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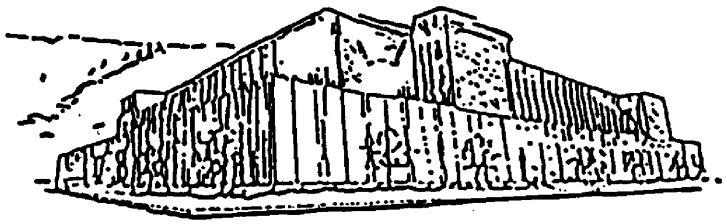
# Depression-like behavioral and endocrine effects of brain glucocorticoid type I receptors in the male rat

Bret Fajans Bessac
*The University of Montana*



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# DEPRESSION-LIKE BEHAVIORAL AND ENDOCRINE EFFECTS OF BRAIN GLUCOCORTICOID TYPE I RECEPTORS IN THE MALE RAT

Bret Fajans Bessac

B.A. Psychology, 1993; B.A. Zoology, 1993
University of Montana.

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The University of Montana

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Dean of the Graduate School

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# Abstract

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Title: Depression-like Behavioral and Endocrine Effects of Brain Glucocorticoid Type I Receptors In the Male Rat

Advisor: Craig A. Johnston, Ph. D.

Much evidence ties decreased brain glucocorticoid receptors together with anxiety and clinical depression. There are two known types of glucocorticoid receptors in the brain, type I (MR) and type II (GR). MR and GR often coexist in hippocampal and other limbic neurons and often cause different effects within these neurons, within the brain and in animal behavior. The present study examined the effects of chronic alterations in the stimulation of brain MR on depression associated behaviors using an open field test, circadian and 24-hour locomotor activity and plasma corticosterone (B). Rats were chronically exposed to an intracerebroventricular infusion of vehicle (propylene glycol), MR agonist (aldosterone) or MR antagonist (spironolactone) using ALZET mini-osmotic pumps. Two days following pump placement locomotor activity was monitored for 24 hours in all rats. Spironolactone decreased in total 24-hour activity from pre-treatment control levels and all other treatments, ($p<0.01$). This effect was most pronounced during the dark phase of the light cycle. There was no difference between any of the treatment groups in plasma B concentration determined by RIA, nor inner nor outer squares entered in the open field test. Intracerebral infusions decreased open field activity, although this was not correlated with the treatment. MR were examined using a monoclonal antibody raised to the renal MR ligand-binding domain and GR were examined using a polyclonal antibody. GR were observed in both kidney and hippocampal tissues, MR were observed only in the kidney. This was true for both immunocytochemical and Western blot analysis.

The results provide evidence that brain MR are linked to locomotor activity, and potentially depression. Furthermore, evidence was collected to suggest that hippocampal MR are different from renal MR.

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# Chapter 1

## Introduction

Almost everyone suffers from depression sometime during their life. Some people feel this emotional state at an extreme level for a prolonged period of time, and to such an extent, that they are unable to carry on a normal and productive life. In the Diagnostic and statistical manual of mental disorders (4th Edition), the American Psychiatric Association has defined a major depression episode in an adult as having four or more of the following criteria for most of every day for at least two weeks, in addition to having a depressed mood or loss of pleasure or loss of interest: 1) a change in weight or appetite, 2) a change in sleep, 3) psychomotor agitation, 4) decreased energy, 5) feelings of worthlessness or excessive guilt, 6) difficulty thinking or concentrating or making decisions, and 7) recurrent thoughts of death, suicidal ideation, or attempted suicide.[1]

Major depression is often associated with an environmental stressor, such as a death of a loved one, loss of employment, etc. Endogenous stressors and several diseases with endocrine malfunctions or changes are often associated with depression. These include diabetes mellitus, Cushing's disease, excessive glucocorticoid therapy, hypothyroidism, post-partum depression, menopause, pre-menstrual syndrome, and oral and subcutaneous contraceptive therapy,

decrease in exposure to sunlight (seasonal affective disorder) .[1 2 3 4 5 6 7 8 9 10]

Depression demonstrates an age and gender-related association. To examine whether gender differences or any particular age group in the population suffers a greater incidence of depression, or more precisely, has a greater incidence of prescribed anti-depressant therapy, a survey of population eligible for Montana MedicAid in 1995 was performed. The MedicAid data base allowed for gender and age demographics of the recipients of antidepressants.

Many neurochemical theories for the etiological basis for depression have been proposed. However most of these have serious problems in time from when therapy was first applied, time when neurochemical changes occur, and time when patient notices a therapeutic benefit. Medical research of the pharmacology of antidepressant drugs, physiological parameters of patients with depression, physiological and behavioral parameters in animal models of depression and the shared characteristics of endocrine stressors associated with depression has provided evidence that a chronic decrease in available brain glucocorticoid type I (MR) or type II receptors (GR), or both, could be involved in the etiology of depression.

In 1967, Seligman et. al., suggested an animal model for modeling human depression known as the learned helplessness model. [11] The methodology of this model is to chronically expose an animal to a stressor which this animal cannot escape. Eventually the animal quits attempting to escape the stressor, even when this animal is provided with a method of escape from the stressor,

the animal is incapable of attempting such an escape, while an untreated animal readily performs the escape.[12] Seligman and others have adapted these results to clinical psychological therapy, such as learned optimism, providing environments and methods for people to have self-control of stressors and reinterpretation of uncontrollable stressors.[13]

Learned helplessness animals, as well as depressed humans, have been shown to exhibit abnormal function of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is the primary pathway by which the brain regulates the secretion of the adrenal glucocorticoids, corticosterone or cortisol (CORT). The paraventricular nucleus of the hypothalamus (PVN) synthesizes many neuropeptides, including corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). These peptides are transported down neuronal axons and secreted from terminals in the median eminence into the blood of the hypophyseal portal blood vessels. These vessels carry blood directly from the median eminence to the anterior pituitary, where the CRH and AVP bind to their respective receptors on corticotroph cells of the anterior pituitary. The binding of CRH and AVP to their membrane G-protein receptors induces the corticotrophs to secrete the hormonal peptide, adrenocorticotropin (ACTH), into the blood. ACTH travels in the blood from the anterior pituitary to the adrenal gland located at the top of the kidney, where it binds to ACTH receptors found in the adrenal cortex and medulla. Here, it stimulates the adrenal gland cells to synthesize and

secrete steroid hormones including CORT from the cortex and stimulates the synthesis of catecholamines within the adrenal medulla.[14]

