Effects of Adrenalectomy and Selective Adrenal Steroid Receptor Agonists on Spatial Memory Performance and Dentate Gyrus Morphology

by

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Abstract

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Adrenalectomy (ADX) causes neuronal degeneration in the dentate gyrus (DG) of the hippocampus as early as 3 days after surgery and extensive granule cell loss 3 to 4 months later. Since lesion of the DG causes deficits in spatial memory performance, the effect of ADX on spatial memory was investigated. Rats were adrenalectomized and tested on a radial arm maze (RAM) shortly after adrenal removal. ADX resulted in impaired spatial memory performance which lasted for up to 71 days. The presence of accessory adrenal tissue was assessed by measuring serum levels of corticosterone (CORT) and daily intake of 3% saline. Saline consumption was higher in ADX rats and was negatively correlated to serum CORT ($r=-0.85$, $p<0.0018$). Persistent deficits in RAM performance occurred without the marked presence of pyknosis or the reduction of DG size suggesting that adrenal hormones themselves exert an activational effect on spatial memory prior to pervasive degeneration of DG neurons. We next assessed the involvement of

Type 1 and Type 2 adrenal steroid receptors in spatial memory function by treating ADX rats with selective receptor analogs. Type 1 and Type 2 adrenal steroid receptors were shown to have opposite effects on spatial memory function; Replacement of the type 1 receptor agonist, aldosterone, restored normal spatial memory function in ADX rats whereas the type 2 agonist, RU28362, further impaired spatial memory function. On this basis, we predict that the dose-response relationship between serum CORT and spatial memory performance is an inverted U-shaped function. Spatial memory function should be optimal at intermediate levels of CORT while performance is diminished when plasma CORT is high or low. The time course of effects suggest that adrenal steroids mediate spatial memory by genomic mechanisms. Further, rats that were first trained to the RAM before adrenal removal performed as well as controls suggesting that ADX impairs learning while leaving memory intact. The contribution of adrenal steroids to learning and/or memory appears complicated, however, since when these same rats were tested on a delay task, performance was improved in ADX rats.

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LIST OF ABBREVIATIONS

ADX	Adrenalectomy
ALDO	Aldosterone
ANOVA	Analysis of variance
CORT	Corticosterone
DG	Dentate gyrus
GCL	Granule cell loss
LTP	Long-term potentiation
RAM	Radial arm maze
RIA	Radioimmunoassay
SHAM	Operated control group

INTRODUCTION

Glucocorticoids, Stress and the HPA Axis

Glucocorticoids are secreted by the adrenal glands and promote gluconeogenesis in response to stress (Sapolsky, 1992). Although stress can be produced by a number of external stimuli, the physiological response to stress is essentially the same. Hans Selye (Selye, 1946; Selye, 1976; Spencer, Miller, Young, and McEwen, 1990) called the body's reaction to prolonged stress the general adaptation syndrome (G.A.S.). The syndrome consists of three phases: alarm, resistance and exhaustion. Stressors can be physical or psychological. The stress response begins when a stressor is perceived by the brain.

The glucocorticoid response to stress is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Guytong, 1986). Stress causes the release of corticotrophin-releasing hormone (CRH) and other stress related secretagogues into the hypothalamic capillary plexus of the hypophysial portal system and is carried to the pituitary gland. Cell bodies of CRH secreting neurons are located in the nucleus of the posterior medial basal hypothalamus. The nucleus receives neuronal inputs from the limbic system and lower brain stem. CRH modulates the secretion of several pituitary hormones including adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH enters the systemic circulation and stimulates the release

of glucocorticoid hormones from the adrenal glands.

Under conditions of stress, CRH is secreted within a few seconds, systemic levels of ACTH rise in perhaps 15 seconds, and glucocorticoids are released within a few minutes of stimulus onset (Guyton, 1986). In the absence of stress, secretion of CRH, ACTH, and glucocorticoids follow a cyclical pattern of release. Levels of these three substances are high in the early morning, and low in the early evening. Elevated levels of glucocorticoids act to suppress further ACTH release. Thus, ACTH is under negative feedback control of glucocorticoids (Yates, Marsh and Maran, 1974). Plasma cortisol levels are high enough after each diurnal secretory burst to suppress further ACTH secretion and inhibition should certainly occur after termination of an environmental stressor. Circulating levels of plasma cortisol then progressively fall until more CRH is released.

The Adrenal Gland

The adrenal glands are pink nodules that are situated on top of the kidneys. Each gland consists of an outer adrenal cortex and inner medulla. Early researchers (Yates et al., 1974) found that steroid hormones of the adrenal cortex had effects on either carbohydrate metabolism (glucocorticoids) or fluid electrolyte and water balance (mineralocorticoids). About 30 different adrenal steroids have been isolated (Guyton, 1986). Mineralocorticoids, the most important of which is aldosterone

(ALDO), are secreted by the outer most zona glomerlosa of the adrenal cortex. Glucocorticoids and androgens are secreted by the middle zona fasiciculata layer and the innermost zona reticularis. Glucocorticoids increase the availability of glucose to muscle and nervous tissue during stress by degradating glycogen, protein and triglycerides into component substrates. Most glucocorticoid activity in humans is attributable to cortisol. Corticosterone (CORT) is the principal glucocorticoid in the rat. The inner medulla is part of the sympathetic nervous system's response to stress and secretes catecholimines, epinephrine (adrenaline) and norepinephrine, in order to prepare the body for fight or flight in the initial alarm phase of the stress response.

Biosynthesis of Adrenal Steroids

Adrenal steroids are all derived from cholesterol (Kaplan, 1992). Low-density lipoproteins are taken into adrenocortical cells by endocytosis. The lipoproteins are converted into cholesterol by enzymatic modification (cleavage of a lipoprotein group and esterification) and stored in cytoplasmic vacuoles. When ACTH is released from the anterior pituitary, cholesterol esterase within the adrenocortical cells are activated. Cholesterol esterase causes the expulsion of cholesterol into the cytosol making the substrate available for steroid biosynthesis. Cholesterol is first converted into pregnenolone by 20,22-desmolase, a cytochrome P-450 side chain cleavage

enzyme. This is the rate limiting step in steroidogenesis. Pregnenolone is taken up by the mitochondria and sequentially modified by dehydrogenases and hydroxylating enzymes to form glucocorticoids, mineralocorticoids, and androgens.

Adrenal Steroid Receptors and the Hippocampus

McEwen, Weiss & Schwartz (1968) originally reported that the major central nervous system target for adrenal glucocorticoids is the hippocampus. This finding was surprising since hormones were not known to have neuronal target sites outside of the hypothalamus and the pituitary. Tritiated analogs were initially used to characterize two types of adrenal steroid receptors in the brain (see McEwen, de Kloet, & Rostene, 1986). These receptors have since been cloned (Arriza, Weinberger, Cerelli, Glaser, Handelin, Housmann & Evans, 1987; Patel, Sherman, Goldman, & Watson, 1989) and their primary structure has been identified (Hollenberg, Weinberger, Ong, Cerelli, Oro, Lebo, Thompson, Rosenfeld, & Evanset, 1985). Type 1 adrenal steroid receptor has also been called the mineralocorticoid receptor (MR), the tonic influence receptor, or the high affinity receptor (de Kloet, Ratka, Reul, Sutanto, Van Eekelen, 1987). This receptor has a high affinity for ALDO and a somewhat lower affinity for CORT and is localized predominantly in the hippocampus (Reul & de Kloet, 1985). Type 2 receptor, also called the glucocorticoid (GR), feedback or low affinity receptor (de Kloet et al., 1987) was identified in studies using

tritiated dexamethasone, a synthetic glucocorticoid (McEwen et al., 1986). Type 2 receptors are more diffusely distributed throughout the brain (Reul & de Kloet, 1985; Fuxe, Wikstrom, Okret, Agnati, Harfstrand, Yu, Granholm, Zoli, Vale, Gustafsson, 1985) and have a lower binding affinity for naturally occurring steroids (Reul & de Kloet, 1985). The type 2 receptor has a 3- to 5- fold lower affinity for CORT and a 10- to 20- fold lower affinity for ALDO than the type 1 receptor (Spencer, Miller, Moday, Stein & McEwen, 1993).

The adrenal steroid receptors form a two tiered system of glucocorticoid action in which the high affinity type 1 receptors are extensively occupied by lower levels of CORT that are present during the diurnal rise and fall of adrenal steroids whereas the low affinity type 2 receptor may become substantially occupied only at high resting and stress levels of CORT (McEwen et al., 1986). The type 1 receptor is believed to be involved in the tonic or permissive actions of adrenal steroids and is about 90% occupied at low basal levels of circulating CORT (Reul et al., 1987). The type 2 receptor becomes about 70% occupied at stress and peak circadian levels of CORT and is thus believed to be important in mediating the feedback actions of CORT such as terminating the glucocorticoid response to stress (Reul et al., 1985; de Kloet et al., 1987).

Neuroanatomy of the Hippocampal Formation

The hippocampal formation is the broader neuroanatomical

area that, in addition to the hippocampus proper, includes the dentate gyrus and the subiculum (Brodal, 1981). The hippocampus proper, also called Ammon's horn, is part of the limbic system. Ammon's horn protrudes into the temporal floor of the lateral ventricle as it folds around the hippocampal sulcus. An early description of the cortical layers and cell types found in Ammon's horn was provided by Santiago Ramon y Cajal (1893, translated 1968). Ramon y Cajal described seven layers. These are, from the deepest to the most superficial, the epithelial zone, alveus, stratum oriens, stratum pyramidale (pyramidal layer), stratum radiatum, stratum lacunosum, and stratum moleculare. The pyramidal layer is a band of pyramidal cell bodies that stain distinctly from its neighboring zones. Ramon y Cajal called the region adjacent to the subiculum with the more compact arrangement of cells the regio superior. The regio inferior begins where the pyramidal cells become less compact. Lorente de No (as cited by Brodal, 1981) subdivided Ammon's horn into four fields according to cytoarchitectonic criteria. Hippocampal fields are designated as Cornu Ammonis (CA) and are numbered 1 through 4. Ramon y Cajal's regio superior approximately corresponds to field CA1. Field CA3 occupies most of the regio inferior and is parcelled into subareas a, b, and c. The small transitional area between CA1 and CA3 is CA2. CA4 is the area that is transitional between CA3 and the dentate gyrus.

The dentate gyrus, or fascia dentata, is a band of cortex

that caps the free margin of Ammon's horn. The region of interface between the two structures is the hilus. On the basis of its U shaped appearance in transverse section, the dentate gyrus can be sub-divided into three areas. The suprapyramidal blade is adjacent to the hippocampal fissure. The limb of the U that lies along the midbrain is called the infrapyramidal blade. Between the two areas is the crestal area. Ramon y Cajal (1893/1963) partitioned the dentate gyrus into the molecular layer, granule cell layer, and the plexiform or polymorphic cell layers. The granule cell layer consists of the cell bodies of the dentate granule cells which stain as a prominent band.

Electrophysiological studies have shown that the entorhinal cortex, granule cells of the dentate gyrus, and the hippocampal pyramids of the inferior and superior regions are connected in a sequential and unidirectional intrahippocampal circuit called the trisynaptic loop (Anderson, Bliss and Skrede, 1970; Skrede & Westgaard, 1971). Stimulating the entorhinal area with a microelectrode activates fibers of the perforant pathway that synapse with the dendrites of the granule cells in the molecular layer of the dentate gyrus. Granule cell mossy fibers project to the pyramidal cells of the regio inferior. Neuronal signals are relayed to the pyramids of the regio superior by the Schaffer colaterals. All of the synapses in the trisynaptic loop are glutaminergic and excitatory. According to Anderson et al. (1970), the trisynaptic loop functions as an independent functional unit. Units are stacked in lamellae in a plane that

is perpendicular to the long axis of the hippocampus. Lamellae might interact through excitatory and inhibitory transverse connections.

Modern tract tracing techniques have greatly advanced our knowledge of intra- and extrahippocampal projections as well as the three dimensional organization of these connections (for review, see Swanson, Wyss, & Cowan, W.M., 1978; Witter, Groenewegen, da Silva, Lohman, 1989; Witter, 1989). Critics of the "lamellar hypothesis" have stated that projections through the long axis of the lamella are as prominent as those in the transverse axis and that "none of the intrinsic connections in the hippocampal formation is organized in lamellar fashion (Amaral & Witter, 1989)." The two-dimensional brain slice might not be an accurate representation of how a three dimensional structure like the hippocampus functions in vivo.

Adrenal Hormones and Pathology

According to Selye (1946) extreme and prolonged stress (exhaustion) can result in diseases of adaptation. Stress related disorders include heart disease, high blood pressure, arthritis, colds and flu (Ross and Glomset, 1976). Cushing's disease is caused by the hypersecretion of adrenal steroids (Christy, 1971; Hadley, 1984). Symptoms include buffalo torso, moon face, diabetes mellitus, hypertension and osteoporosis. The insufficiency of adrenal hormones results in Addison's disease (Knowlton, 1971; Hadley, 1984; Corrigan, 1989). Symptoms of this

disease include increased skin pigmentation, weakness and hypothermia. Stress can cause death in Addison's patients.

Glucocorticoid Toxicity and the Hippocampus

According to the glucocorticoid cascade hypothesis, prolonged stress results in hypersecretion of glucocorticoids that are toxic to the pyramidal neurons of the hippocampus (Sapolsky, Krey & McEwen, 1985; Watanabe, Gould, McEwen, 1992; Woolley, Gould & McEwen, 1992). Landfield (1987) initially found that aging causes pyramidal cell loss in the hippocampus. Sapolsky et al. (1985) subsequently showed that the effects of aging on the hippocampus can be mimicked by prolonged elevations of serum CORT in the high physiological range. Daily injection of CORT for three months resulted in the depletion of CORT receptors in the hippocampus (Sapolsky, Krey & McEwen, 1984), part of which was due to the loss of CORT concentrating cells in area CA3 (Sapolsky et al., 1985). Stress related cell loss in the hippocampus has been shown in primates (Uno, Tarara, Else, Suleman & Sapolsky, 1989).

In addition to pyramidal cell loss in area CA3, aged rats demonstrate an impaired capacity to terminate CORT secretion in response to stress (Sapolsky, Krey & McEwen, 1983; Sapolsky et al., 1984; Sapolsky et al., 1985; Sapolsky, Krey & McEwen, 1986; Sapolsky, 1991). The failure of aged rats to terminate CORT secretion at the end of stress could be caused by cell loss in area CA3 since the hippocampus has been shown to exhibit an

inhibitory influence on adrenal activity and participates in glucocorticoid feedback (Sapolsky et al, 1984; Fuxe et al., 1985). Thus, prolonged elevation of serum CORT would in turn lead to greater hippocampal cell loss and further accentuate the inability of the HPA axis to terminate secretion.

Study into the mechanism of stress-related cell death suggests that glucocorticoids are not in themselves toxic but exacerbate the effects of other insults to the hippocampus (Sapolsky, 1990). The damage to the hippocampus caused by kainic acid, 3-acetylpyridine (Sapolsky, 1985) and hypoxia-ischemia (Sapolsky & Pulsinelli, 1985) are worsened by CORT. The increase in damage can be as much as 10 fold. Thus, glucocorticoids are said to leave neurons on the edge of a metabolic cliff; with no further challenge, the period of elevated glucocorticoids passes uneventfully. However, a co-incident challenge might be less readily survived (Sapolsky, 1990).

Further studies also show that the toxic effects of CORT are indirect. Phenytoin, an anti-epileptic drug that inhibits the release excitatory amino acids, prevents the neurodegenerative effects of stress on CA3 neurons (Watanabe, Gould, Cameron, Daniels & McEwen, 1992). This suggests that mossy fiber input from the dentate gyrus may be involved (Gould, Woolley, & McEwen, 1991). Glucocorticoids facilitate the release of excitatory amino acids from mossy fiber terminals, which in turn, produces morphological changes in CA3 pyramidal neurons. Neurochemical and electrophysiological alterations of neurons

have also been shown after repeated stress or CORT administration (McEwen, 1992; Diamond & Rose, 1993; Diamond & Rose, 1994).

Hippocampus, Stress and Spatial Memory

A wide variety of learning and memory functions have been attributed to the hippocampal formation and its cytological subregions (for review, see Isaacson, 1982; Eichenbaum & Otto, 1992). Spatial memory, the ability to associate exteroceptive discriminative stimuli with a particular location in space, is one memory function affected by ablation of the hippocampus (Olton, Walker, Gage, 1978). Lesion of CA3 alone with the selective neurotoxin kanic acid (Nadler, Perry, Gentry and Cotman, 1980) is sufficient to result in impaired performance on tests of spatial memory (Handelman & Olton, 1981; Sutherland, Whishaw, Kolb, 1983; Whishaw, 1987). Thus, stress-related cell loss in CA3 should also result in impaired memory performance. Stress has been shown to cause deficits in radial arm maze performance, a test of spatial memory (Diamond & Rose, 1993; Luine, Villages, Martinez and McEwen, 1994; Diamond & Rose, 1994).

