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Chronotoxic Effects of Hydrogen Sulfide on Rhythmic Slow Activity in the Rat Hippocampus

By

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Abstract

Several potentially noxious agents have been shown to vary in their effects as a function of the biological time of exposure. The neurotoxic effects of hydrogen sulfide (H_2S) are well documented, however there is no information regarding a circadian rhythm of this toxicant. The objective of this thesis was to determine whether the neurotoxic effects of H_2S were influenced by circadian rhythms.

Rats were exposed to 125 ppm H_2S for 3 hours per day for 5 consecutive days. Hippocampal electroencephalographic activity (theta) was measured during movement. Repeated exposure to H_2S resulted in a decrease in the peak power and total power of theta activity during the light period, and an increase in peak power and total power in the dark period. A decrease in the peak frequency of theta was found in both the light and dark periods. It is suggested that the effects of H_2S are circadian-dependent.

Acknowledgements

I would like to thank my supervisor, Dr. Sheldon H. Roth, for his guidance and patience during my research, and Dr. Robert S. Sainsbury, for providing advice and input along the way. I would also like to thank Ron Bennington, for the many hours of technical support.

Dedication

This thesis is dedicated to my husband, Spenser, my daughter,

Bethany, and to my parents, Leonard and Della MacGuigan.

Thank you for your love and support.

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Introduction

As a result of biological rhythms, the response of an individual to chemical challenges varies dramatically over a 24 hour period. For several toxic substances these biological time-related differences in the intensity of the observed effect is correlated with differences in the capability of the liver and kidney to detoxify or clear harmful substances from the blood as well as differences in the susceptibility of the target system (Reinberg, 1992). The study of time-related changes in toxicity of chemicals is known as chronotoxicology. Chronotoxic effects are relevant to individuals required to work on a rotating shift schedule, who are at risk for exposure to harmful substances throughout the 24 hour day. Variations in toxic effects due to the time of exposure have been demonstrated for several chemicals commonly found in the workplace, using laboratory animal experiments. These include organophosphorous pesticides (Fatranska *et al.*, 1978), trichloroethylene (Motohashi and Miyazaki, 1990), benzene (Starek *et al.*, 1989), toluene (Harabuchi *et al.*, 1993) mercuric chloride (Tsai *et al.*, 1981), and chloroform (Lavigne *et al.*, 1983).

Many industries use or produce hydrogen sulfide (H_2S), a neurotoxic and potentially fatal gas. H_2S is a common hazard in the oil and gas industry, and poses a significant problem in the province of Alberta due to the high concentration of oil and gas fields. Some occupations associated with this industry require continuous 24-hour operations, requiring that individuals work night shifts, in addition to the regular day shift schedule. As a result, they can be at risk for exposure to H_2S throughout the 24 hour day. Therefore, shift-workers can experience exposure to H_2S at a time when they may be

more susceptible to toxic insult. For this reason, it is important to determine whether the effects of H_2S are more or less toxic depending on the time of exposure.

Circadian Rhythms

All living organisms exhibit non-random periodic variation in physiological and behavioral processes. These variations correlate to the daily time period of 24 hours, and are known as circadian (from the Latin "*circa*", meaning around, and "*diem*", meaning day) rhythms. Circadian rhythms are normally entrained by the natural light/dark cycle, with regular and predictable peaks and troughs occurring at specific times during the 24-hour day. A common example of a circadian rhythm is that which occurs in the alternation of rest and activity. Diurnal mammals, such as humans, are most active during the light period and are at rest during the dark. The reverse is true for nocturnal mammals, such as the laboratory rat. This animal is most active during the dark period, and sleeps in the light. In addition to the alternation of rest and activity, circadian rhythms are prevalent in most biological systems. In humans, body temperature and heart rate are higher during the day than during the night (Hakola *et al.*, 1996), and the reverse is true of rats (Gordon, 1990). Rhythmicity also characterizes events occurring at the subcellular level. The synthesis and release of neurotransmitters in the brain exhibit circadian rhythms in mouse and rat (Wirz-Justice, 1987) and receptor binding studies have shown that the number of receptors available for occupancy by neurotransmitters and neuropeptides varies in a circadian fashion (Wirz-Justice *et al.*, 1983). The activities of liver enzymes and functions underlying renal clearance also vary over 24 hours (Reinberg, 1992).

Circadian rhythms are normally entrained to the light/dark cycle, however, it has been observed that in the absence of environmental time cues, circadian rhythmicity persists in physiological and behavioral processes (Meijer and Reitveld, 1989). The suprachiasmatic nucleus (SCN), located in the anterior hypothalamus, is believed to be the internal pacemaker controlling circadian rhythms (Moore, 1983). The SCN receives photic information directly from the retina via a projection known as the retinohypothalamic tract (RHT) (Moore-Ede, Sulzman and Fuller, 1982). This provides a way for information on the external light/dark cycle to influence the functions and rhythmic output of the SCN. The importance of the RHT in entrainment of circadian rhythms is evidenced by the fact that transection of the RHT results in a complete loss of the ability of the environmental light\dark cycle to entrain circadian rhythms (Rosenwasser and Adler, 1985). However, the SCN itself appears to be the major internal pacemaker for circadian rhythms. Lesions of the SCN result in the elimination of the circadian rhythms in many overt physiological and behavioral processes. Studies have shown disruptions in such variables as wheel-running activity, feeding, drinking, sexual activity, body temperature, sleep/wake cycle, and also the synthesis and/or secretion of several hormones including adrenal corticosterone, adrenocorticotropic hormone, pineal melatonin, pituitary prolactin, and gonadotrophin (Rossenwasser and Adler, 1986). In addition, Kafka *et al.*, (1985) showed that SCN lesions abolished circadian rhythms in benzodiazepine and alpha-adrenergic neurotransmitter receptor binding in the rat. These dramatic effects of SCN lesions indicate that this structure is critical for the generation of circadian rhythmicity.

Chronopharmacology

Chronopharmacology is the study of how the effects of drugs vary with the biological timing of administration. It has only been recognized as an important aspect of traditional pharmacology since the early 1970's (Reinberg, 1992). Since then, the effectiveness and toxicity of many drugs have been shown to vary over the 24-hour period. This is exemplified by the fact that many drugs delivered at a constant rate do not provide constant plasma levels over 24 hours. For ketoprofen, a non-steroid anti-inflammatory drug (NSAID), constant venous infusion resulted in predictable changes in plasma concentration, with a peak at approximately 2300h and a trough at approximately 0900h (Reinberg, 1990).

Chronokinetics refers to the influences of rhythmic changes in absorption, distribution, metabolism and excretion on the biological effectiveness of a drug (Nagayama, 1993, Reinberg, 1992; Reinberg 1988;). Circadian rhythms occur in the rate of intake from the stomach, lungs and skin, plasma protein binding, and liver and kidney functions. These factors play a role in determining how quickly the actions of the drug will take effect, how much of the drug will actually become bound in plasma, and how long the drug will remain active. For example, the effectiveness of theophylline varies over a 24 hour period, which is determined by a circadian rhythm in absorption from the gut (Reinberg, 1990). On the other hand, a circadian rhythm in rate of renal clearance is the primary determinant in the physiologic action of lithium (Nagayama, 1993).

The changes in absorption, distribution, metabolism and excretion cannot account for the entire circadian rhythm of drug action. Chronesthesy refers to the rhythmic

changes in the susceptibility or sensitivity of a target system to a drug, which cannot be explained by chronokinetic changes (Reinberg, 1992). Circadian rhythms can occur in a target organ, such as the skin, the bronchial tree, or any of the internal organs that are directly affected by drug administration (i.e. stomach, heart, and pancreas). Circadian rhythms are also present in target sites, located at the molecular level. This can account for the rhythmic nature of the effectiveness of drugs that act by binding to specific receptors in the CNS, or those that alter membrane structure and enzyme activity. Variations in individual susceptibility to a drug over a 24-hour period are a result of rhythms present within the tissue that is the site of drug action. These endogenous rhythms are responsible for circadian changes in the effectiveness of a drug (Nagayama, 1993).

The rhythmic differences in desired effectiveness or toxicity of a drug on the organism as a whole is termed chronergy (Reinberg, 1992). The temporal variations in effectiveness and toxicity of a drug are dependent on its chronokinetics and chronesthesy. This is an important concept in that it attempts to characterize predictable fluctuations in drug effects, which has therapeutic implications. By including reference to dosing time when drugs are administered, it is possible to maximize the desired effect of the drug, while minimizing harmful or toxic side effects (Reinberg and Levi, 1990).

Chronotoxicology

The same principles employed in chronopharmacology can be applied to the study of exposure to harmful substances or pollutants. Just as the effectiveness of drugs can

vary depending on the time of administration, chemicals that people may come into contact with through their daily activities can be more or less toxic depending on the time of exposure. This is known as chronotoxicology. There are many ways in which to come into contact with harmful chemicals. People who live in urban settings are exposed to many sources of air pollution, arising from such things as vehicle emissions and industrial activities. In a study by Stupfel *et al.* (1977), carbon monoxide (CO, 300 ppm), a major constituent of air pollution, was administered through inhalation to Sprague-Dawley rats between 1030h and 1330h, or between 2230h and 0130h. CO_2 emission was measured as an indicator of gaseous metabolism and activity. It was shown that CO was more toxic during the dark period, the middle of the activity span, as the emission of CO_2 was decreased. This suggests that diurnally active mammals, such as humans, are likely to be more sensitive to the toxic effects of CO at the same time as activities contributing to the production of CO in the air are at a peak.

Another source of pollutants and toxicants is the workplace. Many people are employed in occupations which require them to work in rotating shifts, i.e., both at night and during the day, and as such are at risk for exposure to harmful chemicals at all times of their circadian cycle. Several industries commonly use chemicals in their operations, many of which have been examined for chronotoxic effects in laboratory animals (Table 1). The toxicity of trichloroethylene (TRI), widely used as a degreasing agent in industry, has been shown to vary in a circadian manner (Motohashi and Miyazaki, 1990; Motohashi *et al.*, 1990). A single intraperitoneal (i.p) injection of TRI exerted maximal effects during the early dark period (2100h), as assessed by decreased muscle tone on an inclined

TABLE 1. Illustrative Examples of Circadian Rhythms in Toxic Effects of Various Chemicals

Toxic Agent	Index of Toxic Effects	Species (sex)	Synchronization L=light, D=dark	Dose(s) (no. of time points over 24 hours)	Peak time of toxicity	Reference
Styrene	Liver enzymes	Sprague-Dawley rats (M)	L: 07.00-19.00 D: 19.00-07.00	1.0 g/kg (4)	03.00 hr, mid-activity period	Desgagne & Belanger, 1986
Benzene	% Mortality	Wistar rats (M)	L: 08.00-20.00 D: 20.00-08.00	1.1 g/kg (6)	20.00 hr, onset of activity period	Starek et al., 1989
Trichloro-ethylene	Motor activity Muscle tone	Wistar rats (M)	L: 08.00-20.00 D: 20.00-08.00	1.2 g/kg (4)	09.00 hr, early rest period 21.00 hr, early activity Period	Motohashi & Miyazaki, 1990
Trichloro-ethylene	TRI metabolite concentration, Serum enzymes	Wistar rats (M)	L: 06.00-18.00 D: 18.00-06.00	1.2 g/kg (4)	09.00 hr, early rest period 09.00 hr, early rest period	Motohashi et al., 1990
Potassium Cyanide	% Mortality	BALB/cCr mice (M)	L: 06.00-18.00 D: 18.00-06.00	72.5 mg/kg (6)	16.00 and 20.00 hr, late rest and early activity periods	Bafitis et al., 1978
Toluene	Blood and brain concentration, Shock avoidance	Wistar rats (M)	Inverted L: 21.00-09.00 D: 09.00-21.00	2000 or 4000 ppm over 4 hours (2)	22.00-02.00 hr, mid-rest period	Harabuchi et al., 1993
Carbon monoxide	CO_2 emission V CO_2 %	Sprague-Dawley, CFE rats (M,F)	L: 06.00-18.00 D: 18.00-06.00	300 ppm (2)	00.00 hr, mid-activity period	Stupfel et al., 1977

plane test in male Wistar rats (Motohashi and Miyazaki, 1990). It was also shown that i.p. injections of TRI given for three consecutive days induced a disruption in the circadian rhythm of spontaneous locomotor activity, and injections given at 0900h produced a greater disruption than those given at 2100h. Motohashi *et al.* (1990) examined the circadian rhythm of TRI toxicity on serum glutamic-pyruvate transaminase activity (GPT), blood urea nitrogen concentration (BUN), serum total cholesterol, and tryglyceride concentrations. All were maximally increased following TRI administration at 0900h. Assays of TRI metabolites trichloroethanol and trichloroacetic acid in the blood also revealed maximal increases following TRI administration at 0900h.

Toluene, another commonly used organic solvent, was also studied for chronotoxic effects on male Wistar rats (Harabuchi *et al.*, 1993). Animals were exposed to either 2000 or 4000 parts per million (ppm) by inhalation over a period of 4 hours either in the mid-light or mid-dark period. Performance decrements on a shock-avoidance task were greater in the light period, and it was demonstrated that the greatest concentrations of toluene in both blood and brain also occurred following exposure during the light period.

Hydrogen Sulfide

Hydrogen sulfide (H_2S) is an extremely toxic gas to humans and animals (Roth, 1993). Exposures to H_2S can occur in a wide variety of environmental and occupational settings. A number of physical and neurological symptoms have been described following H_2S poisoning. Chronic exposures to low concentrations have been

associated with many problems, such as headaches, anxiety and nervousness, insomnia and somnolence, and learning and memory deficits (Reiffenstein *et al.*, 1992). In many occupations associated with the use or production of H_2S, people work in shifts, most of which include nighttime rotations. It is therefore important to characterize the circadian rhythm of H_2S toxicity.

The first reported incident involving H_2S was described by Ramazzini (1713). He noted painful irritation and inflammation of the eyes of sewer cleaners, and attributed this to the exposure of some volatile acid released from the cleaning process. In 1775, a chemist, Carl Wilhelm Scheele, was credited with discovering H_2S (see Roth, 1993). Beginning in the nineteenth century, and continuing to present day, animal studies have been carried out on many different species to gain more information on the mechanisms of this gas (Glass, 1990, Alberta Health, 1988). In addition, the documented cases of human exposure to H_2S have grown. These consist mainly of exposures that have occurred in the workplace, due to accidental release of the gas at high concentrations.

Physical and Chemical Properties

Hydrogen sulfide is a flammable, colorless gas, heavier than air (d=1.19) at standard temperature and pressure (STP) (Beauchamp et al., 1984). It has a characteristic odor of rotten eggs. Hydrogen sulfide is soluble in water, and in solvents such as alcohol, ether and glycerol. Because of its high lipid solubility, it can easily penetrate biological membranes. In aqueous solutions, H_2S dissociates in two steps. The first step yields a hydrosulfide anion (HS^-) and the second a sulfide anion ($S^=$). The pKa in 0.01-0.1

mol/litre solutions at 18°C is 7.04 for HS^- and 11.96 for $S^=$. At physiologic pH of 7.4, about one third of H_2S is present as undissociated H_2S and the remainder is largely HS^- (Beauchamp *et al.*, 1984).

Sources of Hydrogen Sulfide

Hydrogen sulfide is found in both environmental and industrial settings. It is one of the principal compounds involved in the natural sulfur cycle. It is produced by anaerobic decomposition of organic-rich material (WHO, 1981). H_2S is also a contaminant of natural gas, volcanic gases, and sulfur springs. It is estimated that 90 to 100 million tons of H_2S is produced annually from natural sources (Beauchamp *et al.*, 1984). An additional three million tons/year of H_2S is emitted from industrial pollution (Beauchamp *et al.*, 1984) . There are various sources of environmental H_2S resulting from human activity. Over 70 occupations have been listed that use H_2S or produce it as a by-product (NIOSH, 1977). For example, H_2S is used as a reagent in the production of sulfides, sodium hydrosulfide, and various organic sulfur compounds (WHO, 1981). H_2S is also used as a moderator in some nuclear power reactors in the production of heavy water (WHO, 1981). It is released during extraction and drilling of natural gas and crude oil deposits and is a by-product of the oil refining process. Other examples of occupations with potential for exposure to H_2S include tannery workers, well diggers, papermakers, farmers, coke oven workers, textile printers, and septic tank cleaners (Roth, 1993).

Concentrations of H_2S in natural or urban settings have been estimated. In 1970, Robinson & Robbins estimated the average ambient air level to be 0.0003 mg/m^3 (0.0002

ppm) (WHO, 1981). Much higher concentrations have been measured near sources of H_2S. In California, peak concentrations as high as 0.20 mg/m^3 (0.13 ppm) have been measured in a community near a pulp and paper mill (WHO, 1981). In New Zealand, discharge of industrial and domestic waste into an inlet produced levels of H_2S sufficient to cause paint blackening and complaints of offensive odors. Air monitoring in the area for 21 months indicated that 40 minute average H_2S concentrations ranged from 0.8 to 1.4 mg/m^3 (0.5 to 0.96 ppm) (WHO, 1981).

The US National Institute of Occupational Safety and Health (NIOSH, 1977) have set occupational exposure limits of 15 mg/m^3 (10 ppm) based on an 8 hour time-weighted average (TWA). Under normal conditions, it is believed that levels of H_2S in the air are less than the occupational exposure limits, however it is well documented that accidental release of high concentrations occur (Guidotti, 1994; Roth, 1993; Tvedt *et al.*, 1991a; Tvedt *et al.*, 1991b; Glass, 1990).

Health Effects of Hydrogen Sulfide on Humans

Most organ systems are vulnerable to the effects of H_2S, and for this reason H_2S has been referred to as a broad-spectrum toxicant. The systems most susceptible to H_2S are those with exposed mucous membranes and those with high oxygen demands (Reiffenstein et al, 1992).

The occupational exposure limit set by NIOSH in 1977 was based on the ability of H_2S to produce symptoms of eye irritation, commonly referred to as "gas eye", at levels above 15 mg/m3 (10 ppm) (Beauchamp *et al.*, 1984). Symptoms of exposure to levels of

70 mg/m^3 (50 ppm) or greater results from local inflammation of the conjunctiva and cornea, and include lacrimation, burning sensation and photophobia. In more serious exposures the cornea can become cloudy and cause blurred vision (Tvedt *et al.*, 1991b).

At low concentrations, around 0.0008 to 0.20 mg/m^3 (0.0005 to 0.13 ppm) H_2S has a detectable odor. At 4.2 to 42 mg/m^3 (3 to 30 ppm) the smell intensifies (Beauchamp,1984). Above 42 mg/m^3 (30 ppm) the odor becomes sickeningly sweet (see Roth, 1993). Concentrations above 140 mg/m^3 (100 ppm) can produce olfactory fatigue, with the result that H_2S is no longer detectable by scent. Olfactory fatigue can occur gradually during exposure to low levels of H_2S or rapidly when high levels are present (Beauchamp *et al.*, 1984). For this reason, the odor of H_2S is not a good warning signal to detect H_2S in the atmosphere.

Since the primary route of entry into the body is the lungs, much work has been done to evaluate the effect of H_2S on the respiratory system. Exposure to concentrations as low as 70 mg/m^3 (50 ppm) can cause laryngitis, bronchitis and pneumonia. Concentrations above 350 mg/m^3 (250 ppm) can produce pulmonary edema (Beauchamp *et al.*, 1984). Acute high concentrations of H_2S cause respiratory failure leading to death. Above 700 mg/m^3 (500 ppm), death can occur in a matter of hours or minutes. It is believed that this effect is due to paralysis of the respiratory centre of the brain (WHO, 1981).

The nervous system is the primary target of H_2S toxicity (Roth, 1993). Acute exposure leads to sudden fatigue, vertigo, intense anxiety, convulsions, unconsciousness and respiratory failure (Reiffenstein *et al.*, 1992). Chronic exposure results in a variety of

symptoms, including headache, light-headedness, memory impairments, fatigue, listlessness, weakness of extremities, agitation, emotional disturbances, delirium, and sleep disturbances, either excessive sleepiness (somnolence) or insomnia (Roth, 1993, Beauchamp *et al.*, 1984). A study by Tvedt *et al.* (1991b) examined the neurological symptoms of six adult males after loss of consciousness caused by H_2S exposure. Examinations included electroencephalographic (EEG) recording, neuropsychological testing for learning and retention, and visual reaction time. In all cases but one, learning and memory deficits were evident between five and ten years later. Of the five patients with learning and memory deficits, three were found to have abnormal EEG activity, described as either an increase in theta activity or theta dysrhythmia as measured from surface electrodes.

Toxicokinetics of Hydrogen Sulfide

Absorption, Distribution, Metabolism and Elimination

In humans the lung is the primary route of absorption. Voigt & Muller (1955) demonstrated that inhalation exposure in rats and guinea pigs resulted in distribution of H_2S to the brain, liver, kidneys, pancreas and small intestine. Warenycia *et al.* (1989a) has shown selective uptake of sulfide by the brainstem, as compared to the cerebellum, hippocampus, striatum and cortex in rats. The animals were injected i.p. with sodium hydrosulfide (NaHS), which when administered to animals, generates H_2S in vivo. Analysis of endogenous sulfide levels in the brainstem revealed that this area has significantly lower amounts of sulfide than other brain areas studied. In addition,

subcellular fractionation demonstrated that sulfide was 2-3 times higher than control animals in fractions enriched in myelin, mitochondria and synaptosomes after injections of 50 mg/kg NaHS.

It has been shown that, following i.v. administration of [^{35}S]-sodium sulfide in dogs, less than 0.5% of H_2S was exhaled from the lung, and excretion in this manner was complete within one minute after injection (Guinea, 1957). Similar results have been obtained with rats and rabbits after an i.v. injection of nonradioactive H_2S (Evans, 1967).

There are three pathways responsible for the metabolism of H_2S (Figure 1). Oxidation of sulfide to sulfate and excretion of sulfate by the kidney is the main metabolic and excretory route. Dziewiatkowski (1945) showed that 50% of an oral administration of [^{35}S]-barium sulfide was excreted in the urine as sulfate within 24 hours. Early *in vitro* studies suggested that the liver and kidney preparations catalyzed the oxidation of sulfide to intermediates such as free sulfur, polythionates and thiosulfate prior to formation of sulfate (Beauchamp *et al.*, 1984). It has been proposed that the oxidation of sulfide to thiosulfate is enzymatically catalyzed by sulfide oxidase, preferentially associated with the liver mitochondrial fraction (Baxter *et al.*, 1958). Further *in vitro* experimentation has found that glutathione stimulates mitochondrial oxidation of [^{35}S]-thiosulfate to [^{35}S]-sulfate (Koj *et al.*, 1967, Bartholemew *et al.*, 1980). Glutathione is involved in the reductive cleavage of thiosulphate to give sulfite, which is then oxidized to sulfate and some form of sulfide by sulfite oxidase, which is recycled to give more thiosulfate (Bartholemew *et al.*, 1980).

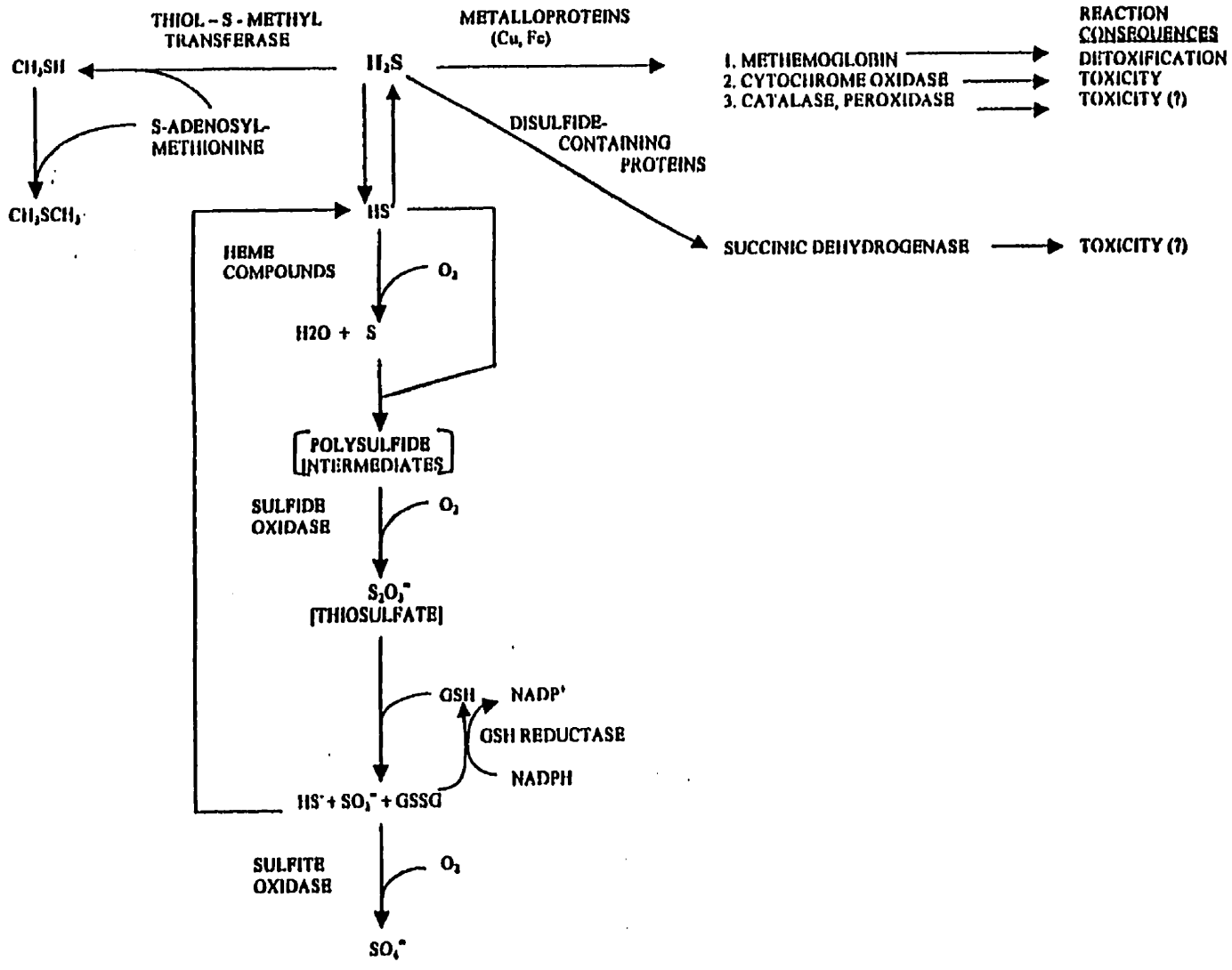


FIGURE 1. Metabolism of H_2S (adapted from Beauchamp *et al.*, 1984)

A second metabolic pathway is the methylation of H_2S to methanethiol (CH_3SH) and dimethylsulfide (CH_3SCH_3), both of which are less toxic than H_2S. This pathway has been identified as important for the metabolism of sulfhydryl-containing protein by anaerobic bacteria in the intestinal tract, which constitutes an endogenous source of H_2S. However, the significance of this route for metabolism and excretion of exogenous H_2S has not been determined (Beauchamp *et al.*, 1984).

