DIETARY CHOLINE AND VITAMIN/MINERAL SUPPLEMENT FOR RECOVERY FROM EARLY CORTICAL INJURY

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A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

MASTER OF SCIENCE

Department of Psychology and Neuroscience
LETHBRIDGE, ALBERTA, CANADA
November, 2003

Abstract

Early cortical injury has been attributed to the consequential effects of various factors, such as alcohol, drug addiction, smoking, and inadequate nutrient intakes during periods of pregnancy and lactation, or delivery of infants by forceps, and premature deliveries. These are only a few examples of circumstances, or "injury", that may result in disorders ranging from mild learning difficulties to aggressive behavior.

Injury to the cortex during the early years of development has been known to result in poor behavioral outcome into adulthood. Presently, the most common form of treatment includes a pharmacological agent, which may be accompanied with behavioral modification therapies supported by families. As an alternative form of therapy towards the treatment of early cortical injury, choline and a vitamin and mineral supplement (EM Power+) were used to determine the possibilities of nutrition intervention in an animal model. The injuries were incurred by aspiration lesion at days three, (Exp.1) and four, (Exp.2) and lesions were localized to the midline medial frontal cortex in some rats, while a different group of rats received lesions in the posterior parietal cortex. The pre- and postnatal choline treated animals showed favorable results for the medial frontal lesions, and the postnatal vitamin supplement treated animals showed favorable results for treatment in both medial frontal and posterior parietal lesions. All animals were tested in adulthood indicating that nutrition intervention is very beneficial for alleviating some of the functional deficits commonly seen from early cortical injury.

Acknowledgements

Firstly, I would like to thank my supervisor, Bryan Kolb for accepting an alien from the field of Nutrition into the field of Psychology and Neuroscience. Bryan was very supportive for me both personally and professionally, giving me the flexibility required to adapt to an overwhelming situation initially, which became an extraordinarily rewarding career path as completion became evident.

I'd also like to thank Rob Sutherland for support personally, as well as with any technical services required.

A number of people in the Kolb and Whishaw group have been extremely helpful towards the completion of these experiments. Robbin Gibb, for her talent of dissection of fresh brain tissue for Western Blot analysis- a technique learned with many years of dissection. Morgan Day, for her talent with the actual Western Blot analysis- a technique requiring experience. Greg Silasi, Dawn Danka, and Grazyna Gorney for assistance with perfusions, histology, and anatomy. Preston Williams for help with the Morris water task and Statsview 5. Omar Gharbawi, Bogdan Gorney, and Claudia Gonzalez for help with behavioral and technical assistance. Paul Whishaw for his expertise in the incorporation of movies into powerpoint presentations.

David Hardy, the formulator of the vitamin supplement, I would also like to thank for managing last minute requests for more supplemented rat feed, and for spending time discussing nutritional aspects of the supplement with me, as well as for providing samples for me to experience the benefits.

The moral support and understanding of my temporary disappearance from family William and Maxine Halliwell, Richard, Pamela, Kevin, Zachery, Bradly, and Meg'n Celeste Halliwell is unquestionably thankful. And last, but not least, I thank my mother Caroline Christiansen for her support of keeping me at her home, but also for being bipolar.

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1. General Introduction

Infant malnutrition has been observed worldwide, enabling nutritionists to study the consequences of inadequate dietary intakes during critical periods of growth and development. Malnutrition during the critical periods of brain development has been reported to result in irreversible functional deficits that impair the learning capacities of these individuals, as discussed by Lewis (1990), and Heuther (1990).

Protein-energy malnutrition in infancy has been reported to alter amino acid profiles, which adversely affect brain development, either through protein accretion, or neurotransmitter formation (Huether, 1990). According to Lewis (1990), prenatal malnutrition affects the proliferation and enlargement of brain cells, whereas malnutrition during the postnatal period can reduce the number of cells necessary for growth and development.

Early cortical injury includes a number of consequential effects from factors such as alcohol, drug addiction, smoking, and inadequate nutrient intakes during periods of pregnancy and lactation, or delivery of infants by forceps, and premature deliveries. These are only a few examples of circumstances, or "injury", known to develop into disorders ranging from mild learning difficulties to aggressive behavior as children grow into adolescence. The increasing demands required by families and communities, to cope with problems of learning disabled children and juvenile delinquency, have necessitated the specialty of child psychiatry (McDermott, 1977). A number of disorders, ranging from organic brain dysfunction to psychosis, have been attributed to a failure of primary development, or an arrest at very early stages of development. Because children have not

matured into their personalities as adults, children express the earlier symptoms of disorders differently. The symptoms most often observed are "hyperactivity, impulsiveness, distractibility, and shortened attention span"(McDermott, 1977), all of which have described astronomical proportions of children in various communities. To what degree these symptoms are actually attributable to developmental circumstances needs to be determined. Many of the developmental consequences of poor nutrition, or early cortical injury, are known to manifest themselves into a variety of disorders into adolescence and adulthood.

Valenstein (1998) has documented theories of circumstances that provide behavioral and cognitive patterns of psychiatric disorders. For example, "prenatal errors in brain development, birth trauma, incompatible immune systems between mother and fetus, slow acting viruses, and genetic factors" have been included in the etiology of mental disorders (Valenstein, 1998). The primary treatment intervention to date is a pharmacological approach to treating a class of diagnosed behavioral and cognitive disorders. Although many of these agents have proven very useful, rarely are some of the consequential effects of nutritional depletions addressed, as a result of a long-term ingestion of a various drugs. The drug effects in the body are known to interfere with nutrient metabolism as secondary malabsorption (Zeman, 1991). As a result, many psychological, or psychiatric disorders can become exacerbated as a result of poor nutritional status, especially inadequate long-term intakes. For example, depletions of various B vitamins due to either inadequate nutrition intakes and/ or using drugs that interfere with the normal metabolism of nutrients, result in numerous peripheral and central nervous system dysfunctions (Groper, Groff, & Hunt, 1995).

As an alternative to pharmacotherapeutic drugs that are currently on the market for treatment, an open-label study on professionally diagnosed bipolar adults using a vitamin and mineral supplement, EM Power +, has been documented. (Kaplan, Simpson, Ferre, Gorman, McMullen, & Crawford, 2001). These studies have inspired interest for the therapeutic application of the same vitamin and mineral supplement toward the treatment for early cortical injuries that can predispose individuals to similar behavioral and cognitive patterns found in many disorders.

Brain injury, and/or early cortical injury, not only affects anatomical structures of the brain, but also the neural circuits that have a specific purpose for the normal physiological functions of the brain. Damage very often has interfered with the learning abilities of the affected individuals. Some of the main effects from brain damage documented include interferences in attention and memory processes (Kolb & Whishaw, 2003). Either perceptual attention of the posterior brain regions, or the control of attention in the prefrontal regions are commonly known to suffer from the effects of brain injury (Kolb & Whishaw, 2003). Attention, in turn, provides the appropriate substrates for mnemonic processes and the ability to learn. A variety of disorders, such as bipolar disorder, attention deficit disorder, and associated comorbid disorders, usually share symptomatologies of deficits of attention (American Psychiatric Association, 1994). If the vitamin supplement treatment intervention can improve behavioral deficits of attention, learning, and memory, there are then reasons to suspect that it can provide the fundamental substrates necessary to support processes of neural plasticity that underlie such behavioral and cognitive improvements.

The goal of nutrition is to correct any nutritional deficiencies, prevent disease and disorders, and to promote maintenance of optimal health. The effects of a well-formulated vitamin and mineral supplement as treatment intervention for brain injury have not been extensively studied, if at all. Similarly, the nutritional requirements for brain injury have barely been addressed in research studies of neuroscience.

The goal of this thesis is to begin to address the potential effects of nutritional intervention as a therapeutic approach to reduce the effects of early cortical injury.

The first experiment used choline as a therapeutic intervention, which also provided a template for which to apply the supplement study, as this supplement has not yet been tested in rats. The second experiment in this study has applied the same vitamin supplement used in the clinical studies, at approximately the same dosages, to determine the potential benefit for treatment intervention. The behavioral tasks used are intended to measure various levels of recovery, or improvement, that can be offered from nutrition intervention. They also take an approach towards a better understanding of attentional processes that are necessary for learning and memory, cognitive deficits that are so commonly seen in people with various forms of brain damage.

Brain Plasticity

The experiences that an individual will endure throughout a lifetime are many and varied, and can be presumed to affect the brain, although likely in different ways. As a result, such various exposures have been proposed to sufficiently enable an individual to adapt to new changes in their environment. The ability to make appropriate behavioral changes in response to a changing environment is considered to be a form of learning,

and learning have been proposed to be accompanied by structural and chemical changes in the brain (Kolb, 1999). If an individual has at some time during their lives suffered from injury to the brain, the behavioral outcome generally changes over time, presumably in association with changes in brain structure and/or activity. These subsequent changes in the brain are known to depend on factors such as locus of lesion, age at injury, extent of injury, gonadal hormones, growth factors, pre- and postinjury experience, and so on (Kolb, 1995).

The idea that brain plasticity might vary with age at injury arose from observations of the effects of early childhood brain injury (Kolb, 1995). Broca noted that his young patients with damage to the language areas of the brain rarely developed aphasia later in life. This led Broca to suspect that there was something different about the effects of brain injury in the infant compared to effects seen in the adult. Kennard supported this hypothesis with her research of brain lesions in monkeys. Infant monkeys with motor cortex lesions showed improved behavioral recovery as opposed to similar injury in adulthood. The argument posed by Kennard (1942), namely that brain injury during childhood has a more favorable behavioral outcome than similar injury later in life, was presumed to be due to the fact that the brain is still developing during these early years. Responses to injury in the early years could be different, possibly by some form of reorganization of neural circuitry. It was therefore postulated that "earlier is better" for recovery from brain injury. In contrast to Kennard's views, Hebb (1949) later suggested that there may be certain periods during development of the cortex that would result in more deleterious effects than that produced by similar injury later in life. Hebb studied children with damage to the frontal lobes and noted that if this damage occurred at certain

periods during development, there were severe cognitive and behavioral consequences in adulthood and considered significant (Hebb, 1949).

To understand these cognitive and behavioral consequences of brain injury, Kolb and colleagues have performed extensive research in rats to determine critical periods for recovery and plasticity in the mammalian brain and to investigate the effects of various treatment interventions. Results of cortical plasticity after brain injury can be quite significant, quantified with measurements taken for changes in the axons, dendrites and glia (see Fig.1). From these data, Kolb and colleagues have constructed charts (see Fig.2) to identify growth periods during cortical development, as well as time dependent periods of maximal and minimal periods of cortical plasticity throughout the lifetime of a rat.

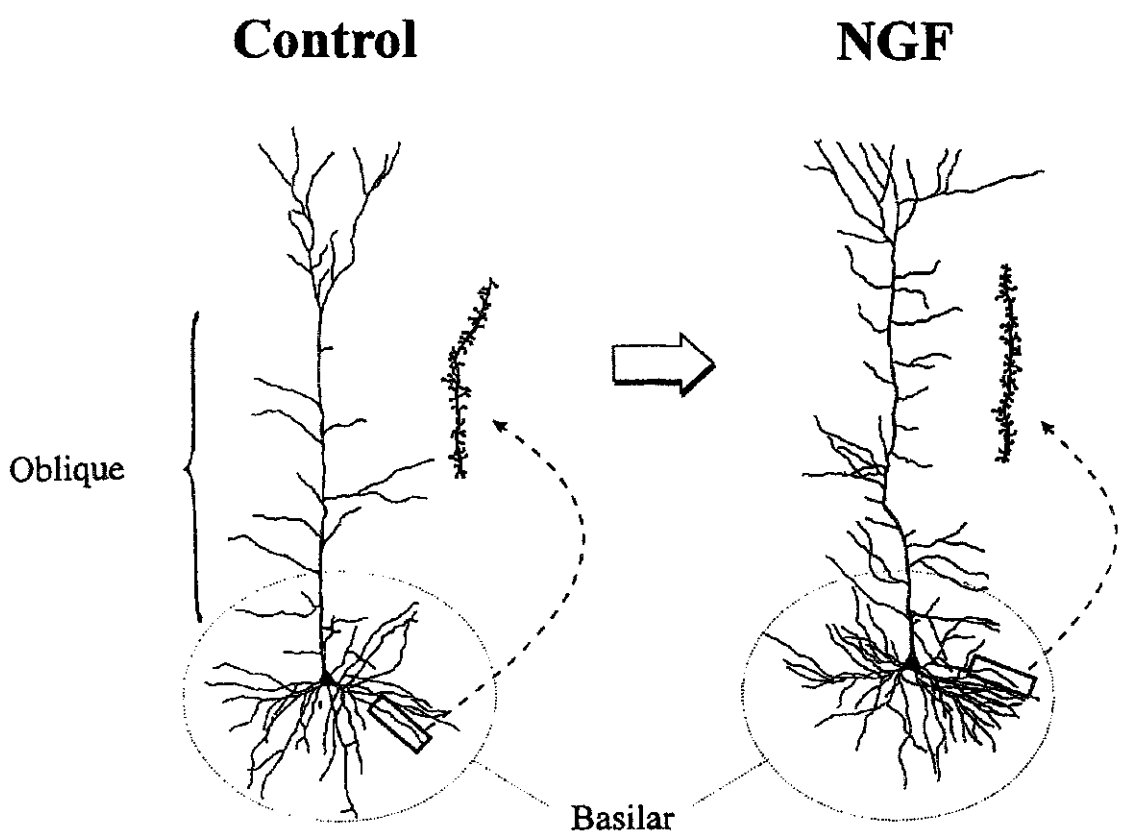


Fig. 1. Example of measures of dendrites, axons, and spines demonstrating neural growth. Illustration reveals the effect of the infusion of nerve growth factor into the lateral ventricle of adult rats. (Extracted from Kolb, 1999).

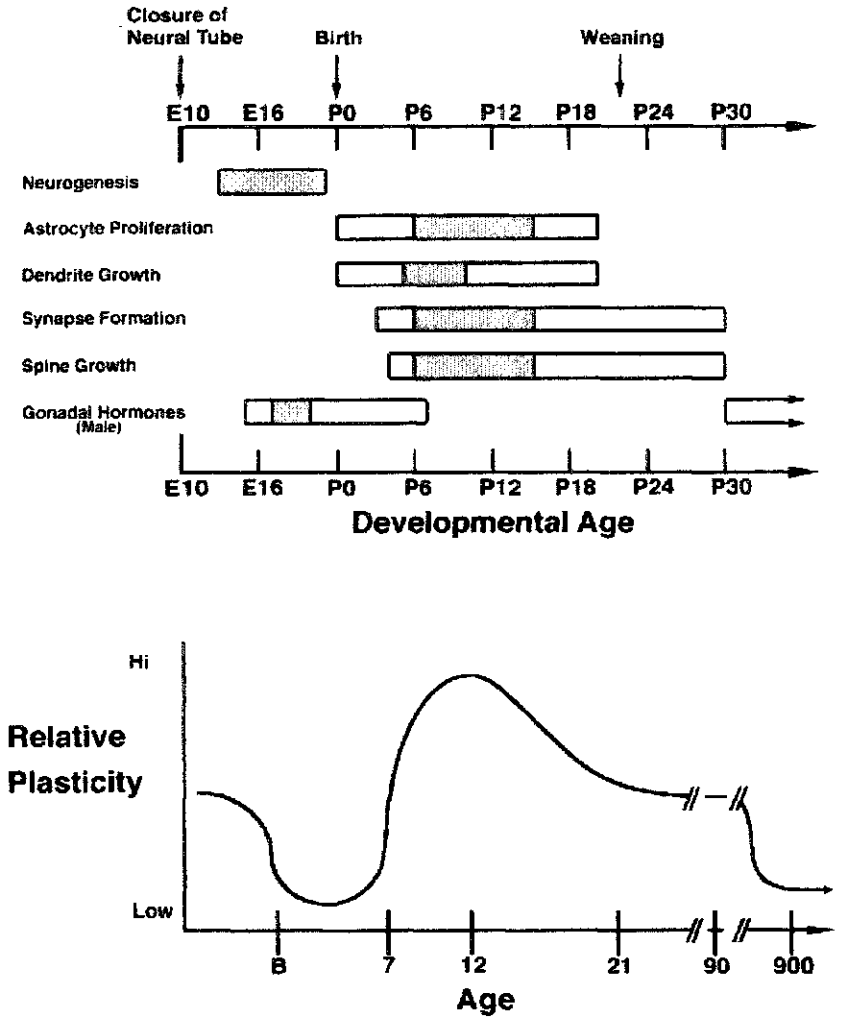


Fig. 2. Cortical plasticity of the developing rat brain. Top- approximate time period of the main cellular events. Bottom- Summary of the time dependent processes of plastic cortical periods during the life of the rat (Extracted from Kolb, 1999).

Development of the vertebrate central nervous system

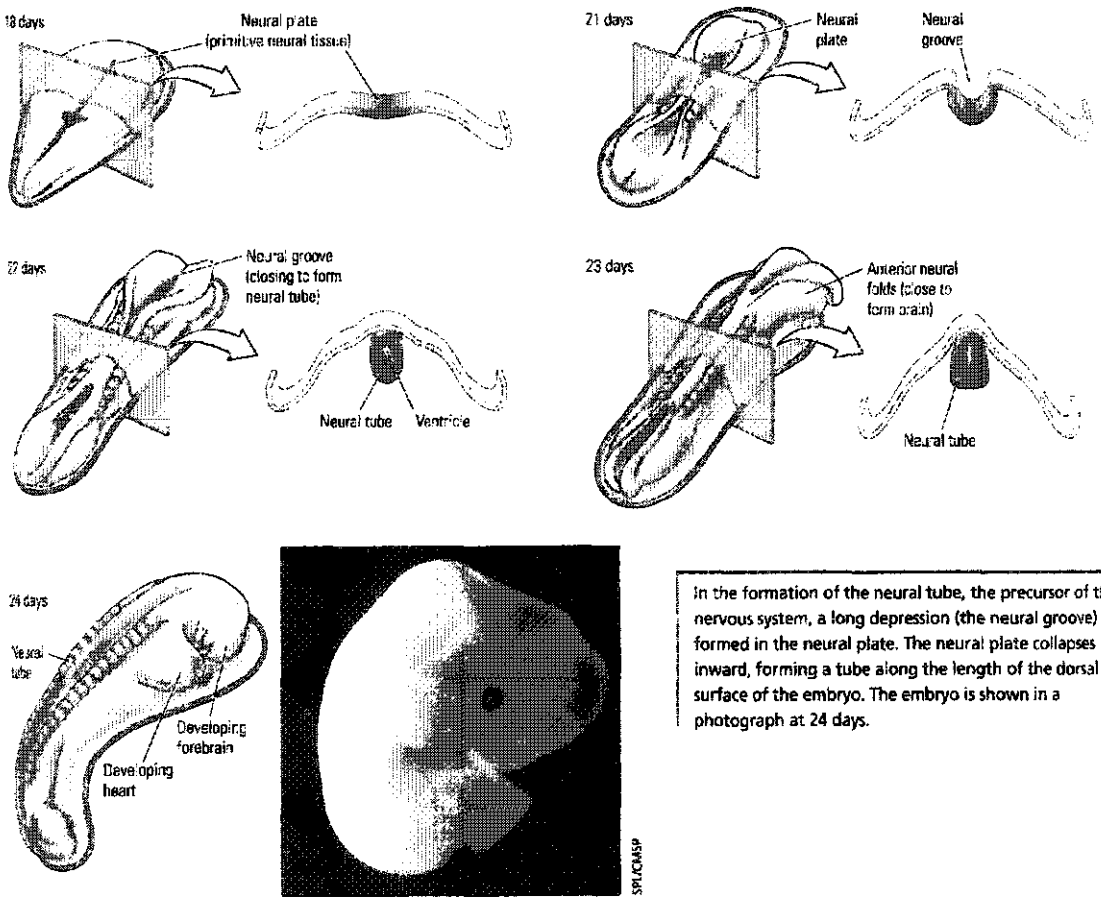
During early embryonic life, embryo cells differentiate into three germinal layers: 1) the ectoderm (skin, nails, brain, and central nervous system) 2) the mesoderm (skeletal

structures, voluntary muscle, heart, kidneys, gonads) and 3) the endoderm (digestive system, respiratory system and glandular organs) (Barr, S, 1998).

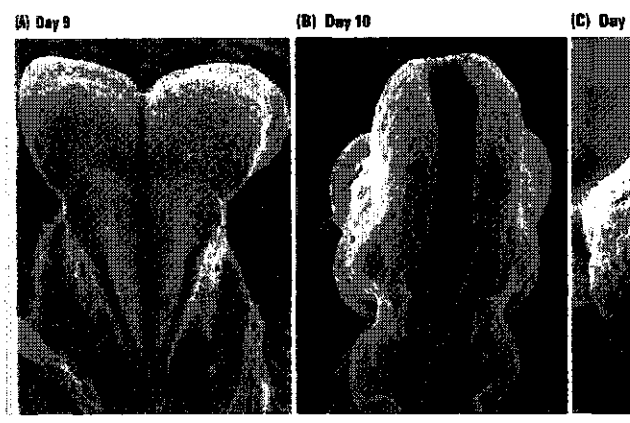
During development of the nervous system, the ectoderm differentiates into the neural plate, and further into the neural tube, neural crest, and ectodermal placodes (Steward, 1989). The neural plate folds into the neural tube, giving rise to the brain and spinal cord. The neural crest gives rise to the sympathetic and parasympathetic system, glial cells of the peripheral system, and some non-neural tissue, such as melanocytes. The rostral end of the neural tube develops three vesicular swellings that give rise to the forebrain, midbrain, and hindbrain of the central nervous system (see Fig.3).

During formation of the neural tube, the epithelial cells that line the wall of the tube become pseudostratified, becoming the germinal neuroepithelium, or the ventricular layer. The cells at the luminal surface of this pseudostratified cell layer are known as precursor, or progenitor cells (Reid & Walsh, 1996). During the earliest stages of cortical development, progenitor cells undergo processes of mitosis and extensive cellular proliferation, prior to migration during the formation of the cerebral cortex (Reid & Walsh, 1996; Steward, 1989).

Cortical cell lineage models of retroviral labeling and in vitro observations have suggested that some progenitor cells do not migrate widely into cortical regions, but undergo multiple rounds of cell division. Other progenitor cells however, are migratory and produce daughter cells of a multipotential migratory cell, and a non-migratory cell in stem-cell fashion (see Fig.4) (Reid & Walsh, 1996).



In the formation of the neural tube, the precursor of the nervous system, a long depression (the neural groove) is first formed in the neural plate. The neural plate collapses inward, forming a tube along the length of the dorsal surface of the embryo. The embryo is shown in a photograph at 24 days.



Scanning electron micrographs show the closing of the neural tube in a mouse embryo.

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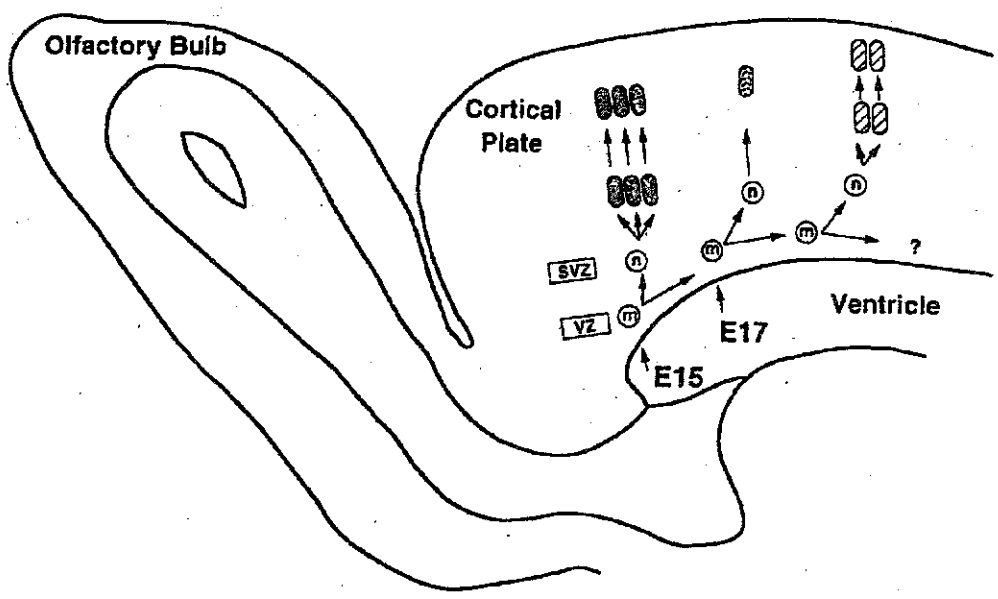
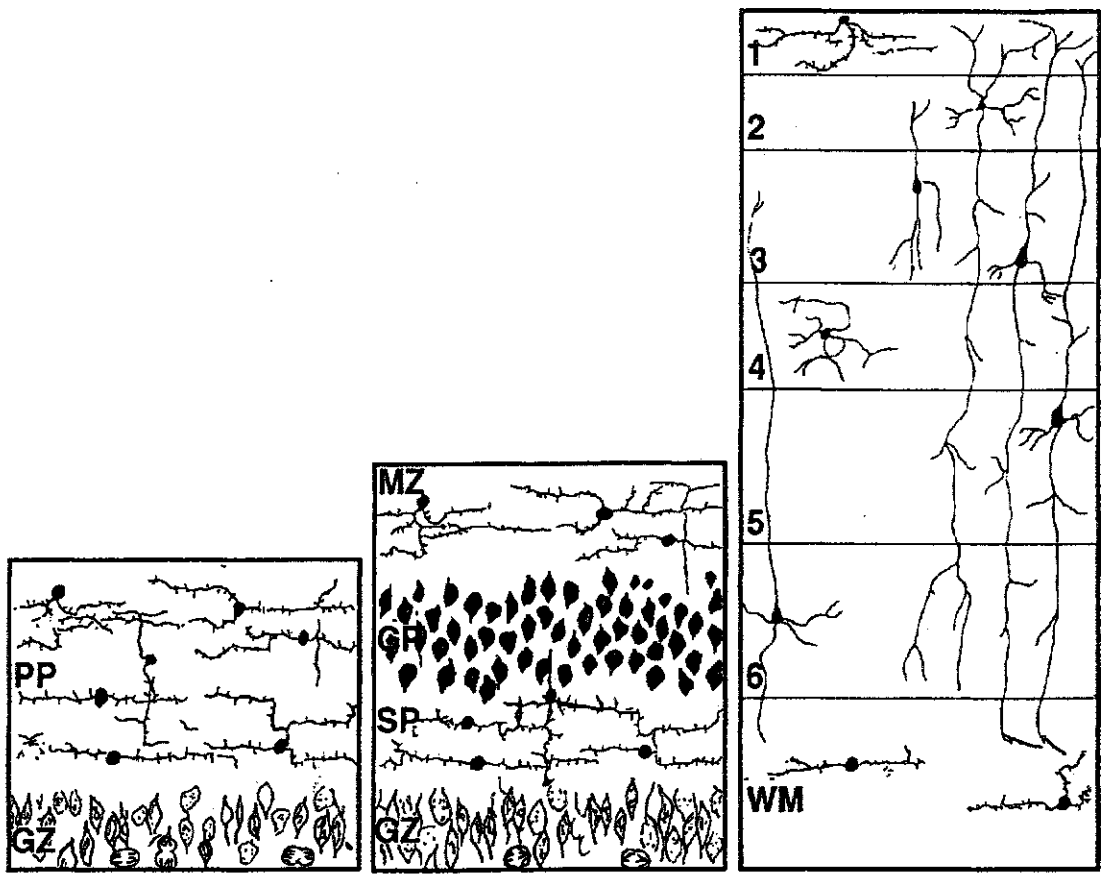
Fig. 3. Development of the central nervous system. (Extracted from Kolb and Whishaw, 2001. Introduction To Brain and Behavior).



A hypothetical model of cell lineage in the mammalian cerebral cortex is depicted along a schematically rendered saggital section through the E22 rat forebrain. Cerebral cortical cells are derived from the two proliferative tones (VZ, SVZ) and most neocortical neurons migrate radially from the proliferative zones to the cortex proper. The behavior of one multipotential cell is illustrated. The multipotential cell (m) migrates as it divides, sequentially producing three non-migratory progenitors at spatio-temporal intervals and regenerating a multipotential cell in "stem cell" fashion. Each non-migratory progenitor then behaves essentially independently, dividing multiple times (shading), directly differentiating (stippling) or dividing once (cross-hatching), to form three distinct cell clusters. Since cells in the rodent cortex are added in a roughly inside-out sequence, the oldest non-migratory progenitors would tend to form deeper neurons, and the newer non-migratory progenitors would tend to form more superficial neurons. Infection of the multipotential precursor at E15 would result, with equal probability, in integration of the viral DNA into the migratory or the non-migratory daughter integration into the non-migratory daughter would label a single clustered clone (or single cell), whereas integration into the migratory daughter would label a widespread clone consisting of several subunits. In contrast, infection at E17 would label a progenitor with fewer remaining cell divisions. Therefore widespread clones would be rare and small following E17 injections.

Fig. 4. Cell lineage model (Extracted from Reid & Walsh, 1996).

The formation of the cortex requires migrating cells to follow an inside-out pattern, while forming distinctive cortical layers. The most superficial layer of the cortex, the preplate is the first layer formed, which differentiates into the subplate and marginal zone. New migratory cells of neurons and glia then form a cortical plate and progressively follow an inside out pattern forming layer six, then five, and so on, until layer two is complete (see Fig.5)(Reid & Walsh, 1996).



A summary of cerebral cortical development represented schematically by drawings of cortical cross-sections at very early (left), intermediate (center) and late stages of development (right). These drawings correspond roughly to the E12 (left), E16 (center), and adult rat (right) sections. The cerebral cortex arises from progenitor cells within the germinal zone (GZ) lining the lateral ventricles. Early in neurogenesis these cells divide to form the preplate (PP) cells. These early formed neurons eventually differentiate to become subplate neurons and the Cajal-Retzius neurons of layer I, also known as the marginal zone (MZ). Over the course of development, other neurons arising from the germinal layer migrate to a position within the preplate, eventually splitting the preplate into layer I (I) superficially and the subplate (SP) subjacently. The intervening neurons arrive to form the cortical plate (CP) in an inside-out fashion. Layer six (6) neurons arrive first, then layer five (5) and so on.

Fig. 5. Summary of cerebral cortical development (Extracted from Reid & Walsh, 1996).

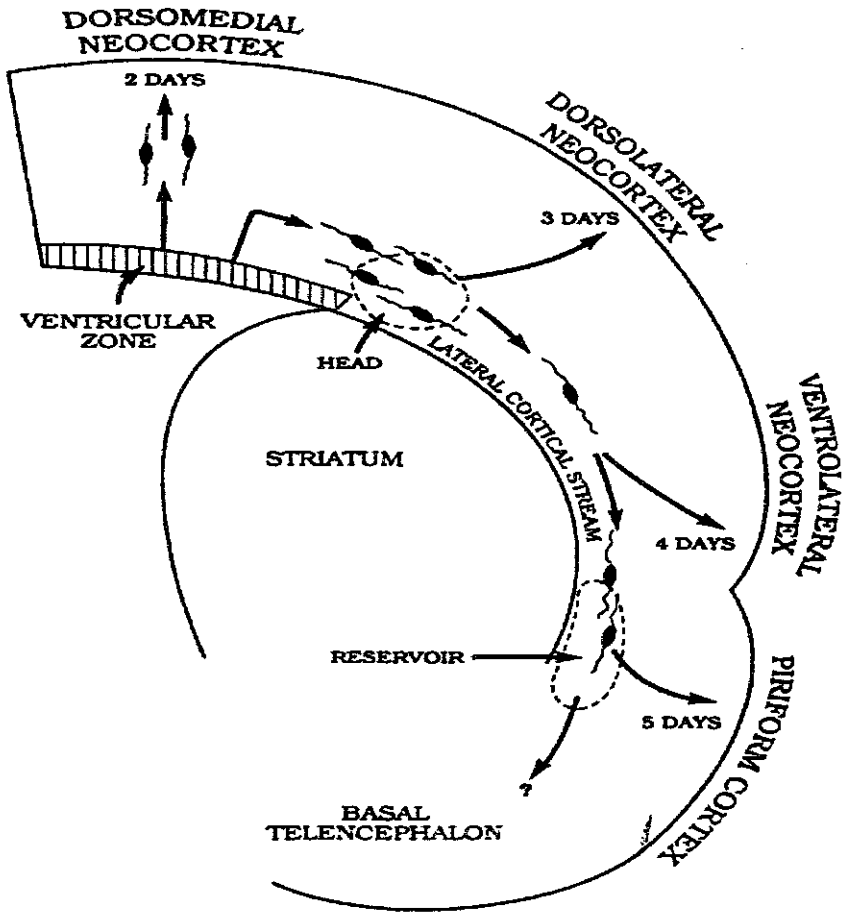


Fig. 6. Lateral migration (Extracted from Bayer & Altman, 1995).

Three-dimensional reconstructions and labeling patterns of the newly generating neocortex indicate that young neurons and glia migrate along radial glia, or co-migrate with glia radially, to reach their destinations. Depending on the distance required for migration and the placement of selective neurons, some neurons are also considered to migrate laterally to reach their destinations (see Fig.6)(Bayer & Altman, 1995).

Most of the newly formed cortical neurons in rats are generated between embryonic days fourteen and twenty (Bayer & Altman, 1995). The Cajal-Retzius neurons

of layer I, originate mainly on embryonic day fourteen, followed by the subplate neurons, originating on embryonic days fourteen and fifteen. Bayer and Altman (1995) consider this time frame of neurogenesis the first epoch. The neurons that form layers VI and V are generated in the second epoch and the final layers, IV to II are generated in the third epoch (Bayer & Altman, 1995).

Critical periods for recovery from brain injury during development

Cerebral neurogenesis in the rat begins on embryonic day twelve and continues until the day of birth, on embryonic day twenty-one. The period of neurogenesis has been designated as the first critical period of development. From the first day of birth to postnatal day six or seven, is a period of neural migration. Astrocyte proliferation occurs through this time period and peaks from around day fifteen to day thirty, depending upon the cortical region examined. This second peak in activity can be considered the second critical period of development and time at which the cerebrum is the most plastic. Shortly after the peak of synapse formation at around day twenty synapse pruning and programmed cell death (apoptosis) begins and stabilized neurons begin to mature.

If the rat cortex is injured during neurogenesis at around embryonic day 18, complete functional recovery is observed in behavioral test results (Kolb et al, 1998). Regrowth of cortical tissue is apparent, although not original in shape. Injuries during the period of intense neural migration, roughly postnatal days one to six, generally results in dendritic atrophy, decreases in brain weight, and poor functional outcome. This period of early life is the least plastic time period for cortical development.

Damage to the brain during postnatal days seven to twelve occurs during a time of astrocyte proliferation, and dendrite and synapse growth. This period is more favorable for recovery of cortical lesions by growth in dendrites and spines in existing neurons, the generation of new neurons at least under some circumstances, and the recovery of function behaviorally.

Injury during adulthood, around postnatal days one-hundred twenty, an initial decrease in dendritic growth is found, but thereafter increases slightly, with a partial recovery of function (Kolb, 1999).

These age-related effects of cortical injury can be seen after lesions across the cerebral cortex with similar effects after lesions of the prefrontal cortex, motor cortex, occipital cortex, temporal cortex, and the posterior parietal cortex of the rat (Kolb, 1995).

Some of the principal mechanisms that influence plasticity appear to be especially active at certain times during development. By enhancing these mechanisms after brain injury it should be possible to make some improvements for recovery processes. Treatment interventions for recovery of function might include pharmacological agents, nutritional support, environmental enrichment or a direct application of growth factors.

Glial cells likely play an important role in supporting brain plasticity after injury. Nutritional support comes from glial cells, which form "end feet" that are attached to blood capillaries, and presumed to derive nutrients through the blood stream. Nutrients that are necessitated by neurons at any given time can then be transferred. Similarly, neuromodulators and nutrients in excess after neural transmission can be absorbed by astrocytes. Astrocytes accompany neurons to provide both structural and nutritional support and express receptors for chemicals, neurotransmitters, and neuromodulators, as

well as produce and respond to trophic factors (Kolb, 1995). Astrocytes are also known to store glycogen and fatty acids to provide the energy sources for metabolic processes of both neuronal and glial cells that are required to operate normal functions (Rouach & Giaume, 2001). The presynaptic influx of calcium, necessary to generate neural transmission, is a response of astrocytes to the extracellular environment produced from the depolarization of neurons. Astrocytes communicate with each other, thereby increasing the neural signals across neighboring astocytes into long-range signaling pathways. These mechanisms increase the modulating capacity of glial cells, which inadvertently result in plastic influences with neural cells (Rouach & Giaume, 2001).

All of these factors are known to increase activity in neural cells, which increases the activity of glial cells and trophic factor production, which in turn, increases the metabolic processes necessary for neurotransmitter synthesis in neural cells. An increase in the number of glial cells generally signifies an increased requirement for neuronal support and therefore, increases in neural activity.

The frontal cortex: Functions in humans and in rats

One of the simplest ways to describe the functions of the frontal lobe is probably as the anterior region of the brain that temporally integrates neuronal input from all regions of the brain, records it into memory, organizes it, and prepares the individual for the execution of a behavioral response. The frontal lobe as a whole consists of the prefrontal cortex, the premotor cortex, and the primary motor cortex. The prefrontal cortex in humans is considered the highest intellect of the mammalian species. The

complexity in connectivity and function gives this area of the brain a recognized characteristic of executive function, which implies that all complicated decisions regarding appropriate behavioral outcome, are assessed in the prefrontal cortex. Another section of the prefrontal cortex, the orbital cortex, is dominant for social awareness and behavior. The premotor cortex prepares the motor cortex for action, which is contingent on the decision-making processes from the prefrontal cortex, during complex and novel experiences.

Injury to the motor cortex in humans is known to cause difficulties in fine finger movements and losses in speed and strength (Kolb & Whishaw, 2003). The premotor cortex provides adequate preparation of voluntary motor movements. It does this as it simultaneously receives and sends signals to the parietal and temporal association areas. An injury to this area of the brain often results in disturbances of voluntary eye gaze, and speech, as well as disturbances with messages to the posterior areas to accommodate for planned movements (Kolb & Whishaw, 2003). Injuries to the orbital frontal region have revealed impulsive, disinhibited behaviors as a result of poor response inhibition, some of which develop into social and sexual disorders (Kolb & Whishaw, 2001; Cummings, 1995). The orbital frontal cortex has also been proposed to contribute to the attention processes by filtering out unnecessary information before entering processes of executive function (Fuster, 2002; Cummings, 1995).

The ventral areas of the prefrontal cortex, as part of a frontoparietal network, have revealed activity during in vivo neuroimaging, when subjects were required to redirect, or shift, their attention to locations of salient, or unexpected stimuli (Corbetta & Shulman, 2002). This area has reciprocal connections to the temporal-parietal junction of the

posterior cortex and is called the ventral frontoparietal network. The parietal connection enables the ventral frontal areas to make spatial shifts of attention. Although the ventral cortical areas are not specialized to focus attention spatially, it is considered to share this feature with the dorsal regions of the frontal cortex.

Prefrontal cortex injuries to the dorsolateral region are known to cause executive dysfunction. Because this set of cognitive functions is very complex, impairments result in an array of behavioral deficits, namely, large deficits in the supervision of attentional control (Fuster, 2002).

The human dorsolateral prefrontal cortex selects and recruits sensory information from the posterior regions of the brain, to integrate and process this information sufficiently. Attention is required to select the appropriate, or relevant, features of a task, which uses working memory to keep information "on line". These processes are necessary for an individual to focus their attention. During working memory processes, the prefrontal cortex can organize and manipulate information to prepare for an appropriate behavioral response according to the task requirements. Impairments with these attention processes generally result in a disorganized plan and inappropriate preparation of actions. In addition, these processes all require monitoring of ongoing cognitive processes and activities to continue producing appropriate behavioral activity and filter out any inappropriate behavioral urges (Fuster, 2002). Fuster's model of executive function operate within long-term memory processes, he calls executive memory. Previously performed actions necessitate past memories for reference, which would progress as an updated version with the present action, and returned back into

long-term memory. Newly acquired behavior would need to undergo processes of rehearsal using working memory.