Adrenal Insufficiency and Cell Death

Adrenal insufficiency has also been shown to cause cell death in the hippocampus (Sloviter, Valiquette, Abrams, Ronk, Sollas, Paul, Neubort, 1989; Roy, Lynn and Bemm, 1990;

Armstrong, McIntyre, Neubort & Sloviter, 1991; McNeill, Masters, Finch, 1991; Conrad & Roy, 1992, Conrad & Roy, 1995). Sloviter et al., (1989) originally reported that adrenalectomy (ADX) can cause as much as a 70% loss of the granule cell layer in the hippocampal dentate gyrus 3-4 months later in rats. Studies of the short term effects of ADX on dentate granule cells have shown neurodegenerative changes as early as two (Jaarsma, Postema, Korf, 1992) and three days (Gould, Woolley, McEwen, 1990) after surgery. These include a decrease in granule cell body area, a decrease in the number of dendritic branch points, and cell death as evidenced by an increase in the number of pyknotic cells (Gould et al., 1990). The type of cell death caused by ADX has been identified as apoptosis (Sloviter, Dean, Neubort, 1993a). ADX is analogous to the adrenal collapse of Addison's disease, and loss of the granule cell layer has been found in the one clinical case studied (Mæhlen & Torvik, 1990).

The granule cell degeneration (Jaarsma et al., 1992; Roy et al., 1990; Sloviter et al., 1989; Sloviter, Sollas, Dean, Neubort, 1993a) and electrophysiological dysfunction (Sloviter et al., 1989) caused by adrenal insufficiency has been prevented in the rat by replacement of CORT. This suggests that adrenal steroids exert a trophic influence on certain cell populations in the DG; Granule cells die if the type 1 or type 2 adrenal steroid receptor is not occupied by endogenous ligand. High concentrations of both Type 1 and Type 2 glucocorticoid receptors are observed in the dentate gyrus (Reul & de Kloet,

1985; de Kloet et al., 1987). Woolley, Gould, Sakai, Spencer and McEwen (1991) found that activation of the Type 1 adrenal steroid receptor completely protected the dentate gyrus against adrenalectomy-induced cell death whereas type 2 receptor activation provides only partial protection.

The dentate gyrus (DG) is unusual in that most of the neurons in this brain area are generated postnatally (Yackel & Puri, 1982). Studies have shown that neurogenesis continues up until at least a year of age in rats (see Gould, 1993; Gould, 1994). It is generally assumed that birth, migration, and programmed cell death occurs only during prenatal development in the mammalian brain. Gould, Woolley & McEwen (1991a, 1991b) have shown that glucocorticoids regulate neuronal birth as well as cell death in the dentate gyrus. Maximum cell death occurs during the stress hyporesponsive period of development when adrenal steroid levels are naturally low (Sapolsky & Meaney, 1986). ADX extends the hyporesponsive period (Gould et al., 1991b) and exogenous adrenal steroids decrease the rate of cell death (Gould et al., 1991a; Gould et al., 1991b; Gould, Cameron, Daniels, Woolley & McEwen, 1992). Thus, neuronal remodeling continues well into adulthood in the DG suggesting that this structure might be important to learning or memory function.

Adrenalectomy and Spatial Memory Performance

Lesions of the dentate gyrus with the selective neurotoxin colchicine (Goldschmidt & Steward, 1982) has been shown to

produce deficits in tests of spatial memory (Sutherland et al., 1983; Walsh, Schultz, Tilson, Schmechel, 1986; Whishaw, 1987; Nanry, Mundy, Tilson, 1989; Tilson, Harry, McLamb, Peterson, Hong, Dyer, 1987; Emerich & Walsh, 1990; McNaughton, Barnes, Meltzer & Sutherland, 1989). Thus, the granule cell loss that results from adrenal insufficiency should also produce spatial memory deficits. In this regard, ADX has been shown to cause deficits in the acquisition of passive (Borrell, Hall & Gold, 1989) and active avoidance tasks (Bialik, Pappas, Roberts, 1984). On the Morris Water Maze, another test of spatial memory, ADX rats showed deficits that were small or short lasting (Armstrong et al., 1993; Conrad & Roy, 1992).

Specific Aims

In this series of studies, I will first determine whether the absence of adrenal steroids causes deficits on the radial arm maze, another test of spatial memory, shortly after ADX. If so, what neuroanatomical or endocrine mechanisms are correlated with performance on a spatial memory tasks. Is impaired performance due to degenerative changes in the dentate gyrus or do adrenal hormones themselves exert an activational effect on spatial memory? Morphological studies of the DG will be completed to answer this question. Further, do Type 1 or Type 2 adrenal steroid receptors contribute to adrenal steroid effects on memory? Drug analogs with high binding specificity will be used to determine the contribution of each receptor to spatial

memory function. Finally, if adrenalectomy causes deficits in radial arm maze performance, are these deficits due to the impairment of learning or memory function?

Study 1: Effects of Adrenalectomy on Spatial Memory Performance and Dentate Gyrus Morphology (Vaher, Luine, Gould, McEwen, 1994).

The purpose of study 1 was to determine if adrenal insufficiency causes deficits on the radial arm maze (RAM), a test of spatial memory, shortly after ADX. Since ADX has been shown to cause loss of granule cell neurons in the dentate gyrus (Sloviter et al., 1989; Roy et al., 1990; Armstrong et al., 1991; McNeill et al., 1991; Conrad & Roy, 1992), measures of dentate gyrus morphology will also be assessed. Intake of 3% saline (Richter, 1941) and serum levels of CORT were measured in all rats as an assay of the completeness of adrenalectomy. Physiological indicators of adrenal function and measures of dentate morphology were tested for correlations to radial arm maze performance in order to determine if deficits in spatial memory performance are due to cell loss in the dentate gyrus or the activational effects of adrenal steroids themselves.

METHODS: STUDY 1

Subjects

Thirty male Sprague-Dawley rats (Charles Rivers Laboratories) were received at two months of age and were allowed free access to food and water for a period of approximately three weeks of acclimation before surgery. They

were individually housed in wire cages and maintained on a reverse 12:12 h light cycle (lights off at 7.00 h) to enable behavioral testing during the dark phase. After recovery from surgery, food intake was restricted so that rats reached 85-90% of free feeding weight. Rats were tested for spatial memory deficits in two cohorts run approximately five months apart.

Adrenalectomy

Animals in each cohort were assigned at random to either ADX or sham operated groups. Proportionately more rats were allocated to the ADX group to compensate for mortality and the elimination of rats that do not meet criteria for complete adrenal removal (see below). After Metofane inhalation, rats were anesthetized with a supplementary IP injection of a PromAce/Ketaset mixture. Adrenals were removed bilaterally along with any surrounding fat that may contain ectopic adrenal tissue. Adrenal glands were merely located and touched with a blunt probe in the sham-operated group. Accessory adrenal tissue, residual tissue not eradicated by adrenalectomy, was assessed by daily intake of 3% saline (Richter, 1941). Saline was made available to all rats after surgery in addition to normal drinking water. ADX rats drinking less than 10 ml/day of saline two weeks after surgery may have accessory adrenal tissue and were excluded from the study (unpublished observation).

Measurement of serum corticosterone

Presence or absence of accessory adrenal tissue was confirmed by RIA measurement of serum corticosterone. RIA was done only on the second cohort of subjects. Blood samples were taken by cardiac puncture at the time of sacrifice prior to perfusion. Rats were deeply anesthetized with PromAce. CORT was measured by using commercially available rabbit antiserum raised against corticosterone-3-oximine BSA (B3-163 Endocrine Science, Tarzana, CA). The antiserum has very low cross-reactivity with other major steroids. Assay sensitivity was 10 pg of CORT and coefficients of variation within and between assays were less than 10%. Serum corticosterone levels were expressed in μg/100 ml or % μg.

Spatial memory testing

Spatial memory was tested on an eight arm radial maze according to the method of Olton et al. (1976), as previously utilized in this lab (Luine & Hearns, 1990; Luine et al., 1990; Luine et al., 1993). Purina dry cat food served as reinforcement. The maze was located in a room that was dimly lit and rich in spatial cues. Rats were shaped to the radial arm maze prior to testing. For the first training trial, several food pellets were placed on the center platform and along the arms of the maze. Rats were placed on the center platform in groups of no more than four and allowed to explore the maze for 10 min. Remaining shaping sessions were completed individually; progressively fewer pellets were used, and pellets were placed

successively further down the arms. By the tenth training trial, all rats were able to traverse the maze and eat the reinforcer from the food cup at the end of each arm.

Immediately following training, rats were tested for 15 trials in which one pellet was placed in the food receptacles at the end of each of the 8 arms and rats were allowed to choose arms in any order until either all eight arms were visited or 10 minutes had elapsed. To begin each trial, the rat was placed on the center platform in a random orientation. Testing was conducted blind without the researcher knowing to which group the subject belonged. A visit to an arm was scored if the rat traversed three-quarters of the length of the arm, if it entered the arm but did not eat, or if it entered the arm and ate the reinforcer. Re-entries into an arm previously visited were counted as errors. Choice accuracy was scored by two measures: number of correct choices in the first eight visits and the number of errors in completing the task.

Spatial memory performance was further tested by introducing a delay in the maze task. After the fourth choice, the rat was removed from the maze and returned to his home cage. When the delay interval had elapsed, the subject was returned to the maze to complete the task. Performance was evaluated at delay intervals of 10 min, 1 h, 2 h and 3 h. Performance was scored as described above.

Histology

At the end of spatial memory testing, rats were transcardically perfused with 10% neutral buffered formalin solution (Sigma) under Metofane and PromAce/Ketaset anesthesia. The brains were dissected from the cranial cavities and placed in a solution of 30% sucrose and perfusate solution until the specimen sank. Brains were then frozen on dry-ice and sectioned coronally in a cryostat to a thickness of 25 μm. Sections taken through the region of the dorsal dentate gyrus were thaw mounted onto gelatinized slides. Slides were stained with cresyl violet and coverslipped under Permount.

Data analysis

Physiological indicators: Measures of serum levels of CORT and daily intake of 3% saline solution were tested for group differences after ADX by independent samples two-tailed t-tests.

Behavioral data: Behavioral data were obtained from two cohorts each containing sham and ADX rats. Data were statistically analyzed as separate replications and then combined and analyzed again as a whole. Three subjects died shortly after surgery and three ADX subjects did not meet criteria for saline consumption. Thirteen ADX subjects and 11 sham-operated rats were shaped and tested. Subjects received 15 maze trials that were grouped into blocks of five trials for statistical analysis. None of the rats developed a consistent response strategy, however, two trials were excluded from the third block of trials because of chaining responses. Three ADX

subjects in the first cohort died prior to maze testing with time delays.

Since preliminary data analysis by two-way repeated measures ANOVA showed an absence of interactions effects (groups x choice accuracy), measures of choice accuracy were analyzed by blocks design ANOVA with repeated measures. The blocks design ANOVA is a more sensitive test than the two-way ANOVA and can applied when there are no interaction effects. Pairwise testing of a significant main effect was done post hoc by the (Sokal & Rohlf, 1981). The GT2 is a robust statistical test when sample sizes are unequal (Sokal & Rohlf, 1981).

Histology: The slides containing brain sections for quantitative analysis were coded and the code was not broken until analysis was complete. Earlier morphological studies of granule cell degeneration after short-term ADX have shown that the highest concentrations of degenerating cells are characteristically distributed in the rostral (Jaarsma et al., 1992) and middle thirds (Gould et al, 1990) of the DG. Sections from the dorsal dentate gyrus qualified for analysis if the suprapyramidal blade and the infrapyramidal blade were joined at the crest and the dentate gyrus was oriented horizontally beneath the corpus callosum. The number of pyknotic cells were counted in the suprapyramidal and infrapyramidal blades at a magnification of 1250x. Pyknotic cells were identified by the presence of darkly stained granules of condensed chromatin, lack of a nuclear membrane and pale or absent cytoplasm (Sengelaub

and Finlay, 1982). Selected sections were always separated by a least 25 μm in order to avoid counting the same cell twice. Cross sectional areas were determined for the suprapyramidal and infrapyramidal blade of the DG from camera lucida drawings (32x magnification) with the aid of a Zeiss interactive digital analysis system. Approximately three brain sections were analyzed per rat. Pyknotic cell counts were expressed as the density of pyknotic cells per 10^6 μm^2. Differences in DG area and pyknotic cell density were tested by independent samples two tailed t-test.

Correlational analysis: The validity of measuring daily saline intake as a method of assessing the presence of accessory adrenal tissue was tested by correlating saline intake with serum CORT level. Physiological indicators and morphological measures were tested for possible relationships to spatial memory behavior by correlation to measures of choice accuracy on the radial arm maze. Behavioral data were averaged across four delay trials for each measure of choice accuracy for three correlational analyses. Finally, correlations between physiological indicators and morphological measures were also assessed.

RESULTS: STUDY 1

Physiological indicators

ADX rats had significantly lower levels of serum CORT than

shams (0.60±0.4 μg% vs. 15.0±2.3 μg%, $t_{4.3}$=-6.24, p<0.003). ADX rats also consumed amounts of saline well above those of the control group (16.9±1.6 ml/day vs. 1.3±0.3 ml/day, $t_{10.9}$=9.49, p<0.0001). Four of 5 ADX subjects assayed had below 0.20 μg% of serum CORT and only 1 of these had a measure of 0 μg%. The correlation between saline intake and serum CORT level was significant (r=-0.85, p<0.0018); i.e. higher saline intake is associated with lower serum levels of CORT. The strong correlation between saline intake and CORT level indicates that measuring daily saline intake is valid for assessing the efficacy of ADX but that high saline intake does not preclude the presence of low levels of circulating adrenal hormones.

Behavioral performance

The effects of ADX on radial arm maze performance are reported in Table 1. ADX rats showed significantly impaired performance during fifteen radial arm maze trials. They had significantly fewer correct choices out of the first eight visits ($F_{1.67}$=12.69, p<0.008) and made more errors in completing the maze task than did shams ($F_{1,67}$=18.03, p<0.0001). Block effects were not significant; i.e. there were no significant improvements during the three blocks of trials.

Following these trials, rats were tested with a delay between the 4th and 5th visits. The second cohort was tested two days following the fifteen maze trials while the first cohort was not tested on delays until three weeks after these three

blocks. During this time, rats of the first cohort were tested on other behavioral tasks that did not involve spatial memory (Vaher et al., unpublished observations). Radial arm maze data for the separate cohorts were similar in that ADX subjects made more errors in completing the task in both replications of this study. When the data for testing with delay were pooled, the ADX group was significantly impaired for the measures of choice accuracy shown in Table 1. They again had significantly fewer correct choices out of the first eight visits ($F_{1,82}=5.14$, $p<0.03$) and made more errors in completing the task ($F_{1,86}=10.83, p<0.0015$). For pooled data, increasing the time delay produced a trend towards poorer spatial memory performance in both groups but this trend was not significant for any measure of choice accuracy.

Dentate gyrus anatomy

Subjects were sacrificed immediately following their last trial; their brains were removed, and histology was performed. There was no difference between groups in cross sectional dentate area of cresyl violet stained sections (Table 2).

Pyknotic cells in the DG were counted per unit area (10^6 μm^2). Since the distribution of pyknotic cell densities in the ADX group were positively skewed ($w_1=1.134$, $p<0.003$), and the variances of the two groups were unequal ($F_{9,10}=24.04$, $p<0.00001$), differences between groups were tested by non-parametric statistics. The pyknotic cell densities were not

significantly different between sham and ADX groups (Table 2; $U=50$, $P<0.67$). Absence of group differences in pyknotic cell densities might be attributable to the large degree of individual variability amongst ADX subjects. Individual differences have been noted in earlier morphological studies (Jaarsma et al., 1992; Roy et al., 1990; Sloviter et al., 1989, Sloviter et al., 1993a). The sham group had a range of pyknotic cell densities from 0.0 to 4.9 whereas ADX subjects showed a broader range from 0.0 to 23.2. The large S.E.M. within ADX subjects contributes to the absence of a statistical difference between groups and obscures the fact that the ADX group had, on the average, twice as many pyknotic cells as controls (Table 2). In addition, the maximum number of 4.9 pyknotic cells/10^6 μm^2 observed in the sham group is not nearly as high as 23.2 in one ADX rat. Thus, some ADX rats showed increased dentate granule cell pyknosis at the time of death.

