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Effect of Pinealectomy on the Behavioural and Neuropathological Consequences of Chronic Hypoperfusion in the Rat

by

Maxine De Butte, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirement for the degree of

Master of Science
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Department of Psychology
and
The Carleton Institute of Neuroscience

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submitted by

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in Partial fulfillment of the requirements for
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Chair

Thesis Supervisor

Carleton University
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ABSTRACT

At ~9 months of age, pinealectomized and non-pinealectomized animals pre-trained on a tactile radial arm maze (RAM), underwent permanent bilateral common carotid artery occlusion (2VO) or non-2VO surgery. Post-surgically, all animals were first tested on the tactile RAM. Pinealectomized animals who underwent 2VO surgery (pinealectomized 2VO) performed worse on this task than non-pinealectomized 2VO (sham 2VO) animals. At 66 days post-surgery, pinealectomized 2VO, pinealectomized non-2VO (pinealectomized sham), sham 2VO, and non-pinealectomized non-2VO (sham sham) animals were given 7 weeks of training on a standard RAM task. No differences were seen among the groups on standard RAM performance. After 122 days, pinealectomized animals had greater hippocampal pathology than non-pinealectomized animals (cell number and glial fibrillary acetic protein). Histological confirmation of pinealectomy revealed cortical damage presumably due to the pinealectomy surgery. Further examination into the effects of these cortical lesions will have to be made before any definitive conclusions can be made regarding the effects of pinealectomy on the consequences of chronic hypoperfusion.

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The pineal gland, located at the top of the midbrain, secretes the hormone melatonin (N-acetyl-5-methoxytryptamine). In mammals, melatonin is involved in a wide range of physiological processes such as controlling seasonal rhythms and seasonal reproduction (Reiter, 1991). More recently, melatonin has been found to be a potent antioxidant and free radical scavenger and as a result, to have seemingly potent neuroprotective properties when administered both *in vitro* and *in vivo*. These findings suggest a deficiency in melatonin would result in increased vulnerability of neurons to insult *in vivo*. The purpose of the present study was to determine the effects of lowering endogenous levels of melatonin by pinealectomy on the behavioral and neural effects of chronic cerebral hypoperfusion, effected by permanent bilateral carotid artery occlusion (2VO) in the rat.

Oxygen Free Radicals

Free radicals are molecules or atoms that contain an unpaired electron in their outermost orbital (Reiter, 1996). This renders them highly reactive and if they interact with a non-radical molecule they can be highly damaging. In order to achieve a level of stability, a free radical is capable of donating or taking an electron from a nearby molecule. If a free radical loses an electron, it becomes a reducing species, while the gain of an electron oxidizes the free radical (Pierrefiche & Laborit, 1995). Once a free radical interacts with a non-radical molecule, another free radical is produced. The generation of new free radicals can then lead to the propagation of free radical chain reactions. These reactions can be detrimental to membrane lipids, DNA, and fundamental cellular proteins (Olanow, 1993).

Free Radical Reactions

Molecular oxygen is capable of generating oxygen-centered free radicals known as oxy radicals. The most damaging oxy radicals are the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$). When molecular oxygen acquires an electron from a free radical, the superoxide free radical anion ($O_2^{\cdot-}$) is formed (Eq. 1) (Pierrefiche & Laborit, 1995). Addition of hydrogen ions results in the dismutation of the superoxide anion which then yields hydrogen peroxide (H_2O_2), a potent oxidizing agent (Eq. 2).

The interaction of the superoxide radical and hydrogen peroxide with a metal ion such as iron produces the highly toxic and reactive hydroxyl radical ($\cdot OH$) in what is known as the Fenton Reaction (Eq. 3) (Reiter, 1996).

The above radical reactions illustrate how newly generated free radicals continue to interact with other molecules to further generate reactive products. The continuation of these radical reactions results in chain reactions that can have a deleterious affect on a variety of tissues.

Lipid Peroxidation

One of the most notable chain reactions is known as lipid peroxidation. This reaction chain is capable of damaging the functional state of lipid membranes and can result in cell death (Olanow, 1993).

When either the superoxide radical or the hydroxyl radical reacts with a polyunsaturated fatty acid, the peroxy (ROO) or alkoxyl (RO) radical is formed (Reiter, 1996). These radicals are then able to initiate the oxidation of further polyunsaturated fatty acids. Thus, once formed, the peroxy and alkoxyl radicals initiate a chain reaction that will continue to damage lipid membranes if it is not terminated by an antioxidant or free radical scavenger.

Antioxidative Defense Mechanisms

The generation of free radicals within organisms is inevitable; however every organism has antioxidative defense systems which either restrict the generation of free radicals or renders them ineffective when they are generated (Reiter, 1996). The escalation of free radical production places a great demand on the antioxidative defense system to limit their damaging reactions. This increased demand on antioxidative mechanisms is termed oxidative stress.

A variety of enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) function to limit the generation of free radicals (Pierrefiche & Laborit, 1995). Superoxide dismutase is an enzyme that functions as a catalyst that dismutates the superoxide radical into hydrogen peroxide. Catalase and glutathione peroxidase then metabolizes hydrogen peroxide into water thus limiting the generation of

the potent hydroxyl radical (Reiter, 1996). These antioxidative enzymes function to convert potentially harmful radicals into inactive substances.

In addition, a variety of molecules such as vitamin C (ascorbic acid) and E (α -tocopherol) function as free radical scavengers that are capable of breaking harmful reaction chains and can interact directly with free radicals to prevent further tissue damage. Recently, much research has focused on the antioxidative and free radical scavenging properties of the pineal hormone melatonin (Marshall et al., 1996; Pieri et al., 1994; Tan et al., 1993).

Antioxidant Actions of Melatonin

The pineal gland located at the top of the midbrain, synthesizes and secretes the hormone, melatonin (N-acetyl-methoxytryptamine). The pineal gland is quite small weighing approximately 120 mg in humans and only 1 mg in the rat (Figure 1). Although located within the brain, the pineal gland is not actually a part of the brain because the blood brain barrier physically separates it from the brain.

The functioning of the pineal gland and thus the production of melatonin is controlled by the photoperiodic environment (Reiter, 1991). The maximal production of melatonin occurs during the daily dark period (nighttime) whereas minimal production occurs during daily light periods (daytime). Melatonin is derived from the amino acid tryptophan. During the light period, the catalyst tryptophan hydroxylase converts tryptophan to 5-hydroxytryptophan (Sugden, 1989). Aromatic L-amino acid decarboxylase then converts 5-hydroxytryptophan to 5-hydroxytryptamine (serotonin). During the daily night period, N-acetyltransferase an enzyme found in the pineal gland,

Figure 1: Top view of a rat brain illustrating the location of the pineal gland. The pineal gland is attached to a short stalk at the posterior border of the third ventricle above the roof of the diencephalon. Figure adapted from Greene (1935) and other sources.

converts serotonin to N-acetylserotonin with the aid of acetyl-coenzyme A. Melatonin is then produced by the action of the pineal enzyme hydroxyindole-O-methyl transferase on N-acetylserotonin.

During the daily dark period, melatonin is secreted from the pineal gland either directly into the bloodstream or into the cerebrospinal fluid prior to entering the bloodstream. Once released by the pineal gland, melatonin is capable of entering all cells of the body because it is both lipid and water-soluble (Reiter, 1996). Moreover, this indole readily crosses the blood brain barrier which enables it to exert its various functions on cells located anywhere in the organism, including the brain.

Melatonin was first described as an indole with hormonal and chronobiological functions (Reiter, 1991). For instance, melatonin is believed to affect the circadian and seasonal timing of various physiological and behavioral processes in mammals such as sexual maturation and seasonal reproduction (Ebling & Foster, 1989). For many mammalian species, sexual maturation, and the ability to reproduce occurs at a time that ensures that future offspring are born at the most ideal time of the year for that species (Ebling & Foster, 1989). The secretion of melatonin, which is controlled by the photoperiodic environment, translates information concerning the light/dark cycle into a signal which affects the neuroendocrine system and controls the timing of sexual maturation in many mammalian species. The key components of the mammalian system are firstly melatonin is synthesized in the both the photoreceptors of the retina as well as the pinealocytes of the pineal gland. Melatonin is secreted locally by the retina and into the bloodstream via the pineal gland. Melatonin then acts on specific high-affinity receptors in the central nervous system as well as the pituitary which regulates a variety

of physiological effects such as the timing of sexual maturation in many mammalian species.