The third metabolic pathway is the reaction of H_2S with metallo- and disulfide-containing proteins (Beauchamp *et al.*, 1984). This is considered to be the primary mechanism by which H_2S exerts its toxic effects. It has been known for some time that H_2S reacts with cytochrome oxidase, inhibiting mitochondrial respiration. Khan *et al.* (1990) found that H_2S produced significant decreases in the activities of cytochrome c oxidase in lung mitochondrial respiratory chain both in vivo and in vitro. This effect is similar to that observed with cyanide, however inhibition by H_2S is more potent (Smith *et al.*, 1977). Recently, Roth *et al.* (1997) have demonstrated inhibition of cytochrome oxidase and carbonic anhydrase in rat brain. Sodium sulfide was shown to inhibit both enzyme systems in a concentration-dependent manner, however, was less effective than specific inhibitors of both enzymes. The dose required to produce an inhibition of 50% of control values (IC_{50}) was calculated to be 0.13 μM for cytochrome oxidase, and 2.17 μM for carbonic anhydrase. The inhibition of enzyme systems responsible for cellular respiration has long been regarded as being responsible for the lethal effects of H_2S.

Hydrogen sulfide also has the potential to reduce disulfide bridges in proteins (Beauchamp *et al.*, 1984). Smith and Abbanat (1966) showed that administration of

oxidized glutathione (GSSG, a disulfide) protected mice against lethality. Scavenging of hydrosulfide by the disulfide linkage of oxidized glutathione and preventing reaction of sulfide with other more critical enzymatic sites was the proposed mechanism of protection.

Neurotoxicology of Hydrogen Sulfide

There is considerable evidence from animal studies that H_2S is a neurotoxic gas. Numerous studies have examined the effects of H_2S on the brain *in vivo*, on both adult and developing organisms, and on *in vitro* neuronal preparations. Hydrogen sulfide has been shown to affect development of the brain in rat pups exposed to H_2S in utero. Repeated exposures of rat pups to 75 ppm H_2S for 7 hours a day from day 5 postcoitus to day 21 postnatal resulted in alterations of amino acid concentrations in the cerebellum and cerebral cortex (Hannah *et al.*, 1989). Aspartate, GABA and glutamate were decreased in the cerebellum on day 21 postnatal, whereas there was an initial increase in taurine which returned to control levels by day 21. In the cerebral cortex, levels of all of the amino acids were altered by the treatment. Aspartate and GABA were reduced on days 7, 14 and 21 postnatal, glutamate was initially reduced on day 7 but returned to control levels by day 21, and taurine was initially increased, but returned to control levels by day 21. Skrajny *et al.* (1992) has demonstrated that exposure to 25 and 75 ppm H_2S alters serotonin (5-HT) and norepinephrine (NE) levels in the developing rat cerebellum and frontal cortex. To determine if the effects of H_2S on the developing organism are long-lasting or are reversible, Roth *et al.* (1995) measured levels of monoamines in various brain regions on days 21, 30, 45 and 60 postnatal, following exposure to low concentrations of H_2S from

day 5 postcoitus to day 21 postnatal. It was found that, in general, tissue levels of monoamines were altered on day 21, then returned towards control values from day 21 to day 60. Chronic exposure has also been shown to alter the growth and morphology of developing cerebellar Purkinje cells (Hannah and Roth., 1991). Cells exhibited increased branch length and vertex path length, and variations in the number of branches in certain areas of the dendritic field. Also, the treated cells displayed nonsymmetrical growth pattern at a time when random terminal branching is normally occurring.

Endogenous H_2S can be formed from cystine by cystathionine β-synthase (Swaroop *et al.*, 1992). Endogenous levels of H_2S have been demonstrated in the brain (Warenycia *et al.*, 1989a), with regional differences occurring in the amount of sulfide detected. The brainstem was found to contain the lowest level of endogenous sulfide, while following administration of 50 mg/Kg NaHS, the brainstem also showed the greatest increase in sulfide levels (Warenycia *et al.*, 1989a). The selective accumulation of sulfide in the brainstem could be explained by the greater solubility of H_2S in lipophilic solvents as compared to water, as the brainstem is composed largely of white matter, which contains more lipids than grey matter. Since the brainstem contains the centers that control central respiratory drive, this selective accumulation of sulfide could account for the rapid respiratory paralysis seen following acute exposure to high concentrations of H_2S in humans.

Biochemical studies examining the acute effects of H_2S administration have demonstrated that amino acid and neurotransmitter levels are altered. Kombian *et al.* (1988) found that levels of aspartate, glutamate, glutamine, GABA, glycine, taurine and

alanine increased in the brainstem, and aspartate and glycine decreased in the cerebellum of rats treated with doses of 10 or 30 mg/kg NaHS. Several of the amino acids that were found to be changed in the brainstem following NaHS are thought to play a role in the control of central respiratory neurons. Warenycia *et al.* (1989b) demonstrated an increase in 5-HT and dopamine levels in the brainstem, and the hippocampus, striatum and brainstem all showed increases in noradrenaline and adrenaline. In addition, monoamine oxidase (MAO) activity was inhibited in both *in vivo* and *in vitro* preparations, although the dose required for this inhibition to occur *in vivo* was significantly higher than *in vitro*.

Biochemical effects of H_2S have also been demonstrated in guinea pigs. Exposure to H_2S at 10 ppm, 1 hour daily for 11 days, has been shown to cause a decrease in total lipids, phospholipids and cholesterol in the cerebral cortex, cerebellum and brainstem, and an increase in lipid peroxidation (Haider et al., 1980). Also, Haider and Hasan (1984) have shown neurotoxic effects in guinea pigs following exposure to sulfur dioxide (10 ppm) and H_2S (20 ppm) one hour daily for 30 days. This combined exposure resulted in a decrease in the levels of total lipids and cholesterol in the cerebral cortex, basal ganglia, brainstem, and spinal cord. Phospholipids were decreased in the cerebral cortex and spinal cord, however they were increased in the cerebellum. Lipid peroxidation was increased in all brain areas and in the spinal cord.

There is some evidence that the effects of repeated exposures to low concentrations of H_2S can be cumulative. Savolainen et al. (1980) demonstrated that exposure of mice to 100 ppm H_2S for 2 hours a day at 4 day intervals for 4 days resulted in cumulative inhibition of cerebral cytochrome oxidase activity. Also, on the fourth day of

exposure, cerebral RNA was decreased, which was accompanied by a reduction of orotic acid uptake in the RNA fraction. Initial exposures caused an increase in glutathione concentration, which returned to baseline values following the third and fourth exposures.

In vitro neuronal preparations have been used to study the mechanisms of action of H_2S neurotoxicity. In 1930, Shmitt and Beck examined the effects of H_2S on nerve irritability using nerve-muscle preparations from green frogs. It was found that H_2S caused a slow, gradual increase in the threshold of responsiveness of the nerve to stimulation, followed by a very rapid rise in the nerve threshold after a period of about 2 hours, with the end result being a complete loss of nerve irritability.

H_2S has also been studied on sciatic nerve bundles from *Rana pipiens* (Beck et al., 1983). Very high concentrations (5300 ppm - 987,000 ppm) were found to cause an initial decrease in compound action potentials in nerves, and resulted in complete unresponsiveness of nerves over a 65 minute testing period. The highest concentrations tested also resulted in a reduction of conduction velocity. Nerves treated with H_2S showed recovery of both the conduction velocity and action potentials after a period of about 1 hour. The action potentials recorded were generally greater than before the exposure, and continued to be greater throughout the stimulation period.

Patch-clamp studies of cultured murine neuroblastoma cells have shown that 5-10 mM NaHS, in combination with either taurine or cysteic acid, reversibly abolished inward sodium currents (Warenycia *et al.*, 1989). The application of NaHS, taurine, or cysteic acid alone had no effect on sodium currents. The sulfhydryl reagents β-mercaptoethanol and dithiothreitol were also shown to reversibly abolish sodium currents, suggesting that

the synergistic action of NaHS with taurine or cysteic acid may result from reduction of the disulfide bonds between subunits comprising the sodium channel. Taurine has been proposed to be an inhibitory transmitter of central brainstem respiratory neurons (Champagnat *et al.*, 1982). These results suggest that reductions in sodium channel function may be at least partially responsible for loss of central respiratory drive during H_2S poisoning.

The brainstem contains several discrete nuclei that are involved in the generation and maintenance of the respiratory rhythm. The serotonergic neurons of the dorsal raphe pontine nucleus have been studied as a model to examine the actions of H_2S *in vitro* (Kombian *et al.*, 1993). Application of toxicologically relevant concentrations (30-400 μM NaHS) resulted in activation of a Cd^{2+}-sensitive inward current and activation of an apamin-sensitive outward current accompanied by an increase in conductance. Sulfide also blocked a TEA-sensitive outwardly rectifying current in some cells, and its removal activated a strophanthidin-sensitive Na^+/K^+ ATPase-generated outward current in all responding cells. In addition, both fast and slow synaptic responses were reversibly inhibited by sulfide in all cells.

Altered neuronal activity has also been demonstrated in the brain stem and medulla of rats *in vitro* following H_2S administration. Greer *et al.* (1995) found that NaHS, acting at the level of the brain stem, caused a dose-dependent decrease in the frequency of rhythmic respiratory bursts within 2 minutes in a brain stem-spinal cord preparation. In the medullary slice preparation, NaHS caused an initial increase in the frequency of rhythmic respiratory bursting, and an increase in the tonic firing on hypoglossal motor

cranial nerves. This was followed by a marked decrease in the frequency and amplitude of respiratory motor neuron discharge, lower than control levels. The same study examined the effects of sulfide on neonatal rats. *In vivo*, i.p. injections of NaHS caused a dose-dependent decrease in the frequency and amplitude of breathing.

Experiments using synaptosomes prepared from the whole brain of mice were carried out to examine the actions of H_2S at the cellular level (Nicholson et al., 1998). Treatment of synaptosomes with 20 - 100 μM NaHS reduced the consumption of oxygen by synaptosomal suspensions in a concentration-dependent manner. Treatment of isolated nerve terminals with NaHS also resulted in a concentration-dependent depolarization of the intraterminal mitochondrial membrane. NaHS caused a reduction in synaptosomal ATP level, but was not completely suppressed even at the highest dose of NaHS (100 μM). In addition, NaHS was found to affect L-glutamate uptake and release (Nicholson et al., 1998). The uptake of L-glutamic acid by synaptosomes was reduced following 100 μM NaHS, and the amount of this neurotransmitter released was increased in a dose-dependent manner. Furthermore, it was found that the increased release of L-glutamate was Ca^{2+} -independent.

It is well-documented that H_2S causes several toxic effects in the brain, both *in vivo* and *in vitro*. The evidence that H_2S is produced endogenously in the brain has sparked some interest in the possibility that it may play a physiological role in normal brain function. At least one group of researchers has examined this possibility. Abe and Kimura (1996) have demonstrated that the H_2S-producing enzyme cystathionine β-synthase (CBS)

is highly expressed in the hippocampus, and specific activators and inhibitors of CBS increase and decrease the concentration of endogenous H_2S in the brain, respectively. They also have found that physiological concentrations of H_2S selectively enhance NMDA receptor-mediated responses, and facilitate the induction of hippocampal long-term potentiation. These results suggest that endogenous H_2S may function as a neuromodulator in the brain.

Circadian Rhythms Influencing Metabolism of H_2S

One of the main functions of the liver is to irreversibly transform drugs and toxic agents into metabolites that are generally more readily excreted into the bile or urine. In the case of H_2S, glutathione plays a role in metabolism, along with other enzyme systems such as sulfide oxidase and sulfite oxidase (Beauchamp, 1984). Glutathione is present in a reduced (GSH) and in an oxidized (GSSG) form in the endoplasm, but the equilibrium between the two states favors a higher concentration of the reduced form, which represents the major non-protein sulfhydryl compound in the cell (Belanger, 1988). Several studies have documented circadian variation in the hepatic concentration of glutathione (Belanger, 1988; Jaeger, 1979). In a review of 19 studies on the circadian rhythm of glutathione concentration in the liver of nocturnal rodents, it was noted that there was much consistency in the times of peak and trough concentrations of GSH (Belanger, 1988). Maximal concentrations were found to occur between 0400 and 1200h, and minimal concentrations were obtained 12 hours later, between 1600 and 0000h.

Jaeger (1979) examined the circadian variation in non-protein sulfhydryl (NPS)

concentrations in rat liver, lungs, kidney and blood. Since GSH is the major NPS compound in the cell, such measurements are indicative of GSH concentration. It was found that all tissues contained a circadian variation of NPS concentration. In the liver, peak concentrations of NPS were obtained between 0400 and 1300h, whereas in the blood, lungs and kidney peak concentrations were found between 1600 and 0100h. Exposures to acrylonitrile, bromobenzene, and 2-chlorobutadiene were also performed (Jaeger, 1979). These compounds were believed to be detoxified by a NPS-dependent pathway, and sulfur-containing metabolites have been identified for acrylonytrile and bromobenzene. It was shown that in each case the chemicals were significantly more toxic to rats (% mortality) when exposures were carried out during the dark period between 1800 to 2200h than 12 hours later, between 0600 to 1000h. This indicates that the toxicity is increased at a time when concentrations of NPS in the liver are lowest, and may be related to a NPS-dependent hepatic detoxification pathway.

In addition to glutathione, circadian rhythms have been documented for many pathways of hepatic drug metabolism. The enzyme reactions of oxidation, reduction, hydrolysis and conjugation have all been demonstrated to vary in their abilities to detoxify substances over a 24-hour period (Belanger, 1988). However, the enzyme systems sulfide oxidase and sulfite oxidase have yet to be characterized for circadian variation.

Hippocampal Theta Activity as a Model System of Hydrogen Sulfide Toxicity

The hippocampus is of particular interest to the study of H_2S, as this structure may relate to the symptoms described by survivors of H_2S poisoning. In particular, the

hippocampus has been implicated in the behavioral states of arousal and anxiety, and seems to be necessary for the functioning of learning and memory, as evidenced by lesions (Olton & Markowska, 1994; Davis & Volpe, 1990) and pharmacological manipulation (Givens & Olton, 1990) of the hippocampus and afferent inputs. The hippocampus is an excellent structure for the study of neurotoxic substances (Stoltenburg-Didinger, 1994). It has been shown to be preferentially susceptible to a wide variety of toxic insults, including environmental toxicants (Alfano & Petit, 1981) and drugs of abuse (Walker *et al.*, 1980), as well as to cerebrovascular insufficiency resulting in hypoxia (Benveniste *et al.*, 1984).

Neuroanatomy of the Hippocampal Formation

The hippocampal formation is a prominent component of the rat nervous system. The three-dimensional position of the hippocampal formation in the brain is rather complex (Figure 2). It appears as an elongated structure with its long axis extending in a C-shaped fashion from the septal nuclei of the basal forebrain rostrodorsally, over and behind the diencephalon, to the temporal lobe caudoventrally (Amaral and Witter, 1995). The term hippocampal formation comprises six distinct regions (Figure 3). These include the dentate gyrus; hippocampus (or hippocampus proper) which is subdivided into three fields (CA1, CA2 and CA3); subiculum, presubiculum, and parasubiculum (the subicular complex); and the entorhinal cortex. (Amaral and Witter, 1995). These divisions are based largely on differing cytoarchitecture. The dentate gyrus, hippocampus proper, and subiculum contain three cortical layers, whereas the presubiculum, parasubiculum and entorhinal cortex are more laminated structures (Amaral and Witter, 1995).

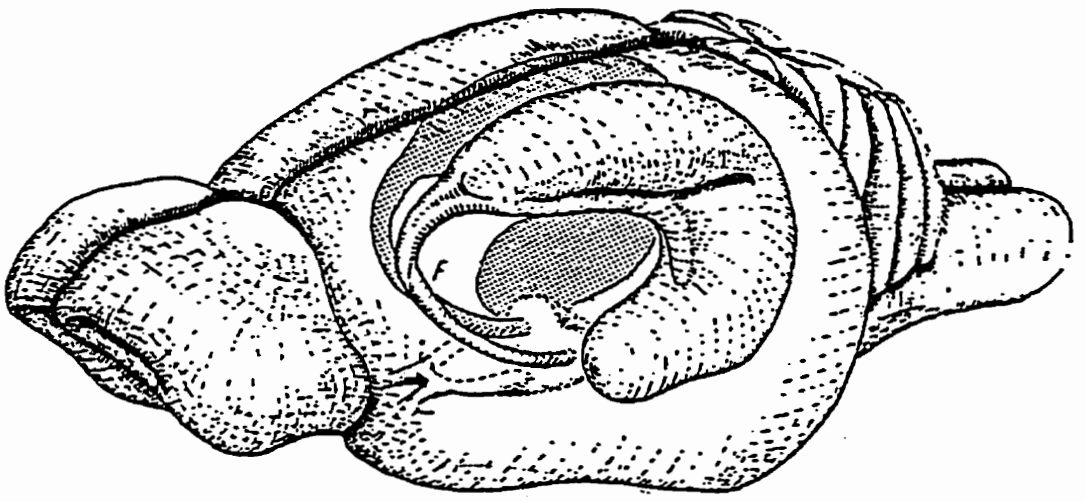


Figure 2. Three-dimensional view of the hippocampus in the rat brain (from Amaral and Witter, 1995)

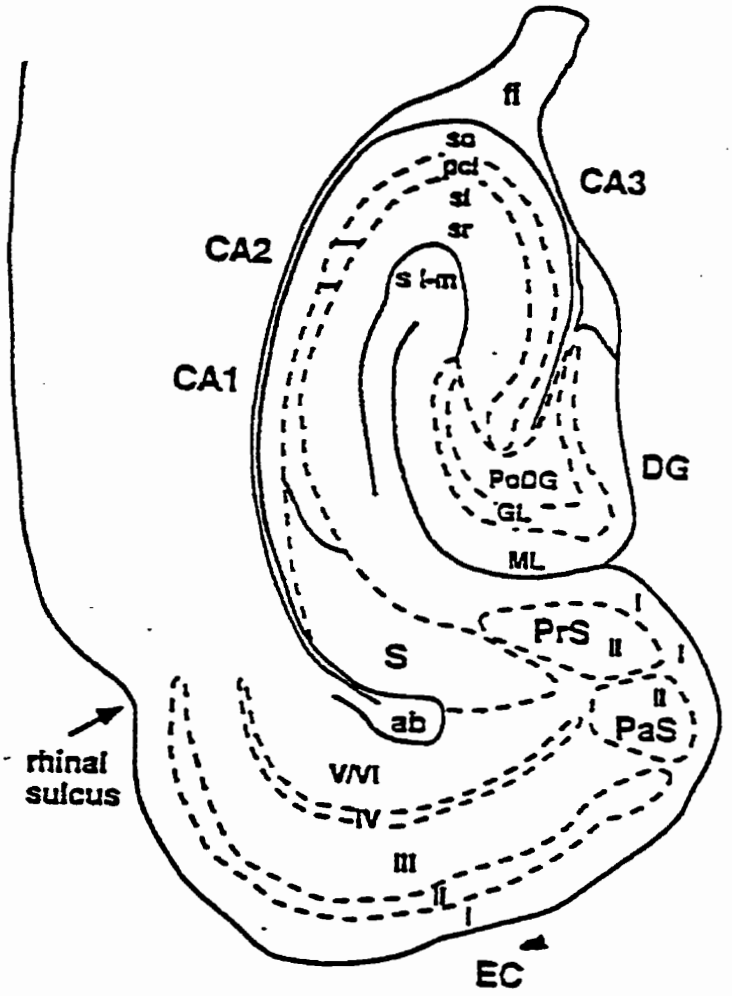


Figure 3. Representative section through the rat hippocampus, showing the various fields of the hippocampal formation. Abbreviations: so, stratum oriens; pcl, pyramidal cell layer; sl, stratum lucidum; sr, stratum radiatum; s l-m, stratum lacunosum-moleculare; PoDG, polymorphic layer; GL, granule layer; ML, molecular layer; PrS, presubiculum; S, subiculum; PaS, parasubiculum; ab, angular bundle; DG, dentate gyrus; EC, entorhinal cortex. (from Amaral and Witter, 1995).

The three layers that make up the dentate gyrus are the molecular layer, granule cell layer and polymorphic cell layer (Lopes Da Silva *et al.*, 1990). The molecular layer lies closest to the hippocampal fissure, and is relatively cell-free. It is occupied mainly by the dendrites of granule cells, basket cells, and various polymorphic cells (Amaral and Witter, 1995). Two neuronal types are found in the molecular layer, one being a type of basket cell (Ribak and Seress, 1983), and the second is a type of axo-axonic or "chandalier" cell, first described in the neocortex (Halasy and Somogyi, 1993). The axo-axonic cells are so named because they send their axons into the granule cell layer, and terminate on the axon initial segments of granule cells (Amaral and Witter, 1995). These cells are immunoreactive for GABA, and it is likely that they provide a means of inhibitory control of granule cell output (Halasy and Somogyi, 1993).

The principal cell type of the dentate gyrus is the granule cell. The granule cell has a characteristic cone-shaped spiny dendritic tree, with all of the branches directed towards the superficial portion of the molecular layer (Clairborne *et al.*, 1990). In addition to the granule cells, basket cells and other cell types are found along the deep surface of the granular cell layer (Amaral and Witter, 1995). The axons of these cells form a basket plexus around the soma of granule cells, and send their dendrites into both the molecular and polymorphic cell layers. The majority of these neurons are GABAergic, and are acting as inhibitory interneurons (Ribak and Seress, 1983). The polymorphic layer is also known as the hilar region of the dentate gyrus, and contains a variety of neuronal cell types (Amaral, 1978). The most common cell type is the mossy cell, whose dendrites remain in the confines of the polymorphic layer (Amaral, 1978). In addition to the mossy

cells, there are also a number of fusiform-type cells and small, multipolar cells (Amaral, 1978).

The granule cells of the dentate gyrus give rise to distinctive unmyelinated axons, called mossy fibers (Amaral and Witter, 1995). The axons run within the polymorphic layer, where they establish contact with the dendrites of mossy cells, pyramidal basket cells, and other unidentified cells (Amaral and Witter, 1995). The mossy fibers project to the area CA3 of the hippocampus proper, where they terminate on the proximal dendrites of CA3 pyramidal cells (Clairborne *et al.*, 1986). Each granule cell influences only 14-28 pyramidal cells, however, each pyramidal cell has been estimated to receive contacts from about 50 granule cells (Amaral and Witter, 1995). The mossy fibers are thought to use glutamate as a primary transmitter substance (Storm-Mathieson and Fonnum, 1972), however some mossy fibers also contain opiate peptides such as dynorphin (McGinty *et al.*, 1983).

Based on the terminology of Lorente de No (1933), the hippocampus proper has been divided into three fields: CA3, CA2 and CA1. The CA3 and CA2 fields contain large pyramidal cells, while the pyramidal cells of CA1 are much smaller. In addition to the differences in size of the pyramidal cells, there is clear connectional differences between the fields. The CA3 pyramidal cells receive input from the dentate mossy fibers, whereas CA2 and CA1 do not (Amaral and Witter, 1995).

The laminar organization of the hippocampus proper is generally the same for all subfields (Amaral and Witter, 1995). The principal cell layer is the pyramidal cell layer. Below the pyramidal cell layer is a narrow, relatively cell-free layer called the stratum

oriens, and below this is the alveus. The alveus contains mainly myelinated efferent and afferent fibers. In part, these are fibers originating from the pyramidal cells of the hippocampus en route to subcortical termination sites or to the contralateral hippocampus. In the CA3 field, a narrow acellualr zone located just above the pyramidal cell layer contains the mossy fiber axons, and is called the stratum lucidum. Above the stratum lucidum in CA3, and above the pyramidal layer in CA2 and CA1, is the stratum radiatum. This layer contains CA3 to CA1 Shaffer collateral connections, as well as associational connections from CA3 to the contralateral CA3. The most superficial portion of the hippocampus proper is called the stratum lacunosum-moleculare. It is in this region that afferents from other brain structures terminate (Amaral and Witter, 1995). The pyramidal cells of area CA1 project to the adjacent subiculum via the stratum oriens (Amaral *et al.*, 1991).

The subicular complex consists of the subiculum, presubiculum and parasubiculum. Together they comprise a conglomerate of cytoarchitectonically different, relatively small cortical fields located between area CA1 and the entorhinal cortex (Amaral and Witter, 1995). The subiculum resembles the dentate gyrus and hippocampus in that it consists of only three cortical layers, whereas the presubiculum and parasubiculum resembles more the entorhinal cortex in that they have a multilaminate cortical structure (Amaral and Witter, 1995). The subiculum is one of the major output regions of the hippocampal formation, and projections are generated to a number of cortical and subcortical regions (Witter at al., 1990), including the entorhinal cortex. The septum also receives a reciprocal innervation by fibers from the entorhinal cortex. (Amaral and Witter, 1995).