CORT (cortisol in humans and corticosterone in rats) is relatively insoluble in the blood, and so, travels through the blood primarily bound to a carrier protein, cortisol binding globulin. However, CORT is soluble in the cellular non-polar lipid bilayer of the plasma membrane, and thus readily enters into the cytosol of the cells. Certain cells express one or two of the known types of receptors for CORT, MR and GR. Both GR and MR share the protein motif of the steroid receptor superfamily, which includes estrogen, retinoic acid, etc.[15] The receptors in this family bind steroids in the cytosol prompting the steroid-receptor complexes to transfer into the cell nucleus where they bind directly to specific sequences of DNA. The steroid-receptor complex binding onto these DNA response elements can directly affect mRNA transcription, and thereby affecting protein synthesis.[16 17]

GR are found thoughout the body and thought to be primarily responsible for the plethora of actions of CORT in the mammalian body including anti-inflammatory, immunosuppressive, metabolic and tubular functions, as well as effects on glomerular filtration, muscle catabolism, bone structure, and actions on many CNS nuclei. In the brain, GR have been found with highest densities in cortex layers (II, III & VI), amygdala (central & posterolateral), hippocampus (CA1, CA2, stratum pyramidalis, dentate gyrus & subfonical organ), bed nucleus of the stria terminalis (medial, lateral & septohypothalamic), hypothalamus

(parvocelluar cells of the paraventricular, medial preoptic, periventricular, dorsomedial and arcuate nuclei; as well as the tuber cinereum), thalamus (laterodorsal), lateral geniculate, Edinger-Westphal nucleus, dorsal raphe nucleus and locus coeruleus.[18]

Brain MR have been found to bind CORT with a much higher affinity than peripheral MR. In the brain, the MR are found predominantly in the hippocampal formation with much lesser concentrations in lateral septum, amygdaloid bodies, olfactory nucleus, cortex layer II, cerebellum and brain stem.[19 20] The rest of the brain appears to be relatively devoid of MR. Rat brain MR receptor mRNA are thought to be identical to the MR mRNA found in the kidney, where aldosterone and other mineralocorticoids act to increase kidney sodium reabsorption.[21] Kidney MR bind mineralocorticoids, such as aldosterone and deoxy-corticosterone, but not CORT, because the MR have a different ligand binding-site than GR and are co-localized with the enzyme 11-beta hydroxysteroid dehydrogenase II (HSD-II).[22] HSD-II oxidizes the 11-carbon hydroxyl of CORT. In physiological conditions, aldosterone forms a hemi-acetyl between the 18-aldehyde and the 11-hydroxyl, which protects the 11-hydroxyl substituent. Deoxycorticosterone does not have an 11-hydroxyl. Therefore, these molecules maintain binding ability.[23]

There has been no evidence of HSD-II in the brain. Therefore brain MR do bind CORT.[24] In fact, these brain MR have a 10-fold higher affinity for CORT than do the GR of the brain.[25] The identical DNA of the kidney and hippocampal

MR implies the proteins are identical. However, the large difference in CORT binding in these two tissues (no binding in the kidney and high affinity binding in the hippocampus) suggests that the proteins comprising the ligand-binding site are different. [159] Splice variants of MR have been identified in the hippocampus and post-translation modifications are possible. [155] [158] To test whether the binding site of both tissue MR are identical a monoclonal antibody specific to the rat kidney ligand binding site was tested in the kidney and hippocampus by immunohistochemistry and Western blot.

CORT is secreted in a circadian manner, with a peak in humans in early morning and a nadir late at night. It is also secreted in response to stressful stimuli and 24-hour CORT levels rise in response to chronic immunological or environmental stress. Brain MR are thought to be always at least 80% bound by CORT. However, the amount of CORT bound to GR is thought to fluctuate between 5% to 90% bound during a 24 hour day, depending on the CORT available. [25]

In order to regulate CORT secretion, CORT receptors directly or indirectly decrease CORT synthesis, as well as the synthesis and secretion of the hormonal peptides of the HPA axis. GR are thought to be primarily involved in mediating the feedback inhibition on the HPA axis peptides (ACTH and CRH).[26] [27] [28] [29] GR activation inhibits the expression of CRH mRNA in the PVN and in the pituitary pro-opiomelanocortin (POMC) peptide mRNA (the pro-peptide of ACTH).[14] MR in the hippocampus are also thought to be involved with regulating

the amount of CORT secreted and the basal circadian rhythm of CORT secretion, although the neurological or biochemical mechanisms underlying this are not yet understood.[30] Decreases of MR or blocking MR binding in the hippocampus, and decreases of GR or blocking GR binding in the hypothalamus (but not GR in the hippocampus) are positively correlated with an increased activation of the HPA axis, resulting in increased plasma CORT.[31 32 33]

Patients with diseases which are associated with chronic alterations in CORT secretion often exhibit symptoms seen in depression.[1 6 7 8] These diseases include Cushing's disease, Addison's disease, diabetes mellitus, autoimmune disorders, multiple sclerosis, and exogenous therapy with CORT analogs. In animals, many of these diseases are associated with a decrease in GR and MR levels.[34 35 36 37] In animal models with an altered HPA axis activity by disease or pharmaceuticals, there is often a presence of behaviors associated with human depression and animal learned helplessness, such as diminished response to a stressor, feeding, cognitive behavior, adaptive behavior, decreased explorative behavior and decreased 24-hour locomotor activity.[38 39 40]

In many people with depression, and in learned helplessness animals, the peaks and valleys of CORT circadian rhythm are diminished and there is an overall increase in total daily plasma CORT levels.[41] Also, learned helplessness animals and depressed people often have a less of decrease in plasma cortisol or pituitary ACTH in response to dexamethasone (a synthetic glucocorticoid) administration.[1 42 43 44 45 46] This diagnostic test is known as the dexamethasone

suppression test. In one study, non-suppression of plasma cortisol level by dexamethasone was noticed in 72% of patients with major depression; 87% of those with melancholia, 100% of mixed bipolars, but only 33% of those with mania only.[47]

The inability of dexamethasone to decrease plasma CORT and ACTH levels and the abnormal CORT circadian rhythm in people with depression implies the CORT receptors in the brain, pituitary and/or adrenal glands (adrenal CORT synthesis is regulated by GR) are low or less responsive. Both brain GR and MR are reduced with chronic immobilization and similar chronic stress (models of learned helplessness).[48 28 49 50 51 52]