Melatonin as a Free Radical Scavenger

In addition to melatonin's role in mammalian chronobiological functions, this indole has also been shown to be a potent antioxidant (Tan et al., 1993). Several studies have provided evidence for its role as a free radical scavenger (Marshall et al., 1996; Pieri et al., 1994). For instance, Tan and colleagues (1993) created hydroxyl radicals ($\text{OH}\cdot$) by exposing hydrogen peroxide (H_2O_2) to ultraviolet light (UV). Because the $\text{OH}\cdot$ radical is short-lived, these investigators utilized a spin-trapping reagent; 5,5-dimethylpyrrolidine-N-oxide (DMPO) which produced $\text{OH}\cdot$ -DMPO adducts. The spin adducts were detected by high-performance liquid chromatography with electron spin resonance.

Tan et al. (1993) found that melatonin was a more effective hydroxyl radical scavenger than either glutathione (GSH) or mannitol. Only 21 μM of melatonin was needed in order to neutralize 50% of the radicals produced whereas 123 μM of GSH and 283 μM of mannitol was required (Tan et al., 1993). The finding that melatonin was a potent scavenger of the $\text{OH}\cdot$ radical was an important one because this radical is thought to be the prime mediator of oxygen toxicity, capable of damaging proteins, carbohydrates, DNA, and lipids (Kehrer, 1993).

Following the finding that melatonin was a scavenger of the toxic $\text{OH}\cdot$ radical, Pieri et al. (1994) examined the ability of melatonin to scavenge the peroxy radical ($\text{ROO}\cdot$). The scavenging ability of melatonin was compared to that of the well known

antioxidants, vitamin C and E, as well as glutathione. Utilizing an *in vitro* system, Pieri and colleagues (1994) found that melatonin was twice as effective in neutralizing the toxic peroxy radicals than vitamin E and C. Moreover, melatonin was three times as effective in scavenging the $\text{ROO}^\cdot$ radical than glutathione.

Antioxidative Properties of Melatonin against DNA Damage

Following their finding that melatonin was an effective free radical scavenger, Tan and colleagues (1993) investigated melatonin's antioxidant properties *in vivo*. Nuclear DNA is highly vulnerable to damage caused by oxygen free radicals. Thus, Tan et al., (1993, 1994) conducted two studies to determine whether melatonin was capable of protecting hepatic nuclear DNA from damage inflicted by free radicals generated by the carcinogen safrole. These investigators found extensive DNA adducts in the hepatic tissue of rats that were administered 300 mg/kg of safrole. Administration of melatonin at concentrations of 0.2 mg/kg and 0.4 mg/kg dose-dependently reduced DNA-adduct formation (Tan et al., 1993). These results demonstrated that melatonin afforded protection against DNA damage caused by safrole.

In a follow-up study, Tan et al. (1994) examined the effectiveness of physiological concentrations of melatonin in protecting hepatic DNA from damage inflicted by safrole. Rats were administered safrole at a dosage of 100 mg/kg either during the day, when melatonin levels are at their lowest, or at night when melatonin levels are high. Tan and colleagues (1994) found that rats who had received the safrole at night exhibited less DNA damage than rats that had received the safrole during the day.

Thus, the nighttime rise in melatonin levels was enough to reduce the damage due to the carcinogen safrole.

In addition, Tan et al. (1994) found that rats that were subjected to pinealectomy (surgical removal of the pineal gland) exhibited the highest DNA damage following the administration of safrole. DNA damage was lessened in the pinealectomized animals when they were given exogenous melatonin. Collectively, their results suggested that even at physiological levels, melatonin is an effective antioxidant *in vivo*.

Melatonin Protects against Lipid Peroxidation In Vivo

In addition to its ability to protect against DNA damage, melatonin has also been found to protect against lipid peroxidation *in vivo* (Melchiorri et al., 1995). Melchiorri et al. (1995) treated rats with paraquat, a herbicide that causes damage to the lungs, liver, and kidneys of both animals and humans. Lipid peroxidation is believed to occur in paraquat toxicity. Thus, Melchiorri and colleagues (1995) examined the effectiveness of melatonin against paraquat-induced oxidative damage in rat lung and liver. Two indices, concentration of malonaldehyde (MDA) and 4-hydroxyalkenals were used in order to measure lipid peroxidation. Melchiorri et al. (1995) found that rats treated with paraquat at a concentration of 20 or 70 mg/kg exhibited elevated tissue levels of both MDA and 4-hydroxyalkenals. These investigators also administered 10 mg/kg of melatonin to rats prior to the administration of paraquat and every 6 hours thereafter, and found that melatonin eliminated the rise in MDA and 4-hydroxyalkenal levels seen in paraquat treated rats. These results demonstrated that melatonin was effective in protecting against lung and liver oxidative damage inflicted by paraquat.

Neuroprotective Effects of Melatonin

Recently, Carneiro and Reiter (1998) examined both the *in vivo* and *in vitro* effect of melatonin on aminolevulinic-induced lipid peroxidation in rat cortex, cerebellum, and hippocampus. An increase in aminolevulinic acid is found during acute intermittent porphyria and results in neuropsychiatric manifestations such as epileptic-type seizures and motor neuropathy.

Concentrations of MDA and 4-hydroxyalkenals were used in order to assess aminolevulinic acid induced membrane oxidative damage. Rats were randomly assigned to one of three groups: saline-treated controls, aminolevulinic acid treated rats and aminolevulinic acid treated animals that also received melatonin. Control animals were injected with saline (0.9% i.p.) three times a day at 6-hour intervals. Aminolevulinic acid treated rats received aminolevulinic injections (40mg/kg) every other day. Following aminolevulinic acid injections, rats received two saline injections at 6-hour intervals. On the days that they did not receive aminolevulinic acid, rats were given saline injections three times a day at 6-hour intervals. Rats in the aminolevulinic acid/melatonin group were given melatonin (10mg/kg) injections 30 minutes prior to aminolevulinic treatment. Following the aminolevulinic acid injection, melatonin was injected twice at 6-hour intervals. Aminolevulinic acid was given every other day similar to the previous group however, these rats received melatonin three times a day at 6-hour intervals. All treatments continued for seven days.

Carneiro and Reiter (1998) found that rats treated with aminolevulinic acid had elevated MDA and 4-hydroxyalkenal levels in the cerebellum and hippocampus but not in cortex. By contrast, rats treated with both aminolevulinic and melatonin exhibited

MDA and 4-hydroxyalkenal levels comparable to control rats. These results demonstrated that melatonin afforded protection against aminolevulinic acid induced oxidative damage.

In the same report, Carneiro and Reiter (1998) also examined melatonin's effectiveness to protect against aminolevulinic acid induced lipid peroxidation *in vitro*. In this study, brain homogenates were taken from cortex, hippocampus, and cerebellum. Aminolevulinic acid (3mM) was added to cortical, cerebellar, and hippocampal homogenates for 1, 2, 3, or 4 hours. These investigators found increased levels of MDA and 4-hydroxyalkenals due to the administration of aminolevulinic acid in cerebellar, cortical, and hippocampal homogenates. This elevation of lipid peroxidation products was both concentration-dependent and time-dependent.

In addition, vitamin E (0.5, 1.0, or 4.0 mM) or melatonin (0.1, 0.5, 1.0, 3.0, or 4.0 mM) was administered in conjunction with aminolevulinic acid in order to examine their antioxidative properties *in vitro*. It was observed that both melatonin and vitamin E dose-dependently protected against lipid peroxidation (Carneiro & Reiter, 1998).

Melatonin Protects against Kainic Acid Neurotoxicity

Other evidence supporting the neuroprotective effect of melatonin has come from studies utilizing the excitotoxin, kainic acid (Dyken et al., 1987; Melchiorri et al., 1996). Kainic acid is a non-degradable analog of glutamate that is both neuroexcitatory and neurotoxic. Administration of kainic acid to rodents produces brain damage comparable to that seen in a number of neurodegenerative conditions such as Huntington's disease and reperfusion tissue injury (Dyken, Stern, & Trenkner, 1987;

The tissue injury observed after the administration of kainic acid is believed to involve free radicals.

Several studies have examined whether melatonin affords protection against kainate-induced toxicity. For instance, Melchiorri et al. (1995) examined whether melatonin protected against kainate-induced toxicity in brain homogenates. Tissue concentrations of MDA and 4-hydroxyalkenals were used as indices of lipid peroxidation. Brain homogenates from cerebral cortex, cerebellum, hippocampus, hypothalamus, and corpus striatum were exposed to kainic acid (11.7 mM) for 20 minutes. Kainic acid treatment resulted in an elevation in lipid peroxidation products. By contrast, brain homogenates incubated with kainic acid combined with melatonin (from 0.1 to 4mM) showed reduced accumulations of lipid peroxidation products in a dose-dependent manner.