The entorhinal cortex consists of six layers, first described by Ramon y Cajal (1911). There are four cellular layers (layers II, III, V and VI), and two acellular or plexiform layers (layers I and IV). Layer IV is also known as the lamina dissecans (Amaral and Witter, 1995). The entorhinal cortex can be subdivided into two general areas, the lateral entorhinal area (LEA) and the medial entorhinal area (MEA). (Lopes Da Silva *et al.*, 1990).

The major input to the dentate gyrus and a prominent input to the hippocampus and subiculum arises from the entorhinal cortex, through a pathway known as the perforant path (Kohler, 1985). The projection to the dentate gyrus arises mainly from layer II of the entorhinal cortex (Ruth et al, 1982), and terminates on dendrites of dentate granule cells in the outer two-thirds of the molecular layer (Amaral and Witter, 1995). Projections of the entorhinal cortex to areas CA3/CA2 also originate in layer II, and terminate in the stratum lacunosum-moleculare. In contrast, the perforant path projection to area CA1 originates in layer III, however also terminates in stratum lacunosum-moleculare (Sterward and Scoville, 1976). Area CA1 has a reciprocal projection to the entorhinal cortex, and is in this way also different from the dentate gyrus and areas CA3/CA2 (Amaral and Witter, 1995). The projection from the entorhinal cortex to the subiculum originates in layers II and III, and terminates either in the proximal part of the subiculum, close to area CA1, or in the distal region, closer to the presubiculum (Witter, 1993). The major transmitter of the perforant path is most likely glutamate (Fonnam, 1970), however, terminals of the perforant path within the dentate molecular layer are also immunoreactive for enkephalin and cholecystokinin (CCK) (Fredens *et al.*, 1984).

Several cortical and subcortical structures innervate the entorhinal cortex, including the olfactory bulb, the adjacent perirhinal cortex, the medial septum, the amygdaloid complex, the thalamus, and various structures in the hypothalamus and brain stem (Amaral and Witter, 1995). Many of these same strucutures also innervate the dentate gyrus, hippocampus and subiculum directly. In particular, a major input to the dentate gyrus and hippocampus arises from cells of the medial septal nucleus and the nucleus of the diagonal band of Broca (Amaral and Kurtz, 1985). In the dentate gyrus, fibers heavily innervate the polymorphic layer, and terminate more lightly in the molecular layer (Amaral and Kurz, 1985). The projection from the medial septum/diagonal band of Broca has been shown to be largely cholinergic in nature (Lewis and Shute, 1967). It has been demonstrated that 30-50% of the cells in the medial septal nucleus and 50-75% of the cells in the nucleus of the diagonal band that project to the hippocampal formation are cholinergic (Amaral and Kurz, 1985). The majority of the cells in the septohippocampal projection that are non-cholinergic have been found to contain GABA, and also terminate in the dentate gyrus and hippocampus (Kohler *et al.*, 1984), innervating hippocampal inhibitory interneurons (Freund and Antal, 1988).

Circadian rhythms in the hippocampal formation

It is well documented that circadian rhythms exist in most, if not all brain regions, and the hippocampal formation is no exception. For example, it has been demonstrated by several authors that electrophysiological characteristics of principle cells vary in their responsiveness to excitatory input over a 24 hour period (Harris and Teyler, 1983; Barnes

et al., 1977; West and Deadwyler, 1980; Cauller *et al.*, 1985; Brunel and DeMontigney, 1987). One of the first demonstrations of this came from Barnes and colleagues in 1977, who showed that synaptic responses in hippocampal dentate granule cells to stimulation of their afferent fibers from the entorhinal cortex fluctuated over a 24 hour period. The population excitatory post-synaptic potential (EPSP) and population action potential (or population spike, representing the firing of many granule cells) were measured in male rats and male squirrel monkeys once every half hour for a period of 24 hours. It was found that in male rats, the EPSP and population spike amplitudes were approximately 30% larger in the dark period than the light period. For squirrel monkeys, a circadian rhythm was also observed but was opposite to that seen in rats; the amplitude was highest during the light periods and lowest during darkness. These results suggested that the activity of dentate granule cells is highest during an animal's active phase, which could influence the performance of a number of behaviors, and possibly be important for normal memory function.

Since the initial findings of Barnes *et al.* (1977), several authors have tried to replicate and expand the information regarding a possible circadian rhythm of synaptic transmission in the dentate gyrus, and to include other subfields of the hippocampus. West and Deadwyler (1980) examined the circadian variation in the amplitude of field potentials in dentate granule cells elicited by stimulation of the perforant path during different levels of behavioral arousal, and following bilateral adrenalectomy. They found an increase in spike amplitude during the light period, and a decrease during the dark period, which was not dependent on behavioral state or levels of circulating

corticosterone. However, Dana and Martinez (1984) found that adrenalectomy reversed the circadian rhythm of long-term potentiation (LTP) in hippocampal granule cells. LTP is an enduring form of synaptic plasticity found in several pathways of the hippocampus. Following stimulation of the perforant path, intact control rats showed more LTP during the dark period, while adrenalectomized rats demonstrated more LTP during the light period. These data suggest that adrenal hormones play a role in regulating the circadian rhythm of LTP. Cauller *et al.* (1981) have found that the dentate field EPSP was largest during the animal's dark period, while the population spike was largest during the animal's light period. This would indicate that dentate granule cells were more excitable during the light period. Harris and Teyler (1983) extended these findings to determine whether any light/dark variations existed in either the incidence or magnitude of long-term potentiation (LTP) in area CA1 or in the dentate gyrus of the rat hippocampal slice preparation. They found that the dentate region was more likely to show LTP when the slice was prepared during the animal's dark period, while the CA1 area showed LTP more frequently during the light period. The same pattern of light/dark differences were seen for the magnitude of the LTP generated: the dentate had a larger LTP response during the dark period, while area CA1 had a larger response during the light.

These results indicate that there is a circadian rhythm in synaptic transmission and cell excitability in the hippocampus, which is preseved *in vitro,* and may be under the influence of circulating adrenal hormones. Hippocampal neurons contain high concentrations of specific receptors for adrenal corticosteriods (Kloet *et al.*, 1993). Selective occupation of these receptors can lead to either an increase or decrease in cell

excitability, depending on the type of receptor occupied (Kloet *et al.*, 1993). Furthermore, it has been shown that there is a circadian rhythm in corticosteriod receptor binding in the hippocampus (Holmes *et al.*, 1995; Kloet *et al.*, 1993).

Levels of certain neurotransmitters and neurotransmitter receptors also show a significant circadian rhythm in the hippocampus. The serotonin (5-HT) receptors 5-HT_1, 5-HT_{1A}, and 5-HT_2 all show a circadian rhythm, with maximal binding sites occurring in the light period (Weiner *et al.*, 1992). 5-HT_{2C} receptor mRNA expression was also found to be greatest in the light period (Holmes *et al.*, 1995). The release of 5-HT in the hippocampus has also been studied for circadian variation. Extraneuronal 5-hydroxyindoleacetic acid (5-HIAA) was measured as an index of 5-HT release, using *in vivo* voltammetry (Weiner *et al.*, 1992). It was found that 5-HT release in the hippocampus was greatest in the dark period.

Measures of acetylcholine (ACh) release in the hippocampus also show a significant difference over a 24 hour period. Using the microdialysis method, Mizuno *et al.* (1991; 1994) showed that ACh release in the hippocampus of rats is about 70% greater in the dark period than the light period. Furthermore, it was shown that the amount of ACh release was positively correlated with motor activity. An increase in motor activity was accompanied by an increase in ACh release. Acetylcholinesterase activity in the rat brain is reduced in the dark period (Moudgil et al, 1973), which may contribute to the increased levels of ACh in the dark. Receptor binding studies have shown that muscarinic receptor binding is highest in the light period, and lowest in the dark (Jenni-Eiermann *et al.*, 1986), which suggests a reciprocal relationship between number of binding sites and

ACh release.

Extracellular ascorbate concentrations in the hippocampus have also been measured for circadian variation. Glutamate and aspartate cause the release of ascorbate from synaptosomes (Grunewald and Fillenz, 1984), and it has been shown that electrical stimulation of the perforant path, which is glutamatergic, causes a release of ascorbate in the dentate gyrus (O'Neil *et al.*, 1984). For these reasons, measures of ascorbate in the brain can be used as an index of excitatory amino acid release. It has been found that ascorbate release in the rat hippocampus was maximal in the dark period (O'Neil and Fillenz, 1985), and the increase in ascorbate was correlated with an increase in motor activity.

Further neurotransmitter rhythms in the hippocampus include an increase in norepinephrine (NE) concentrations in the dark period (Moore and Traynor, 1976).

It has also been demonstrated that rat hippocampal CA3 pyramidal cells vary in their response to microiontophoretic applications of 5-HT, NE, GABA and ACh over a 24-hour period (Brunel and DeMontigney, 1987). Circadian rhythms were found for CA3 pyramidal cells responsiveness to 5-HT and ACh, which were maximal in the dark period, and for NE, which was maximal in the light period. No rhythm was found in the responsiveness of pyramidal cells to GABA. In addition, rhythms in the spontaneous firing rate of pyramidal neurons were found, which were highest in the light period and lowest in the dark.

Hippocampal Theta Activity

Characteristics

The electrical activity of hippocampal granule cells in the dentate gyrus is a 4-12 Hz sinusoidal waveform, commonly referred to as theta or rhythmic slow activity (RSA). In animals, theta is associated with certain types of movement, that which allows the animal to discover information about its surroundings. Theta also occurs, or can be elicited, during immobility. The frequency of this rhythm can range from 3 to 12 Hz depending on the species studied and the behavioral state of the animal (Robinson, 1980). Theta activity in the hippocampus has been found to be relatively stable across species, occurring in rats, rabbits, guinea pigs, gerbils, cats and dogs (Sainsbury *et al.*, 1986; Montoya & Sainsbury, 1984;Vinogradova *et al.*, 1992; Ambrosini *et al.*, 1993; Bland, 1986; Black *et al.*, 1970). Hippocampal theta has also been found in human (Arnolds *et al.*, 1980) and non-human primates (Stewart & Fox, 1991).

A number of studies have found evidence for two types of theta in the awake animal, based on behavioral and pharmacological characteristics. These have been labeled type 1 and type 2 theta. Type 1 theta is associated with voluntary movements, such as walking, running, swimming, rearing, jumping, manipulation of objects, and shifts in posture (Bland, 1986). Type 1 theta has a frequency range of about 7 to 12 Hz, and is not attenuated with cholinergic antagonists such as atropine sulfate. It is sensitive to anesthetics such as ether, urethane and pentobarbitol (Bland, 1986; Vanderwolf *et al.*, 1978), which is indicative of the relationship between type 1 theta and movement.

Type 2 theta is associated with immobility. It has a lower frequency than type 1

theta, with a range of about 4 to 9 Hz (Bland, 1986). In the presence of anesthetics, animals will still produce type 2 theta, however it is completely abolished by atropine, and can be elicited by administration of cholinergic agonists (Vanderwolf, 1978). In the rat, rabbit, guinea pig and cat, type 2 theta can be elicited by presentation of an arousing stimulus (Sainsbury *et al.*, 1986; Bland 1986; Montoya & Sainsbury, 1985; Sainsbury, 1985). Type 2 theta can become habituated to repeated presentations of a stimulus, suggesting that it is associated with a certain level of arousal in the hippocampus (Sainsbury, 1985).

Behaviors other than type 1 voluntary movements are associated with large amplitude irregular activity (LIA) and small amplitude irregular activity (SIA) in the hippocampus. Movement associated with LIA includes licking, chewing, teeth chattering, shivering, face-washing, scratching, and vocalizations, and are sometimes referred to as "automatic" behaviors (Bland, 1986). In the rat, LIA occurs during automatic behavior and during immobility (Robinson, 1980).

Theta activity is also prevalent during rapid eye-movement (REM) sleep. During REM sleep, two types of theta activity occur, called phasic and tonic theta. Tonic theta has a frequency of about 4-7 Hz, while phasic theta has a frequency of about 7-9 Hz (Robinson *et al.*, 1977). These two types of theta have been compared to type 1 and type 2 theta occurring during wakefulness. Theta activity associated with movement during REM sleep (muscle twitches, rapid eye-movements) has a higher frequency than the tonic component, which is associated with the period between phasic movements and atonia, or complete loss of muscle tone (Robinson, 1977).

It has been suggested that theta occurring during exploratory activity, immobility and REM sleep represent the processing of information in the hippocampus and subsequent consolidation into memory. Evidence supporting this notion comes from the findings that the elimination of theta rhythm in the rat produces retrograde or anterograde spatial memory deficits (Winson, 1978). Additional evidence for the role of theta in memory comes from the consideration of long term potentiation (LTP). LTP is considered a model for synaptic plasticity underlying memory processing in the hippocampus. Stimulation pulses applied at theta rhythm frequency are more effective in inducing LTP in the dentate gyrus and CA1 regions of the hippocampus than pulses of any other frequency (Larson *et al.*, 1986), suggesting that theta rhythm is the normal means by which LTP occurs.

Pharmacology

The two types of theta appear to be pharmacologically distinct. Vanderwolf (1978) examined the pharmacologic characteristics of type 1 and type 2 theta using a number of different drugs. It was found that type 2 theta was blocked by atropine and stimulated by eserine, and resistant to anesthetics such as urethane. Hemicholinum-3, which blocks the uptake of choline, has been shown to attenuate type 2 theta (Robinson & Green, 1980), and systemic injection of choline restored the activity. Tonic theta occurring during REM sleep is also sensitive to cholinergic manipulation (Robinson *et al.*, 1977).

The cholinergic nature of type 2 theta is well established, however the

pharmacology of type 1 theta is not well understood. It is known that anesthetics such as urethane and ether can attenuate type 1 theta. There is evidence to suggest that serotonin may exert a modulatory effect on type 1 theta. A study by Robertson *et al.* (1991) demonstrated that increases in central serotonergic activity with IP injections of tranylcypromine plus tryptophan, or with *p*-chloroamphetamine, elicited theta activity in the hippocampus. Furthermore, the theta activity was shown to be scopolamine resistant, suggesting that type 1 theta was selectively affected. However, Vertes *et al.* (1994) demonstrated that procaine and 5-HT_{1A} agonists, injected into the median raphe nucleus, inhibit its activity and elicit theta in the hippocampus. This indicates that serotonin-containing neurons in the median raphe normally suppress hippocampal theta activity. The noradrenergic $alpha_2$ agonist detomidine has been shown to attenuate type 1 theta when infused into the hippocampus (Sainsbury & Partlo, 1993). This effect was attributed to the inhibitory action of detomidine on the serotonergic neurons in the hippocampus.

In addition, the role of GABA in mediating type 1 theta has been examined. Both GABAergic and cholinergic projections to the hippocampus from the medial septum have been demonstrated, and GABAergic synapses of septal origin have been found on inhibitory interneurons in the hippocampus, as well as on pyramidal and granular neurons (Vinogradova, 1995). Rhythmic activity in the medial septum is retained and is able to generate hippocampal theta at high frequencies associated with type 1 theta after complete blockade of cholinergic influences (Stewart and Fox, 1989). It has been suggested that ACh and GABA coexist in the same neuronal synapses (Bonanno and Raiteri, 1986). Septal cholinergic terminals were shown to have a GABA uptake system, and application

of GABA to synaptosomes induced prolonged enhancement of ACh release. Vinogradova (1995) suggests that the transmitter released and the physiological effect may vary at different levels of excitation of the medial septal neurons, and at different frequencies of theta.

Theta activity associated with phasic phenomena in REM sleep is also poorly understood. Like type 1 theta, phasic theta during REM sleep is selectively abolished by anesthetics such as urethane (Robinson *et al.*, 1977). It has also shown to be sensitive to diazepam (Monmaur, 1981) and tetrahydrocannabinols (Moreton and Davis, 1973), implicating the GABAergic, serotonergic and cholinergic systems in its regulation.

Mechanisms of Theta Generation

Two internal generators of hippocampal theta activity have been described. One is located in the stratum oriens of the CA1 region and one in the stratum moleculare of the dentate gyrus (Bland & Whishaw, 1976). The two generators were shown to be 180° out of phase with one another. There was agreement between the two areas in relation to the frequency of theta activity during behavior. In addition, two major classes of cells in the hippocampus have been identified that change their rate of firing in synchrony with theta activity. They have been termed theta-on and theta-off cells (Colom and Bland, 1987).

The medial septum and diagonal band of Broca seem to be of critical importance to the generation of theta in the hippocampus. Lesions of the medial septum-diagonal band (MS-DB) complex completely abolish both type 1 and type 2 theta activity in rabbits (Andersen *et al.*, 1979), as does transection of the septo-hippocampal pathway

(Vinogradova, 1995). In addition, there is a high correlation between rhythmic neuronal discharge in the MS-DB and hippocampal theta waves (Stewart & Fox, 1989). Isolated cells in MS-DB slices retain rhythmic bursting activity (Vinogradova, 1995). As well, disruption of the connections between the hippocampus and the MS-DB in the intact animal do not eliminate rhythmic bursting in the MS-DB (Vinogradova, 1995). Pharmacological examination of the septo-hippocampal pathway indicates that it is largely cholinergic in nature (Bland, 1996). Stimulation of the MS-DB results in a release of acetylcholine (ACh) from the hippocampus, while lesions of the MS-DB result in a reduction of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) in the hippocampus (Bland, 1986).

The brainstem reticular formation is also important for the generation of hippocampal theta. The reticular neurons have an increased tonic level of spontaneous activity during hippocampal theta states, such as arousal, exploratory activity, or REM sleep (Vinogradova, 1995). Stimulation of the reticular formation can trigger theta in the hippocampus and rhythmic bursting in the medial septum over the whole range of theta frequencies (Vinogradova, 1995). Vertes (1981) described three ascending pathways responsible for theta synchronization from the reticular formation: (1) the medial longitudinal fasiculus, (2) the medial forebrain bundle and (3) the central tegmental tract. Although in the absence of input from the reticular formation, rhythmic bursting neurons in the MS-DB are still present (Vinogradova, 1995), theta activity in the hippocampus is altered (Gottesman, 1992).

There is evidence that the raphe nuclei may exert an inhibitory effect on

hippocampal theta activity (Vertes *et al.*,1994). The median raphe projects to the septum and the hippocampus, and stimulation of this nucleus suppresses hippocampal theta (Vertes, 1981), and decreases the regularity and frequency in MS-DB neurons (Vinogradova, 1995). Additional evidence for the suppressing influence of raphe nuclei on theta activity comes from the fact that the activity of raphe neurons is minimal during REM sleep and exploratory behavior, two behavioral states which are characterized by continuous occurrence of hippocampal theta (Fornal *et al.*, 1985). However, neurons in the raphe nuclei are highly activated during automatic behaviors such as chewing and grooming, which are characterized by LIA in the hippocampus (Jacobs & Fornal, 1991).

Cortical input to the hippocampus also modulates theta activity. Bilateral lesions of the entorhinal cortex in guinea pigs resulted in changes of amplitude, onset and frequency of both type 1 and type 2 theta (Montoya and Sainsbury, 1985). Lesions of the entorhinal cortex reduced the correlation between type 1 and type 2 theta and behavior. Furthermore, it was shown that atropine sulfate abolished theta activity during both movement and immobility, suggesting that the type of theta occuring subsequent to entorhinal lesions is cholinergically mediated.

Cellular Basis of Hippocampal Theta

The neuronal mechanisms underlying hippocampal theta generation are not well understood, despite extensive investigation. It has been shown that continuous application of the cholinergic agonist, carbachol, can induce rhythmic neuronal activity in hippocampal slices, which resemble the hippocampal theta rhythm recorded from the intact

animal (Konopacki *et al.*, 1987; Bland *et al.*, 1988). MacVicar and Tse (1989) examined the carbachol-driven rhythmic activity in hippocampal CA3 pyramidal cells *in vitro.* It was found that the carbachol-induced activity was reversibly blocked by a broad spectrum excitatory amino acid antagonist, but not by specific NMDA, $GABA_A$ or $GABA_B$ antagonists. Their results suggested that this activity occurs synchronously among a large population of pyramidal neurons, which is driven by a polysynaptic recurrent excitatory circuit, involving non-NMDA, but not GABAergic synapses. However, Heynen and Bilkey (1991) demonstrated that, in adult hippocampal slices, the application of carbachol alone was not sufficient to produce theta activity, but required the simultaneous blockade of GABAergic IPSPs by picrotoxin. Furthermore, the co-application of glutamate and picrotoxin also resulted in the production of theta activity. One characteristic of these experiments is that in the hippocampal slice preparation, the input from the septum, which is considered to be a "pacemaker" for the *in vivo* generation of theta (see Bland, 1986), is absent. A recent study by Toth et al (1997) examined the influence of septal stimulation on hippocampal inhibitory circuits using slices from the rat forebrain which included both the septum and hippocampus. It is known that the septohippocampal pathway contains both cholinergic and GABAergic fibers, and the GABAergic terminals innervate the inhibitory interneurons, but not the pyramidal cells, of the hippocampus (Freund and Antal, 1988). The results of Toth *et al.* (1997) demonstrated that stimulation of septohippocampal afferents resulted in a decrease in the frequency of spontaneous IPSPs recorded from CA3 pyramidal neurons, and initiated IPSPs in hippocampal inhibitory interneurons. Also, in the presence of antagonists of excitatory amino acid and muscarinic

receptors, stimulation of septal afferents at theta frequency (5 Hz) initiated rhythmic oscillations in pyramidal cells. It appears that disinhibition, arising from GABAergic input to inhibitory interneurons, causes excitation in pyramidal cells, sufficient to produce rhythmic oscillations in the absence of excitatory input.

Although it is possible to produce rhythmic activity in the hippocampus under blockade of excitatory input, generation of theta activity in the whole animal under normal conditions has been shown to depend on several types of neurochemical inputs, both excitatory and inhibitory in nature. Two types of theta (type 1, atropine resistant, and type 2, atropine-sensitive) are believed to be controlled by distinct mechanisms, however some overlap is also believed to occur. In 1983, Busaki *et al.* proposed a mechanism for the generation of hippocampal theta *in vivo*. The model is based on a feed-forward (cholinergic) excitation from the medial septum to GABAergic interneurons in the hippocampus proper and dentate gyrus, which inhibits the pyramidal and granule cells at their somata. The dendrites of the pyramidal and granule cells receive a synchronous excitatory (glutamatergic) input from the entorhinal cortex. The relative strength of the somatic inhibition and dendritic excitation determines the probability of cell discharge. Smythe *et al.* (1992) suggested that the inputs from the medial septum (cholinergic and GABAergic) were the critical determinant of the theta rhythm. Theta may be generated in the hippocampus by an increase in cholinergic activity, which stimulates the principle or "theta-on" cells, accompanied by a reduction of hippocampal inhibitory interneuron activity via the GABAergic projection, achieving disinhibition of principle cells.

When the medial septum input is abolished by intraseptal application of the

anesthetic procaine, theta activity can be generated *in vivo* following microinfusions of carbachol and glutamate, provided some degree of disinhibition is also induced, by the co-application of picrotoxin (Heynen and Bilkey, 1991). The cholinergic nature of type 2 theta is well established, however the generation of type 1 theta is less well understood (Bland, 1986). The findings of Heynen and Bilkey (1991) suggest that coactivation of the disinhibitory component of the septohippocampal pathway and the glutaminergic perforant path input to the hippocampus may result in type 1, atropine resistant theta.

The data regarding the serotonergic control of type 1 theta is unclear. Some studies suggest that serotonin facilitates the occurrence of theta (Vanderwolf, 1988; Robertson et al., 1991; Peck and Vanderwolf, 1991), while others suggest that serotonin inhibits the production of theta in the hippocampus (Richter-Levin and Segal, 1990; Piguet and Galvan, 1994; Staubli and Xu, 1995). In the dentate gyrus, serotonin was found to hyperpolarize granule cells via postsynaptic 5-HT_{1A} receptors, and increase spontaneous GABA release from inhibitory interneurones via the activation of 5-HT_3 and/or 5-HT_2 receptors (Piguet and Galvan, 1994). It has also been shown to suppress theta-burst induced NMDA currents and reduce the LTP generated in CA1 neurons (Staubli and Otaky, 1994). Presynaptic 5-HT_{1A} receptors decrease the release of serotonin, and agonists of this receptor type induce hippocampal theta activity in freely moving cats (Marrosu *et al.*, 1996). Studies suggesting that serotonin facilitates hippocampal type 1 theta (e.g. Robertson *et al.*, 1991) have been confounded by secondary effects, namely the induction of stereotyped movements and general arousal in the freely moving animal.

Effects of H_2S on Hippocampal Activity

The *in vitro* hippocampal slice preparation has been used successfully to study the mechanism of action of H_2S (Baldelli *et al.*, 1989; Baldelli *et al.*, 1990). Intracellular recordings of hippocampal CA1 neurons were studied in response to 27-200 uM NaHS (Baldelli *et al.*, 1990). It was shown that NaHS caused a concentration-dependent membrane hyperpolarization and reduction in membrane resistance of CA1 neurons, a maximal effect reached at 160 uM NaHS. It was suggested that the reduction in membrane resistance was probably due to activation of Na^+/K^+ ATPase, whereas the hyperpolarization was the result of the opening of K^+ channels. Extracellularly recorded population spikes, EPSP field potentials, and intracellular EPSPs revealed a depression of synaptic transmission produced by NaHS (Baldelli *et al.*, 1989).

In the case of H_2S, the primary route of entry into the body is through the lungs via inhalation exposure. The study of H_2S toxicity *in vivo* provides a model that preserves the naturally occurring processes of uptake, distribution, metabolism and excretion, all of which have been shown to influence the biological actions of drugs and toxicants in a circadian manner (Reinberg, 1992). It also allows for the investigation of the effects of chronic exposure, a situation common in many industries. There is evidence that chronic exposure to low concentrations of H_2S result in alterations of the hippocampal EEG activity. Skrajny *et al.* (1996) demonstrated that inhalation exposure to 25, 50, 75 and 100 ppm to freely moving rats resulted in an increase in the total power of hippocampal theta activity in a dose-dependent manner. This effect was shown to be cumulative, as

successive exposures over a period of 5 days produced a gradual increase in the total power, the maximum effect being noted on the 5th day. In addition, this effect was shown to be reversible, as the increase in total power had returned to baseline levels after two weeks.