Cognitive and behavioral inflexibility is common in prefrontal cortex injuries and revealed by individuals having difficulty in shifting attention set. Inflexibility can be seen when an individual has achieved an attention set with a particular rule for a task, but then is unable to shift to another rule. For example, some people who are accustomed to cooking a meal one particular way, with the same seasonings and methods, struggle to try a new recipe with new flavor combinations. They may want to try a new recipe and understand what is required to go through with a new plan of cooking, but cannot shift to a new method. Rules can also be considered as plans necessary to adapt to a new desired behavior.

Injury to the frontal cortex in rats indicates some similarities to that of humans (Kolb, 1984). Brain lesions including the motor cortex impair the serial ordering of digit and forelimb movements and the execution of a chain of voluntary movements. Effects of damage can also be seen in tasks requiring strength or speed.

Injury of the orbital regions, or the ventral portions of the rat prefrontal cortex, produces abnormal social interactions, hyperactivity, and an inability to initiate spontaneous behaviors, such as in generating new strategies for task solutions. Defective odor discriminations are also characteristic of orbital frontal lesions, and Kolb (2003) recently proposed that orbital lesions interfere with processes that integrate olfactory information into working memory. Maintaining stimuli "on line" is necessary to make the appropriate associations among stimuli and to attach the appropriate meanings to stimuli. This type of deficit could provide numerous complications for rats, as olfaction is one of

their primary sensory modalities necessary for orientation within their environment and among peers (Kolb, 1984). Lesions to the orbital frontal cortex in rats have also indicated impairments in delayed response of low reinforcement tasks that measures bar-pressing extinction (Kolb, Nonneman, & Singh, 1974). Deficits in extinction behaviors have been considered as impairments in shifting response strategies. The rat is required to extinguish a previously rewarded response that was no longer rewarded on subsequent trials. Spatial reversal tasks also indicate some problems for rats with orbital lesions, but the impairment is actually one of perseverative tendencies to the previously rewarded arm, not one of solving spatial problems. Brain lesions of the medial frontal regions of the prefrontal cortex do reveal spatial problem solving deficits and delayed-response type solving tasks, but not in bar-pressing extinction tasks (Kolb, Nonneman, & Singh, 1974).

The serial ordering, or temporal integration, of movements has been determined to be a function of the medial frontal cortical areas in the rat (Kolb, 1984). Measures associated with the serial organization of behavior have been determined in delay-response type tasks that determine capabilities in working memory, or in spatial aspects of that task (Kolb, 1984).

In humans, these processes are considered as executive functions, housed in the dorsolateral areas of the prefrontal cortex. In consideration of the possibility that rats might possess executive functions, many researchers have devised various experiments in attempts to isolate certain functions with particular regions of the prefrontal cortex. Kolb (1984) has defined impairments in behavior of temporal ordering of movements, response inhibition, spatial processing, and habituation as a result of prefrontal cortical damage.

Although these impairments have not been labeled as "executive dysfunction", the resulting behavior may be comparable.

Fuster's (2002) model of executive function, or supervision of attention, includes four contingencies of known prefrontal cortex functions: 1) attention, 2) working memory, 3) prepatory set, and 4) monitoring. These processes however do not function in a linear form from the first to the last process, but rather operate in an overlapping manner (Fuster, 2002). To adequately respond to a stimulus, one must first be able to attend to the relevant aspects of that stimulus, while disregarding the irrelevant aspects of the stimulus. During this time, working memory would be required to maintain "online" information to determine necessary preparation for behavioral responses. Monitoring records the feedback of information held online in working memory for prepatory set of the next behavioral response. Attention throughout these overlapping contingencies determines appropriate encoding of information and planning of responses.

Researchers who have attempted to delineate the separate effects of executive processes in rats have isolated certain aspects of these functions through brain lesion experiments. A "supervisory attention system" residing in the prefrontal cortex of rats has been proposed by Delatour and Gisuet-Verrier (2000). Attention, behavioral flexibility or attention shift, spatial attention and working memory were examined. By using chemically induced excitotoxic lesions of the prelimbic-infralimbic region, they were able to determine behavioral deficits of these executive functions defined by attention and working memory abilities. One paradigm tested for flexibility was navigation on a circular arena. Starting positions changed with each trial to assess the ability of rats to shift with each new starting position (Delatour & Gisuet-Verrier, 2000). Birrell and

Brown (2000) maintain that executive function of attention shift does exist in the rat, and resides in the prelimbic-infralimbic region of the prefrontal cortex. This was demonstrated using excitotoxic lesions and a behavioral task that did not require spatial shifts of attention, but was intended to measure only a shift of attention, using inhibition of previous responses. (Birrell and Brown, 2000). Behavioral tasks measuring spatial attention and working memory have been indicated as part of the prelimbic functions, as determined by Fritts et al (1998). Fritts, performed studies of cortical lesions from surgical removal of the medial frontal cortical region, some or which included the prelimbic areas, some did not. The results of this experiment suggests that the anterior cingulate, infralimbic and medial precentral (premotor) areas are required for attention with working memory, and temporal sequencing of information in rats (Fritts, Asbury, Horton & Isaac, 1998).

Table1. Summary of effects of medial frontal and orbital frontal lesions in rats

	Behavioral Impairment	Basic Reference
Medial Frontal: (including motor)	Serial ordering of digit and forelimb movements, Excecution of a chain of voluntary movements, Decreases in strength and speed.	Kolb et al., 1984
Medial Frontal	Spatial problem solving, Delay-response type solving tasks, Working Memory, Habituation.	Kolb, Noneman & Singh, 1974
	Spatial attention, Attention shift, or behavioral flexibility.	Delatour & Gisett-Verrier, 2000
	Non-spatial attention shift.	Birrell & Brown, 2000
	Attention with working memory, Temporal sequencing of information.	Fritts, Ashbury, Horton & Isaac, 1998
Orbital Frontal	Social interaction, Hyperactivity, Response inhibition, Inability to initiate spontaneous behavior, Defective odor discriminations,	Kolb, 2003
	Extinction, Perseveration.	Kolb, Noneman & Singh, 1974

Functions of the parietal cortex in humans and in rats

The primary function of the posterior parietal cortex is to provide sensory guidance of movements in space. The dorsal stream of visual information arrives to the parietal cortex directly from the occipital cortex. This information is known as the "where" stream of information. The ventral stream of visual information from the occipital cortex flows to the temporal lobes and provides the "what" stream of information. Reciprocal connections between the parietal and temporal lobes enable the individual to make associations of objects, or landmarks, in space with other objects or landmarks.

The visual-spatial information of the posterior parietal lobe is that of extrapersonal cues, or landmarks, which enables the individual to monitor its place in space. It is believed by some that this lobe provides the individual with a "cognitive map" of representations of their environment, as was originally suggested by O'keefe and Nadel (1978). Recent research, however, suggests that the parietal lobes trace a series of representations (Kolb & Whishaw, 2003). These representations must be continually updated in memory, according to the changes within the environment as the individual moves from one location to another. A mental map would be formed to trace the previous path, but the individual would also need to consider what it is approaching. The prefrontal cortex monitors these cognitive processes, holds them in working memory, while new information is presented and kept on line to make a trace of spatial events. Both the posterior parietal and prefrontal cortexes also have reciprocal connections with the hippocampus in the medial temporal lobe, which plays an important role in the generation of certain forms of long-term memory.

Another approach to understanding spatial behavior is the accuracy of hand-eye coordination of movements within the proximal area of the individual. The parietal lobes are responsible for visually guided movements, which also require a continual update of behavioral actions just performed. The premotor cortex receives this information and prepares for the next behavioral action. This information is again projected to the posterior parietal cortex forming a re-entry loop.

Injuries to the parietal lobes in humans and laboratory animals can seriously interfere with the normal updating processes necessary to maintain accurate representations in space. Whether these representations are of distal cues and landmarks, or of proximal movements of the individual, there is often a disorientation of extrapersonal space.

An extraordinary example seen in humans is the experience of contralateral neglect with injury to the posterior parietal regions of the brain, particularly if the damaged area is in the right hemisphere. The resulting behavior indicates an inability to acknowledge the information presented to the visual field opposite the side of the brain lesion location. This tends to interrupt the formulation of associations with distal cues within their environment, but also of movements within proximal areas of the body. Many of these patients do not realize that they have the other side of their body to dress, or the other side of their plate of food to eat and so on (Kolb, & Whishaw, 2003).

A number of researchers have proposed possibilities for the underlying deficits of behavior resulting from brain damage to the posterior parietal cortex. One primary deficit proposed is an inability to selectively attend to the relevant stimuli required to maintain

accurate representations and associations of environmental cues. The end result could be a confusion of appropriate and inappropriate representations into memory.

Imaging of brain activity has revealed heightened activity in the parietal cortex during shifts of attention and has been implicated as a region for spatial attention control, as well as to maintain the current state of attention (Yantis et al., 2002). The extrastriate areas of the adjacent visual cortex are also proposed to be involved in attention-related changes by mediating the type of information that is communicated with the motor systems of the frontal lobe. During shifts of attention required to perform changes in eye movements, or arm reaches, the posterior regions of the brain perform the necessary shifts of attention (Yantis et al., 2002).

The temporal-parietal junction is reciprocally connected to the ventral areas in the prefrontal cortex (Corbetta & Shulman, 2002). Activity in these areas is also heightened during periods of orienting attention towards oddball or infrequent stimuli that are distractors from relevant stimuli. If the distracting stimuli have similarities to the relevant stimuli, such as color, attention would transiently shift toward peripheral distractors, resulting in loss of attention at the relevant spatial location. For example, if you were looking for a friend wearing a red hat in a crowd of people, anyone wearing red would activate your attention. The activation, in this case, would reside in the temporal-parietal junction. It is suggested that this cortical area is differentially engaged by task-relevant stimuli and determines the their behavioral valence (Corbetta & Shulman, 2002).

To determine the possibility that rats may have some similarities in behavioral deficits from posterior parietal lesions, to those experienced by humans, a region of similar characteristics in the posterior cortex needed to be identified (Kolb, & Walkey,

1987). Kolb and Walkey used tracer techniques to identify areas of the posterior cortex that would indicate the same characteristic connectivity patterns of the parietal cortex to that of primates. Kreig's area 7 was the area identified that closely resembles the primate posterior parietal cortex. Injections of tracer into area 7 indicated staining in the border areas of the precentral (premotor) and anterior cingulate. Similarly, injections into the frontal regions of the precentral and anterior cingulate produced staining in Kreig's area 7. Counterstaining with another tracer revealed an overlap of neural connections in both of these regions, indicating reciprocal connectivity between the posterior parietal areas with the anterior cingulate and precentral (premotor) areas of the frontal cortex in the rat. The behavioral results of cortical lesions in Kreig's area 7 included tactile sensory impairments on a tactile discrimination task that required rats to chose the rewarded alley according to tactile textures. Impairments were also found in the Morris water task where rats were required to navigate their way to a hidden platform using distal cues. Their impairments in this task were of a poor heading angle, or trajectory, to find the platform after each new starting point.

Some interpretations have extended poor initial trajectory as an impairment in the ability to use the extrapersonal cues as indicators necessary to orient the head and body in the correct direction before initiating a motor response (Ward, & Brown, 1997). To determine whether deficits are attentional in nature, Ward and Brown used a nine-hole apparatus, employing a covert orienting task, with rats tested before and after surgery to produce unilateral lesions in the posterior parietal cortex. There was no evidence for asymmetry of responses, but the incidence of incorrect responses toward invalid cues was higher. Ward and Brown concluded that using unilateral lesions of the posterior parietal

cortex do not reveal impairments in covert orienting, or in signal detection, or vigilance (Ward & Brown, 1997). The impairment in the attentional task of covert orienting was considered one of response impairment, which suggests impulsive responses if inhibition to respond to irrelevant cues was impaired.

The neural circuit considered responsible for directing spatial attention in rodents thus involves the medial agranular insular cortex (Zilles AI), the ventrolateral orbital frontal cortex, and the posterior parietal cortex (Ward, & Brown, 1997).

Disturbances in orientation that are necessary for maze solving in rats may also be due, in part, to impairments in the activity of head-direction cells. These cells have been proposed to aid the rat in maintaining awareness of direction according to a series of body orientations; therefore, if damage should include these cells, impairments in an innate form of orientation would ensue (Golob, Stackman, Wong, & Taube, 2001).

Table 2. Summary of the effects of posterior parietal lesions in rats.

Behavioral Impairment	Basic Reference
Tactile sensory	Kolb & Walkey 1987
Spatial navigation	Kolb & Walkey 1987
Poor initial trajectory	Ward, Brown, 1997
Spatial attention	
Head-direction cells for orientation	Golob, Stackman, Wong, & Taube, 2001

Choline Treatment

Choline is an example of a vital amine. "Vital amine" was a term to describe organic compounds necessary in small quantities for normal health, coined by Casmuir Funk in 1912 (Blusztajn, 1998; Goff, Gropper, & Hunt, 1995). Although choline is synthesized naturally in the human body, this amount is too small to sustain optimal health and requires supplementation from sources of food. Choline has important functions in metabolic processes due to the interrelationship it has with methionine, folate, vitamin B_6 (pyridoxine) and vitamin B_{12} (cyanocobalamin). It is essential for lipid-cholesterol transport and metabolism in the liver, necessary for the synthesis of phospholipids in cell membranes and neural glia. Choline is also a precursor for two signaling lipids, platelet activating factor and sphingophosphorylcholine, as well as for acetylcholine, an excitatory neurotransmitter in the brain (Zeisel, 2000; Blusztajn, 1998).

The Institute of Medicine recently has designated choline as an essential nutrient for humans and for animals (Zeisel, 2000; Blusztajn, 1998). Its essentiality as a nutrient has been determined from cases of choline deficient diets, which lead to liver dysfunction, growth retardation, renal dysfunction, and bone abnormalities in baboons. As a result, choline is generally added to animal feeds. In humans, liver damage has been reported in those receiving total parenteral nutrition devoid of choline. These effects are reversible with the addition of choline to the formulation. Dietary deficiency of choline in rats has revealed the development of hepatocarcinomas in the absence of any known carcinogen (Zeisel, 2000).

Female rats have been noted to be less sensitive to choline deficiency than males, potentially due to the effects of estrogen with choline synthesis. Pregnant and lactating

female rats however, are considered as sensitive to deficiency as males, because large amounts of choline are delivered to the fetus via the placenta. These results could imply that choline is essential during critical periods of development. Studies in women, children and infants have not been carried out to determine recommended daily intakes for choline (Zeisel, 2000).

Dietary choline administered prenatally and postnatally has revealed lifelong increases in attention, learning, and memory in rats (Meck, Smith, & Williams, 1988). An extension of this research found cognitive enhancements of working memory and reference memory to be correlated with neurochemical measures of brain activity. These results were found in both the frontal lobe and hippocampus of male rats tested in adulthood.

Developmental factors are proposed to produce this enhanced effect of learning and memory into adulthood (Albright, Freidrich, Brown, Mar, & Zeisel, 1999). Changes in the timing of mitosis, differentiation, migration, and apoptosis as a result of maternal choline availability have been determined in the rat hippocampus and septum. Albright and colleagues suggests that interferences in the natural timing between stages of neural development could influence neural patterns of organization (Albright, Freidrich, Brown, Mar, & Zeisel, 1999). During the prenatal and postnatal periods of brain development, choline is metabolized in the maternal liver into phosphorylcholine and betain. These are the active forms of choline during periods of fetal brain development until migrating neurons reach their target cells and mature into cholinergic neurons (Garner, Mar, & Zeisel, 1995). According to Garner et al., choline in the form of phophorylcholine is the

essential messenger that can induce DNA synthesis, thereby increasing the rate of cell division.

The effectiveness of dietary choline as a precursor for acetylcholine comes from the advantageous endogenous relationship that cholinergic neurons have with nerve growth factor (NGF). By increasing the plasma choline and the subsequent storage of choline as phosphatidylcholine in mature cholinergic neurons, neurons would have an available supply for acetylcholine synthesis, which increases nerve growth factor synthesis. Although NGF is largely committed to cholinergic neurons it may also enhance trophic activity for other neurons.

The dietary and metabolic characteristics during the pre- and postnatal period of development that give long-lasting effects in learning and memory indicate that dietary choline could provide some benefit for the treatment of early cortical injury.

Vitamin and mineral supplement treatment

The discovery of vitamins came about in the early years of the twentieth century Researchers who became interested in diet composition realized that nutrients from food consist of more than fat, carbohydrate, protein, minerals and water (Goff, Gropper, & Hunt, 1995).

The minerals identified as required for maintenance of normal health are numerous with various functions and are classified as macrominerals and microminerals (Goff, Gropper, & Hunt, 1995). The discovery of minerals began as knowledge of body fluid and body tissue composition, which eventually became more elaborate with the improvement of assay techniques. There is no concrete definition for either

macrominerals or microminerals. The two classifications can be distinguished by their occurrence in the normal healthy body, as a normal amount necessary to sustain health. Definitions used for macrominerals are: 1) present in a constitution of .01 % total body weight, or 2) present in a minimum quantity of 5 grams for 60 kilograms of body weight. A less ambiguous suggestion is a requirement of amounts greater than 100milligrams daily. Trace minerals are generally defined as a requirement of less than 100milligrams daily (Goff, Gropper, & Hunt, 1995).

Vitamins and minerals are also a necessary dietary requirement for plants and animals (Morris, 1991). There is apparently a high degree of uniformity among all living organisms in their need of dietary essential minerals, although the necessary quantities vary between species. Morris (1991) has discovered the mineral requirement for most animals to be approximately five percent of dry weight food intakes. Vitamins, as well as essential fatty acids, are also nutrient requirements for animals with suggested intakes of approximately one percent and one to two percent dry matter, respectively (Morris, 1991).

The metabolic characteristics of vitamins and minerals and their effects on physiological function are well known for peripheral systems of the body. Their effects in the central nervous system, however, are not as well documented and many deficiency studies have been restricted to animal studies (Dreyfus, 1988; Goff, Gropper, & Hunt, 1995).

What is known and pertinent to the study of nutrition in the central nervous system, is that vitamin deficiencies generally result in bilateral "biochemical lesions", which affect certain cell populations as well as the neural connections associated with

them. The characteristic clinical signs of many deficiencies, such as dry hair, scaly skin, and brittle nails, are the late stages following already present neurological changes (Dreyfus, 1988; Goff, Gropper, & Hunt, 1995).

A few of the known neurological dysfunctions caused by vitamin deficiencies have been attributed largely to deficiencies of the B vitamins (Dreyfus, 1988). Human studies of vitamin B_1, or thiamin, deficiencies have been numerous since its discovery. Beriberi results in polyneuropathy in the peripheral nervous system, characterized by progressive weakness and muscle wasting. Wernicke's encephalopathy has many characteristics of mental symptoms, such as global confusion, profound disorientation, inattentiveness and decreases in spontaneous speech. Korsakoff's psychosis is a known syndrome from excessive intakes of alcohol, characterized as an amnesic-confabulatory syndrome with impaired perceptual and conceptual mental abilities.

Deficiencies of vitamin B_6 (pyridoxine), has been reported to result in peripheral neuropathy in humans. In the human neonate, acute deprivation of vitamin B_6 is known to lower the seizure threshold of the immature brain. Pregnant rats fed a vitamin B_6 deficient diet resulted in a delayed or reduced rate of myelination along with significant structural alterations in the brains of the offspring rats.

Pernicious anemia has been commonly recognized in vitamin B_{12} (cyanocobalamin) deficient humans. These signs of deficiency are generally treated before observations of neurological abnormalities become apparent. A severe deficiency of vitamin B_{12} has been noted as degeneration of cerebral white matter, optic nerves, the spinal cord and of the peripheral nerves.

Vitamin B3 (niacin) deficiencies have caused Pellagra, an encephalopathy that is associated with mental symptoms. Symptoms are also seen in peripheral nerves and spinal cord. Deficient humans and animals administered niacin have shown increases in nucleotide synthesis and increases of norepinephrine and dopamine tissue concentrations to well above normal (Dreyfus, 1988).

Folic acid is probably the best known B vitamin that has deleterious effects if deficient during pregnancy. Folic acid is essential to complete the formation of the neural tube during development. Deficiency during pregnancy is known to cause spina bifida, or simply, neural tube defects.

The neurological deficits mentioned as a result of vitamin deficiencies are only a few examples of the importance of vitamins and minerals for optimal physical, emotional, and mental health.

Prenatal protein malnutrition, during neurogenesis, has been noted to interfere with the normal proliferation of cells and the normal subsequent enlargement of brain cells (Lewis, 1990). Among a number of deleterious effects of malnutrition postnatally, during late neurogenesis of the dentate gyrus and cerebellar cells, Lewis has reported a decrease of ten to twenty percent in cell population with severe deficits in total DNA. These findings revealed reductions in germinal cells, and an increase in the number of degenerated postmitotic cells, among other changes from normal developmental processes (Lewis, 1990).

During the developmental processes cells require sufficient supplies of vitamins and minerals to sustain all of the nutritional demands of the growing fetus. All organs

have characteristic "critical periods" during development and are apparently very susceptible to nutrient alterations (McArdle & Ashworth, 1999).

McArdle and Ashworth have reported Vitamin A as essential for reproduction, embryogenesis, development and growth. Vitamin E intakes are important to prevent peroxidation reactions and to maintain the integrity of newly formed cell membranes. Vitamin K supplementation improves the mineralization processes for bone, cartilage and tooth growth and development. Of the water-soluble vitamins, vitamin B_6 is known to prevent alterations in brain N- methyl D- aspartate receptor function, necessary for learning and memory. Folic acid increases nucleic acid sysnthesis, necessary for cell growth and replication during gestation and for rapid tissue growth periods. Vitamin B_{12} is necessary for the myelination processs in the brain (McArdle & Ashworth, 1999).

The B vitamins, B_1, B_3, and B_6 in particular, are cofactors that are essential in metabolic pathways linked to energy and protein synthesis, which require increases in vitamin intakes for increases in energy and protein accretion. Vitamin C levels generally decrease during pregnancy and require vitamin increases to maintain pooled supply (Barr, 1998).

Aside from collagen synthesis and its antioxidant properties, vitamin C is also necessary to synthesize brain neurotransmitters serotonin, dopamine, and norepinephrine (Goff, Gropper, & Hunt, 1995).

Vitamin D is often present in low (serum) amounts in infants, and is necessary to maintain neonatal homeostasis. Vitamin D aids in the prevention of bone malformations (rickets) during growth periods. During pregnancy iron is necessary for red blood cell expansion. Zinc is also essential, as it is part of enzyme complexes in numerous

metabolic pathways. Protein synthesis requires zinc, and therefore is necessary for normal weight gain during pregnancy (Barr, 1998).

The studies mentioned exemplify the importance of a nutritionally balanced diet that reflect the body's present physiological status and metabolic requirements. Hence, during sensitive periods of pregnancy and lactation, maternal nutrient requirements change to prevent any unnecessary consequences. Importance has also been stressed on interactions among nutrients. Higher levels of one vitamin and/or mineral could interfere with the absorption of others. For example high magnesium intakes could affect the absorption of zinc and calcium. Similarly, synergistic effects could occur with other nutrients. Vitamin C is known to enhance the absorption of iron. A properly balanced vitamin and mineral supplement, formulated for pregnancy would be highly recommended to prevent potential imbalances in the various nutrients.

2. General Organization of the Thesis Experiments

Two experiments were conducted to investigate the role of choline (Experiment 1) and a vitamin/mineral supplement (Experiment 2) in facilitating recovery from early cortical injury in rats. In both experiments, rats were given suction lesions of the medial frontal or posterior parietal cortex on postnatal day 3 (Exp. 1) and postnatal day 4 (Exp. 2). As noted earlier the functional outcome after injury at this age is very poor relative to similar damage occuring one week later. The general hypothesis was that it ought to be possible to facilitate recovery from day 3 or 4 lesions by providing factors that either might potentiate the activity of endogenous factors necessary for plastic changes that could support functional recovery, or by some direct action of the factors themselves. The experiments were designed to run in parallel to other studies by Kolb and his colleagues that were looking at the effects the application of various pharmacological (eg., psychomotor stimulants, neurotrophic factors) and environmental (eg., tactile stimulation, complex housing) factors on recovery from similar brain injuries.

Behavioral Measures

The behavioral tasks chosen to study the effects of treatment intervention are summarized here. All animals in both experiments were tested on three behavioral tests; 1) open field (to measure activity in a novel environment), 2) the Morris water task, (a spatial learning task), and 3) tray reaching, (a skilled forelimb motor task). A subset of each treatment group from both experiments participated in the attention shift task to measure proposed executive functions.

Open Field:

The open field apparatus is commonly used to measure a variety of locomotor movements in rodents (see Fig.7). Using the open field as the first exposure to behavioral testing also gives this apparatus the opportunity to reveal activity in a novel environment. Sensors that surround the box for this test can measure horizontal and vertical activity, the distance covered and time spent in the margins, or the center of the field. Some experimenters also analyze fixed action patterns with the open field behavioral task. Habituation to a novel environment is generally indicated by a pattern of a decline in overall activity with each timed interval that is repeated for a designated time frame.

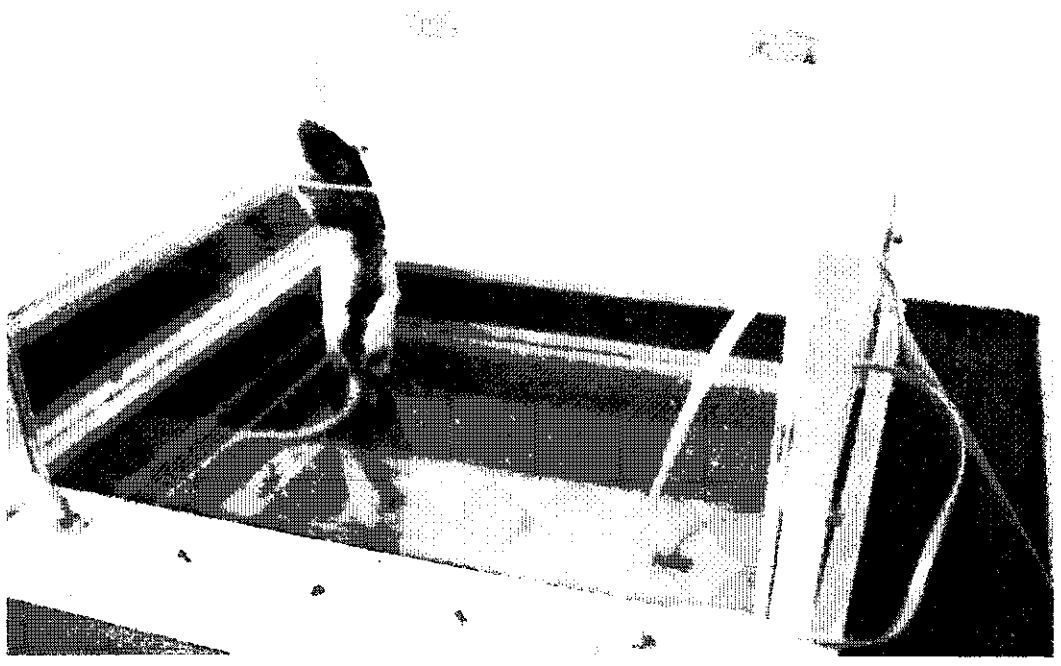


Fig. 7. Open Field Apparatus

Morris Water Task:

In their natural habitats, animals must learn to construct a cognitive map of their surroundings to remember specific locations, such as food sources, areas of safety from predators, and the route back to home base.

The Morris water maze task has been a widely used behavioral task to measure spatial memory in rodents. It requires rats to form cognitive representations of their world by navigating within their local environment. To construct a cognitive representation, the rats must learn to make associations with distal cues in its environment with that of a hidden platform to escape the cool water (see Fig.8). The latency, or time, that the rat requires to find the platform is the most commonly used measure to indicate any learning deficits. Additional measures considered for testing in this task are: 1) heading angle, or initial direction of orientation towards the platform 2) speed, to assess motor capacity 3) path distance to determine the length of distance required for each trial, and 4) thigmotaxis to determine the amount of time an animal spends at the edges of the pool trying to get out, rather than learning to find the platform.

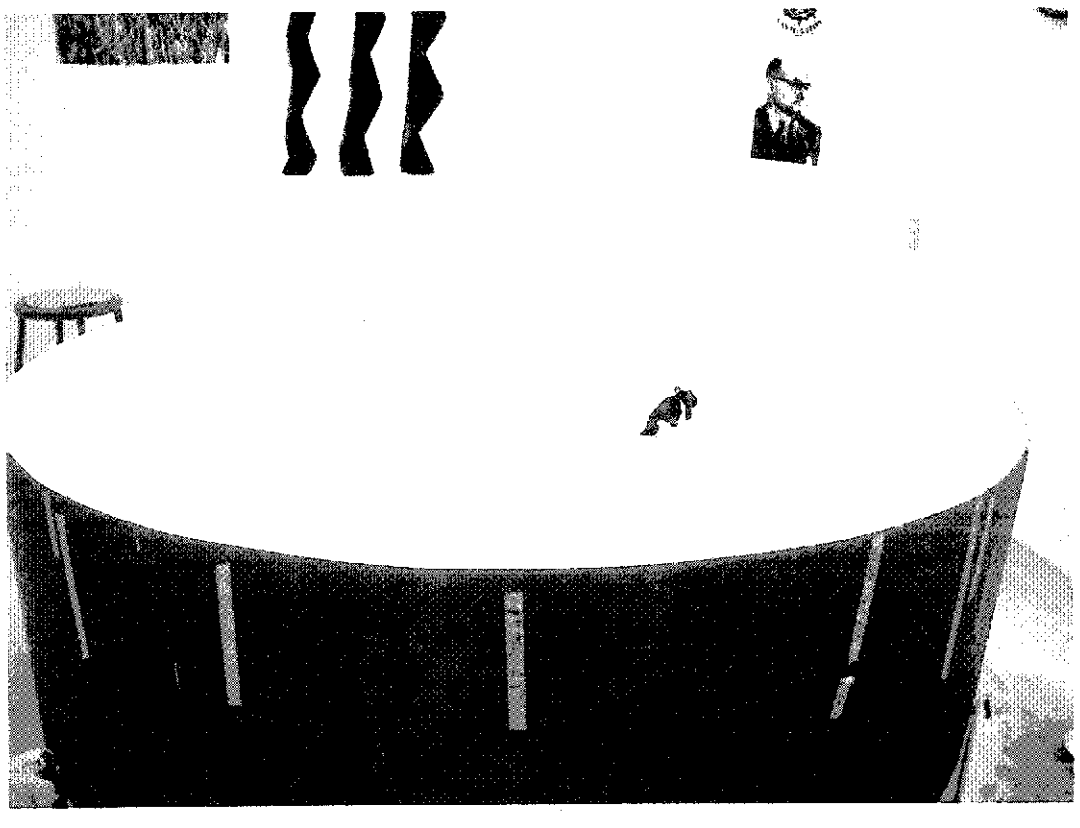


Fig.8. Morris Water Maze

Tray Reaching:

The tray-reaching task is useful to assess limb and grasp function of motor control. Rats are required to reach through the steel bars on the face of the cage to a tray which holds food pellets, grasp a pellet, and bring it back to their mouths to eat (see Fig.9). Once they are trained until consistently successful to their capacity, each rat is then filmed individually for assessment and scoring. Various lesions of the rat brain have revealed similar deficits to those seen in humans with brain damage in similar areas (Whishaw, Pellis, & Gorney, 1992). To assess a simplified form of a skilled reach, the tray-reaching task has been very useful to determine the effects of various treatment interventions under these conditions.

Fig.9. Tray Reaching Apparatus.

Attention Shift:

The attention shift task for rodents is proposed to be somewhat analogous to the Wisconsin Card Sorting Test used in human neuropsychology studies. In the card sorting task patients are required to sort a deck of cards according to certain characteristics presented (Kolb, & Whishaw, 2003). These characteristics vary according to color, shape, and number of elements. The patient does not know the rule of the task, but is only informed whether or not the response is correct. For example, the first rule might be color. Patients must sort the cards according to color. Without notification, the rule changes to shape. The patient is then required to inhibit the previous rule of the task of color, and adopt the new rule of shape (Kolb, & Whishaw, 2003). It has been designed to assess prefrontal function of executive processes, or higher cognition, of patients with frontal lobe damage.

This behavioral task proposes to determine the ability to maintain and shift attention set, when the rule of a task changes, without prior notification (Birell, & Brown, 2000). Humans and monkeys with frontal lobe damage have difficulty with this task. Therefore, if rats also have difficulty shifting attention set it may be possible to presume that rats have the fundamental capability to learn and adapt to new situations, using executive functions of cognition. The main difference with this task used for rodents is that the stimuli for which to detect a rule change is within the rats' preferred modes of learning- olfactory or tactile. The paradigm for this task, with fixed dimension pairs, was based upon Birrell and Brown (2000). The differences between the paradigm used by Birrell and Brown, to the one used here were: 1) They used a maze that was not considered to have a spatial feature to it, this maze does have spatial features, 2) they

used three exemplars for rats to discriminate during each dimension, only two were used here, and 3) Birell and Brown used excitotoxic lesions to the prelimbic and infralimbic areas of the prefrontal cortex, this experiment used aspiration lesions of the entire midline frontal cortex. The apparatus used for this task has two arms for rats to enter to obtain a food reward (see Fig.10). The baited arm is randomly chosen in such a way that it might also be possible to tease out any spatial reversal deficits.

The purpose for the investigation of attention shift in these experiments was to determine the capacities of the rat's cognitive processes of attention, and potential executive function, and the underlying anatomical regions in the brain that rats use to solve difficult problems.

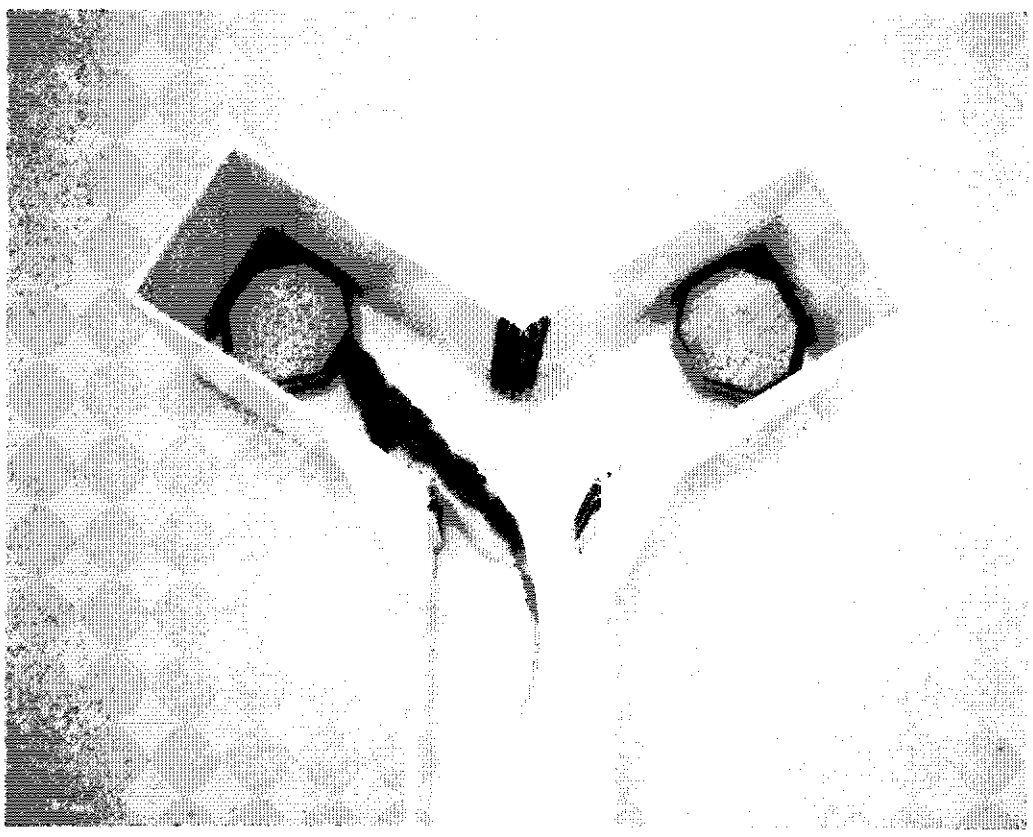


Fig. 10. Attention Shift Maze

3. Experiment 1

The experimental design used for treatment preparation was based upon one used by Richard Tees and Ehsan Mohammadi (1999). They introduced choline water solution, which was the only source of drinking water to females, two days prior to conception. Tees and Mohammadi then cross-fostered their rat pups at birth to mothers who no longer received choline water. The pups that had received prenatal choline also received postnatal choline by subcutaneous injections. The current experiment differed in that choline treatment continued in pups postnatal via their mother's milk for thirty days until weaned at postnatal day thirty.

Preliminary studies (B. Kolb & R. Tees, unpublished observations) had shown that the new procedure was effective in altering neuronal morphology, so it was expected that it should be able to influence synaptic organization of animals with perinatal cortical injuries.

In order to simplify the presentation of the results from the effects of the treatments on both frontal and parietal lesions, respectively, the frontal and parietal lesions are reported as separate experiments.

Experiment # 1A

The Effects of Choline Treatment on Recovery from Perinatal Medial Frontal Injury

Method

Subjects

A total of sixty-four Long Evans rats prepared using a crossed litter design were used for experiment 1A (see Table 3). This group of rats was used in three behavioral studies. Not all the rats were tested in the attention shift task. A subset of twenty-one rats from a total of fifty-eight, were selected for the attention shift task based on which rats were participating during the training session.

Any sex differences that are significant to the experimental results are reported.

Table 3. Number of rats used in experiment 1A.

	Untreated		Choline Treatment	
Sex	Female	Male	Female	Male
Unlesioned	n= 5	n= 12	n= 7	n=13
Frontal Lesion	n= 5	n= 11	n= 4	n= 7

Treatment procedures

Choline was added to regular tap water for drinking water ad libitum. Concentrations of choline are 5mL/L of 70% choline chloride in 0.02M saccharin solution.

Surgical procedures

The animals were all anesthetized by means of cooling in a Thermatron set at -5°C on postnatal days two and three. Cooling occurred for approximately 10 minutes, or until the rat pups were immobile and whitish in color. These parameters of immobility and color have been correlated with appropriate rectal temperatures, ranging from 18-20°C by Kolb and colleagues. Once the skin was incised, the bone of the skull was removed from bregma to lambda using iris scissors. Frontal cortex lesions required removal of the midline region of the cortex, anterior to bregma, by gentle aspiration. Suturing of the wound was done using silk thread. The sham control animals underwent all procedures except for bone incision and lesion aspirations. After completion of surgery, animals were slowly warmed using body heat by holding the pups in warm hands until they began moving, then were returned to their mothers.

Behavioral procedures

Open field Activity:

The open field test was performed in a plexiglass box (42 cm×42 cm×32 cm *h*), which has a lid on the top of the box. At the base of the box and approximately 1/3 in height, is a band of sensors around the perimeter of the box to record a variety of activity measures. A printer was connected to the open field apparatus, which resided in the same room, and was programmed to print activity behavior at two-minute intervals for ten minutes. At the end of the ten-minute period, the rats were placed back into their holding tubs and the number of defecations remaining in the apparatus were noted, which has

previously been used, in part, as observations of the stress response. Data collection was completed for each rat at the end of the ten- minute period.

Morris Water Task:

The maze used in the water task is a circular pool has a diameter of 1.5m and a height of 0.5m deep. The walls of the pool were painted white and the water rendered opaque with powdered milk. The platform (11×12 cm.) was placed in the northwest quadrant of the pool and submerged about 1.5 cm. below the water's surface. Each rat was placed into the pool facing the wall in one of four random starting positions (east, west, north, or south).