Correlational analysis

While ADX rats showed consistent and significant impaired performance as compared to intact rats, there was heterogeneity in the performance of the two groups. The worst performance was exhibited by five ADX rats and the best performance was exhibited by two shams. The performance of the remaining rats was distributed between these two extremes. Since the behavioral performance was heterogeneous, correlational analyses were applied to determine the extent to which physiological

indicators of adrenal function and/or brain morphological measures may have contributed to behavioral variability (Table 3).

Serum CORT levels at the time of sacrifice were negatively correlated to the number of errors in completing the task (Table 3), suggesting that lower levels of serum CORT are associated with impaired radial arm maze performance. Significant correlations were also found between intake of 3% saline and number correct in first eight choices and number of errors to complete the task (Figure 1). Thus, higher saline consumption was associated with poorer spatial memory performance overall. Table 3 shows that the morphological measures, cross sectional dentate area and pyknotic cell count, were not correlated to behavioral performance. Pyknotic cell counts were also not significantly correlated to physiological indicators of ADX, i.e. levels of serum CORT ($r=-0.41$, $p=0.27$, $n=9$) or intake of 3% saline solution ($r=0.36$, $p=0.11$, $n=21$).

As expected, the body weight of rats on food restriction was not correlated to any measure of choice accuracy (data not shown).

TABLE 1. Effect of adrenalectomy (ADX) on radial arm maze performance.

	Regular Trials			Trials with a Delay			
Group	Block 1	Block 2	Block 3	10 min	1 hr	2 hr	3 hr
	NUMBER CORRECT IN FIRST EIGHT CHOICES						
SHAM	7.16 ±.09	7.18 ±.14	7.02 ±.16	6.73 ±.27	6.82 ±.22	6.40 ±.22	6.18 ±.26
ADX	6.60 ±.17	6.71 ±.14	6.79 ±.16	6.27 ±.24	6.27 ±.30	5.91 ±.25	6.00 ±.36
	NUMBER OF ERRORS IN COMPLETING THE TASK						
SHAM	1.62 ±.29	1.60 ±.36	1.62 ±.18	2.27 ±.54	2.36 ±.58	2.40 ±.43	2.82 ±.50
ADX	3.11 ±.61	2.85 ±.42	2.90 ±.49	3.36 ±.66	3.82 ±.86	3.82 ±.60	5.09 ±1.0

Entries are means ± S.E.M. Eleven sham and thirteen ADX rats started maze testing but two ADX rats died prior to testing with delays. Since interaction effects were not significant, group effects were tested by blocks design ANOVA with repeated measures. Data for the initial 15 maze trials were averaged into blocks of 5 trials for analysis. The ADX effect was significant for both the number correct in the first eight choices ($p<0.008$) and the number of errors in completing the task ($p<0.0001$) but blocks effects were not significant. For trials with a delay, the ADX effect was also significant for the number correct ($p<0.03$) and the number of errors ($p<0.0015$). Delay effects were not significant; Thus, increasing the delay interval did not significantly alter maze performance.

Table 2. Effect of ADX on the morphology of the dentate gyrus.

Morphological variable:	Group Sham (n=11)	 ADX (n=10)
Cross Sectional Dentate Area (10^6 μm^2)	0.083 ±0.003	0.087 ±0.003
Number of pyknotic cells / 10^5 μm^2	2.1 ±0.5	5.2 ±2.3

Entries represent mean ± S.E.M. There were no significant differences between groups on any morphological measure. See text for details.

Table 3. Correlation of RAM performance to physiological and morphological measures.

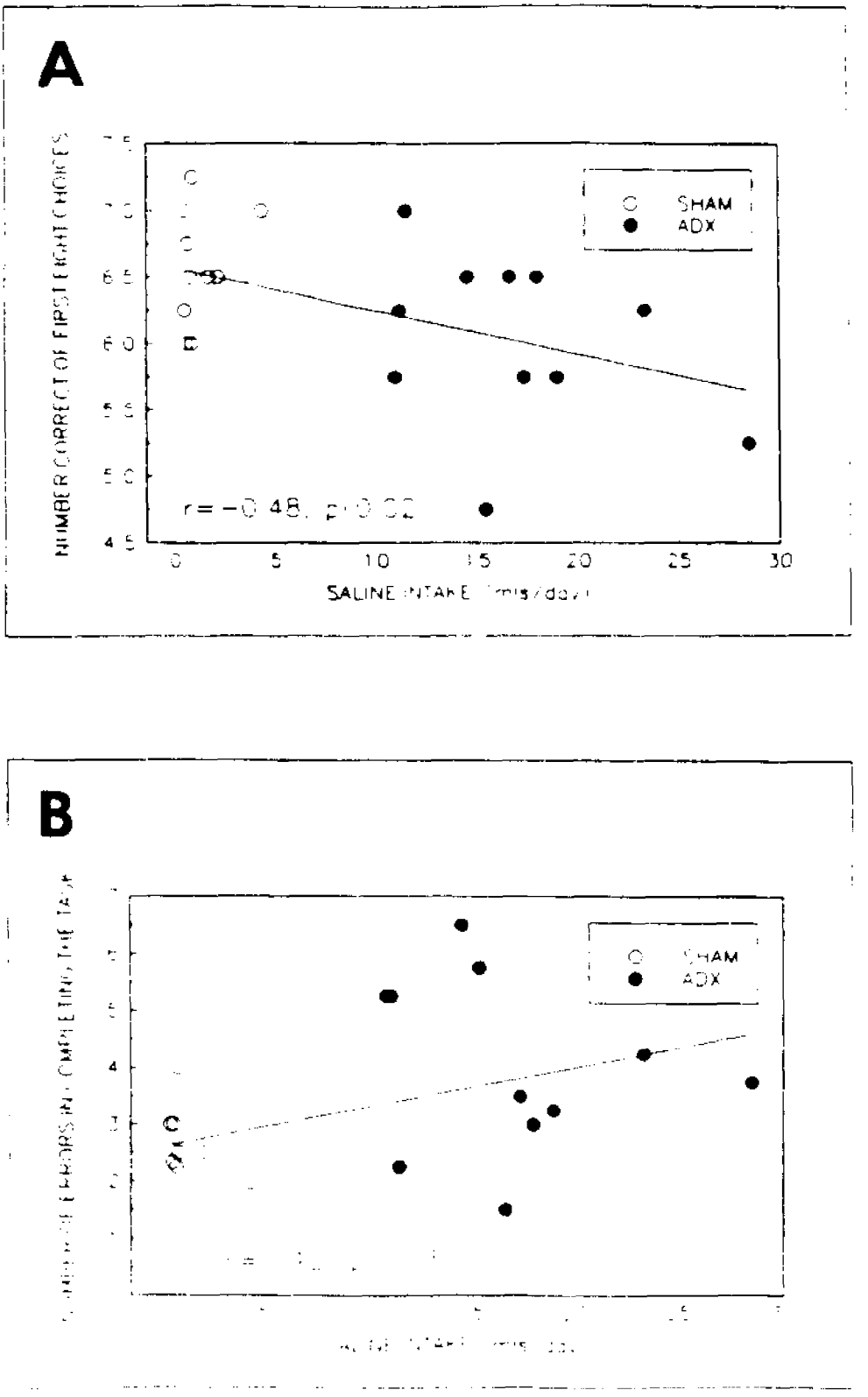
	CHOICE ACCURACY	
	NUMBER CORRECT IN FIRST EIGHT CHOICES	NUMBER OF ERRORS
PHYSIOLOGICAL INDICATOR:		
3% Saline Intake (ml) n=22	-0.48* p<0.02	0.42* p<0.04
Serum CORT Levels (%µg) n=10	0.57 p<0.09	-0.75* p<0.008
MORPHOLOGICAL VARIABLE:		
Cross Sectional Dentate Area (10^6 μm^2) n=21	0.16 p<0.09	0.07 p<0.82
Number of pyknotic cells / 10^6 μm^2 n=21	0.03 p<0.88	0.17 p<0.47

Entries are Pearson's product moment correlation coefficients and exact probabilities of significance. Significance is indicated by an asterisk if $p<0.05$. Choice accuracy measures used in these correlations are the average performance across the four delay intervals tested.

FIGURE LEGENDS

Figure 1. Regression plots of saline intake vs measures of choice accuracy on the 8-arm radial maze for 11 sham (open circles) and 11 ADX (closed circles) rats. Entries represent average saline intake and performance during the delay trials. A, the number correct of first eight choices, B, the number of errors in completing the task.

Figure 1



Study 2: Effects of Aldosterone Replacement on the Radial Arm Maze Performance of Adrenalectomized Rats (Vaher, 1995a).

Study 2 attempted to determine if type 1 receptor activation abates spatial memory deficits in ADX rats. Aldosterone (ALDO) is a mineralocorticoid that binds to the type 1 receptor with high specificity (McEwen et al., 1986; Reul & de Kloet, 1985; Spencer et al., 1993). Selective activation of the type 1 receptor by replacement of ALDO in ADX rats has been shown to protect against ADX-induced granule cell death (Woolley et al., 1991) and to facilitate long-term potentiation (LTP), a model for the neuronal basis of learning (Pavlides, Watanabe, Margarinos & McEwen, 1992; Pavlides, Watanabe, Margarinos & McEwen, in press). In order to determine whether type 1 receptor activation abates spatial memory deficits in ADX rats, RAM performance was tested in intact, ADX, and ADX rats treated with ALDO. Indicators of adrenal function and measures of dentate gyrus morphology were tested for correlations to measures of spatial memory performance to determine if any facilitation is due to the effects on dentate neuroanatomy or the activational effects of adrenal steroids themselves.

METHODS: STUDY 2

Methods were the same as in our first study (Vaher et al.,

1994) except for the changes listed below.

Subjects

Thirty one male Sprague-Dawley rats, received at three months of age, were housed and food deprived as described in study 1 (Vaher et al., 1994). Rats were tested for spatial memory deficits in four cohorts run approximately two months apart.

Adrenalectomy

Rats in each cohort were assigned at random to either sham, ADX or ADX+treatment groups and adrenalectomized bilaterally as described in our first study (Vaher et al., 1994). Saline intake was again measured daily in order to assess the presence of ectopic adrenal tissue, detect adrenal regrowth and assay circulating levels of ALDO (Richter, 1941; Vaher et al., 1994). ADX rats drinking less than 10 ml/day of saline two weeks after surgery are likely to have accessory adrenal tissue and were excluded from behavioral analysis. Our earlier study, using the same criteria for exclusion, showed that saline intake and serum levels of CORT are highly correlated ($r=-0.85$, $p<0.002$) in ADX rats (Vaher et al., 1994).

Spatial Memory Testing

Spatial memory was tested on an eight arm radial as described in our first study (Vaher et al., 1994). The

experimental protocol is summarized in Figure 2. After shaping and minipump implantation (see below), rats were tested with all eight arms of the maze baited. Testing was done no more than once a day, four times a week, in order to keep performance from reaching asymptotic levels before steroid replacement could be shown to be effective. Rats were tested until steroid effects subsided. Measures of choice accuracy chosen for analysis in this experiment are the same as in study 1: number of correct choices in the first eight visits and the number of errors in completing the task.

Aldosterone Replacement

ALDO was administered by osmotic minipump (Alzet). Pumps were filled with ALDO dissolved in propylene glycol (10 mg/ml). Control rats were implanted with a "dummy pump," a nylon screw spacer of about the same size and weight as the minipump. After shaping to the radial arm maze, ADX rats were subdivided into dummy pump (ADX control) and minipump (ADX+ALDO) groups by matching them on the basis of their average saline consumption. Rats were first anesthetized with Metofane inhalant and then injected with a PromAce/Ketaset mixture. Pumps were implanted subcutaneously through a half inch incision made just below the juncture of the shoulder blades. These pumps secreted ALDO for seven days at a rate of 10 μg/hr, a dose that provides 85-90% type 1 receptor occupancy in the hippocampus (Spencer, R.L., personal communication). Maze testing began one day after

minipump implantation.

Histology

At the end of spatial memory testing, rats were sacrificed and perfused under pentobarbital anesthesia as described in study 1. Brains were frozen and sectioned coronally through the region of the dorsal dentate gyrus to a thickness of 25 μm. Sections were thaw mounted onto gelatinized slides. Slides were stained with cresyl violet and coverslipped under Permount.

DATA ANALYSIS

Physiological Indicators: Measures of daily saline intake were grouped according to weeks for statistical analysis. Saline data was tested for group differences by one way between groups ANOVA. Pairwise testing of a significant effect was done post hoc by the GT2 test for unequal sample sizes (Sokal & Rohlf, 1981).

Behavioral Data: Data from four cohorts were pooled for analysis. Each cohort contained three groups: sham adrenalectomized with dummy pump implant (Sham), ADX with dummy pump (ADX) and ADX with ALDO minipump (ADX+ALDO). Ten rats completed the study in each group. Of the rats that started the study, four rats were excluded because they were not adequately shaped to the radial arm maze in time. Four rats died after ADX and 11 after minipump implant. Rats received 12 maze trials that were grouped into blocks of four trials for statistical

analysis. None of the rats developed a consistent response strategy. However, ten trials were excluded from analysis because of chaining responses. Five more trials were excluded because rats did not make eight choices to complete the task. Data from one rat was excluded because it was sick and two other trials were not completed because rats were ill. Seven ADX rats given ALDO replacement were excluded because saline intake remained low three weeks after minipump surgery. Of the ten remaining ADX+ALDO rats, data from three were excluded from analysis of the second block of trials (n=7) and three from the third (n=7) because saline intake was below 10 ml per day.

Measures of choice accuracy were analyzed by blocks design ANOVA with repeated measures. Preliminary data analysis by two way repeated measures ANOVA showed an absence of interaction effects (groups X weeks). Since the ANOVA analysis of main effects ignores the pattern of scores in the data (Plutchik, 1983), treatment effects were further tested on a weekly basis by one-way between groups ANOVA. Pairwise testing of a significant effects was done post hoc by the GT2 test for unequal sample sizes (Sokal & Rohlf, 1981).

Histology: Sections from the dorsal dentate gyrus were analyzed as described in study 1 (Vaher et al, 1994). The number of pyknotic cells were counted in the suprapyramidal and infrapyramidal blades at a magnification of 1000x. Pyknotic cells were counted according to strict criteria. Only "late stage" pyknotic cells, consisting of smaller cells with darkly

stained condensed chromatin and an absence of cytoplasm, were counted (Sloviter et al., 1993a). Cross sectional areas were determined for the suprapyramidal and infrapyramidal blade of the DG from camera lucida drawings (25x magnification) with the aid of a Zeiss interactive digital analysis system. Both left and right sides were analyzed in two brain sections from each rat. Differences in DG area were tested by one way between groups ANOVA. Study 1 showed that the distribution of pyknotic cell densities in ADX rats is positively skewed (Vaher et al., 1994). The distribution of pyknotic cell densities was tested for skewedness and analyzed for group differences by nonparametric means (Sokal & Rohlf, 1981).

Correlational Analysis: The effects of ALDO were tested for possible relationships to spatial memory performance by correlating daily measures of saline intake to measures of choice accuracy on the radial arm maze. Previous studies have shown that the saline intake of ADX rats is correlated to serum levels of CORT and radial arm maze performance as well (Vaher et al., 1994). The effects of ALDO on dentate gyrus morphology was assessed by correlating saline intake to measures of dentate area and pyknotic cell density. Finally, the relationship between dentate gyrus morphology and spatial memory was tested by correlating dentate morphology measures to measures of choice accuracy on the radial arm maze.

RESULTS: STUDY 2

completing the task (groups x weeks). Analysis of main effects by blocks design ANOVA with repeated measures shows that weeks effects were significant for both the number of correct choices ($F_{2,314}$=6.03, p<0.003) and the number of errors ($F_{2,314}$=6.36, p<0.0014). There was significant improvement in overall maze performance during the twelve trials tested. Groups effects were also significant for both measures of choice accuracy; the number of errors ($F_{2,314}$=12.88, p<0.0001) and number correct ($F_{2,314}$=11.23, p<0.0001). Further analysis of groups differences was done by one way between groups ANOVA on each of the three weeks of maze trials.

For the first week of maze trials, one way analysis of measures of choice accuracy were significant for the number of errors in completing the task ($F_{2,109}$=3.81, p<0.02) but not for the number of correct choices in the first eight visits ($F_{2,109}$=2.39, p<0.10). Number correct might not be as sensitive a measure of choice accuracy as the number of errors. Pairwise testing of the number of errors by GT2 analysis showed that sham and ADX+ALDO rats did not differ from each other in the number of maze errors and both of these groups made fewer errors than ADX controls (see Figure 4A, week 1). Thus, ALDO replacement was effective in reducing the number of maze errors in ADX rats.