Dexamethasone non-suppression, loss of CORT circadian rhythm with chronic CORT hypersecretion, and depressed mood often normalizes after about ten to fourteen days of antidepressant drug therapy, even though neurochemical changes occur within minutes to hours. Historically, antidepressant drugs were derived from plants, such as *Hypericum perforatum* (St. John's Wort) and *Erthroxylan Coca sp.* (cocaine). In the early 1950's, investigations by Zeller, by Crane and by Kline noticed alleviation of depression with a synthetic drug originally used for treating tuberculosis, isoniazid. In the late 1940's, Haeflinger and Schindler synthesized imipramine, which Kuhn characterized in 1958 as having antidepressant actions. Imipramine led to the synthesis of other similar molecular tricyclic antidepressant drugs (TCAs). In order to remove many of the side effects of TCAs, chemical blockers of the

presynaptic neuronal reuptake transport systems of serotonin (SSRI) have been synthesized and used successfully to treat depression.[53]

Antidepressants are not the only biological manipulators successful in depression treatment. Electroconvulsive shock therapy (ECT) has been used as a treatment for the immediate resolution of depression. In seasonal affective disorder, exposure to intense UV light for about an hour per day is an effective therapy. Lithium has been successfully prescribed for bipolar disorder.

Isoniazid and extracts of St. John's Wort inhibit the enzyme monoamine oxidase (MAO).[54] MAO oxidizes the amine on serotonin and catecholamines, thereby making these molecules inactive at their receptors. Inhibitors of MAO allow serotonin, dopamine and norepinephrine to remain active in the neuronal synaptic cleft for a greater time and concentration. The antidepressant TCAs and cocaine competitively inhibited the neuronal reuptake transport systems for serotonin and/or catecholamines. Competitive inhibition refers to the fact that the drugs directly block the site were catecholamines or serotonin interact. SSRIs are designed to only inhibit the reuptake of serotonin. Neuronal reuptake transporters act physiologically to remove neurotransmitters from the neuronal synaptic cleft by transporting them back into the pre-synaptic neuron. Similar to MAO, reuptake inhibitors increase the time and concentration of catecholamines and/or serotonin in the synaptic cleft. All of these drugs result in an increase of catecholamines and/or serotonin in the neuronal synapse within minutes of intake. However, the maximal therapeutic antidepressant benefits are not

noticed for about 10-14 days, which confounds theories suggesting that only low synaptic serotonin and/or catecholamines are directly responsible for the onset of depression. [55]

Drugs which reduce serotonin secretion or synthesis and/or catacholamine secretion or synthesis, such as reserpine, 3,4-methylenedioxy-methamphetamine, 5,7-dihydroxytryptamine, p-chloroamphetamine, or 6-hydroxy-dopamine all decrease hippocampal GR and MR.[56] [57] [58] [59] [60] [61] [62] Serotonin within the hippocampus acts to increase GR and MR expression. Both hippocampal MR and GR levels increase after chronic treatment with SSRIs, TCAs, MAO inhibitors, lithium, or following ECT in a time course which is similar to the observed effect of these therapies on human mood.[63] [64] [65] [66] [67] [68] [69] [70] These results coupled with HPA axis anomalies of patients with depression directed our attention toward GR and MR in the etiology of depression.

Acute administration of pharmacological GR antagonists have induced depression-like animal behaviors.[71] [72] [73] [74] [75] Chronic low GR have been demonstrated in mice with depression-like behaviors and endocrine parameters. Antidepressants attenuate the HPA axis hypersecretion and depression-like behaviors in a mouse model expressing GR mRNA anti-sense.[76] The characteristics of these mice, including hypersecretion of the HPA axis, dexamethasone non-suppression and learned helplessness-like behaviors, provide evidence that depression may involve a decrease in brain GR levels.[77] [78] [79] [80] [81] [82]

To directly examine the consequences that a chronic decrease in available brain MR would have on the HPA axis and depression-like behaviors, an animal model of chronic low MR has been explored.[83] An 18-base end anti-sense to MR was delivered into the third cerebral ventricle of a rat with an osmotic mini-pump for 7 days. Changes were examined in hippocampal GR, MR, MR mRNA, a stress behavioral test, morning (nadir) plasma ACTH and CORT, and plasma ACTH and CORT after the behavioral test stressor. MR anti-sense treatment did not affect hippocampal GR levels, morning plasma ACTH or CORT concentrations nor anxiety-like behavior from the stress test (social defeat and then an elevated plus-maze). As would be expected, hippocampal MR levels decreased (21%) and MR mRNA increased from the treatment. There was a slight but significant increase of plasma ACTH, but not CORT, in response to the behavioral test in the treated rats.

The MR antisense treatment only produced a 21% decline in hippocampal MR concentrations. In the present experiments, a similar model was examined but using continuous administration of a MR antagonist, which theoretically would provide a greater decrease in the available hippocampal MR. In this model six days of constant administration of the MR antagonist, spironolactone, directly into a cerebral ventricle of a rat by an osmotic mini-pump was used. Chronic administration of a MR antagonist given centrally should increase basal plasma CORT concentration. Plasma CORT, 24-hour locomotor activity (to

monitor circadian rhythm), and open field tests (a behavioral test of anxiety) were all measured in this model.

In another experiment the chronic administration of a MR agonist, aldosterone was utilized, to determine the effects of chronic MR activation on the same parameters. An agonist is a drug which binds to the receptor and elicits the receptor to respond in a manner similar to the endogenous ligand, in this case the agonist-steroid receptor complex binding onto DNA. Unlike CORT, neither aldosterone (nor spironolactone) have a high affinity for GR.[84] Chronic administration of a MR specific agonist given centrally should decrease basal CORT and blunt CORT circadian peak levels. Since MR are always about 80% bound, increasing the amount of available ligand may not produce noticeable behavioral/neurochemical effects.

Changes in weight, dexamethasone non-suppression, loss of CORT circadian rhythm and high 24-hour CORT in people with depression, and the higher incidence of depression in people having diseases or hormonal therapies which are associated with abnormal CORT levels implies that CORT and it's receptors may be involved in the etiology of depression. The loss of normal CORT circadian rhythm, dexamethasone non-suppression, decreased hippocampal MR and GR in animal models of learned helplessness, the anxiety and depression-like behaviors of animals demonstrating decreased brain GR and the increased animal hippocampal MR and GR seen in animals tested with TCAs, SSRIs, lithium or ECT in a time course similar to their therapeutic effect,

provide further evidence that chronic decreases in hippocampal GR is involved in the etiology of depression. The present experiments examined the behavioral and hormonal effects of chronic alterations in central MR binding and MR's possible role in the etiology of depression.