In addition to finding that melatonin was protective against kainic acid treatment *in vitro*, this finding has also been shown *in vivo*. For instance, Guisti and colleagues (1996) injected rats with kainic acid (10 mg/kg i.p.) alone, or combined with melatonin (2.5 mg/kg). Rats were given melatonin 20 minutes prior to kainic acid treatment, immediately following, and 1 and 2 hours following the administration of kainic acid. Rats treated with kainic acid exhibited neurobehavioral changes such as head nodding, wet dog shakes and masticatory movements. Moreover, these rats exhibited marked neuronal damage in the hippocampus, pyriform cortex, and amygdala. Biochemical changes, such as a decrease in norepinephrine and an elevation in 5-hydroxy-indoleacetic acid were also observed.

The administration of melatonin prevented the neurobehavioral changes seen in rats treated with kainic acid. Moreover, no hippocampal cell loss was observed in the melatonin-kainic acid treated rats. In addition, Guisti et al. (1996) did not find any decreases in noradrenaline or increases in 5-hydroxy-indoleacetic acid in melatonin treated animals. Based on these findings, Guisti et al. (1996) concluded that melatonin, due to its antioxidative properties, affords protection against kainic-induced brain damage.

In a similar study, Manev and colleagues (1996) administered intraperitoneal injections of kainate to pinealectomized or control rats at a dosage of 10 mg/kg. Following the administration of kainic acid, both the pinealectomized and sham-pinealectomized rats experienced wet dog shakes, chronic convulsions, and tonic-clonic convulsions. There were no differences in the severity or duration of the seizures between the pinealectomized and sham-pinealectomized animals. These investigators found neuronal damage in the hippocampus, entorhinal cortex, and amygdala in both the pinealectomized and sham-pinealectomized rats. However, the damage was more extensive in the pinealectomized rats.

Free Radicals, Melatonin, and Neurodegenerative Diseases

Collectively, all of the studies reviewed have provided strong evidence that melatonin is a potent antioxidant and free radical scavenger. This finding has been of interest to researchers interested in neurodegenerative disorders. Damage to cells and tissues due to toxic free radicals is believed to occur in a variety of neurodegenerative disorders such as Alzheimer's disease and as well in cerebrovascular ischemia

(Braughley & Hall, 1989; Kehrer, 1993). Several studies have provided evidence for the increase of oxygen radical reactions during ischemia (Siesjo, 1988; Zini et al., 1992). For example, Zini and colleagues (1992) subjected rats to 30 minutes of transient forebrain ischemia. Using intracerebral microdialysis coupled to the spin trapping technique, these investigators detected extracellular free radicals.

The finding that free radicals may be involved in the neuropathology seen in ischemia and other neurodegenerative disorders, has led investigators to explore the efficacy of melatonin for the treatment of neurodegenerative conditions. Significantly, it has been found that melatonin production is reduced with increasing age (Reiter, 1995). Thus, melatonin levels are lowest when the incidence of neurodegenerative disorders is at its peak. It is important to ascertain if there is a causal relationship between age-associated reduction of endogenous melatonin and the incidence of neurodegenerative diseases.

Recent research has assessed the role of melatonin in neurodegeneration using an acute stroke animal model. Manev et al. (1996) examined the effects of melatonin deficiency in rats who underwent middle cerebral artery occlusion (MCAO). Fifteen days following pinealectomy, both common carotid arteries and the left middle cerebral artery were occluded with microsurgical clips for 1 hour. Following 1 hour of occlusion, blood flow was restored by removal of the clips. Melatonin deficiency was produced by pinealectomy.

Manev et al. (1996) found that stroke induced by MCAO resulted in greater brain damage in the pinealectomized rats compared to the sham operated animals. Using a computer assisted image analysis system, Manev et al. (1996) measured infarct volumes

on Nissl stained coronal sections (40 μ m thick). A modest loss of Nissl staining in the left frontoparietal cortex was observed 4 hours following MCAO. Quantitative analysis revealed that the pinealectomized animals exhibited greater lesion volume than sham-pinealectomized animals. Thus, lowering the level of endogenous melatonin exacerbated the neural effects of acute stroke.

Present Study

To date, no study has examined the effects of pinealectomy on other experimental models of stroke. Moreover, previous studies have only examined the neural and not the behavioral effects of pinealectomy on stroke consequences. The purpose of the present study was to examine the effects of pinealectomy on the behavioral and neural sequelae of chronic cerebrovascular hypoperfusion (ischemia), as created in the rat by chronic bilateral carotid artery occlusion (2VO) in the rat.

Neuropathology of Permanent Carotid Occlusion

Chronic carotid artery ligation in the rat produces a 25-50% reduction of cerebral blood flow 2.5 hours post-occlusion (Tsuchiya et al., 1992). Tsuchiya et al. (1992) found that local cerebral blood flow as measured by ^{14}C -iodoantipyrine autoradiography was significantly lessened in many subcortical structures. For instance, these investigators found a 60% reduction in local cerebral blood flow in the caudate nucleus, 61-75% in cortical areas, 48% in the hippocampus, and 42-51% in thalamic areas 2.5 hours following 2VO. Moreover, it has been found that one-week following chronic 2VO,

several areas such as the hippocampus and cortex exhibit significantly reduced (~30%) cerebral blood flow (Tsuchiya et al. 1992).

The reduction in cerebral blood flow that accompanies 2VO has been found to result in hippocampal damage particularly in the CA1 region (Bennett et al., 1998; Ni et al., 1994; Pappas et al., 1996). For instance, Ni et al. (1994) observed a small loss in the number of neurons in the CA1 region of the hippocampus 1-month following 2VO. Four months following 2VO, these investigators observed a significant loss of hippocampal cells. Similarly, Pappas et al. (1996) found a significant loss of cells and increased glial fibrillary acidic protein immunoreactivity in the CA1 region of the hippocampus. Interestingly, no hippocampal damage was observed 2-weeks following 2VO. Taken together, it can be postulated that damage to hippocampal CA1 pyramidal cells occurs sometime between 1-month and 4-months post-occlusion.

Behavioral Consequences of 2VO

Of the structures affected by 2VO, the hippocampus is of particular interest because of its important role in learning and memory (Olton & Papas, 1979). It is well known that hippocampal damage in rats impairs spatial learning and memory (Olton & Papas, 1979). One of the memory systems particularly vulnerable to hippocampal damage is working memory, which is the memory system responsible for remembering trial-specific events. Several studies have found that working memory is impaired among 2VO animals (Bennett et al., 1998; Ni et al., 1994; Pappas et al., 1996). For instance, Bennett et al. (1998) assessed the working memory performance of 2VO animals using a radial arm maze task. Briefly, animals were required to obtain a sucrose reward that was

located in a recessed well at the end of each maze arm. Once an animal obtained a reward he was required to refrain from re-entering that arm. A re-entry into an arm in which he had already received a reward was recorded as a working memory error. Bennett et al. (1998) found a working memory impairment among their 2VO animals that correlated with hippocampal CA1 neuronal loss.

Current Study

Taken together, it can be seen that the neural and behavioral changes following 2VO are slow to emerge and are relatively modest. Hence, 2VO is a seemingly ideal model to examine the potentiating effects of pinealectomy. Thus, in the present study, spatial and non-spatial working memory were assessed using a radial arm maze task that is sensitive to hippocampal damage (Olton & Papas; 1979; Olton & Feustle, 1981). A tactile version of the radial arm maze task was also utilized in the present study because of the recent finding that among some 2VO rats, there is a loss of the pupillary reflex to light and as well, retinal degeneration (Davidson et al. Submitted). The tactile version of the radial arm maze task developed by Olton & Feustle (1981) enables the rat to solve the working memory component without having to rely on visual extra-maze cues. Rather, the rat can solve the task using various intramaze cues in the form of tactile inserts within each maze arm.

It was predicted in the present study that pinealectomy would exacerbate the behavioral and neural effects of permanent 2VO. Specifically, it was predicted that pinealectomized 2VO rats would exhibit a greater working memory impairment compared to non-pinealectomized animals. Moreover, it was predicted that

pinealectomized rats would have increased neuronal damage particularly in the hippocampus, than sham-pinealectomized animals, similar to that seen by Manev et al. (1996). Stereological techniques were utilized in order to obtain unbiased estimates of total cell number. Stereological techniques compared to manual counting techniques are more appropriate for the purpose of detecting and quantifying neuron loss (West, 1994).