Results for the second block of maze trials were similar to the first (Figure 4). One way analysis was significant for the number of errors ($F_{2,99}$=4.42, p<0.01) and ADX+ALDO and sham rats still made fewer errors in completing the task than ADX controls

(Figure 4A, week 2). Thus, ALDO continued to be effective in improving RAM performance in ADX rats even though minipumps were presumably depleted before the start of the second week of maze testing. The ALDO effect was paralleled by a continued suppression of saline intake. Groups differences were also significant for the number of correct choices in the first eight visits during the second week of maze trials ($F_{2,99}=3.05$, $p<0.05$). Pairwise testing of the number correct showed that ADX+ALDO rats made as many correct choices as shams but the number correct was not statistically different from ADX controls (see Figure 4B, week 2). ALDO treated rats should not be expected to perform as well as shams since ALDO at this dose did not completely eliminate salt appetite.

The trend towards continued improvement in ALDO treated rats was lost during the third week of maze trials and was accompanied by an increase in salt appetite. One way ANOVA's were again significant for the number of errors ($F_{2,103}=5.39$, $p<0.006$) and the number correct ($F_{2,102}=7.56$, $p<0.0009$) for the third week of trials. Figure 4A shows that ADX rats treated with ALDO made more errors than sham control rats during the third week of maze trials. Although statistical testing showed that the number of errors made by ADX+ALDO rats did not differ from shams, the number of errors made by ADX+ALDO rats did not differ from ADX controls either. Pairwise testing of the number correct showed that ALDO treated rats made as many correct choices as ADX controls and both of these groups made fewer correct choices

than shams (Figure 4B, week 3). The saline intake of ADX+ALDO rats also increased during the third week of maze trials but intake was still not as high as ADX controls (Figure 3, week 3); Thus, the performance of ALDO supplemented ADX rats would not be expected to returned to the more impaired level of ADX controls during the third week.

The ALDO effect shown in Figure 4 is more impressive when data from the first week of maze trials is plotted by days. Figure 5 shows the saline intake of sham, ADX and ADX+ALDO rats plotted together with the average daily number of maze errors in completing the task. Differences between days were tested by one way repeated measures ANOVA. Only ADX+ALDO rats showed significant differences in the number of maze errors for the first week of testing ($F_{3,34}=6.62$, $p<0.001$). Pairwise testing by GT2 showed that ADX+ALDO rats made fewer errors three and four days after ALDO replacement than on days one and two. The decrease in maze errors coincides with a decrease in saline intake, a measure of type 1 receptor occupancy. Thus, ALDO replacement requires three days to achieve a maximum effect in ADX rats.

Dentate Gyrus Anatomy

Morphological results are shown in Table 4. Several ADX rats excluded from the behavioral data were included in this analysis. Two way analysis of area measurements (group x blade) showed no interaction effects. ADX effects were not significant;

ADX did not alter dentate gyrus area. Blade effects were not significant but this finding is irrelevant in the absence of treatment effects.

The distribution of pyknotic cell densities were found to be positively skewed in ADX rats (w_1=1.7889, $p<0.002$ in ADX control rats and w_1=1.1778, $p<0.008$ in ADX+ALDO rats). Cell densities were analyzed by nonparametric statistics. The Kruskal-Wallis test between groups was significant (adj. H=9.9908, $p<0.05$). Multiple comparisons were tested by non-parametric STP (Sokal & Rohlf, 1981). ADX and ADX+ALDO rats differed from sham adrenalectomized rats but pyknosis did not differ between these two groups. Adrenalectomy increased pyknosis in ADX and ADX+ALDO groups but ALDO replacement could not be shown to abate pyknosis at the time of sacrifice since minipumps were depleted at the time of sacrifice.

Table 4 shows that ADX rats have a higher density of pyknotic cells in the suprapyramidal blade of the DG than in the infrapyramidal blade. Pyknotic cell densities of ADX and ALDO treated rats were pooled and tested by Wilcoxon's Signed-Ranks tested for paired observations (Sokal & Rohlf, 1981). There were no significant differences between blades; i.e., there was no difference in pyknosis between the suprapyramidal and infrapyramidal blades in our rats.

Pyknotic cells were observed in the hilus but only rarely (Table 4). The Kruskal-Wallis test between groups was not significant. ADX was not shown to increase pyknosis in the hilar

region.

Correlational Analysis

Since the behavioral performance of ADX rats exhibits a large degree of variability, correlational analysis was used to help determine the extent to which adrenal function and/or brain morphology contributes to spatial memory function. Addition of the fourth cohort, perfused one week after behavioral testing was completed, did not significantly alter behavioral results and were included in this analysis.

Intake of 3% saline was found to be significantly correlated to measures of choice accuracy: the number of correct choices in the first eight visits and the number of errors in completing the task (Table 5). Higher saline intake is associated with poorer spatial memory performance overall. Regression plots of this relationship were published earlier (see Figure 1). Table 6 shows that the morphological measures, cross sectional dentate area and pyknotic cell density, were not correlated to measures of choice accuracy.

Saline intake was positively correlated to pyknotic cell density ($r=0.39$, $p<0.04$). Higher saline intake, and therefore lower levels of serum adrenal steroids, were associated with increased pyknosis suggesting that adrenal insufficiency causes cell loss in the dentate gyrus. The arrangement of points in Figure 6 suggests a difference between groups in the level of saline intake and pyknosis. As expected, saline intake was not

correlated to granule cell layer area ($r=0.13$, $p<0.52$).

Table 4. The effect of ALDO replacement on dentate gyrus morphology in ADX rats.

	GROUP		
	Sham (n=10)	ADX (n=10)	ADX+ALDO (n=11)
	Dentate Area (10^6 μm^2)		
Suprapyramidal Blade	0.146±0.013	0.160±0.008	0.166±0.007
Infrapyramidal Blade	0.121±0.006	0.115±0.004	0.123±0.008
Pooled Area	0.267±0.020	0.272±0.011	0.286±0.011
Pyknotic Cell Density (Number of Pyknotic Cells / 10^6 μm^2)			
Suprapyramidal Blade	0.3±0.2	19.0±9.1	17.6±8.0
Infrapyramidal Blade	0.2±0.2	7.0±2.8	15.0±6.6
Pooled Area	0.1±0.1	8.5±4.2*	8.1±3.3*
	Number of Pyknotic Cells in the Hilus		
Total	0.1±0.1	0.1±0.1	0.2±0.1

Entries are the mean ± the S.E.M. Several rats excluded from the behavioral data were included in the ADX+ALDO group. ADX significantly increased pyknotic cell density in ADX rats five weeks after surgery but dentate area was not reduced. Pyknosis did not differ between ADX and ADX+ALDO rats since ALDO pumps had been depleted at the time of sacrifice. Asterisk indicates significant difference from sham ($p<0.05$).

Table 5. Correlation of saline intake to radial arm maze performance.

	Week		
Measure of choice accuracy	1 (n=30)	2 (n=27)	3 (n=27)
Number correct in first eight choices	-0.22 p=0.24	-0.39* p=0.046	-0.58* p=0.002
Number of errors	0.44* p=0.01	0.56* p=0.001	0.47* p=0.01

Entries are Pearson's product moment correlation coefficients and exact probabilities of significance. Asterisk indicates a significant correlation ($p<0.05$).

Table 6. Correlation of DG morphology to radial arm maze performance.

	Choice Accuracy	
Morphological Measure	Number correct in first eight choices	Number of errors
Cross sectional DG area (10^6 μm^2)	0.18 p=0.37	-0.17 p=0.39
Number of pyknotic cells / 10^6 μm^2	-0.20 p=0.32	0.32 p=0.10

Entries are Pearson's product moment correlation coefficients and exact probabilities of significance. Morphological measures were correlated to choice accuracy measures from the third block of trials. Correlations were not significant.

FIGURE LEGENDS

Figure 2. Protocol of procedural events in ALDO replacement experiment. Rats were acclimated for one week prior to ADX. After recovery, rats were shaped to an eight arm radial maze. ALDO was administered by osmotic minipump (Alzet) implanted subcutaneously 18 days after ADX. Test trials with all eight arms baited began the following day. Rats were tested four times a week for three weeks before sacrifice. Histology was performed as described in the text.

Figure 3. Effects of ALDO replacement on the consumption of 3% saline in ADX rats. Intake of 3% saline was significantly higher in ADX rats prior to ALDO replacement. Supplemental ALDO was given to ADX+ALDO rats one day before radial arm maze testing by Alzet minipump at a dose of 10 μg/hr, producing 85-90% type 1 adrenal steroid receptor occupancy. ALDO replacement at this dose significantly decreased saline intake in ADX+ALDO rats but intake was not statistically as low as that of sham control rats. Saline data from rats in the fourth cohort are plotted for the week after maze testing. Saline intake of ADX+ALDO rats increased slowly after ALDO replacement and does not return to pre-treatment levels until four weeks later.

Figure 4. Effects of ALDO replacement on radial arm maze performance in ADX rats. ALDO was administered to ADX+ALDO rats by Alzet minipumps implanted subcutaneously one day before testing. Pumps lasted for 7 days and secreted ALDO at a rate of 10 μg/hr. Rats received four maze trials a week for three weeks. Trials were grouped into blocks of four for statistical analysis.

Figure 5. The number of maze errors (closed circles) are plotted together with saline intake (open circles) for the first week of ALDO replacement. Points represent the daily mean of ten rats. Analysis of the number of errors by one way repeated measures ANOVA was significant only for ADX+ALDO rats. Asterisk indicates a significant difference from days one and two. ALDO has a maximum effect three days after replacement in ADX+ALDO rats.

Figure 6. Regression plot of saline intake vs. density of pyknotic cells for 10 sham (open circles) 10 ADX (gray circles) and 7 ADX+ALDO (black circles) rats. ALDO minipumps were depleted at the time of sacrifice, three weeks after minipump implant. Saline data is from the third week of behavioral testing. The correlation between saline intake and pyknotic cell density was significant ($r=0.39$, $p<0.04$).

EXPERIMENTAL PROTOCOL

Male Sprague-Dawley rats (3 mo. old), run in four cohorts

Acclimate for one week, singly housed

Surgery; 2 ADX groups and 1 sham operated group
(Saline and normal drinking water were made available
to all rats after surgery)

Recovery period of one week

Weeks 2-3 post surgery: Shaping on the radial arm maze

Subcutaneous implants:
*ADX group - Alzet minipumps
(10 µg/hr of aldosterone for seven days)
*ADX group - nylon dummy pumps
*Sham ADX - nylon dummy pumps