# Chapter 2

# Materials and Methods

## Procedure

Acclimate to cages

-4 Day: 24hr Cage activity

-3 Day: Open field activity

-2 Day: Intra-jugular cannula- leftside

-1 Day: Pre-Treatment Plasma samples at 8:00, 14:00, 18:00

0 Day: Set up osmotic pumps

1 Day: Insert brain cannulae and osmotic pumps

2 Day: Rest from surgery

3 Day: 24hr Cage activity

4 Day: Open field activity/ Intra-jugular cannula- rightside

5 Day: Treatment Plasma samples at 8:00, 14:00, 18:00

20:00 PBS perfusion; remove brains; cannula patency assessed

### *Animals*

Forty 250g-300g adult male Spague-Dawley rats were obtained from Harlan Labs.[85] They were housed singly in the Pharmacy/Psychology Vivarium, an AAALAC accredited facility, with rat chow pellets and water *ad libitum*, in standard environmental conditions (white light on 7:00-19:00 and 23°C +/- 1°C). Rats were housed and maintained according to guidelines published in NIH Principles for Use of Animals, Guide for Care and Use of Laboratory Animals.[86]

***Materials***

Rat chow (Purina), Micro-osmotic pumps and brain infusion kit (Alzet 1007D, Alza, Palo Alto, CA), sodium laurel sulfate, d-aldosterone, spironolactone, glycine, diaminobenzene, triton-X 100, para-formaldehyde, xylene, peroxidase conjugated rabbit anti-mouse and anti-rabbit antibodies (Sigma, St. Louis, MO), Tween-20, TRIS base, propylene glycol, sodium hydroxide pellets (J.T.Baker, Phillipsburg, NJ), #0-80 x 1/8" screws and #56 drill bits (Small Parts, Miami Lakes, FL), dental cement Fastray powder and liquid (Harry J. Bosworth, Inc., Stokie, IL), 2,2,2-tribromoethanol (TBE) (Fluka, Ronkonkoma, NY), silicone plastic (Silastic) tubing and non-reinforced silicone plastic sheeting 0.25 x 3.25 x 0.03 in. (Konigsberg Instruments, Inc., Pasadena, CA), 100% silicone aquarium sealant (Dow Corning Corp., Dayton, OH), cardiovascular 4-0 silk (Ethicon, Inc., Somerville, N. J.), wound clips, 23G1 needles, polyethylene tubing PE50, microscope slides and 5cc, 3cc and 1cc syringes (Becton Dickinson, Inc., Parsippany, NJ), heparin sodium, sodium phosphate monobasic, sodium phosphate dibasic, 30% hydrogen peroxide, microscope cover glass (Fisher, Fair Lawn, NJ), sodium chloride (EM Industries, Inc., Gibbstown, NJ), $I^{125}$corticosterone radioimmunoassay kit (Amersham Life Science Inc., Arlington Heights, IL), Glucocorticoid receptor II and mineralocorticoid receptor mouse monoclonal antibodies (Affinity BioReagents, Golden, CO), biotinylated anti-rabbit antibody, Reagent A and Reagent B (Vector Laboratories, Burlingame, CA), DPX mountant for microscopy (BDH

Laboratory Supplies, Poole, England, UK), 8 x 10 wells WHO trays, 5.25% sodium hypochlorite (Shurfine, Northlake, IL), 10% CAP gel TRIS SDS 8x10cm (ICN Biomedicals, Aurora, OH), 15 gauge trochar (Innovative Research, Rockville, MD), immobilon-P transfer membranes (Millipore, Bedford, MA) were purchased commercially. Reagent A and B (Transduction Laboratories, Lexington, KY) was a gift from Dr. Charles Eyer. Biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA) was a gift from Dr. Diana Lurie. Protein Assay Dye (Bio-Rad, Hercules,CA) was a gift from Dr. Keith Parker. Film developer (GBX) and fixative reagents were gifts from Dr. Michael Minnick.

### *Apparatuses*

Stereotaxic apparatus (Kopf, Tunga, CA), a cryostat (HistoStat, Reichert-Jung, Buffalo, NY), drill (Model 750, Dremel, Racine, WI), submini lever switches (Radio Shack, Fort Worth, TX), 386 computers (Dell), pipetmans (Gilson, France), a gamma counter (ICN Micromedic Systems, Huntsville, AL), a centrifuge (J-68, Beckman, Redmond, WA), a -80°C refrigerator (Environmental Equipment, Cincinnati, OH), a camera (Nikon, Japan), Spectrometer (Bausch & Laumb), a microscope (Nikon, Japan), homogenizer (Tissue Tearor), electrophoresis power supply (ISCO, Lincoln, NE), an electrophoresis transphor tank, and an electrophoresis gel tank (Hoefer, San Francisco, CA) were used.

## *Methods*

*Intracerebroventricular Cannulation and Pump Implantation:*

Surgeries were conducted with the rats under tribromoethanol (TBE) anesthesia (1ml of a 2.5% solution in saline/ kg body weight). The methods were similar to those suggested by Alza.[87] The scalp was shaved and washed with betadine solution. The animal was then placed in a stereotaxic device (SD), a 2.5 cm long incision was made to expose the cranium, a periosteal elevator scraped tissue from the skull. Blunt-nose scissors were used to make a subcutaneous tunnel from the scalp incision to the mid-scapular area. At the end of the tunnel a pocket was made large enough to house the pump and permit some pump movement. Two holes were drilled through the skull (one -4 mm posterior (AP) from Bregma, -2mm lateral from midline and one +2 mm AP, +2 mm lateral). Into these holes small stainless steel screws were placed. The elevator arm of the SD was altered, so that the end not connected to the SD was attached to an alligator clip. The placement tab of the brain cannula was clipped and held in place with the alligator clips. The cannula was connected and glued with epoxy to 7 cm of catheter tubing (0.69 ID) with 28G needle/syringe filled with solution at the other end of tubing. The brain cannula was filled with solution. At -1.3 mm AP, a hole was drilled through the skull and the brain infusion L-shaped cannula was lowered -4.5 mm from dura into the third ventricle.[88] A bolus of 50 μl was injected into the brain to flush any tissue which could form a clog and to insure the cannula was patent. The cannula was affixed

to the skull using dental cement. Once the cement hardened, the tubing on the osmotic pump was removed and the pump was attached to the catheter tubing of the brain cannula. Then the osmotic pump was inserted into the subcutaneous pocket. The placement tab was cut from the top of the cannula and the scalp wound closed with small wound clips.

