light–dark environments that have an impact on cancer development and/or growth (i.e. stimulation) are constant light exposure (two positive of three studies, five positive of six initiation–promotion studies, five positive of five tumour-growth studies), dim light during darkness (five positive of five studies), experimental chronic jet lag (two positive of two studies), and circadian timing of carcinogens (four positive of four studies). Two conditions that produced no clear effects or even slowed tumour growth were light pulses during the dark period (two of two studies), and constant darkness (two of two studies). Mechanistically oriented animal studies specifically aimed at investigating the role of the pineal gland (i.e. pinealectomy-induced stimulation of cancer development and/or growth) and the nocturnal melatonin profile (i.e. inhibition of cancer proliferative activity) also had a major impact on cancer (18 positive of 26 studies). Furthermore, a limited number of studies on suprachiasmatic nuclei or clock genes yielded important results with respect to increased tumorigenesis (two positive of three studies).

5.4 Other relevant data

The evidence that relates laboratory investigations and mechanistic considerations to shiftwork-induced carcinogenesis can be divided into two basic fields: disturbance of the circadian system due to light at night with alteration of the sleep–activity pattern leading to potential melatonin suppression and circadian gene alterations; and sleep deprivation that results from the need to sleep when it is not readily possible and misaligned with the surrounding active daytime social environment.

The disturbance of the circadian system is studied at the level of the molecular circadian oscillation. Genes that are responsible for maintaining circadian rhythms have been identified, and may function as transcriptional factors and regulate expression of genes in cancer-related pathways, such as cell cycle, DNA repair, and apoptosis. Animal studies have shown that knockout of the circadian *Period* gene, *Per2*, promotes tumour development. Some evidence in humans links genetic polymorphisms in circadian genes to breast cancer and non-Hodgkin lymphoma, and functional loss of the *PERIOD* genes has been observed in various human tumours. Exposure to artificial light during the night has been demonstrated to disrupt circadian gene expression in mice and humans, which in turn, may alter circadian-regulated biological pathways. Because of their possible roles in tumorigenesis, the light-mediated dysfunction of circadian genes may provide a possible mechanism for the putative carcinogenic effect of light that may or may not involve melatonin.

The light-induced alteration of the circadian system is in part linked to the suppression of melatonin, which is secreted by the pineal gland and acts throughout the organism as a time signal. The suppression of melatonin leads to changes in the gonadotropic axis that specifically involves estrogens and androgens in experimental animals, and may be stimulatory or inhibitory depending on the species and the situation. In humans treated with low pharmacological doses of melatonin, few melatonin-induced changes were documented except a stimulation of prolactin. In animal studies, melatonin in the target sites interferes with the metabolism of estrogen through several metabolic pathways. No data clearly link nocturnal blood levels of endogenous melatonin with endogenous production of estradiol in women.

The decrease in endogenous melatonin may lead to diminished free radical scavenging that may induce local tissue damage, the extent and importance of which is not entirely clear at present.

Melatonin is a direct and indirect immunostimulant; its suppression leads to a state of immunodeficiency that is aggravated by the pronounced effects of sleep deprivation upon the immune system. Prolactin, a strong immunostimulant, is decreased during sleep deprivation.

Direct inhibitory effects of melatonin on tumour cell proliferation have been shown in several animal models not only at pharmacological but also at physiological concentrations.

Sleep deprivation is a common feature in most forms of shiftwork that involves night and/or early morning hours. Changes in the immune system have been shown to occur in

partial (early or late night) sleep deprivation and comprise changes in the cytokine pattern that favours the Th2 group of cytokines and decreases Th1 cytokines (e.g. interferon γ) which act in cellular immune defence and in immune surveillance to counteract tumour growth. In the majority of studies of sleep deprivation, suppression of natural-killer-cell activity has been shown, and this also leads to a decrease in anti-tumour surveillance.

The evidence in support of shiftwork-induced carcinogenesis thus links events at the cellular level that affect cell proliferation and endocrine changes with hormonal constellations that promote endocrine-dependent cancers with defects in the immune surveillance that enhance tumour development and growth.

None of these changes stands in isolation; they are all linked to the disruption of the circadian system of shiftworkers and, in combination, may alter the risk of cancer through both tumour induction and promotion.

The experimental data from animal studies in several inter-related physiological systems are strongly suggestive of a causal link between circadian disruption and all its consequences and the development of malignant tumours. Human studies are suggestive of physiological effects that are possibly relevant to carcinogenesis.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *limited evidence* in humans for the carcinogenicity of shiftwork that involves night work.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of light during the daily dark period (biological night).

6.3 Overall evaluation

Shiftwork that involves circadian disruption is *probably carcinogenic to humans (Group 2A)*.