Week 5-7: 12 radial arm maze trials

Sacrifice and histological analysis

Figure 3

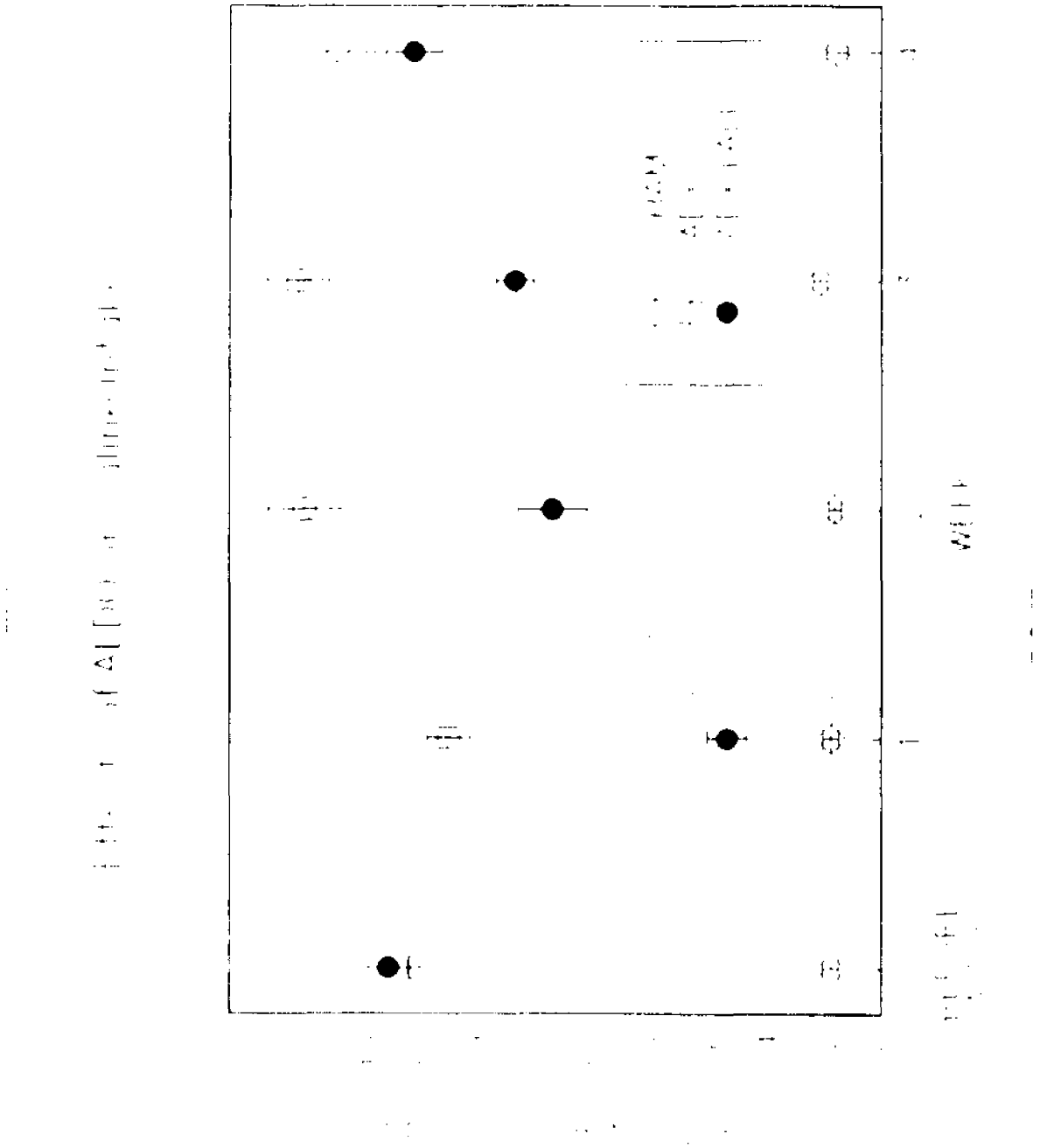


Figure 4

Figure 5

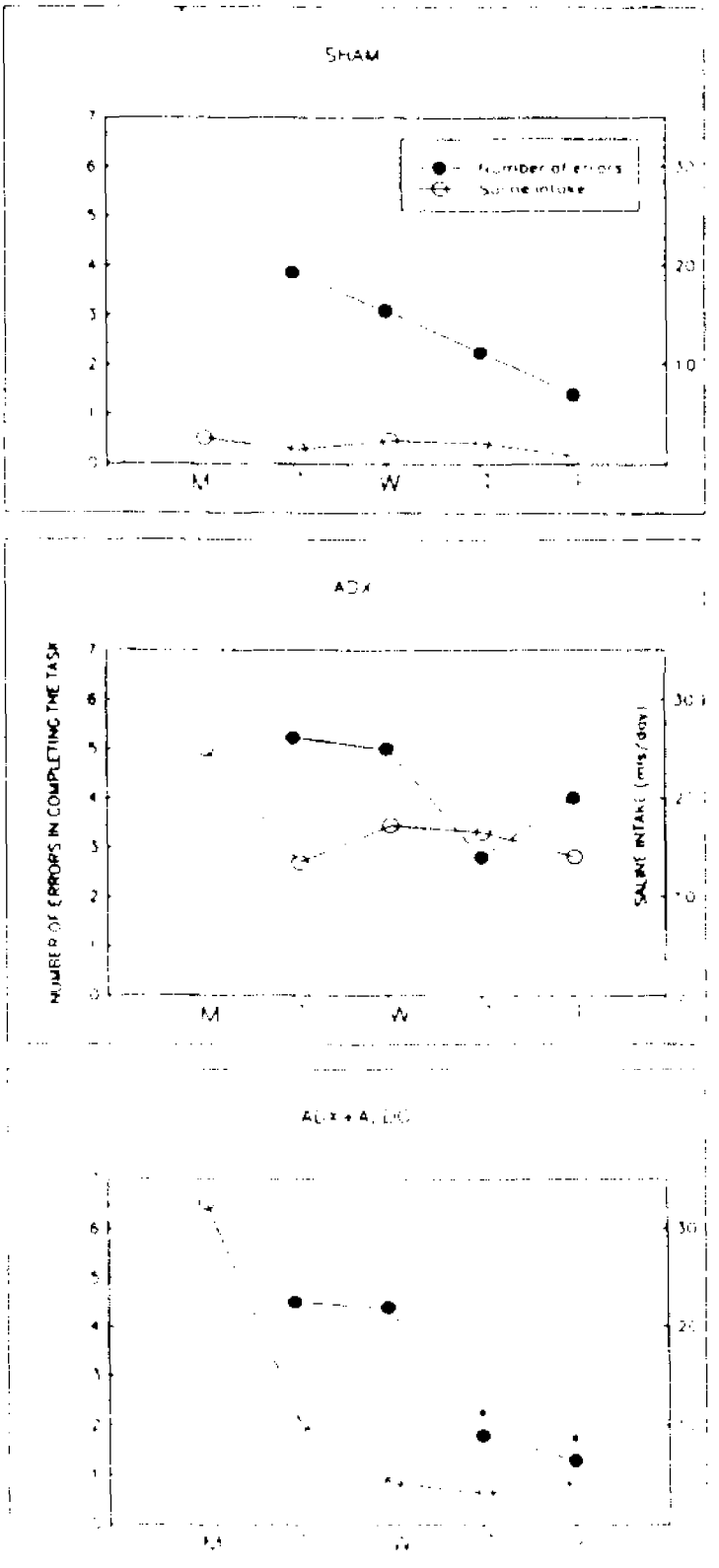
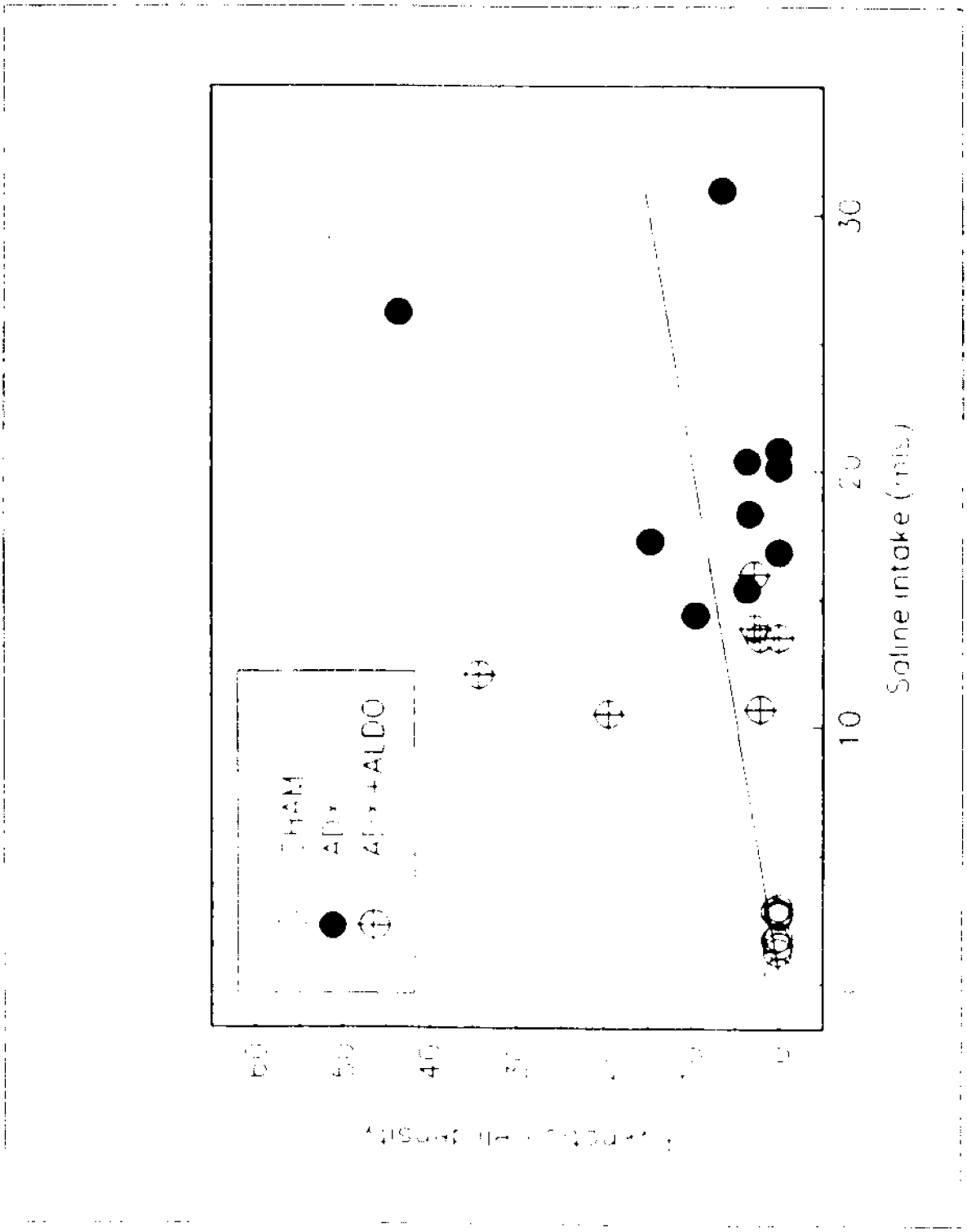


Figure 6



Study 3: Effect of Type 2 Adrenal Steroid Receptor Agonist RU28362 on the Radial Arm Maze Performance of Adrenalectomized Rats (Vaher, 1995b).

Study 3 tests the effects of RU28362 on the radial arm maze performance of ADX rats. Earlier studies suggest that the type 2 receptor may affect spatial memory function. Treatment of ADX rats with RU28362, a selective type 2 receptor agonist, inhibited the formation of LTP (Pavlides et al., 1992; Pavlides et al., in press). Occupation of type 2 receptors has also been shown to depress neuronal excitability in the hippocampus (Joëls et al., 1994). In contrast to these findings, Oitzl et al. (1992), found that rats treated with RU38486, a type 2 receptor antagonist, took longer to relocate a submerged platform in the Morris water maze, another test of spatial memory. Intake of 3% saline will be measured as an index of serum levels of adrenal steroids (Richter, 1941; Vaher et al., 1994; Vaher, 1995a). Since ADX has been shown to cause loss of granule cell neurons in the dentate gyrus (Gould et al., 1990; Jaarsma et al., 1992; Sloviter et al., 1989; Sloviter et al., 1993a; Sloviter et al., 1993b), measures of dentate gyrus morphology will also be assessed. Measures of serum steroids and DG morphology will tested for correlations to RAM performance to determine if any effects on spatial memory are due to RU28362 or changes in the dentate gyrus neurcanatomy.

METHODS: STUDY 3

Methods were the same as in study 2 (Vaher, 1995a) unless otherwise noted.

Subjects

Thirty six male Sprague-Dawley rats, received at three months of age, were housed and food deprived as described in our first study (Vaher et al., 1994). Rats were tested for spatial memory deficits in two cohorts run approximately two months apart.

Adrenalectomy

Rats in each cohort were assigned at random to either sham, ADX, or ADX+treatment groups and adrenalectomized as described in study 1 (Vaher et al, 1994). Saline intake was again measured daily in order to assess the presence of circulating steroids (Richter, 1941; Vaher et al., 1994; Vaher, 1995a). ADX rats drinking less than 10 ml/day of saline two weeks after surgery are likely to have accessory adrenal tissue and were excluded from the behavioral analysis. Study 1 (Vaher et al., 1994) and study 2 (Vaher, 1995a), using the same criteria for exclusion, have shown that saline intake is negatively correlated to serum levels of corticosterone (CORT) in ADX rats.

Spatial Memory Testing

Spatial memory was tested on an eight arm radial maze as described in study 1 (Vaher et al., 1994). The experimental protocol is summarized in Figure 7. After shaping and minipump implantation (see below), rats were tested with all eight arms of the maze baited. Like our earlier study (Study 2: Vaher, 1995a), testing was done no more than once a day, four times a week for three weeks. Choice accuracy was scored by two measures: number of correct choices in the first eight visits and the number of errors in completing the task.

RU28362 Treatment

RU28362 was administered by osmotic minipump (Alzet) as described in our second study 2 (Vaher, 1995a). Pumps were filled with RU28362 dissolved in propylene glycol (10 mg/ml). Control rats were implanted with a "dummy pump," a nylon screw spacer of about the same size and weight as the minipump. After shaping to the radial arm maze, ADX rats were subdivided into dummy pump (ADX control) and minipump (ADX+362) groups by matching them on the basis of their average saline consumption. These pumps secreted steroid s.c. for seven days at a rate of 10 µg/hr, a dose that provides maximum saturation of the type 2 receptor in the brain (Sakai, R., personal communication). Maze testing began one day after minipump implantation.

Histology

At the end of spatial memory testing, rats were sacrificed and perfused under pentobarbital anesthesia as described in our first study (Vaher et al., 1994). Brains were sectioned coronally through the dorsal dentate gyrus and stained with cresyl violet as described before.

Data analysis

Physiological Indicators: Measures of daily saline intake were grouped according to weeks for statistical analysis. Saline data was tested for groups differences each week by one way between groups ANOVA. Pairwise testing of a significant effect was done post hoc by GT2 analysis for unequal sample sizes (Sokal & Rohlf, 1981).

Behavioral Data: Behavioral data were obtained from two cohorts each containing three groups: sham adrenalectomized rats with a dummy pump implant (Sham, n=10), ADX with dummy pump (ADX, n=7), and ADX with RU28362 minipump (ADX+362, n=8). Of the rats that started the study, five died after ADX, three ADX rats were excluded on the basis of low saline intake, two were not adequately shaped to the RAM in time, data from one rat was excluded because it was ill, and one died after minipump surgery. Rats received 12 trials that were grouped according to weeks for statistical analysis. None of the rats developed a consistent response strategy. However, 46 trials were excluded from analysis because responses were chained. Anorexia was a problem with the dose of RU28362 tested. Rats received a high

calorie supplement (Nutrical) after testing and weights were closely monitored. Only ten trials were removed because rats did not make eight choices to complete the task.

Preliminary data analysis by two way repeated measures ANOVA showed an absence of interaction effects for either measure of choice accuracy (groups X weeks). Choice accuracy measures were analyzed by blocks design ANOVA with repeated measures. Treatment effects were further tested on a weekly basis by one-way between groups ANOVA. Pairwise testing of significant effects were done post hoc by the GT2 test for unequal sample sizes (Sokal & Rohlf, 1981).

Histology: Sections from the dorsal dentate gyrus will be analyzed as described in study 2 (Vaher, 1995a). The number of pyknotic cells were counted in the suprapyramidal and infrapyramidal blades at a magnification of 1000x. Cross sectional areas were determined for the suprapyramidal and infrapyramidal blade of the DG from camera lucida drawings (25x magnification) with the aid of a Zeiss interactive digital analysis system. Differences in DG area were tested by one way between groups ANOVA. Our earlier studies, Study 1 (Vaher et al., 1994) and Study 2 (Vaher, 1995a), have shown that the distribution of pyknotic cells densities is positively skewed. Pyknotic cell densities were tested for groups differences by non-parametric means.

Correlational Analysis: The effects of RU28362 were tested for possible relationships to spatial memory performance by

correlating daily measures of saline intake to measures of choice accuracy on the radial arm maze. Earlier studies in this series have shown that the saline intake of ADX rats is correlated to serum levels of CORT (Vaher et al., 1994) and to radial arm maze performance as well (Vaher et al, 1994; Vaher, 1995a). The effects of RU28362 on dentate gyrus morphology will be assessed by correlating daily measures of saline intake to measures of dentate area and pyknotic cell density. Finally, the relationship between dentate gyrus morphology and spatial memory will be tested by correlating morphological measures of the dentate gyrus to measures of choice accuracy on the radial arm maze.

RESULTS: STUDY 3

Physiological Indicators

RU28362 significantly and unexpectedly reduced saline intake in ADX rats (Table 7). The saline intake of ADX+362 rats was lower than that of ADX controls for the first week of treatment. However, the intake was not as low as that in shams or as that in ADX rats treated with ALDO (Vaher, 1995a). The effect lasted for at least two weeks, one week beyond the expected life of the pump. Saline intake did not completely return to ADX levels until three weeks after treatment. ADX controls consumed amounts of saline well above 10 ml/day throughout the three weeks of maze testing.

Behavioral Performance

Preliminary analysis of radial arm maze data by two way ANOVA showed that interaction effects were not significant for either measure of choice accuracy, the number of correct choices in the first eight visits and the number of errors in completing the task (groups X weeks). Analysis of main effects by blocks design ANOVA with repeated measures showed that weeks effects were significant for both the number of correct choices ($F_{2,223}$=7.90, p=0.0005) and the number of errors ($F_{2,227}$=3.54, p=0.03). There was significant improvement in overall maze performance during the 12 trial tested. Groups effects were also significant for both measures of choice accuracy; the number correct ($F_{2,223}$=13.49, p=0.0001) and the number of errors ($F_{2,227}$=23.83, p=0.0001). Further analysis of group differences was done by one way between groups ANOVA on each of the three weeks of maze trials.

For the first week of maze trials, one way analysis of measures of choice accuracy were significant for the number of errors in completing the task ($F_{2,82}$=4.98, p=0.009). Pairwise testing by GT2 analysis showed that ADX+362 rats made significantly more errors than shams (Figure 8A, week 1). Poorer maze performance was accompanied by a somewhat decreased saline intake (Table 7). An ADX effect was significant for the first week of testing; The number of errors made by ADX controls did not differ significantly from ADX+362 rats (Figure 8A, week 1).

Groups differences were also significant for the number of correct choices in the first eight visits ($F_{2,82}$=6.18, p=0.03). Pairwise testing also showed that ADX+362 rats made fewer correct choices in the first eight visits than shams (Figure 8B, week 1). Thus, RU28362 further impaired RAM performance in ADX rats. The effect was evident in both measures of choice accuracy.

One way ANOVAs were again significant for the number of errors ($F_{2,75}$=13.07, p=0.0001) and the number correct ($F_{2,76}$=10.64, p=0.0001) for the second week of testing. Pairwise testing showed that ADX+362 rats made more errors and fewer correct choices than ADX and sham controls (Figure 8, week 2). RAM performance was impaired in ADX+362 rats two weeks after treatment. Like our earlier study (Study 2: Vaher, 1995a), the steroid effect lasted beyond the expected life of the pump and was paralleled by a continued suppression of salt appetite (Table 7). An ADX effect was not shown after the second week of maze testing.

ADX+362 rats showed a trend towards improved maze performance during the third week of steroid treatment. The trend was apparent in the number of errors made (Figure 8A, week 3) and coincided with an increase in saline intake (Table 7). One way ANOVAs were again significant for the number of errors ($F_{2,70}$=6.25, p=0.003) and the number of correct choices ($F_{2,69}$=5.30, p=0.007) but pairwise testing showed that ADX+362 rats still made significantly more errors and fewer correct

choices than sham and ADX controls. Thus, the saline intake of ADX+362 rats returned to the amounts consumed by ADX controls during the third week of treatment but the reduction in the number of maze errors was not yet significant.

Earlier studies in this series showed a prolonged ADX effect on radial arm maze performance (Vaher et al., 1994; Vaher, 1995a). ADX was shown to impair spatial memory performance in this study but the effect was not robust. The effect of RU28362 on behavioral performance, however, was pronounced enough as to be apparent in both measures of choice accuracy and significant in both of the cohorts tested. Since ADX controls and ADX+362 rats were initially matched on the basis of saline intake after adrenal removal, differences in choice accuracy measures must be due to RU28362. The effect of the substance tested had been demonstrated and further replication with anorexic rats was not warranted.

Dentate Gyrus Morphology

The density of pyknotic cells was tested for differences between groups by non-parametric statistics. Analysis by Kruskal-Wallis showed a significant difference between groups (adj. $H=7.78$, $p<0.05$). Pairwise comparisons were tested by non-parametric STP (Sokal & Rohlf, 1981). ADX rats treated with RU28362 differed significantly from shams (Table 8) suggesting that RU28362 further increased pyknosis in ADX rats. This seems to be in contrast to the finding that RU28362 partially protects

against ADX-dependent cell death in the DG (Woolley et al., 1991). ADX produced a small increase in pyknosis in ADX control rats but the increase did not reach significance. A more pronounced increase in pyknosis was not shown in ADX controls because of the absence of a robust ADX effect in this experiment. There was no significant ADX effect on behavior at the time of sacrifice. Study 1 (Vaher et al., 1994) and study 2 (Vaher, 1995a) have shown that ADX produces a small increase in pyknosis which is not always detected by statistical analysis. Thus, the increase in pyknosis shown in ADX rats treated with RU28362 might be the result of an ADX effect.

Cross sectional DG area was tested for differences between groups by one way ANOVA (Table 8). ADX effects on cross sectional area were not significant.