*Osmotic Pump:*

The Alzet mini-osmotic pumps provided the animals with chronic exposure for seven days to intracerebroventriclular (icv) administration of one of three treatments: MR antagonist (0.6 mg spironolactone/ 1 ml propylene glycol), MR agonist (0.05 mg aldosterone/1 ml propylene glycol) or propylene glycol vehicle. The pumps had a mean pumping rate of 1 μl/hour. Therefore, the drug solution infusion rates were 600 ng spironolactone/hour or 50 ng aldosterone/hour. The solutions were carefully injected through the 25-gauge fill tube and into the osmotic pump. The clear plastic top of the universal flow moderator was removed. The flow monitor was attached to a 5 mm piece of tubing with a 28-gauge needle/syringe filled with solution at the other end of the tubing. Solution was pushed into the flow monitor until it was filled with solution. Then the flow monitor was carefully inserted into the pump. The pumps were incubated overnight in 0.9% saline at room temperature to assure activation of the infusion mechanisms.

*Open Field Activity:*

Before and four days after surgery, in the late morning (10:00 to 12:00), animals were placed in an open field apparatus (an open-top box with 46 cm walls and a 122 x 122 cm floor, with a 7.6 x 7.6 cm grid pattern on the floor). A video camera recorded the activity in the field for 10 minutes. The time to initiate movement, number of total squares entered and the number of inner squares versus peripheral squares (three rows along the walls) entered were assessed. Animals were also monitored for rearing, grooming and defecations. The field was cleaned with dilute ethanol between each session.

*24 Hour Cage Activity:*

The apparatuses for locomotor activity measurement were constructed by the investigator. Each rat was individually housed in 42x23 cm opaque plastic rat cages covered by two 21x 23 cm wire cage tops. Each cage top contained a similar quanity of water and food, with both the bottles in the middle of the cage. Each top, also, held a similar quantity of food. Each cage sat on a fixed round rod in such an orientation that the two ends of the cage were balanced, like a scale or teeter-totter with the rod being the fulcrum. Under one end of the cage was a submini lever switch, so when the rat was on that end of the cage the switch was closed, but when the rat moved to the other end of the cage, the cage teetered to that side and the switch was opened. The changes of a switch from open to closed and from closed to open were quantified for every 15

minutes for 24 hours through a PC computer interface.[89 90]

*Chronic intra-jugular cannula placement:*

Prior to surgery, cannulae were made by making a small hole in a 1x2 cm silastic sheet at 1/3 of the longer length and the middle of the shorter length, as previously described.[91] A piece of 20 cm silastic tubing (0.02 in. ID) was threaded through the hole until 5 cm had been threaded through the hole. Then silicone glue was dabbed to the 1/3 section of the sheet, this glue covered section was folded onto the middle third of the sheet, covering a portion of the tubing held tight by binding clips until the glue set. After the glue dried, the glued portion of the cannula was trimmed close to the tubing, but the third which was not glued remained untrimmed. The portion of the tubing below where the sheet was glued to the tubing was cut to a length of 2.8 cm.

Cannulae were surgically implanted under anesthesia of TBE (1 ml/0.1 kg BW, of a 2.5% solution in saline) as previously described. [91] The surgical site was sterilized with 70% ethanol. An incision was made in the chest to expose the pectoral muscle and the jugular vein. The prepared cannula was flushed by a syringe filled with heparin (10 kU/ml) attached at the open end with the long length of tubing. The other end was attached to an L-shaped 20-gauge/23-gauge specially designed needle. [92] The needle was shaped, so that the attached cannula and needle were inserted into the jugular vein just superior to the pectoral muscle and then exited the jugular vein beneath the pectoral

muscle, consequently exiting through the muscle. The needle was removed from the cannula and the cannula was pulled back under the muscle and carefully guided into the vein. The pressure of the pectoral muscle sealed the exit hole. The cannula was threaded through the vein into the right atrium of the heart. The untrimmed section of the sheet acted like a tab, which allowed the cannula to be anchored to the pectoral muscle by silk thread suture. A 15 gauge trochar was pushed subcutaneously from the middle of the back of the neck to the wound site. The syringe was removed and the exposed end of the silastic cannula was threaded into and through the trochar. The trochar was removed, leaving the cannula, which exited the back of the neck. The open end of the cannula was cut so that a 5 cm portion was exposed and sealed with a knot under pressure with heparinized saline.

*Blood Retrieval:*

The animals were individually housed in opaque cages with stainless steel tops. At least one hour prior to sampling, the knot closing the cannula was cut. A 0.75 m PE 50 tube filled with 100 U Heparin/ 1 ml 0.9% saline was connected to the cannula by a ~5 cm stainless steel tube (28 gauge). The PE 50 tubing connecting tube was strung to the outside of the cage, where it was attached to a another 0.25m piece of PE 50 tubing by ~5 cm stainless steel tube (28 gauge). The open end of the 0.25m PE 50 tubing was connected to a 3 cc syringe containing at least 1 cc of the heparin/ saline. Plasma samples were

retrieved by drawing blood up the 75 cm PE 50 tubing and into about a third of the 0.25 PE 50 tubing by the 3 cc syringe. The 75 cm and 25 cm PE 50 tubing were detached and a 1 cc heparinized syringe with a 28 gauge needle was attached to the open end of 0.75m PE 50 tubing and 0.7 ml of blood was slowly drawn into the heparinized syringe without disturbing the animal. The two pieces of PE 50 tubing were reattached and the blood flushed back into the animal with heparin/ saline (100 U/ml). Blood samples were removed at 9:00, 14:00 and 18:00 hours, before treatment and after 5 days of treatment. Plasma was separated by centrifuge at 15,000 rpm and stored at -80°C for hormone analysis.

*Plasma Corticosterone Radio Immunoassay:*

Plasma corticosterone (B) was measured using the protocol of the $I^{125}$-corticosterone radioimmunoassay kit.[93] Plasma samples were placed in a water bath at 60°C for 30 minutes to displace B from cortisol binding globulin. One hundred microliters of each plasma sample was diluted with 400 µl 0.02M borate buffer pH 7.4 with 0.1% sodium azide in a polystyrene tube.

Standards were prepared by pipetting 500 µl of buffer into 18 polystyrene tubes. Five hundred microliters of a standard of 400 ng B/ml buffer was pipetted into two of the tubes and vortexed. Five hundred microliters of the contents of these two tubes was transferred into two other tubes and this double dilution was repeated successively with the remaining tubes giving rise to 2 sets of tubes with

9 different concentrations of B. Aliquots of 100 µl from each of the serial dilutions were pipetted into 2 sets of 9 tubes with respective dillutions equivalent to 0.78, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, and 20 ng/ml.