Method

Animals

One-month old pinealectomized (N=22) and sham-pinealectomized (N=20) male Sprague-Dawley rats were obtained from Harlan-Sprague Dawley (Indianapolis, IN). The pinealectomy surgery was performed 1 week prior to receiving the animals. Briefly, the pinealectomy was performed as follow. Animals were anaesthetized with a ketamine (5mg/kg) and xylazine (1mg/kg) combination. Animals were then placed in a stereotaxic instrument and a 1-1.5 midline incision was made. The underlying connective tissue was removed by scraping with a blunt probe. A small hole was then drilled above the right cerebral hemisphere near the junction of the superior sagittal sinus and the transverse sinus. A small cut was made in the exposed dura. The pineal gland was then removed using a pair of blunt-tipped curved iris forceps. The incision was closed with silk suture. Animals who received the sham pinealectomy procedure underwent the identical procedure except the pineal gland was not removed.

Animals were individually housed in a 12-hour light/dark cycle (lights on 20:30, off 8:30). During radial arm maze testing, the animals were food deprived to 85% of their free feeding weight. Water was available *ad libitum*.

Apparatus

Tactile Radial Arm Maze Task (Tactile RAM)

The apparatus was a grey plastic five arm RAM modified to emphasize intra-maze rather than extra-maze cues. The maze consisted of a central hub enclosed by a 30 cm wall with guillotine-type doors which allowed access to each arm. Each arm was 67

cm long, 10 cm wide, and 30 cm high and contained a unique tactile cue that lined its floor. These cues consisted of 1 inch thick grey PVC plastic inserts with different tactile characteristics. These were (a) smooth surface (b) rough sandblasted surface, (c) longitudinal strips, each 1/4 inch wide and 1/4 inch high separated by 1/4 inch (d) horizontal strips, each 1/4 inch wide and 1/4 inch high separated by 1/4 inch (e) holes drilled 1/4 inch deep, each 1/2 inch wide with a 1/2 inch space separating each hole. A recessed food well was at the end of each maze. All of the arms had a removable top made of white bench coat and sides covered with brown opaque paper that prevented the rats from seeing any extra-maze cues.

Standard Radial Arm Maze Task (Standard RAM)

The apparatus consisted of a grey plastic 10-arm radial arm maze that had a 75 cm diameter central hub and 10 equally spaced arms radiating out from the hub. Each arm was 66 cm long, 12 cm wide, and had 25 cm high clear side walls. At the end of the maze arms was a recessed food well. The maze room contained various constant extra-maze stimuli such as a large colored poster, tables, and ceiling lights.

Preoperative Training

At approximately 9 months post-pinelectomy, rats were presurgically trained on the tactile RAM to enter all of the arms in the maze and obtain the food reward, Honey Nut Cheerios. On the first day of shaping, the Cheerios were scattered throughout the maze. Each rat was placed on the center platform and was allowed to explore the maze for 15 minutes. Shaping then consisted of placing one Cheerio at the end of each arm.

The rat was then placed onto the center platform and the session continued until he had eaten all 5 food rewards or until 10 minutes had elapsed.

Following shaping, the rats received 2 daily training trials for 5 consecutive days each week until they met a strict performance criterion after which they underwent 2VO surgery. The performance criterion for surgery was that in a 5 day training week, the rat had to make no working memory errors on 7 of the 10 training trials.

For each daily training trial, the orientation of the tactile arms was changed so that the topographical relationship among the arms was different for each trial. A Cheerio was placed in the food well at the end of each arm. The rat was placed onto the center platform with the guillotine doors closed. The doors were then opened and the rat was allowed to choose among the maze arms until he had obtained all five food rewards or 10 minutes had elapsed. Visitation of an arm more than once within a trial was considered to be a working memory error. The correct entry of a baited arm was deemed to occur only when the rat ate the food reward. Re-entry into a baited arm was recorded whenever the rat went to the empty food well at the end of the arm.

Permanent Bilateral Carotid Artery Occlusion

All rats were quasi-randomly assigned to one of 4 treatment groups: pinealectomized 2VO (N=10), pinealectomized sham (N=12), sham 2VO (N=10), or sham sham (N=10). All rats were anaesthetized with 2% halothane and given 0.1 ml of atropine (i.m.) in order to prevent respiratory distress.

A ventral mid-line incision was made to expose the common carotid arteries bilaterally. They were then carefully separated from the carotid sheath and vagus nerve.

Animals in the 2VO group had their carotid arteries bilaterally ligated with 5-0 silk suture. Animals in the sham group underwent the identical surgical procedure except their carotid arteries were not ligated. All animals were given 10 days to recover before behavioral testing resumed.

Vision Assessment

Due to the previous finding by Davidson et al. (Submitted) of a loss of pupillary reflex among 44% of 2VO animals, pupillary constriction was examined every day following surgery. This was done by individually exposing the animal's dark adapted eyes to a beam of light from a hand-held otoscope.

Post-Operative Behavioral Testing: Tactile RAM

Testing sessions were identical to training sessions except that the rats received 4 trials per week. Beginning 10 days post-2VO rats were tested on this task for 7 weeks. Testing then ceased for 1 week and rats were then trained on a standard RAM.

Standard Radial Arm Maze Testing

The rats received 7 weeks of post-operative training on the standard radial arm maze task, beginning on day 66 following 2VO. On the first training day, Cheerios were scattered around the maze. The rats were individually allowed 15 minutes to explore the maze. Training trials consisted of baiting all 10 arms with the Cheerios. Each rat was positioned onto the center platform and allowed to choose among the maze arms until he had obtained all 10 food rewards or until 10 minutes had elapsed.

The entry of an arm was recorded only when the rat ate the food reward. A re-entry into a baited arm (working memory error) was recorded whenever the rat placed all four feet into an arm where he had already eaten the food reward.

Histological Methods

Following radial arm maze testing, approximately 122 days post-occlusion, all rats were deeply anaesthetized by ketamine (100 mg/kg i.m) and methohexital sodium (40 mg/kg i.p.) overdose. Rats were then transcardially perfused with 75 ml heparinized saline followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.

Upon removal of the brain, the presence or absence of the pineal gland was verified by visual inspection. The brains were postfixed in the fixative solution for 24 hours at 4°C. Following post fixation, tissue was rinsed in 0.1 M phosphate buffer + azide, pH 7.4. The first rinse was for 4 hours and the second rinse overnight. The brains were then dehydrated in graded alcohols, cleared in clearane and then paraffin embedded.

Tissue Sectioning and Staining

The brain was first cut coronally separating the cerebellum, and then the two cerebral hemispheres were separated at the midline. Forty micron (μm) sagittal sections containing the hippocampus were sectioned using a rotary microtome. Forty μm coronal sections containing the pineal gland were also sectioned with a rotary microtome.

In order to visualize hippocampal pyramidal cells, 40 μm sections were stained with hematoxylin. Briefly, sections were de-paraffinized and then incubated in Mayer's hematoxylin (1.25% hematoxylin, 10% ethanol, 4.5% aluminum ammonium sulphate,

0.02% sodium iodate, 0.1% citric acid) at 60°C for 30 minutes. Sections were then placed under running lukewarm water for an additional 30 minutes and then dehydrated, cleared and coverslipped. The coronal tissue sections were also stained with hematoxylin in order to histologically verify the pinealectomy.

In addition, 40 μ m left sagittal sections containing the hippocampus were stained immunohistochemically with a monoclonal antibody to glial fibrillary acidic protein (GFAP). Tissue sections were first de-paraffinized and rehydrated and then rinsed in 10 mM (PBS) pH 7.2 for 5 minutes. Sections were then reacted with 1:600 mouse anti-GFAP (Sigma) in a blocking buffer, followed by biotinylated goat anti-mouse antibody (1:100) (Amersham) then streptavidin biotinylated horseradish peroxidase complex (1:100) (Amersham). Tissue sections were placed in a solution containing 0.02% 3,3 diaminobenzidine, 0.6% ammonium nickel sulphate and 0.76% trizma pre-set crystals (pH 7.4) for 5 minutes after which 40 μ l of hydrogen peroxide was added to the solution. The tissue sections remained in this solution for 7 minutes after which another 40 μ l of hydrogen peroxide was added and tissue sections incubated for an additional 7 minutes. Tissue sections were then counterstained with 0.085% pyronin Y in a 0.05N acetate buffer, pH 4.8. Slides were then dehydrated, cleared and coverslipped.