It has been suggested that EEG activity may be used as an indicator of cognitive processing relating to specific behaviors (Fox *et al.*, 1982). Theta is the most characteristic EEG activity of the hippocampus (Bland, 1986), and disruptions in theta activity have been correlated with memory impairments (Markowska *et al.*, 1995; Winson, 1978). Disruptions in theta activity following exposure to H_2S may account for some neurological deficits attributed to H_2S poisoning. Therefore, the investigation of hippocampal theta activity in the freely moving animal may be a useful tool to examine a possible circadian rhythm in H_2S neurotoxicity.

Hypothesis

Using a laboratory rat model, it is hypothesized that the effects of H_2S on hippocampal theta activity will be different when administered during the dark period than during the light period, varying in a circadian fashion.

Objectives

The objectives of this proposal are:

1. to record hippocampal theta activity during exposure to 125 ppm H_2S in the early light (rest) period and in the early dark (active) period of rats

2. to determine if repeated exposures to H_2S over 5 consecutive days alters the power spectrum of hippocampal theta

3. to determine if repeated exposures to H_2S results in a cumulative effect on hippocampal theta activity

4. to establish whether the effects of H_2S on hippocampal theta activity are still evident after a period of two weeks following the last day of exposure

5. to determine if H_2S shows a circadian rhythm in its effect on hippocampal theta activity.

Materials and Methods

Subjects

Male Sprague-Dawley albino rats (Charles River; St. Constant, Quebec) weighing between 250-300 g at the beginning of the experiment were used. They were housed individually in plastic cages, with free access to food and water, except during the exposure to H_2S or control gas. The animals in each group were on a 12 hour light-dark cycle, with lights on at 0700h and off at 1900h for the light condition, and lights on at 1900h and off at 0700h for the dark condition. Room temperature was maintained around 21° C, and room lighting intensity was approximately 490 lux during the lights-on period.

It is generally accepted that animals should be adapted to a new light schedule for a period of two weeks prior to initiation of any experimental treatment (De Prins et al., 1986). It has been demonstrated previously that shifting light-dark cycles by a period of 8 hours results in reentrainment of the circadian rhythms of wheel running activity in about 6 days (range 4.1-6.8 days; Takamure et al., 1991). Furthermore, there is evidence that albino rats reentrain more rapidly than pigmented rats. Kennaway (1994) has shown that after an 8 hour shift in light/dark cycle, the rhythms in melatonin metabolite excretion were restored in about 4 days (range 2-10 days) in albino rats, whereas pigmented rats took greater than 10 days to reentrain. Activity rhythms were also restored more rapidly in albino rats. The animals used in the dark condition in this study were placed on a reversed light cycle for a minimum of two weeks prior to beginning the experiment.

Surgical Procedure

All surgeries were carried out while the animals were on a standard 12 hour L/D schedule. Animals were anaesthetised with an intramuscular injection of a ketamine and xylazine solution (85 mg/mL:15 mg/mL; dose 0.1 mL/100 g body weight, U of C Animal Health Unit guidelines for rat anaesthesia) and placed in a custom stereotaxic frame. Ketamine/xylazine was supplied by the University of Calgary Biosciences Vivarium. During the surgery, maintenance doses of ketamine/xylazine (0.1 mL) were administered as required to ensure the animal remained in an anesthetized state. The animal was placed in the stereotaxic frame and secured with earbars. Once the head was level, a small incision was made, and membranes were removed to expose the skull. Measurements of lambda and bregma were taken, and bregma served as the reference point for placement coordinates of the electrodes. Holes were drilled into the skull with a dental drill (model NCL-35SH/H, Healthco Inc.) to expose the surface of the brain. One electrode was placed in the right frontal cortex, and one in both the contralateral and ipsilateral dentate gyrus of the hippocampus. Coordinates for electrode placement were determined using The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 1986). All depth measurements were taken from the surface of the brain. The coordinates for frontal cortex were: 1.2 mm anterior to bregma, 2.4 mm lateral to midline and 1.5 mm ventral to dural surface, and for bilateral dentate gyrus were: 3.4 mm posterior to bregma, 1.8 mm lateral to midline, and 2.7 mm ventral to dural surface. Tungsten wire insulated with Kynar (vinylidene flouride resin; The Plastic Dept., Pennwalt Corporation, Philadelphia) and the tip electrolytically etched in a saturated potassium nitrate solution served as the

recording electrodes. An uncoated tungsten wire placed in the right parietal cortex was used as an indifferent electrode, and a gold-plated amphenol micro-miniature connector (Richey Electronics, Calgary, Alberta) screwed into the skull over the left frontal cortex served as a ground. The electrodes and ground were held in place with dental acrylic affixed to stainless steel screws (M 1.6 x 0.35 x 3 mm; Spae-Naur Inc., Kitchener, Ontario). Prior to suturing, topical antibiotic (Gentacin™) was applied to the area around the headset. The animals in the light condition were allowed to recover for at least 7 days prior to initiation of experimental treatment, whereas the animals in the dark condition were placed on the reversed light cycle immediately following surgery, and two weeks elapsed before initiation of experimental treatment.

Exposure

Rats were exposed to H_2S concentrations of 125 ppm, 3 hours/day for 5 consecutive days. The exposures took place in a sealed plexiglass chamber, designed to permit observation of the animal during exposure. Filtered room air was drawn through the chamber with a vacuum pump and combined with certified H_2S (1990 ppm of H_2S in nitrogen; Linde, Union Carbide Canada Limited). The mixture was passed through an orifice plate to measure flow rate, then through a diffuser in the top of the chamber. An air flow of 11 litres/minute allowed a complete chamber replacement every three minutes. The volume of the H_2S/nitrogen mixture did not exceed 5% of the total volume of the air drawn into the chamber. The concentration of H_2S inside the chamber was continuously monitored through an H_2S transmitter (H_2S CiTicel 4-20 transmitter; City Technology

Centre, Portsmouth, England). The transmitter relayed the gas concentration via a 4-20mA input signal to an H_2S monitor (Gastech Model SAFE T NET –2000; Gastech Canada, Alberta), which displayed the concentration of H_2S on a LED read-out. Low and high alarms were activated on the monitor, set at 115 and 135, respectively. Control exposures to nitrogen/air were carried out, since the H_2S used in this study was balanced with nitrogen. The flow rate of nitrogen was similar to that of the H_2S/N_2 mixture during the exposure to hydrogen sulfide (125 ppm).

Experimental Design

Two groups (light vs. dark) of 5 animals each were exposed to 125 ppm H_2S, while another two comparable groups were exposed to the nitrogen/air control mixture. For a minimum of 7 days following surgery, the animals in the dark condition were adapted to the reversed light cycle. All exposures were conducted between 9:00 am and 12:00 pm. Since the two groups were on opposite light/dark schedules, the time the exposures took place can be expressed in terms of the 24 hour clock, with the time of lights on defined as 0100h. Therefore, in the light condition, exposures took place during 0300h-0600h. In the dark condition, exposures were conducted from 1500h-1800h. The schedule of testing was as follows: Two animals were run simultaneously, one in the light condition and one in the dark condition. For example, on one week the animal in the dark condition would receive H_2S and the animal in the light condition would receive nitrogen/air. The following week the animal in the dark condition would receive nitrogen/air and the light condition would receive H_2S.

To elicit theta activity in the hippocampus, a motorized rotating circular platform (5-6 rpm) was used to initiate movement of the animal (Figure 4). The platform was constructed with plexiglass with small holes drilled into it so as not to obstruct the flow of gas in the exposure chamber. A plexiglass bar was placed across the center, approximately one inch above the platform to prevent the animal from remaining motionless while the platform was activated. On each day of testing, the animals were placed in the chamber, and prior to exposure a ten-minute baseline recording of theta activity was taken. The animals were then treated with either H_2S or nitrogen/air for three hours. Ten-minute recordings of EEG activity were taken between 50 -60, 110-120, and 170-180 minutes during the three-hour exposure period. The behavior of the animal was continuously observed and comments made by the investigator were recorded on videotape using an audio channel. Behavior was classified into four categories: walking, associated with type 1 (high frequency) theta; immobility, associated with large amplitude irregular activity ; head movements in the absence of walking behavior, associated with theta; and grooming, associated with a mixture of LIA and theta (Table 2).

Two weeks following the fifth day of exposure, theta activity was reassessed in the H_2S exposed animals for a three hour period for one day to determine if theta activity recovered to pre-exposure values. All recordings followed the procedures outlined above.

Power Spectral Analysis of Hippocampal Theta

Light Condition

Recording equipment used during the experiments were different between the

Table 2. Classification of rat behavior during testing based on activity and EEG

Category	Behavior	Activity in Hippocampal EEG
Still	Immobile, eyes open,head up off the floor	Large amplitude irregular slow waves (LIA), occasional (rare) low frequency theta
Walking	Coordinated gross body movements of the limbs and trunk considered walking, large postural shifts, turning, resisting to walk by struggling against the horizontal bar	High (5.5-8.5 Hz) frequency theta, no LIA
Head	Minor movements of the head and neck in the absence of large limb and trunk movements	High frequency theta similar to that during walking, mixed with LIA
Grooming	Cleaning fur, paws and face by licking, scratching or chewing	LIA with intermittent occurrence of theta

light and dark experimental conditions. This was due to availability of equipment. Recordings of hippocampal EEG activity in the light condition were made using a Grass model 7D Polygraph, with filter settings at 0.3 Hz for the half amplitude low filter and 35 Hz for the half amplitude high filter. The signals from the polygraph were amplified with a Wide Band A.C. EEG pre-amplifier model 7P5B (Grass Medical Instruments Corporation, Quincy, Massachusetts), and displayed on a digital oscilloscope (model R5103N; Tektronix Canada Inc., Weston, Ontario). The data was stored on videocassette using a Neurodata interface (Model DR –484; NeuroData Instruments Inc., New York N.Y.) and VCR (RCA) for subsequent computer analysis. During the recording sessions, the behavior of the animal was continuously observed, and comments were stored on videocassette using an audio channel (Figure 4).

Dark Condition

Recordings of hippocampal EEG activity were made using a Grass model 79C Polygraph, with filter settings at 0.3 Hz for the half amplitude low filter and 35 Hz for the half amplitude high filter. The signals from the polygraph were amplified with a Wide Band A.C. EEG pre-amplifier model 7P5B (Grass Medical Instruments Corporation, Quincy, Massachusetts). The signal was stored on videotape using a Vetter PCM Data Recorder (model 200; A.R. Vetter Co., Rebersburg, PA). The EEG data were recorded digitally at a sampling rate of 44.1 KHz per channel. A voice channel was recorded on the normal audio track of the VCR. Real-time visualization of EEG data was displayed on an oscilloscope (model TDS-340; Tektronix Canada Inc., Weston, Ontario) (Figure 5).

Figure 4. Diagram of exposure chamber and EEG recording equipment for animals in the light condtion.

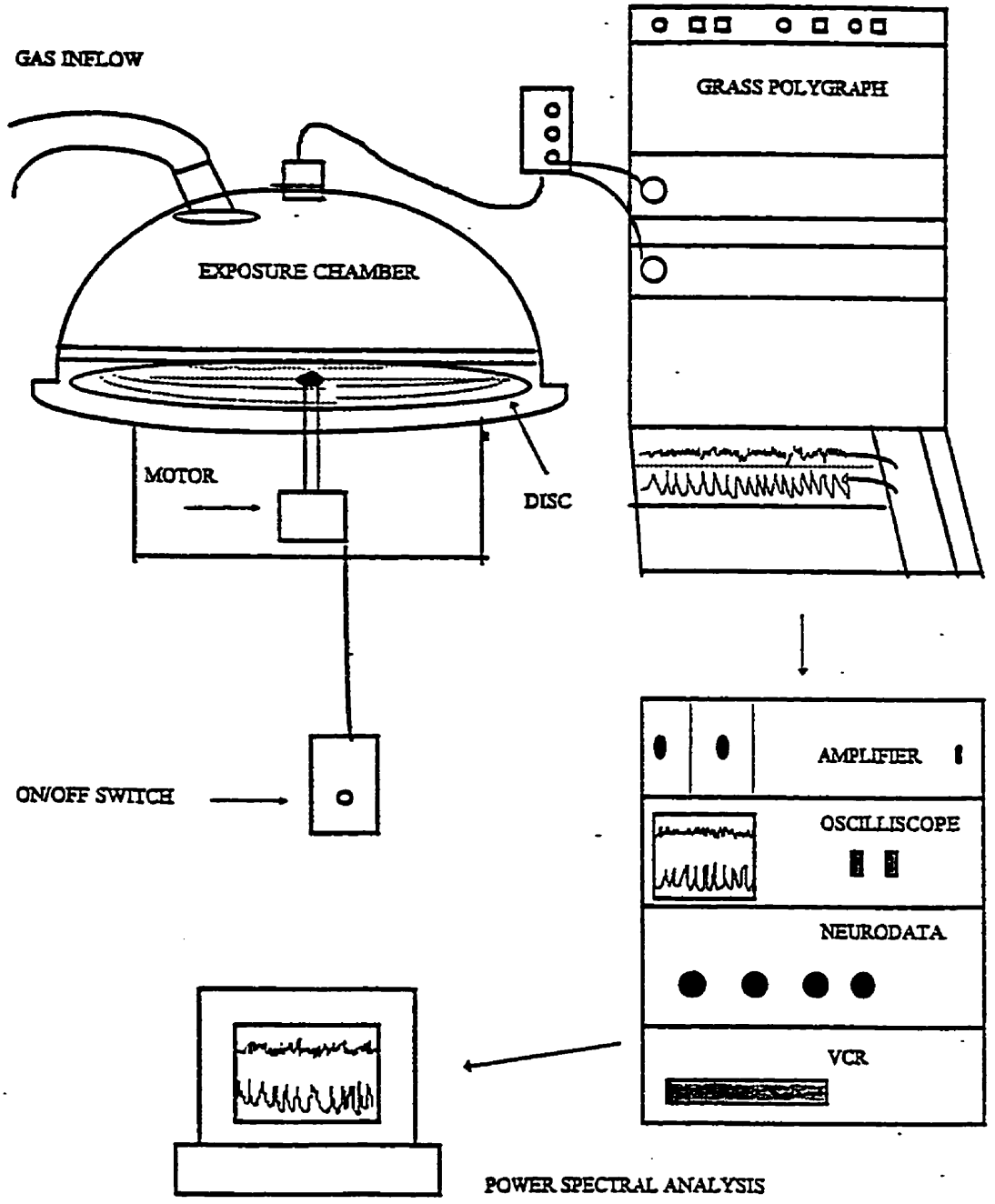
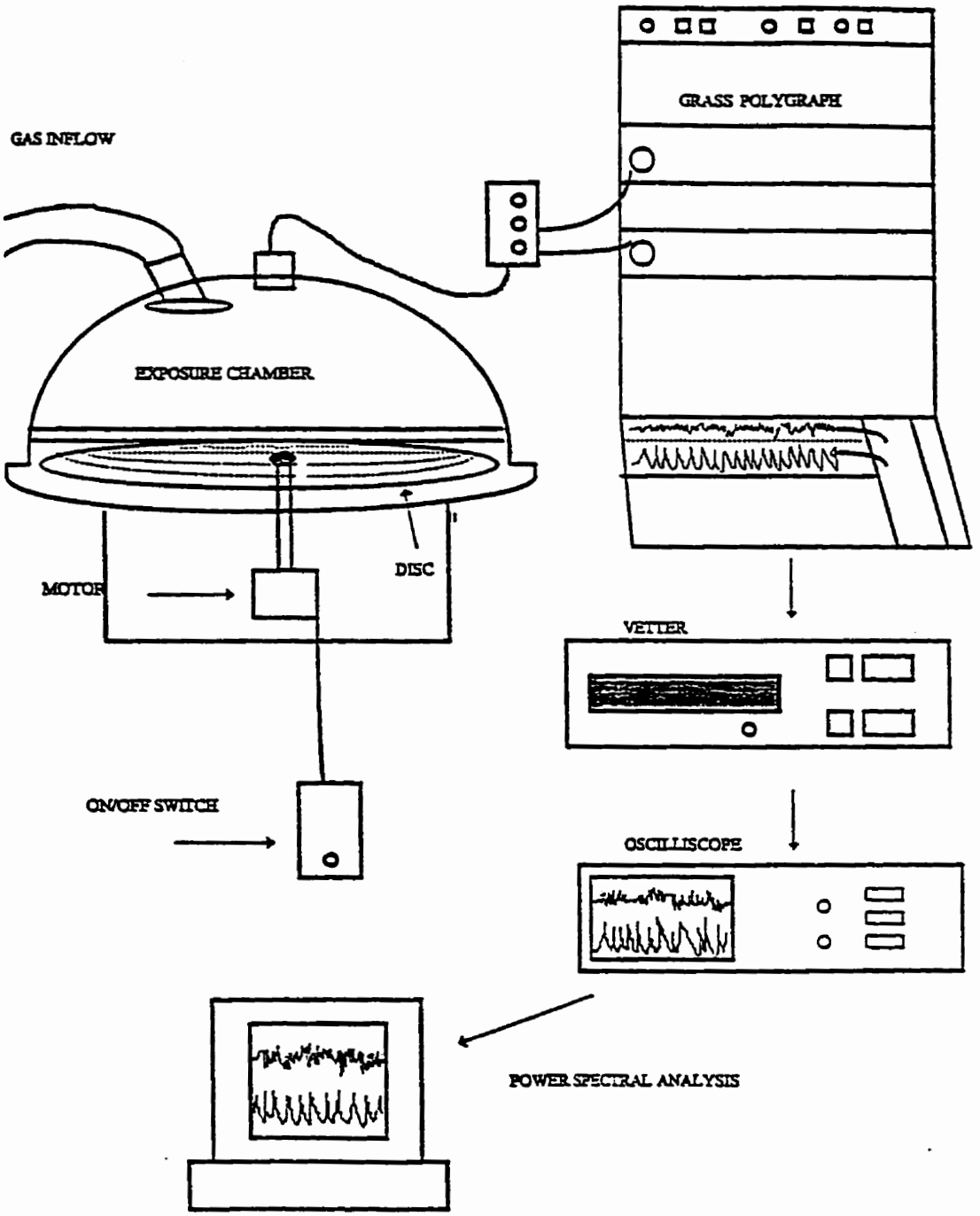

GAS INFLOW
GRASS POLYGRAPH
EXPOSURE CHAMBER
MOTOR
DISC
ON/OFF SWITCH
AMPLIFIER
OSCILLISCOPE
NEURODATA
VCR
POWER SPECTRAL ANALYSIS

Figure 5. Diagram of exposure chamber and EEG recording equipment for animals in the dark condition.


GRASS POLYGRAPH
GAS INFLOW
EXPOSURE CHAMBER
DISC
MOTOR
VETTER
ON/OFF SWITCH
OSCILLISCOPE
POWER SPECTRAL ANALYSIS

Data was acquired from videocassette to a computer software program designed for this laboratory, based on LabView 4.0. (Advanced Measurements Inc., Calgary, Alberta), at a sampling rate of 200 Hz. During acquisition the data from each 10 minute sample was categorized according to behavioral state of the animal to allow for discrete analysis of EEG activity occurring within a specific behavioral state. The data from each 10 minute recording period (pre-treatment, 1st, 2nd and 3rd hour) were analyzed in 2.5 second epochs. Measures of peak frequency (Hz), power at peak frequency ($Vrms^2/Hz$ or power density) and total power (V^2/Hz) of hippocampal theta activity during walking, and LIA during immobility, were assessed.

Statistical Analysis

EEG data was initially imported from the spectral analysis program onto a Microsoft Excel Spreadsheet (version 7.0 for windows 95; Microsoft Inc.) and statistical analyses performed using Sigma Stat (version 2.0; Jandel Scientific, San Rafael, CA).

The values obtained for each 2.5 second epoch during the 10 minute recording period were averaged to obtain the mean ± standard error of the mean (S.E.M.). This gives the mean ± S.E.M.. for each hour, for each dependent variable. For each day, the mean values of the 1st, 2nd and 3rd hour were averaged, which resulted in a single mean number representing the day of treatment. Since these numbers vary greatly between individual subjects, the data were represented as a percentage of the baseline recording obtained prior to exposure on day 1. This number was obtained by dividing the mean number from each day (1 through 5) by the baseline value on day 1, then multiplying by

100. Standard errors were obtained by combining the percentages from all subjects within a group. Differences between control and exposed groups, and differences between light and dark groups were calculated by two-way ANOVA, while one-way ANOVA was used to determine if there were any differences due to the day (1-5) of treatment. Tukey multiple comparison tests were used to follow up any significant main effects and interactions (Sigma Stat, version 2.0, Jandel Scientific, San Rafael, CA).

Results

Histology

Upon completion of the experiment, animals were deeply anaesthetized with sodium pentobarbitol and perfused with physiological saline to flush the blood from the brain, followed with 10% formalin. Brains were carefully removed and stored in 30% glucose/formalin solution. The fixed tissue was embedded in O.C.T. (embedding medium for frozen tissue specimens, Canlab) and 40 μm sections were cut on a cryostat. Sections were mounted on glass, gelatin-coated slides and stained with cresyl violet. Placement of the electrode was determined by locating electrode tracks. A representative example is shown in figure 6. In each case, the electrode was located within the dentate gyrus of the hippocampus.

Within Groups

Control Light Condition

Control exposures to a nitrogen/air mixture were carried out in order to exclude the possibility that effects observed during exposures to H_2S were caused by anoxic conditions which may have occurred in the exposure chamber due to the presence of nitrogen. The control exposure to nitrogen/air did not result in any significant differences in the hippocampal EEG activity of the animals exposed in the light condition. Hippocampal theta activity occurred during the performance of voluntary movements, such as walking, whereas immobility and automatic behavior, such as grooming, were accompanied by large amplitude irregular activity (LIA). Example recordings of hippocampal theta activity taken during

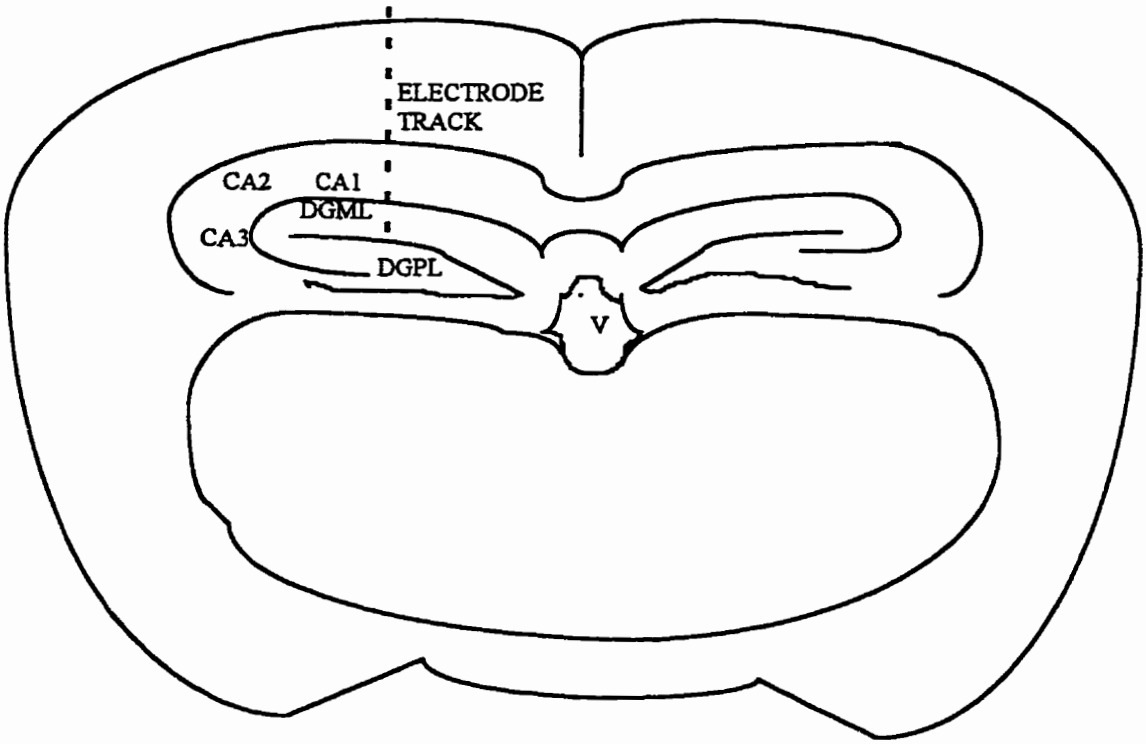


Figure 6. Schematic representation of a coronal section through the rat hippocampus showing the placement of electrodes in the dentate gyrus. Abbreviations: Hippocampus proper areas CA1, CA2, CA3; DGML, dentate gyrus molecular layer; DGPL, dentate gyrus plexiform layer; V, ventricle.

walking and LIA during immobility are presented in Figure 7. Representative power spectra are also presented for hippocampal theta (Figure 8) and LIA (Figure 9), on day 1 and day 5 of exposure. There were no significant changes in the variables of power at the peak frequency and total power of hippocampal theta over the 5 days of exposure (Figure 10a and 10b). The peak frequency of theta did not show any differences over the 5 days of exposure (Figure 10c). In addition, there were no differences in LIA over the 5 days of exposure.

Control Dark Condition

The control exposure to nitrogen/air in the dark condition had a different effect on animals than the control exposure in the light condition. Example recordings of hippocampal theta activity and LIA are presented in Figure 11, and the accompanying power spectra are presented in Figures 12 and 13. There was a significant difference over the 5 days of exposure in the power at the peak frequency of theta ($F(5,24)=5.842$; $p<0.001$) (Figure 14a), and in the total power of theta($F(5,24)=5.050$; $p<0.003$) (Figure 14b). Both the power at the peak frequency and total power decreased over the 5 days of exposure. There was no significant difference in the peak frequency of theta on any day of exposure (Figure 14c), nor were there any differences found in LIA over the 5 days of exposure.