All animals were selected randomly into groups of eleven, to prevent any potential litter effects. The animals swam four trials each day for five days. Each trial had a different starting position, using all four points of direction each day, and changed in order of direction each day. Each trial consisted of a maximum of ninety seconds during which the rats had to find the hidden platform. After ninety seconds, the trial ended and rats that did not find the platform within that time were placed on the platform for ten seconds. Rats who did find the platform within the ninety-second interval were able to stay on the platform for ten seconds before being removed from the pool. All swim trials were recorded by means of a video camera mounted from the ceiling directly above the pool. Data collected from the camera were analyzed by a computer program (Water 2020). On the sixth day, a probe trial was given. During the probe trial, the platform was removed and the rats started at the north position. Rats swam for twenty seconds and each trial was recorded into the computer program for analysis.

Tray Reaching:

The procedure for the tray reach task was devised from Whishaw's skilled reaching task (Whishaw, O'Connor, & Dunnet, 1986). The test cages consist of clear plexiglass walls with a closable lid (20× 28× 26 cm). The front wall and floor of each cage was constructed of stainless steel bars. The floor was cross-linked with 2mm bars, and the front had vertical 3mm bars, extending 9mm apart from one edge of the cage to the other. Mounted in front of each set of three cages was a stainless steel tray, which held small pellets of chicken feed. The tray was suspended on runners at the sides of each cage set, and is positioned about 5mm away from the faces of the cage.

Rats were food deprived daily by feeding 15g. to each female and 20g. to each male. These food portions have been determined to slowly reduce body weight, which should not exceed in a loss of 15% body weight. The rats were fed a standard lab chow diet and were food deprived for six consecutive days. On the sixth day, after training, rats were fed ad libitum to prevent any potential further weight loss. Food deprivation began again after one day off.

Reaches were considered unsuccessful if the rats did not maintain a successful grasp at the tray, or during transport of the pellet back to the rat's mouth. If the rat had extended an arm towards the tray, but did not touch the food pellets, the reach attempt was not considered for scoring. When the animals had sufficiently learned to reach for the pellets, the rats were filmed by a video camera individually for ten minutes. Scoring of reach attempts and successes were determined by replay of the video for seven minutes

per rat. Attempts included both successes and failures. Overall success was determined as a percentage of successes verses attempts.

Attention Shift:

A wooden Y-shaped box (27 cm *l*×14.5 cm *w*×21 cm *h*) was used and two glass octagonal bowls were placed at each of the two arms of what was designated the top portion of the Y maze. Each bowl contained sawdust during the training, or habituation, period. Rats were trained to dig for one half of a Froot Loop as a baited reward. The rats were then required to enter each arm to visit each bowl to learn to dig for a food reward. Once rats were trained to dig in bowls for a Froot Loop, the scent and medium in the bowls varied as the task progressed and only one of two bowls was baited. The baited arm was also randomly chosen, not exceeding more than two to three arm reversals throughout testing (Appendix B). Each rat was allowed four free visits to either of two bowls to determine which bowl was baited. Thereafter, errors were recorded as defined by a rat digging, or poking its head into the unbaited bowl, in an attempt to find the Froot Loop. A criterion of six correct consecutive trials was required to complete each discrimination combination. Approximately ten seconds was required to re-bait the bowls. The number of distractions were also recorded, because it seemed directly linked to the frequency of errors. A distraction was defined as having to remove the rat from the maze due to failure to participate in the task for that trial. The distracted trial was recorded as a distraction and another trial began after discontinuation.

Anatomical procedures

At the completion of behavioral testing, rats were given an overdose of sodium pentobarbital (0.5ml-0.7ml) until they reached a comatose state. At this point, they were perfused intracardially, using 0.9 % saline solution. Rat brains destined for Cresyl Violet staining, perfusions of 4% paraformaldehyde followed saline. Removal of the brains were done using Rongeurs and weighed immediately following removal from the skull. Forty-two rat brains were designated for Cresyl Violet stains. The remaining 22 male rats were perfused for Golgi staining. For Cresyl Violet preparations, the brains were placed in small bottles containing 4% paraformaldehyde in 30% sucrose solution.

For Golgi preparations, brains were weighed at the end of saline perfusions and placed in small bottles containing Golgi solution. These brains were processed following procedures devised by Gibb and Kolb (1998).

The brains for Cresyl Violet staining were sliced on a Cryostat 2800 Frigocut, which maintains tissue at -21°C. Every 7th slice was taken at 40 μm thickness and placed on slides fixed with a film consisting of 1.0% gel and 0.2% chromatin. Once the brains were dried to the slides, the slides were placed in slide trays, which hang on a Fisher Histomatic Slide Stainer (model 172). This apparatus runs through a programmed series of solutions required to complete Cresyl Violet staining.

Brain Weight:

The measures of brain weight are obtained by weighing each brain directly after removal from the skull of the perfused rat. Thereafter, brain weights are compared between groups to determine an estimate of tissue lost as a result of the lesions. Care was

taken to ensure consistency of brain removal before weighing. The spinal cord was cut along the caudal edge of the cerebellum, the cerebellar paraflocculi were removed, the optic nerve was cut 1-2 mm anterior to the optic chiasm, and remaining dura was stripped off the brain's surface. At the front end of the brain, the olfactory bulbs are cut at 1.5-2.5 mm from the frontal cortex.

Cortical thickness:

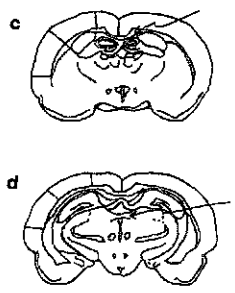
After slide preparation, the brains were viewed and assessed at a magnification of 17.5 × with a Zeiss DL 2 POL petrographic projector.

Cortical measurements were made using a clear plastic ruler for both the choline treated group and the no treatment control group. Three different cortical measurements, for each hemisphere, were determined at five planes. A standard technique was followed:

Zilles (1995) (see Fig.11)

Plane 1: AID, Par1, Fr2
(most anterior plane
of caudate putamen)

Plane 2: Par2, Par1, Fr1
(anterior commissure)



Plane 3: GI/DI, Par1, Fr1
(hippocampus)

Plane 4: Te1, Oc2L, RSA
(posterior commissure)

Plane 5: Te1, O1B, Oc2ML
(hippocampus)

Fig. 11. Zilles (1995) Lateral, central, and medial regions measured across five planes for cortical thickness.

Thalamic width:

Thalamic width was measured at two planes based upon previous studies by Kolb and Whishaw (1981). The first was the anterior plane, at the point of a predominant MD nucleus, and when the hippocampus CA fields and dentate gyrus had become full. The second plane was at the last slice that had a full thalamus, which is at the beginning of the posterior commissure. Degeneration of thalamic nuclei is commonly seen as a result of cortical injury. Degeneration is generally characterized by darker stained cells in thalamic nuclei, shrunken nuclear areas, as well as abnormally shaped cells.

Results

Anatomical Results

The frontal lesion consistently removed the midline region of cingulate 1 and 2 (Cg1, Cg2) areas with primary motor regions M1 and M2, or Zilles Fr1 and Fr3. The prelimbic (PrL), or Zilles (1995) cingulate 3 (Cg3), and the infralimbic (IL) areas were usually gone, as well as variable amounts of the frontal association (FrA), or Zilles (1995) frontal (Fr2) areas. Sections of the medial orbital (MO), ventral orbital (VO) and lateral orbital (LO) cortex of the ventral frontal areas were also removed. A visual interpretation of comparisons between brains of animals with choline treatment, with brains of animals without treatment, suggests that some of the lesion cavities may have been slightly smaller in the choline treated rats.

Thalamic degeneration was observed as a reduction in size of the thalamus, and an apparent increase in cell density, most likely from the compression of nuclei. The anterior areas most affected were the paraventricular nucleus (PVN) and possibly, to some mild extent the medial dorsal nucleus. Thalamic degeneration appeared more extensive in the no treatment group than that of the choline treatment group, indicating some beneficial treatment effects, although, this was not quantified (see Fig. 12).

Choline Treatment

Untreated

Fig. 12. Frontal cortical lesions of the choline treated animals.

Brain Weight:

The overall result was that the choline treatment increased brain weight in female rats, whereas the frontal lesions decreased brain weight in both sexes.

Because the Cresyl Violet and Golgi perfusion preparations yield different brain weights, they were analyzed separately.

Cresyl Violet Preparation:

Increases in brain weight were observed in the female choline treatment groups and the animals with frontal lesions that showed the greatest benefit from the choline treatment (see table 4).

A three-way ANOVA found a main effect of lesion ($F(1,34)= 72.900$, $P< .0001$), of sex ($F(1,34)= 4.500$, $P=.0400$), and of the interaction ($F(1,34)= 9.900$, $P= .0030$). There was a main effect of choline treatment ($F(1,34)= 7.300$, $P= .0100$), and for the interaction between sex and choline treatment ($F(1,34)= 5.900$, $P= .0200$), but not for the interaction between lesion and choline treatment ($F(1,34)= 3.700$, $P= .0600$), or for the interaction among all three groups ($F(1,34)= .4000$, $P= .5200$).

Table 4. Brain Weight for Cresyl Violet Preparations:

Group	Female	Male
Untreated lesion	1.706 ± .050 (n=5) *	1.768 ± .063 (n=5) *
Choline lesion	1.940 ± .020 (n=4)	1.815 ± .045 (n=2)
Untreated unlesioned	1.948 ± .024 (n=5)	2.162 ± .035 (n=6)
Choline unlesioned	2.026 ± .024 (n=7) **	2.131 ± .035 (n=8)

* Denotes statistically different from the unlesioned groups ($p< .05$, or better).
** Denotes statistically different from untreated groups ($p< .05$, or better).

Golgi Preparation:

It appears that male brains may have received some benefits of choline treatment when comparing with Golgi preparation brains, although the effect did not reach statistical significance. (Only male rats were prepared for Golgi).

A two-way ANOVA indicated a main effect of lesion ($F(1,19)= 21.600$, $P= .0002$), but not of treatment ($F(1,19)= 3.340$, $P=.0800$), nor for an interaction ($F(1,19)= 2.250$, $P= .1500$).

Table 5. Brain Weight for Golgi Preparations:

Group	Male
Untreated lesion	1.764 ± .024 (n=6) *
Choline lesion	1.958 ± .121 (n=5)
Untreated unlesioned	2.123 ± .033 (n=7)
Choline unlesioned	2.142 ± .023 (n=5) **

*Denotes statistical difference from unlesioned groups ($p<.05$, or better).
** Denotes statistically different from untreated groups ($p<.05$, or better).

Cortical thickness:

The brains that were used to measure and analyze cortical thickness and thalamus were those that were prepared for Cresyl Violet staining.

Data for all five planes were analyzed by a two-way ANOVA collectively to determine the overall effects on cortical thickness between treatment and no treatment groups.

A repeated measures ANOVA across the five planes measured found the frontal lesion groups to have the lowest cortical thickness, those with choline treatment showing the greatest benefit (see Fig.13). A significant interaction was found between mean cortical thickness and lesion therefore, two-way ANOVAs between lesion and treatment groups were performed for each plane to determine the plane(s) affected.

ANOVA for the first plane indicated a main effect of lesion ($F(1,73)= 40.676$, $P< .0001$), as well as for the interaction ($F(1,73)= 4.304$, $P=.0415$), but no significant effects of treatment ($F(1,73)= 1.691$, $P=.1976$).

ANOVA for the second plane did not indicate any main effects of lesion ($F(1,73)= 22.020$, $P< .0001$), of treatment ($F(1,73)= .302$, $P= .5842$), nor for the interaction between groups ($F(1,73)= 2.164$, $P=.1455$).

ANOVA for the third plane indicated a main effect of lesion ($F(1, 73)= 8.416$, $P=.0049$), but no effects of treatment ($F(1,73)= 2.932$, $P=.0911$), nor for the interaction ($F(1,73)= 2.130$, $P=.1487$).

ANOVA for the fourth plane indicated a main effect of lesion ($F(1,73)= 14.785$, $P= .0003$), but not of treatment ($F(1,73)= .217$, $P=.6430$), nor for the interaction ($F(1,73)= 2.933$, $P= .0910$).

ANOVA for plane five indicated a main effect of lesion ($F(1,73)= 4.796$, $P= .0317$), but not of treatment ($F(1,73)= 3.154$, $P= .0799$), nor of the interaction ($F(1,73)= 1.104$, $P= .2969$.

Overall, the frontal lesion group treated with choline had thicker cortices than the animals that did not receive choline, throughout all five planes measured (see Fig.14).

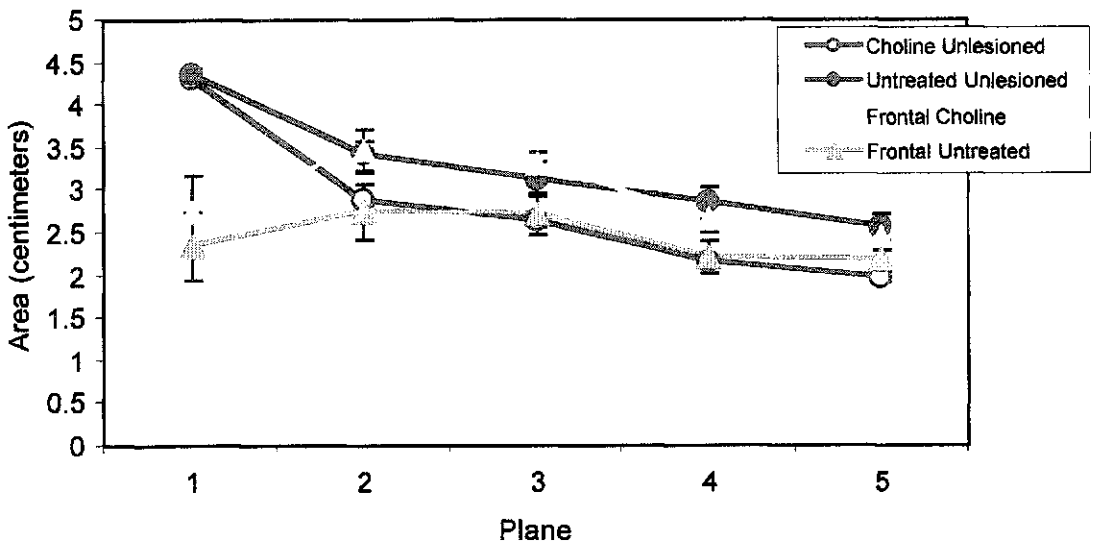


Fig. 13. Cortical thickness for each of five planes measured in animals with frontal lesions treated with choline.

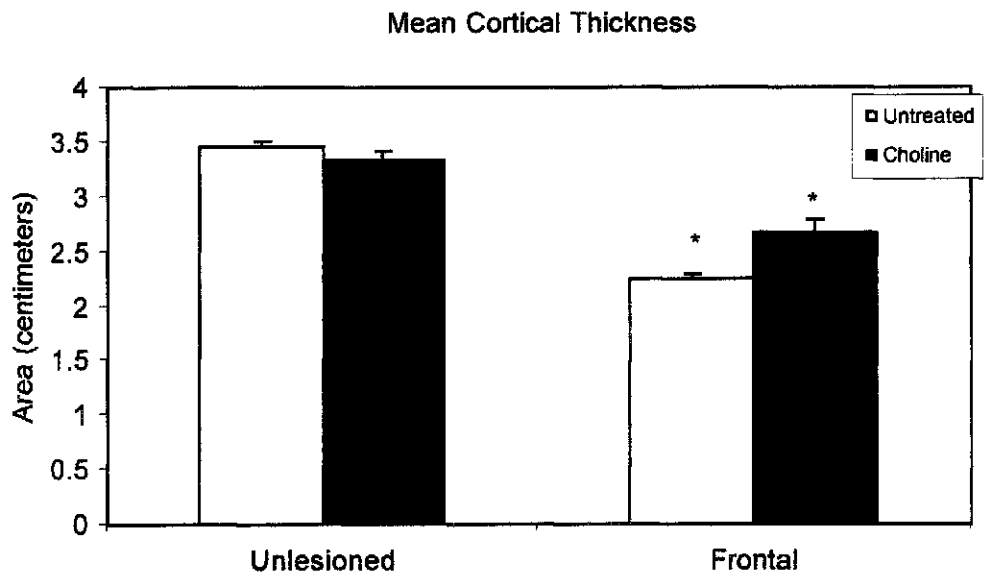


Fig. 14. Mean cortical thickness for the five planes measured in animals with frontal lesions treated with choline. * Denotes statistically different from unlesioned groups ($p < .05$, or better)

Thalamic width:

The anterior portions of the thalamus of the lesion brains showed a trend to have increased in thalamic width with the choline treatment. There was relatively high variance however, which prevented any statistical support for this impression.

A two-way analysis of variance (ANOVA) for the anterior plane of measure between the lesion and treatment groups did not reveal a main effect of lesion ($F(1,25)= .266$, $P=.6103$), of treatment ($F(1,25)= 2.441$, $P= .1308$), nor for the interaction between groups ($F(1,25)= 1.791$, $P=.1928$).

ANOVA for the posterior plane of the thalamus between the lesion and treatment group indicated a main effect of lesion ($F(1,25)= 28.344$, $P< .0001$), but not of treatment ($F(1,25)= 1.042$, $P=.3172$), nor for the interaction ($F(1,25)$, $P=3.319$, $P= .0805$).

Table 6. Mean measures of thalamic width.

	Anterior Plane (mm)	Posterior Plane (mm)
Untreated lesion	11.120 ± .282	12.940 ± .282 *
Choline lesion	12.340 ± .223	13.660 ± .223 **
Untreated unlesioned	11.900 ± .400	14.750 ± .400
Choline unlesioned	11.994 ± .239	14.547 ± .239

* Denotes statistically different from unlesioned groups ($p<.05$, or better).
** Denotes statistically different from untreated lesion group ($p<.05$, or better).

Behavioral results

Open Field:

Both the frontal lesion and unlesioned groups that received choline treatment displayed less activity than the untreated groups in the measures of horizontal activity and distance, with both males and females showing a treatment effect.

Horizontal activity:

Because there was an effect for sex, a three-way ANOVA between lesion, sex, and treatment for horizontal activity was performed. ANOVA found a main effect of treatment ($F(2,44)= 38.200$, $P< .0001$), lesion ($F(2,44)= 15.00$, $P< .0001$), and sex ($F(1,44)= 15.200$, $P< .0001$).as well as for an interaction between lesion and treatment ($F(2,44)= 23.900$, $P< .0001$), but not between sex and treatment ($F(2,44)= 3.200$, $P= .0823$), sex and lesion ($F(1,44)= 0.400$, $P= .8300$), nor of an interaction among all three groups ($F(2,44)= 1.00$, $P= .3200$) (see Fig.15).

Distance:

A three-way ANOVA for distance between lesion, treatment and sex groups indicated a main effect of treatment ($F(2, 36)= 53.700$, $P< .0001$), lesion ($F(1,36)= 19.100$, $P= .0001$), sex ($F(1,36)= 4.600$, $P= 0.400$), and for an interaction between lesion and treatment ($F(2,36)= 7.100$, $P= .0100$), but not for an interaction between sex and treatment ($F(2,36)= 2.200$, $P= .1500$), nor for sex and lesion ($F(1,36)= 2.400$, $P= .1283$), nor among all three groups ($F(2,36)= 1.100$, $P= .3100$) (see Fig.16). The interaction between treatment, sex, and lesion reflects that the choline treatment benefit was found in females with frontal lesions.

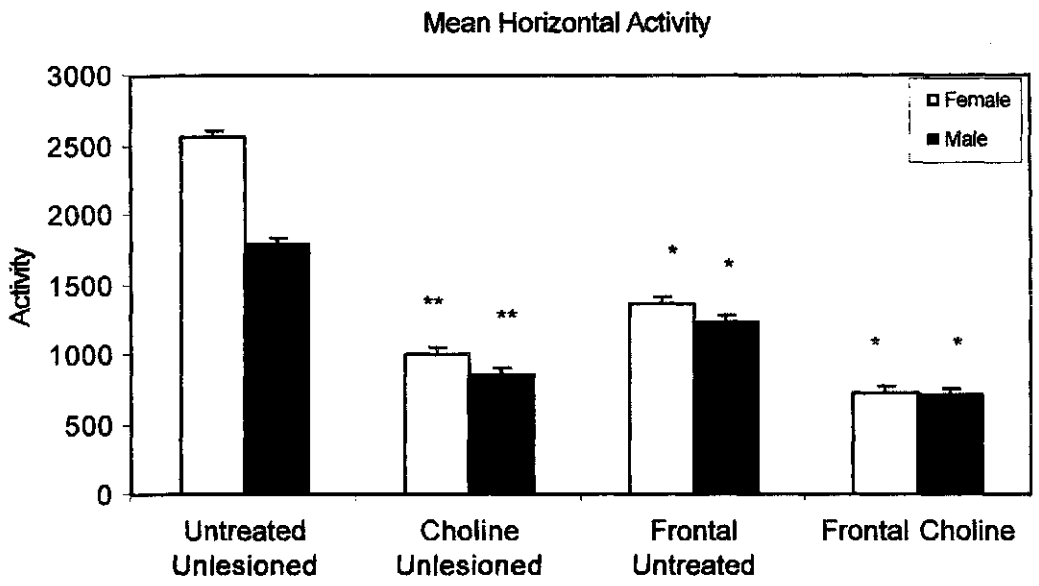


Fig. 15. Mean horizontal activity of animals with frontal lesions treated with choline. * Denotes statistically different from unlesioned groups (p<.05, or better). ** Denotes statistically different from untreated unlesioned groups (p<.05, or better).

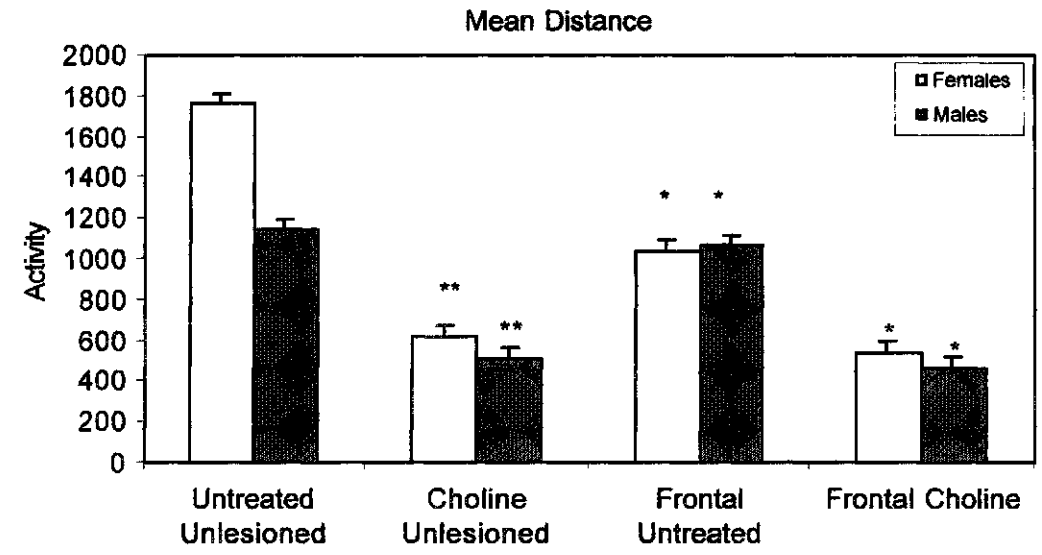


Fig. 16.Mean distance in open field of animals with frontal lesions treated with choline. * Denotes statistically different from unlesioned groups (p<.05, or better). ** Denotes statistically different from untreated unlesioned groups (p<.05, or better).

Morris water task:

A two-way analysis of variance (ANOVA) between lesion and treatment indicated a main effect of lesion ($F(1,54)= 5.914$, $P= .0184$), a main effect of choline treatment ($F(1, 54)= 3.998$, $P= .0509$), as well as for the interaction ($F(1,54)= 6.866$, $P= .0114$). The interaction reflected the selective effect of the choline treatment on the frontal lesion animal's performance (see Fig.17 & Fig.18).

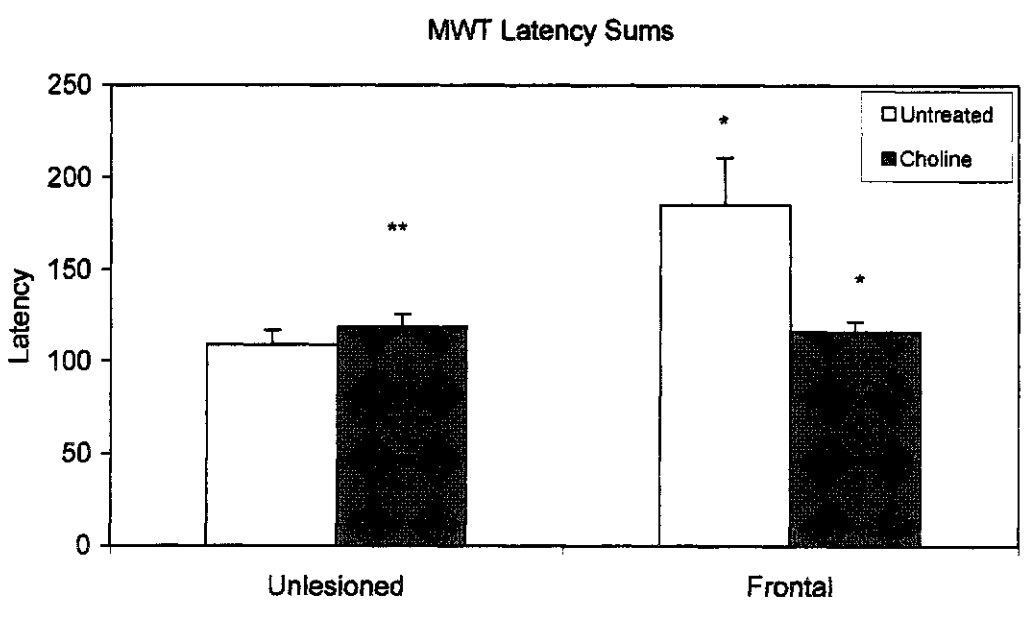


Fig. 17. Morris water task sums for frontal lesion choline-treated animals. * Denotes statistically different from unlesioned animals ($p< .05$, or better). ** Denotes statistically different from untreated animals ($p< .05$, or better).

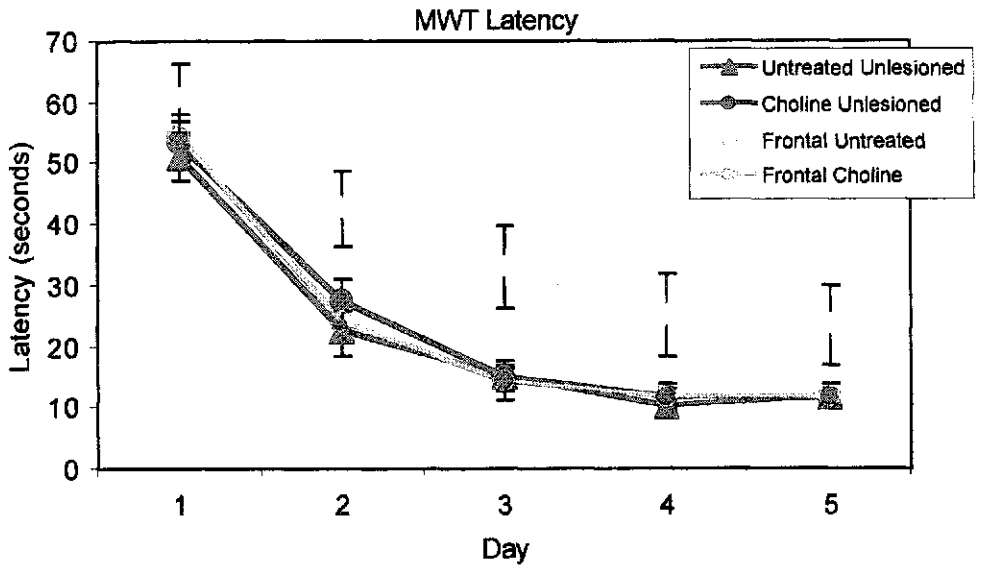


Fig. 18. Morris water task latency over the five-day trial period. Animals had frontal lesions and choline treatment.

Probe trials at the end of training revealed that the choline-treated frontal lesion animals spent the most time in the correct quadrant, quadrant one. All groups did however, spend time in quadrant one, sharing time in quadrant four (see Fig.19).

A two way ANOVA indicated a main effect of lesion ($F(1,34)= 4.718$, $P= .0369$) and of treatment ($F(1,34)= 7.400$, $P= .0102$), but not for the interaction ($F(1,34)= .850$, $P= .3630$).

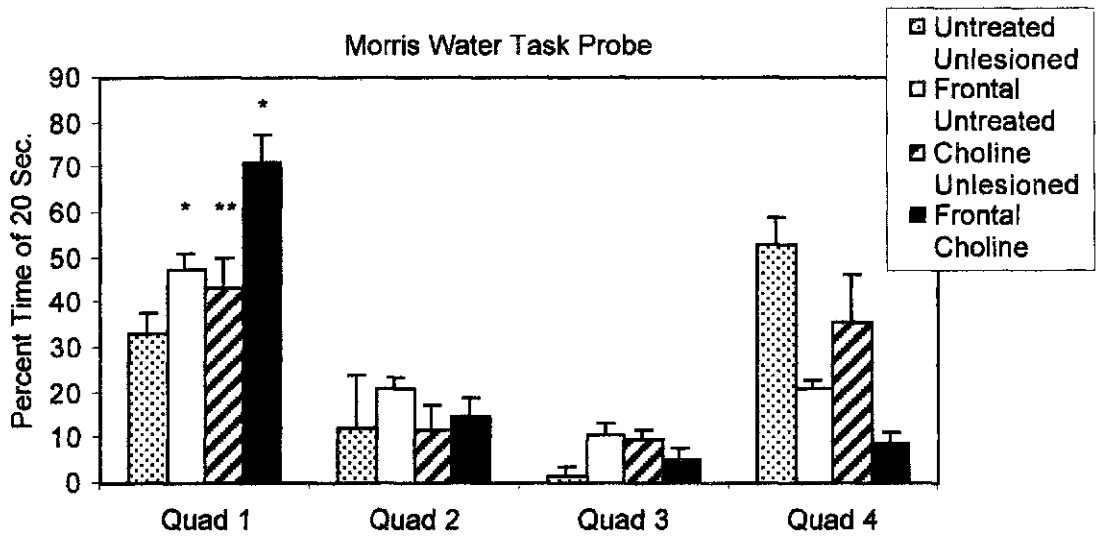


Fig. 19. Probe trials of Morris water task for frontal lesion animals treated with choline. Quadrant 1 is the correct quadrant. * Denotes statistically different from unlesioned animals (p<. 05, or better). ** Denotes statistically different from untreated animals (p<. 05, or better).

Tray Reaching:

Rats with frontal lesions were impaired at the task, but treatment with choline successfully reversed the impairment (see Fig.20). In fact, the success rate for the choline-treated frontal lesion group was similar to that of the unlesioned choline treated group.

A two-way analysis of variance (ANOVA) for lesion and choline treatment indicated a main effect of lesion ($F(1,30)= 11.246$, $P= .0022$), as well as for the interaction between groups ($F(1,30)= 10.714$, $P= .0027$), but not of choline treatment

(F(1,30)= 1.194, P= .2831). The interaction reflects the selective effect of the choline treatment on the frontal lesion group.

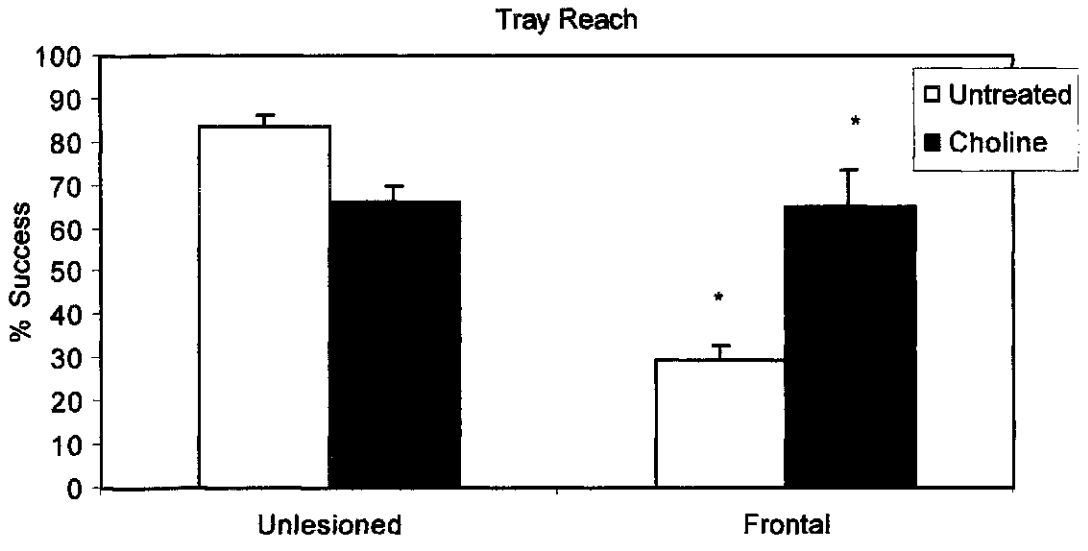


Fig. 20. Summary of tray reaching task for frontal lesion choline-treated group. * Denotes statistically different from unlesioned animals (p<. 05, or better).

Attention Shift:

Birrell & Brown (2000) proposed their task apparatus task to be different from those that have a spatial component to it, such as found in a Y-, or T-maze. Their purpose for not using a Y-, or T-maze is because it also measures spatial deficits that might confound the variable of attention shift. The anatomical area Birrell and Brown have claimed to house executive functions of cognition in the rat is in the prelimbic (Zilles Cg3) and infralimbic areas of the prefrontal cortex. This task did use a spatial Y-maze and midline frontal lesions in rats includes the cingulated cortex, which is known to be

responsible for spatial problem solving. Reversal of baited arm was also noted to account for spatial reversal deficits. Overall, this task proved to be difficult for all animals (see fig.20). For example, the unlesioned animals made errors at the beginning of the task during the simple dimension of odor, the complex dimension of odor and medium as well as during the first reversal (Rev1) of odor-medium pairs (see Appendix B). It appears that learning of the dimension changes, as well as baited arm changes, provided some challenges during the beginning stages of the task. All animals eventually learned the task however, and towards the end of the task, were making fewer errors. The animals that indicated the greatest impairment were in the frontal lesion untreated group and seemed to perform less well throughout the series of dimensions. The greatest number of errors performed during the extradimensional shift (ED), were from the frontal lesion animals, particularly the animals treated with choline. The ED is the dimension proposed to measure the ability for rats to shift attention from the previously rewarded dimension of odor, to a new dimension of medium. All rats but the unlesioned animals expressed much confusion during the ED shift and required more time to solve this dimension indicating the possibility that rats are capable of solving complex tasks, and that this task possibly could measure attention shift. Both frontal lesion groups, especially the choline-treated animals, were the most distracted and inattentive, which was noted during testing.

Surprisingly, the choline treatment did not indicate any statistically significant benefit as measured by decreases in errors throughout each dimension. It could be possible that choline treatment may have actually been detrimental to the performance of this task, but it also should be noted that the treated animals were run as a larger group, and for a longer period of food deprivation, potentially increasing the animals' tendency

for distraction. Anatomically, however, the rats indicating the greatest number of errors in the choline treatment group were also missing the proposed areas of the prefrontal cortex responsible for the ability to shift attention. The fact that the untreated group appeared to perform this task with fewer errors than the choline treatment group could also be attributed to the rats resorting to simpler modes of cognition to resolve this task. A visual inspection of the untreated animals indicated that they were missing larger portions of the prefrontal cortex, particularly the infralimbic/prelimbic region proposed responsible for attention shift.

A two-way analysis of variance was performed for each dimension. ANOVA on the simple dimension found a main effect of choline treatment (SD) ($F(1,38)= 6.265$, $P= .0167$), but not of lesion ($F(1,38)= .204$, $P= .6543$), nor for the interaction ($F(1,38)= .204$, $P= .6543$). The choline treated animals exhibited the greatest number of errors in this dimension.

ANOVA for the compound dimension (CD) also found a main effect of choline treatment ($F(1,38)= 6.293$, $P= .0165$) and no significant effects for lesion ($F(1,38)= .400$, $P= .5309$), nor an interaction ($F(1,38)= 1.252$, $P= .2703$). For this dimension, the choline treated animals exhibited the least number of errors.

ANOVA for the first reversal (Rev1) of exemplar pairs indicated no significant effects of lesion ($F(1,38)= .869$, $P= .3572$), treatment ($F(1,38)= 1.496$, $P= .2289$), nor the interaction ($F(1,38)= .273$, $P= .6043$). The statistical results suggest that all groups performed reasonably well in this dimension, although the frontal lesion group did make more errors.

ANOVA for the intradimensional shift (ID) also did not reveal any significant findings. Thus, there were no main effects of lesion ($F(1,38)= 1.766$, $P= 1918$), or of treatment ($F(1,38)= .004$, $P= .9489$), nor for the interaction ($F(1,38)= .004$, $P= .9489$).

ANOVA for the second reversal (Rev2) found a marginal effect of lesion ($F(1,38)= 3.960$, $P= .0538$), but no significant effects of treatment ($F(1,38)= 2.021$, $P= .1633$, or an interaction ($F(1,38)= 2.021$, $P= .1633$).

ANOVA for the extradimensional shift (ED) found no significant effects of lesion ($F(1,38)= .845$, $P= .3639$), of treatment ($F(1,38)= .904$, $P= .3476$), nor for an interaction ($F(1,38)= 1.824$, $P= .1824$). Errors were evident during this dimension, although not in substantial quantities.

ANOVA for the third reversal (Rev3) also did not indicate any significant effects of lesion ($F(1,38)= .845$, $P= .3639$), treatment ($F(1,38)= .904$, $P= .3476$), or for the interaction between groups ($F(1,38)= 1.824$, $P= .1848$). The frontal lesion animals were the only group to commit any errors in this dimension (see Fig.20).

Finally, I should note that although the measures of mean errors during the ED shift were not statistically obvious, all lesion animals in both treatment groups became very confused and distracted during this dimension, requiring much more time to figure out the changes. Unfortunately, time was not measured, but the behavior of the animals does suggest that the prefrontal cortex does play a role in the efficient solution of the task.

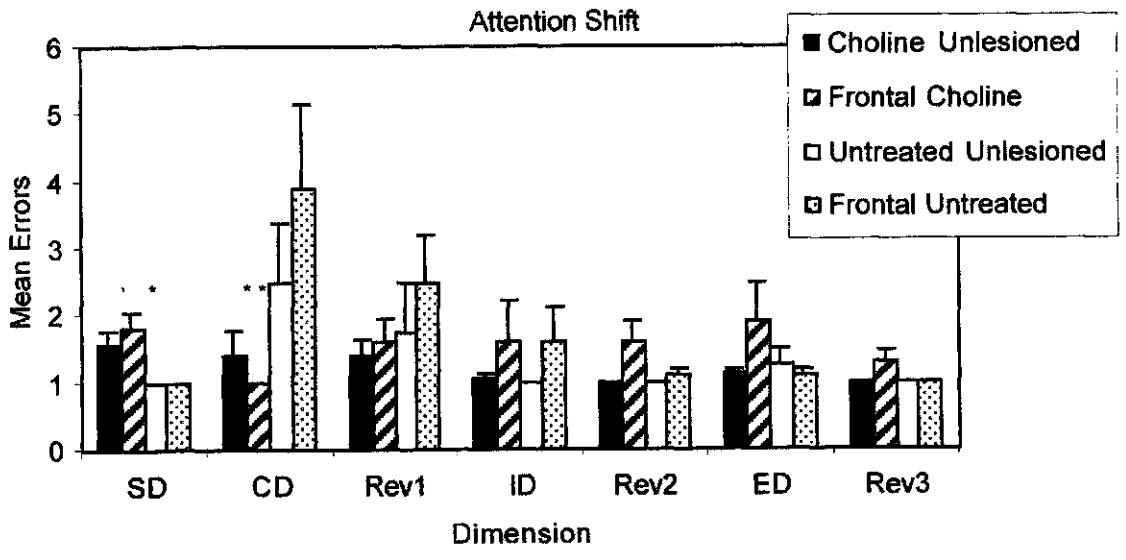


Fig. 21. Summary of all dimensions measured in the attention shift task. Animals had frontal lesions and choline treatment.