Hippocampal Measures

Measures for the determination of left hippocampal CA1 and CA4 pyramidal cell counts as well as GFAP immunoreactivity were derived using systematic random sampling techniques. Briefly, this involved selecting sections at a regular interval (6) along the entire extent of the hippocampus after the first section had been selected at

random from the first interval. All microscopic analyses were conducted on an Olympus BH-2 microscope.

Hippocampal cell counts of both the CA1 and CA4 regions were carried out using standard stereological techniques (West, Slomianka, & Gundersen, 1991). Briefly, stereological techniques allow for the determination of an unbiased estimate of total cell number in a specified neuroanatomical region such as the hippocampus. Estimation of total cell number can be made without having to make assumptions regarding cell size, orientation or distribution. Thus, any tissue shrinkage that may occur during the processing of the tissue has no affect on the estimate. Neurons were counted using the optical fractionator method consisting of the use of a three-dimensional probe known as the optical disector, and systematic sampling of the region of interest. Thus, stereology produces accurate and reliable cell counts of an area such as the hippocampus. The present study utilized this technique with the use of stereo investigator software (Microbrightfield,) at 1000X magnification.

GFAP immunoreactivity was determined by measuring the percentage of the area that exhibited GFAP reactivity in both the left CA1 and CA4 oriens layers of the hippocampus. A threshold was set to detect GFAP containing glial cells and this was used to measure optical density. This was accomplished using image analysis software (Imaging Research, St. Catherines) at 200X magnification. All histological and behavioural measures were conducted by an observer who was blind to group membership.

Results

Results

Animals

Of the 42 animals that underwent 2VO or sham 2VO surgery, 4 animals were dropped from the study. One pinealectomized sham animal died during tactile RAM posttesting due to ill health. Moreover, one sham sham animal died during standard RAM testing due to ill health. Two additional animals (pinealectomized sham and sham 2VO) were dropped from standard RAM testing due to an inability to acquire any of the food rewards. Thus, statistical analyses were performed on tactile RAM data from 41 animals (10 pinealectomized 2VO, 11 pinealectomized sham, 10 sham 2VO, and 10 sham sham). Moreover, statistical analyses were performed on standard RAM data from 38 of the 41 animals that had underwent tactile testing (10 pinealectomized 2VO, 10 pinealectomized sham, 9 sham-2VO, and 9 sham sham).

Pupillary Reflex

Following surgery, all animals had their pupillary reflex examined every day by exposing the animal's dark adapted eye to a beam of light from a hand held otoscope. None of the 2VO animals exhibited loss of pupillary reflex.

Tactile Radial Arm Maze

Animals were required to reach a performance criterion prior to surgery and were subsequently tested on the tactile RAM for 7 weeks. The number of working memory errors were calculated for each post-surgical week and was used for statistical analysis. These data are shown in Figure 2.

Figure 2. Tactile maze baited arm re-entry errors. Overall, the trials differed ($F(6,222)=2.77$, $p<.02$) with animals exhibiting improvement across post-surgical weeks. Error bars represent S.E.M.

Working Memory Errors on Tactile RAM

A 2 X 2 X 7 mixed analysis of variance (ANOVA) with pinealectomy (pinealectomized and sham pinealectomized) and 2VO (2VO and sham 2VO) as between subjects factors and trial (1-7) as a repeated measures variable revealed a significant pinealectomy by 2VO interaction ($F(1,37)= 4.08, p<.05$). This interaction is shown in Figure 3. A t-test revealed that pinealectomized 2VO animals made more working memory errors per week than sham 2VO animals ($t(18)=2.09, p<.05$) (Figure 2). Moreover, the ANOVA revealed a significant trial effect ($F(6,222)=2.77, p<.02$) (See Figure 2). It was observed that animals improved across post-surgical weeks. No significant trial by pinealectomy interaction was found, nor was there a significant trial by 2VO interaction. Moreover, there was no significant main effect of pinealectomy or 2VO.

Standard Radial Arm Maze

Following tactile RAM testing, all rats received 7 weeks of training on a standard RAM task. The average number of consecutive correct arm entries was calculated for each daily session. The average for each post-surgical week was then used for statistical analyses.

A 2 X 2 X 7 mixed analysis of variance with pinealectomy (pinealectomy and sham pinealectomy) and 2VO (2VO and sham 2VO) as between subject variables and trials (1-7) as a repeated measures variable found a significant trial effect ($F(6,204)=14.34, p<.001$), with all groups exhibiting improvement across post-surgical weeks (Figure 4). No significant trial by pinealectomy interaction was found, nor was there a significant trial by 2VO interaction. Moreover, no significant main effect of

Figure 3. Pinealectomy by 2VO interaction. Overall, there was a significant pinealectomy by 2VO interaction ($F(1,37)=4.08$, $p<.05$), with the pinealectomized 2VO animals making more working memory errors than the sham 2VO animals

Pinealectomy by 2VO Interaction

Figure 4. Number of consecutive correct arm entries across 7 post-surgical weeks. Overall, a significant trial effect was found ($F(6,204)=14.34$, $p<.001$) whereby all groups improved across post-surgical weeks. Error bars represent S.E.M.

Correct Consecutive Entries on Standard RAM

pinealectomy was found, nor was there a significant main effect of 2VO. Similarly, no significant pinealectomy by 2VO interaction was found.

Hippocampal Pathology

CA1 and CA4 Pyramidal Cell Counts

Tissue sections containing the hippocampus were stained with Mayer's hematoxylin. Cells were counted using stereological techniques which yielded unbiased estimates of the hippocampal CA1 and CA4 regions. Results were analyzed using a 2 X 2 analysis of variance with pinealectomy (pinealectomized and sham pinealectomized) and 2VO (2VO and sham 2VO) as between subjects factors. Analyses of variance revealed a significant effect of pinealectomy for both CA1 ($F(1,16)=17.46$, $p<.001$) and CA4 ($F(1,16)=11.63$, $p<.005$) regions (Figure 5 and 6). Pinealectomized animals had fewer cells in both hippocampal CA1 and CA4 regions compared to sham animals. Although no effect due to 2VO was found for both CA1 and CA4 regions, the analysis of variance narrowly missed reaching significance for both CA1 ($p<.07$) and CA4 ($p<.09$) sectors.

GFAP Immunohistochemistry

Hippocampal GFAP was quantified as the percentage of GFAP staining in the CA1 and CA4 oriens layers. Two by two (pinealectomy X 2VO) ANOVAs with pinealectomy and 2VO as between subjects factors, revealed a significant main effect of pinealectomy in both CA1 ($F(1,16)=13.27$, $p<.002$) and CA4 ($F(1,16)=8.99$, $p<.01$) sectors. The pinealectomized animals exhibited a higher percentage of GFAP staining in

Figure 5. Estimated hippocampal CA1 cell counts. Overall, a significant effect of pinealectomy was found ($F(1,16)=17.46$, $p<.001$). ** denotes significantly fewer pyramidal cells relative to sham animals. Error bars represent S.E.M.

Estimated Hippocampal CA1 Cell Counts

Figure 6. Estimated hippocampal CA4 cell counts. Overall, a significant effect of pinealectomy was found ($F(1,16)=11.63$, $p<.005$). ** denotes significantly fewer cells relative to sham animals. Error bars represent S.E.M.

Estimated Hippocampal CA4 Cell Counts

both the hippocampal CA1 and CA4 regions compared to sham animals. This is illustrated in Figures 7 and 8. No main effect of 2VO was found nor was there a significant pinealectomy X 2VO interaction.

Correlational Analyses of Hippocampal Cell Number and GFAP Staining

Correlational analyses were performed on the hippocampal measures. Overall, a positive relation was found between the total number of CA1 pyramidal cells and the total number of CA4 cells ($r=.74$, $p<.01$). Animals with higher cell counts in the CA1 also had higher CA4 cell numbers. Moreover, animals that exhibited neuronal loss in the CA1 also exhibited neuronal loss in the CA4 region. This relationship is illustrated in Figure 9. A positive correlation was also found between percent of area exhibiting GFAP immunostaining in the CA1 and percent of area exhibiting GFAP immunostaining in the CA4 ($r=.93$, $p<.01$). Animals with a higher percent area staining in the CA1 also had an elevation of GFAP immunoreactivity in the CA4 (See Figure 9).