Correlational analysis

Ccrrelational analysis was used to determine the extent to which adrenal function and/or brain morphology contributes to spatial memory function. A statistical correlation between indexes of serum adrenal steroids and maze performance were not shown in this study (Table 9). This finding was unexpected since the maze performance of ADX+362 rats was paralleled by changes in salt appetite. Study 1 (Vaher et al., 1994) and study 2 (Vaher, 1995a) in this series have shown that physiological indicators of serum levels of adrenal steroids, serum CORT and saline intake are significantly correlated to radial arm maze

performance. The lack of a statistical correlation could be due to the absence of a pronounced ADX effect and the reduction of saline intake in the more impaired ADX+362 rats. Higher levels of saline intake are usually associated with poorer maze performance.

Morphological measures, the number of pyknotic cells in the DG and cross sectional DG area, were not correlated to measures of choice accuracy (Table 10). An association between saline intake and cell death was not evident.

Table 7. Effect of type 2 adrenal steroid agonist RU28362 on daily intake of 3% saline.

	Week		
Group	1	2	3
Sham	1.31±0.30	1.63±0.19	0.81±0.25
ADX	17.23±1.38	21.25±1.65	17.27±1.62
ADX+362	9.65±1.33*	7.31±1.86*	16.09±0.99

Entries are means ± S.E.M. ADX and ADX+362 rats were matched according to daily saline intake prior to treatment with RU28362. RU28362 was administered by osmotic minipump at a dose of 10 µg/hr for seven days. Treatment effects were tested on a weekly basis by one way between groups ANOVA. Pairwise testing showed that ADX significantly increased saline intake in ADX rats for three weeks of testing. RU28362 significantly lowered saline intake in ADX rats for the first two weeks of treatment (asterisk). Saline intake of ADX+362 rats was not significantly different from ADX controls by the third week.

Table 8. Effect of RU28362 on the morphology of the dentate gyrus in ADX rats.

Morphological variable	Group		
	Sham (n=7)	ADX (n=7)	ADX+362 (n=7)
Cross sectional dentate area (10^6 μm^2)	0.2755±0.01	0.2485±0.01	0.2848±0.01
Number of pyknotic cells / 10^6 μm^2	0.39±0.31	1.45±0.8	14.82±4.03*

Entries represent mean ± S.E.M. Measurements of DG area were tested for groups differences by one way between groups ANOVA. Pyknotic cell densities were tested by non-parametric statistics. Asterisk indicates a significant difference from sham ($p<0.05$).

Table 9. Correlation of daily intake of 3% saline intake to radial arm maze performance.

Measures of Choice Accuracy	Week 1 (n=24)	Week 2 (n=9)	Week 3 (n=23)
Number correct in first eight choices	-0.05 p=0.82	-0.23 p=0.54	-0.21 p=0.34
Number of errors	0.19 p=0.36	0.03 p=0.93	0.16 p=0.48

Entries are Pearson's product moment correlation coefficients. Saline intake was not significantly correlated to measures of choice accuracy ($p<0.05$).

Table 10. Correlation of radial arm maze performance to measures of dentate gyrus morphology.

Morphological variable	Choice Accuracy: Number correct in first eight choices	Choice Accuracy: Number of errors
Cross sectional dentate area (10^6 μm^2)	-0.25 p=0.29	0.21 p=0.39
Number of pyknotic cells / 10^6 μm^2	-0.40 p=0.09	0.15 p=0.53

Entries are Pearson's product moment correlation coefficients and exact probabilities of significance. Morphometric measures from 19 rats were tested for correlations to measures of choice accuracy from the third week of testing. Correlations were not significant ($p<0.05$).

FIGURE LEGENDS

Figure 7. Protocol of procedural events in RU28362 experiment. Rats were acclimated for one week prior to adrenalectomy (ADX). After recovery, rats were shaped to an eight arm radial maze. RU28362 was administered by osmotic minipump (Alzet) implanted subcutaneously 18 days after ADX. Test trials with all eight arms baited began the following day. Rats were tested four times a week for three weeks before sacrifice. Histology was performed as described in the text.

Figure 8. Effects of RU28362 on the radial arm maze performance of ADX rats. RU28362, a selective type 2 adrenal steroid receptor agonist, was administered to ADX rats by osmotic minipumps implanted subcutaneously one day before testing. Pumps lasted for seven days and secreted steroid at a rate of 10 μg/hr. Sham (n=10), ADX (n=7) and ADX rats treated with RU28362 (n=8) received 12 maze trials over three weeks. Trials were grouped into blocks of four according to weeks for statistical analysis. RU28362 further impaired radial arm maze performance in ADX rats. The effect lasted at least two weeks, one week beyond the supposed life of the pump. ADX rats treated with RU28362 showed a trend towards improvement during the third week of testing when minipumps are depleted. A: the number of errors in completing the task. B: the number of correct choices in the first eight visits.

EXPERIMENTAL PROTOCOL

Male Sprague-Dawley rats (3 mo. old), run in four cohorts

Acclimate for one week (singly housed)

Surgery: 2 groups of ADX rats, 1 group sham operated
(Saline and normal drinking water were made available after surgery)

Recovery period of one week

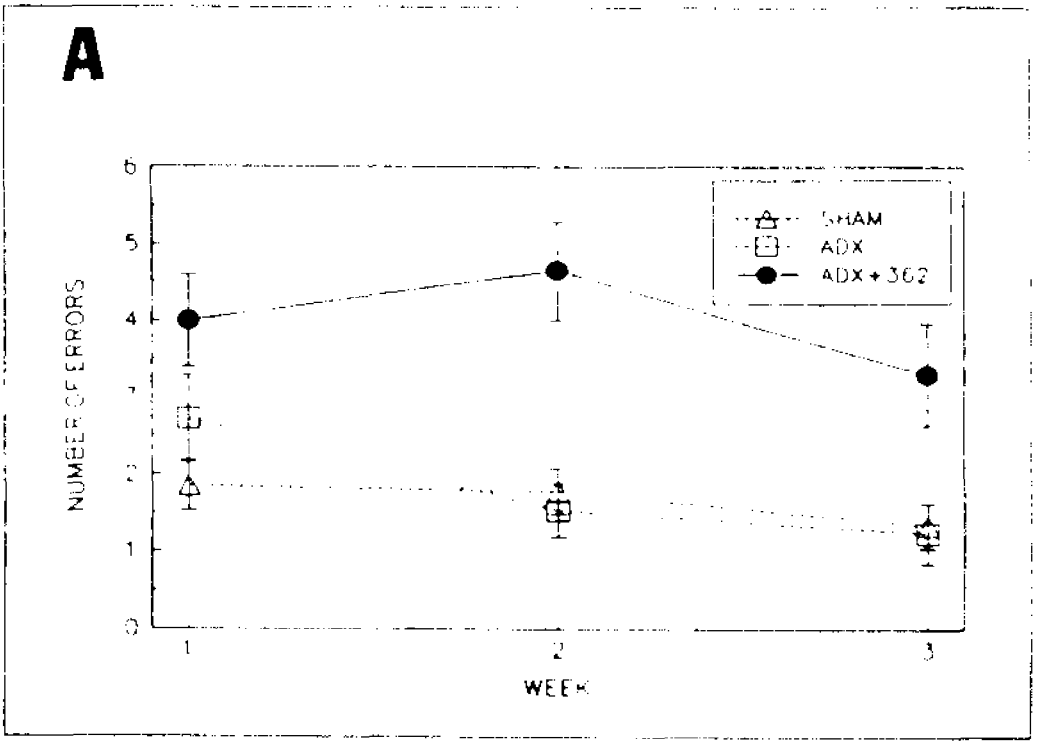
Weeks 2-3 post ADX: Food restriction and shaping to the radial arm maze

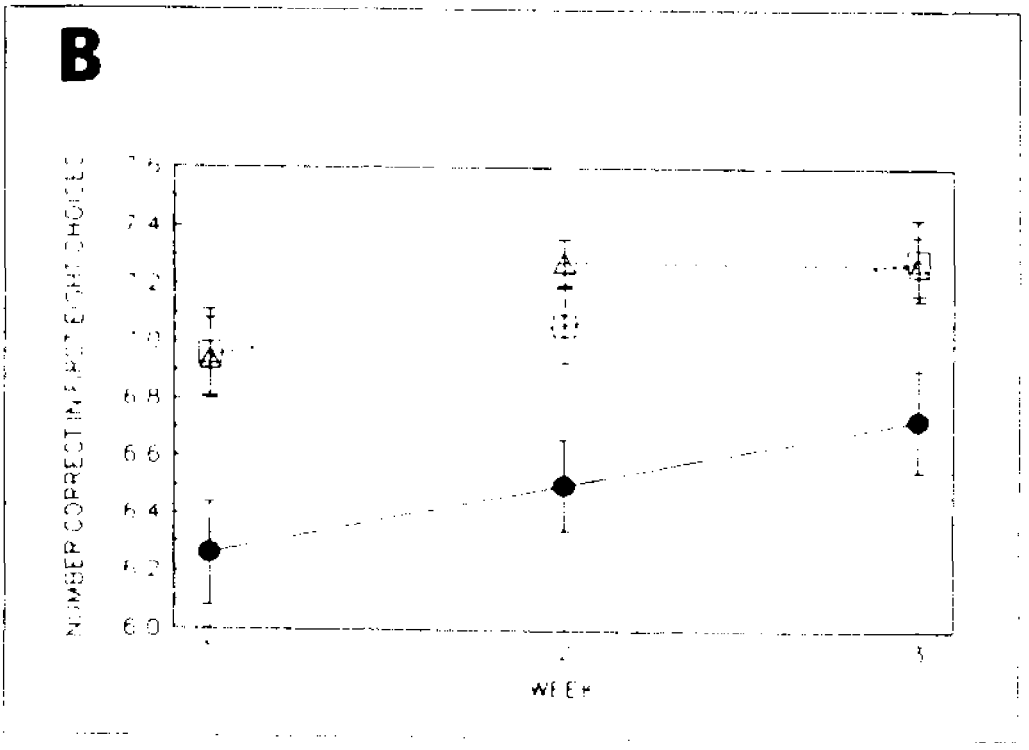
Subcutaneous implants:
ADX group - Alzet minipumps
(10 μg/hr of RU28362 in propylene glycol for seven days)
* ADX group - nylon dummy pumps
* sham ADX - nylon dummy pumps

Weeks 5-7 post ADX: 12 radial arm maze trials

Sacrifice and histological analysis

Figure 8


A
NUMBER OF ERRORS
0
1
2
3
4
5
6
WEEK
SHAM
ADX
ADX + 3G2


B
6.0
6.4
6.8
7.4
WEEK

Study 4: Effects of Adrenalectomy on Rats First Trained to the Radial Arm Maze Task

Study 4 investigated whether ADX-dependent deficits in spatial memory performance are due to the impairment of learning or memory. Rats were first trained to the radial arm maze task before ADX and then tested again. If ADX affects learning but not memory, the requirements of the radial arm maze task would have already been incorporated into memory prior to surgery and ADX rats would not be impaired when tested again. If ADX affects memory, performance after surgery would be impaired since rats would not be able to remember experiences prior to surgery. Although the evidence of this experiment would not be considered conclusive (see discussion), these findings provide a basis for determining whether ADX-dependent impairments in the acquisition of the radial arm maze task shown in study 1 are due to deficits in learning or memory function.

(Acknowledgements: This study was a summer project for undergraduate students that was supervised by myself in Dr. Luine's laboratory. The technical assistance of Hijoung Yu and Jenny Yee are gratefully acknowledged).

METHODS: STUDY 4

Methods are the same as in study 1 (Vaher et al., 1994) unless otherwise noted.

Subjects

Sixteen male Sprague-Dawley rats (Charles Rivers Laboratories) were received at two months of age and were allowed to acclimate for two weeks before shaping to the radial arm maze. Rats were trained to the radial arm maze in two cohorts run approximately one month apart.

Surgery

After shaping to the radial arm maze and five maze trials with all arms baited (see below), rats were allowed free access to food and adrenalectomy was performed on about half of the rats as explained in study 1 (Vaher et al., 1994). The remaining rats were sham operated. Saline was again made available to all rats after surgery in addition to normal drinking water. A solution of 3% saline was made available to all rats after surgery in addition to normal drinking water. Saline intake was measured daily in order to assess the presence of ectopic adrenal tissue and detect adrenal regrowth (Richter, 1941; Vaher et al., 1994; Vaher, 1995a). Saline intake has been shown to be highly correlated to serum levels of CORT and is a valid index of adrenal function (Conrad & Roy, 1992; Vaher et al., 1994). ADX rats drinking less that 10 ml/day of saline are likely to have accessory adrenal tissue

and were excluded from behavioral analysis.

Spatial Memory Testing

Spatial memory was tested on the radial arm maze as described in Study 1 earlier (Vaher et al., 1994). Choice accuracy was again scored by two measures: number of correct choices in the first eight visits and the number of errors in completing the task.

Data analysis

Physiological indicators: Measures of daily intake of 3% saline were tested for group differences after ADX by independent samples two-tailed t-tests.

Behavioral Data: Behavioral data were obtained from two cohorts each containing sham and ADX rats. Data from the two cohorts were combined and analyzed as a whole. Rats received ten shaping trials and five maze trials with all eight arms baited. Adrenalectomy was performed on nine rats. Seven rats were sham operated. Ten days after surgery, rats received five trials with all arms baited, then four trials with a time delay after the fourth choice. Delays were of 10 min., 1 hr., 2 hr., and 3 hr. All ADX rats met criteria for saline consumption. Measures of maze accuracy were again analyzed by blocks design ANOVA with repeated measures.

RESULTS: STUDY 4

Saline intake

As expected, ADX rats consumed amounts of saline well above that of shams during the week of delay testing (12.40±2.51 ml/day in ADX rats vs. 2.75±1.10 ml/day in shams, t_{14}=-9.52, p<0.0000).

Behavioral performance

Radial arm maze performance before and after ADX is shown in Table 5. There were no initial differences between groups before surgery in either the number of correct choices in the first eight visits (t_{14}=-1.04, p<0.32) or in the number of errors in completing the task (t_{14}=1.34, p<0.20). Since interaction effects were not significant, main effects were tested by blocks design ANOVA with repeated measures. The ADX effect was not significant for either the number of correct choices in the first eight visits ($F_{1,29}$=1.97, p<0.17) or the number of errors in completing the task ($F_{1,29}$=0.90, p<0.35). Blocks effects were significant for both accuracy measures; maze performance improved after surgery overall.

Following these trials, rats were tested with a delay between the 4th and 5th visits. Maze performance was significantly improved in ADX rats for the measures of choice accuracy shown in Table 11. They made significantly more correct choices out of the first eight visits ($F_{1,58}$=5.33, p<0.02) and made fewer errors in completing the task ($F_{1,58}$=12.14, p<0.0009). Increasing the time delay did not

significantly alter maze performance for either group.

Table 11. Effect of adrenalectomy (ADX) on rats first trained to the radial arm maze.

	REGULAR TRIALS		DELAY TRIALS
GROUP	BEFORE	AFTER	
	NUMBER CORRECT IN FIRST EIGHT CHOICES		
SHAM	6.43 ±0.20	6.88 ±0.16	6.54 ±0.12
ADX	6.71 ±0.11	7.02 ±0.14	6.94* ±0.12
	NUMBER OF ERRORS IN COMPLETING THE TASK		
SHAM	3.03 ±0.38	2.21 ±0.44	3.68 ±0.37
ADX	2.91 ±0.33	1.67 ±0.27	2.08** ±0.27

Entries are means ± S.E.M. Seven sham and nine ADX rats were tested on the radial arm maze. There were no initial differences between groups in the number correct in the first eight or the number of errors in completing the task. After surgery, there was no ADX effect during regular trials. For trials with a delay, the ADX effect was significant for the number correct (* $p<0.02$) and the number of errors (** $p<0.0009$). ADX rats performed better on the delay task than shams.

DISCUSSION

Studies in this series have shown that adrenal steroids affect spatial memory. The results of study 1 showed a persistent impairment of radial arm maze performance in ADX rats for up to 71 days when rats were tested shortly after adrenal removal. Further studies with selective agonists showed that the Type 1 and Type 2 adrenal steroid receptors have opposite effects on spatial memory function; Study 2 showed that type 1 receptor activation by replacement of ALDO in ADX rats abated deficits in radial arm maze performance whereas in study 3 we found that type 2 receptor activation with RU28362 further impaired the radial arm maze performance of ADX rats. Steroid effects lasted for two weeks, one week beyond the expected life of the minipump vehicle, and were paralleled by a continued suppression of saline intake. The effects on behavior were lost in the absence of steroid three weeks after hormone replacement and was accompanied by an increase in saline intake. Steroid effects on radial arm maze performance occurred in the absence of any marked reduction of dentate gyrus granule cell volume or in a substantial increase in dentate gyrus granule cell pyknosis at the time of death. In study 4, rats that were first trained to RAM before adrenal removal performed as well as controls suggesting that ADX impairs learning while leaving memory intact. The contribution of adrenal steroids to learning and/or

memory appears complicated since when a delay is added, performance is improved in ADX rats. These findings must be considered in relation to other studies on adrenal steroids and memory, physiological indicators of adrenal function, and the effects of ADX on dentate gyrus morphology.