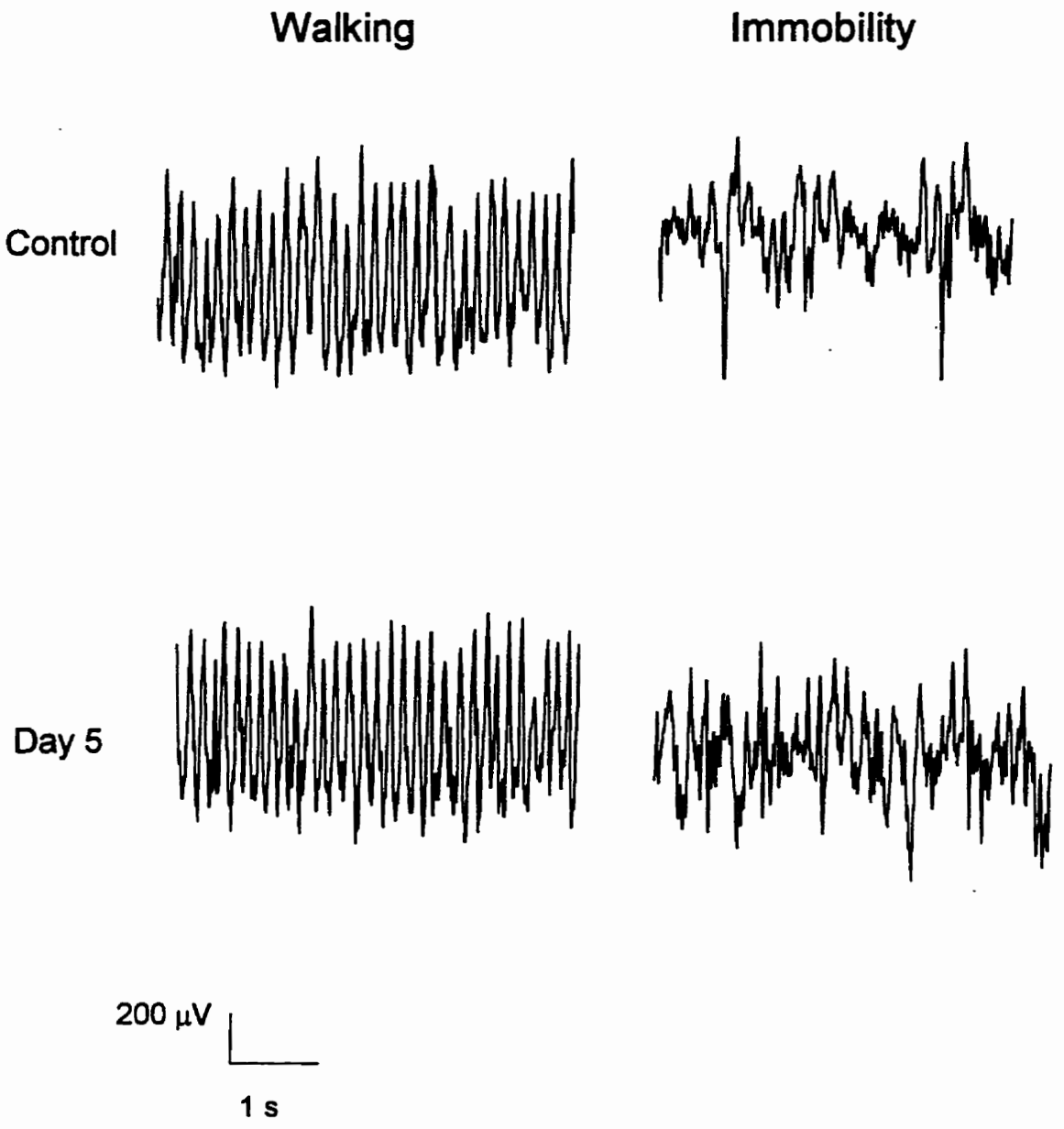


Figure 7. Example recordings of hippocampal EEG activity recorded from dentate gyrus of a rat in the light control condition during walking and immobility prior to and after exposure to nitrogen/air for 5 days.

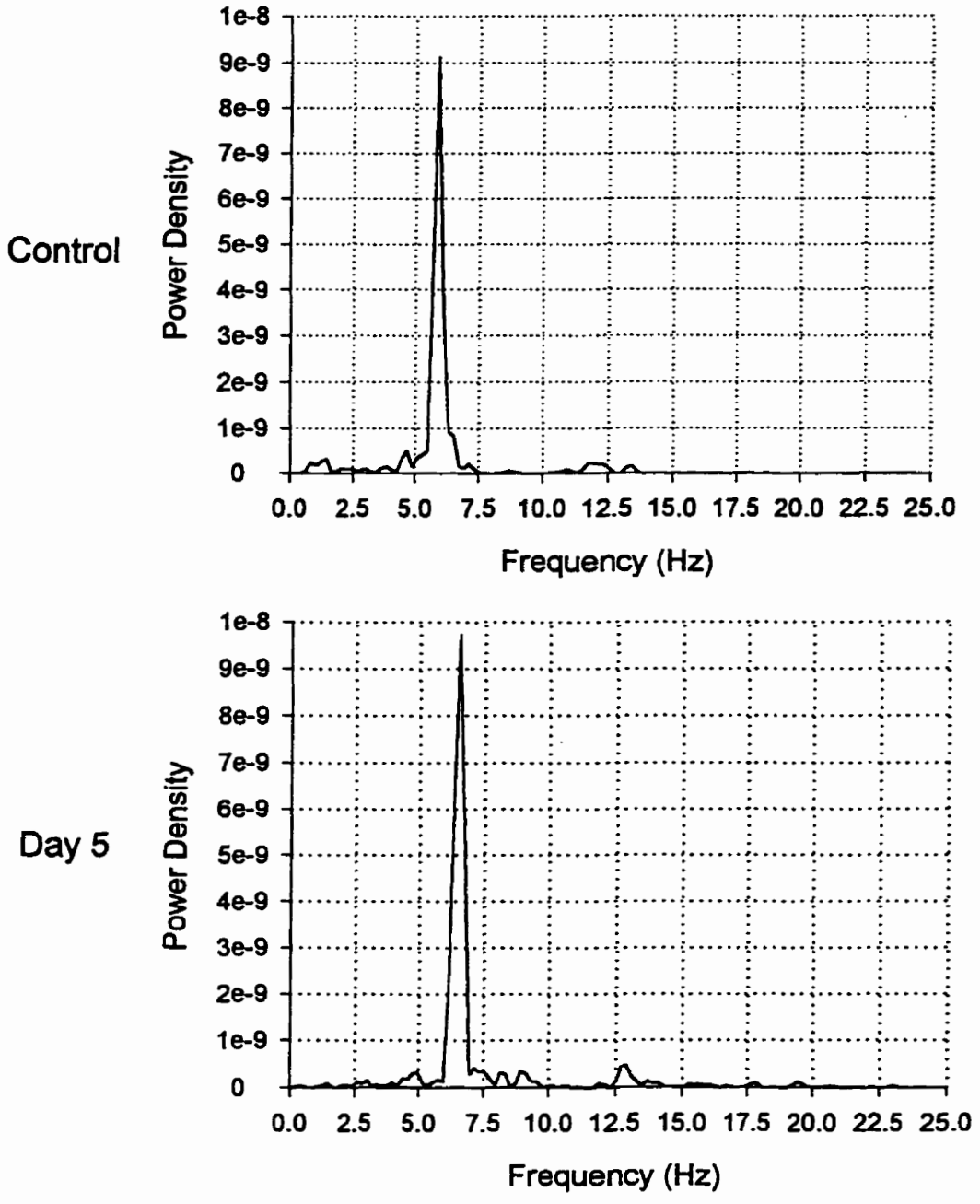


Figure 8. Example power spectra of hippocampal theta activity recorded from dentate gyrus of a rat in the light control condition while walking prior to and following exposure to nitrogen/air on day 5.

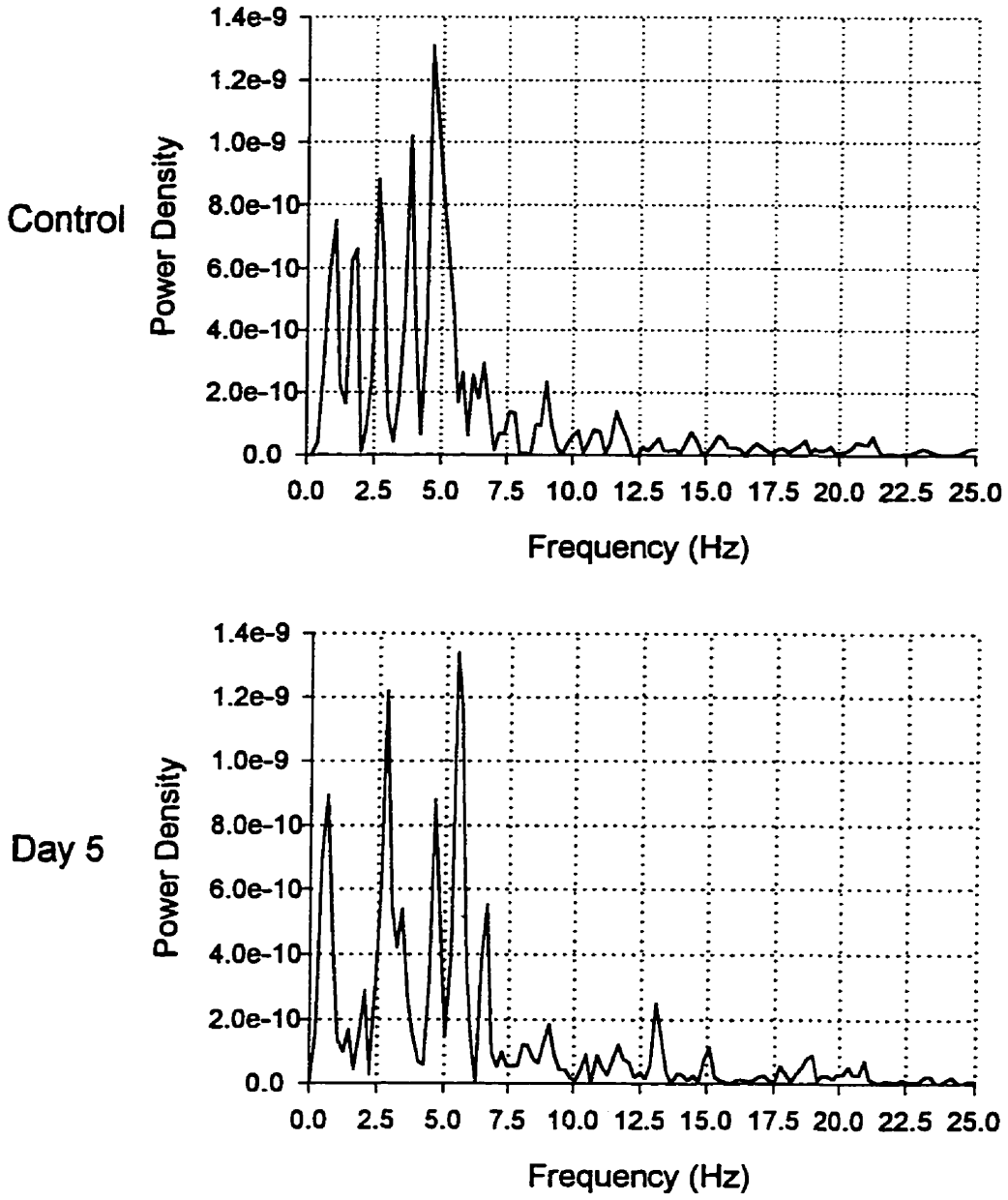
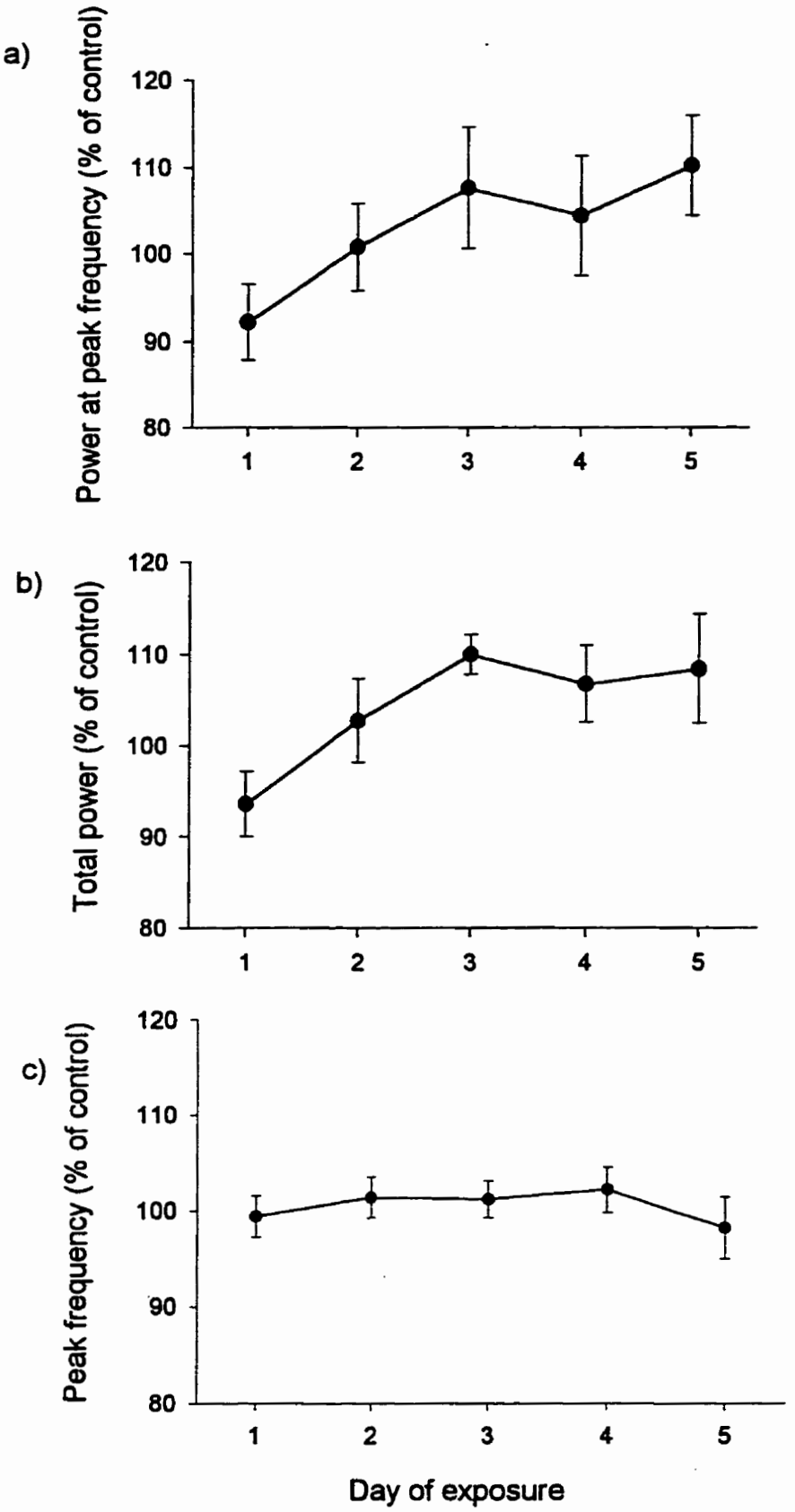


Figure 9. Example power spectra of LIA recorded from the dentate gyrus of a rat in the light control condition during immobility prior to and following exposure to nitrogen/air on day 5.

Figure 10. Graphs demonstrating a change in a) power at the peak frequency, b) total power, and c) peak frequency recorded from the dentate gyrus during walking over the 5 days of exposure to nitrogen/ air for animals in the LIGHT CONTROL condition. Each point represents the mean ± S.E.M. of 5 animals.


a)
Power at peak frequency (% of control)
120
110
100
90
80
1
2
3
4
5
b)
Total power (% of control)
120
110
100
90
80
1
2
3
4
5
c)
Peak frequency (% of control)
120
110
100
90
80
1
2
3
4
5
Day of exposure

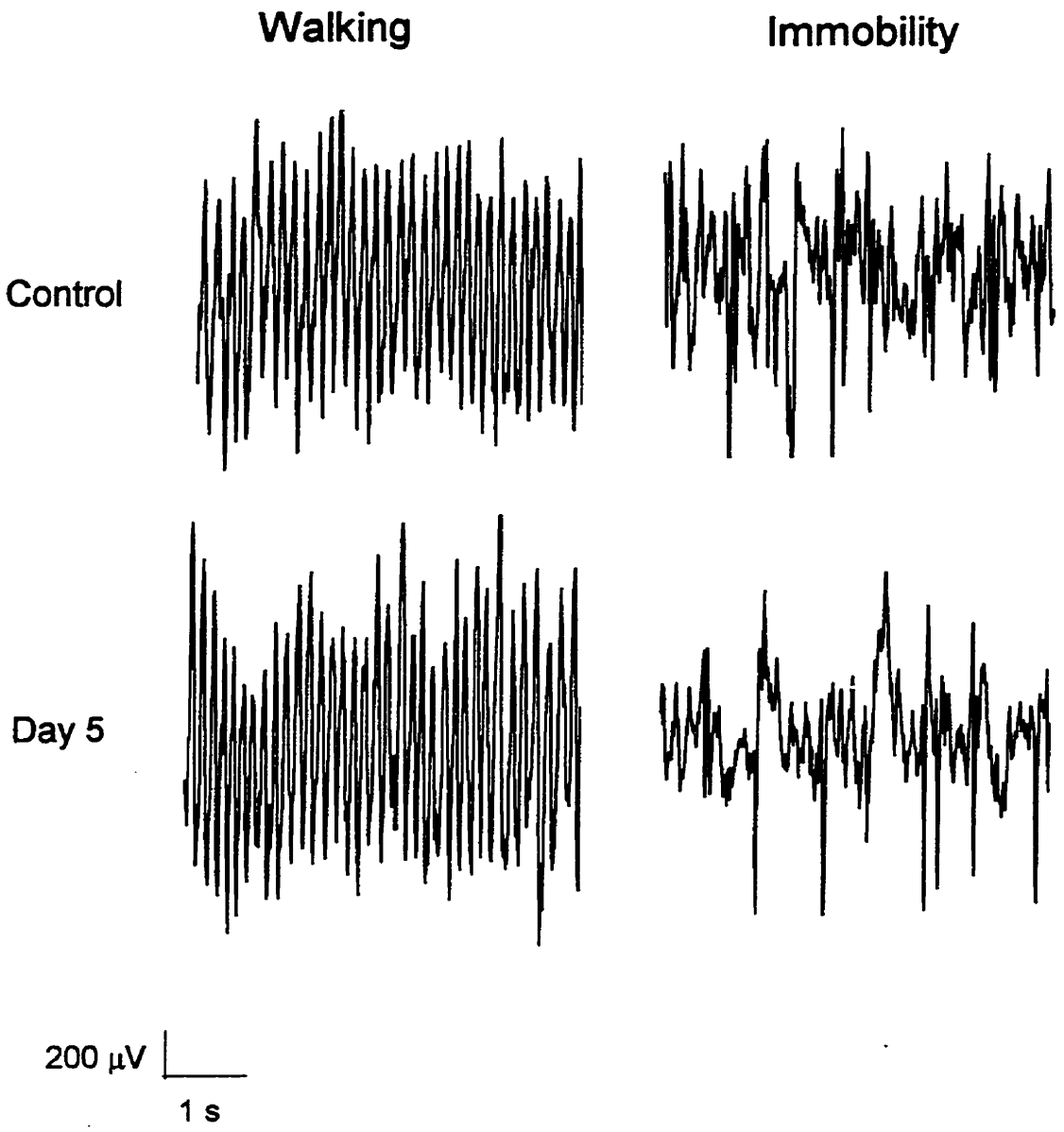


Figure 11. Example recordings of hippocampal EEG activity recorded from dentate gyrus of a rat in the dark control condition during walking and immobility prior to and after exposure to nitrogen/air on day 5.

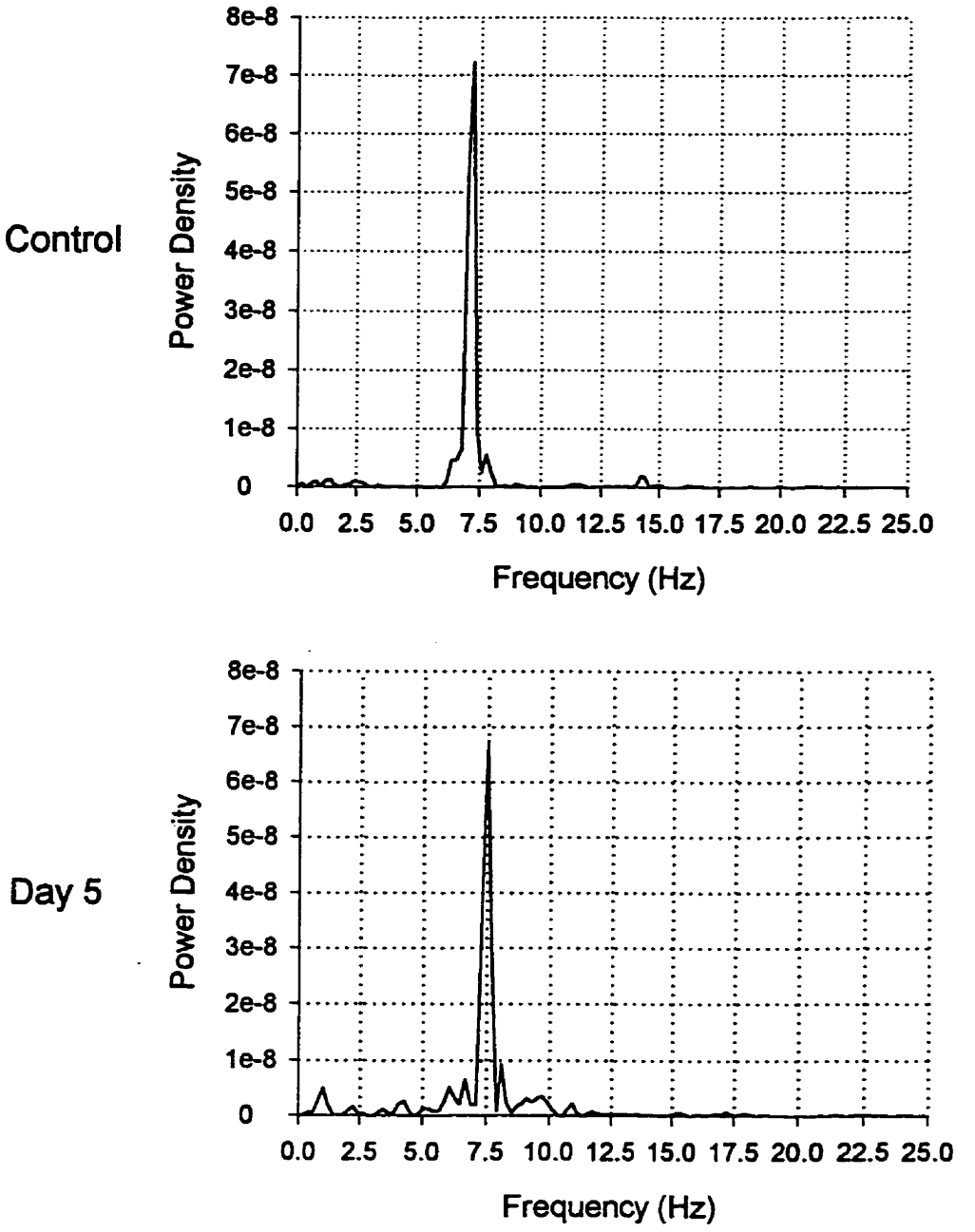


Figure 12. Example power spectra of hippocampal theta activity recorded from dentate gyrus of a rat in the dark control condition while walking prior to and following exposure to nitrogen/air on day 5.