Experiment #1B

The Effects of Choline Treatment on Recovery from Perinatal Posterior Parietal Injury

The methods used for experiment 1B were essentially identical to those used in 1A. The differences were only in subject numbers and surgical procedures.

Method

Subjects

A total of seventy-three Long Evans rats were used for Experiment 1B, all of which participated in three behavioral tasks (see table 7). A subset of twenty-two rats was selected for the attention shift task. At the age of 60 days the animals began behavioral testing. Any sex differences that are significant to the experimental results are reported.

Table 7. Number of rats used in Experiment 1B.

	Untreated		Choline Treatment	
Sex	Female	Male	Female	Male
Unlesioned	n= 5	n= 12	n= 7	n=13
Parietal Lesion	n= 5	n= 11	n= 7	n=13

Surgical procedures

The animals were all anesthetized by means of cooling in a Thermatron set at -5°C as in Experiment 1A. Once the skin was incised, the middle third of the skull between bregma and lambda was removed using iris scissors. Laterally, the removal began about 1.5 mm lateral to the saggital suture and went laterally to make a square removal of the bone. The exposed tissue was removed by gentle aspiration. The skin was sutured with sterile silk thread. The sham control animals underwent all procedures except for bone incision and lesion aspirations. After completion of surgery, animals

were slowly warmed using body heat by holding the pups in warm hands until they began moving, then were returned to their mothers.

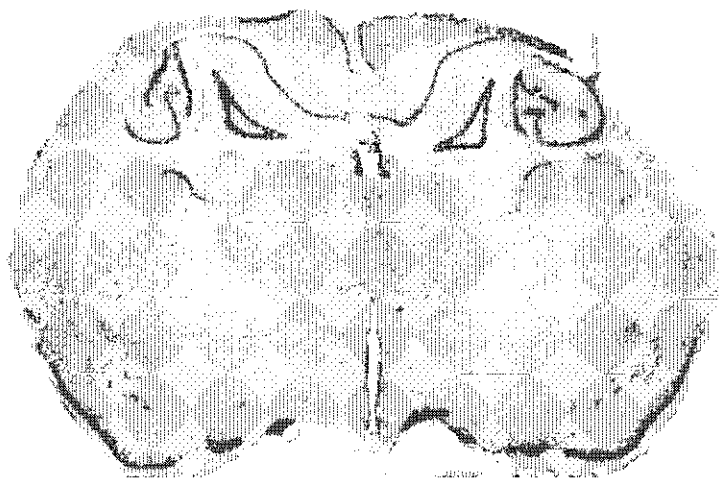
Results

Anatomical Results

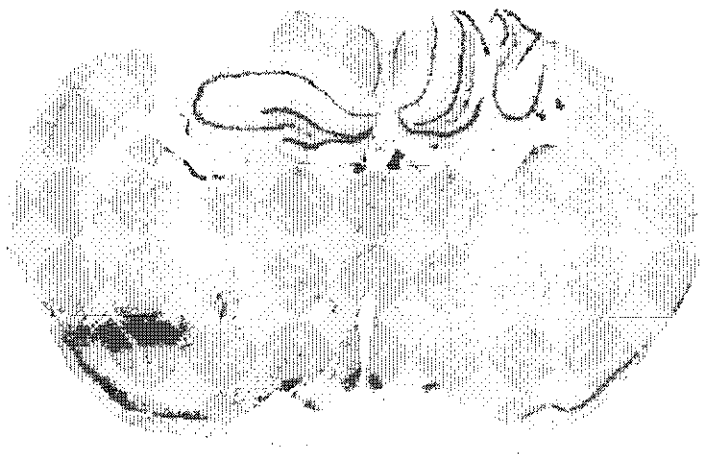
The lesions removed Kreig's area 7, in addition to the posterior part of the primary parietal cortex (Par1), and the anterior part of Zilles' occipital 2 areas (Oc2). Occasional unilateral damage was apparent in the posterior cingulate regions (RSA, RSG). There was no direct damage to the hippocampus, or to other subcortical regions. The hippocampus had an unusual shape however, as it often grew through the lesion cavity, leading to an appearance of a smooth cortical surface (see Fig. 21).

Thalamic degeneration, as characterized by a reduction in nuclear size, was apparent in all brains observed and was variable in extent. Most posterior parietal lesions resulted in degeneration of anterior nuclei, especially the anterior dorsal (AD) and lateral dorsal (LD) and paraventricular thalamic nuclei (PVN). In some cases the cells of the medial dorsal (MD) nucleus also appeared to be sparse.

The brains of rats in the untreated lesion group appeared to have more extensive damage than the choline treatment group. The lesions appeared larger, the remaining cortex was thinner, and degeneration in the thalamus was more obvious.



Choline Treatment



Untreated

Fig. 22. Posterior parietal cortical lesions of the choline treatment animals.

Brain Weight:

Posterior parietal lesions reduced brain weight in both sexes, but the choline treatment didn't have significant effects on brain weight. Differences in brain weight are usually found between fixation methods, therefore, the analysis has been done separately for each fixation technique (see Table 8).

Cresyl Violet Preparation:

A three-way ANOVA found a main effect of lesion ($F(1,43)= 58.700$, $P< .0001$), sex ($F(1,43)= 10.100$, $P= .0030$), but not for the interaction between lesion and sex ($F(1,43)= 2.200$, $P= .1400$). Choline treatment did not reach significance ($F(1,43)= .2000$, $P= .6600$), nor did the interactions between lesion and choline treatment ($F(1,43)= 1.300$, $P= .2700$), between sex and choline treatment ($F(1,43)= 2.800$, $P= .1000$), nor for the interaction among all three groups ($F(1,43)= .0100$, $P= .9300$).

Table 8. Brain Weight for Cresyl Violet Preparations:

Group	Female	Male
Untreated lesion	1.772 ± .055 (n=5) *	1.890 ± .040 (n=5) *
Choline lesion	1.779 ± .040 (n=7)	1.776 ± .070 (n=8)
Untreated Unlesioned	1.948 ± .024 (n=5)	2.162 ± .035 (n=6)
Choline Unlesioned	2.026 ± .024 (n=7)	2.131 ± .035 (n=8)

* Denotes statistically different from unlesioned groups ($p<.05$, or better).

Golgi Preparation:

A two-way ANOVA indicated a main effect of lesion ($F(1,19)= 38.300$, $P< .0001$), but no effects of treatment ($F(1,19)= .1300$, $P=.7300$), or for the interaction ($F(1,19)= .8400$, $P= .3700$). (Only male rats were used for Golgi preparation) (see Table 9).

Table 9. Brain Weight for Golgi Preparations:

Group	Male
Untreated lesion	1.942 ± .010 (n=6) *
Choline lesion	1.898 ± .060 (n=5) *
Untreated Unlesioned	2.123 ± .030 (n=6)
Choline Unlesioned	2.142 ± .020 (n=5)

* Denotes statistically different from unlesioned groups ($p<.05$, or better).

Cortical thickness:

Overall, the lesion groups had thinner cortices than animals without lesions and choline treatment showed no beneficial effect on cortical thickness. The mean cortical thickness across all five planes was analyzed first.

Repeated measures ANOVA for all five planes did not reveal any significant interactions between mean cortical thickness and lesion (see Fig. 22).

A two-way ANOVA indicted a main effect of lesion ($F(1,29)= 26.650$, $P< .0001$), but no significant effects of treatment ($F(1,29)= 2.846$, $P= .1023$), or for the interaction ($F(1,29)= .522$, $P= .4759$) (see Fig.23).

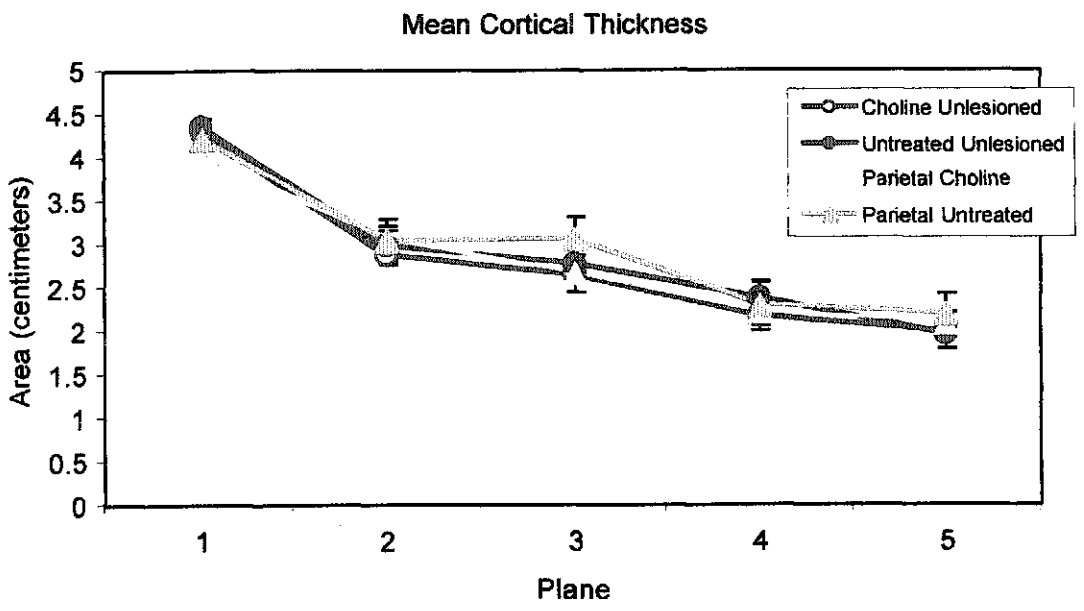


Fig.23. Cortical thickness for each of five planes measured in animals with parietal lesions treated with choline.

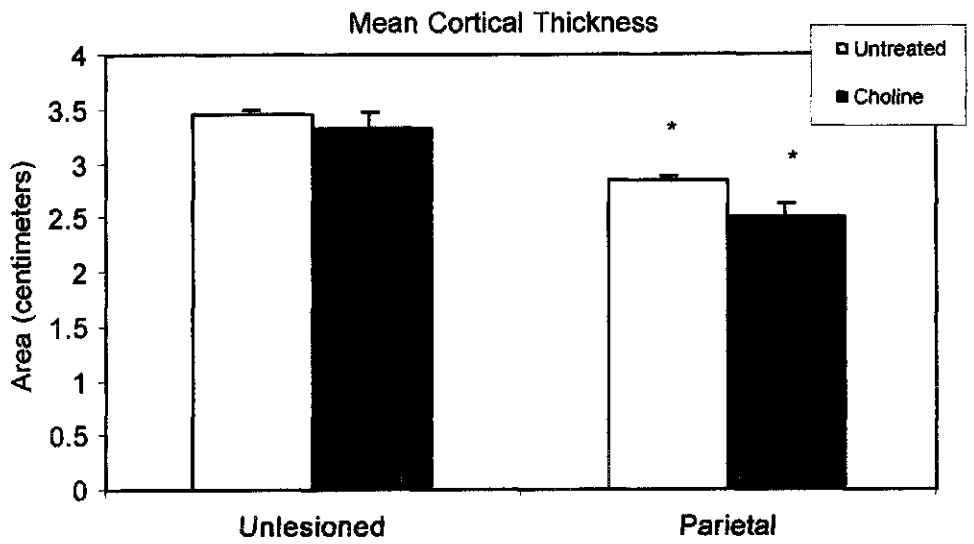


Fig.24. Mean cortical thickness for the five planes measured in animals with parietal lesions treated with choline. * Denotes statistically different from unlesioned groups ($p < .05$, or better).

Thalamic width:

The thalamus was smaller in both planes measured, and especially in the anterior plane. The choline treatment had no effect (see Table 10).

A two-way analysis of variance (ANOVA) for the anterior plane of measure revealed significant effects of lesion ($F(1,34)= 23.008$, $P< .0001$), but not of choline treatment ($F(1,34)= .254$, $P= .6175$), or for the interaction ($F(1,34)= .394$, $P=.5346$).

ANOVA for the posterior plane also found a main effect of lesion ($F(1,34)= 32.246$, $P< .0001$), but not of treatment ($F(1,34)= 3.270$, $P=.0794$), or for the interaction ($F(1,34)$, $P=1.150$, $P= .2910$.

Table 10. Mean measures of thalamic width.

	Anterior Plane (mm)	Posterior Plane (mm)
Untreated Lesion	8.720 ± .346 *	13.480 ± .128 *
Choline Lesion	7.857 ± .651	12.686 ± .199
Untreated Unlesioned	11.900 ± .400	14.750 ± .250
Choline Unlesioned	11.994 ± .239	14.547 ± .132

* Denotes statistically different from unlesioned groups ($p<.05$, or better).

Behavioral results

Open Field:

The choline treated animals displayed a lower amount of activity in horizontal activity and distance of movement than the untreated group; the largest effects were seen in the females. The parietal lesions also decreased activity and this was potentiated with the choline treatment, especially in the females.

Horizontal activity:

Because there were sex differences in activity, a three-way ANOVA was performed with lesion, sex and treatment factors. There was a main effect of choline treatment ($F(1,36)= 61.900$, $P< .0001$), lesion ($F(1,36)= 11.700$, $P= .0020$), and sex ($F(1,36)= 5.300$, $P= .0300$). There was no interaction between lesion and treatment groups ($F(1,36)= 2.400$, $P= .1300$), between sex and treatment groups ($F(1,36)= 2.400$, $P= .1300$), between sex and lesion ($F(1,36)= 2.600$, $P= .1200$), or for an interaction among all three factors ($F(1,36)= 1.300$, $P= .2600$) (see Fig.24).

Distance:

A three-way ANOVA between lesion, sex, and treatment groups indicated a main effect of choline treatment ($F(1,44)= 52.600$, $P< .0001$), lesion ($F(1,44)= 4.900$, $P= .0300$), and sex ($F(1,44)= 52.600$, $P< .0001$), as well as interaction between lesion and treatment groups ($F(1,44)= 11.100$, $P= .0017$), and between sex and treatment groups ($F(1,44)= 5.500$, $P= .0240$). There was no effect of the sex by lesion interaction ($F(1,44)= .0300$, $P= .6100$), or for an interaction among all three groups ($F(1,44)= .500$, $P= .4900$) (see Fig.26).

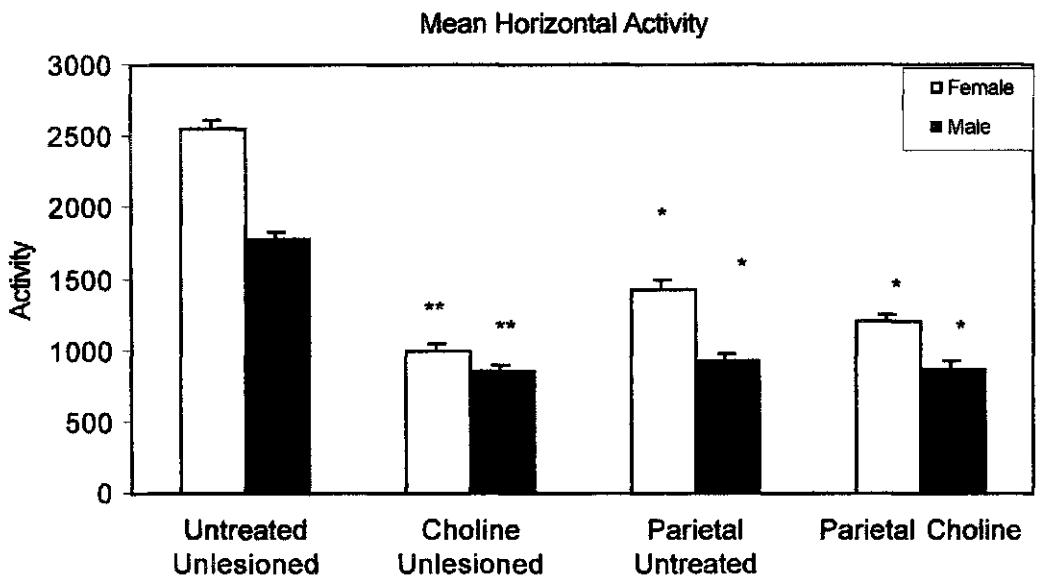


Fig. 25. Mean horizontal activity in the open field task of parietal lesion animals treated with choline. * Denotes statistically different from unlesioned groups (p<. 05, or better). ** Denotes statistically different from untreated groups (p<. 05, or better).

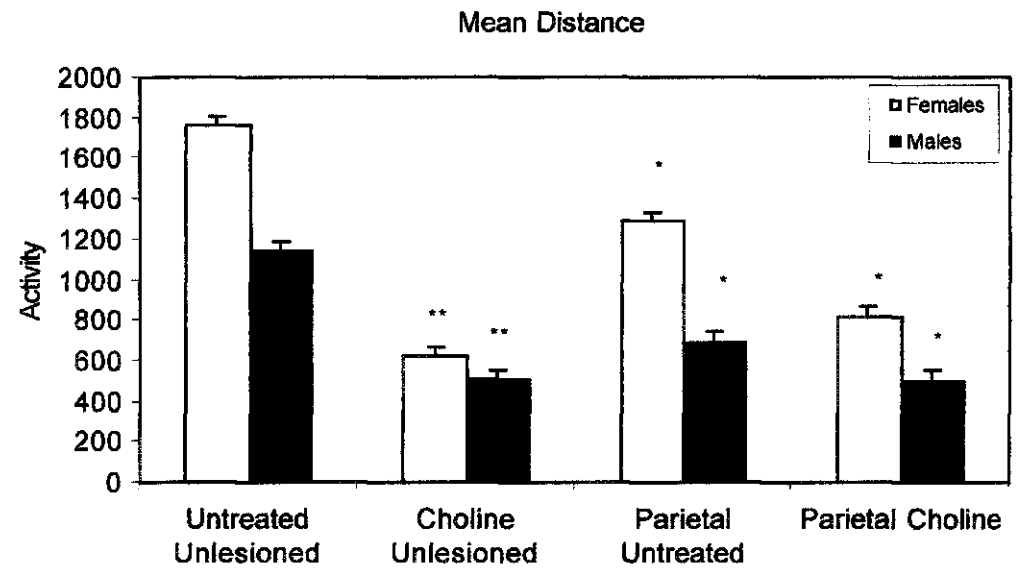


Fig.26. Mean distance in open field task of parietal lesion animals treated with choline.
* Denotes statistically different from unlesioned groups (p<. 05, or better).
** Denotes statistically different from untreated groups (p<. 05, or better).

Morris Water Task:

The general finding was that the parietal lesion groups were impaired relative to the unlesioned groups and choline treatment was without benefit (see Fig.26). The lesion effect persisted to the final trial block as the animals with lesions asymptote at around twenty seconds on days four and five.

A two-way ANOVA between lesion and choline treatment indicated a main effect of lesion ($F(1,52)= 16.400$, $P=.0002$), but not of treatment ($F(1,52)= .400$, $P= .5300$), or for the interaction ($F(1,52)= 0$, $P= 1.0$) (see Fig 27).

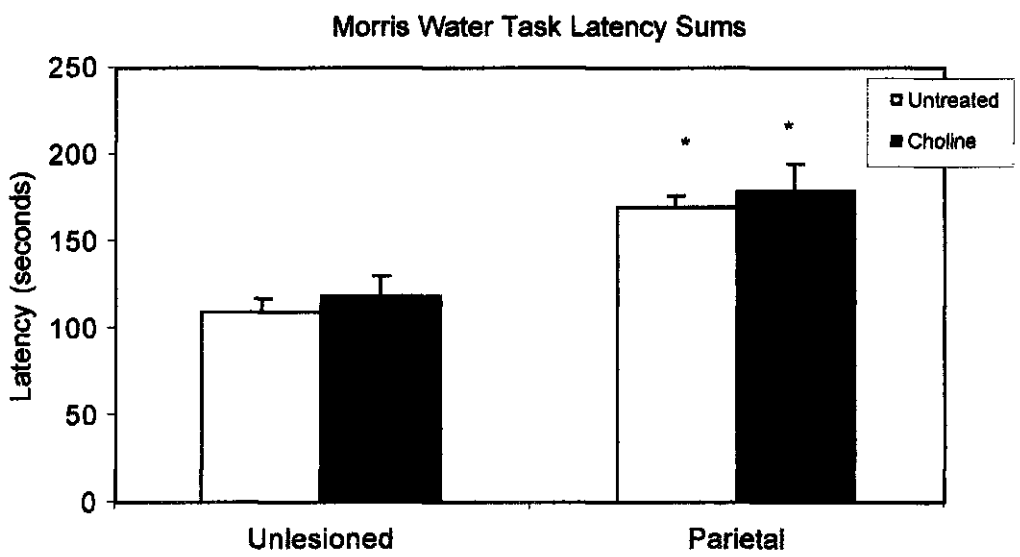


Fig.27. Morris water task sums of animals with parietal lesions treated with choline.
* Denotes statistically different from unlesioned groups ($p<.05$, or better).

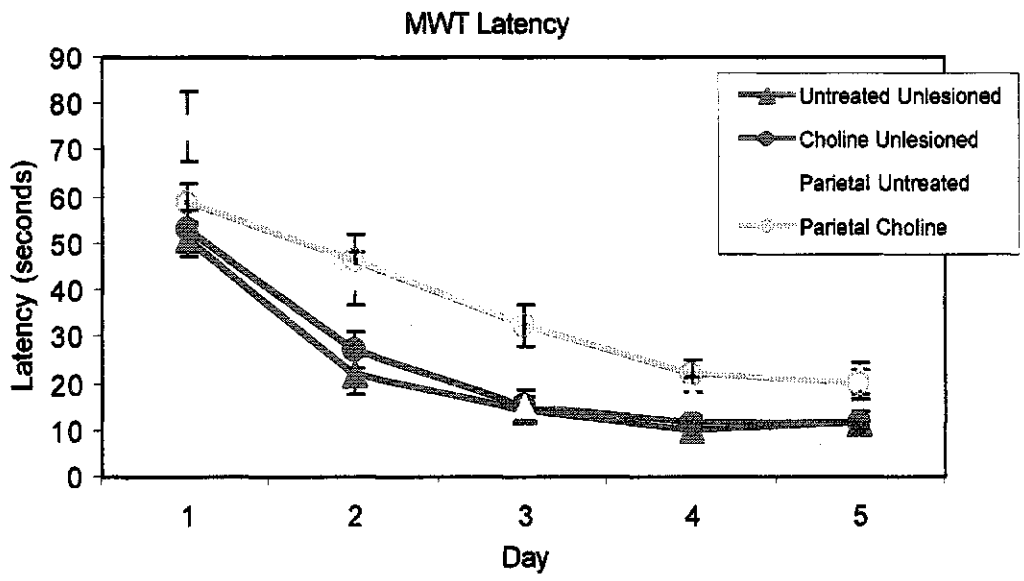


Fig.28. Morris water task latency over the five-day trial period. Animals had parietal lesions and choline treatment.

The probe trials at the end of training revealed that none of the groups was very accurate in their swim patterns. It appears that all groups spent most of their time in both quadrants one and four (see Fig.28).

A two-way ANOVA between groups for lesion and treatment did not reveal a main effect of lesion ($F(1,52)= .020$, $P= .8891$), or of treatment ($F(1,52)= .289$, $P= .5936$), or the interaction ($F(1,52)= 1.784$, $P=.1887$).

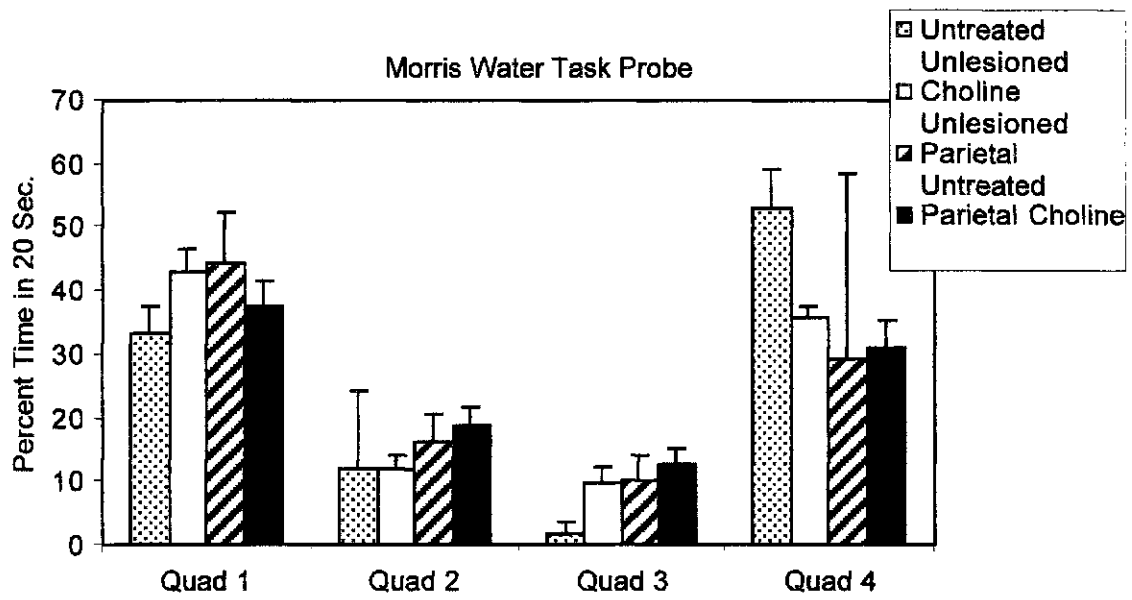


Fig. 29. Probe trials of Morris water task for parietal lesion animals treated with choline. Quadrant 1 was correct quadrant.

Tray Reaching:

No significant effects of lesion, or of choline treatment were found in the reaching task, for the parietal lesion animals (see Fig.29).

A two-way ANOVA was used between the lesion and treatment groups, which did not indicated a main effect of lesion ($F(1,40)= 2.631$, $P= .1126$), of treatment ($F(1,40)= 2.252$, $P= .1413$), or of an interaction ($F(1,40)= 2.575$, $P= .1164$).

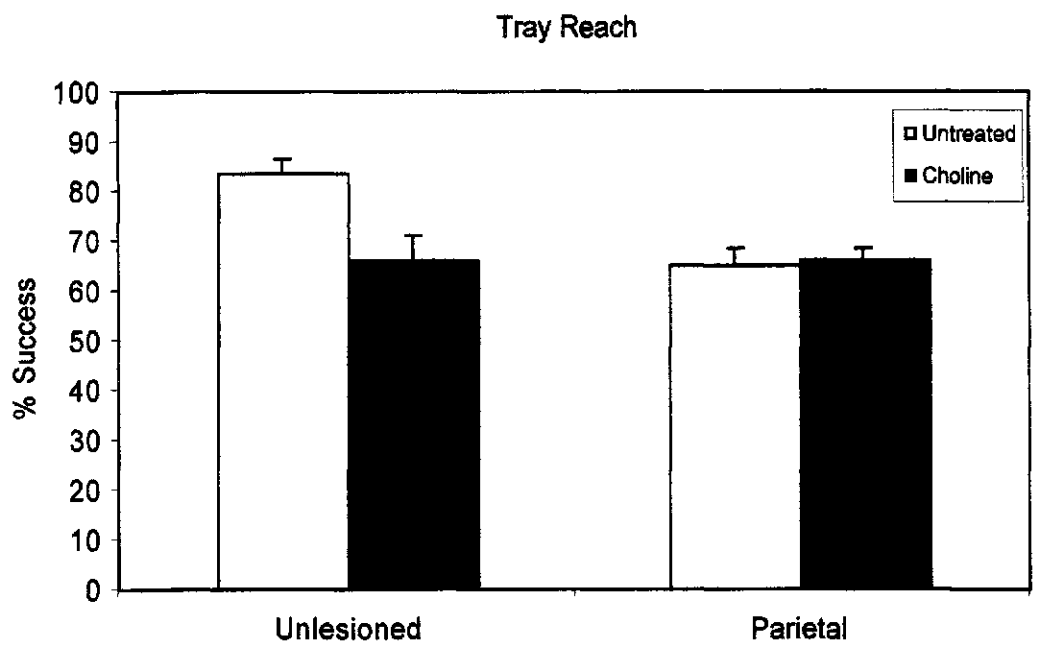


Fig.30. Summary of tray reaching task for parietal lesion choline-treated group.

Attention Shift:

As in Experiment 1A, the Attention Shift task did not reveal either lesion or choline treatment effects. The parietal animals did appear to be more impaired during the reversal of exemplars and distracted by baited arm changes, but this was not reflected in the statistical analysis of performance (see fig.31).

Two-way ANOVA was performed between lesion and treatment groups for each dimension in the attention shift task, which did not reveal any significant effects of lesion, or treatment, nor for interactions in all dimensions.

ANOVA for simple dimension (SD) indicated no effects of lesion ($F(1,36)= .353$, $P= .5561$), treatment ($F(1,36)= 2.454$, $P= .1260$), nor for the interaction ($F(1,36)= .353$, $P= .5561$).

ANOVA for compound dimension (CD) indicated no significant effects of lesion ($F(1,36)= 1.990$, $P= .1669$), of treatment ($F(1,36)= 2.678$, $P= .1105$), nor the interaction ($F(1,36)= .385$, $P= .5387$).

ANOVA for the first reversal (Rev1) indicated no significant effects of lesion ($F(1,36)= 2.164$, $P= .1500$), of treatment ($F(1,36)= 3.133$, $P= .0852$), or of an interaction ($F(1,36)= 1.791$, $P= .1892$).

ANOVA for the intradimensional shift (ID) indicated no significant effects of lesion ($F(1,36)= .785$, $P= .3815$), of treatment ($F(1,36)= 1.426$, $P= .2402$), or for the interaction ($F(1,36)= .785$, $P= .3815$).

ANOVA for the second reversal (Rev2) did not indicate any significant effects ($F(1,36)= 2.090$, $P= .1569$), of treatment ($F(1,36)= .354$, $P= .5554$), for the interaction ($F(1,36)= .354$, $P= .5554$).

ANOVA for the extradimensional shift (ED) did not indicate any significant effects of lesion ($F(1,36)= .024$, $P= .8781$), of treatment ($F(1,36)= .230$, $P= .2748$), or for the interaction ($F(1,36)= .166$, $P= .6864$).

ANOVA for the third reversal (Rev3) did not indicate any significant effects of lesion ($F(1,36)= 2.120$, $P= .1540$, treatment ($F(1,36)= 2.120$, $P= .1540$), or for interaction ($F(1,36)= 2.120$, $P= .1540$).

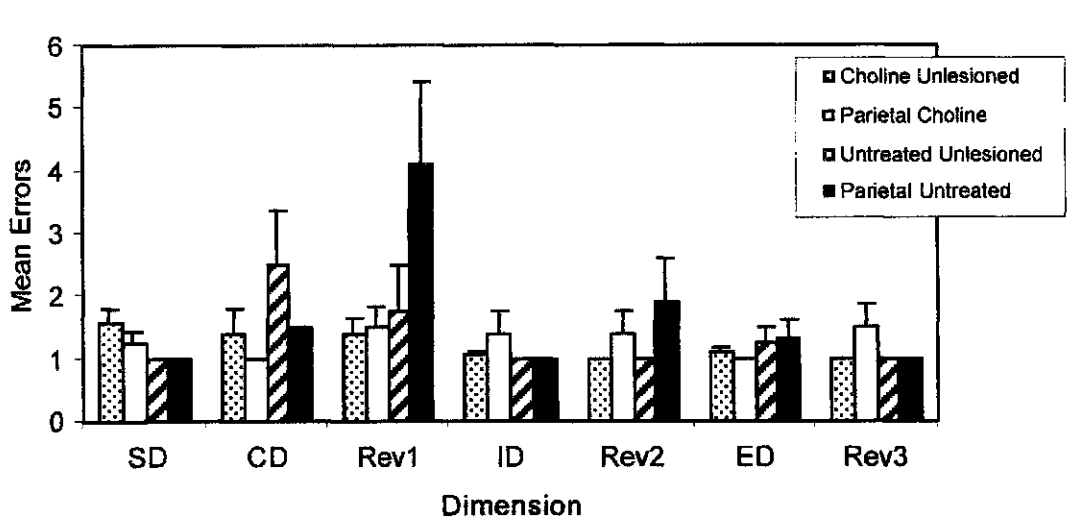


Fig. 31. Summary of all dimensions measured in the attention shift task. Animals had parietal lesions and choline treatment.

Discussion

The main results of the choline treatment studies were: 1) the perinatal lesions produced a decrease in the thickness of the remaining cortical mantle following both frontal and parietal lesions, 2) treatment with choline increased cortical thickness in the frontal lesion animals, which was not apparent in the parietal lesion animals, 3) both perinatal medial frontal and posterior parietal lesions produced deficits in spatial navigation. Medial frontal lesions also produced deficits in skilled reaching, and, 4) the choline treatment partially reversed the behavioral impairments in the frontal, but not the parietal operates. I will consider the behavioral and anatomical results separately.

Anatomical effects of early lesions and choline treatment

A consistent finding of studies of the effects of early cortical lesions in rats is that there is always a reduction in cortical thickness across the remaining cortical mantle. The current study confirms this finding. It was also shown that choline partially reversed the cortical thinning in the frontal operates, but not in the parietal operates and was correlated with functional recovery in the former, but not the latter group. Thus, it appears that this increase in cortical thickness might represent some reorganization of cerebral circuitry that can support functional recovery. The most likely explanation for the increase in cortical thickness is an increase in dendritic arborization in the remaining pyramidal and/or stellate cells in the cortical mantle. Previous studies have shown increased dendritic arborization (and increased cortical thickness) in rats with early medial frontal lesions that were later placed in complex environments (Gibb, 2000). Kolb & Tees (in

submission) also found an increase in dendritic arborization in a preliminary study of rats with medial frontal lesions that were treated with the same regime of choline as that in the current study.

An additional explanation for the improvement in behavioral performance of the choline-treated medial frontal lesion animals regards the visibly smaller lesion cavity size, which suggests that there potentially may have been a neurogenic response in the choline-treated rats. Previous studies have shown that basic fibroblast growth factor (FGF-2) does produce neurogenesis in the day three medial frontal lesion rats (Waite, 2003), giving a precedent for postinjury treatments stimulating neurogenesis. Demonstration of this possibility will require studies using a mitotic marker to identify new neurons. In addition, studies will also need to demonstrate that new neurons integrate with the rest of the brain and are functional.

In sum, the choline treatment, both prenatally and postnatally during lactation, facilitated functional recovery and morphological changes after perinatal medial frontal lesions, but not after posterior parietal lesions.

Behavioral effects of early lesions and choline treatment

If the cerebral cortex is injured during the first few days of life there is generally a poor functional outcome in adulthood, which is reflected in the impairments in a wide range of behavioral tasks (eg. Kolb, 1995). Although the behavioral analysis was limited to only a few measures in the current study, the present results generally confirm the findings of Kolb's group. One of the goals of the current study was to determine if treating infant rats with dietary choline could facilitate recovery from the early cortical

injuries. There is considerable literature showing that choline can influence cerebral organization and function in animals given choline during the pre- and postnatal periods of development. Meck, Smith and Williams (1988), and Kolb and Tees had shown in a preliminary unpublished study that choline treatment was beneficial to rats that had frontal lesions on postnatal day four. The current results showed a similar benefit in rats with day three frontal lesions, however, there was no significant benefit for animals with day three posterior parietal lesions.

The failure to find a therapeutic effect of choline in animals with posterior parietal lesions was disappointing, but is consistent with parallel evidence that rats with perinatal injury to the posterior parietal region have more extensive behavioral deficits (eg. Kolb, Holmes & Whishaw, 1987). Furthermore, unlike day ten lesions elsewhere in the cortex, rats with posterior parietal lesions do not show increases in dendritic morphology, a result that may account for the relatively poorer functional outcomes (Kolb & Cioe, 1999).

The reason for the differential effect of choline on the frontal- versus parietal-injured brains remains a matter of speculation. It is known for example, that choline supplementation increases the endogenous production of nerve growth factor (NGF) and it is possible that the effect of choline on the frontal lesion animals is via its actions on NGF. Nerve growth factor is known to stimulate dendritic length and an increase in spine density after cortical lesions in adult rats (Kolb et al., 1997), which could provide a mechanism for its beneficial effects after frontal lesions. It is not clear however, why animals with posterior parietal lesions did not show the same beneficial effects supported by NGF. Treating rats with perinatal lesions of the medial frontal, or posterior parietal

lesions with FGF-2 has shown to have greater recovery with medial frontal lesions than posterior parietal lesions (Waite, 2003).

4. Experiment 2

The Effects of Vitamin Supplement Treatment on Recovery From Perinatal Cortical Lesions

The general plan of Experiment 2 was similar to Experiment 1. Pregnant rats were to be given a vitamin supplemented diet prior to conception and continued throughout their pregnancy. As in Experiment 1, approximately half of the pups would receive medial frontal, or posterior parietal lesions on postnatal day 4. Unfortunately, this did not prove possible. The supplement appeared to retard pregnancy, as the initial pairing of males and females for two to three months did not result in successful pregnancies.

"Plan B" thus was to introduce the supplement approximately one week after the introduction of males to females, potentially to allow conception to occur before treatment. The result of this plan was only one pregnancy and the litter consisted of only three pups (2males, 1 female). These animals were maintained on the supplement throughout life and as adults, the animals remained paired. This resulted in two pregnancies and two litters of animals. The second generation of animals remained on the supplement throughout their lives as well and one litter had behavioral testing in adulthood (see Appendix F).

"Plan C" was to begin supplement administration on day of birth of the pups and continue the supplemented diet throughout the life of the pups (see Fig.31).

This strategy was successful. As in Experiment 1, the analysis of the results for the medial frontal and posterior animals is presented separately.

Plan A: 2 pairs male/female. Introduced fortified diet 2 days prior to gestation

→ No pregnancy

Plan B: 2 pairs male/female. Introduce fortified diet approximately 1 week after gestation

→ 3 pups: 2 Males, 1 Female

↘ ↙

→ Pregnancy 1: 10 pups: behavioral tasks (Appendix F)

→ Pregnancy 2: 10 pups: Western Blot (Incomplete)

Plan C: Introduce fortified diet on day of birth of full litters

→ 4 full litters: 3 litters: brain lesions and behavior

1 litter: no lesions and Western Blot (Appendix D)

Fig. 32. Flowchart of subjects in the vitamin supplement experiment.

Experiment 2A

Vitamin Supplement Treatment with Frontal Lesions

The Effects of Diet Supplementation on Recovery from Perinatal Medial Frontal Injury

Method

Subjects

A total of seventy-seven Long Evans rats were used for Experiment 2A. This group of rats was used in three behavioral studies. At the age of 60 days the animals began behavioral testing. Any sex differences that are significant to the experimental results are reported.

Table 11. Number of rats used in Experiment 2A.

	Untreated Unlesioned		Supplement Treatment	
Sex	Female	Male	Female	Male
Unlesioned	n= 5	n=12	n= 8	n=14
Frontal Lesion	n= 6	n=11	n= 8	n=13

Treatment preparation

The vitamin supplement was incorporated into food pellets for the rats to eat as their regular diet ad libitum. The supplemented diet was the only food source available to the treatment groups. The concentration of the supplemented food pellets was determined according to a similar method used in the clinical studies. A mean daily intake of thirty capsules per day was applied to an average daily food intake of the human subjects, of approximately two to three pounds per day. This concentration was then extrapolated to a theoretical mean intake of a maximum of thirty grams of food per day, for each rat. (Hardy, 2002). The vitamin and mineral supplement was prepared into rat feed by the research laboratories at the Lethbridge Research Station. The standard laboratory rat

chow used is 5001 Rodent Diet, prepared by Purina Laboratories (see analysis in Appendix D).