In addition, total cell number in the CA1 and CA4 was negatively correlated with GFAP immunoreactivity in the CA1 and CA4 respectively ($r=-.56$, $p<.05$; $r=-.55$, $p<.05$). Animals who exhibited neuronal loss in the CA1 and/or the CA4 also exhibited an elevation of GFAP staining in that area. By contrast, animals with normal neuron numbers in the CA1 and CA4 did not show an elevation in GFAP immunostaining. This finding is illustrated in Figure 10.

Correlational analyses were also performed on the hippocampal measures for 2VO/non-2VO and pinealectomized/non-pinealectomized groups (Table 1 and 2). Among

Figure 7. Percentage of GFAP immunostaining in the CA1 oriens layer. An elevation in GFAP immunostaining was observed in the CA1 of pinealectomized animals compared to sham animals ($F(1,16)=13.27$, $p<.002$). ** represents differences between pinealectomized and sham groups. Error bars represent S.E.M.

GFAP Immunoreactivity in CA1 region

Figure 8. Percentage of GFAP immunostaining in the CA4 oriens layer. An elevation in GFAP immunostaining was observed in the CA4 of pinealectomized animals compared to sham animals ($F(1,16)=8.99$, $p<.01$). ** represents differences between pinealectomized and sham groups. Error bars represent S.E.M.

GFAP Immunoreactivity in CA4 region

Figure 9. **Top Panel:** Scatterplot illustrating the correlation between total hippocampal CA1 and CA4 cell number. Overall, a significant positive correlation was found ($r=.74$, $p<.01$). **Bottom Panel:** Scatterplot illustrating relationship between GFAP immunostaining in CA1 and CA4 regions of the hippocampus. A significant positive correlation was found ($r=.93$, $p<.01$).

Figure 10. Correlations between hippocampal cell numbers and percent area GFAP immunoreactivity in hippocampus. **Top Panel:** Relationship between total CA1 cell number and percent area GFAP immunostaining. A significant negative correlation was found ($r=-.56$, $p<.05$). **Bottom Panel:** Relationship between total CA4 cell number and percent area GFAP immunostaining. A significant negative correlation was found ($r=-.55$, $p<.05$).

Table 1: Intercorrelations between hippocampal measures for 2VO and non-2VO animals

Group	Measure	CA1	CA4	GFAP CA1	GFAP CA4
2VO (n=10)	CA1	-----	.662**	-.872**	-.810**
	CA4		-----	-.388	-.548
	GFAP CA1			-----	.909**
	GFAP CA4				-----
Non-2VO (n=10)	CA1	-----	.801**	-.258**	-.137
	CA4		-----	-.560	-.456
	GFAP CA1			-----	.974**
	GFAP CA4				-----

* Correlation is significant at the .05 level

** Correlation is significant at the .01 level

Table 2: Intercorrelations between hippocampal measures for the pinealectomized and non-pinealectomized animals

Group	Measure	CA1	CA4	GFAPCA1	GFAPCA4
Pineal (n=10)	CA1	-----	.492	-.141	-.178
	CA4		-----	-.172	-.367
	GFAP CA1			-----	.927**
	GFAP CA4				-----
Non Pineal (n=10)	CA1	-----	.705*	-.362	-.239
	CA4		-----	.127	-.049
	GFAP CA1			-----	.747
	GFAP CA4				-----

* Correlation is significant at the .05 level

** Correlation is significant at the .01 level

the 2VO animals, total cell number in the CA1 and CA4 was positively correlated ($r=.66$, $p<.05$). Moreover, a positive correlation was found between the percentage of GFAP staining in the CA1 and percentage of GFAP staining in the CA4 ($r=.91$, $p<.01$). A negative correlation was also found between total cell number in the CA1 and CA4 and percent area GFAP staining in the CA1 and CA4 respectively ($r=-.87$, $p<.01$; $r=-.81$, $p<.01$).

Similarly, among non-2VO animals, a positive relationship was found between total cell number in the CA1 and CA4 ($r=.80$, $p<.01$). Moreover, an increase in percent area GFAP staining in the CA1 was associated with an increase in GFAP staining in the CA4 ($r=.97$, $p<.01$). No relation was found between total cell numbers in the CA1 and CA4 and GFAP staining.

Among the pinealectomized and sham-pinealectomized animals, percent area GFAP staining in the CA1 was positively correlated with percent area GFAP staining in the CA4 ($r=.93$, $p<.01$; $r=.75$, $p<.05$). Only the non-pinealectomized group had a significant correlation between neuron number in the the CA1 and the CA4 ($r=.71$, $p<.05$). No relationship was found for both pinealectomized and non-pinealectomized animals between cell number and GFAP staining for both the CA1 and the CA4 sectors.

Correlational Analyses: Behavior and Hippocampal Pathology

Tactile RAM

A correlational analysis was performed between the number of working memory errors made by all animals and hippocampal pathology. Overall, no significant correlation was found between number of errors and total cell number in the CA1 and CA4 ($r=.36$,

n.s.; $r = -.08$, n.s.). Moreover, no significant correlation was found between number of errors made per week by all animals and GFAP immunoreactivity in the CA1 and the CA4 regions of the hippocampus ($r = .04$, n.s.; $r = .003$, n.s.).

In addition, intercorrelations between number of working memory errors and hippocampal pathology for 2VO/non-2VO and pinealectomized/non-pinealectomized groups did not reveal any relationship between number of working errors made by the groups and hippocampal pathology (Table 3).

Standard RAM

A correlational analysis was performed between the number of consecutive correct arm entries and hippocampal pathology for all animals. Overall, a significant positive correlation was found between the number of consecutive correct arm entries and total cell number in the CA1 ($r = .55$, $p < .05$). This relationship is illustrated in Figure 11. Removal of an outlier however, resulted in the correlation losing its statistical significance.

In addition, intercorrelations between the number of consecutive correct arm entries and hippocampal pathology for the 2VO/Non-2VO and pinealectomized/non-pinealectomized groups revealed a significant correlation only for the non-2VO group (Table 4). Again, removal of an outlier resulted in the correlation losing its statistical significance.

A significant positive correlation was found among the sham sham animals between number of correct arm entries and total cell number in the CA1 ($r = .892$, $p < .05$). Animals with higher cell counts in the CA1 sector also made more correct consecutive

arm entries than animals with lower cell counts. This relationship is illustrated in Figure 12.

Histological Confirmation of Pinealectomy

Coronal tissue sections containing the pineal gland were stained with Mayer's hematoxylin and then histologically examined to confirm pinealectomy or sham pinealectomy. Examination of these tissue sections revealed the absence of a pineal gland in all pinealectomized animals. Moreover, all sham animals, except for one sham 2VO, had an intact pineal gland.

Upon histological examination it was further observed that all pinealectomized animals as well as one sham sham and one sham 2VO animal sustained damage in the right visual association cortex due to the pinealectomy surgery. Lesion size varied from animal to animal. Figures 13 to 24 illustrate the size and location of the lesion for each animal that sustained cortical damage.

In order to determine if there was a relationship between the cortical damage and the hippocampal pathology found among the pinealectomized animals, a correlational analysis was performed. The twelve animals were ranked on a 12-point scale where 1 represented the smallest lesion and 12 the largest. Once ranked, correlational analyses were performed between the rankings and the hippocampal measures. No significant correlation was found between ranking and total cell number in the CA1 and the CA4 regions ($r = .16$, n.s.; $r = .13$, n.s.). This is illustrated in Figure 25. Moreover, no significant correlation was found between ranking and GFAP staining in the CA1 and CA4 sectors ($r = -.06$, n.s.; $r = -.13$, n.s.). This is illustrated in Figure 26.

Table 3. Intercorrelations between number of working memory errors on tactile RAM and hippocampal pathology for 2VO/non-2VO and pinealectomized/non pinealectomized animals.

Group	Measure	CA1	CA4	GFAP CA1	GFAP CA4
2VO	Errors	-.474	-.175	.200	.070
(n=10)					
Non- 2VO	Errors	-.226	.168	-.223	-.158
(n=10)					
Pineal	Errors	-.469	-.047	-.257	-.285
(n=10)					
Non-Pineal	Errors	.189	.447	.250	.371
(n=10)					

* Correlation is significant at the .05 level

** Correlation is significant at the .01 level

Figure 11. Scatterplot illustrating correlation between number of consecutive correct arm entries and total cell number in the hippocampal CA1 region. Correlation lost significance when an outlier was removed from the analysis.