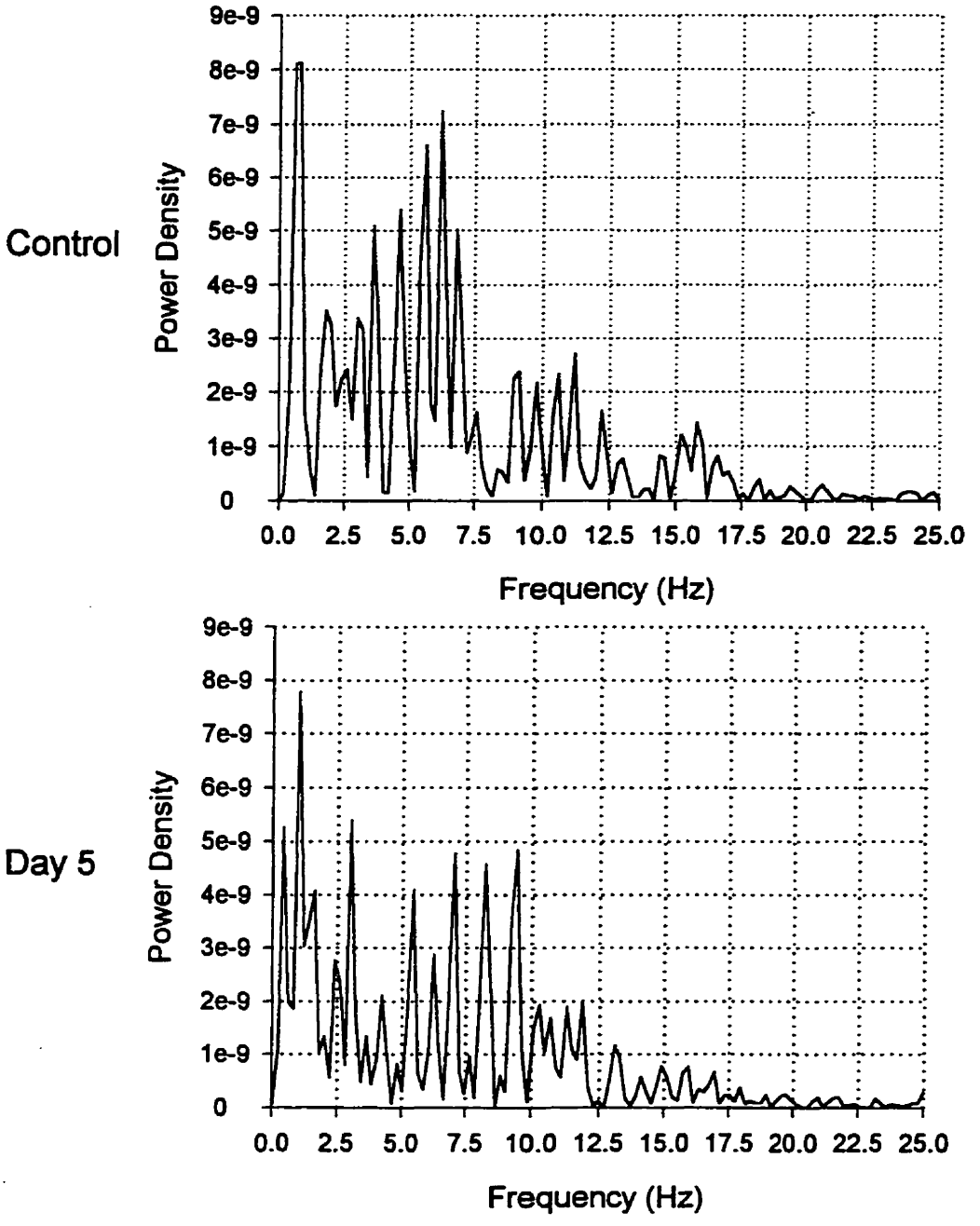
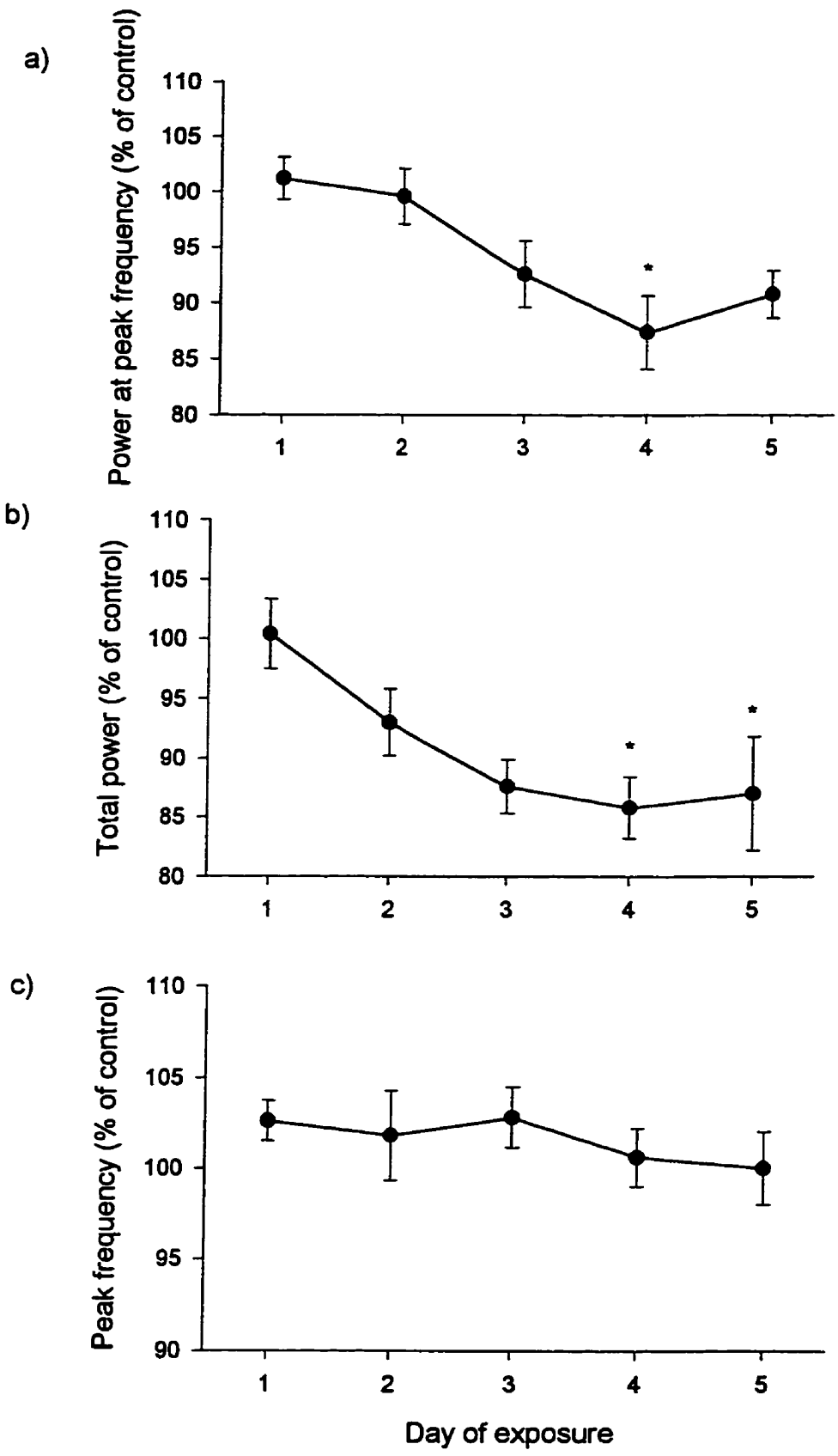


Figure 13. Example power spectra of LIA recorded from the dentate gyrus of a rat in the dark control condition during immobility prior to and following 5 days of exposure to nitrogen/air.

Figure 14. Graph demonstrating the change in a) power at the peak frequency, b) total power, and c) peak frequency of hippocampal theta activity recorded from the dentate gyrus during walking over the 5 days of exposure to nitrogen/air for animals in the DARK CONTROL condition. Each point represents the mean ± S.E.M. of five animals. Asterisks denote significant difference from day 1 pre-exposure control values.


a)
Power at peak frequency (% of control)
110
105
100
95
90
85
80
1
2
3
4
5
*
b)
Total power (% of control)
110
105
100
95
90
85
80
1
2
3
4
5
*
*
c)
Peak frequency (% of control)
110
105
100
95
90
1
2
3
4
5
Day of exposure

Repeated Exposure to 125 ppm H_2S

Light Condition

Animals exposed to 125 ppm H_2S showed significant differences in hippocampal theta activity over the 5 days of exposure in all variables tested. Figure 15 shows example recordings of hippocampal theta and LIA on day 1 and day 5 of exposure, and the representative power spectra are shown in Figures 16 and 17. There were significant differences in power at the peak frequency ($F(5,24)=5.727$, $p<0.001$) (Figure 18a), and in total power of hippocampal theta activity ($F(5,24)= 3.101$, $p<0.027$) (Figure 18b). In each case, the values decreased over the 5 days of exposure. Comparison of the values obtained prior to exposure on day 1 (control exposure) and following each day of exposure revealed that the power at the peak frequency of theta was significantly reduced on days 2, 3, 4, and 5 ($p<0.05$), which was maximally reduced on day 4 at 74.40 ± 1.86%, and the total power was significantly reduced on day 5 ($p<0.05$), at 77.60 ● 2.36% of control. The power at peak frequency and total power of theta recorded just prior to the exposures to H_2S on each day also decreased in a cumulative fashion, similar to what was seen during exposure to H_2S. The peak frequency of theta was decreased compared to pre-treatment values over the 5 days ($F(5,24)=3.155$, $p<0.025$) however this effect was significant only on day 2 of exposure, at 89.60 ± 2.42% (Figure 18c). There was no decrease in peak frequency observed during the recording taken just prior to exposure to H_2S on any of the 5 days. There were no significant differences in LIA recorded over the 5 days of exposure.

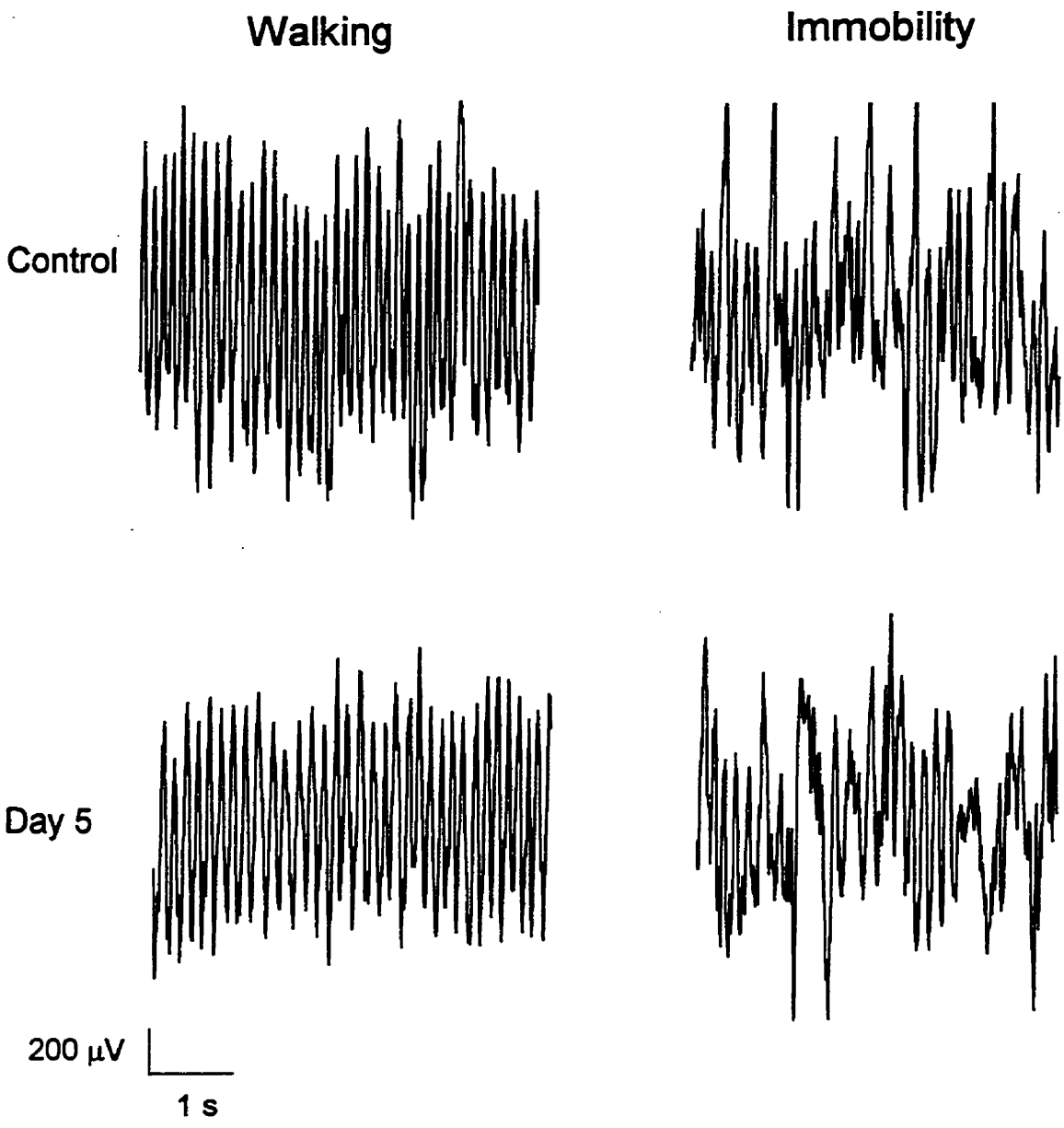


Figure 15. Example recordings of hippocampal EEG activity from dentate gyrus of a rat in the light H_2S condition during walking and immobility prior to and after exposure to 125 ppm H_2S for 5 days.

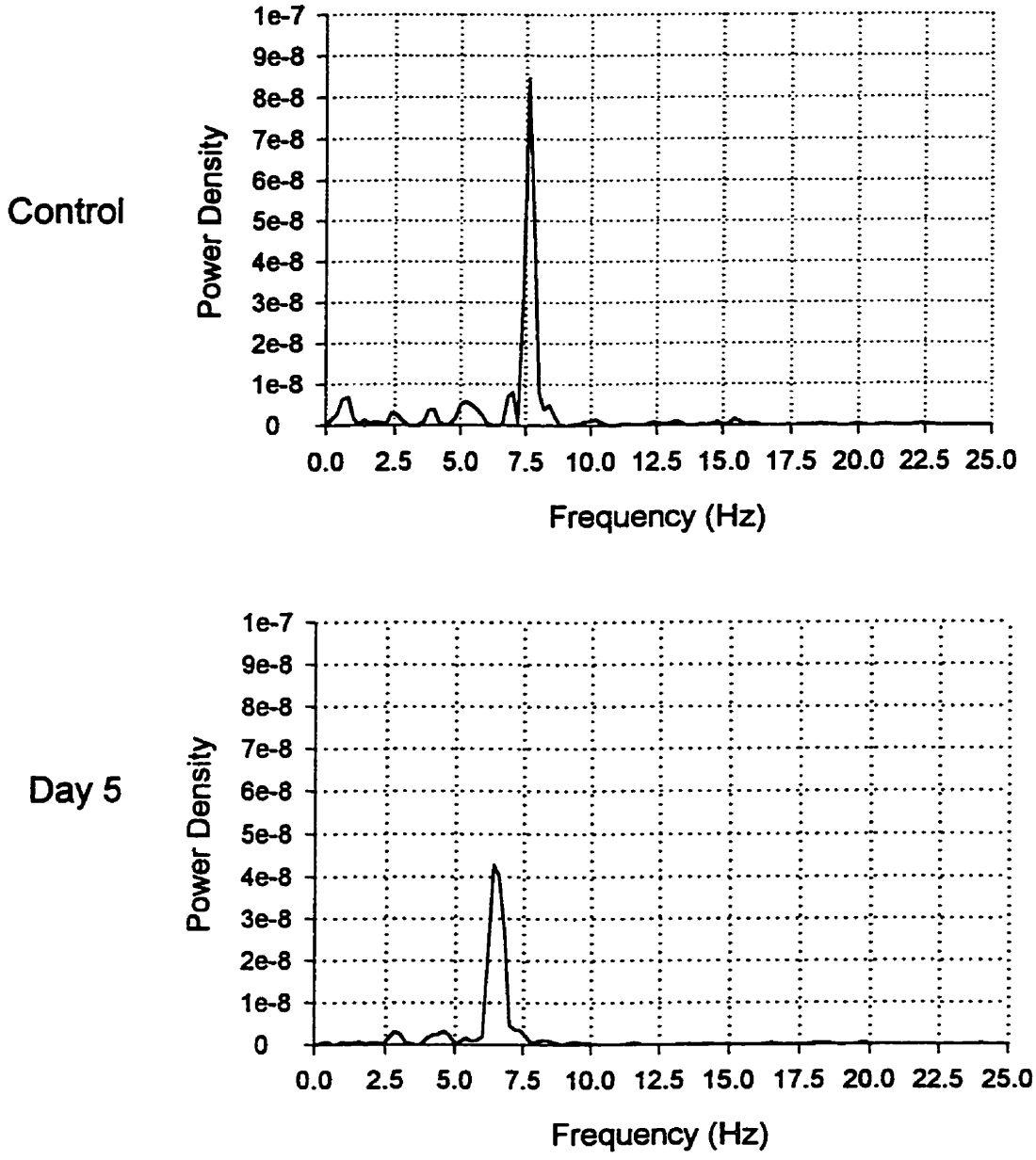


Figure 16. Example power spectra of hippocampal theta activity recorded from dentate gyrus of a rat in the light H_2S condition while walking prior to and following exposure to 125 ppm H_2S for 5 days.

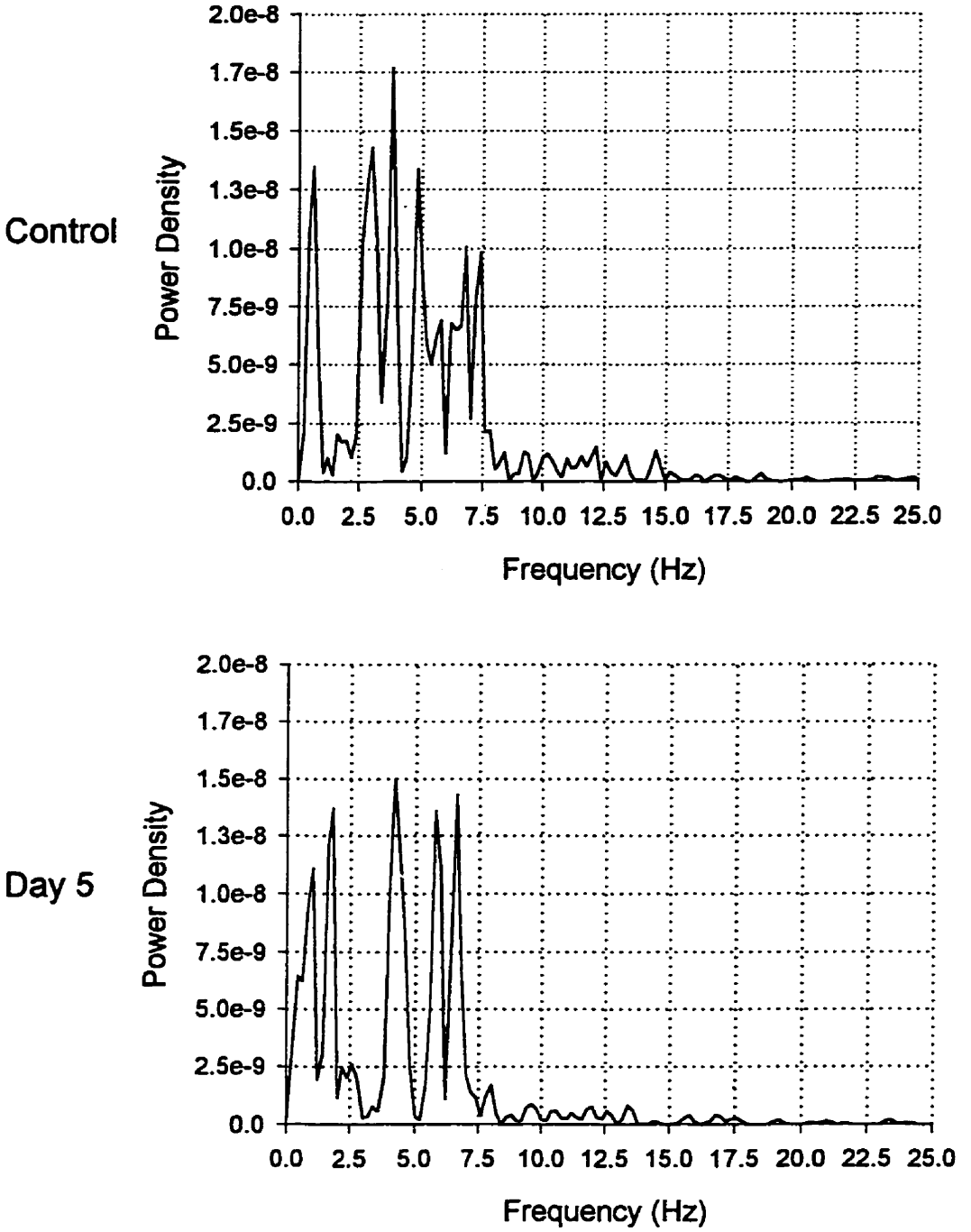
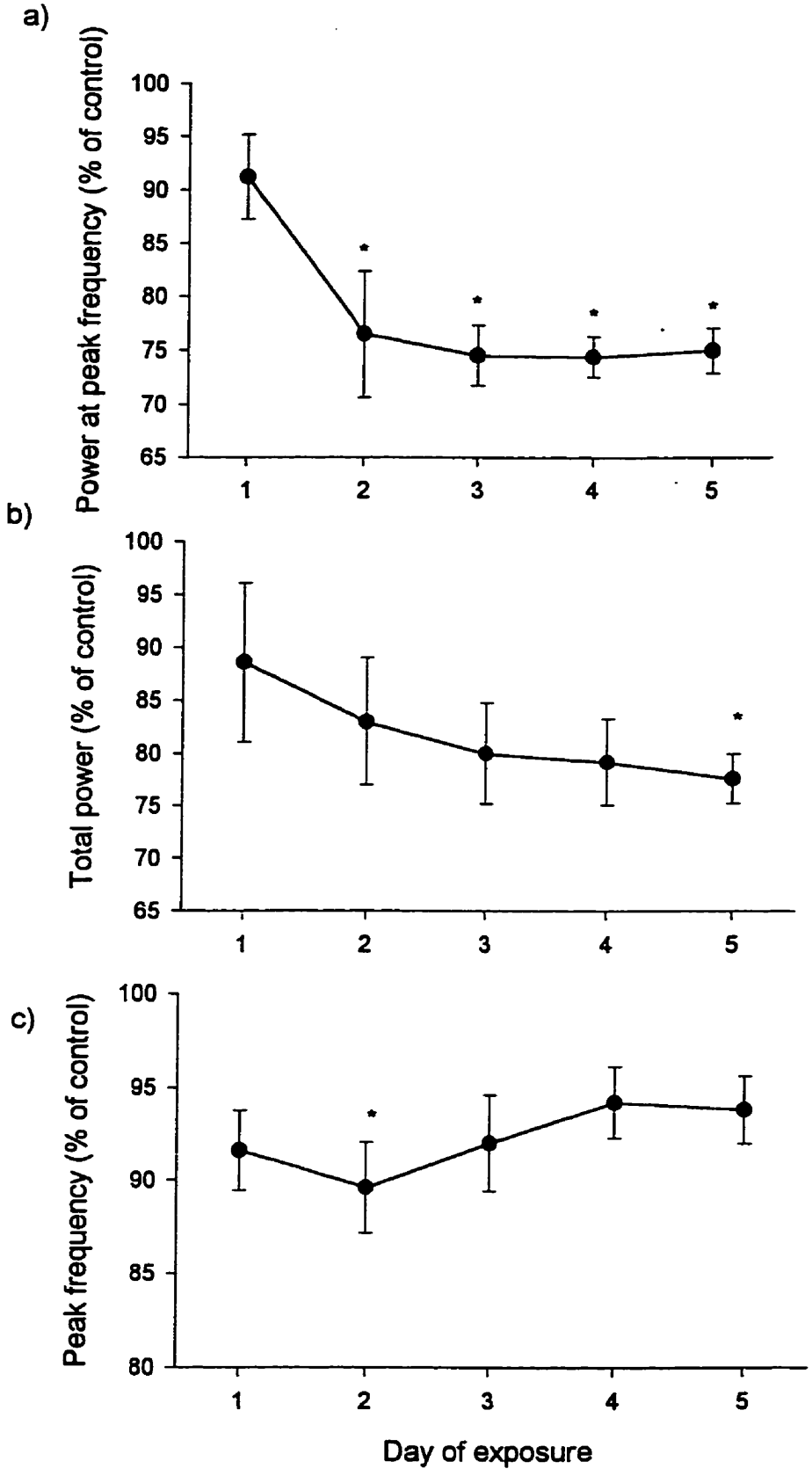


Figure 17. Example power spectra of LIA recorded from the dentate gyrus of a rat in the light H_2S exposed condition during immobility prior to and following 5 days of exposure to 125 ppm H_2S.

Figure 18. Graphs demonstrating the change in a) power at the peak frequency, b) total power, and c) peak frequency of hippocampal theta activity recorded from the dentate gyrus during walking over the 5 days of exposure to 125 ppm H_2S for animals in the LIGHT EXPOSURE condition. Each point represents the mean ± S.E.M. of five animals. Asterisks denote significant difference from the day 1 pre-exposure control.


a)
Power at peak frequency (% of control)
100
95
90
85
80
75
70
65
1
2
3
4
5
b)
Total power (% of control)
100
95
90
85
80
75
70
65
1
2
3
4
5
c)
Peak frequency (% of control)
100
95
90
85
80
1
2
3
4
5
Day of exposure

Dark Condition

Exposure to 125 ppm H_2S to animals in the dark condition also resulted in significant changes in hippocampal theta activity over the 5 day exposure period. Example recordings of theta activity and LIA are presented in Figure 19. The representative power spectra are shown in Figures 20 and 21. Exposure to H_2S resulted in an increase in the power at the peak frequency ($F(5,24)=6.11$, $p<0.001$), which was significantly different from control on days 2, 3 and 4. The maximal increase occurred on day 2, at 142.20 +/- 13.95%, then gradually decreased (Figure 22a). There was a significant increase in the total power of theta ($F(5.24)=3.21$, $p<0.023$), which was significantly different from control on day 2, at 130.00+/- 11.66% (Figure 22b). The power at peak frequency and total power of theta recorded just prior to exposure on each day also increased in a manner similar to that during the exposure to H_2S. There was also a significant decrease in the peak frequency of theta activity during exposure ($F(5,24)=4.475$, $p<0.005$). The decrease was significantly different from control on all days of exposure, and was maximal on day 5 at 87.80 +/- 2.84% (Figure 22c). The peak frequency of theta also decreased over the 5 days of exposure during the recording taken prior to exposure. There were no significant differences in LIA following repeated exposure to H_2S.