The percentage of dietary fat, carbohydrate and protein between both diets were very similar, both of which have sources of vitamins, minerals, and essential fatty acids. The main difference between the two diets is the additional vitamins and minerals, but importantly that the minerals in the supplement E.M. Power+ are chelated to increase their bioavailability. In addition to vitamins and minerals are amino acids and herbal ingredients. Grape seed (extract) is included in the formulation and known to provide essential omega-3 fatty acids (see Appendix D).

Drinking water was regular tap water ad libitum.

Surgical procedures

The animals were all anesthetized by means of cooling in a Thermatron set at -5°C on postnatal days two and three. Cooling occurred for approximately 10 minutes, or until the rat pups were immobile and whitish in color. These parameters of immobility and color have been correlated with appropriate rectal temperatures ranging from 18-20°C. Once the skin was incised, the bone of the skull was removed from bregma to lambda using iris scissors. Frontal cortex lesions required removal of the midline region of the cortex, anterior to bregma, by gentle aspiration. Suturing of the wound was done using silk thread. The sham control animals underwent all procedures except for bone incision and lesion aspirations. After completion of surgery, animals were slowly warmed using body heat by holding the pups in warm hands until they began moving, then were returned to their mothers.

Behavioral procedures

Open field Activity:

The open field test was performed in a plexiglass box (42 cm×42 cm×32 cm *h*) attached to a Digiscan Analyzer, and has a lid on the top of the box. At the base of the box and approximately at 1/3 in height, is a band of sensors around the perimeter of the box to record a variety of activity measures. A printer was connected to the open field apparatus, which resided in the same room, and was programmed to print activity behavior at two -minute intervals for ten minutes. At the end of the ten-minute period, the rats were placed back into their holding tubs and the number of defecations remaining in the apparatus were noted. Data collection was completed for each rat at the end of the 10-minute period.

Morris Water Task:

The maze used in the water task is a circular pool has a diameter of 1.5m and a height of 0.5m deep. The walls of the pool were painted white and the water rendered opaque with powdered milk. The platform (11×12 cm.) was placed in the northwest quadrant of the pool and submerged about 1.5cm. below the water's surface. Each rat was placed into the pool facing the wall in one of four random starting positions (east, west, north, or south). All animals were selected randomly into groups of eleven, to prevent any potential litter effects. The no treatment control group swam at a separate date.

The animals swam four trials each day for five days. Each trial had a different starting position, using all four points of direction each day, and changed in order of direction each day. Each trial consisted of a maximum of ninety seconds during which

the rats had to find the hidden platform. After ninety seconds, the trial ended and rats that did not find the platform within that time were placed on the platform for ten seconds. Rats that did find the platform within the ninety-second interval were able to stay on the platform for ten seconds before being removed from the pool. All swim trials were recorded by means of a video camera mounted from the ceiling directly above the pool. Data recorded from the camera were analyzed by a computer program (Water 2020). On the sixth day, a probe trial was given. During the probe trial, the platform was removed and the rats started at the north position. Rats swam for twenty seconds and each trial was recorded into the computer program for analysis.

Tray Reaching:

The procedure for the tray reach task was devised from Whishaw's skilled reaching task (Whishaw, O'Connor & Dunnet, 1986). The test cages consist of clear plexiglass walls with a closable lid (20× 28× 26 cm). The front wall and floor of each cage was constructed of stainless steel bars. The floor was cross-linked with 2mm bars, and the front had vertical 3mm bars, extending 9mm apart from one edge of the cage to the other. Mounted in front of each set of three cages was a stainless steel tray, which held small pellets of chicken feed. The tray was suspended on runners at the sides of each cage set, and is positioned about 5mm away from the faces of the cage.

Rats were food deprived daily by feeding 15g. to each female and 20g. to each male. These food portions have been determined to slowly reduce body weight, which should not exceed in a loss of 15% body weight. The rats were fed a standard lab chow diet and were food deprived for six days at a time. On the sixth day, after training, rats

were fed ad libitum to prevent any potential further weight loss. Food deprivation began again after one day off.

Reaches were considered unsuccessful if the rats did not maintain a successful grasp at the tray, or during transport of the pellet back to the rat's mouth. If the rat had extended an arm towards the tray, but did not touch the food pellets, the reach attempt was not considered for scoring. When the animals had sufficiently learned to reach for the pellets, the rats were filmed by a videocamera individually for ten minutes. Scoring of reach attempts and successes were determined by replay of the video for seven minutes per rat. Attempts included both successes and failures. Overall success was determined as a percentage of successes verses attempts.

Anatomical procedures

At the completion of behavioral testing, rats were given an overdose of sodium pentobarbital (0.5ml-0.7ml) until they reached a comatose state. At this point, they were perfused intracardially, using 0.9 % saline solution. Rat brains destined for Cresyl Violet staining, were then perfused with 4% paraformaldehyde. Rongeurs were used to remove the brains and were weighed immediately following removal from the skull. Fifty-five rat brains were designated for Cresyl Violet stains. The remaining twenty-two male rats were perfused for Golgi staining. For Cresyl Violet preparations, the brains were placed in small bottles containing 4% paraformaldehyde in 30% sucrose solution.

For Golgi preparations, brains were weighed at the end of saline perfusions and placed in small bottles containing Golgi solution. These brains were processed following procedures devised by Gibb and Kolb (1998).

The brains for Cresyl Violet staining were sliced on a Cryostat 2800 Frigocut, which maintains tissue at -21°C. Every 4th slice of the treatment brains was taken at 40 μm thickness throughout the frontal lesion site. Thereafter, every 7th slice was taken. All slices were placed on slides fixed with a film consisting of 1.0% gel and 0.2% chromatin. Once the brains were dried to the slides, the sections were stained with Cresyl Violet.

Brain Weight:

The measures of brain weight are obtained by weighing each brain directly after removal from the skull of the perfused rat. Thereafter, brain weights are compared between groups to determine an estimate of tissue lost as a result of the lesions. Care was taken to ensure consistency of brain removal before weighing. The spinal cord was cut along the caudal edge of the cerebellum, the cerebellar paraflocculi were removed, the optic nerve was cut 1-2 mm anterior to the optic chiasm, and remaining dura was stripped off the brain's surface. At the front end of the brain, the olfactory bulbs are cut at 1.5-2.5 mm from the frontal cortex.

Cortical Thickness:

After slide preparation, the brains were viewed and assessed at a magnification of 17.5 × with a Zeiss DL 2 POL petrographic projector.

Cortical measurements were made using a clear plastic ruler for both the supplement treated groups and the untreated groups. Three different cortical measurements, for each hemisphere, were determined at five planes. A standard technique was followed:

Zilles (1995) (see Fig. 32)

Plane 1: AID, Par1, Fr2
(most anterior plane
of caudate putamen)

Plane 2: Par2, Par1, Fr1
(anterior commissure)

Plane 3: GI/DI, Par1, Fr1
(hippocampus)

Plane 4: Te1, Oc2L, RSA
(posterior commissure)

Plane 5: Te1, O1B, Oc2ML
(hippocampus)

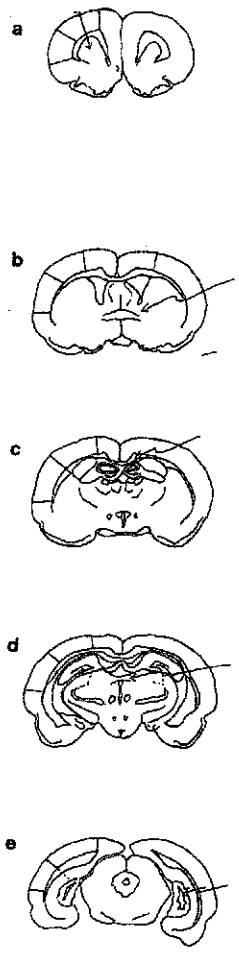


Fig. 33. Zilles (1995) Lateral, central, and medial regions measured across five planes for cortical thickness.

Thalamic width:

Thalamic width was measured at two planes. The first was the anterior plane, at the point of a predominant MD nucleus, and when the hippocampus CA fields and

dentate gyrus had become full. The second plane, was at the last slice which had a full thalamus and at the beginning of the posterior commissure.

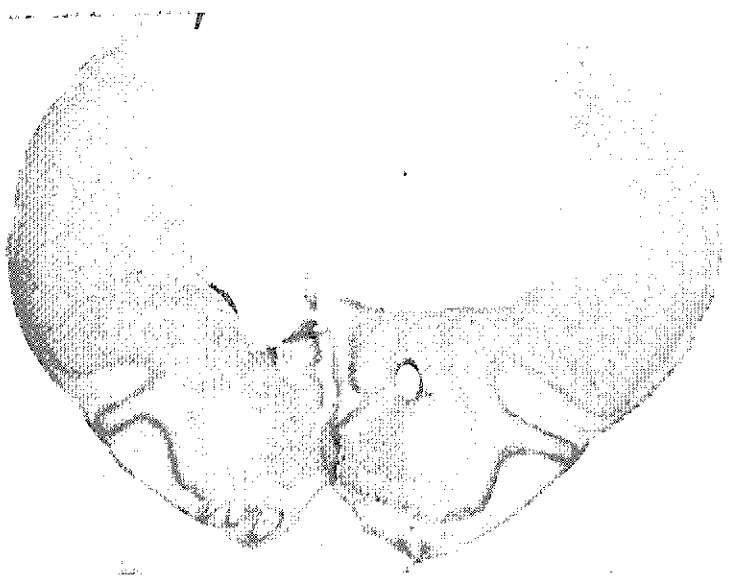
Results

Anatomical results

The frontal lesion consistently removed the midline region of cingulate 1 and 2 (Cg1, Cg2) areas with motor regions M1 and M2, or Zilles (1995) Fr1 and Fr3. The prelimbic (PrL), or Zilles (1995) cingulate 3 (Cg3), and the infralimbic (IL) areas were usually gone, as well as variable amounts of the frontal association (FrA), or Zilles frontal 2(Fr2) areas. Sections of the medial orbital (MO), ventral orbital (VO) and lateral orbital (LO) cortex of the ventral frontal areas were also removed.

The vitamin supplement treatment had an obvious effect upon the lesion site, as most brains showed filling in of cortical tissue, including some of the most anterior areas of what was a presumptive FrA region (Zilles Fr2), PrL (Zilles Cg3) and IL (see Fig. 33). Very often, the ventral areas, containing portions of the orbital cortex, was present as a fuller cortex, than what is normally seen. The caudate putamen also appeared larger in the supplement treatment brains that regenerated large amounts of cortical tissue.

Thalamic degeneration, observed as a reduction in size of the thalamus, was consistent in the anterior sections. Animals that had received the supplement however, had larger nuclear areas and many more cells than in the brains of untreated animals. Thus, cortical tissue loss and thalamic degeneration appeared less extensive in the brains of animals that received the supplement treatment.



Untreated

Supplement Treatment

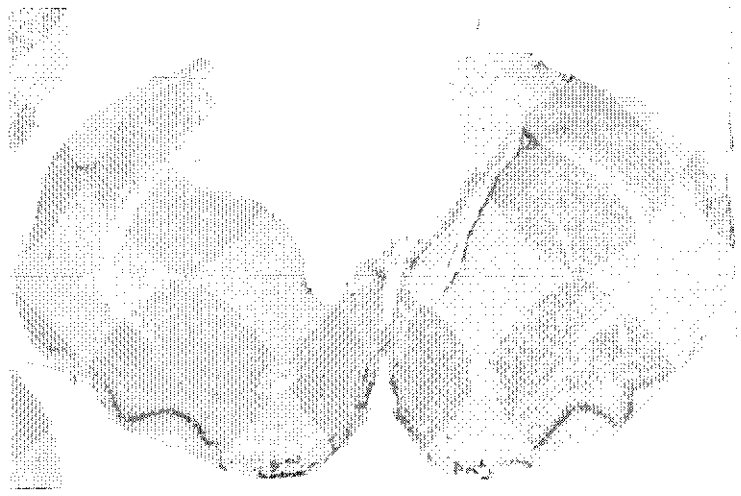


Fig. 34. Examples of frontal cortical lesions in animals treated with the vitamin supplement.

Brain Weight:

The vitamin supplement appeared to selectively increase brain weight in the lesion animals relative to the control animals in which there was little effect of the supplements. Differences in brain weight are usually found between fixation methods therefore, the analysis has been done separately for each method.

Cresyl Violet Preparation:

A three-way ANOVA with lesion, supplement treatment, and sex as factors showed a main effect of lesion ($F(1,37)=41.7$, $P<.0001$) and sex ($1,37)=10.3$, $P<.003$) but not of treatment ($F(1,37)=1.35$, $P=.25$. There was, however, a significant lesion × treatment interaction ($F(1.37)=7.7$, $P=.009$), a result that reflects the selective effect of the treatment on the lesion brains. The other interactions were not significant (p's>.2).

Table 12. Brain Weight for Cresyl Violet Preparations:

Group	Female	Male
Untreated lesion	1.730 ± .030 (n=6) *	1.768 ± .107 (n=5) *
Supplement lesion	1.761 ± .030 (n=8)	1.843 ± .020 (n=10)
Untreated Unlesioned	1.948 ± .020 (n=5)	2.162 ± .035 (n=6)
Supplement Unlesioned	1.879 ± .040 (n=8)	1.970 ± .078 (n=9)

* Denotes statistical significance from unlesioned groups (p<.05, or better).

Golgi Preparations:

The supplement treatment indicates an increase in brain weight in animals with frontal lesions. (Only male rats were prepared for Golgi-Cox staining).

A two-way ANOVA indicated a main effect of lesion ($F(1,19)= 61.100$, $P< .0001$), as well as significant effects for the interaction ($F(1,19)= 7.800$, $P= .0100$), but not of treatment ($F(1,19)= 1.670$, $P=.2100$). These results reflect the treatment benefits were found in male animals with frontal lesions.

Table 13. Brain Weight for Golgi Preparations:

Group	Male
Untreated lesion	1.764 ± .020 (n=6) *
Supplement lesion	1.902 ± .030 (n=5) **
Untreated Unlesioned	2.123 ± .030 (n=6)
Supplement Unlesioned	2.072 ± .050 (n=5)

* Denotes statistical significance from unlesioned groups (p<.05, or better).
** Denotes statistically different from untreated lesion group (p<. 05, or better).

Cortical thickness:

The brains that were used to measure and analyze cortical thickness and thalamus were those that were prepared for Cresyl Violet staining.

Data for all five planes were analyzed by a two-way ANOVA collectively to determine the overall effects on cortical thickness between treatment and no treatment groups.

Data for all five planes were analyzed collectively by repeated measures ANOVA to determine the overall effects on cortical thickness and lesion (see Fig.34). A significant interaction between mean cortical thickness and lesion was found in the analysis therefore, two-way ANOVAS were performed for each plane.

A two-way ANOVA for the first cortical plane showed a main effect of lesion ($F(1,83)= 51.256$, $P<.0001$), and of treatment ($F(1,83)= 4.419$, $P=.0386$), but not for the interaction ($F(1,83)= 2.748$, $P=.1011$).

ANOVA for the second plane revealed no main effect of lesion ($F(1,83)= .891$, $P= .3479$), a main effect of treatment ($F(1,83)= 63.567$, $P< .0001$), but not for the interaction ($F(1,83)= 1.032$, $P= .3126$).

ANOVA for the third plane indicated a main effect of lesion ($F(1, 83)= 10.303$, $P=.0019$), but not of treatment ($F(1,83)= 1.874$, $P= .1747$), and not for the interaction ($F(1,83)= .192$, $P=.6620$).

ANOVA for the fourth plane indicated a main effect of lesion ($F(1,83)= 22.010$, $P<.0001$), as well as for the interaction ($F(1,83)= 5.356$, $P= .0231$), but not of treatment ($F(1,83)= 1.355$, $P=.2512$).

ANOVA for plane five indicated a main effect of lesion ($F(1,83)= 8.463$, $P= .0046$), and of treatment ($F(1,83)= 14.707$, $P= .0002$), but not for the interaction ($F(1,83)= 2.312$, $P= .1322$).

Overall, the frontal lesion group treated with the supplement had thicker cortices than the untreated animals throughout all five planes measured (see Fig.35).

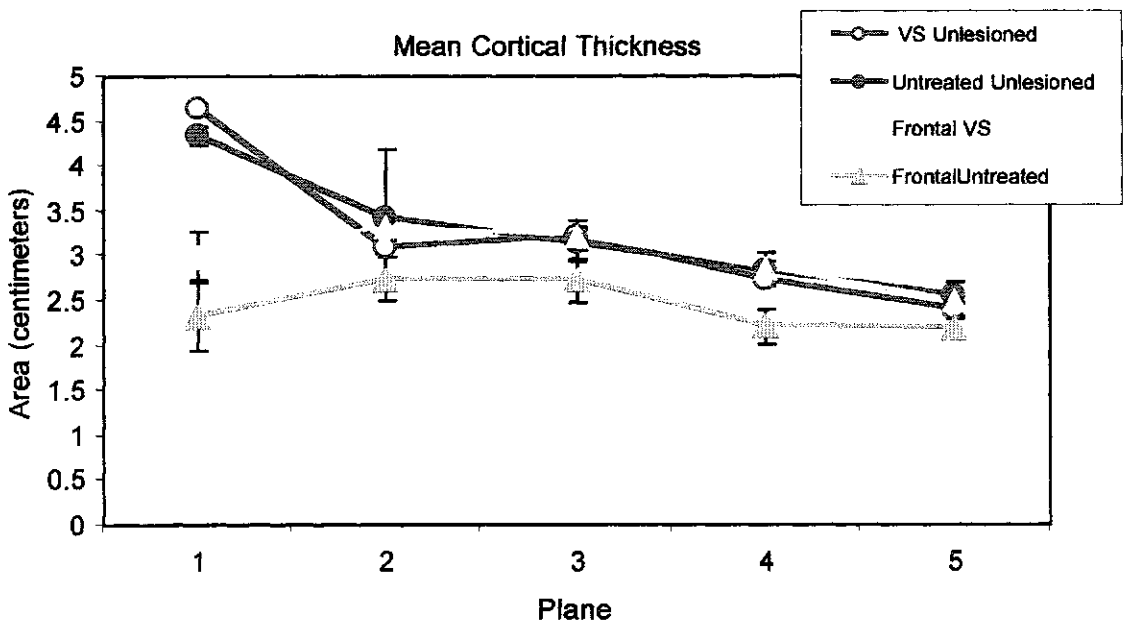


Fig. 35. Cortical thickness for each of five planes measured in animals with frontal lesions treated with the vitamin supplement.

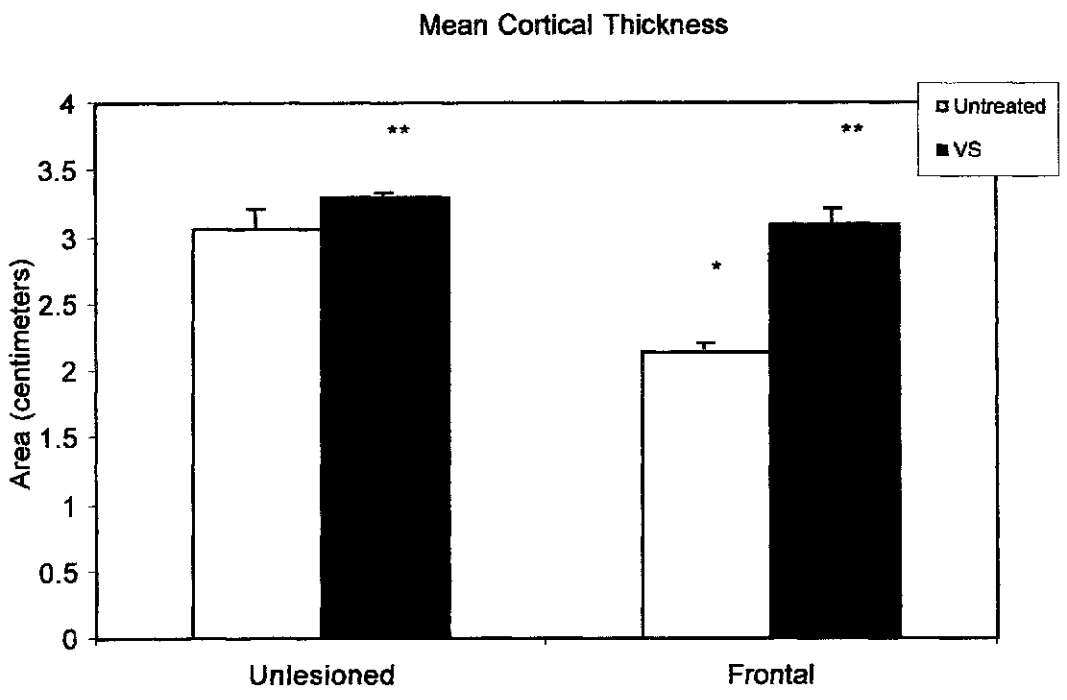


Fig. 36. Mean cortical thickness for the five planes measured in animals with frontal lesions treated with the vitamin supplement. * Denotes statistically significant from unlesioned groups (p<. 05, or better). ** Denotes statistically different from untreated groups (p<. 05, or better).

Thalamic width:

The frontal lesions produced a reduction in thalamic width in the posterior, but not the anterior plane. There was no effect of supplement treatment (see Table 14).

ANOVA for the anterior plane of measure did not indicate any significant effects of lesion ($F(1,27)= 2.032$, $P=.1655$), of treatment ($F(1, 27)= .305$, $P= .5850$), or for the interaction ($F(1,27)= .712$, $P=.4063$).

ANOVA for the posterior plane of the thalamus did indicate a main effect of lesion ($F(1,27)= 25.167$, $P< .0001$), but not of treatment ($F(1,27)= .943$, $P=.3401$), or for the interaction ($F(1,27)= 1.455$, $P=.2382$).

Table 14. Mean measures of thalamic width.

	Anterior Plane (mm)	Posterior Plane (mm)
Untreated Lesion	11.120 ± .282	12.940 ± .277 *
Supplement Lesion	11.600 ± .284	13.008 ± .238
Untreated Unlesioned	11.900 ± .400	14.750 ± .250
Supplement Unlesioned	11.800 ± .284	14.117 ± .104

* Denotes statistical significance from unlesioned groups ($p<.05$, or better).

Behavioral results

Open Field:

The frontal lesion decreased the activity levels in the animals, which was further reduced by the supplement treatment. The supplement treatment reduced activity levels in the unlesioned group as well.

Horizontal activity:

ANOVA between lesion and treatment groups for horizontal activity did have a main effect of treatment ($F(1,49)= 61.000$, $P< .0001$), as well as significant effects for the interaction between groups ($F(1,49)= 9.200$, $P= .0040$), but did not indicate a main effect of lesion ($F(1,49)= 2.800$, $P= .1000$) (see Fig.36).

Distance:

ANOVA between lesion and treatment groups for distance also found main effects of treatment ($F(1,49)= 76.800$, $P< .0001$), as well as for interaction ($F(1,49)= 4.500$, $P= .0400$), but not of lesion ($F(1,49)= 0.700$, $P= .3900$). The interaction reflects

the fact that the supplement treatment effects were significant for the lesion group, but there was no lesion effect because the supplement effect was larger in the unlesioned animals (see Fig.37).

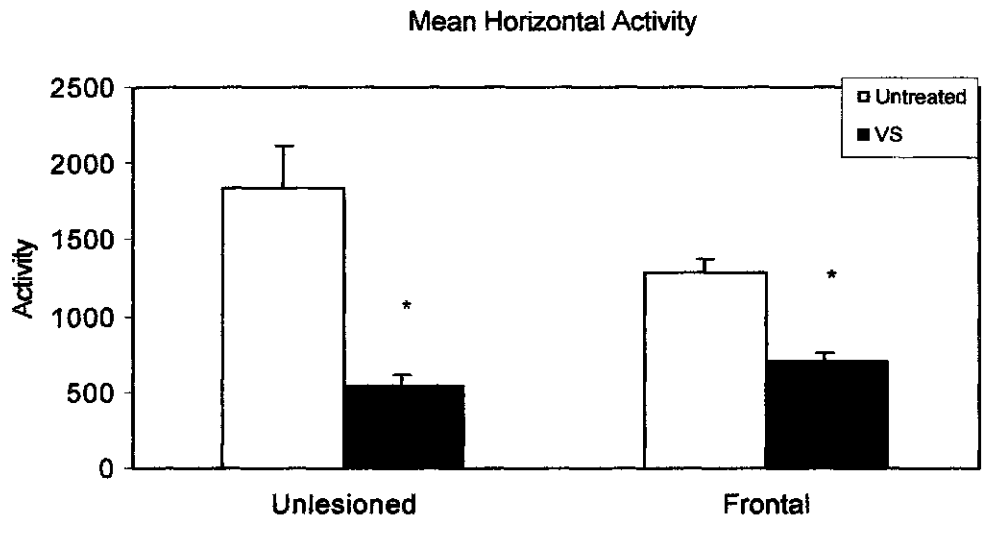


Fig. 37. Mean horizontal activity of frontal lesion animals treated with the vitamin and mineral supplement. * Denotes statistically different from untreated groups ($p < .05$, or better).

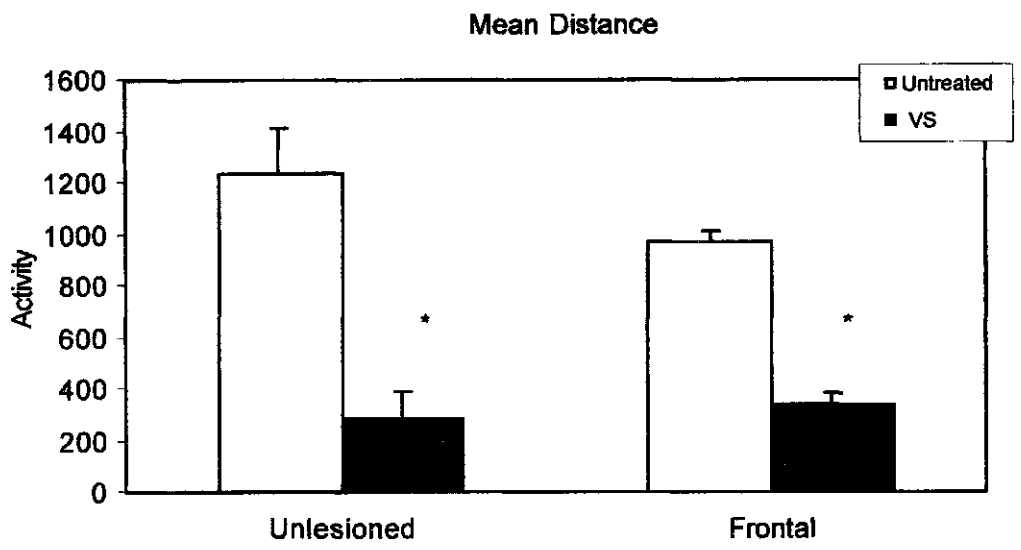


Fig. 38. Mean distance of frontal lesion animals treated with the vitamin and mineral supplement. * Denotes statistically different from untreated groups (p<. 05, or better).

Morris water task:

The rats with frontal lesions were impaired at the task, and this impairment was reversed with the supplement treatment, suggesting a complete recovery of function (see Fig.38).

Repeated measures ANOVA indicated significant effects of lesion ($F(1,59)= 9.531$, $P= .0031$, treatment ($F(1,59)= 12.018$, $P= .0010$, and an interaction between lesion and treatment groups ($F(1,59)= 4.868$, $P= .0313$, as well as a main effect for the category of latency ($F(4,236)= 89.004$, $P< .0001$. All other interactions were not significant ($p> .10$).

A two-way ANOVA found main effects of lesion ($F(1,59)= 9.531$, $P= .0031$, treatment ($F(1,59)= 12.018$, $P= .0010$ and for an interaction between groups ($F(1,59)=$

4.868, P= .0313. The interaction reflects the greater treatment effects of the supplement in the animals with lesions (see Fig.39).

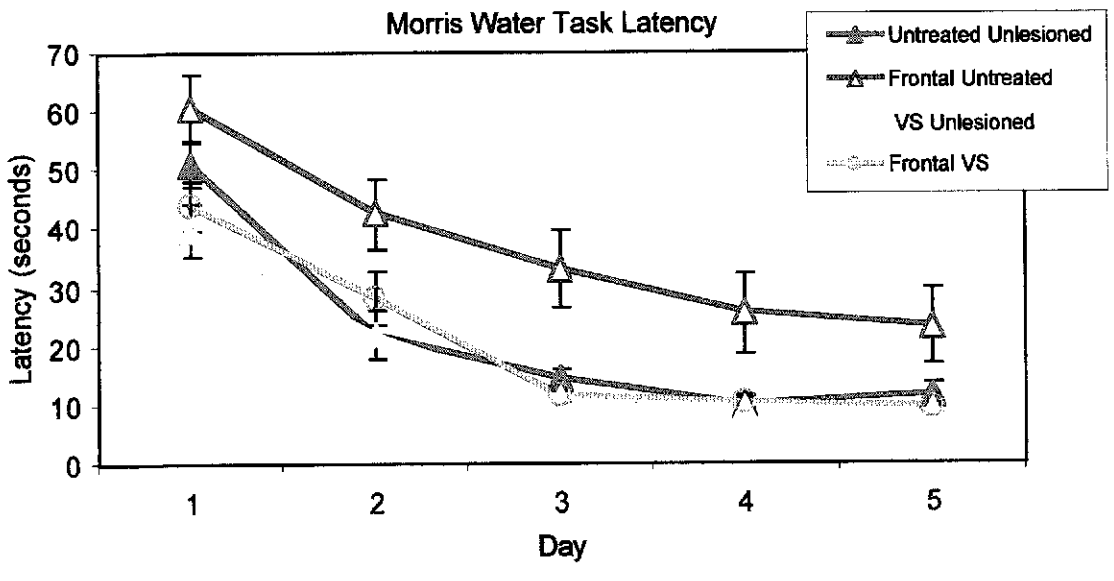


Fig. 39. Morris water task latency over five-day training period. Animals had frontal lesions and were treated with the supplement.

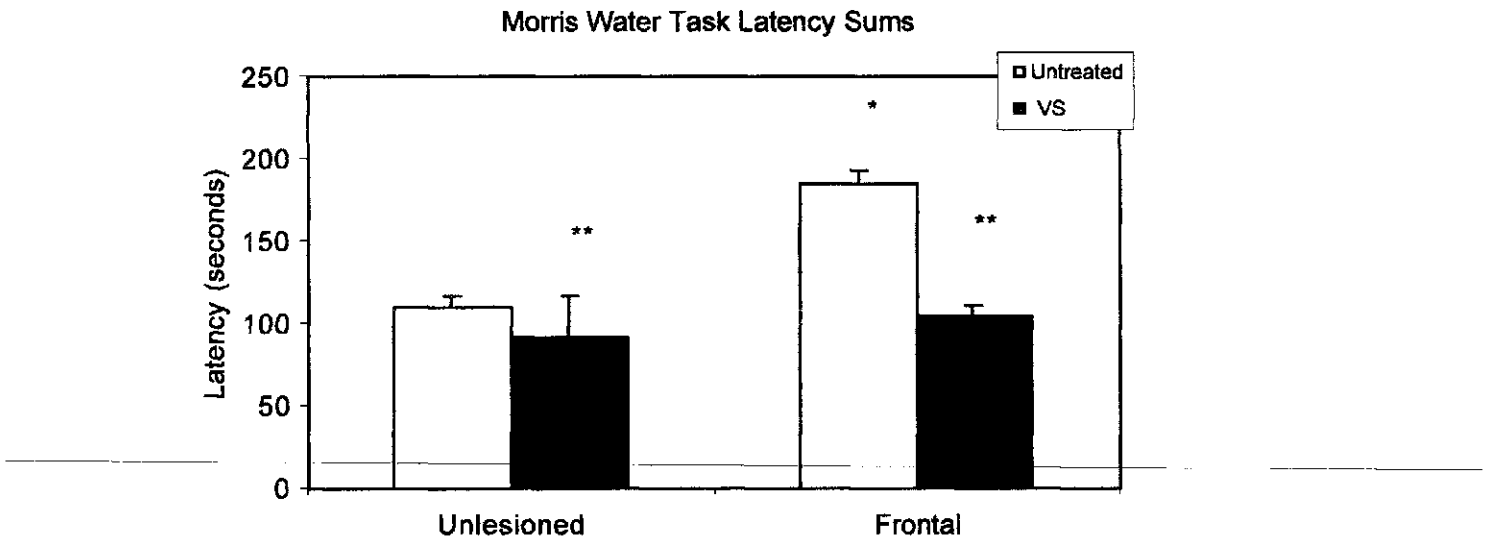


Fig. 40. Morris water task latency sums of frontal lesion animals treated with the vitamin supplement. * Denotes statistically different from unlesioned groups (p<. 05, or better). ** Denotes statistically different from untreated groups (p<. 05, or better).

The probe analysis for the second experiment was not completed by the computer data processes due to computer malfunction in tracking of the rats' swim patterns. The probe was therefore graded from scores of one to four, according to a hand drawn trace of the swim path (see Fig.40). These results were analyzed by a two-way ANOVA, which indicated that both lesion groups spent less time than the control group in the correct quadrant, quadrant one.

ANOVA for the lesion groups was significant and found a main effect of lesion ($F(1,29)= 5.255$, $P= .0293$, but no significant effects of treatment ($F(1,29)= .250$, $P= .6206$, or the interaction ($F(1,29)= .730$, $P= .3999$.

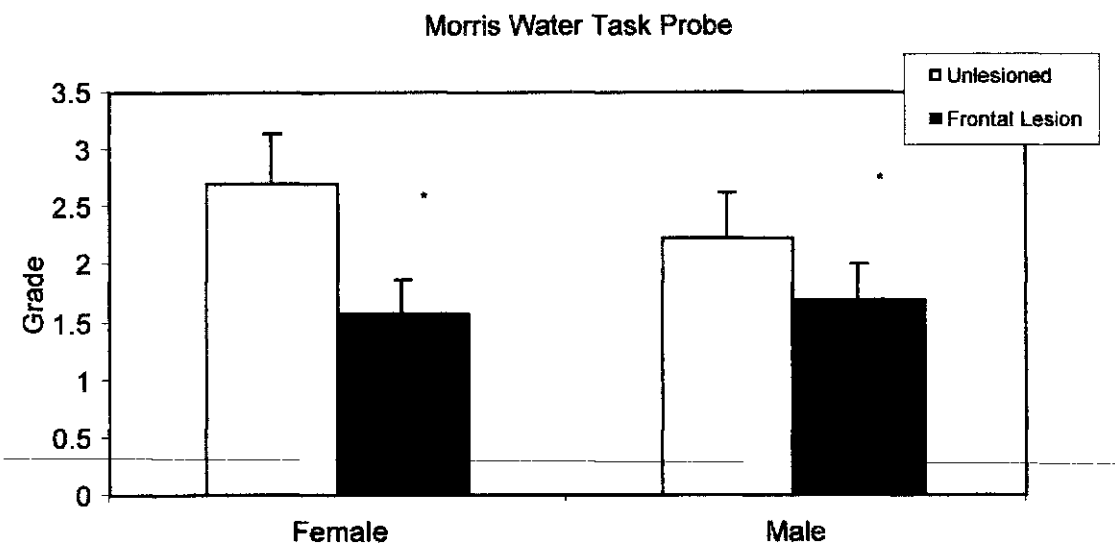


Fig. 41. Probe trials of frontal lesion animals treated with the vitamin supplement. A grade of1 indicates least amount of time spent in correct quadrant, quadrant 1. A grade of 4 indicates most time spent in quadrant 1. All animals are within the treatment group. * Denotes statistically different from unlesioned groups (p<. 05, or better).

Tray Reaching:

Rats with frontal lesions were impaired with the reaching task and this deficit was partially reversed with the supplement treatment (see Fig. 41).

A two-way ANOVA between revealed a main effect of lesion ($F(1,59)= 88.740$, $P< .0001$, and the interaction ($F(1,59)= 10.437$, $P= .0020$. There was no effect of treatment ($F(1,59)= 1.676$, $P=.2004$. The interaction reflects the selective effect of the supplement treatment.

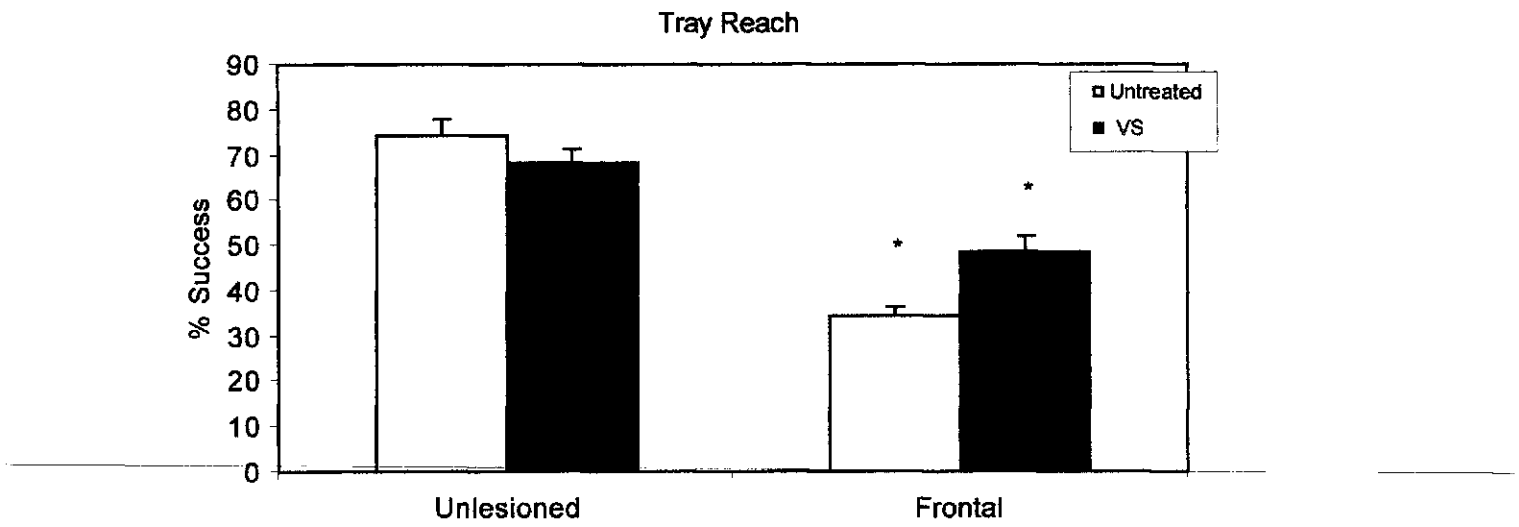


Fig. 42. Summary of tray reaching task of frontal lesion animals treated with the vitamin supplement. * Denotes statistically different from unlesioned groups ($p < .05$, or better).

Experiment # 2B

The Effects of a Dietary Vitamin and Mineral Supplement on Recovery from Perinatal Posterior Parietal Injury

The methods used for Experiment 2B were essentially identical to those used in 2A. The differences were only in subject numbers and surgical procedures.

Method

Subjects

A total of seventy-four Long Evans rats were used for Experiment 2B. These rats participated in three behavioral tasks (see Table 15). At the age of 60 days the animals began behavioral testing. Any sex differences that are significant to the experimental results are reported.

Table 15. Number of rats used in Experiment 2B.

	Untreated		Supplement Treatment	
Sex	Female	Male	Female	Male
Unlesioned	n= 5	n=12	n= 8	n=14
Parietal Lesion	n= 5	n=11	n= 8	n=11

Surgical, anatomical and behavioral procedures

These procedures were identical to those in Experiment 1B.