In addition to the labeled standard and sample tubes, 3 tubes were labeled TC for total counts, 3 tubes were labeled NSB for non-specific binding, and 5 tubes were labeled Bo for zero binding standard. Two hundred microliters of buffer was pipetted into the NSB tubes and 0.1 ml of buffer was pipetted into the Bo tubes. One hundred microliters of rabbit anti-B (400 ng/ml) was pipetted into all tubes except for NSB and TC tubes. One hundred microliters of [$^{125}$I]B (approximately 11,570 cpm) was pipetted into all of the tubes. Then the TC tubes were removed for counting. All other tubes were vortexed, covered and incubated for two hours at 22°C. After the incubation, 0.4 ml of the Amerlex-M goat anti-rabbit second antibody was pipetted into each tube except TC tubes. All tubes except the TC tubes were then centrifuged for 10-20 minutes at 3000 rpm, 4°C. These tubes' supernate was aspirated off from leaving a precipitate.

A gamma counter was connected to a computer with Isodata software, a protocol was written for a log-logit standard curve: the counts per minute of sample binding minus non-specific binding divided by maximum binding (Bo) standard minus non-specific binding times 100, versus an X-axis: log dose B concentration (ng/ml). The gamma counter read the radioactive counts per minute of the standards, NSB, Bo and TC and provided this information to the computer, which provided a standard curve. (See Figure 7, page 45) By applying

this curve to the unknown samples' counts per minute minus NSB an estimate of B concentration in ng/ml could be estimated for each sample. All plasma B was measured in the same assay, interassay coeffient of varation=0.02, 50% effective dose=2.5 ng B/ml and sensitivity=0.16 ng B/tube

*Brain and Kidney Removal:*

In order to remove blood cells from brain and kidney samples for western blot analysis or immunohistochemical analysis (ICC), the rats were given TBE anesthesia and transaortically perfused with 0.1M phosphate buffer saline (PBS), pH 7.4 for 5 minutes. Chest hair was scalped off exhibiting subcutaneous tissue, an incision was made at the xyphoid process and with a hemostat the xyphoid process was pulled through the incision. This separates the peritoneal muscle wall from the vital organs, allowing an incision to be made all along the diaphragm base of the rib cage. The diaphragm was cut from the ribs, without damaging vital organs. Two cuts were made 2-3 inches to the side of, and parallel to, the sternum. The hemostat clamped to the xyphoid process was pulled to the head of the animal and pericardial tissue was cut from the sternum.

This surgery was performed on a refrigerator rack mounted over a sink. Above the sink sat two 10 gallon barrels containing 5% para-formaldehyde (PFA) or PBS. A 3 foot piece of 1/4 inch x 1/16 inch class VI plastic tubing was attached by an adapter to the spigot of each of the barrels. The tubes were connected together by a Y glass adapter, which had 2 foot tube on the third arm.

Each tube had a clamp near it's end. The last tube had a dull-end 13G needle attached at the end.

Both the spigots were open and the clamps on the PBS tube and the third tube were opened to clear out the tubes with PBS. Then the third tube was clamped closed and the needle was inserted into the left cardiac ventricle and threaded up the aorta. The third tube was unclamped again and PBS flowed into the animal. The right cardiac atrium was cut to allow the PBS/blood to exit. When the blood had been replaced by PBS, the liver and eyes turned white and the exiting fluid became clear. If the tissues were to be analyzed by western blot, the needle was then removed. The kidneys and brain were retrieved. The cerebral cortex was pealed off and cut from the corpus callosum of the freshly retrieved brain. The cerebellum was also removed. The different brain sections were then frozen at -80°C.

If the tissues were to be analyzed by ICC, the PFA tube clamp was opened and the PBS clamp was closed. PFA flowed into the rat for about 5 min. causing the muscles of the rat to constrict and the limbs to curl. The brain and kidneys were retrieved and post-fixed in PFA for 4 hours, followed by cryoprotection in 30% sucrose in PBS until the tissues sunk (about 2 days).

*Immunohistochemistry (ICC) of Brain and Kidney MR and GR:*

The cerebellum was removed with a straight-edge razor blade. The different brain sections were then frozen at -80°C. A razor blade was used to

make coronal cuts just caudal to the mamallary bodies and just rostal to the optic chiasm. The rostal and caudal sections were discarded.

A room temperature cryostatic chuck had tissue freezing medium placed on top of it, so that all the indented grooves, except the outer most groove were coated with medium. The caudal end of the middle section of frozen brain tissue was placed in the freezing medium on top of the chuck. The chuck stem was placed in a pre-bore hole in the dry ice, so that the brain platform sat flat on dry ice. The brain dorsal and ventral sides were maintained perpendicular to the chuck platform with cold forceps. Once the medium solidified, the chuck/brain was placed in the cold (-12°C) cryostat. The brain was trimmed to the optic chiasm (Bregma) using the microtome blade. At about -2.0 mm AP from Bregma, the hippocampal folds began to appear. From this point to the hippocampal fissure, 40 μm coronal sections were obtained.

The 40 μm coronal sections were placed in 0.01 M PBS filled WHO tray wells with a #2 round artificial horsehair paintbrush. After five minutes, sections were moved from one well of PBS to another. Endogenous peroxidase activity was removed by immersion of the sections for 10 minutes in hydrogen peroxide (500 μl 30% $H_2O_2$ in 30 ml PBS) filled wells followed by two 5 minute washes in PBS wells. Sections were blocked with normal goat serum (NGS) in wells filled with 200 μl NGS/ 10 ml Day1B (0.5 g bovine serum albumin/ 50 ml PBS/ 150 μl Triton-X). After one hour, the sections were placed in wells containing rabbit polyclonal antibody to GR (Dilution 5μg/ml Day1B) or mouse monoclonal

antibody to MR in Day1B (Dilution 1:100).[94]

Microscope slides were placed in a slide tray. One gram gelatin and 0.5g chromic potassium sulfate was dissolved in 1000 ml flask containing 500 ml distilled water and gently heated until mostly dissolved. The insoluble gelatin was then filtered out. The slides were immersed in a glass tray containing 0.1% acid 70% alcohol (290 ml distilled water + 1 ml HCl + 700 ml absolute methanol) for two minutes, followed by two minutes in a glass tray with distilled water. The slides were then placed for two minutes in gelatin subbing solution and stored overnight in an oven at 37°C.