Spatial Memory Performance

The results of study 1 have shown that ADX rats are impaired in the performance of a spatial memory task, the eight arm radial maze. This impairment was reflected in all aspects of choice accuracy performance and lasted throughout the period tested, from three to ten weeks after ADX. Previous studies of the effects of ADX on spatial memory have shown transient deficits after long-term adrenalectomy. Conrad and Roy (1992) tested the effects of ADX on the Morris water maze, another spatial memory task. Their testing began 12 weeks after ADX, and they found that ADX rats were slower in locating a platform hidden in a pool of cloudy water. During reversal testing, the location of the platform was changed and performance of the ADX group did not differ from controls. Radial arm maze testing was done 22 weeks after ADX. For six weeks of testing, every other arm was baited. Performance was then assessed on maze reversal (arms that previously had not been baited were now the baited arms). ADX rats were not impaired on either test of the radial arm maze.

Armstrong, McIntyre, Neuport and Sloviter (1993) tested

subjects 4 months after ADX in the Morris maze. Differences in maze behavior were found in those ADX subjects with moderate to extensive granule cell loss (GCL). Like the Conrad and Roy (1992) study, these rats required a longer time to find the hidden platform and performance between groups was alike when the location of the platform was changed. Short-lived impairments were seen in GCL rats when the pool was rotated 90^0 and also when all extramaze cues were removed. Thus in both studies, ADX rats were slightly impaired in acquisition of the Morris water maze task.

ADX should result in spatial memory deficits by causing granule cell death and opening the trisynaptic circuit in the hippocampus. It has been suggested that the absence of robust ADX effect is due to the presence of auxiliary pathways that circumvent the dentate granule cells (for review, see Armstrong et al., 1993). First, tract tracing studies have shown that the longitudinal projections in the hippocampus are just as prominent as those in the transverse direction (Amaral & Witter, 1989). Direct connections from the entorhinal cortex and other parahippocampal structures to pyramidal neurons in the hippocampus have been shown. McNaughton et al. (1989), showed that cells in area CA3 respond to spatial stimuli even if the granule cells have been destroyed by colchicine. Thus, neuroanatomists have argued that the few remaining granule cells could distribute information throughout much of the hippocampus. However, electrophysiological studies have shown that

stimulation of one of these entorhinal projections to the hippocmpus, the perforant projection to the pyramidal cells of CA1, rarely elicits firing of hippocampal neurons even when synaptic potentials are quite large (Colbert & Levy, 1992a; Colbert & Levy, 1992b; Anderson, Holmquist & Voorhoeve, 1966; Desmond, Heydenreich & Levy, submitted). More importantly, colchicine lesions of the dentate gyrus do cause pronounced deficits in spatial memory function (Sutherland et al., 1983; Walsh et al., 1986; Whishaw, 1987; Nanry et al., 1989; Tilson et al., 1987; Emerich & Walsh, 1990; McNaughton et al., 1989). This suggests that the integrity of the intrahippocampal circuit is necessary for normal spatial memory function. Thus, the tract tracing studies of Amaral and Witter (1989) do not negate the lamellar hypothesis but augment our knowledge of hippocampal projection systems.

The more pronounced effect of ADX on spatial memory function in our studies could be due to a number of factors. First, there are differences between the Morris water maze and radial arm maze tasks. Transient effects might be a characteristic of the Morris water maze because it is an easier task. The Morris water maze requires that the rat remember the location of only one submerged platform whereas completion of the radial arm maze needs memory of eight locations visited. Once the location of the submerged platform is learned, even affected rats soon reach asymptotic performance and deficits can no longer be discerned.

Second, there was a shorter time interval between ADX and behavioral testing in our study. Testing memory performance 4 months after ADX, when extensive cell loss is known to occur in some rats, may also allow time for compensatory changes to take place. The DG is especially of interest to the study of learning and memory because of the marked degree of neuronal plasticity exhibited by this structure. Lesions of the DG result in an increase in acetylcholine activity in hippocampal area CA3 (Drust & Crawford, 1985; Emerich & Walsh, 1990; McKeon, Viete & Wells, 1989; Tilson et al., 1987). More importantly, Gould et al. (1991b), has shown that ADX triggers cell birth as well as cell death and that neuronal restructuring continues well into adulthood as part of normal development. Thus, recovery of function is less likely to occur if testing is done shortly after ADX. This may be relevant to Conrad and Roy (1992) since radial arm maze testing was done 22 weeks past ADX. The absence of a robust ADX effect in study 3 of this series (Vaher, 1995b) might be better explained by the marked degree of plasticity exhibited by this area.

The differences in conclusions do not appear to be due to differences in criteria for selecting ADX rats. Other researchers studying the effects of ADX on spatial memory have used changes in body weight as the principal selection criterion (Armstrong et al., 1993; Conrad & Roy, 1992; Conrad & Roy, 1995) whereas our studies use saline intake for evaluating the completeness of ADX (Vaher et al., 1994; Vaher, 1995a). Levels

of serum CORT are significantly correlated to both body weight ($r=0.79$, $p<0.01$) (Conrad & Roy, 1992) and saline intake ($r=-0.85$, $p<0.01$) (Vaher et al., 1994). Differences in our conclusions with respect to cell loss and spatial memory deficits are better explained by the factors just discussed.

The results of study 2 have shown that replacement with ALDO, a specific type 1 adrenal steroid agonist, abates ADX-dependent impairments of a spatial memory task, the radial arm maze. The ALDO effect was especially effective in reducing the number of maze errors in completing the task. In this context, icv microinjection of the intact rats with Spironolactone, a specific type 1 receptor antagonist, increased the latency to relocate a submerged platform during session 3 of Morris water maze testing (Oitzel & de Kloet, 1992). ALDO also produced a marked enhancement of LTP in ADX rats (Pavlides, Watanabe, Margariós, & McEwen, 1992; Pavlides, Watanabe, Margariós, & McEwen, in press). The effect was blocked when RU28318, a type 1 receptor antagonist, was administered prior to ALDO replacement. Further, electrophysiological recording studies have shown that occupation of the type 1 receptor optimizes synaptic transmission in hippocampal neurons (Joëls, Hesen, Karst, & de Kloet, 1994). These studies together suggest that type 1 adrenal steroid receptor activation improves spatial memory function in ADX rats.

Study 3 showed that RU28362, a selective type 2 receptor agonist, further impaired ADX rats in the performance of a

spatial memory task, the radial arm maze. The effect was pronounced as was evident in both measures of choice accuracy, the number of errors in completing the task and the number of correct choices in the first eight visits. In this context, treatment of ADX rats with RU28362 produced a marked decrement in LTP, a model for the neuronal basis of learning (Pavlides et al., 1992; Pavlides et al., In press). The effect was blocked by prior injection of RU38486, a type 2 receptor antagonist. Further, electrophysiological recording studies have shown that occupation of the type 2 receptor in addition to the type 1 receptor resulted in a large voltage gated and transmitter-dependent responses that result in a gradual failure of hippocampal neurons to respond to repeated stimulation (Joëls et al., 1994); i.e., activation of the type 2 receptor depressed the excitability of hippocampal neurons. Taken together, these studies suggest that type 2 receptor activation further impairs spatial memory function in ADX rats.

In contrast to these findings, Oitzl and de Kloet (1992) found that icv microinjection of type 2 receptor antagonist RU38486 increased the latency to relocate a hidden platform during the second day of Morris water maze testing, another test of spatial memory. Oitzl and de Kloet (1992) tested the effects of adrenal steroid receptor antagonists on intact rats, not ADX. Although the effects of adrenal steroid receptor activation on spatial memory function in ADX rats has become clear (see below), the interactions between the two receptor types in the

intact rat have not been studied.

In both studies 2 and 3, adrenal steroid effects on RAM performance lasted two weeks, one week beyond the expected life of the pump, and was paralleled by a continued suppression of saline intake. Steroid effects subsided three weeks later and were accompanied by a increase in salt appetite. This suggests that the effects of adrenal steroid replacement on spatial memory performance are reversible and dependent on the presence of serum steroids in ADX rats and that effects appear to be long-lasting.

In study 1 (Vaher et al., 1994), rats were adrenalectomized, then shaped to the radial arm maze shortly after. ADX rats were impaired in maze performance. It is not clear whether their performance was worse than controls because they were unable to learn as well as controls or because their memory was compromised after ADX. In study 4, we trained the rats on the task before adrenalectomy so that we could begin to asses whether learning and/or memory contributes to ADX-dependent impairments in performance. If ADX affects learning but not memory, the requirements of the radial arm maze task would have already been encoded into memory prior to surgery and ADX rats would not be impaired when tested again. If ADX affects memory, performance after surgery would be impaired since rats would not be able to remember experiences prior to adrenal removal. In trials with all eight arms baited, the ADX groups performed as well as controls. This result suggests that ADX

rats do not learn as well as intact rats. This finding is consistent with case studies that have shown that bilateral removal of the hippocampus results in an anterograde amnesia, an inability to learn anything new (Milner, 1970).

It should be noted that learning and memory are related processes. In order to acquire the radial maze task, rats must remember the reference memory aspects of the task. That is, the rat must remember what is required to complete the maze. Task acquisition also requires working memory, memory of which arms had already visited. Lesion of the hippocampus produces deficits in RAM performance which have also been interpreted as an impairment in working memory function (Olton et al., 1978). Although the evidence is not conclusive, the absence of a performance deficit in study 4 suggests that ADX impairs learning while leaving memory intact.

Physiological Correlates of Behavior

We monitored saline intake and body weight in all animals. Levels of serum CORT were measured only in the second cohort of the first study. CORT was correlated to one of two measures of choice accuracy on the radial arm maze suggesting that lower levels of adrenal steroids are associated with poorer behavioral performance. Saline intake was highly correlated to serum CORT levels and thus appears to be a sensitive index of adrenocortical function, but does not preclude the presence of sub-detectable levels of circulating titers.

Previous researchers studying the effects of ADX on Morris water maze performance (Armstrong et al., 1993; Roy et al., 1990) used body weight change after surgery as a criterion for selecting subjects with granule cell loss. Roy et al. (1990) showed that body weight gain after ADX is positively correlated with granule cell layer area three months after surgery. Further, short-term loss of body weight is negatively correlated to the degree of degeneration of the granule cell layer when supplemental saline is replaced with tap water. Free feeding weight cannot be assessed in rats tested on the radial arm maze since the task requires food deprivation. However, saline intake was correlated to radial arm maze performance and can be used when food serves as reinforcement. Thus, saline intake provides a simple and inexpensive means for evaluating the relative completeness of ADX.

Study 1 showed that saline intake was highly correlated to both serum levels of CORT and to radial arm maze performance (Vaher et al., 1994). Thus, saline intake was measured as an assay of serum levels of adrenal steroids in the remaining studies in this series. As expected, ALDO replacement significantly decreased the saline intake in ADX rats. Saline intake was significantly correlated to measures of choice accuracy throughout 3 weeks of testing overall. This was true even though the amount of saline intake varied as ALDO minipumps were depleted over the period of testing. Saline intake declined within four days after ALDO replacement but requires four weeks

to return to ADX levels. Since saline intake was correlated to maze performance, we would expect that the performance of ADX rats treated with ALDO would continue to decline if tested for a fourth week. Thus, measuring saline intake would seem to be a valid method of assessing serum levels of adrenal steroids.

The reduction in saline intake in ADX rats treated with RU28362 was unexpected (Study 3). It is well established that the reabsorption of sodium is regulated by the type 1 adrenal steroid receptor in the kidney (Richter, 1941; McEwen, Lambdin, Rainbow, 1986; Burg, 1986) and RU28362 is believed to be a "pure" type 2 receptor agonist (Reul & de Kloet, 1985). RU28362 could affect saline intake if the type 2 receptor activation produces K+ wasting. Glucocorticoid receptors have been found in the kidney (Lee, Chekal & Katz, 1983) and replacement of CORT has been shown to reduce hyperkalemia in ADX rats (Mujais, Chekal, Jones, Hayslett & Katz, 1984).

Dentate Gyrus Morphology

Previous studies have shown that short term ADX (3-7 days) results in dramatic increases in the number of degenerating cells as indexed by pyknotic cell density (Gould et al., 1990; Jaarsma et al., 1992; Woolley et al., 1991) and that long term ADX results in almost complete cell loss (Sloviter et al., 1989; Roy et al., 1990; Armstrong et al., 1991; McNeill et al., 1991; Conrad & Roy, 1992, Conrad & Roy, 1995). It would be expected that neuronal loss would continue until there is "nearly

complete loss" (Sloviter et al., 1989) of the granule cell layer 3 or 4 months later. Given these observations, it was surprising that, despite a substantial impairment in radial arm maze performance (Studies 1 and 2), the correlation of physiological indicators of adrenal function to radial arm maze performance (Studies 1 and 2), and the correlation of serum adrenal steroids to pyknosis (Study 2), no sizable increase in the number of pyknotic cells was observed in any of the studies in this series (Studies 1, 2 or 3). Indeed, there was no evidence five and ten weeks after ADX that the dentate gyrus had atrophied, and the pyknotic neuron density was not substantially higher, on the average, than the control group. The pyknotic cell count was much lower five (8.5 ± 4.2 pyknotic cells/10^6 μm^2, study 2) and ten (5.19 ± 2.3 pyknotic cells/10^6 μm^2, study 1) weeks post-ADX than found previously in similar age rats 3 to 7 days after adrenal removal (2163.8 ± 556 pyknotic cells/10^6 μm^2, Gould et al., 1990). On the basis of earlier work with the same strain of rat it is reasonable to assume all of these rats had shown increased pyknosis 3-7 days post-ADX.

The density of pyknotic cells in ADX rats was low but highly variable in our study and did not correlate with measures of adrenal function or radial arm maze performance. Previous authors have also noted the high degree of individual variability in dentate morphology amongst ADX rats (Conrad & Roy, 1992; Roy et al., 1990; Sloviter et al., 1989; Sloviter et al., 1993a). Complete granule cell loss is actually a rare event

with only a minority of cases resulting in complete destruction of the granule cell layer. Only 2-30% of ADX rats exhibit this phenomenon (Sloviter et al., 1989; Sloviter et al., 1993b). In agreement with this observation, our distribution of pyknotic cell densities was positively skewed. Sloviter et al., (1993a) conducted a long-term analysis of ADX-dependent cell death in the DG. One year after adrenal removal, 35% exhibited obvious cell loss, 33% exhibited no cell loss in the presence of adrenal insufficiency (also called partial ADX rats by Conrad & Roy, 1992) and 32% exhibited no cell loss and no adrenal insufficiency. Almost all of our ADX rats exhibited a slight increase in pyknosis five and ten weeks after adrenalectomy and about half would be expected to develop obvious cell loss after one year. Sloviter et al (1993a) attributed the absence of complete cell loss in ADX rats to the presence of subdetectable levels of adrenal steroids secreted by nodules of accessory adrenal tissues.

In study 1, RIA analysis of serum levels of CORT showed that many of our ADX rats had some accessory adrenal tissue capable of producing CORT (only 1 of 5 ADX subjects had no detectable CORT) and this CORT may have been sufficient to suppress granule neuron pyknosis, yet not sufficient to suppress salt intake. This situation could arise if the accessory adrenal tissue produced CORT and not aldosterone. CORT is known to suppress dentate gyrus neuronal pyknosis via Type 1 receptors (Woolley et al., 1990), but it is unable to reach kidney Type 1

receptors because of the enzyme 11-ß-hydroxysteroid dehydrogenase (Steward & Edwards, 1990). In contrast, aldosterone reaches hippocampal Type 1 receptors and also kidney Type 1 receptors (Steward & Edwards, 1990). Aldosterone levels were not directly measured in our studies.

Studies 1, 2 & 3 have all shown only a low frequency of pyknosis in the presence of high saline intake five and ten weeks after surgery. The consistency of this finding suggests that ADX-dependent cell death might temporarily slow at a time point several weeks after ADX. It has been shown that low levels of serum adrenal steroids trigger cell birth as well as cell death in rats less than one year of age (Gould et al., 1991). Further study is needed to determine if cell death subsides and/or compensatory cell birth occurs at a higher rate five to ten weeks after ADX. Excitatory amino acids, peptide trophic factors, and regulatory genes have also been shown to mediate mitosis and apoptosis in the DG (for review, see Gould et al., 1993). The time course of these effects has not been studied.