Results

Anatomical results

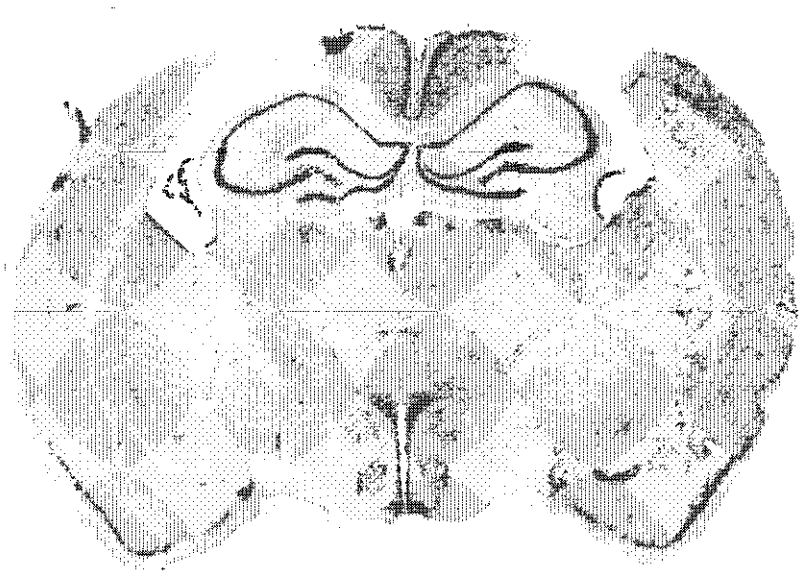
The lesions removed Kreig's area 7, in addition to the posterior part of the primary parietal cortex (Zilles Par1), and the anterior part of occipital 2 area (Oc2).

Occasionally, unilateral damage was apparent in the posterior cingulate regions (RSG, RSA). There was no direct damage to the hippocampus or other subcortical regions.

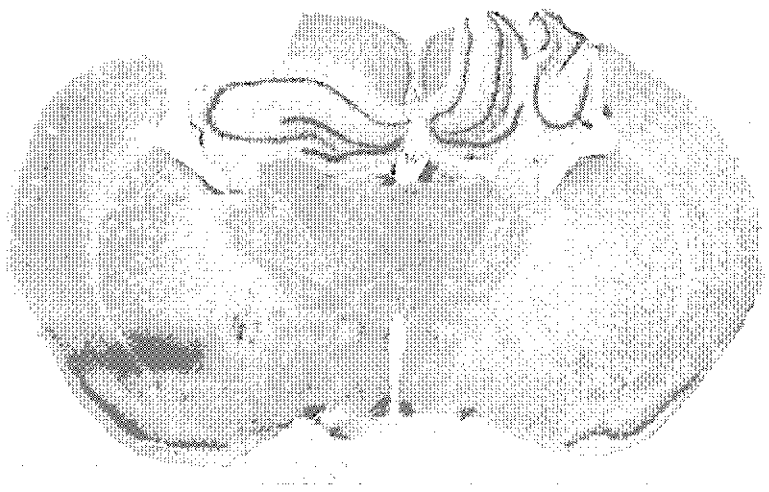
The hippocampus usually had an abnormal shape and often grew through the lesion cavity. A dorsal view of the cortex, therefore often indicated the appearance of a smooth exterior cortical surface (see Fig. 42).

As in Experiment 2A, the lesion cavities appeared to be smaller in the supplement-treated animals with the hippocampus growing to the cortical surface in fewer brains.

Most posterior lesions resulted in some degradation in the anterior nuclei, but overall had larger nuclei and more cells than brains in the untreated group. An interesting effect observed in the caudate putamen of the supplement-treated brains was an increase in white matter, as well as cell density (see Fig. 43).



Supplement Treatment



Untreated

Fig. 43. Parietal lesions of the vitamin supplement treated group.

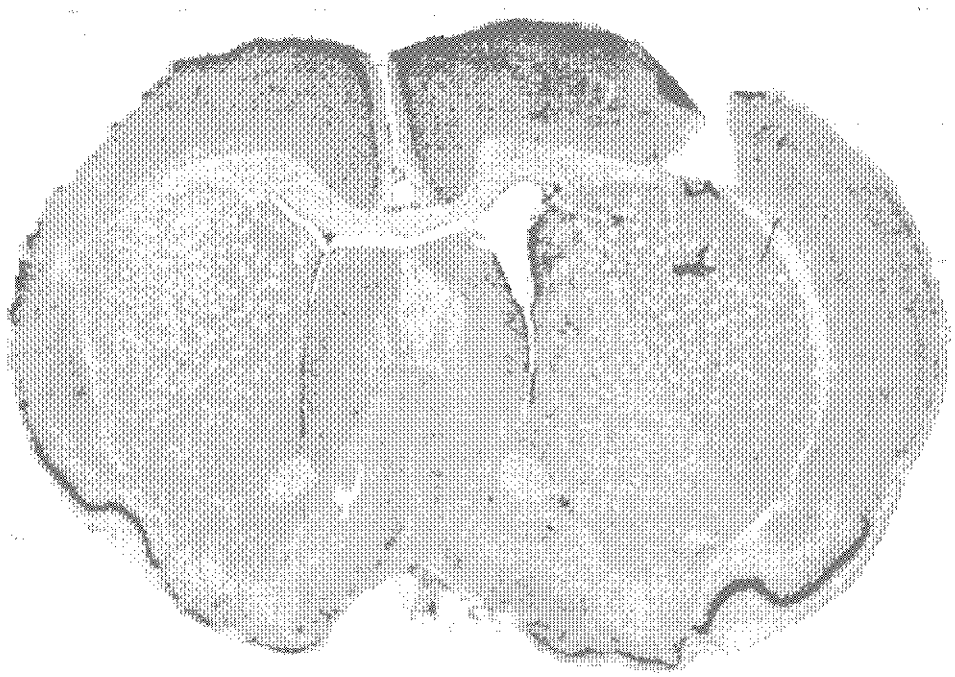


Fig. 44. The caudate putamen in parietal lesion brains.

Brain Weight:

The parietal lesion resulted in a decrease in brain weight, which was unaffected by the vitamin supplement. Differences in brain weight are usually found between the sexes, as well as with fixation method, therefore, the analysis has been done separately for each sex and fixation technique.

Cresyl Violet Preparations:

The parietal lesion resulted in a decrease in brain weight, which was reversed in both males and females that received the vitamin supplement treatment (see Table 16).

A three-way ANOVA with lesion, supplement treatment, and sex as factors showed a main effect of lesion ($F(1,38)= 4.8$, $P <.04$) and sex ($1,38)=16.6$, $P <.0002$) but not of treatment ($F(1,38)= 0.24$, $P=.25$). There was, however, a significant lesion × treatment interaction ($F((1.38)=14.3$, $p=.0005$), a result that reflects the selective effect of the treatment on the lesion brains. The other interactions were not significant (p's>.2).

Table 16. Brain Weight for Cresyl Violet Preparations:

Group	Female	Male
Untreated lesion	1.772 ± .050 (n=5) *	1.890 ± .040 (n=5) *
Supplement lesion	1.892 ± .038 (n=8)	2.077 ± .100 (n=6)
Untreated Unlesioned	1.948 ± .020 (n=5)	2.162 ± .073 (n=6)
Supplement Unlesioned	1.879 ± .040 (n=8)	1.970 ± .040 (n=9)

*Denotes statistical significance from unlesioned untreated group (p<.05, or better).

Golgi Preparation:

The parietal lesion resulted in lower brain weight, which did not indicate a benefit with the vitamin supplement. (All golgi analysis were done for male rats).

ANOVA revealed a main effect of lesion ($F(1,19)= 32.100$, $P< .0001$), but not of treatment ($F(1,19)= 3.210$, $P= .0900$), or of an interaction ($F(1,19)= .0800$, $P= .7800$) (see Table 17).

Table 17. Brain Weight for Golgi Preparations:

Group	Male
Untreated lesion	1.942 ± .010 (n= 6) *
Supplement lesion	1.872 ± .040 (n= 5)
Untreated unlesioned	2.123 ± .030 (n=6)
Supplement Unlesioned	2.072 ± .050 (n=5)

* Denotes statistical significance from unlesioned groups ($p<.05$, or better).

Cortical thickness:

The parietal lesion resulted in thinner cortices, which was significantly increased in the supplement-treated parietal lesion animals. (see Fig.44).

An overall ANOVA on cortical thickness between lesion and treatment groups indicated significant effects of lesion ($F(1,22)= 6.083$, $P= .0219$), and treatment ($F(1,22)= 9.182$, $P= .0061$), but not of an interaction ($F(1,22)= 1.484$, $P= .2361$).

A repeated measures ANOVA for all five planes did not reveal significant interactions between mean cortical thickness and lesion (see Fig.45).

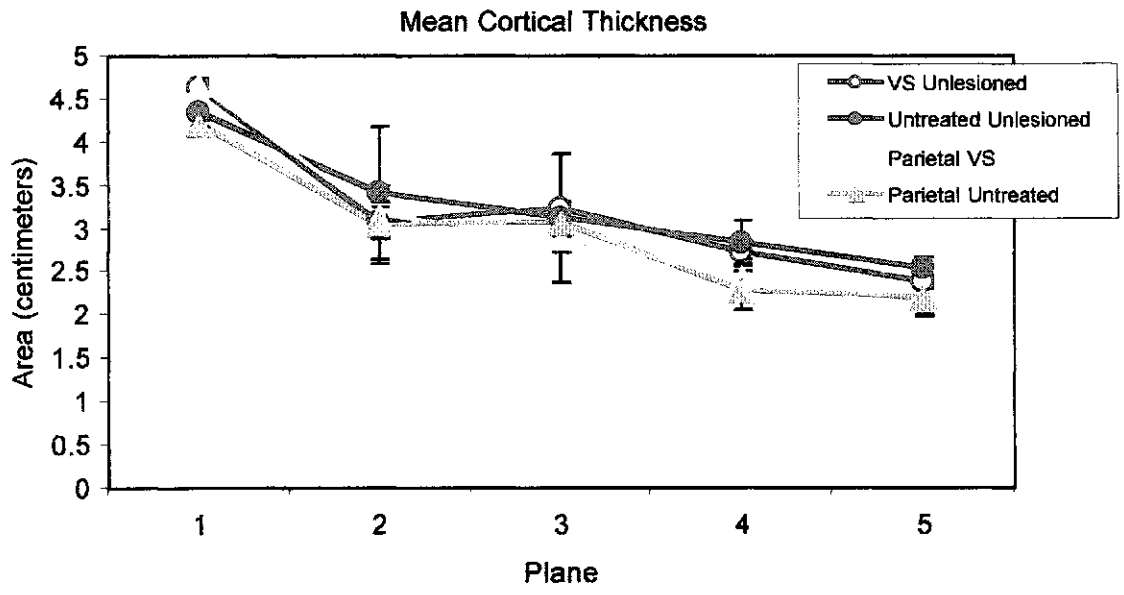


Fig. 45. Cortical thickness for each of five planes measured in animals with parietal lesions treated with the vitamin supplement.

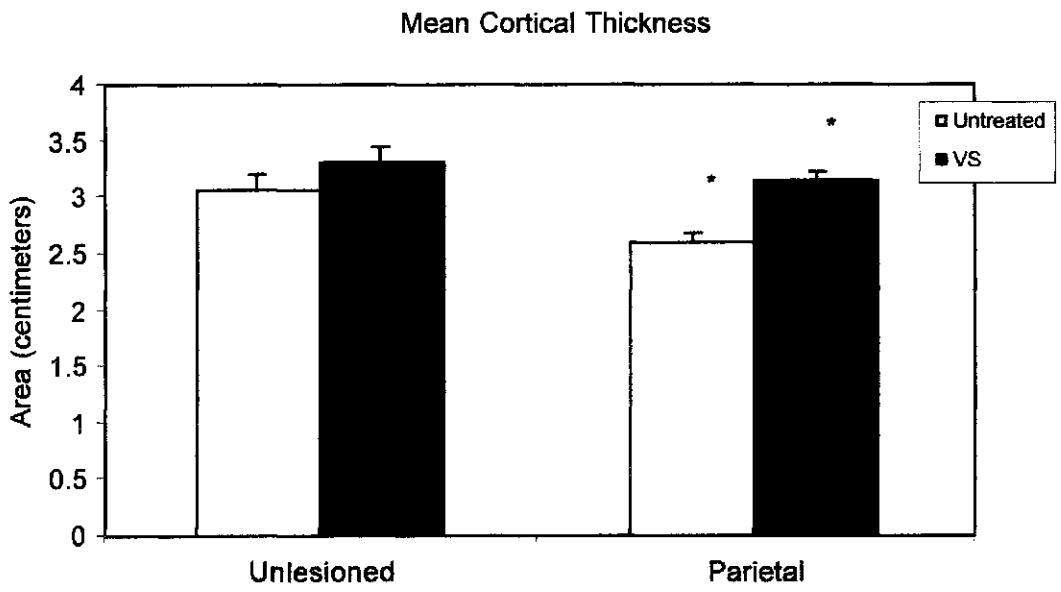


Fig. 46. Mean cortical thickness for the five planes measured in animals with parietal lesions treated with the vitamin supplement. * Denotes statistically different from unlesioned groups ($p < .05$, or better).

Thalamic width:

Nuclear atrophy in the anterior plane of the thalamus was found for both parietal lesion groups with the appearance of less cell damage in the supplement treated brains (see Table 18).

A two-way analysis of variance (ANOVA) of the anterior plane for thalamus indicated a main effect of lesion ($F(1,23)= 57.820$, $P< .0001$), but not of treatment ($F(1,23)= .144$, $P= .7081$), nor for the interaction ($F(1,23)= .407$, $P= .5296$).

ANOVA for the posterior plane of the thalamus indicated a main effect of lesion ($F(1,23)= 54.024$, $P< .0001$) and of treatment ($F(1,23)= 10.899$, $P= .0031$), but not for the interaction ($F(1,23)= .527$, $P= .4752$).

Table 18. Mean measures of thalamic width.

	Anterior Plane (mm)	Posterior Plane (mm)
Untreated Lesion	8.720 ± .346 *	13.480 ± .128 *
Supplement Lesion	9.112 ± .651	13.075 ± .105
Untreated Unlesioned	11.900 ± .400	14.750 ± .250
Supplement Unlesioned	11.800 ± .129	14.117 ± .104

* Denotes statistically different from unlesioned groups ($p<.05$, or better).

Behavioral results

Open Field:

The Parietal lesion resulted in decreased activity levels in the animals, which was further reduced by the supplement treatment. The supplement treatment reduced activity levels in the unlesioned animals as well.

Horizontal activity:

ANOVA between lesion and treatment groups for horizontal activity found main effects of lesion ($F(1,47)= 4.800$, $P= .0400$, of treatment ($F(1,47)= 46.600$, $P< .0001$), as well as for an interaction between groups ($F(1,47)= 11.700$, $P= .0010$) (see Fig.46). The main effect of the supplement treatment was evident in both the lesion and unlesioned groups, as well as having a direct effect with the parietal lesion animals.

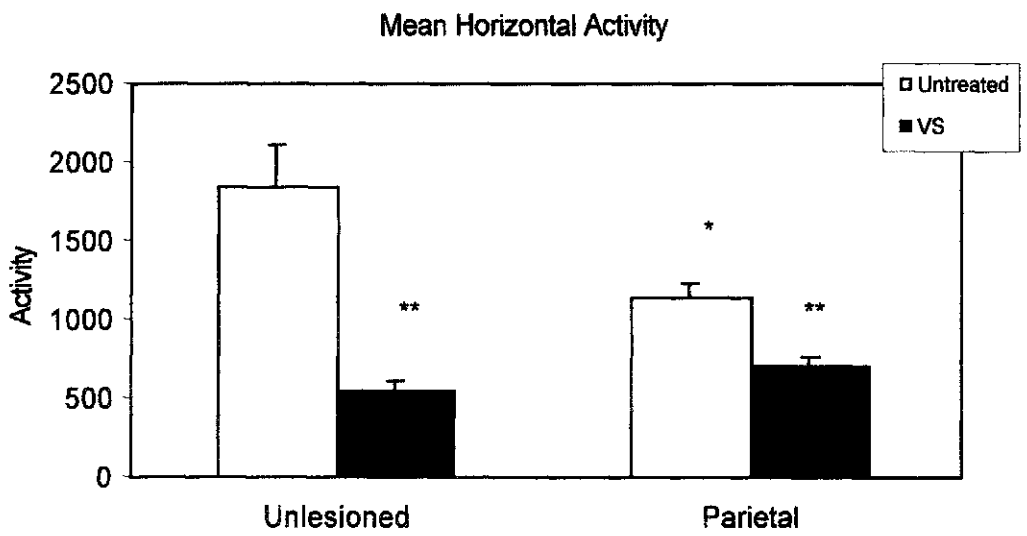


Fig. 47. Mean horizontal activity in open field of parietal lesion animals treated with the supplement. * Denoted statistically different from unlesioned groups (p<. 05, or better). ** Denotes statistically different from untreated groups (p<. 05, or better).

Distance:

ANOVA between lesion and treatment groups for distance also found main effects of treatment ($F(1,47)= 61.600$, $P< .0001$), as well as of interaction ($F(1,47)= 5.100$, $P= .0200$), but not of lesion ($F(1,47)= 0.960$, $P= .3300$) (see Fig.47).

The interaction reflects the fact that the supplement treatment effects were significant for the lesion group, but there was no lesion effect because the supplement effect was larger in the unlesioned animals.

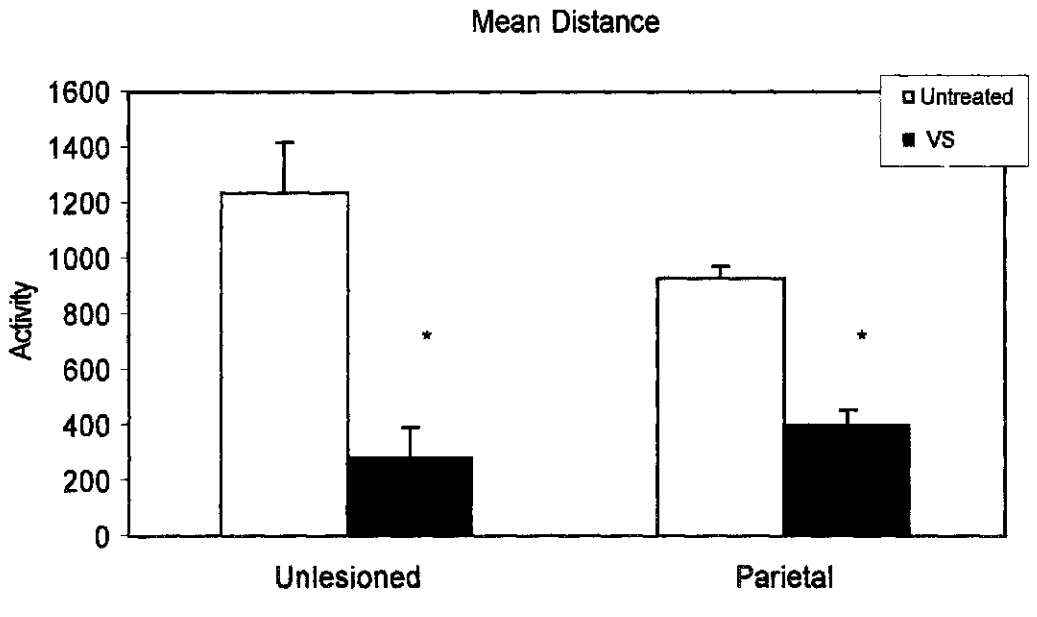


Fig. 48. Mean distance in open field of parietal lesion animals treated with the supplement. * Denotes statistically different from untreated groups ($p<.05$, or better).

Morris Water Task:

Rats with parietal lesions were impaired at the water task, which was reversed with the supplement treatment.

A repeated measures ANOVA for the water task indicated many significant effects for all measures of lesion, treatment and category of latency (see Fig.48).

There were main effects of lesion ($F(1,42)= 12.560$, $P= .0010$), and of treatment ($F(1,42)= 10.407$, $P= .0024$), but not for an interaction between lesion and treatment ($F(1,42)= 2.880$, $p= .0971$). There was also a main effect of category latency ($F(4, 168)= 110.754$, $P< .0001$), an interaction between latency and lesion ($F(4,168)= 3.338$, $P= .0108$), and an interaction between latency and treatment ($F(4,168)= 4.614$, $P= .0015$).

A two-way ANOVA between lesion and treatment for latency found main effects of lesion ($F(1,42)= 12.560$, $P= .0010$, and of treatment ($F(1,42)= 10.407$, $P= .0024$, but not for an interaction ($F(1,42)= 2.880$, $P= .0971$, indicating that the supplement treatment provided a benefit for all animals, especially those with parietal lesions in the water task (see Fig.49).

The probe analysis for the second experiment was not completed by the computer data recording processes, due to a computer malfunction in tracking the swim patterns of the rats. The probe was therefore graded from scores of one to four, according to a hand drawn trace of the swim path (see Fig.50). These results were analyzed by a two-way ANOVA, which indicated that the no treatment parietal lesion group spent less time than the other groups in the correct quadrant, quadrant one.

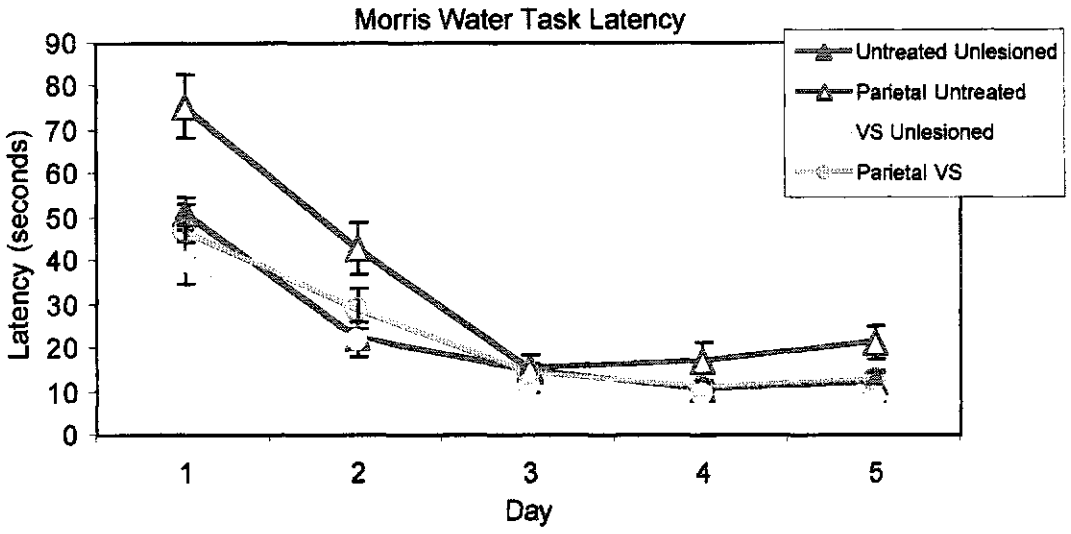


Fig. 49. Latency in Morris water task over five-day training period. Animals had parietal lesions and treated with the supplement.

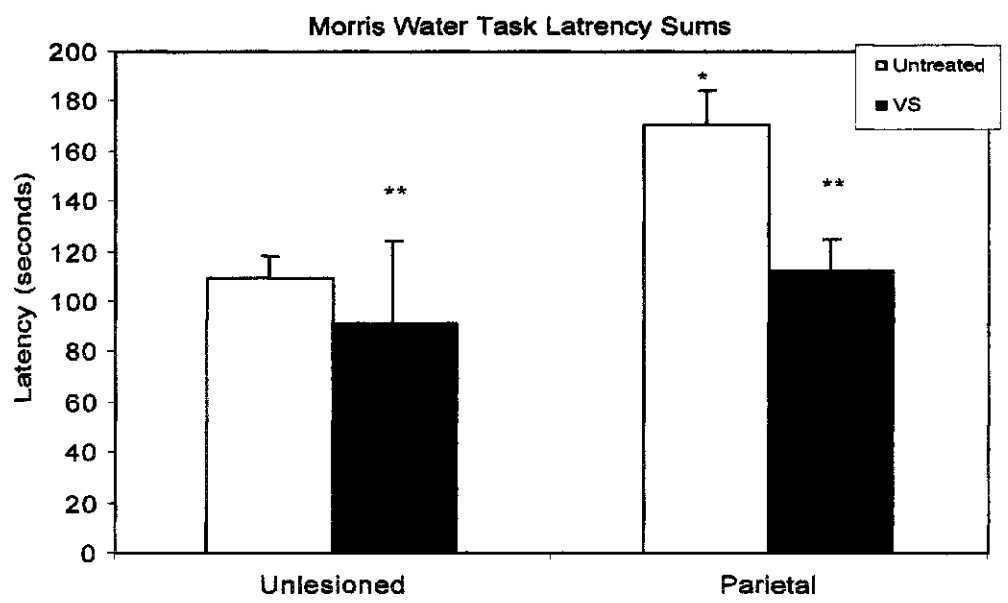


Fig. 50. Morris water task latency sums of parietal lesion animals treated with the supplement. * Denotes statistically different from unlesioned groups (p<. 05, or better). ** Denotes statistically different from untreated groups (p<. 05, or better).

A two-way ANOVA between lesion and treatment groups did not reveal any significant effects of lesion ($F(1,22)= 1.389$, $P= .2512$, of treatment ($F(1,22)= .008$, $P= .9314$, nor for an interaction ($F(1,22)= 1.103$, $P= .3051$. According to this analysis, there were no significant effects with the parietal lesion group.

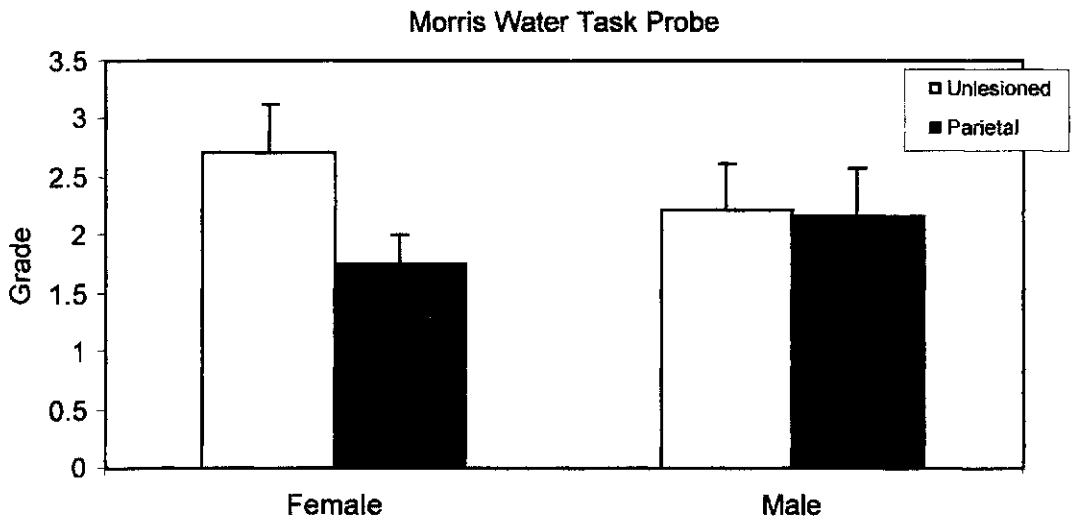


Fig. 51. Probe Morris water task trials for parietal lesion animals treated with the supplement. . A grade of1 indicates least amount of time spent in correct quadrant, quadrant 1. A grade of 4 indicates most time spent in quadrant 1. All animals are within the treatment group.

Tray Reaching:

The rats with parietal lesions were impaired compared to unlesioned animals. The supplement treatment partially reversed this impairment (see Fig. 51).

A two-way ANOVA between lesion and treatment groups indicated a main effect of lesion ($F(1,33)= 9.711$, $P= .0038$), and of treatment ($F(1,33)= 6.308$, $P= .0171$), but not for an interaction ($F(1,33)= .076$, $P= .7842$).

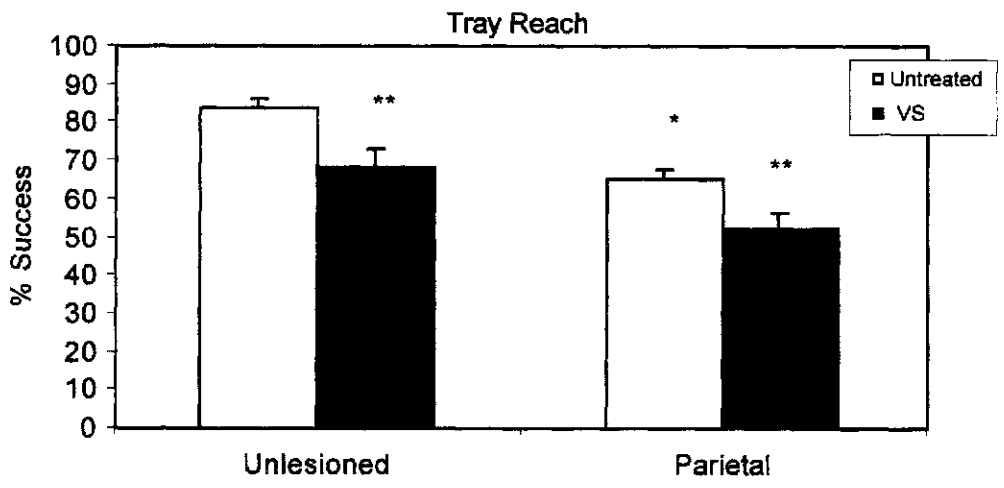


Fig. 52. Summary of tray reaching task of parietal lesion animals treated with the supplement. * Denotes statistically different from unlesioned groups (p<. 05, or better). ** Denotes statistically different from untreated groups (p<. 05, or better).

Discussion

The main anatomical findings of the current experiment were: 1) perinatal frontal and posterior parietal lesions produce a smaller than normal brain and reduced cortical thickness; 2) the supplement treatment significantly increased cortical thickness in both the lesion and control animals; 3) the supplement treatment may have reduced the chronic lesion size normally seen in the lesion animals.

Behaviorally, the main findings of the current studies were: 1) both perinatal medial frontal and posterior parietal lesions reduced open field activity relative to controls and produced impairments in both spatial navigation and skilled reaching relative to controls, and; 2) the supplement completely reversed the spatial deficit in the medial frontal lesion animals and partially reversed the deficits found in spatial learning in the posterior parietal lesion animals. Partial reversals of skilled reaching impairments were found for both frontal and posterior lesion animals.

Anatomical effects of the vitamin/mineral supplement treatment

Over the past twenty years or so, Kolb and colleagues have repeatedly demonstrated that perinatal cortical lesions produce a reduction in brain size and cortical thickness (Kolb, 1995; Kolb & Whishaw, 1989). One goal of postinjury therapies is to stimulate an increase in cortical thickness because it can be presumed that this should facilitate behavioral outcome. Placing animals with perinatal lesions into complex

environments reduces the behavioral impairments, which is correlated with an increase in cortical thickness (Kolb & Elliott, 1987). The results of the current studies are consistent with the same general notion. The supplement treatment partially reversed the behavioral deficits and increased cortical thickness. The question is, why did this treatment have these effects?

The thinner cortical mantle that invariably follows perinatal cortical injury is consistently associated with a reduction of dendritic arborization in cortical neurons, the actual cause for this however, is not yet established (Kolb, 1995). It is also likely that there are fewer cortical neurons than normal, possibly because of an enhancement in apoptosis (Kolb & Gibb, 1990). Thus, it seems reasonable to suggest that the supplement treatment may have stimulated dendritic growth, which is normally associated with synaptogenesis, and in addition, may have reduced apoptosis, or stimulated neurogenesis. A Golgi analysis is in progress to explore the first hypothesis. The second possibility has not yet been examined, but evidence suggests that this may be the case.

The lesion cavities in both the medial frontal and posterior parietal experiments were visibly smaller in the supplement-treated lesion animals. Several studies have demonstrated that neurogenesis is possible after perinatal cortical injuries, although is normally seen only if the injury occurs during the second week of life in the rat (Dallison & Kolb, 2003; Kolb et al., 1998). Treatment with FGF-2 decreased cavity size in lesion brains and stimulated neurogenesis (Waite, 2003). As noted earlier, if the expression of FGF-2 is affected by the supplement treatment, this could provide a mechanism for stimulating neurogenesis. Naturally, a direct test for neurogenesis with respect to the findings of the supplement treatment is necessary for the identification of newly

generated neurons, and to determine if the new neurons integrate anatomically and functionally with the brain.

One unexpected finding in the current study was that the supplements not only increased cortical thickness in the lesion animals, but also in the animals that did not receive lesions. Aside from the impressive results of the frontal lesion animals in the Morris water task, we do not have an obvious behavioral correlate with this increase in cortical thickness. It is likely that the behavioral tasks employed in the current studies were not particularly sensitive to determine these results, or perhaps not extensive enough to identify behavioral effects in the normal animals. If the animals were perturbed, or stressed, such as that seen during cerebral injury, or with a dementing disease, the behavioral advantages could appear. The thicker cortical mantle, with presumably more synapses, would be able to support a better functional outcome than that observed in untreated animals.

Behavioral effects of the vitamin/mineral supplement treatment

Rats with perinatal medial frontal or posterior parietal lesions typically have deficits in spatial navigation and the medial frontal animals also have deficits in skilled reaching. The current experiment further supports this finding. All of the lesion effects that are usually observed in lesion studies were reduced with the supplement treatment. Furthermore, even the unlesioned animals benefited from the supplement. The actual effect in the tray-reaching task is uncertain, as the results indicate that the supplement-treated animals had lower success rates. This could be due to the rats not being hungry enough to make assertive attempts to reach and maintain a pellet grasp. The animals on

the supplement diet were more sensitive to food deprivation than those animals on the standard diet as observed by increases in inattentiveness and hyperactivity.

The benefit of the supplement on functional recovery after perinatal cortical lesions is exciting because it is a simple treatment that can be initiated in a noninvasive manner. The critical time period for the administration of the supplement, however, is unknown because treatment was continued throughout the lifetime of the rat, since the day of birth in the current study. Future studies should determine if there are particular periods post-injury when the treatment is most beneficial.

To date, there have been very few other post-injury treatments that have been shown to affect the outcome of early cortical injury. One treatment that has been found, however, is post-injury tactile stimulation (Gibb & Kolb, in submission). Gibb and Kolb have proposed that the mechanism for the tactile stimulation effect is via an upregulation of Fibroblast Growth Factor (FGF-2). Thus, the stimulation increases FGF-2 both in skin and in brain, and direct administration of FGF-2 has been found to stimulate functional recovery. Increases in FGF-2 expression have been associated with other forms of cortical plasticity as well, including the effects of psychoactive drugs (Flores & Stewart, 2000). By administering psychomotor stimulant drugs, cocaine and amphetamine, the neural activity that is associated with sensitization to the drugs was found to produce long-lasting structural changes in the brain, which was correlated with increased FGF-2 expression. It is therefore possible that the supplement treatment could act via some similar mechanism, either on FGF-2, as well as other neurotrophic factor(s).

5. General Discussion

The fundamental goal behind the experiments performed was to enhance pre-and postnatal factors and mechanisms that are normal processes during the developmental stages, in an attempt to promote recovery of function following early cortical injury. Two different treatments were chosen, dietary choline and a vitamin and mineral supplement. Choline intervention has been studied for many years as therapeutic treatment during development. The majority of this research indicates that perinatal choline intervention can produce long-lasting behavioral benefits (Tees, & Mohammadi, 1999; Albright, Friedrich, Brown, Mar & Zeisel, 1999; Meck, Smith & Williams, 1988; Blusztajn, 1998; Zeisel, 2000). The vitamin and mineral supplement, on the other hand, has not previously been investigated as a therapeutic agent in laboratory animals.

The principal findings of the experiments were that both the choline and vitamin supplement treatments facilitated functional recovery and reversed some morphological sequelae of perinatal medial frontal lesions. In contrast, only the vitamin supplement had a similar effect on these measures in rats with perinatal posterior parietal lesions. Furthermore, both treatment interventions appeared to exert some kind of an anxiolytic effect, as indicated by observed stress responses of behavior and defecations in the open field task. Subjective observations of the animals are they were calmer and had a more relaxed behavior with handling and with the task training. In addition, they displayed a reduction of activity in the open field test, possibly because they were less anxious.

I should note parenthetically, that there were some initial difficulties in using the supplement in dams prior to conception, and possibly even during gestation, but it was possible to administer the diet postnatally. In addition, a second generation of animals

was successfully bred on the diet and the second generation of pups appeared healthy and performed normally on the behavioral tests (Appendix E).

The focus of this discussion will be on the possible mechanisms underlying the beneficial effects of the dietary treatments, first at a molecular level and then at a behavioral level. I then consider how this research might be extrapolated to other disorders and where future research should head.

Molecular Actions of the Choline and Vitamin/Mineral Supplement

When the brain is injured there are both degenerative processes engaged as well as a continuation of the genetically prescribed developmental processes. To successfully treat injury of the developing brain, the progression of degenerative effects of the injury would need to be retarded and reversed as quickly as possible, in order to encourage the continuation of normal development and to promote the reparative processes. Treatments such as choline can be administered prenatally to counter some of the damaging effects of injury incurred at a later stage of development. Phosphorylcholine is a metabolite of choline found in the fetal brains of pregnant dams, which also shows higher concentrations in dams supplemented with dietary choline (Garner, Mar & Zeisel, 1995). Garner and colleagues have proposed that phosphorylcholine may serve as an essential messenger for the induction of DNA synthesis, thereby promoting cell division.

Dietary choline and its metabolites have also been implicated in the prevention of apoptosis, a naturally occurring form of cell death and cell turnover in the brain (Holmes-McNary, Loy, Mar, Albright & Zeisel, 1997). Cells deficient of choline show characteristic signs of cellular atrophy with intracellular degenerative processes, which

are known to occur prior to apoptotic cell death. The synthesis of choline metabolite, phosphatidylcholine (cell membrane phospholipid), has been proposed to be necessary to maintain the progression of the cell cycle, from the first growth phase (G1) to the synthesis phase (S) of DNA nucleotide synthesis (Holmes-McNary, Loy, Mar, Albright & Zeisel, 1997).

During the developmental period of neural migration at postnatal days one to six, cortical injury would result in losses of cortical target cells necessary to guide neuritic outgrowth, particularly in the case of cholinergic cells. In the brain, cholinergic neurons have an intimate relationship with nerve growth factor. Nerve growth factor is synthesized by the target cells in the cortex, which is then transported in a retrograde fashion to the nucleus basalis magnocellularis in the basal forebrain of the rat (Hefti, Hartikka, Knusel, LaPlume & Mash, 1990). Nerve growth factor is activated by neural activity, which could also promote cholinergic activity around the lesion site if sufficient growth factors exist in the area. Increasing the dietary choline content has been reported to increase the size of cholinergic cells, thereby preventing cell shrinkage as a result of cortical lesions. The subsequent effects of cholinergic activity increase growth factor activity to retard the degenerative processes of lesions, and potentially promote neuritic sprouting (Loy, Heyer, Williams & Meck, 1991). During development, neurite outgrowth is suggested to increase in activity from electrical activity occurring in neighboring cells (van Pelt, Ooyen & Corner, 1996). Cortical lesions remove a portion of cortical tissue, so any new tissue that could regenerate would need to rely on neighboring cells for electrical activation, trophic support, and guidance. There would also be a loss of neural and glial cells during migration. The cortex would suffer losses of early migrating neural cells,

which would not normally be replaced. Glia, on the other hand, can proliferate throughout development and the peak time for astrocyte genesis is not until the second week of life. Therefore, if treatment could enhance the proliferation of glial cells, there would be an increase in trophic support for neural cells. Furthermore, because it has been proposed that under some circumstances astrocytes can redifferentiate into neurons, it is possible that a treatment that enhanced glial proliferation could enhance the likelihood of neurogenesis. Radial glia that are proposed to guide neural cells during migration have been found to exhibit molecular characteristics that are typical of neural cells and express various transcription factors involved in cell fate in different brain regions (Reid, & Walsh, 1996). Any direct damage to migrating neurite elongation processes (axons) would result in losses of cytoplasmic constituents that are required to provide growth cones with cell signaling molecules, energy substrates and cytoskeletal constituents to continue elongating protrusions.