The next day, the sections received two 5 minute washings in wells containing Day2B (20 ml Day1B + 40 ml PBS). The sections were then placed in wells of biotin conjugated goat anti-rabbit or horse anti-mouse secondary antibody in Day2B (Dilution 1:1000). One half hour after the application of antibody, a solution to express the biotin made of 50 μl reagent A, 50 μl reagent B and 20 ml Day2B (A+B) was made. One hour after immersion in secondary antibody, the sections received two 5 minute washings in wells containing Day2B, followed by immersion in wells of A+B. One hour after the application of A+B, the sections received two 5 minute washings in PBS.

Sections were developed using the chromagen, diamino benzene diluted 20 mg in 15 ml PBS (DAB+B). The sections were immersed in 1 ml DAB+B + 19 ml PBS + 6.6 μl 30% $H_2O_2$ until sections turn a suntan brown. DAB contaminated material other than the sections and the DAB solution were placed

in 5.25% sodium hypochlorite to destroy the carcinogenic DAB.

The developed sections were placed in a petri dish filled 3/4 full with PBS and mounted onto gelatin coated microscope slides with the paintbrush. The slides were air dried, then dehydrated for coverslipping using a series of immersions in increasing concentrations of ethanol. First, slides were immersed for 5 minutes in 70% ethanol, followed by 5 minutes in 95% ethanol, followed by two 5 minute immersions in 100% ethanol and finally two 5 minute immersions in xylene. The slides then had DPX mountant applied and a coverslip was secured by the DPX. The coverslipped slides dried for 12 hours.

The slides were examined with a Nikon microscope at 10x, 40x and 50x powers and photos taken with an attached camera.

*SDS Gel Electrophoretic Western ImmunoBlot of Brain and Kidney MR and GR:*

The frozen sections were removed from the refrigerator. A razor blade was used to make coronal cuts just caudal to the mamallary bodies and just rostal to the optic chiasm. A room temperature cryostat chuck had tissue freezing medium placed on top of it, so that all the indented grooves, except the outer most groove were coated with medium. The caudal end of the middle section of brain tissue was placed in the freezing medium atop the chuck. The chuck stem was placed in a pre-bore hole into dry ice, so that the brain platform sat flat on dry ice. Once the medium solidified, the chuck/brain was placed in the cryostat. The brain was trimmed to the optic chiasm (Bregma). At about -2.0

mm AP from Bregma, the hippocampal folds began to appear. A cut was made with a razor blade separating the hippocampus and the hypothalamus from this point to the end of the section. The hippocampus was stored at -80°C in pre-weighed vials on dry ice for Western blot analysis.

The hippocampal and kidney tissues were weighed and 5 ml TRIS buffer/ gram tissue sample (200g/l) was added into the vials. The tissues were homogenized until completely homogenous to the eye. A Bradford protein analysis was conducted on each tissue sample. Samples of 10-300 μg/ well were used for Western blot analysis. This amount of protein was pipetted from the homogenized sample into an eppendorf tube and q.s. to 40 μl with (6x buffer). A pin hole was placed into the top of each tube.

A CAPS pre-poured gel (Amersham) was placed onto the gel electrophoretic unit (GEU) with the shorter gel glass plate facing towards the electrodes and forming a seal with rubber gasket.[95] The gel plates were clipped onto the GEU. A 1 ml glass pipette washed the gel by pouring tank buffer (9.075g TRIS, 43.2 g glycine, 3g sodium laurel sulfate in 3L de-ionized water) on top of wells. The inner holding area (behind the gel) was loaded with tank buffer until level with the top of the shorter glass plates. The seal between the glass plate and the gasket was examined for any leaks. If no leaks were observed then the lower holding area was filled with tank buffer.

The tissue sample/ 6x buffer tubes were put into a styrofoam float and placed in boiling water for 150 seconds. The bottom half of the tubes were

exposed in the water. Into the first gel well 30 μl stained protein standard was pipetted with a capillary pipette tip. The rest of the wells had the samples/6x pipetted into them.

The electrophoretic power supply had it's parameter's set; meter scale: volts, current: 100 mA on 600 V, voltage limit: max., wattage limit: max., current limit: 20. The EGU top was connected with the EGU and the power supply. The EGU water cooling system and the power supply were turned on.

When the samples' dye had migrated to bottom of the gel, a membrane was placed in 100% methanol and the transphor electrophoretic unit (TEU) was filled with transphor buffer (20% methanol in tank buffer) to the start fill level, the power supply was turned off and the gel was removed. The gel was removed from the glass plates and gently placed in transphor buffer. A gel sandwich was made in transphor buffer; black side down, sponge, 2 pieces of paper, gel, membrane, 2 pieces of paper, sponge, gray side. The sandwich was transferred into the TEU with black side facing black electrode end and the TEU was filled to minimum buffer level with transphor buffer. The TEU and power supply were moved to a cold room and the power supply had it's parameters set; wattage limit: max., current limit: max., voltage limit: 20. The TEU was placed on a stir plate and a stir bar was spun in the TEU. The power supply was turned on and the proteins in the gel transferred into the membrane overnight.[96]

The next day, the membrane was removed and air dried. If the stained protein standard was noticed on the membrane, the transphor was considered

successful. The dry membrane was soaked in a blocking solution of (TTBS) with 5% non-fat milk for an hour. This was followed by placing the membrane in fresh TTBS + 5% non-fat milk and a mouse monoclonal MR antibody (Dilution 1:250) or a rabbit polyclonal GR antibody (Dilution 1μg/1ml) overnight at 4°C. The next day the membrane was washed by soaking it in fresh TTBS every 5 minutes for 30 minutes, then in TTBS + 5% non-fat milk and horseradish peroxidase conjugated secondary anti-body, anti-mouse (1:4000) or anti-rabbit (1:8000) for an hour. The membrane was then washed as previously described.

In order to detect and quantify the target protein on a permanent media, the membrane was soaked in luminol based chemiluminescent reagents for 2-10 minutes. Under a red light, the membrane was sandwiched between plastic wrap and had electro-chemiluminescent film placed on top of it for 10 seconds to 5 minutes. The film was developed in GBX developer for 5 minutes, washed for a minute in running tap water and fixed for 5 minutes. If the target protein, MR or GR, was present, the film had a black band from the luminescence of the peroxidase on the secondary antibody.