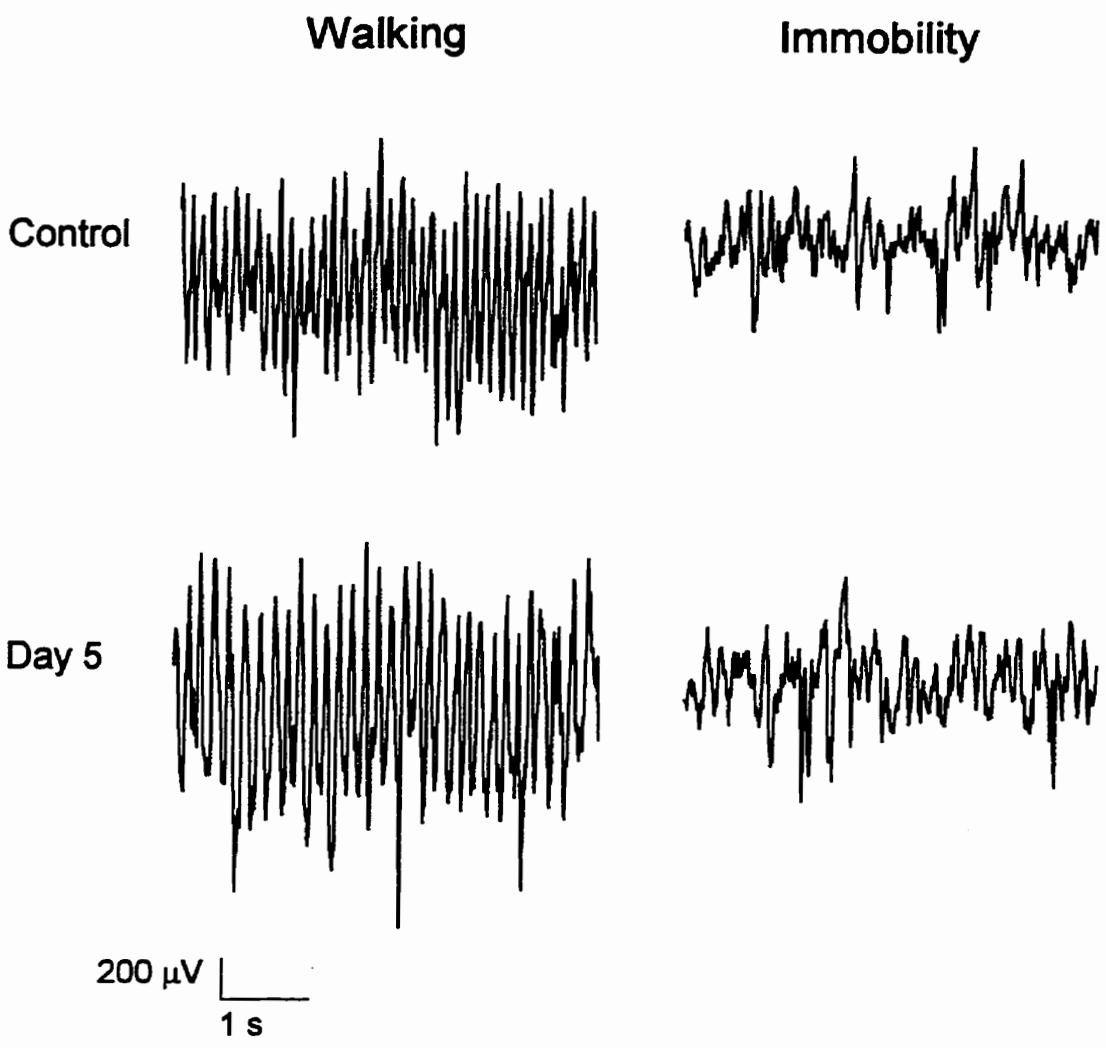


Figure 19. Example recordings of hippocampal EEG activity recorded from dentate gyrus of a rat in the dark exposure condition during walking and immobility prior to and after exposure to 125 ppm H_2S on day 5.

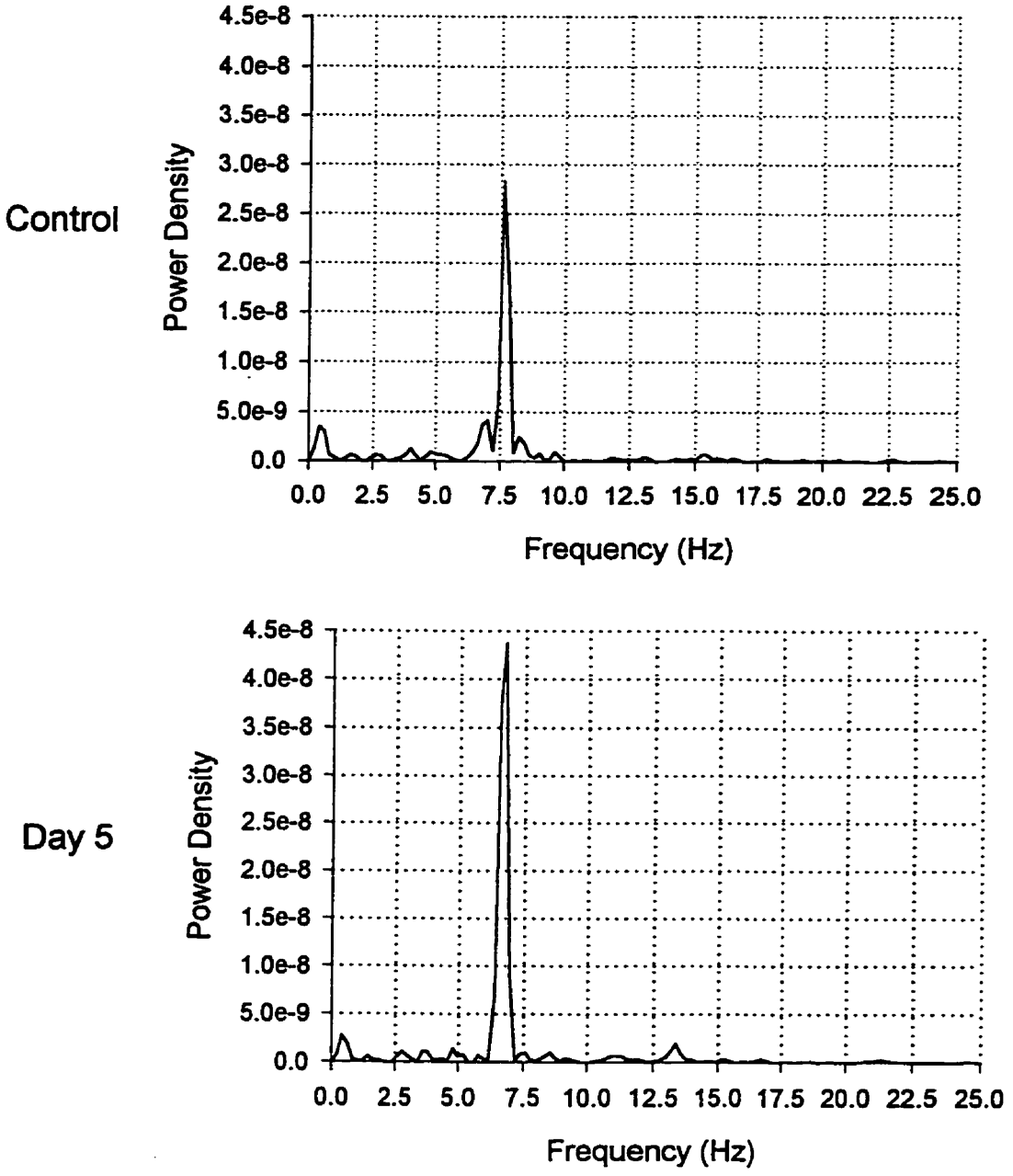


Figure 20. Example power spectra of hippocampal theta activity recorded from dentate gyrus of a rat in the dark H_2S exposed condition while walking prior to and following exposure to 125 ppm H_2S on day 5.

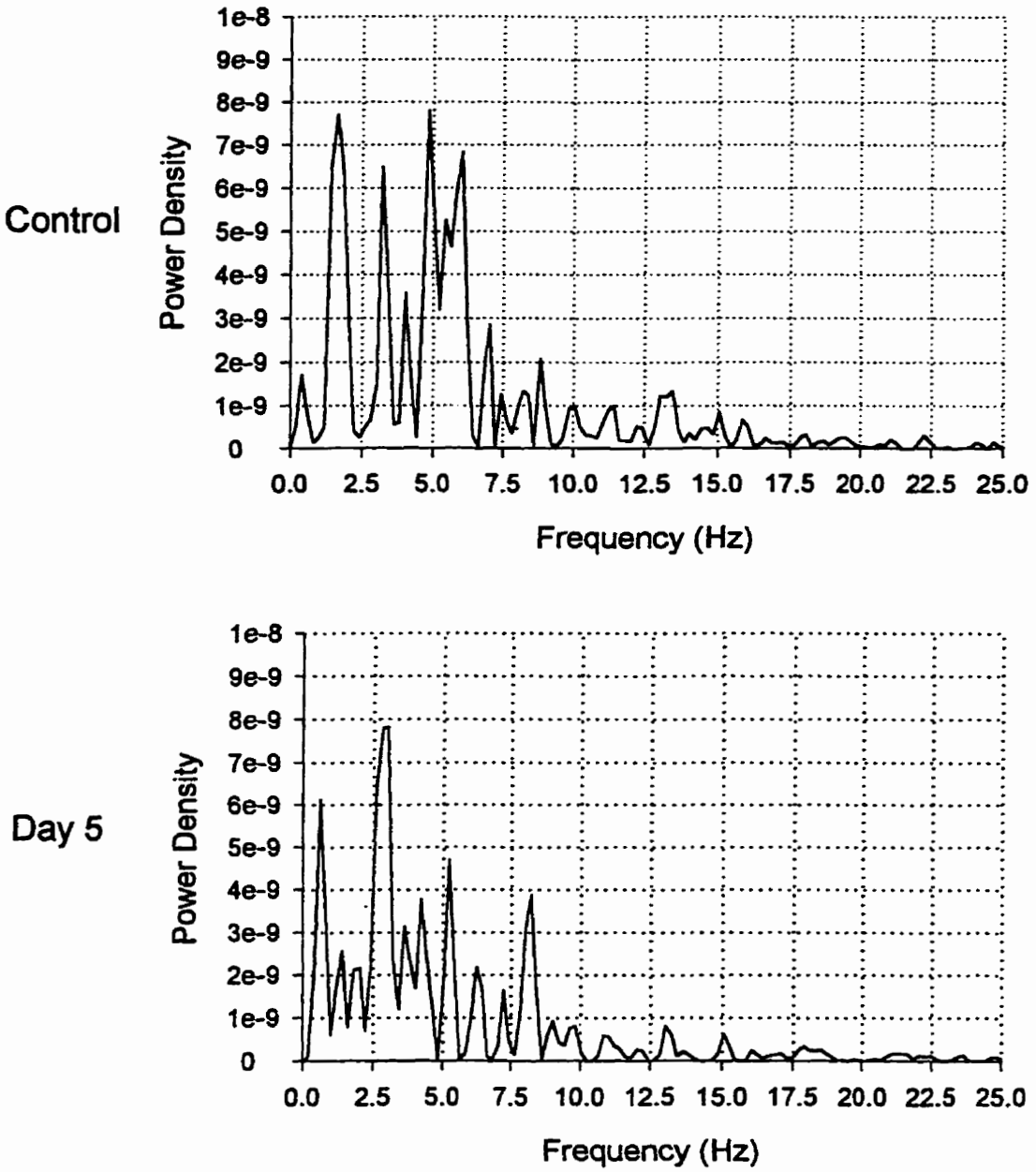
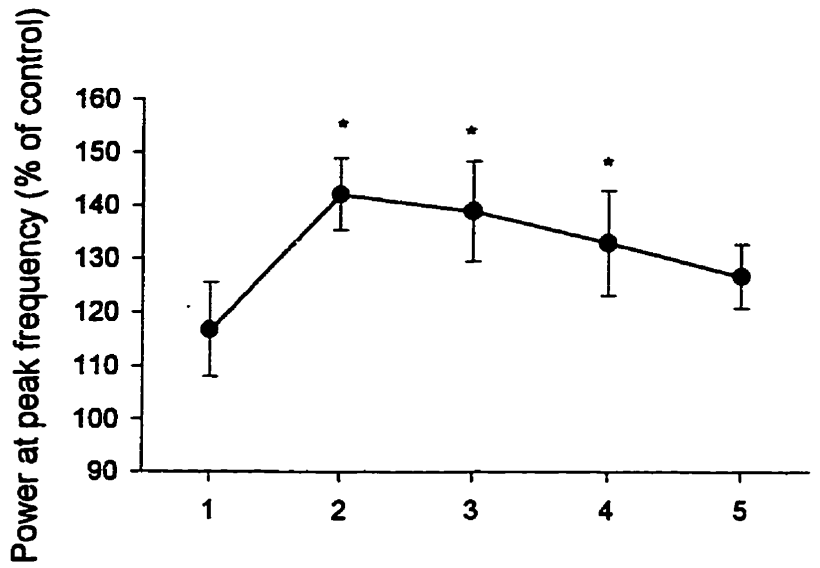


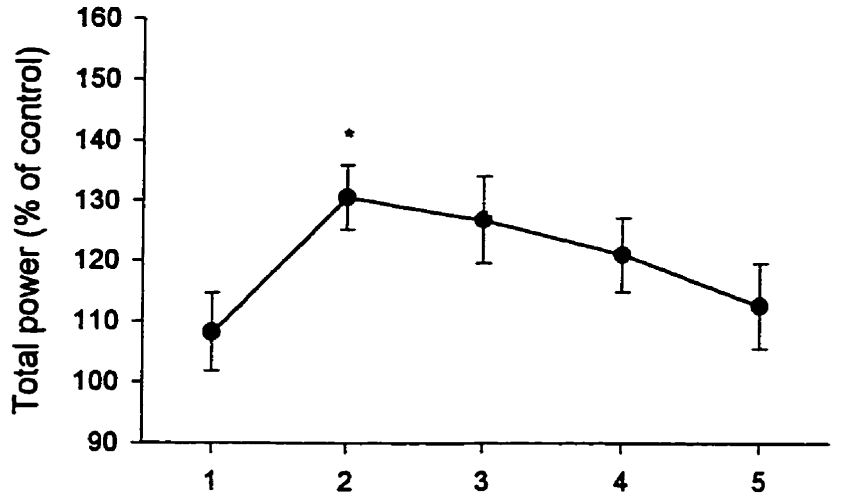
Figure 21. Example power spectra of LIA recorded from the dentate gyrus of a rat in the dark H_2S exposed condition during immobility prior to and following exposure to $1_2$5 ppm H_2S for 5 days.

Figure 22. Graphs demonstrating the change in a) power at the peak frequency, b) total power, and c) peak frequency of hippocampal theta activity recorded from the dentate gyrus during walking over the 5 days of exposure to 125 ppm H_2S for animals in the DARK EXPOSURE condition. Each point represents the mean ± S.E.M. of five animals. Asterisks denote significant difference from the day 1 pre-exposure control values.

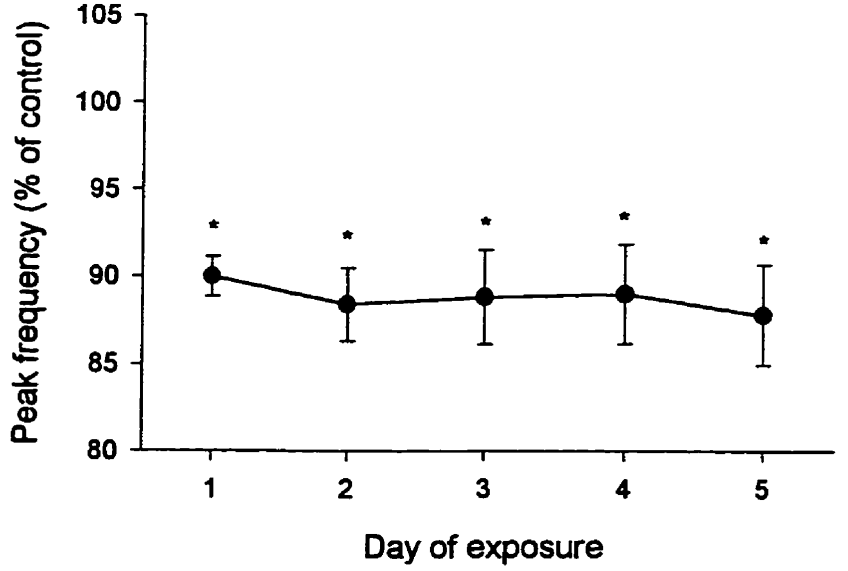
a)


Power at peak frequency (% of control)
160
150
140
130
120
110
100
90
1
2
3
4
5
*
*
*

b)


Total power (% of control)
160
150
140
130
120
110
100
90
1
2
3
4
5
*

c)


Peak frequency (% of control)
105
100
95
90
85
80
1
2
3
4
5
*
*
*
*
*
Day of exposure

Between Groups

Control (Light) vs. Control (Dark)

Theta activity in the control groups in the light and dark condition differed in their response to the 5 day exposure. Animals in the light condition showed a slight increase in the variables of power at the peak frequency and total power of theta activity, while animals in the dark condition showed a tendency towards decreasing values. The difference between the light and dark control groups was significant for total power ($p<0.001$) (Table 3). There were no significant differences between the two groups in the peak frequency of theta.

H_2S Exposed (Light) vs. H_2S Exposed (Dark)

The effects of H_2S on theta activity were different when exposures were conducted in the light condition than in the dark condition. Animals in the light condition showed a decrease in the power at the peak and total power of theta, however animals in the dark condition showed an increase in these variables. The differences between the light and dark groups were significant for both measures ($p<0.001$) (Table 3). Animals exposed to H_2S in both groups showed a decrease in the peak frequency of theta activity. There was a statistically significant difference between the two groups, however, in that the decrease observed in peak frequency of theta was more profound in the dark condition than the light condition ($p<0.001$) (Table 3).

Table 3. Means ± S.E.M. expressed as a percentage of day 1 pre-exposure control values for each of the 4 treatment groups (n=5/group)

	LIGHT			DARK		
	CONTROL	H_2S	Δ	CONTROL	H_2S	Δ
Power at Peak Frequency	103.04 ± 3.35	78.33 ± 3.35[a]	-24.71	93.23 ± 3.35	131.56 ± 3.35[a,c]	+38.33
Total Power	104.44 ± 3.10	81.68 ± 3.10[a]	-22.76	90.71 ± 3.10[b]	119.72 ± 3.10[a,c]	+29.01
Peak Frequency	100.48 ± .063	92.24 ± 0.63[a]	-8.24	101.45 ± 0.63	88.8 ± 0.63[a,c]	-12.65

Note:

[a] denotes significant difference between H_2S exposed group and control group for the light and dark conditions ($p<0.001$)

[b] denotes significant difference between the control groups in the light and dark ($p<0.001$)

[c] denotes significant difference between the H_2S treated groups in the light and dark ($p<0.001$).

Light Condition

There was a significant difference in the control and H_2S exposed animals in the light condition. Control animals showed a slight increase in power at the peak frequency, and total power. Animals exposed to H_2S showed a significant decrease in all variables over the 5 days of exposure. There was a significant difference between the control and H_2S groups in both power at peak frequency and total power ($p<0.001$) (Table 3). In addition, there was a significant difference between the control and H_2S groups in the peak frequency of theta ($p<0.03$). The control group showed no change in peak frequency, whereas exposure to H_2S caused a decrease in peak frequency (Table 3).

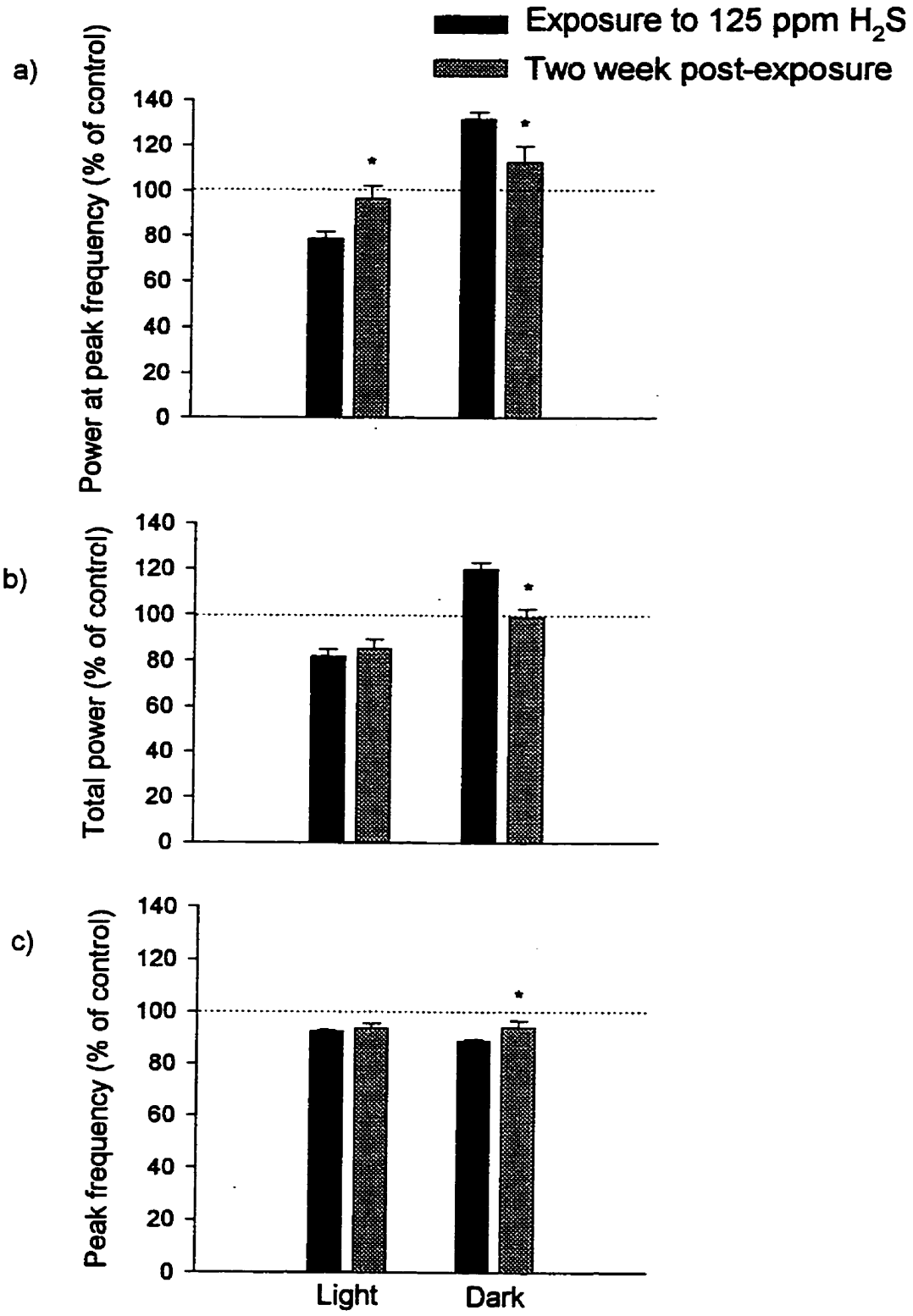
Dark Condition

The control and H_2S groups in the dark condition were significantly different from one another. Animals in the control group showed a decrease in power at the peak frequency and total power. Exposure to H_2S had the opposite effect, resulting in an increase in both measures. The differences between the control and H_2S exposed animals were significant for power at the peak frequency and total power ($p<0.001$) (Table 3). There was also a significant difference in the peak frequency of theta between the control and H_2S groups ($p<0.001$). The control group showed no change in peak frequency, whereas the H_2S exposed group showed a decrease in peak frequency (Table 3).

Two week post-exposure recovery

Two weeks following the 5 day exposure period, the animals were tested again to determine if the effects of H_2S on hippocampal theta activity were reversible. In some cases, for both the light and dark exposure groups, the recovery was not complete after a two week period (Figure 23). The dark group showed recovery of power at the peak frequency ($F(1,7) = 6.623$, $p < 0.037$), and total power ($F(1,7) = 19.549$, $p < 0.003$). The light group showed significant recovery of power at the peak frequency only ($F(1,7) = 8.724$, $p < 0.025$).

Figure 23. Graphs demonstrating the change in a) power at the peak frequency, b) total power, and c) peak frequency from values during exposure to 125 ppm H2S and those obtained two weeks following the exposure period for animals in the dark and light exposure groups. The dark bars represent values obtained during exposure, and the light bars represent the two week recovery values. Each bar represents the mean ± S.E.M. of five animals. Asterisks denote significant difference from values during the exposure period.


Exposure to 125 ppm H_2S
Two week post-exposure
a)
b)
c)
Power at peak frequency (% of control)
Total power (% of control)
Peak frequency (% of control)
140
120
100
80
60
40
20
0
*
Light
Dark

Discussion

Summary

This study has shown that the effects of H_2S on theta activity in the hippocampus are different depending on the time of day the exposures take place. When rats were exposed to 125 ppm H_2S during the light period, the amplitude, power at the peak theta frequency, and total power of theta activity decreased over a 5 day exposure period in a cumulative fashion. However, when animals were exposed during the dark period, the opposite occurred. There was an increase in all three variables, also in a cumulative manner over the 5 days. The peak frequency of theta did not show a similar difference. In both the dark and the light period, the peak frequency of theta decreased, however the magnitude of this decrease was greater in the dark period.

An unexpected finding of this study is that the animals in the control groups differed from one another in theta activity recorded over the 5 days of exposure to the nitrogen/air mixture. Rats exposed in the light period showed an increase in the amplitude, power at the peak, and peak frequency of theta, while in the dark period animals showed a decrease in all three variables. These changes seen in the control animals over the 5 days of treatment were less dramatic than those seen in the H_2S groups, and in fact were opposite in direction from the H_2S treated groups for both the light and dark periods. Also, in contrast to the H_2S exposed groups, the peak frequency of theta did not change over the 5 days of exposure. These differences suggest that the nitrogen used to balance H_2S in the treated groups did not significantly affect the outcome of H_2S treatment, and the effects seen were a direct result of H_2S actions on the central nervous

system. The changes in theta activity seen in the control and exposed groups will be dealt with separately in the discussion to follow.