Table 4. Intercorrelations between number of correct consecutive arm entries and hippocampal measures for 2VO/non-2VO and pinealectomized/non-pinealectomized groups

Group	Measures	CA1	CA4	GFAP CA1	GFAP CA4
2VO (n=10)	# Correct	.375	-.115	-.382	-.015
Non-2VO (n=10)	# Correct	.767 **	.458	.178	.246
Pineal (n=10)	# Correct	.457	-.119	.374	.490
Non-Pineal (n=10)	# Correct	.616	.050	-.439	.067

** Correlation is significant at .01 level.

Figure 12. Scatterplot illustrating correlation between the number of consecutive correct arm entries and total CA1 neuron number for the sham sham animals.

Figure 13. Sham 2VO animal that was given a lesion ranking of 12. This animal exhibited the largest lesion. Plate numbers from Paxinos and Watson (1986).

Figure 14. Pinealectomized sham animal that received a lesion ranking of 11. Plate numbers from Paxinos and Watson (1986).

Figure 15. Pinealectomized 2VO animal that received a lesion ranking of 10. Plate numbers from Paxinos and Watson (1986).

Figure 16. Pinealectomized 2VO that received a lesion ranking of 9. Plate numbers from Paxinos and Watson (1986).

Figure 17. Pinealectomized 2VO animal that received a lesion ranking of 8. Plate numbers from Paxinos and Watson (1986).

Figure 18. Pinealectomized sham animal that received a lesion ranking of 7. Plate numbers from Paxinos and Watson (1986).

Figure 19. Sham sham animal that received a lesion ranking of 6. Plate numbers from Paxinos and Watson (1986).

Figure 20. Pinealectomized 2VO animal that received a lesion ranking of 5. Plate numbers from Paxinos and Watson (1986).

Figure 21 Pinealectomized sham animal that received a lesion ranking of 5. Plate number from Paxinos and Watson (1986).

Figure 22. Pinealectomized 2VO animal that received a lesion ranking of 3. Plate number from Paxinos and Watson (1986).

Figure 23. Pinealectomized sham animal that received a lesion ranking of 2. Plate number from Paxinos and Watson (1986).

Figure 24. Pinealectomized sham that received a lesion ranking of 1. This animal had the smallest lesion. Plate number from Paxinos and Watson (1986).

Figure 25. **Top Panel:** Correlation between ranking of lesion size and total cell number in CA1. **Bottom Panel:** Correlation between ranking of lesion size and total cell number in the CA4.

Figure 26. **Top Panel:** Scatterplot illustrating the correlation between lesion ranking and GFAP staining in CA1. **Bottom Panel:** Scatterplot illustrating the correlation between lesion ranking and GFAP staining in the CA4.

- Pineal 2VO
- Pineal sham
- ▼ Sham sham
- ▽ Sham 2VO

Discussion

Following 2VO surgery, none of the animals lost the pupillary reflex. Subsequent testing on a tactile version of the radial maze revealed that the pinealectomized 2VO animals made significantly more working memory errors on the tactile RAM than the sham 2VO animals. No differences between groups was found on the number of consecutive correct arm entries on the standard RAM task.

Approximately 122 days following 2VO, the pinealectomized animals exhibited significant neuronal loss in both CA1 and CA4 regions compared to the sham animals. Moreover, pinealectomized animals were found to have significantly higher levels of GFAP immunoreactivity in both the CA1 and CA4 regions compared to the non-pinealectomized animals. An unexpected finding was that all pinealectomized animals had sustained damage in the right visual association cortex due to the pinealectomy surgery. Moreover, one sham sham and one sham 2VO animal also sustained damage in the same area due to the sham pinealectomy surgical procedure. Extent of the damage varied and was not related to hippocampal pathology.

Pupillary Reflex

The finding that no animals lost their pupillary reflex at any time following 2VO, is not consistent with previous findings in this laboratory that have found a permanent loss of pupillary reflex among some 2VO animals (Davidson et al. Submitted). For instance, Davidson et al.(Submitted) found a loss of the pupillary reflex in 44% of 2VO animals, presumably due to retinal ischemia. The pterygopalatine artery, which is the principle source of retinal perfusion in the rat, branches from the internal carotid. It is reasonable to

assume that when the common carotid is blocked, there would be reverse perfusion of the internal carotid and thence the pterygopalatine, via the circle of Willis. This seems to be the case in the 2VO rats in the current study. However, reverse perfusion either did not occur or was insufficient in about half of the rats in the Davidson et al. study. The reason for this discrepancy is unclear. Surgical procedures were similar with the exception of anaesthetic. Halothane was used in the present study whereas Davidson et al. (Submitted) used a barbiturate and ketamine combination. Both the present and the Davidson et al experiment used Sprague-Dawley rats. However, they were purchased from different breeders and it is feasible that the rat lines differ in their adaptation to 2VO, as has been noticed in an earlier study which found marked variation among rat strains in the lethality of 2VO (Payan, Levine, & Strebel, 1965).

Behavioral Findings

Tactile RAM

Overall, the pinealectomized 2VO animals made significantly more working memory errors than the sham 2VO animals. No difference was found in the performance of the sham 2VO, pinealectomized sham, and sham sham animals. In order to interpret this finding it is important to take into account the unexpected cortical damage (to be discussed in a later section). It could be argued that the pinealectomized 2VO animals performed worse than the sham 2VO animals because of the cortical lesion and not pinealectomy. If this were the case however, then the pinealectomized sham animals who also sustained cortical damage, should have made more working memory errors as well. This was not the case. In fact, mean error score for the pinealectomized sham group across

the 7 weeks of testing was found to be lower than the corresponding mean for sham sham group mean. Thus, the pinealectomized sham did not have any difficulty solving the task. Hence, a plausible explanation for the impairment seen in the pinealectomized 2VO animals is that pinealectomy exacerbated the behavioural effects of permanent 2VO.

The finding that working memory was not impaired among pre-trained sham 2VO animals was consistent with previous research (Ni et al., 1994; Pappas et al., 1996). For instance, Ni et al. (1994) found that rats who were pre-trained on a standard RAM task prior to 2VO surgery did not exhibit a working memory impairment. Similarly, Pappas et al. (1996) found that 2VO animals that had received pre-training prior to surgery did not exhibit a working memory impairment on a standard 12-arm RAM even at 24 weeks post-occlusion.

Standard RAM

Approximately 66 days following 2VO, rats were tested on a standard 10-arm RAM task. No differences were found between groups on the average number of correct consecutive arm entries. Consistent with the tactile maze data, the pinealectomized 2VO group mean was lower at all 7 post-surgical weeks compared to all other groups but statistically this was not significant. Perhaps with a larger sample size, this would have reached significance.

Consistent with the tactile maze, sham 2VO animals did not differ from sham sham animals in this task. Hence, it could be argued that working memory was not impaired in these animals. This is consistent with the previous finding by Davidson et al. (submitted) who found that pretrained 2VO animals do not exhibit working memory

deficits. This investigator found that only 2VO rats with a visual impairment were impaired on the RAM task. The RAM is a visual based spatial task thus having visual deficits greatly impacts upon successfully solving the task. Although the present study did not pre-train animals on the standard RAM they did receive extensive training on the tactile RAM. Thus, these animals did receive training on a RAM task prior to standard RAM training.

Pathology

Hippocampal Pathology

At 122 days post-surgery, unbiased stereological counting indicated that the pinealectomized animals exhibited a significant loss of cells in the CA1 compared to the non-pinealectomized animals. The pinealectomized 2VO animals exhibited 24% CA1 cell loss compared to the sham sham animals. Moreover, the pinealectomized sham animals showed a 17% reduction in CA1 pyramidal cells compared to the sham sham animals. Although not statistically significant, sham 2VO animals were found to have 10% fewer cells in the CA1 compared to the sham sham animals.

Similar to the CA1, a significant reduction in CA4 neuron number was found in all pinealectomized animals compared to all non-pinealectomized rats. Pinealectomized 2VO animals exhibited a 30% reduction of cells in the CA4 compared to the sham sham animals. One of the five pinealectomized animals, had complete cell loss in the CA4. Pinealectomized sham animals exhibited a 21% loss of cells in the CA4 region compared to the sham sham animals. Consistent with the CA1 region, sham 2VO animals had 10% fewer cells in the CA4 sector than sham sham animals.

in agreement with the data found on total cell number, pinealectomized animals showed a significant elevation of GFAP immunoreaction in both the CA1 and CA4 sectors compared to the non-pinealectomized animals. Unlike a previous report that found an elevation of GFAP immunoreaction in the CA1 of sham 2VO animals (Pappas et al., 1996), no difference was found between percent area GFAP staining in both the CA1 and CA4 of sham 2VO and sham sham animals.