The first two weeks of postnatal life in the rat begins a period of developing dendrites and synaptic connections. The firing patterns of immature cells are considered different from the patterns of their mature counterparts (Erzurumlu & Guido, 1996). Rather than a summation in electrical activity that produces a single burst of neural activity, as in mature neurons, immature neurons display smaller, but continuous firing patterns of electrical activity. Erzurumlu and Guido (1996) suggest that these new neurons have the capacity to generate a wide variety of responses based on the transduction pathways generated postsynaptically. These responses, in turn, could induce changes in neuronal differentiation, structurally at new synapses, and processes of plasticity (Erzurumlu & Guido, 1996). This concept of plasticity during development can

be applied to any type of treatment, provided that it could increase activation of neural cells. The supplement treatment would supply not only precursor amino acids for neurotransmitter synthesis, but also the necessary precursors for the enzymatic reactions. By influencing all systems, neural activity would experience a higher amount of electrical activity, which could recover some of the debilitating effects of a lesion.

The activation of trophic factors that are necessary for neural support and survival depend upon the activity of the neurons supported by glial cells. Glial cells contain receptors for neurotransmitters, which can trigger trophic responses to support ongoing neurotransmission. Immature glial cells have also been identified as carriers of growth factors and extracellular proteins (Acarin, Gonzalez & Castanello, 2001). In the advent of injury to the brain, glial cells provide the injured area with factors necessary for repair and growth. One of the primary factors upregulated after injury is nerve growth factor (Acarin, Gonzalez & Castanello, 2001). Nerve growth factor then is important for injury repair, but has also been shown to provide mechanisms necessary for cortical plasticity. The direct infusion of trophic factors, particularly nerve growth factor, into the lateral ventricle (ventricular zone) has been correlated with functional plasticity (Kolb, 1999). Increases in dendritic arbor and spine density in neurons were observed after cortical lesions in rats, potentially as a result of stimulated neurogenesis to replace some of the lost cortical tissue. Removal of the newly grown tissue resulted in elimination of functional recovery, as measured in behavioral tasks, implying that this new tissue was functional.

Cholinergic systems of the brain have a beneficial and endogenous relationship with nerve growth factor, giving dietary choline treatment a great advantage. Nerve

growth factor is suspect to support other neural systems, and there are a number of trophic factors in the brain aside from nerve growth factor. The vitamin supplement treatment could derive the benefits of other trophic factors, as well as nerve growth factor, through increases in neural activity of all neural systems postnatally. This increase in activity stimulates the growth in glial cells, which in turn provide additional neuroprotective factors and nutritive support.

In addition to various beneficial factors necessary for the healing and reparative processes, such as antioxidants, a potential mechanism that has been considered advantageous postsurgery, is the vitamin A content of the supplemented diet. An increase in wound strength has been reported from supplementation in megadoses of retinyl acetate and beta-carotene (vitamin A) at five days after surgery (Gerber, Erdman, 1982). The mechanisms proposed to enhance wound strength are enhancements of normal processes during the initial phase of wound healing. Gerber and Erdman (1982) have suggested that the proliferative effect of vitamin A on cells could potentiate the mitogenic effects normally induced by epidermal and mesodermal growth factors, which would increase the fibroblast activity necessary to heal the wound.

Enhancements of neurotransmitter system conductive abilities could potentiate the regenerative capacities in the damaged cortex and their functional outcome. The ability for neurons to synthesize neurotransmitters, and release neurotransmitters upon stimulation, is largely dependent on the availability of the amino acid precursors of these neurotransmitters (Heuther). Although the immature blood brain barrier is more permeable to nutrients during development, its destruction as a result of injury would further increase the amount of available supply of all nutrients via the blood stream.

The vitamin supplement provides amino acid precursors of neurotransmitters (choline, phenylalanine, glutamine), thereby providing large increases in overall neural activity and glial activity, into neural maturation. The functional consequences could potentially increase the processes of attention, learning and memory in rats. The catecholamines (norepinephrine and dopamine) have been previously implicated to influence alerting brain systems, but not orienting systems. Conversely, the cholinergics have been implicated to alter orienting abilities, but not alerting mechanisms (Marrocco & Davidson, 1998).

During development some migrating neural cells can become differentiated depending on certain characteristics in their local extracellular environment. Cells that are initially designated to be noradrenergic cells have been suggested to change course to become cholinergic cells, if the conditions in their environment influence such a change (Purves & Lichtman, 1985). All neuotransmitter systems are known to modulate each other, and therefore, a higher increase of acetylcholine activity in the brain may depress the activity of other neurotransmitters, such as norepinephrine. A hypothesis for the treatment of anxiety using choline in the form of lecithin has been suggested to involve prostaglandin synthesis (Vergrosen, 1979). Based on a number of studies, the pharmacological effects of prostaglandins in the E series (PGE's) and F series (PGFα) are noted to include effects of "sedation, tranquilization, analgesia, reduction of brain catecholamines, and reductions in cardiac arrhythmia via a central action on brainstem neurons" (Vergrosen). A keynote with this hypothesis includes the fatty acid content of the source (lecithin), as well as the tissues stimulating prostaglandin synthesis. Grape seed has a high content of linolenic acid (omega-3 fatty acid) and is included in the

vitamin and mineral supplement. Omega-3 fatty acids contribute to the production of prostaglandin series 3, such as PGE3. According to Vergrosen's hypothesis, the prostaglandins produced by the grape seed might contribute to the anxiolytic effects seen in rats treated with the supplement. Anxiolytic effects have also been attributed to therapeutic quantities of gingko biloba, which is also included in the vitamin supplement and might have contributed to decreases in activity. Gingko biloba extract has been shown to restore restraint stress-induced elevations of catecholamines, norepinephrine, dopamine, serotonin, and plasma corticosterone levels to near normal in rats (Shah, Sharma, & Vohora, 2003).

The increase in activity of newly formed excitatory acetylcholine is accompanied by natural increases in neurite outgrowth of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Cholinergic activation may thereby be accompanied with appropriate activations of GABA, dependent upon the circumstances requiring activation. Some drugs formulated as anti-anxiety agents are known to depress the stress-induced increases of norepinephrine and dopamine. The anxiolytic characteristics that counter these neurotransmitter actions are considered to be an effect of GABA, a predominantly occurring inhibitory neurotransmitter (Cooper, Bloom, Roth, 2001).

The results seen in Experiments 1 and 2 of this thesis are valuable and could be applied for clinical treatment of injured patients. In the clinical setting treatment of injury also necessitates an aggressive nutrition intervention. The vitamin supplement used in Experiment 2 would be very beneficial for the treatment of injury through numerous mechanisms, and although only a few have been highlighted, many factors must be considered with treatment. The purpose of noting some of the physiological responses to

injury, or stress, is to emphasize the importance of an aggressive nutrition intervention for recovery.

The body responds to a variety of signals within hours of injury to begin the healing and tissue regeneration processes. An increase in protein synthesis is paramount, as well as the energy required, to support the repair processes. Hormones are activated to meet the metabolic demands during the initial stages of injury and stress. Glucagon, insulin, catecholamines, gluccocorticoids, and growth hormone are the primary hormones activated, based on the present level of circulating amino acids. Increases in protein turnover accompany stress, sepsis, surgery and trauma in attempts to synthesize all of the necessary proteins for repair. Unfortunately, the rate of protein degradation via amino acid catabolism is significantly higher than protein accumulation. The amino acid substrates from muscle protein catabolism are required for glucose production. In addition, adipocytes (fat cells) also undergo lipolysis to provide the body with glucose and ketones. The energy demands during the natural healing processes are very high, thus the increases in blood glucose is necessary to provide all active tissue with energy sources. Insulin secretion is generally increased during periods of stress to distribute the available increases of blood glucose.

During prolonged periods of stress, however, tissues requiring glucose become resistant to insulin. Because tissues are not receiving glucose, the glucagon-to-insulin ratio changes to favor glucagon release, which signals a continuation of protein degradation and glucose synthesis. The brain can also utilize ketones for energy during periods of stress and starvation (glucose deprivation), but during prolonged periods of stress, ketones also become resistant to the effects of insulin. Cortisol (gluccocorticoid)

concentrations generally remain elevated, further increasing degradation processes to synthesize glucose.

The net result of these catabolic processes is a gradual and continual overload of blood glucose and a slow recovery of damaged tissue. Furthermore, if there is a considerable lack of digestible nutrient intake into the gastrointestinal tract as a result of prolonged periods of stress or injury the intestinal mucosa becomes susceptible to atrophy, which contributes to risks of sepsis. The demands for intestinal cell's needs are usually much higher than can be afforded from amino acid catabolism (Goff, Gropper, & Hunt, 1995).

Cerebral metabolism after head injury has been noted to necessitate increases in glucose and amino acids for energy production to accommodate the metabolic demands during repair processes (Robertson et. al., 1988). Although the metabolic substrates of glucose are present in the blood stream, the brain cannot extract what it needs and some of the cerebral amino acids circulating, such as glutamate, may actually contribute to further neural damage. Other circulating aromatic amino acids that include tryptophan, phenylalanine, and tyrosine, apparently have not been equated to neurotransmitter production. Robertson and colleagues report that the initial purpose for these substrates would be for energy production to aid the reparative processes; however, they are rather associated with neuronal dysfunction, emphasizing the importance of regulating energy metabolism in the brain (Robertson, et. al., 1988).

Head-injured children have even greater metabolic demands to ameliorate some of the physiological effects of stress, to prevent weight loss, and provide for increases in protein synthesis (Phillips, Ott, Young & Walsh, 1987).

The vitamin and mineral supplement provides a number of factors that could retard long-term effects of stress and begin supporting demanding metabolic systems with the appropriate substrates and enzymes. The antioxidants vitamins A, E, and C, and selenium, as well as, mineral complexes of antioxidant enzymes, copper-zinc superoxide dismutase and manganese-zinc superoxide dismutase would be essential to prevent neural excitotoxic reactions. Others worth mentioning are chromium, glutamine, and vitamin B_6.

One of many nutritional roles of glutamine is for the maintenance of the integrity of the intestinal mucosal cells, and is required in large amounts during periods of stress, which often leads to limited dietary intake. Aside from the prevention of sepsis, the necessity of healthy intestinal cells is to be able to absorb and transport nutrients across the intestinal barrier, as is necessary to provide the body with nutrients for the reparative processes and prevent malnutrition.

After the initial period of tissue repair, when cortisol release is beneficial by inducing neuroprotective factors, the debilitating activity of chronic cortisol release is known to downregulate the expression of neuroprotective factors in the brain. Systemically, chronic cortisol in circulation results in the continuous attempt to provide glucose for energy substrates, but instead begins accumulating in the blood stream. The debilitating processes of long-term cortisol release could be reversed, by enabling insulin to transport blood glucose through capillaries into peripheral tissues and the brain. Glucose would then be oxidized, generating sufficient energy sources required to reverse catabolic (degrading) metabolism to anabolic (synthesis) metabolism that usually results with insulin activity.

A primary factor that could retard the chronic degradative processes of metabolism is vitamin B6 in its cofactor form pyridoxal phosphate (PLP) (Groff, Groper, & Hunt, 1995). Pyridoxal phosphate has been shown to prevent, or interfere with steroid hormone binding on target cells and can also bind to the steroid hormone receptors, thus diminishing the normal activities of steroid hormones on cells. The termination of glucocorticoid activity can then influence protein, carbohydrate, and lipid metabolism. Ironically, some drugs that are often prescribed post-surgery, such as corticosteroids, anticonvulsants, penicillamine, and isoniazid are known to deplete the body of vitamin B6 (Groff, Groper, & Hunt, 1995).

Chromium, as a glucose-tolerance factor, is provided in large amounts in the supplement, along with antioxidants to prevent the damaging effects of any surplus byproducts of oxidative metabolism. Chromium is known to enhance the effects of insulin. The subsequent clearing of high blood glucose levels should at some point trigger an appetite to provide the body with nourishment, particularly protein, to increase protein synthesis. Each metabolic pathway that is higher in activity during periods of stress, sepsis, injury, or trauma, requires a variety and abundance of vitamins and minerals with their diet to sustain prolonged nutrient depletion. The vitamins and minerals provide substrates and enzyme cofactors for the primary metabolic pathways, but also support a number of other pathways that are interlinked with these primary pathways to contribute to such a demanding task.

Retardation of the chronic effects of stress, and subsequent reversal of catabolic metabolism associated with chronic stress could decrease the duration for healing and any nutritional losses could be significantly reduced.

Collectively, the metabolic demands that the body processes as a result of injury are numerous, complicated, and usually exceed the body's capabilities without nutrition intervention. Treatment of brain injury necessitates much more than caring for the wound alone. By addressing the issue using aggressive nutritional measures with a well-formulated vitamin supplement and an adequate dietary intake, the body as a whole would receive substantial support to enhance the healing processes.

If all the necessary substrates are available, through the diet and the vitamin and mineral supplement, the long-term effect of treatment in physiological systems of the brain and the body could possibly become balanced and operate under "normal" homeostatic circumstances.

Behavioral Effects of the Choline and Vitamin Treatments

Although I have considered a variety of molecular mechanisms whereby the treatments might influence brain function, it is not immediately obvious how these effects would translate into behavior. There are few clues about this in the existing behavioral literature so I have taken the liberty of speculating about possible ways that the treatments could alter behavior. I consider both the anatomical and cognitive substrates of the behavior.

Anatomical Effects of Treatments

The animals that showed beneficial behavioral effects of the treatments also showed several gross morphological correlates of the behavior. Thus, the lesions appeared to be smaller in the choline-treated frontal animals and in the supplement-treated frontal and parietal animals. It would have been ideal to quantify the lesion

volumes in these animals but this is very difficult to do in neonatally injured brains because there is so much distortion of the brain (e.g., Kolb, Sutherland & Whishaw, 1983). Nonetheless, the apparently smaller lesions are reminiscent of the brains reported by Kolb et al (1999), in which they showed partial regeneration of lost cerebral tissue after day 10 medial frontal lesions, and by Waite (2003) in her animals with prenatal injections of FGF-2 in rats who later received day 3 medial frontal or posterior parietal lesions. If we assume that the brains in the current study really are similar to those in the parallel studies in the Kolb lab, then it seems likely that the molecular changes discussed above could have stimulated neurogenesis and gliogenesis as proposed above. The new tissue would then be presumed to have influenced behavior.

In addition to the lesion size, there was also a reduction in the shrinkage of the cortical mantle in the choline- and supplement-treated animals with lesions. Changes in cortical thickness can be presumed to reflect changes in the intrinsic organization of the cortex, which in turn can be presumed to influence cortical functioning (e.g., Kolb, 1995). Although it is not known exactly how such changes influence cognitive processing, there is an extensive literature showing the relationship between changes in cortical organization and behavior (e.g., Kolb & Whishaw, 1999).

Finally, although again difficult to quantify, it did appear that there was less extensive retrograde degeneration in the thalamus of the treated versus untreated lesion animals. If the thalami were in fact larger in the treated animals, then presumably there would be more thalamo-cortical and cortico-thalamic connections. These connections could be presumed to benefit cortical functioning.

Cognitive Effects of the Treatments in Cognitive Functioning

Habituation to novel events is considered the basis of selective attention, which forms the fundamental processes for all forms of learning (Rose, & Rankin, 2000). In the open field task, selective and sustained attention would be required to filter out the distracting auditory stimulus throughout each interval in order to habituate to a novel environment. Rats that have difficulty with the suppression of distractions would have difficulty habituating in that each subsequent presentation of the auditory stimulus would be perceived as novel.

One cognitive explanation for enhanced performance of animals in the water task is that there could be improvements in sustained and selective attention. In order to acknowledge and make associations with distal cues to a platform in the Morris water task, the rats need to be able to filter out irrelevant and distracting stimuli to make specific and relevant associations meaningful. For example, Delatour and Gisuet-Verrier proposed that navigation from different start positions as an ability to shift attention requires behavioral flexibility, which in turn, enables the animal to shift attention with novel starting positions (Delatour & Gisuet-Verrier, 2000). An extrapolation of this proposal would imply that the treated frontal lesion animals were successful in shifting their attention, by inhibiting previous responses from previous starting positions, and adapting to new starting positions. Parietal lesion animals in the supplement treated group may also have shown an improved ability to attend to relevant cues, although in this case to use them to choose an appropriate spatial trajectory towards the platform and inhibit previous responses.

One reason for using the attention-shift task was to test directly the idea that changes in the ability to shift and sustain attention might be influenced by the dietary treatments. Although subjectively it appeared that the treatments were beneficial to the brain-injured rats, the statistical measures used did not provide support for this impression. The task proved to be very difficult for all animals and the animals selected for the task may not have been a representative sample in each group. Specifically, a larger group of animals treated with choline were pretrained in the task and only those animals that were relaxed and adapted to the task well enough were chosen to perform in it. A subset of animals could have been selected and trained in smaller numbers. This task had previously not been performed at this laboratory; therefore, all rats were pretrained to select participants. In retrospect, this may have biased the results. The difficulty however, is that it seems likely that many of the parietal and frontal operates were not going to be able to succeed even in the early stages of the task. In addition, even with the preselection of subjects for the task in the subsequent groups tested, there was considerable intragroup variability, which made the statistical analyses very difficult.

Broader Implications of Using Dietary Treatments for Behavioral Disorders

Since the advent of magavitamin approaches for the alleviation of health symptoms by Linus Pauling, Abram Hoffer and others, a number of studies have been performed in humans using a variety of vitamins for treatment. Many results have indicated some short comings due to a lack of longitudinal studies, no significant treatment effects, or adverse effects of toxicity. The treatment interventions employed however, only used one, two, or a few vitamins, such as vitamin B_6, vitamin B_{12}, vitamin

B_3, Vitamin C, Vitamin A, or combinations of these vitamins. The problem with this type of intervention is that vitamins and minerals should not be used singularly for therapeutic purposes because they work together, in some cases synergistically.

Using vitamin B_6, for example, increases only one B vitamin, producing decreases in the levels of the other B vitamins, thereby potentially amplifying toxic effects. The B vitamins are interlinked metabolically therefore would operate more efficiently if all substrates and enzymes are present in relatively proportional amounts. With regard to using a vitamin supplement, in therapeutic quantities, having balanced proportions of all nutrients available would produce a more favorable outcome, as opposed to imbalances of nutrient intakes.

For example, deficiency of vitamin B_2 (riboflavin) rarely occurs in isolation, because it is accompanied by other nutrients (Goff, Gropper, & Hunt, 1995). Chronic deficiency of riboflavin however, has been known to impair the synthesis of vitamin B_6, in its coenzyme form pyridoxal phosphate (PLP), as well as, the synthesis of vitamin B_3 (niacin) from tryptophan. Impairments in PLP synthesis could in turn, affect a number of necessary reactions involved in amino acid metabolism. Pyridoxal phosphate is also a required component for enzymatic activity in the catabolism of glycogen to form glucose. Neurotransmitter synthesis of dopamine and norepinephrine, as well as the conversion of glutamate to gamma-aminobezoic acid (GABA), also necessitate PLP as a cofactor to complete their respective reactions (Goff, Gropper, & Hunt, 1995).

The vitamin and mineral supplement used for treatment intervention in the second experiment revealed significant effects both anatomically and behaviorally. The animals had a very calm demeanor, were easy to handle, and performed all tasks very well. Not

one incidence of illness was observed in any rat that would have been an effect of vitamin toxicity, as measured by comparisons with animals that did not receive supplement treatment. This product (EM Power +) is different than most in that the minerals are chelated to provide for more efficient absorption and utilization. The minerals are generally a prosthetic (inorganic) component to enzyme complexes that are essential to fulfill biochemical reactions. By chelating minerals and increasing their bioavailability they could more effectively manage the metabolic demands of the higher amounts of vitamins ingested with them. In other words, if the enzymatic activity is not sufficient for the adequate metabolism of vitamins, a pooling of substrate could result, requiring the body to dispose of it, and/or resulting in toxic accumulations in tissues.

Future Directions for Research

The present study supported the advantages of pre-and postnatal administration of choline treatment to enhance the learning and memory processes into adulthood. The vitamin supplement treatment was administered postnatally, and continuous throughout the lifetime of the rat. It would be interesting to determine any critical periods for treatment administration with the supplement also to see if pre-and postnatal administration would have similar functional outcome as the choline treatment.

The female rats seemed to require some time to adapt to the supplement before becoming impregnated. It would therefore be very beneficial to be able to arrive at a dosage, or a time frame that would favor successful reproductive processes, and be able to deliver a therapeutic dosage of nutrients.

Kolb has pursued research in animal models of stroke. Because stroke is known to generally occur in adulthood, it would be interesting to see the effects of the vitamin supplement in the reparative processes of adult rats. The anatomical and behavioral effects could be measured, and would also be advantageous to determine the time required for recovery processes for functional recovery.

The experimental use of bromodeoxyuridine (BrdU) and other mitotic markers could reveal some potential implications of neurogenesis for both choline and supplement treatments. Administering these markers at different time frames throughout development, and thereafter, could determine some of the growth patterns and changes that could be occurring during each period.

The Western Blot technique is very useful for revealing the proteins that are expressed after cortical injury and the effects that choline and the supplement treatment have during the reparative processes. Glial fibrillary acidic protein (GFAP) and its receptor Flg are markers for glial cells. Proliferation of these cells is normal after cortical injury indicating reparative processes. Gluccocorticoid type 1 receptor (GR1) may be useful to reveal any increases, or decreases, in stress hormones the animals have been subject to. Basic fibroblast growth factor (bFGF) resides in glial cells and is known to increase in expression after cortical injury. Its increase is generally an indicator of factors supporting the process of neurogenesis. Structural proteins can also be assessed with microtubule associated protein-2 (MAP-2) to quantify the density of any newly formed dendrites. Synaptophysin is a marker for structural proteins that are associated with newly formed neural synapses.

Using a variety of lesions would also be useful to determine these treatment effects with various injuries to different areas of the brain. Hippocampal lesions are commonly used to measure treatment effects on memory; lesions of the occipital cortex, or visual system, can determine disturbances in visual functions.

Dietary supplementation of the vitamin supplement into senescence would be very interesting to determine a potential treatment that could retard the aging processes of learning and memory, and possibly retard the overall effects of aging, as is proposed with anti-oxidants.

Using a variety of behavioral tests is always advantageous to arrive at a clearer picture of what types of processes can recover functionally. The motor cortex appears somewhat resistant to full functional recovery, as is demonstrated in Whishaw's skilled reaching task. By improving motor function on this type of motor task, the supplement treatment may be able to contribute therapeutic benefit to people with motor disorders, such as Parkinson's Disease.

I would also like to determine if the vitamin A content in the supplement could improve visual discriminations in the afoveate rat, using the visual discrimination maze.

I also did enjoy working with my rats in the attention shift task. I do believe that it is possible to arrive at some accurate subjective judgments of rat behavior by carefully examining their moves and spending many days with them. The supplement did appear to improve attention processes in the attention shift task, but it should be repeated with several scoring techniques, such as filming, timing during each trial, etc. Other behavioral tests of attention, such as the five-hole task could support other findings.

The radial arm maze is often employed for tests of memory, which produces various results depending on the paradigm used, but also the lesion and treatments employed.

If possible, it would be interesting and beneficial to observe differences in behavior and cognition in animals using different diets. By employing a diet that might resemble an imbalanced, or slightly deficient diet, in comparison with the vitamin supplemented diet, with a long duration of stress to determine the consequences of inadequate nutrient intakes might resemble a situation of chronic fatigue, or chronic burnout. Tasks would include the running wheel, and various cognitively demanding tasks, such as water maze, radial arm maze and others. Dietary intake and consumption would have to be carefully controlled to gain some insight into average caloric and nutrient intakes. The underlying proposal for this type of experiment is to provide a greater understanding of the determinants for depression. I believe that many cases of depression are due to nutritional burnout, and these people afflicted are being treated with antidepressants, while not seriously addressing nutritional issues.

6. Epilogue

Aside from the advantages of therapeutic nutrition intervention for treatment of injury, studies in humans, such as adults with Bipolar Disorder also report to benefit from a this vitamin and mineral supplement, EM Power+ (Kaplan, Simpson, Ferre, Gorman, McMullen, Crawford, 2001). Kaplan and colleagues have reported patients' increased feelings of well being, as well as feeling more normal, while taking the vitamin supplement. Patients also have reduced their psychotropic medications by more than fifty percent since beginning treatment intervention with the supplement.

Because my mother is bipolar and my academic background is in nutrition, I have been able to make careful dietary observations in relation to the characteristic mood swings of mania and depression, including the effects that perceived stressful events play into dietary behavior. Based on some known general characteristics of Bipolar Disorder and characteristics of my mother's behavior, I realize that there are some very important nutritional consequences of this disorder. Although this concept is basically an outline to the actual complexities that exist in Bipolar Disorder, I do feel that it can begin to provide some explanations for the benefits that this well formulated vitamin and mineral supplement has brought to many people who are presently receiving it for therapeutic intervention.

One of the primary issues normally addressed in Bipolar Disorder is of a dysregulation of the hypothalamic-pituitary-adrenal axis (HPA axis). The stress response of the HPA axis is thought abnormal due to inappropriate feedback mechanisms in the brain required to turn off the HPA axis response. As a result of inefficient feedback to

gluccocorticoids in circulation, increases in cortisol begin to alter metabolic processes in the body and the brain, as has been discussed previously for injury treatment. Patients diagnosed with chronic (major) depression, or bipolar patients, not experiencing a manic episode, are considered to have a higher predisposition to experiences of stress.

The limbic region of the brain contains a structure, the amygdala that is primarily associated with assigning the emotional significance to stimuli (Kolb, & Whishaw, 2003). Kolb and Whishaw have noted that in patients who have major depressive disorder the amygdala is unusually overactivated, even under resting conditions. Since the amygdala is also strongly connected to the hypothalamus, its activity has been proposed to stimulate cortisol release, thereby activating the stress response of the HPA axis (Kolb, & Whishaw, 2003).

If you can recall some of the metabolic consequences of stress mentioned earlier, the abundance of circulating hormones, including cortisol and catecholamines, serve to mobilize metabolic processes necessary to meet the higher metabolic demands required to begin the reparative processes of stress and injury. The perceived stress of depressive patients would differ from injury responses in magnitude, at least initially, however, depressive patients generally are known to undergo chronic episodes of stress because of the length of time they must endure it. Bipolar patients have been known to endure a stressful episode for periods of weeks to months. I have seen my mother in serious episodes of stress for as long as one to two months. Her dietary intake is often limited, but more notably irregular and at times imbalanced nutritionally. Some of the metabolic consequences under stressful conditions lead to limited food intake and resemble the stress response (Groff, Groper & Hunt, 1995).

During periods of mania, bipolar patients are again undergoing these metabolic demands to provide the person with the energy used during this period, and to compensate for the lack of food and nutrient intake. My mother and undoubtedly many other bipolar patients love their manic and euphoric phases. Food intake is not a concern to her at this time; in fact, eating rarely crosses her mind. Periods of mania are known to occur for at least one week (American Psychiatric Association, 1999). Laboratory findings associated with mania include increases in cortisol secretion and potential abnormalities in functions of neurotransmitter systems norepinephrine, acetylcholine, serotonin, dopamine and gama-aminobutyric acid (American Psychiatric Association, 1999). The major depressive episodes are also characterized by decreases in appetite and nutrient intakes, and people are noted to lack the motivation necessary to perform regular daily chores including preparing regular and nutritionally balanced meals. During most every depressive period, my mother would engage in carbohydrate bingeing to provide the energy-depleted body with quick sugars that her body craved and restore some "normal" level of functioning. The large surplus of carbohydrates would bring her out of her depressive phases.

To gain a better understanding of metabolic demands, the brain is the last organ in the body to be deprived of glucose, and with other central nervous system tissues, uses a very large amount of glucose to perform normal processes (Groff, Groper & Hunt, 1995). Groff et al. have indicated that during an overnight fast, nearly all liver and muscle glycogen has been depleted shifting the body into a fasting state after forty-eight hours of food deprivation (Groff, Groper & Hunt, 1995). Needless to say, Bipolar Disorder patients spend a lot of energy during their phases.

To summarize some of the potential nutritional consequences experienced by patients with bipolar disorder, the primary energy substrate required by the body and brain under various conditions in an attempt to maintain homeostatic conditions, is glucose. A number of catabolic processes of glycogen, protein, and fatty acids, can provide the organism with glucose for energy production. What is important however is that these metabolic processes are ongoing and also require the necessary vitamins and minerals to ensure activity of enzymes and their cofactors to support metabolism. The B vitamins in particular are noteworthy in this situation, as they are required for the final processes of glucose oxidation (see Fig. 53).

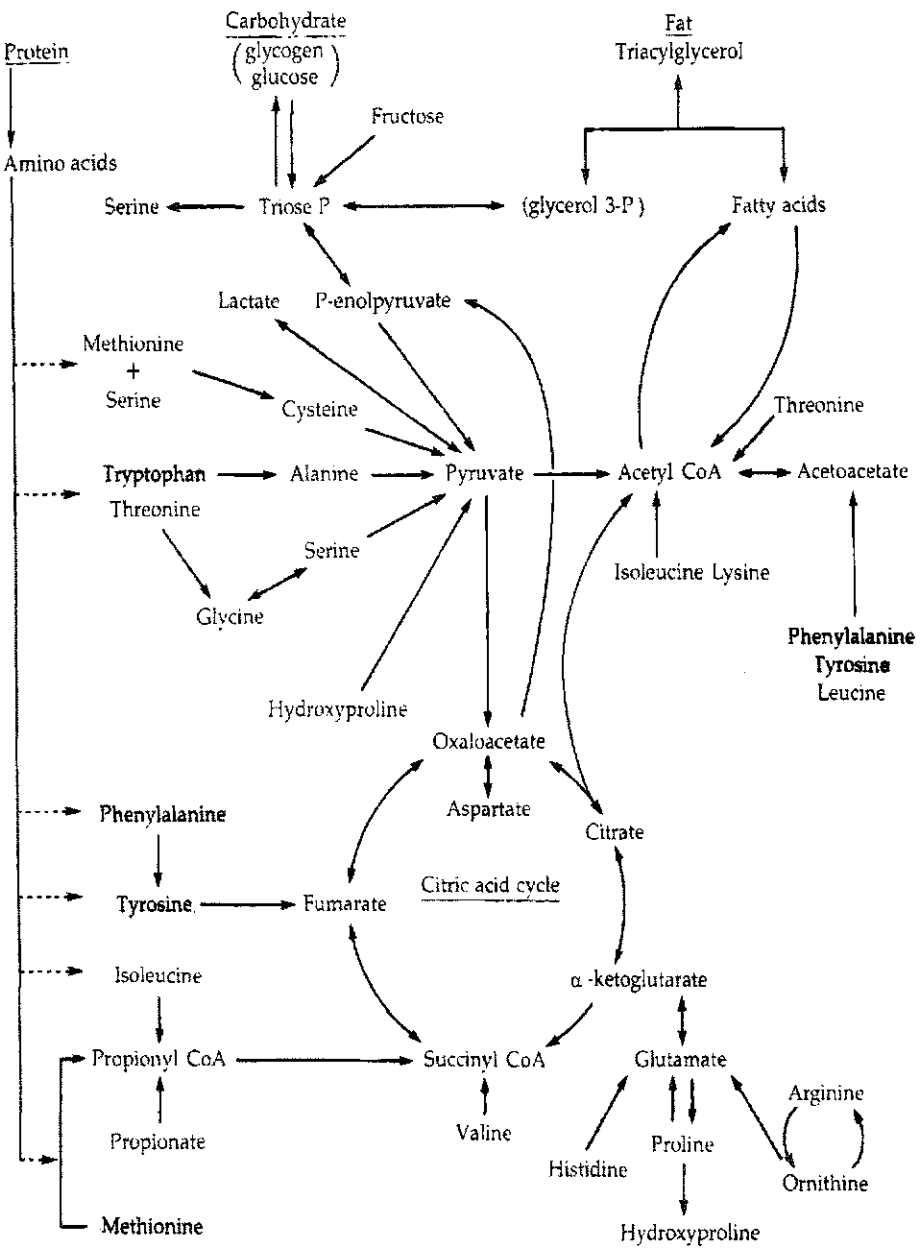


Fig. 53. The interconversion of nutrients required for energy metabolism (Extracted from Groff, Groper & Hunt, 1995).

An association of Bipolar Disorder and nutritional deficits may not seem likely, or significant, because after each phase of the cycle the individual begins to consume food again. Within time, the patient will overcome the event, and regain his/her appetite and energy. What should be considered however is that people with this disorder undergo these cycles for their entire lives. The B vitamins are very susceptible to depletion because they are water-soluble, have relatively quick clearance rates, and are in higher demands in people that are undergoing recurrent episodes of catabolic states. Furthermore, the numerous medications that are taken by these people every day also necessitate nutrients to metabolize these agents (see Fig. 54).

To compound the potential imbalances of nutrient intakes in Bipolar Disorder these patients have been characterized as carbohydrate cravers, and unfortunately, some have developed Bulimia Nervosa. As I have previously mentioned, my mother would crave quick (refined) sugars, and eat only large amounts of carbohydrate, which inadvertently imbalances dietary intakes for a number of days. The carbohydrates in turn, have been shown to increase the effectiveness of tryptophan to cross the blood brain barrier for serotonin production (Chaftetz, 1990).

Finally, it should be noted that niacin (vitamin B_3) plays a number of important roles in this situation. Niacin is necessary to complete the oxidation process of glucose to provide the body and brain with energy sources. Tryptophan is also an amino acid precursor for the synthesis of niacin. If dietary intakes of direct sources (meat, fish) of niacin are low, the body will use tryptophan to produce niacin, which may initiate depletions of serotonin synthesis. Chaftez has also noted that the consumption of various proteins, such as that found in meats, provides competition for the transport of tryptophan

across the blood brain barrier making it more difficult for these people to obtain brain sources of tryptophan (Chaftetz, 1990). Also, during metabolically demanding periods of stress and mania, circulating amino acids are used for the production of glucose, compromising the availability of phenylalanine, tryptophan, and tyrosine for neurotransmitter synthesis (see Fig. 53). The long-term duration of nutrient imbalances and inadequate nutrient replenishment that are prone to result from recurrent periods of stress, mania, and depression, could produce a condition of "nutritional burnout". It is this type of nutritional imbalance then that could either initiate, or exacerbate, the chemical imbalances that contribute to some of the cognitive deficits that are characterized in mood disorders.

Nutrients Used in Drug Oxidation and Conjugation

Nutrient	Oxidation	Conjugation
Carbohydrate		Glucose
Lipid	Lecithin	Acetyl (also from protein and carbohydrate)
Amino acids and derivatives	Glycine Protein	Glycine Glutamic acid, glutamine Cysteine, cystine Methionine Serine Arginine Alanine Some peptides
Minerals	Iron, copper, calcium, zinc, magnesium	
Vitamins	Pantothenate Niacin Riboflavin Ascorbic acid(?)	Pantothenate Niacin Folate Vitamin B_{12}

Fig. 54. Nutrients required for drug metabolism (Extracted from Zeman, F.J., 1991, in Clinical Nutrition and Dietetics).

Overall, nutrition is extremely important for recovery from illness, regardless of etiology; therefore, the therapeutic intervention of nutrition requires that all physiological and metabolic systems are effectively supported. Producing deficiencies at one stage of metabolism would most likely interfere with other metabolic processes, thereby beginning a cascade of effects as a result. Eventually, the catabolic processes of various nutrient sources are required, to "borrow" some necessary compounds and/or enzymes from other biological reactions to complete any further reactions. In this way the body can compensate metabolically for short-term duration of nutritional losses. Long-term duration however, could lead to a variety of deficiencies and result in some serious health consequences.

A well-formulated vitamin and mineral supplement in therapeutic quantities could be a very beneficial agent for health care. The applications that have been noted for its use in the treatment of brain injury and bipolar disorder are only examples of its benefits.

The body and brain is a unit that is integrated together. By keeping systemic systems functioning efficiently, and having the appropriate nutrient supply, the body can more effectively support the demands of central nervous system, which in turn effectively provides the organism with the appropriate learning capabilities to better adapt to its environment.

7. Appendixes

Appendix A: Anatomy and connectivity of the rat

Appendix B: Attention Shift (Exp.#1)

Appendix C: Western Blot

Appendix D: Vitamin Supplement/ Standard Rat Chow Analysis

Appendix E: Behavioral Results of the Second Generation Animals Treated with the Vitamin and Mineral Supplement

Appendix A

Anatomy and Connectivity of the Rat Cortex

The importance for an animal (or human) to be able to continually adapt to a changing environment requires the integrity of not only anatomical regions of the brain, but also the neural connections among them that makes the brain functional.

In the mature rat cortex, some basic neural connections should be addressed between the two cortexes subject to lesions: the frontal cortex and the parietal cortex, along with some connections to other cortical regions.

Zilles & Wree (1995) have revised previous editions of the rat cortical and subcortical connections and cytoarchitecture. To view past and present changes in nomenclature for the rat cortex, see table 19.

The frontal cortex:

The nomenclature for the frontal cortical areas are mainly from Zille's (1995) most recent research.

The medial prefrontal cortex consists of cingulate areas, Cg1, Cg2, and Cg3, collectively make up the anterior cingulate cortex. The infralimbic region is also considered by some as part of the medial prefrontal cortex. The Cg3 is also known as the prelimbic (PrL) area. The infralimbic area (IL) lies ventral to Cg3 (PrL). The medial precentral area frontal 2 (Fr2) lies dorsal to the cingulate cortex (Cg2), and is an association area involved in premotor functions.

Frontal areas Fr1 and Fr3 are situated dorsal to Fr2 (premotor) and are involved in the primary motor functions (see Fig.55).

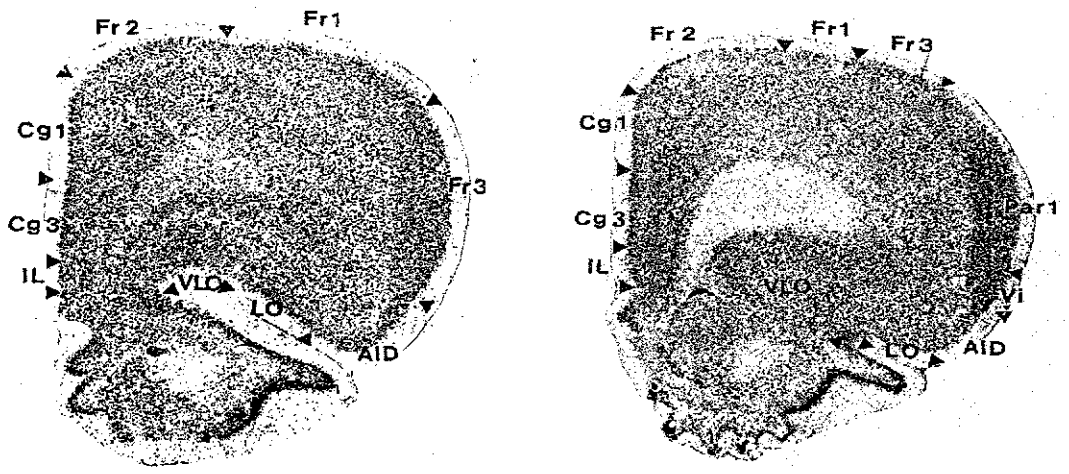


Fig. 55. Anatomical areas of the frontal cortex in the rat.

Connections of the frontal cortex:

Fr1 (motor) receives neural afferents from Fr2 (premotor) and Parietal area 1 (Par1) (sensory association) from both the contralateral (opposite) and ipsilateral (same) hemispheres of the brain.

Fr1 (motor) also receives afferents from the contralateral (motor) and from ipsilateral forelimb (FL) and hindlimb (HL) areas (sensory association). Fr2 (premotor) receives additional afferents from the retrosplenial cortex, an area specific to the posterior cingulate cortex.

Efferent connections from Fr1 and Fr3 (motor) terminate in Par 1 (somatosensory).