Circadian Changes in Control Animals

Studies concerning a circadian rhythm of hippocampal cellular activity are limited. None have examined a possible rhythm in the mechanisms of theta generation, however there is evidence of a circadian rhythm in synaptic transmission and cell excitability. There is a circadian cyclicity in the sensitivity of the granule cells of the dentate gyrus to perforant path input (Barnes *et al.*, 1977; West and Deadwyler, 1980; Harris and Teyler, 1983; Cauller *et al.*, 1985). These changes may be mediated by the behavioral state of the animal, as Cauller *et al.* (1985) have shown that circadian variations in EPSPs were found within the active awake, still alert, slow-wave sleep and paradoxical sleep states with a maximum in the dark period, while a rhythm in population spike amplitude was found during the still alert and paradoxical sleep states, with a maximum in the light period. However, West and Deadwyler (1980) demonstrated that population spike amplitudes elicited in dentate granule cells recorded during alert and still alert during the dark period were significantly reduced as compared to spikes recorded during the identical behavioral states prior to the onset of darkness. It was suggested that circadian modulation of granule cell sensitivity to perforant path synaptic input may be due to a rhythmic variation in granule cell resting membrane potential produced by increased inhibitory input during the dark period. In agreement with these findings, Winson and Abzug (1978) demonstrated an inverse relationship between behavioral arousal and granule cell

population spike amplitude in the rat. Population spikes were larger during slow wave sleep and smaller when the animal was alert. A decrease in the population spike amplitude would then be expected during the dark period, since rats are more alert at this time. Interestingly, the serotonin-releasing drug d-fenfluramine enhanced the population spike elicited in the dentate gyrus following perforant path stimulation, while decreasing the spontaneous extracellular single unit activity and reducing the magnitude of hippocampal EEG (Richter-Levin and Segal, 1990). Unfortunately, the behavioral state of the animal or the time of day were not examined.

Circadian Changes in Theta Activity in Control Animals Possibly Mediated by Stress

In the present study, it was found that the theta activity of the control animals in the light and dark groups differed from one another. The amplitude and power of theta increased in the light period, while it decreased in the dark period. A possible explanation for this effect could be the relative levels of corticosterone induced by creating a stressful situation. The method used to induce type 1 theta, forced walking on a rotating platform, is likely to have acted as a chronic stressor. Furthermore, the animal's ability to adapt to the stressor may have contributed to the differences seen between the light and dark periods. Rats are nocturnal animals, which means they are more active during the dark period, and are at rest during the light period. Animals subjected to the repeated stimulation of forced walking during the light period may have experienced increased stress, whereas during the dark period animals may have been better able to adapt to the forced walking. In the hippocampus, there are two types of corticosteriod receptors (de

Kloet *et al.*, 1993). Mineralcorticoid receptors (MRs), which serve to maintain excitability, and glucocorticoid receptors (GRs), which suppress excitability. MRs bind corticosterone with a 10-fold higher affinity than GRs, however during the circadian peak or after stress, GR binding increases. An animal's sensitivity to stress is highest at the trough of basal corticosterone activity. The circadian peak of corticosterone in the rat is in the dark period, while lowest levels are found in the beginning of the light period (de Kloet *et al.*, 1993). A circadian rhythm in MR receptor number is also found, with a peak in the dark period (Reul *et al.*, 1987). An important consideration for the relative effects of MR and GR is that the two receptor systems interact. In the hippocampus, chronic GR activation down-regulates GR number and increases MR (Reul *et al.*, 1987). MR activation down-regulates both receptor types (Sutanto and de Kloet, 1991). The effects of corticosterone have been studied on hippocampal pyramidal cells (Reiheld *et al.*, 1984). It was found that elevations of corticosterone produced increases in population spike amplitudes following stimulation of the Shaeffer collaterals. It has also been shown that following acute stress (tail-shock), two hours or 24 hours later the amplitude of a theta burst was increased (Shors and Dryver *et al.*, 1994). Levels of glutamate in the hippocampus are increased following stress (Lowy *et al.*, 1993), and following corticosterone treatment (Stein-Behrens *et al.*, 1994). If the animals in the light group were indeed more stressed, it is possible that this could account for an increase in theta activity. On the other hand, since all measures of theta were based on the pre-exposure baseline, it is possible that an initial increase in the stress response was seen in the dark phase during the first exposure, but decreased due to the animal's ability to adapt more

quickly to the stressor.

Chronokinetic Factors Influencing the Bioavailability of H_2S

Any discussion of the chronotoxicity of a substance, regardless of the substance in question or its route of administration, must include a consideration of the possible influences of circadian rhythms in the processes regulating absorption, distribution, metabolism and excretion. Circadian rhythms influencing these factors will determine the amount of a substance available to exert a biological effect. The effectiveness and toxicity of a number drugs and chemicals have been shown to vary over a 24 hour period (Labreque and Belanger, 1991), and for many of these substances a direct correlation can be observed with a circadian rhythm in kinetic processes.

The evidence concerning a temporal variation in absorption from the lungs, as is the case with H_2S exposure, is limited. It is possible that a circadian rhythm in H_2S absorption from the lungs does exist, as such a rhythm has been demonstrated for other inhaled substances. One study by Munson *et al* (1970) demonstrated a circadian rhythm in the minimum alveolar concentration of halothane needed to keep rats anesthetized. The lowest concentration was needed at 12:00 pm, and the highest at 8:00 pm. This could reflect a circadian rhythm in absorption, suggesting that absorption from the lungs is greatest in the animal's light period. However, it could also be the result of the natural activity level of the rat, as they are more active in the dark period. More experimental evidence is needed before any conclusions are made as to the influence of absorption on the circadian rhythm of H_2S.

It has been demonstrated that, *in vitro*, [^{35}S]-sodium sulfide is rapidly and extensively bound to blood proteins (Curtis *et al.*, 1972). Addition of glutathione or cysteine to the serum liberated ^{35}S from the proteins, suggesting that ^{35}S-sulfide binds to proteins by a reduction of disulfide bridges. Circadian variations in the amount of protein binding have been shown for several drugs, by determining the amount of free plasma levels (Brugerolle and Lemmer, 1993). Temporal changes in protein binding are often reported to be dependent on the amount of plasma proteins. Changes in the amount of plasma proteins available to bind H_2S *in vivo* would result in varying concentrations of H_2S reaching the CNS. No information is currently available on a circadian rhythm in H_2S binding to plasma proteins, therefore conclusions regarding the peak time of H_2S binding cannot be made. There is, however, evidence that the regional blood flow to the hippocampus reaches a peak during the dark period in rats (Endo *et al.*, 1990). This could result in an increased concentration of H_2S reaching the hippocampus via the blood stream during the dark period.

The metabolism of H_2S is dependent on a number of hepatic enzymes (Beauchamp *et al.*, 1986), and at least one of these, glutathione, has been shown to vary in its concentration over a 24 hour period (Brugerolle and Lemmer, 1993; Belanger, 1988). Glutathione is an important enzyme in the detoxification process of several toxic chemicals, as it forms an adduct with electrophilic compounds, free radical species and with reactive intermediates formed by the p450 mono-oxegenase system (Belanger, 1997). The oxidation of H_2S is catalyzed by sulfide oxidase, which results in the production of thiosulfate as an intermediate. Glutathione stimulates the mitochondrial oxidation of

thiosulfate to sulfate (Bartholomew *et al.*, 1980). A circadian rhythm in hepatic glutathione concentration has been detected in several studies, with a minimum concentration at the beginning of the animal's dark period, and a maximum at the beginning of the animal's light period (see Belanger, 1988). Also, the toxicity of several compounds have been shown to be increased during the time corresponding to the minimal concentrations of hepatic glutathione (Jaeger *et al.*, 1973; Hanson and Anders, 1978; Lavigne *et al.*, 1983; Desgagne and Belanger, 1986). It is possible that H_2S would have increased toxicity during the dark period, due to a reduction in the hepatic glutathione concentration.

It has been shown that administration of [^{35}S]-sodium sulfide is rapidly and extensively excreted as sulfate in urine in rats (Gunina, 1957a; Curtis *et al.*, 1972), and dogs (Gunina, 1957b). Information on circadian rhythms in the rate of renal clearance from the kidneys is sparse, however what is known suggests that the processes of glomerular filtration and tubular secretion are highest during the active phase in both humans and rats (Labreque *et al.*, 1997). If the main influence on the bioavailability of H_2S is the rate of excretion from the kidneys, it is possible that H_2S is excreted more rapidly during the dark period in rats, suggesting an increased toxicity during the day.

It is obvious that in order to fully understand the chronokinetics of H_2S further studies must be done. Currently there is no information available on the rhythmic nature of the absorption, distribution, metabolism and excretion of H_2S, or on the relative importance of any of these factors on the amount of H_2S available to tissues to exert a biological effect.

Possible Neurochemical Mechanisms For a Circadian Rhythm of H_2S-Induced Changes in Hippocampal Theta Activity

Several neurotransmitters and neuromodulators have been shown to alter the characteristics of the hippocampal theta rhythm and cellular excitability. In the hippocampal slice preparation, the blockade of $GABA_A$ mediated fast inhibition caused an increase in the amplitude and decrease in the frequency of synchronized activity (Shneiderman, 1986). It has also been shown that baclofen, a $GABA_B$ receptor agonist, reduces the frequency of theta elicited by stimulation of the reticular formation (Coop *et al.*, 1991). The amplitude or power characteristics were not examined in their study, however it is possible that a decrease in the amplitude could occur, due to increased inhibition of the principle cells. In any case, the properties of hippocampal theta activity are sensitive to changes in GABA receptor mediated responses. Furthermore, $GABA_A$ receptors are maximal in rat forebrain during the dark period (Kafka *et al.*, 1982), reflecting an increase in the number of receptors, and not an increase in receptor affinity. Since an increase in amplitude was seen following exposure to H_2S in the dark period, and a reduction in frequency was seen in both the light and the dark groups, it is possible that H_2S exerts an effect on $GABA_A$ or $GABA_B$ function.

There is evidence that serotonin may modulate the occurrence and characteristics of theta activity. It has been shown that H_2S reduces serotonin levels in the hippocampus (Skrajny, 1994). Based on previous studies, it may be postulated that H_2S may increase the amplitude and power of hippocampal theta activity by reducing serotonergic neurotransmission in the hippocampus. Marossou *et al.* (1996) demonstrated that

decreasing serotonin release by activating 5-HT_{1A} autoreceptors resulted in an increase in the relative theta power, while producing no change in mean theta frequency. The 5-HT_3 receptor antagonist odansetron, however, was found to significantly increase the mean theta frequency (Staubli and Xu, 1995). Pharmacological blockade of 5-HT_3 receptors reduces the excitability of a class of interneurons that control the $GABA_B$ -mediated inhibition of principle cells (Freund *et al.*, 1990). Levels of serotonin in rat brain show a daily variation, with the highest levels found in the light period (Bhaskaran and Radha, 1984). Although a reduction in the levels of endogenous serotonin in the dark period, and a further reduction by H_2S could possibly result in an increase in the amplitude and power of type 1 theta, it is not likely that this is the mechanism whereby H_2S exerts its effects. First, a reduction in serotonergic activity in the hippocampus could also result in reduced activation of $GABA_B$ -mediated inhibition of principle cells, resulting in an increase in the frequency of theta, while in the present study a decrease in the frequency was found. Second, a reduction in hippocampal serotonin levels cannot account for the decrease in the amplitude and power of theta activity which occurred during the light period.

It has been demonstrated that glutamate levels are increased in the hippocampus following H_2S exposure (Skrajny, 1994; Nicholson *et al.*, 1998). Glutamate transmission in the hippocampus is believed to be at least partly responsible for the generation of type 1 theta (Destrade and Ott, 1980). In the hippocampal slice preparation, blockade of non-NMDA mediated excitatory synaptic transmission reduced the amplitude of rhythmic activity (MacVicar and Tse, 1989). The effects of glutamate on type 1 theta have also

been demonstrated *in vivo*. Fontani *et al.* (1984) found that the glutamate antagonist GDEE inhibited the production of theta and reduced the frequency during movement. The NMDA receptor antagonist APV has been shown to attenuate the theta rhythm during movement (Leung and Desborough, 1988). Low doses of glutamic acid induced theta activity in freely moving rabbits (Smialowski, 1983). It is also significant that LTP generated in the hippocampus shows a circadian rhythm in rats, with greater LTP produced during the dark phase, as LTP is thought to depend largely on glutamatergic transmission mediated by NMDA receptors (Harris and Teyler, 1983). Increased levels of glutamate in the hippocampus may account for the increase in amplitude and power observed following H_2S exposure in the dark period, however if the actions of H_2S were solely dependent on an increase in glutamate, based on previous studies it would be expected that an increase in frequency may also occur, and this was not the case. An increase in glutamatergic transmission can also not account for the decrease in amplitude and power of theta activity seen in animals in the light period.

Taurine is also proposed to be a neuromodulator/neurotransmitter in the hippocampus (Taber *et al.*, 1986). H_2S has been shown to increase the level of taurine in the brainstem of rats following high doses of NaHS (Kombian *et al.*, 1988), and in the cerebral cortex and cerebellum of developing rats following inhalation exposure to low concentrations (Hannah *et al.*, 1989). It has been demonstrated that taurine is released in the hippocampus by increasing the extracellular concentration of K^+ ions (Solis *et al.*, 1986). This may be of significance to H_2S poisoning, as Baldelli *et al.* (1990) found that H_2S resulted in the opening of a K^+ channel and activation of Na^+/K^+ ATPase in

hippocampal neurons, producing inhibition. The release of taurine is also stimulated by elevated levels of glutamate (Lehmann *et al.*, 1984). In the hippocampus, taurine has been shown to have a number of neuromodulatory functions. It is believed that taurine could act as an inhibitory transmitter, as application of taurine to hippocampal neurons in a slice preparation caused hyperpolarization, decrease in membrane conductance and inhibition of spontaneously occurring action potentials (Taber *et al.*, 1986). It is likely that this inhibitory action of taurine is mediated by activation of $GABA_A$ or glycine receptors, as it is blocked by specific antagonists for both receptors (Horikoshi *et al.*, 1988). It also regulates CA^{2+} fluxes in brain nerve terminals, and has been shown to reduce the synthesis and release of acetylcholine in the hippocampus (Kleinrok *et al.*, 1980). More recently, a new neuromodulatory role for taurine has been proposed. It has been found that taurine perfusion in hippocampal slice induced a long-lasting potentiation in synaptic transmission in the hippocampus (Galarreta *et al.*, 1996). This effect was not due to activation of GABA or glycine, and lasted at least 3 hours after taurine washout. It is interesting to note that this effect occurred with high levels of taurine. It has been shown that taurine content in rat brain undergoes circadian changes, with the highest levels occurring during the dark period (Iwata *et al.*, 1978). Exposure to H_2S could elevate taurine levels sufficiently in the dark period to result in this type of effect in the hippocampus. During the light period, the levels of taurine may not be sufficiently elevated to produce such a potentiation, and therefore act mainly to inhibit hippocampal neurons.

The neuronal mechanisms underlying the generation of theta frequency are not well understood. Several neurochemical manipulations have been shown to alter the

frequency of theta (see above), however the precise mechanisms whereby they exert such effects have not been elucidated. It has been suggested that changes in theta frequency could reflect a change in the motor activity of the animal, as type 1 theta (movement-related) has a higher frequency than type 2 theta (immobility-related) (Bland, 1986). It has been demonstrated that theta frequency increases with increased walking speed on a motorized treadmill (Rivas *et al.*, 1996). A common complaint of human exposure to H_2S is fatigue. Interestingly, it has been shown that intracerebroventricular application of taurine depresses motor behavior, lowers body temperature, and causes muscular weakness (Kleinrok *et al.*, 1980). In the present experiment, walking and type 1 theta were induced in rats by activating a rotating platform, which was maintained at a constant speed. However, a depressant effect on the motor activity of rats by H_2S could explain the reduction in frequency of theta, even though the animals were observed to walk whenever the platform was activated.

Possible Role of Enzymes In H_2S Toxicity

It is well known that acetylcholine regulates type 2 (immobility-related) theta, however there is evidence to suggest that it also plays a role in type 1 theta (Fontani *et al.*, 1984; Vanderwolf, 1988). H_2S has been shown to inhibit acetylcholinesterase, an enzyme responsible for the degradation of acetylcholine (see Roth *et al.*, 1992), therefore, the possibility exists that H_2S exerts its effects on type 1 theta via an action on acetylcholine. Administration of the muscarinic agonist oxotremorine resulted in an increase in the peak theta power and a decrease of peak theta frequency in freely moving rats (Markowska *et*

al., 1995). Oxotremorine has also been shown to decrease the population spike in CA1 evoked from stimulation of the Shaffer collaterals, however it enhanced the amplitude of the population spike following tetanic stimulation, facilitating the generation of LTP (Iga *et al.*, 1996). Acetylcholine release in the hippocampus is increased in the dark period, and is positively correlated with increases in motor activity (Mizuno *et al.*, 1991; Mizuno *et al.*, 1994). One study found that forced running on a treadmill resulted in the instantaneous appearance of theta waves in the hippocampus in the rat, and that this response was blocked by the muscarinic antagonist scopolamine (Teitelbaum *et al.*, 1975). It has also been shown that acetylcholine release is positively correlated with the amplitude of theta activity in the hippocampus (Monimaur *et al.*, 1997). Muscarinic receptors in the hippocampus exhibit a circadian variation, with maximal binding occurring in the light phase (Jenni-Eiermann *et al.*, 1986). There are two types of muscarinic receptors in the hippocampus, the M_1 receptor decreases K^+ conductance postsynaptically and induces depolarization, and the M_2 receptor inhibits synaptic transmission presynaptically (Iga *et al.*, 1996). The increase in theta amplitude following H_2S exposure in the dark period could be due to the increased concentrations of endogenous acetylcholine at this time, which may be further increased by H_2S. It is also possible that the increase in muscarinic receptor binding during the light phase may be responsible for a decrease in theta amplitude mediated by M_2 inhibitory autoreceptors.

Inhibition of monoamine oxidase (MAO) and increased levels of catecholamines in the hippocampus have been demonstrated in rats following treatment with NaHS (Warencycia *et al.*, 1989). Elevated levels of catecholamines and/or serotonin resulting

from the inhibition of MAO may be involved in the alteration of hippocampal theta activity following H_2S exposure. Norepinephrine infused into the hippocampus resulted in a decrease in the peak theta power and a reduction of peak theta frequency during movement (Sainsbury and Partlo, 1993). Dopamine has been shown to produce a depression of the excitatory synaptic transmission in the hippocampus, possibly mediated by presynaptic D_2 receptors which decrease glutamate release from presynaptic terminals (Hsu *et al.*, 1996). MAO activity in the rat brain exhibits a circadian variation, with peak levels occurring at around 6:00 am (Bhaskaran and Radha, 1984). An increase in catecholamines and serotonin during the light period may result in the decrease of amplitude and power observed following H_2S exposure.

An action of H_2S on MAO may also explain the time required for the effects of H_2S to be reversed. The half-life for the recovery of rat brain MAO after irreversible inhibition was reported to be up to 30 days (Arnette *et al.*, 1987). Thus, the fact that H_2S in the present study produced effects that were not completely reversed after a period of 2 weeks provides further support for the possibility that H_2S is producing an effect at least in part through the inhibition of MAO.

H_2S has been proposed to be a non-cumulative toxicant because of its rapid metabolism and excretion (Curtis *et al.*, 1972; Beauchamp *et al.*, 1986). However, in the present study, the effects of H_2S were cumulative, as demonstrated by a progressive increase and decrease in the power and amplitude of theta activity in the dark and light period, respectively. Previous studies have also demonstrated that the effects of H_2S can be cumulative following repeated exposure (Skrajny *et al.*, 1996; Savolainen *et al.*, 1980).

It has been suggested that a cumulative inhibition of cytochrome oxidase may account for these results. The slow dissociation of the haem-sulfide complex and the rate of synthesis of new haem may be the limiting factor for the recovery of H_2S toxicity (Savolainen *et al.*, 1980). The half-life of rat brain cytochrome haem has been estimated to be longer than 24 hours (Schanley *et al.*, 1977). This could account for a cumulative effect of H_2S following repeated exposures over successive days. It cannot, however, explain the prolonged recovery time after exposure to H_2S was terminated.

General Conclusions

H_2S has been postulated to affect memory, and reports of memory deficits following H_2S exposure are common (Kilburn and Warshaw, 1995). The hippocampal formation is believed to be important for the normal functioning of memory processes (Zola-Morgan and Squire, 1993). The hippocampal theta rhythm has also been considered to play a significant role in memory. Alterations in theta rhythm produced through pharmacological treatment can produce either an impairment (Markowska *et al.*, 1995) or an improvement (Staubli and Xu, 1995; Iga et al, 1996) in memory performance. Recently it has been suggested that theta represents the inhibition of irrelevant sensory stimuli, allowing an animal to focus on cues which are important to the execution of an appropriate behavioral response (Sainsbury, 1998). At least one study has shown that an increase in the power of theta was associated with an improvement in choice accuracy on a spatial working memory task, while a decrease in power was accompanied by a decrease in choice accuracy (Markowska *et al.*, 1995).

The present study has shown that H_2S, at a concentration about 1/4 that of the LD_{50} reported for rats (Prior *et al.*, 1988), produced a significant effect on hippocampal theta activity. The main toxic effect of H_2S is generally regarded as its ability to inhibit cytochrome oxidase, thereby inhibiting cellular respiration and oxidative metabolism (Beauchamp *et al.*, 1986). It is interesting to point out that cytochrome oxidase activity is decreased in the dentate gyrus and hippocampal subfields in the brains of Alzheimer's patients (Simonian *et al.*, 1993), a disease characterized by significant memory impairments.

The results presented here, however, are likely due to multiple mechanisms and sites of action of H_2S, which is consistent with the categorization of this gas as a broad spectrum toxicant (Roth, 1993). The effects of H_2S were different depending on the time of day the exposures took place. H_2S is a hazard in many industries, and shift-workers are likely to come into contact with H_2S not only during the day, but at night as well. The effects of H_2S on hippocampal theta activity may explain some of the neurological deficits seen following human H_2S intoxication, and also suggests that a circadian rhythm in either the kinetics or the dynamics of H_2S will result in a circadian rhythm of individual susceptibility to this toxicant.

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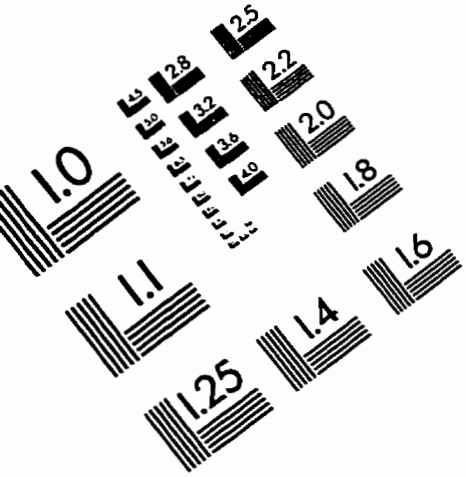
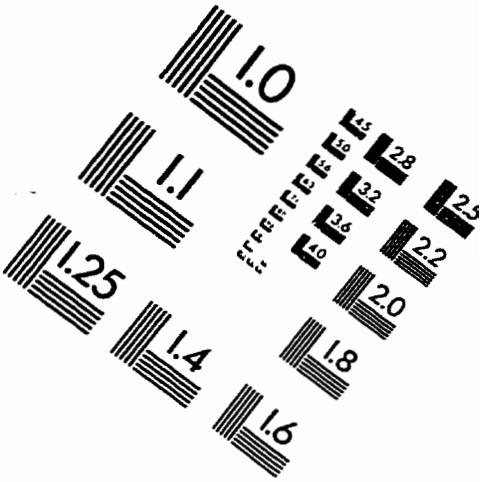
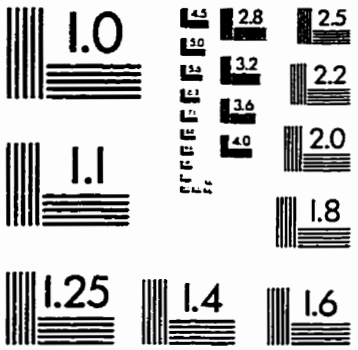
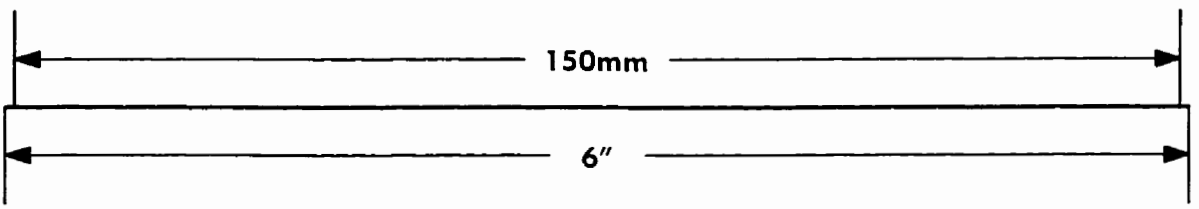
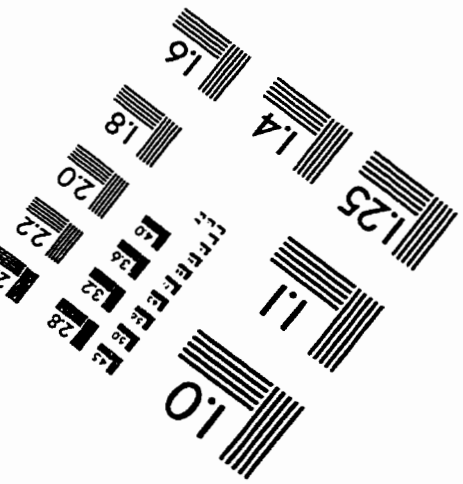
IMAGE EVALUATION TEST TARGET (QA-3)

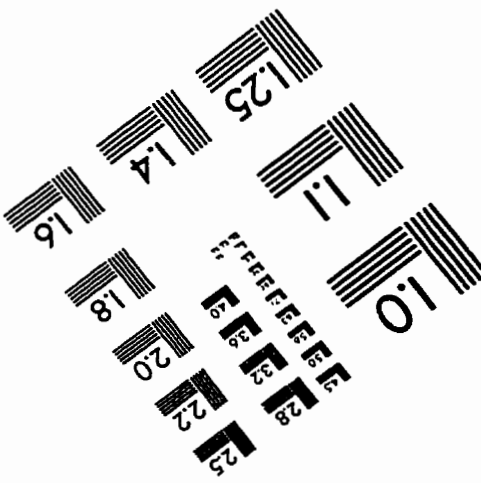












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