These results for pinealectomy are consistent with research by Manev et al. (1996) who reported that pinealectomy increased the neuropathology after acute, severe ischemic episode. However, unlike previous studies which found significant neuronal loss among 2VO animals 3-6 months following 2VO (Ni et al., 1994; Pappas et al., 1996) the current study did not find a significant loss of neurons in the CA1 sector of the hippocampus among the sham 2VO animals. The 2VO rats did show a 10% decrease in cell number in both CA1 and CA4 and with the addition of more subjects, this could reach statistical significance. Unlike previous studies that determined cell counts manually, from a few sections, the current study used stereology to determine total cell number in the CA1 and the CA4. Stereological techniques require that an entire structure such as the hippocampus, be systematically sampled (in this experiment, about 20 sections) allowing for a more accurate unbiased estimate of total cell number. A problem with manual counting techniques is that they are based on numerical density. Thus, cell counts can be greatly affected by tissue shrinkage that may occur during tissue processing and by the repeat counting of cells which may occur when serial sections are enumerated. The application of the optical disector technique in this study avoided this problem in that any

shrinkage or expansion of the tissue during processing does not affect the estimate of total neuron number (West, 1994).

Correlational Analysis

Significant correlations were found between the different hippocampal measures. Overall, animals that exhibited a significant loss of cells in the CA1 also exhibited elevated GFAP immunoreaction in the CA1. Similarly, animals that showed a significant reduction in total CA4 cell number also exhibited an increase in GFAP density in that area. Moreover, animals that showed a significant loss of cells in the CA1 sector also lost cells in the CA4 sector.

No correlation was found between hippocampal pathology and tactile RAM performance. In addition, while a significant correlation was found between total cell number in the CA1 and the number of consecutive correct arm entries on the standard RAM, this was found to be not significant when an outlier was removed from the correlational analysis. Hence overall, there was no relation between the pathology seen among the pinealectomized animals and performance on these RAM tasks.

Cortical Damage

In addition to hippocampal pathology, all pinealectomized animals as well as one sham sham and one sham 2VO animal were found to have sustained damage in the right visual association cortex. Apparently, the damage was a result of the blunt dissection technique used to remove the pineal gland. Due to the proximity of midline blood vessels in the area where the pineal gland is located, the skull was not opened directly above the

pineal gland. Thus, removal of the gland was done blindly. Sham animals also had a hole drilled into the skull above the right cerebral hemisphere and this presumably accounted for the damage among the 2 sham animals.

The precise consequences of this cortical damage are not known but it is unlikely that vision was affected because the lesions were small. It is well established that small lesions to the visual cortex have little effect on rat vision (Sautter & Sabel, 1993).

Prior research on pinealectomized rats, confirmed melatonin deficiency by determining blood levels of melatonin rather than by histologically examining for the absence/presence of the gland (Manev et al. 1996). Similar to the current study, these investigators obtained animals that underwent pinealectomy/sham-pinealectomy surgery by the supplier. A similar blunt dissection technique was used on these animals. Following brain removal for the histological examination of the hippocampus, no examination of the pineal area was undertaken by Manev et al. (1996). Our finding illustrated the importance of ascertaining whether the surrounding tissue in the pineal area was also damaged in order to be able to make precise conclusions regarding the effects of pinealectomy on neuropathologic conditions such as ischemia.

The lesions that were found among the pinealectomized animals were unilateral and small and because of their location in the visual association cortex, it seems unlikely that they could cause the substantial loss of CA1 and CA4 cells and the elevation of GFAP immunostaining seen in the pinealectomized animals. In addition, one sham sham animal had a large lesion but also had high cell counts in both the CA1 and CA4 and did not exhibit elevated GFAP staining in these areas. A sham 2VO animal had the largest cortical lesion among all the animals but his GFAP was equal to that of the controls. This

animal did exhibit cell loss in both sectors of the hippocampus but the loss was comparable to that seen among the non-lesioned sham 2VO animals. In contrast, pinealectomized animals with very small lesions exhibited substantial cell loss in both CA1 and CA4 sectors and had elevated GFAP staining. Thus, no correlation was found between lesion size and cell loss. While these findings argue against a role for these cortical lesions in the effects observed following pinealectomy, this possibility can only be definitively ruled out by examining whether a cortical lesion alone, caused the effects observed in the present study.

Conclusions

The current study found significant hippocampal pathology among the pinealectomized animals. Moreover, similar to the finding by Manev et al. (1996) pinealectomized ischemic animals exhibited greater neuropathology compared to non-pinealectomized ischemic animals. This evidence suggests that melatonin deficiency may lead to increased brain vulnerability to ischemia. The finding that the pinealectomized 2VO animals unlike the sham 2VO animals, exhibited significant hippocampal pathology and behavioural impairment provides further evidence to suggest that endogenous melatonin plays a neuroprotective role. This is consistent with evidence that melatonin is an effective and potent free radical scavenger (Reiter, 1996).

It would be desirable to develop less invasive ways to study melatonin deficiency in animals. If surgical techniques must be used, a better technique other than blunt dissection, should be utilized. Research should also examine the possibility of utilizing an

antagonist that could block melatonin receptor function to dissociate its antioxidant from its receptor-mediated neuroprotective effects.

The finding that the pinealectomized 2VO animals were impaired on the tactile maze suggests this task is sensitive to the pathology seen in these animals. Perhaps making the task more difficult would result in greater impairments on this task. For instance, the task could be made more difficult by increasing the memory requirement from five arms to eight. In addition, confining the rat to the center of the maze between arm choices for a pre-determined amount of time could render this task more difficult. These modifications to the maze may also allow for the detection of deficits among rats such as the sham 2VO who exhibit mild to moderate cell loss.

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APPENDIX A.1

ANOVA Summary Table for working memory errors on the tactile RAM

Source	SS	df	MS	F	P
Pineal	5.90	1	5.90	3.14	.085
2VO	2.83	1	2.83	1.50	.228
Pineal X 2VO	7.67	1	7.67	4.08	.050
Error	69.52	37	1.88		
Trial	13.94	6	2.32	2.76	.013
Trial X Pineal	4.76	6	.79	.95	.464
Trial X 2VO	5.25	6	.88	1.04	.398
Trial X Pineal X 2VO	8.09	6	1.35	1.60	.146
Error (Trial)	186.25	222	.84		

APPENDIX A.2

ANOVA Summary Table for Number of Consecutive Correct Arm Entries

Source	SS	df	MS	F	P
Pineal	44.38	1	44.38	2.35	.134
2VO	15.40	1	15.40	.82	.373
Pineal X 2VO	21.73	1	21.73	1.15	.291
Error	641.19	34	18.86		
Trial	69.93	6	11.66	14.34	.001
Trial X Pineal	8.45	6	1.41	1.73	.115
Trial X 2VO	5.20	6	.87	1.07	.383
Trial X PinealX 2VO	4.51	6	.75	.93	.477
Error (Trial)	641.19	34	18.86		

APPENDIX A.3

ANOVA Summary Table for Total Number of Hippocampal CA1 and CA4 Cells*CA1*

Source	SS	df	MS	F	P
Pineal	1.38E+ 10	1	1.38E+10	17.45	.001
2VO	2.96E+09	1	2.96E+09	3.75	.071
Pineal X 2VO	3828125.0	1	3828125.0	.01	.945
Error	1.26E+10	16	7.90E+08		

CA4

Source	SS	df	MS	F	P
Pineal	5.15E+08	1	5.15E+08	11.63	.004
2VO	1.47E+08	1	1.47E+08	3.32	.087
Pineal X 2VO	1914697.2	1	1914697.2	.04	.838
Error	7.09E+08	16	44314272		

APPENDIX A.4

ANOVA Summary Tables for Percent area GFAP Staining in the CA1 and CA4*CA1*

Source	SS	df	MS	F	P
Pineal	46.67	1	46.67	13.27	.002
2VO	1.99	1	1.99	.57	.463
Pineal X 2VO	.27	1	.27	.08	.785
Error	52.29	16	3.52		

CA4

Source	SS	df	MS	F	P
Pineal	49.46	1	49.46	8.99	.009
2VO	8.57	1	8.57	1.56	.230
Pineal X 2VO	.32	1	.32	.06	.812
Error	88.03	16	5.50		