Lasting impairments in radial arm maze performance without the marked presence of granule cell pyknosis or reduction in DG size suggests that levels of circulating adrenal steroids themselves exert an important influence on spatial memory function prior to pervasive degeneration of DG granule cells. Several studies have shown that adrenal steroids acutely affect LTP (Diamond & Rose, 1994; Diamond et al., 1995; Pavlides, Watanabe, & McEwen, 1993; Pavlides, Watanabe, Margariños &

McEwen, 1992; Pavlides, Watanabe, & McEwen, 1993; Pavlides, Watanabe, Margariños & McEwen, in press) and regulate neuronal excitability in the hippocampus (Joëls, Hesen, Karst, & de Kloet, 1994). These effects occur within hours of application before any sizable cell loss can occur. This conclusion remains tentative in the absence of studies that correlate the morphology of granule cell dendrites to measures of spatial memory performance.

Earlier studies with the Morris water maze, another test of spatial memory, would seem to contradict our conclusion; deficits on the Morris water maze occurred only in those rats with measurable loss of granule cell neurons (Armstrong et al., 1993; Conrad & Roy, 1992). Recently however, Conrad & Roy (1995), suggested that the absence of adrenal steroids is responsible for at least some of the water maze impairment seen in ADX rats. In this study, ADX rats received either chronic CORT replacement (chCORT) or acute CORT replacement during the two weeks of water maze testing three months after surgery (acCORT). Rats in the chCORT condition performed better than acCORT rats and ADX controls. More importantly, acCORT rats performed slightly better than ADX controls. Since there was no difference between these two groups in the amount of granule cell loss and the only difference between acCORT and ADX control rats was the exogenous CORT given at the time of testing, the slight improvement in water maze performance could be due to an endocrine effect. Thus, adrenal steroids themselves appear to

exert activational effects on spatial memory prior to pervasive degeneration of DG granule cells in ADX rats.

Woolley et al. (1991), showed that type 1 receptor activation protects against ADX-induced cell death. Adrenalectomy increased pyknosis in ADX rats but ALDO replacement could not be shown to prevent cell death in study 2 since ALDO pumps had already been depleted at the time of sacrifice. If rats had been perfused at the time of ALDO replacement, we would expect to find that ALDO reduced pyknosis in adrenalectomized rats.

Morphological results in study 3 suggested a higher rate of pyknosis in the DG of ADX rats treated with RU28362. This seems to be in contrast to the finding that RU28362 partially protects against ADX-dependent cell death (Woolley et al., 1991). Further, Joëls et al. (1994) suggests that the cell death in hippocampal area CA3 resulting from prolonged stress or high circulating titers of CORT shown by Sapolsky (Sapolsky et al., 1985) can be accounted for by an influx of calcium caused by type 2 receptor activation. Prolonged stress or high levels of CORT have not been shown to cause granule cell death in the dentate gyrus. Earlier studies in this series have shown that ADX resulted in a small increase in pyknosis at the time of sacrifice that are not always detected by statistical analysis (Studies 1 & 2). Thus, the increase in pyknosis shown in ADX rats treated with RU28362 might be the result of an ADX effect.

Conrad and Roy (1995) showed that middle aged

adrenalectomized rats lost granule cells only in the suprapyramidal blade of the dentate gyrus. Although rats in study 2 showed more pyknosis in the suprapyramidal blade than in the infrapyramidal blade, the increase was not significant. The trend may not have been detected in our study because we had less cell loss five weeks after adrenalectomy than did Conrad and Roy (1995). Conrad and Roy (1995) also showed that older rats exhibit less ADX dependent cell loss than do younger rats and concluded that mature granule cells are less affected by adrenalectomy.

Several researchers have noted degeneration staining in the area of the hilus in adrenalectomized rats (Jaarsma et al., 1992; Pavlides, Margariños, Gould, Hsu, Pierre, Watanabe, McEwen & Buzsaki, 1993; Sapolsky, Stein-Behrtens, Armanini, 1991; Sloviter et al, 1993). The number of pyknotic cells in the hilus were counted in Study 2 (Vaher, 1995a). Pyknotic cells were found in this region but only rarely. Sapolsky et al. (1991), originally reported that these were pyramidal cells in area CA4. Others have stated that these cells were always near the lateral aspect of the suprapyramidal blade of the granule cell layer (Jaarsma et al., 1992; Sloviter et al., 1993). Sloviter et al. (1991), believes that these cell bodies are displaced granule cells since the cell bodies were found to be calbindin-D28K-immunoreactive in normal sections. Pavlides et al. (1993) also found degeneration staining in the hilus but concluded that these were dentate polymorphic cells and interneurons of area

CA3. Unlike dentate granule cells, these cells were not protected by CORT replacement in ADX rats. One pyknotic cell in a sham ADX rat was located lateral to the suprapyramidal blade thus suggesting apoptosis.

Mechanism for ALDO Effects on Memory Performance

Traditional adrenal steroid receptors are found in the neuronal cytosol. Binding of the ligand to the receptor initiates translocation to the nucleus (McEwen, Gerlach & Micco, 1975) where protein synthesis can be regulated by genomic mechanisms. Recently, a nongenomic steroid receptor has been postulated (McEwen, 1994). Whereas genomic effects take several days, nongenomic effects are more rapid in onset. The time course of ALDO effects suggests a genomic mechanism of action; ALDO required three days to take a maximum effect and several days to revert. The expression of several types of mRNA has been shown to be regulated by adrenal steroids (Hiremagalur, Kvetnansky, Fukuhura, Fleischer, Geertman, Nankova, Viskupic & Sabban, 1993; Holmes, French, Yau, Secki, 1993; Liao, Miesak, Azmitia, 1993; Watanabe, Weiland, & McEwen, 1993). Since ALDO has been shown to facilitate spatial memory performance, type 1 receptor activation most likely includes an initiation of changes in protein synthesis that will improve memory.

One of many means by which protein synthesis can regulate memory is by receptor upregulation. Learning can be defined as the enhancement of synaptic efficacy in certain neurons as the

result of experience. Serotonin (5-HT) receptors might be upregulated in this way since 5-HT_{1A} receptor labeling was decreased by ADX and increased by treatment with dexamethasone (Liao et al., 1993). The time course of effect was shown to take three days, just as did the ALDO effect in study 2. Further, levels of serotonin are significantly correlated to spatial memory performance (Luine, Spencer & McEwen, 1993). The NMDA glutamate receptor might also be upregulated by adrenal steroids. NMDA receptor agonists inhibit the formation of long-term potentiation (Collingridge & Bliss, 1987).

Adrenal steroids might also affect spatial memory by regulating the synthesis of structural proteins. ADX has been shown to decrease the number of dendritic branch points in dentate gyrus granule cells (Gould et al., 1990). Study 1 and study 2 have shown that ADX causes impairment in spatial memory function (Vaher et al., 1994; Vaher, 1995a). Thus, it is possible that the number of dendritic branches and/or the number of synapses formed by dentate gyrus granule cells might be correlated to spatial memory performance. The process of dendrite formation requires the activation of genes that increase the synthesis of proteins needed for the construction of dendrites. Examples of structural proteins that might be synthesized in the process of dendrite formation are MAP2, NCAMs and GAP. Adrenal steroids could regulate spatial memory function by initiating the activation of genes important to dendrite formation.

Theory of Adrenal Steroid Receptor Regulation of Spatial Learning

Selective activation of type 1 and type 2 adrenal steroid receptors has been shown to produce opposite effects on spatial memory function in ADX rats. Study 1 showed that replacement of the type 1 receptor agonist, aldosterone, restored normal spatial memory function in ADX rats (Vaher, 1995a) while study 2 showed that administration of the type 2 agonist, RU28362, further impaired spatial memory performance (Vaher, 1995b). These findings are consistent with selective activation studies on LTP (Pavlides et al., in press) and neuronal excitability in the hippocampus (Joëls et al., 1994).

Our series of studies suggest that the type 1 and type 2 adrenal steroid receptors, with different affinities for corticosterone (CORT), form a two tiered system for the regulation of spatial memory function. The type 1 receptor has a high affinity for CORT and is extensively occupied by lower levels of CORT that are present during the diurnal rise and fall, whereas type 2 receptor occupancy may become substantial only at high resting and stress levels of CORT (McEwen et al., 1986; Reul et al., 1987; Spencer et al., 1993). This suggests that spatial memory performance should vary as an inverted U-shaped function as serum levels of adrenal steroids are increased (Figure 9). Type 1 receptors are occupied and spatial memory is facilitated when levels of adrenal steroids are

initially low. Rising serum CORT would activate type 2 receptors which impairs spatial memory performance and counters the faciliatory effects of type 1 receptor activation. Thus, spatial memory function should be optimal at an intermediate level of CORT when type 1 receptors are maximally saturated while performance is diminished when plasma CORT is either too high (Diamond & Rose, 1994; Diamond et al., 1994; Luine et al., 1994) or too low (Vaher et al., 1994; Vaher et al., 1995a). In this context, Diamond et al., (1992) showed that the overall relationship between plasma CORT and hippocampal primed burst potentiation is an inverted U-shaped function. Similarly, Joels et al. (1994), showed a U-shaped dose-response relationship between the relative ionic conductances and transmitter responses in hippocampal neurons as a function of the Type 1/Type 2 occupancy ratio. Study 2 showed that adrenal steroids require several days of tonic receptor activation to affect spatial memory function.

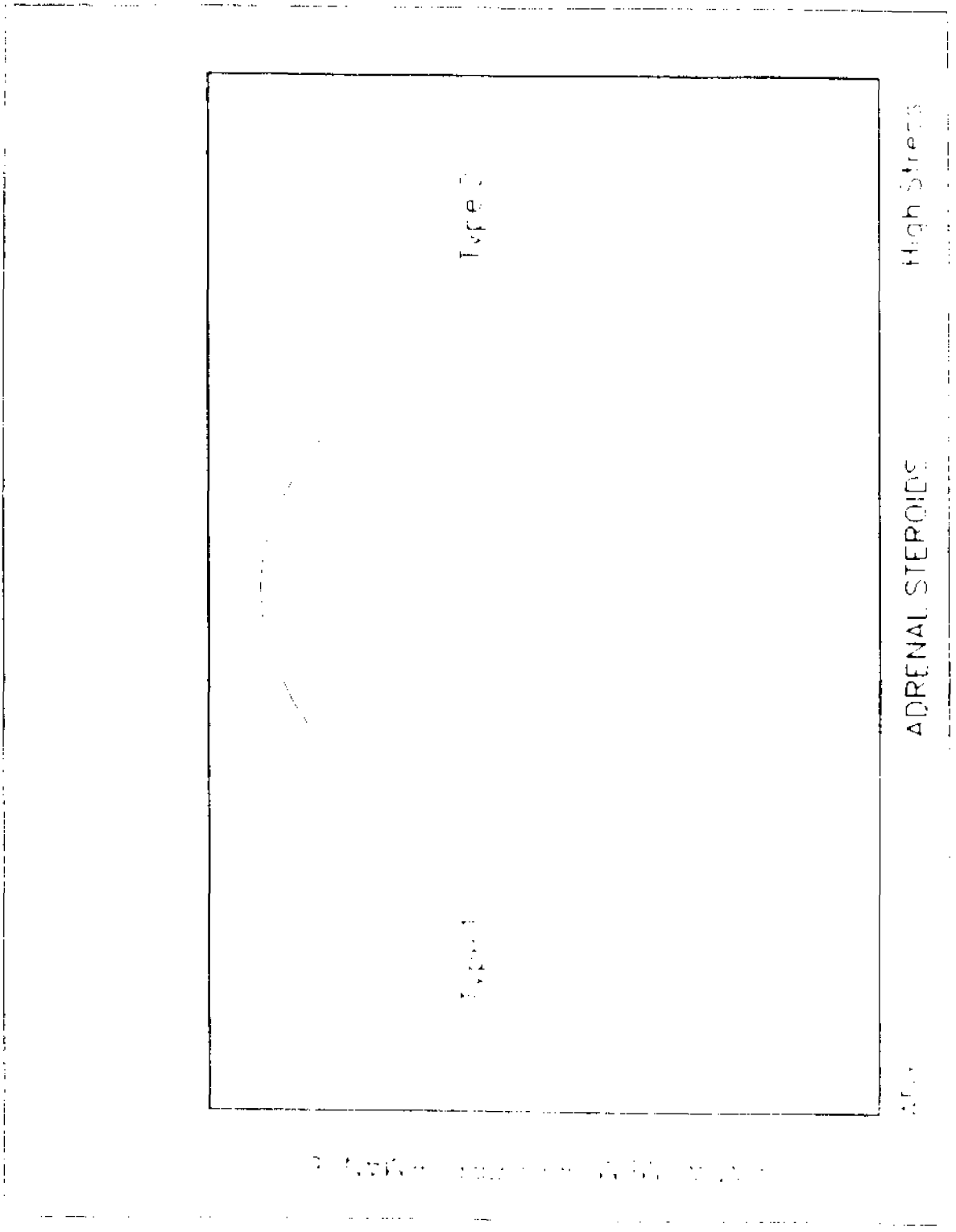
The contribution of adrenal steroids to learning and/or memory appears complicated, however. In study 4 we showed that ADX rats performed better at a delay task when rats were first trained on the RAM before adrenal removal. Similar improvements in performance have been found in ADX rats in other laboratories (Diamond, D., personal communication). Explanation of this anomalous finding might be found in studies on the effects of stress and arousal.

Release of adrenal steroids are part of the sympathetic

nervous system's response to stress. Similar to findings with rats, studies on human performance have shown that performance is very poor when levels of physiological arousal are either very high or very low and is best when arousal is at some intermediate level (Yerkes & Dodson, 1908; Hebb, 1955). Hyperarousal has particularly aversive effects on complex mental tasks. Improved performance was seen only when the standard maze task was made more difficult by adding a time delay between choices. It could be speculated that adding a time delay to the standard maze task is not only more difficult but also more stressful. The time delay in our study could be a stressor that impairs performance on a "complex mental task," the radial arm maze. ADX rats might perform better than shams on a well learned radial arm maze task because they are deficient in adrenal hormones that are released in high levels in response to stress and interfere with spatial memory performance. Further investigation is needed to see if adding a time delay to the standard radial arm maze task is a stressor and whether the adverse effects of stress on the performance of a well learned memory task are abated by ADX.

Figure 9. Theoretical dose-response relationship between serum corticosterone and spatial memory performance. This series has shown that the type 1 and type 2 adrenal steroid receptors have opposite effects on spatial memory performance in ADX rats; type 1 receptor activation facilitates spatial memory whereas type 2 activation further impairs performance. As serum CORT is increased, only the high affinity type 1 receptor are initially occupied and spatial memory performance is improved. At higher doses, the low affinity type 2 receptors become occupied and inhibit spatial memory. Optimal performance is obtained at intermediate levels of CORT while performance deteriorates when serum levels are high or low.

Figure 9



SUMMARY

Adrenalectomy (ADX) resulted in lasting deficits in the performance of a spatial memory task, the radial arm maze (RAM) when rats were tested shortly after surgery. While a wide range of individual differences amongst ADX subjects might have obscured any neuroanatomical differences or correlations, persistent deficits in radial arm maze performance without the marked presence of pyknosis or the reduction of DG size suggests that adrenal hormones themselves exert an activational effect on spatial memory prior to pervasive degeneration of DG neurons. Selective activation of type 1 and type 2 adrenal steroid receptors was shown to produce opposite effects on spatial memory function in ADX rats; Replacement of the type 1 receptor agonist aldosterone restored normal spatial memory function in ADX rats while administration of the type 2 agonist RU28362 further impaired spatial memory function.

On the basis of these findings, we predicted that the dose-response relationship between serum corticosterone (CORT) and spatial memory performance is an inverted U-shaped function in ADX rats. The type 1 receptor has a high affinity for CORT whereas the type 2 receptor binds with low affinity. Low affinity receptors become occupied only after high affinity receptors are saturated. Spatial memory function in

ADX rats should initially improve as CORT levels are increased and more type 1 receptors are occupied. Further rasing serum CORT would activate type 2 receptors which impair spatial memory performance and counter the faciliatory effects of the type 1 receptor. Spatial memory function should be optimal when type 1 receptors are maximally saturated while performance is diminished when plasma CORT is high or low.

Further investigation showed that ADX-dependent impairments in radial arm maze performance are actually due to deficits in learning and not memory; ADX rats do not learn as well as intact rats. The contribution of adrenal steroids to learning and/or memory appears complicated, however, since ADX rats performed better at a delay task when rats were first trained on the RAM before adrenal removal. Study beyond this thesis is needed to explain how adrenal steroids produced this effect.

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