Efferents from the infralimbic (IL) area terminate in the cingulate (Cg1-3), and agranular insular (AID/AIV) areas, as well as the piriform and perirhinal cortices of the limbic system.

Efferent fibers from the AI cortex reach the prefrontal areas, IL, Cg1-3, the presubiculum, perirhinal, piriform, and entorhinal areas of the limbic system, and to the retrosplenial cortices of the posterior brain regions.

The orbital frontal cortex has been called the transition zone by Krettek and Price and is the region of frontal cortex that lies dorsal to the agranular insular cortex (AI) and ventral to the cingulate cortex Cg3. The orbital cortex of the ventral frontal areas are difficult to identify by cronal (front to back) sections, and are usually viewed using horizontal and saggittal (lengthwise) sections. Efferents from many cortical areas have been identified as connected with the orbital regions. The orbital frontal cortex is also known to have connections with the limbic system (see Fig.56).

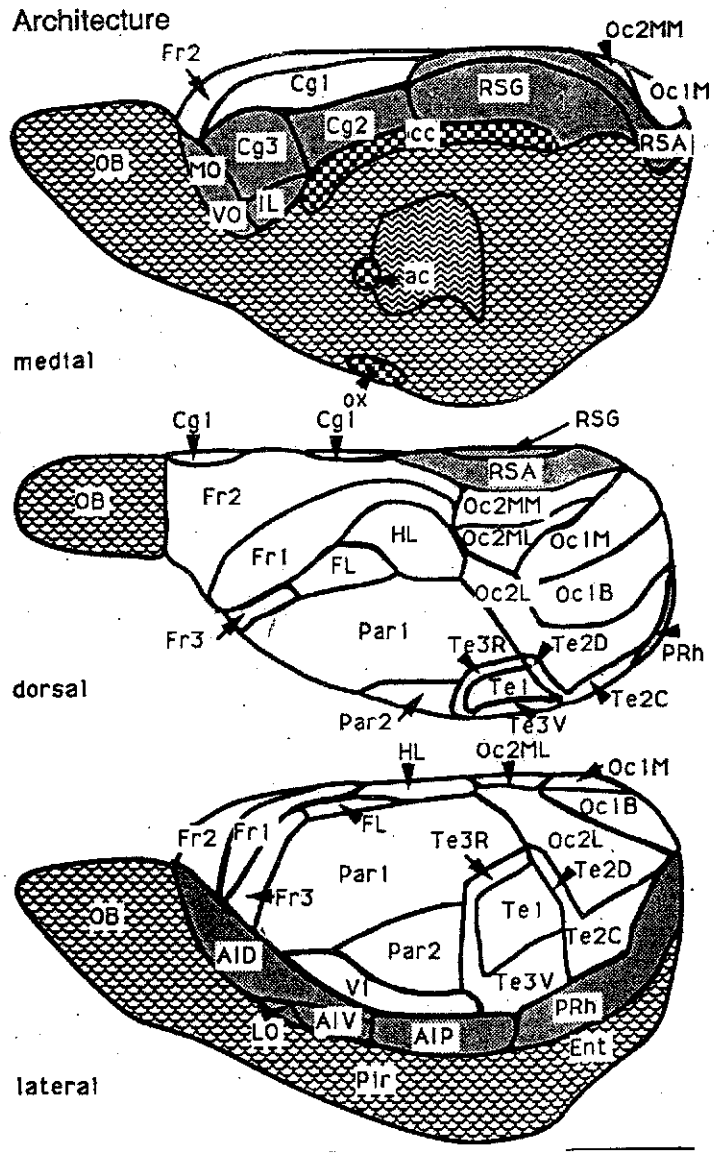


Fig. 56. Lateral, medial, and dorsal views of the rat brain.

The parietal cortex:

Some anatomical correlates of the posterior parietal cortex in primates have been determined for rats using True Blue tracing techniques (Kolb & Walkey, 1987). Kreig's area 7 in the rat's posterior cortex is the region of the brain that has some of the same connectivity characteristics as the posterior parietal cortex in primates.

Injection of True Blue tracer into Kreig's area 7 also revealed traces of dye into the adjacent somatosensory cortex (head and limb areas), extrastriate (association visual) cortical areas, (Vogt & Peters;18a,18b), striate (primary visual) areas (17), and frontal regions included the anterior cingulate (Cg2), the frontal eye fields in the precentral medial (PrCm/premotor) areas, and in some cases in the medial orbital, ventral orbital, and prelimbic areas (ventral frontal). To determine any reciprocal connections with Kreig's area 7, Nuclear Yellow was injected into areas in the frontal cortex. Labeling in area 7 was evident from connections with the anterior cingulate and the precentral (PrCm) region. The fronto-parietal connections labeled with True Blue overlapped with the parieto-frontal connections labeled with Nuclear Yellow, which indicated a convergence of connections in the same area (Kolb & Walkey, 1987).

Zille's recent map of the posterior parietal cortex include Parietal 1 (Par1), forelimb (FL), and hindlimb (HL) areas as the primary somatosensory cortex, and parietal 2 (Par2) as the secondary somatosensory cortex.

The surgical lesions performed in Experiments 1B and 2B are from Kreig's area 7, which is somewhat equivalent to Zille's occipital 2 lateral (Oc2L), therefore, connections involving Oc2L will be discussed (see Fig.57).

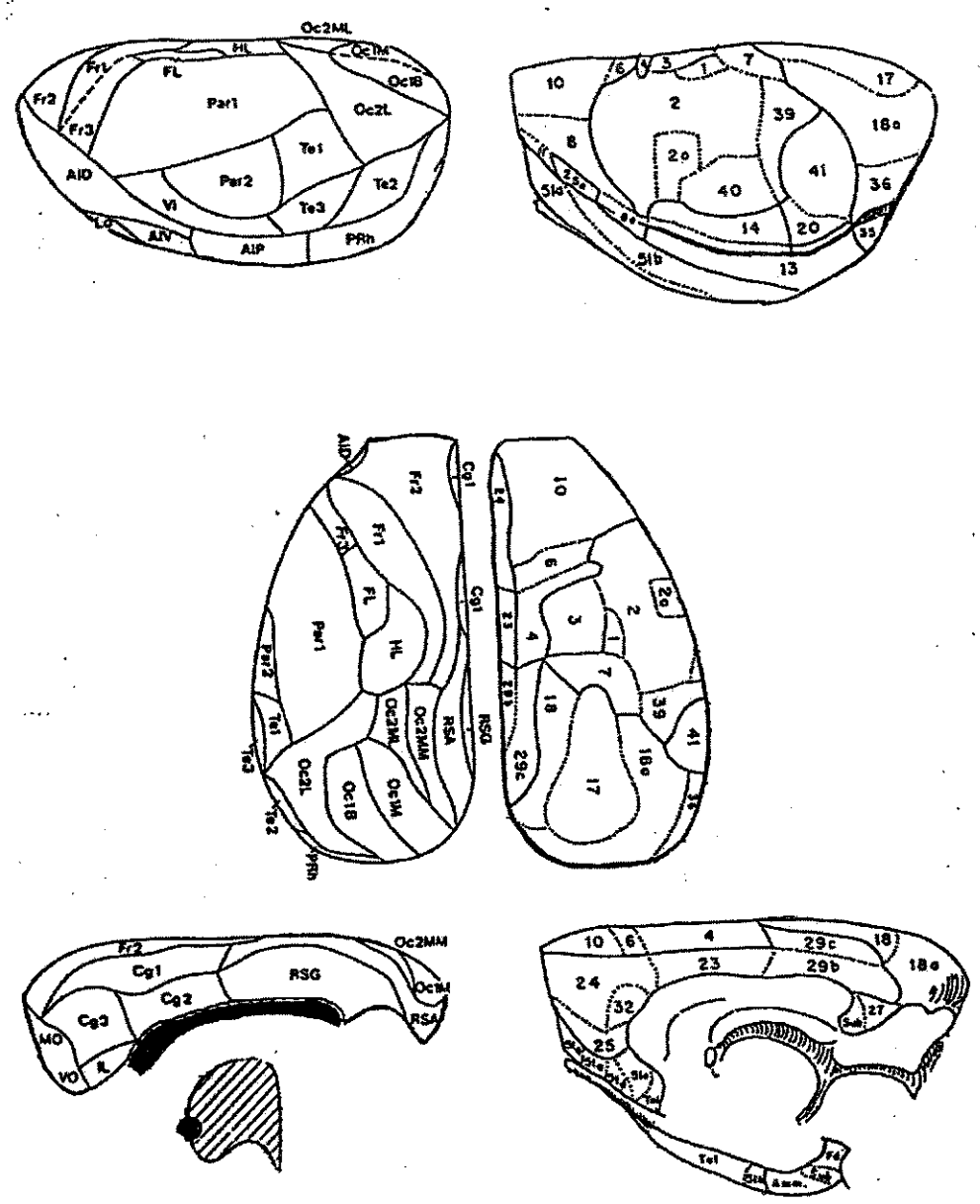


Fig. 57. Comparisons of Zilles and Kreig's areas of the rat brain.

Target efferent connections from the Oc2L include the primary visual cortex Oc1 (ipsilateral and contralateral), and the secondary Oc2M (medial) and the contralateral Oc2L areas.

The posterior cingulate areas of the retrosplenial granular cortex (RSG) and the retrosplenial agranular cortex (RSA) receive efferents from Oc2L

Oc2L also extends efferents into the hindlimb (HL) area of the primary somatosensory areas, as well as, the secondary auditory cortex Te2 in the temporal regions.

The Fr2 of the frontal cortex is the only area in the anterior sections of the cortex to receive efferents from Oc2L.

Other cortical areas:

The temporal and perirhinal cortices interlink many of the cortical areas discussed, all of which are necessary to carry out the numerous functions of the brain. The perirhinal cortex is part of the limbic system and receives efferents from the frontal cortical areas cingulate 1-3 (Cg1-3), frontal association Fr2, the agranular insular ventral and posterior regions (AIV,AIP), the secondary sensory area parietal 2, the agranular restrosplenial area (RSA), the secondary auditory cortex (Te2,Te3) and the lateral secondary visual cortex (Oc2L).

Additional areas of the hippocampal proper innervate the perirhinal cortex, such as the presubiculum, parasubiculum, subiculum, and the CA1 region of the hippocampus. Efferent fibers from the perirhinal cortex are the entorhinal (ipsilateral), subiculum, and CA1 of the hippocampus. The CA1 and subiculum, in turn, project fibers to the infralimbic (IL) area of the prefrontal cortex.

The temporal cortex lies dorsal to the perirhinal cortex. Most of the identified afferents to the temporal cortex are from various thalamic nuclei. Similarly many thalamic nuclei send projections to the temporal cortex. The cortical areas that have been

noted to project to the temporal cortex are the retrosplenial cortices; RSG and RSA (see Fig.58).

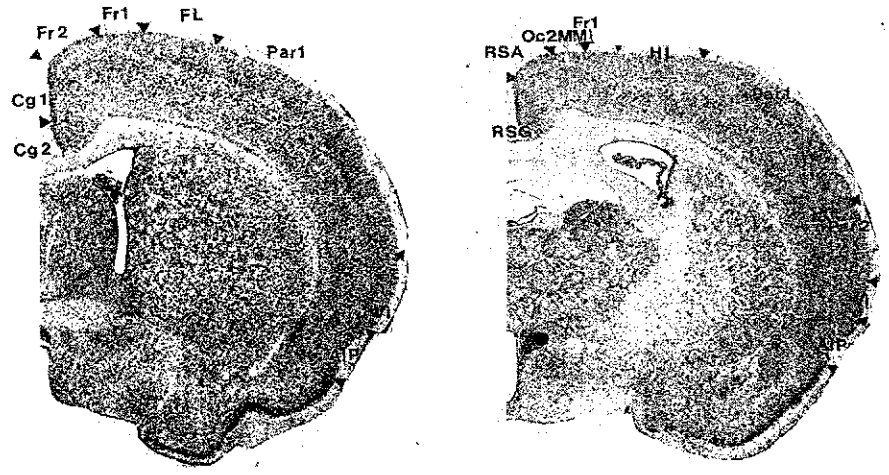


Fig. 58. Anatomical areas of the posterior regions of the brain in the rat.

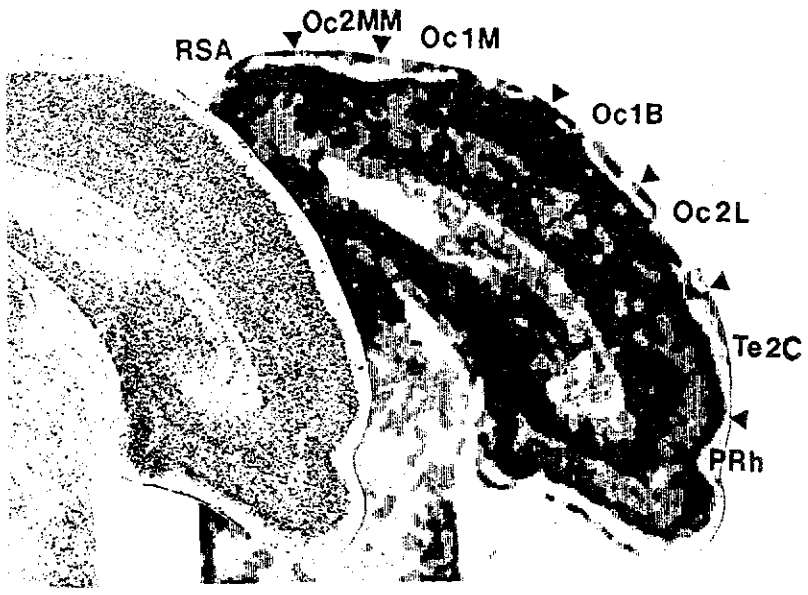


Fig. 59. Anatomical areas of occipital cortex and posterior regions of the rat brain.

Table 19. Nomenclature of equivalent cortical areas.

Zille's 1995	Zille's 1980	Krettek and Price, 1977	Kreig, 1946
Cg1,Cg2,IL,CG3	C1,C2,C3,C4	ACd , Acv, IL	Areas 23,24, & 32 (IL/PL)
Fr2	Prcm, Prc 3	PrCm	Areas 4,6,8,8a,10 and 11
Fr1, Fr3	Prc 1-2	PrCl	
Par 1	SmI exclude dorsomedial section		
FL, HL	SmI dorsomedial		
Par2	SmII		
Oc2MM	Part of Rag	Part of RsAg	
Oc2L	Oc2L		Area 7,18a

The reciprocal projections between the frontal and parietal cortexes serve as a very important connection functionally. The posterior regions of the brain communicate certain messages of sensory information received from the body, which is communicated as "bottom up" information to the prefrontal cortex. The prefrontal cortex operates at a "top-down" level of control over sensory input, as it must organize the information for behavioral output. Needless to say, brain injury to either of these regions disrupts a number of cognitive processes and behavioral responses related to both cortexes.

APPENDIX B

Attention Shift

Arm Baited	**Habituation**	**Dimension**	**Odor/Medium**
Left and Right	Bait both bowls		Sawdust
Left	Series of 2 discriminations	Odor	Cloves vs. Rosemary
Right		Medium	Styrofoam vs. Shredded paper
Right	**Testing**	Simple (SD): Odor O1 vs.O2	Cinnamon vs. Basil
Left		Compound (CD): O1/M1 vs. O2/M2	Cinnamon Sawdust vs. Basil Corncob
Left		CD: O1/M2 vs. O2/M1	Cinnamon Corncob vs. Basil Sawdust
Right		Reversal (Rev 1) O2/M1 vs. O1/M2	Basil Sawdust vs. Cinnamon Cob
Right		Rev 1 O2/M2 vs. O1/M1	Basil Cob vs. Cinnamon Sawdust
Right		Intradimensional Shift (ID): O3/M3 vs. O4/M4	Cumin Beads vs. Thyme Glass Stones
Left		ID: O3/M4 vs. O4/M3	Cumin Glass Stones vs. Thyme Beads
Left		Reversal 2 (Rev): O4/M3 vs. O3/M4	Thyme Beads vs. Cumin Glass Stones
Right		Rev 2: O4/M4 vs. O3/M3	Thyme Stones vs. Cumin Beads
Left		Extradimentional Shift (ED): M5/O5 vs. M6/O6	Curry Stones vs. Allspice Woodchip
Left		ED: M5/O6 vs. M6/O5	Curry Woodchip vs. Allspice Stones
Right		Reversal 3 (Rev): M6/O5 vs. M5/O6	Curry Woodchip vs. Allspice Stones
Left		Rev 3: M6/O6 vs. M5/O5	Allspice Woodchip vs. Curry Stones

Discrimination Pairs:

	1	2	3	4	5	6
Odor	Cinnamon	Basil	Cumin	Thyme	Curry	Allspice
Medium	Sawdust	Corncob	Beads	Glass Stones	Stones	Woodchip

Appendix C
Western Blot

Procedures:

Brain tissue was removed from decapitated animals and placed on ice. Following rapid dissection of cortical regions, brain samples were placed in microcentrifuge tubes and frozen at -75° C, until ready for preparation. Preparation of a homogeneous sample consisted of sonicating the frozen tissue in the tubes with 800μL of 1% sodium dodecyl sulfate (SDS). Sample was then aliquoted into new microcentrifuge tubes and diluted 1/20 to determine the protein concentration (Bradford Assay) before resolving labeled proteins on 12% acrylamide gels (5μg of protein per well) using Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis. Prepared gels were blotted on polyvinylidine difluoride (PVDF) membranes.

The membranes were blocked for non-specific binding proteins for one hour with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween 20. Primary antibodies to proteins of interest were diluted (1:1000) with the same solution used for blocking.

The membranes were incubated in the primary antibody for two hours, followed by five more washes with PBS. Proteins of interest was revealed with an ECL + detection kit from Amersham (RPN2132) and the resulting image was captured on Hyperfilm ECL (Amersham #RPN1647K).

The membranes were stained with 0.1% Coomassie Blue (Omnipure EM Science) to reveal all proteins in order to ensure pipeting consistency. Exposed film was imaged with a Kodak digital camera and the blot density was then analyzed using NIH Image software.

Primary anitibodies used: GR, Santa Cruz, SC-1004
bFGF, Santa Cruz, SC-7911
GFAP, Sigma, G3893
Flg, Santa Cruz, SC-121

Analysis was performed for proteins; gluccocorticoid receptor type 1 (GR)
Basic fibroblast growth factor (bFGF)
Glial fibrillary acidic protein (GFAP)
GFAP receptor protein Flg

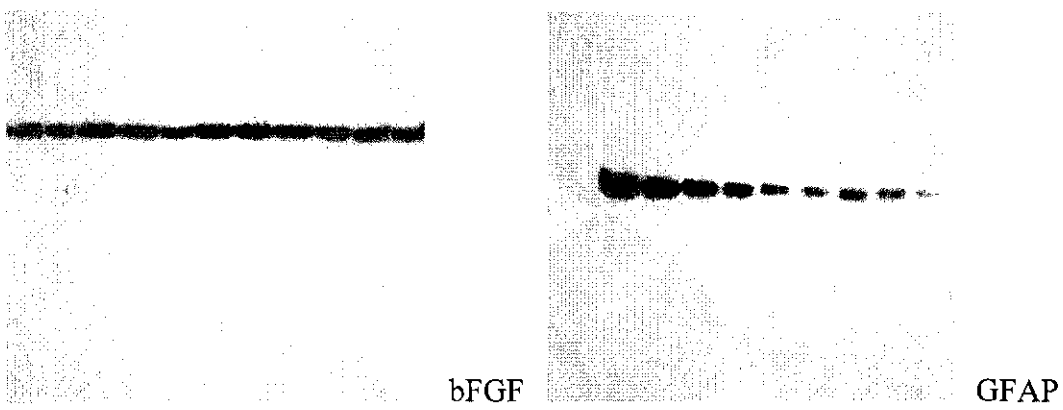


Fig. 60. Proteins analyzed by Western Blot technique.

Results: All brain tissue analyzed for specific proteins were present in normal quantities, as indicated by the control group (see Table 20).

Table 20. Summary of Statistical Analysis for Western Blot.

Protein	Group	F-value (1,11)	P-value	Mean	Std. Error
GR	Supplement	.263	.6179	1.037	.081
	Control			1.087	.044
bFGF	Supplement	.608	.4520	2.235	.181
	Control			2.422	.151
GFAP	Supplement	.211	.6548	1.523	.297
	Control			1.690	.182
Flg	Supplement	.379	.5415	2.012	.148
	Control			2.161	.187

Appendix D
Analysis of Vitamin Supplement Diet vs. Analysis of Standard Laboratory Rat Chow

The analysis of the vitamin supplement diet is a sample of some of the nutrients provided by the base diet, as well as the diet with the vitamin and mineral supplement added.

Table 21. Nutritional analysis of the base diet composition in the vitamin supplemented diet.

Analyte	Units	As Fed	Dry Basis
Moisture	%	7.2	
Ash	%	6.74	7.26
Crude Protein	%	22.9	24.6
Crude Fiber	%	7.4	7.9
Total Digestible Nutrients (Crude)	%	72.62	78.22
Digestible Energy (Crude)	Mcal/kg	3.27	3.53
Gross Energy (Crude)	Mcal/kg	4.3	4.63
Metabolizable Energy (Crude)	Mcal/kg	3.09	3.33
Net Energy For Growth	Mcal/kg	2.16	2.33
Crude Fat	%	5.5	5.9
Nitrogen Free Extract	%		54.3

Analysis provided by Northwest Labs.

Table 22. Mineral content of vitamin supplemented diet.

Analyte	Units	As Fed	Dry Basis
Calcium	%	1.02	1.10
Phosphorus	%	0.67	0.72
Potassium	%	1.08	1.16
Magnesium	%	0.22	0.24
Sodium	%	0.24	0.26
Salt	%	0.62	0.67
Sulphur	%	0.33	0.36
Zinc	ppm	78.7	84.7
Manganese	ppm	44.6	48.0
Copper	ppm	14.5	15.7
Iron	ppm	290	313

Analysis provided by Northwest Labs.

The vitamin supplement provided approximately 1/2 to 2/3 of the mineral content in addition to the nutrients present in the base diet (Hardy, 2003). The minerals that are measured in parts per million (ppm), are a measure of milligrams per kilogram (mg/kg). The nutrient analysis of the standard lab diet fed to control animals is very similar to the vitamin supplement fortified diet in measures of crude protein, crude fat and crude fiber (see Table 23).

The Amino acid and essential fatty acid composition of base diet of the vitamin supplement diet, and for the standard Rat Chow diet are also similar and present in similar quantities. The standard rodent diet (control) and the base diet for the fortified diet (supplement) differ in many of the vitamins and mineral contents. They are in higher quantities in the control standard diet than to those in the base diet of the fortified diet (see Table 25). Addition of the vitamin and mineral supplement to the base diet increased the amount of micronutrients (see Table 22) to levels just slightly above that in the control standard diet (see Table 24).

Table 23. Nutritional analysis of the standard diet composition in the control diet.

Analyte	Units	Dry Basis
Moisture	%	
Ash	%	6.9
Crude Protein	%	not less than 23%
Crude Fiber	%	Not more than 6%
Total Digestible Nutrients (Crude)	%	76.0
Digestible Energy (Crude)	Mcal/kg	n/a
Gross Energy (Crude)	Mcal/kg	4.00
Metabolizable Energy (Crude)	Mcal/kg	3.04
Net Energy For Growth	Mcal/kg	n/a
Crude Fat	%	4.5 to 5.5
Nitrogen Free Extract	%	49.9

Analysis provided by Purina Labs.

Table 24. Mineral content of control standard diet.

Analyte	Units	Dry Basis
Calcium	%	.95
Phosphorus	%	0.67
Potassium	%	1.10
Magnesium	%	0.21
Sodium	%	0.28
Cloride	%	0.65
Sulphur	%	0.36
Zinc	ppm	70.0
Manganese	ppm	64.0
Copper	ppm	13.0
Iron	ppm	270

Analysis provided by Purina Labs.

Table 25. Base nutrient profiles of the control and vitamin supplement diets.

Dietary Constituents	Standard Lab Diet (control)	Base Diet for Supplement
Amino Acids		
Arginine	1.38 %	1.47 %
Cystein	0.32 %	0.72 % (Met & Cys)
Glycine	1.20 %	1.19 %
Histidine	0.55 %	0.57 %
Isoleucine	1.18 %	1.19 %
Leucine	1.70 %	1.87 %
Lysine	1.42 %	1.42 %
Methionine	0.43 %	0.40 %
Phenylalanine	1.03 %	1.08 %
Tyrosine	0.68 %	
Threonine	0.91 %	0.93 %
Tryptophan	0.29 %	0.30 %
Valine	1.21 %	1.22 %
Serine	1.21 %	
Aspartate	2.83 %	2.94 %
Glutamate	4.54 %	4.75 %
* note: not all amino acids	included in table.	
Fat		
Omega-3 fatty acids	0.33 %	0.24 %
Omega-6 fatty acids	1.16 %	1.23 %
Arachidonic acid	< 0.01 %	0.11 %
Vitamins		
Carotene	4.5 ppm.	0
Vitamin K (menadione)	0.5 ppm.	0.54 ppm.
Vitamin B1 (thiamine)	17.0 ppm.	7.01 ppm.

Vitamin B2 (riboflavin)	8.0 ppm.	3.23 ppm.
Vitamin B3 (niacin)	124.0 ppm.	43.44 ppm.
Pantothenic acid	24.0 ppm.	10.64 ppm.
Choline chloride	2250.0 ppm.	0
Folate	5.9 ppm.	1.21 ppm.
Vitamin B6 (pyridoxine)	6.0 ppm.	4.18 ppm.
Biotin	0.2 ppm.	0.2 ppm.
Vitamin B12	22 mcg./kg.	3.52 mcg./lb.
Vitamin A	22.0 I.U./g.	0
Vitamin D3	4.5 I.U./g.	0
Vitamin E	49.0 I.U./g.	11.85 %
Vitamin C	0	0
Minerals		
Calcium	0.95 %	0.66 %
Phosphorus	0.67 %	0.62 %
Potassium	1.10 %	1.16 %
Magnesium	0.21 %	0.21 %
Sulfur	0.28 %	
Sodium	0.40 %	0.13 %
Chloride	0.65 %	0.23 %
Fluoride	18.0 ppm.	13.77 ppm.
Iron	270 ppm.	238.73 ppm.
Zinc	70.0 ppm.	40.98 ppm.
Manganese	64.0 ppm.	28.86 ppm.
Copper	13.0 ppm.	8.35 ppm.
Iodine	0.8 ppm.	0.31 ppm.
Chromium	2.0 ppm.	1.08 ppm.
Selenium	0.27 ppm.	0.23 ppm.
Cobalt		0.68 ppm.

The vitamin supplement added to the base diet includes some ingredients that were not included in the base diet analysis. Listed in Table 6 are label claims of the vitamin and mineral supplement EM Power.

Table 26. Vitamin Supplement. Serving Size is 8 Capsules. (Label Claim)

Dietary Constituent	Amount Per Serving	% Daily Value
Vitamin A (retinyl palmitate)	2400 I.U.	48 %
Vitamin C (ascorbic acid)	250 mg.	417 %
Vitamin D (cholecalciferol)	400 I.U.	100 %
Vitamin E (d- α tochopherol succinate)	100 I.U.	333 %
Vitamin B1 (thiamine mononitrate)	5 mg.	333 %
Vitamin B2 (riboflavin)	5.5 mg.	324 %
Vitamin B3 (niacinamide)	25 mg.	125 %
Vitamin B6 (pyridoxine hydrochloride)	7 mg.	350 %
Vitamin B9 (folic acid)	400mcg.	100 %
Vitamin B12 (cyanocobalamin)	250 mcg.	416 %
Vitamin H (biotin)	25 mcg.	8 %
Vitamin B5 (d-calcium pantothenate)	6 mg.	60 %
Calcium (amino acid chelate)	550 mg.	55 %
Iron (amino acid chelate)	6 mg.	33 %
Phosphorous (phosphorous complex)	350 mg.	35 %
Iodine (from kelp)	75 mcg.	50 %
Magnesium (amino acid chelate, magnesium complex)	250 mg.	63 %
Zinc (amino acid chelate, zinc complex)	20 mg.	133 %
Selenium (amino acid chelate, selenium complex)	100 mcg.	143 %
Copper (amino acid chelate, copper complex)	3 mg.	150 %
Manganese (amino acid chelate, manganese complex)	4 mg.	200 %
Chromium	250 mcg.	208 %
Molybdenum (amino acid chelate, molybdenum complex)	66 mcg.	88 %
Potassium (potassium complex)	100 mg.	3 %

The central nervous system proprietary blend as part of this supplement consists of dl-phenylalanine, glutamate, citrus bioflavinoids, grape seed, choline, inositol, ginkgo biloba, methionine, organic germanium, boron, vanadium, and nickel.

Appendix E
Behavioral Results of the Second Generation Animals Treated with the Vitamin/Mineral Supplement

One female and two males were born to a mother rat that received the vitamin supplement administration approximately one week after introduction to the male (Plan B). The three rats remained housed together until the birth of ten pups. Although this pregnancy was not planned, it had come as a pleasant surprise knowing that a second generation of rats on this supplement had been born. Two rats died during their early days, leaving eight rats to run on behavioral tests. Surgeries were not performed with this group. The new pups were calm and very easy to handle during behavioral tests. A few analyses were performed and compared to controls to measure the effects of this supplement with a second-generation litter.

Method

Subjects

All rats participated in three behavioral tasks and were tested at 60 days of age.

Table 27. Number of second-generation supplement treated animals.

	Animals
Supplement Treatment	N= 8
Control	N= 8

Treatment, and behavioral procedures

Performed the same as for Experiments 1 and 2.

Results

Behavioral results:

Open Field:

As in the second experiment, the supplement treatment significantly reduced the activity level in both the horizontal activity measure (see Fig. 62), and in the distance measure (see Fig. 63).

Analysis of variance for horizontal activity reveal a main effect of treatment ($F(1,14)= 24.088$, $P= .0002$),

ANOVA for distance also indicated a main effect of treatment ($F(1,14)= 25.667$, $P= .0002$).

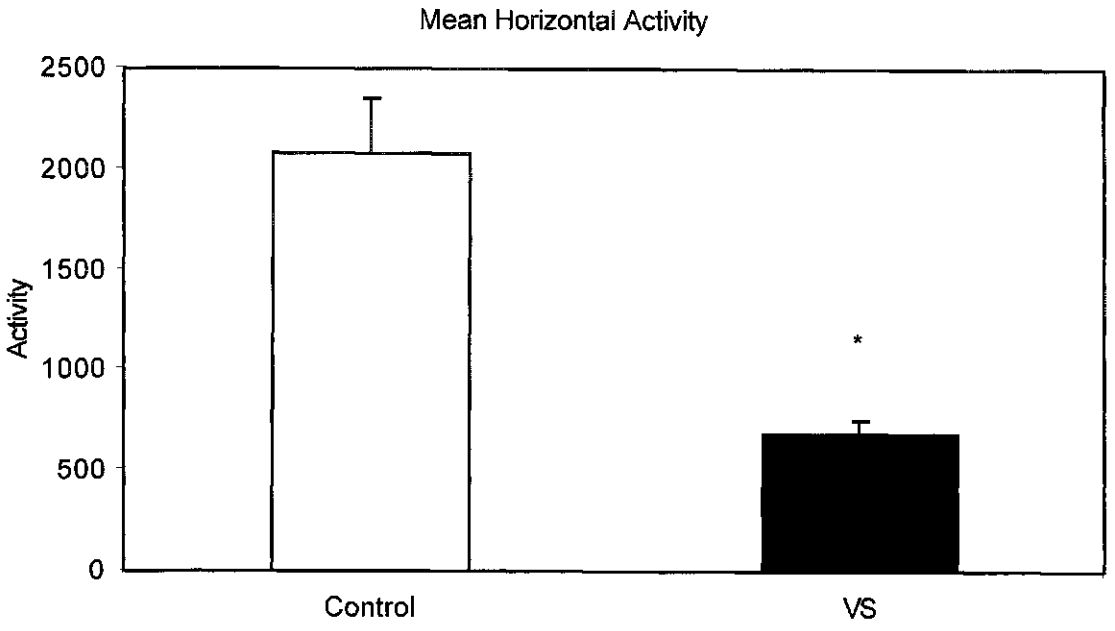


Fig. 61. Mean horizontal activity in the open field task of the second-generation supplement treated animals.

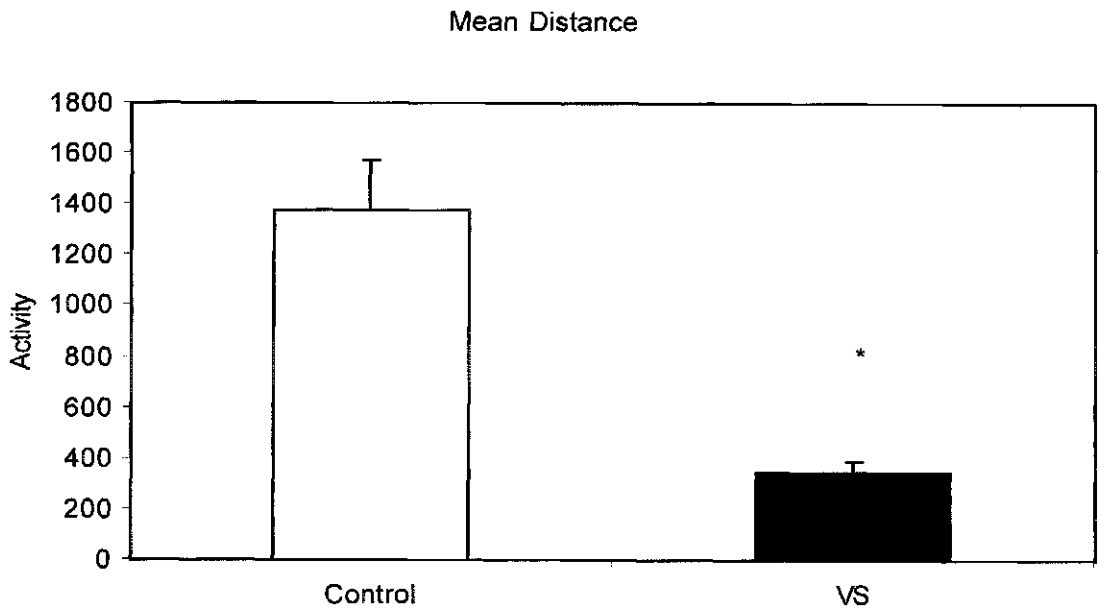


Fig. 62. Mean distance for second-generation supplement treated animals.

Morris water task:

Latencies for the supplement-treated females were lower than males throughout the 5-day trial period (see Fig. 64). Of the 3 males that participated in this task, only one rat did not realize the goal of the task until day 3, possibly skewing good results of the other 2 males.

Analysis of variance (ANOVA) between sexes for total sums of latency in the second generation group did not indicate a main effect for sex ($F(1,13)= 3.019$, $P= .1059$), latency ($F(1,13)= 2.812$, $P= .1174$), or for the interaction ($F(1,13)= .988$, $P= .3384$. The results indicate that the supplement treatment during pregnancy and lactation produces animals that function as normal as control animals on the standard diet.

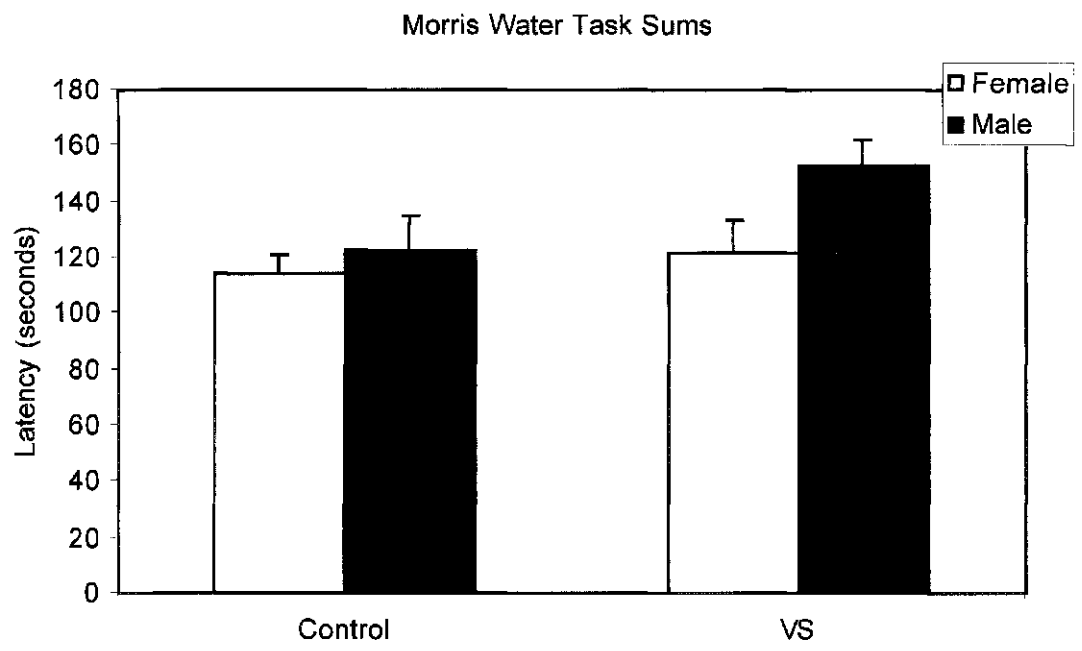


Fig. 63. Mean latency of second-generation supplement treated animals.

Tray Reaching:

The supplement-treated animals showed similar success rates to the control animals (see Fig. 65). A two-way ANOVA for treatment did not indicate a main effect ($F(1,16)= 1.424$, $P= .2502$).

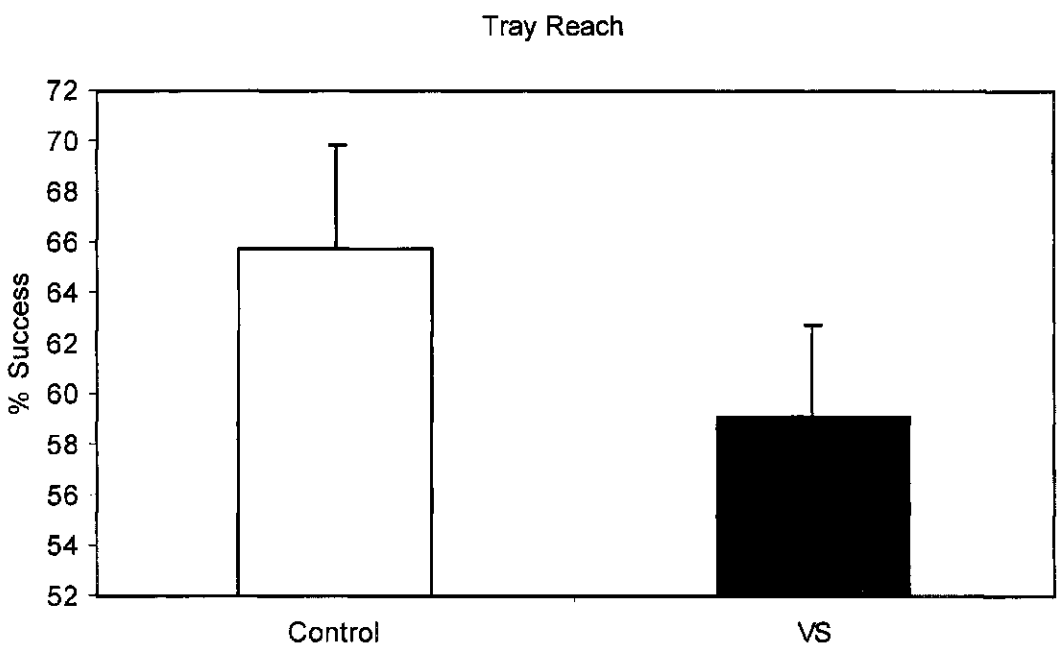


Fig. 64. Summary of tray reaching task for the second-generation supplement treated animals.

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