*Bradford Protein Analysis*

A standard curve of pure protein concentration was created with bovine serum albumin (BSA). First, 25 mg BSA were mixed in 10 ml of TRIS buffer, which is a concentration of 2.5 mg/ml. 0.1 ml of the standard of 0.25 mg/ml was pipetted into two of the tubes containing 0.9 ml buffer and vortexed. 0.5 ml of

the contents of these two tubes was transferred into the two tubes with 0.5 ml buffer and this dilution was repeated successively with the remaining tubes giving rise to 2 sets of tubes with 6 different concentrations of BSA. Aliquots of 0.1 ml from each of the serial dilutions were pipetted into 2 sets of 6 tubes. Next, 0.7 ml buffer were pipetted in each of the appropriately labeled polystyrene tubes, followed by 0.2 ml of Bradford Reagent and vortexing each of the respective dilutions of 0.78, 1.56, 3.12, 6.25, 12.5, and 25 μg/ml.[97] Three blank tubes were made with 0.8 ml buffer + 0.2 ml Reagent.

A spectrometer was set at wavelength of 595 nm and zeroed with distilled water. The absorbance of the blank tubes was read versus the distilled water and then the fluid of the blanks was combined together. The combined blank fluid was placed in the reference beam cuvette and the absorbance of each of the samples containing tissue was found. The absorbance was compared to previous experiments using BSA.

A standard curve was then made for the tissue sample to find how the slope of absorbance of a tissue sample versus the of concentration of the tissue compared to the BSA protein analysis slope. This comparison provided an estimation of protein concentration in the sample. Twelve labeled tubes were used, the first three contained 0.8 ml TRIS buffer, the next three tubes contained 800 μl buffer + 1μl homogenized tissue, the next three tubes, 800 μl buffer + 2 μl tissue and the final tubes, 800 ml buffer + 4μl tissue. The rest of the procedure was identical to that described above. (See Figure 11, page 49)

*Statistical Analysis:*

Between-within split plot analysis of variance and multiple comparison procedure in a Bonfronni recognition procedure were computed with SPSS software available available through the University of Montana mainframe computer.[98] Dunnett's test for comparison to a control baseline were computed with Minitab software also available through the mainframe.[99] [100] The $p<0.05$ was used as a minimum criterion of significance in all experiments.

*Montana MedicAid Population Survey:*

Montana MedicAid is a state program to help low income Montanas pay for medical bills. The supervising officers, Mr. Jeff Ireland and Ms. Dorothy Paulson, provided the 1995 anonymous data with a software package.

# Chapter 3

## Results

*24-Hour Activity:*

On each test day, at least one rat from each treatment group was represented. Prior to treatment and after three days of intracerebral Alzet mini-pumps, the total 24-hour activity (indicated by total cage switch closures) of each animal was recorded. To remove the error associated with cage differences, each treatment activity was subtracted from baseline cage activity (average of all pre-treatment recordings of the cage apparatus that measured the treatment rat's activity). The rats receiving spironolactone displayed a significant decrease in total 24-hour activity. Rats receiving aldosterone or vehicle did not show any significant difference in total 24-hour activity from baseline cage activity, and were not significantly different from each other. Rats are nocturnal animals, and the difference in cage activity was noticed primarily during dark (active) hours (See Figure 1 and Table 1). There was very little cage activity by the rats in any treatment group during the "lights on" period (See Figure 2 & Table 2). The ability of spironolactone to decrease activity supports the hypothesis that brain MR are involved, at least in part, for behaviors indicative of depression. That continuous agonist treatment did not alter activity may not be surprising as more than 80% of brain MR are thought to be bound even in the absence of the exogenous agonist treatment.

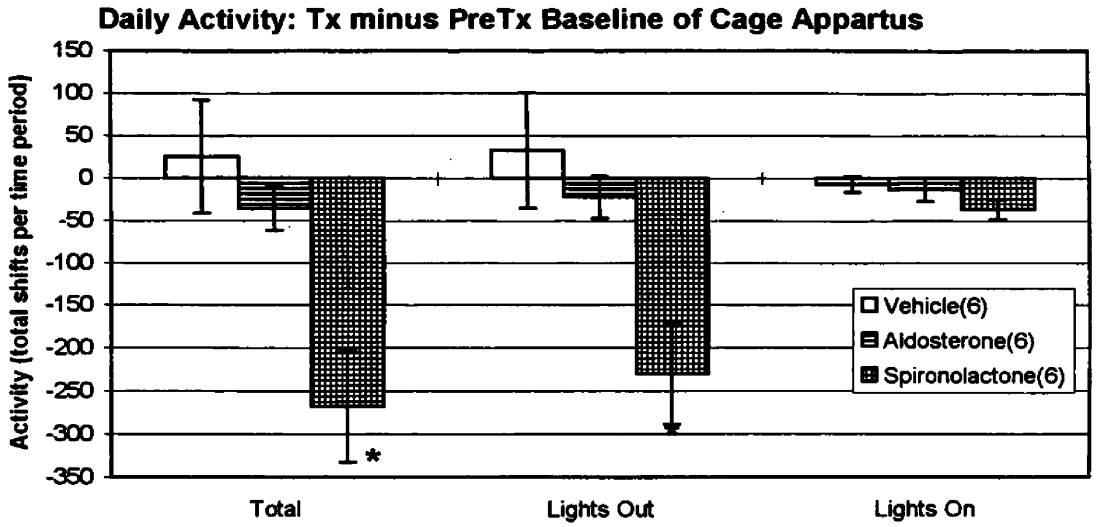



**Figure 1: Total 24-hour activity: Treatment minus Baseline of Cage Apparatus.** Height of each bar represents the mean total activity per time period for all rats within each treatment group with baseline cage activity subtracted, and the vertical lines represent the SEM obtained from n=6 animals per group. *, significantly different $p<0.05$.

**Table 1: Analysis of Variance 24-hour activity: Treatment minus Baseline of Cage Apparatus.** Split plot analysis of variance examined difference in activity over time, activity of treatments for the sum of all the times, as well as an interaction of activity of treatments and time. There is a significant difference in activity depending on the time of day and total activity for 24-hours between the treatment groups.

| Source | DF | SS | MS | F | p |
|---|---|---|---|---|---|
| Treatment Groups | 2 | 11901 | 5950 | 8.94 | 0.003 |
| Rats Nested Within Groups | 15 | 9985 | 666 | | |
| Hours | 23 | 15793 | 687 | 1.99 | 0.005 |
| Treatment Groups X Hrs | 46 | 27427 | 596 | 1.72 | 0.004 |
| Rats Nested Within Groups X Hrs | 345 | 119308 | 346 | | |
| Total | 431 | 1184415 | | | |

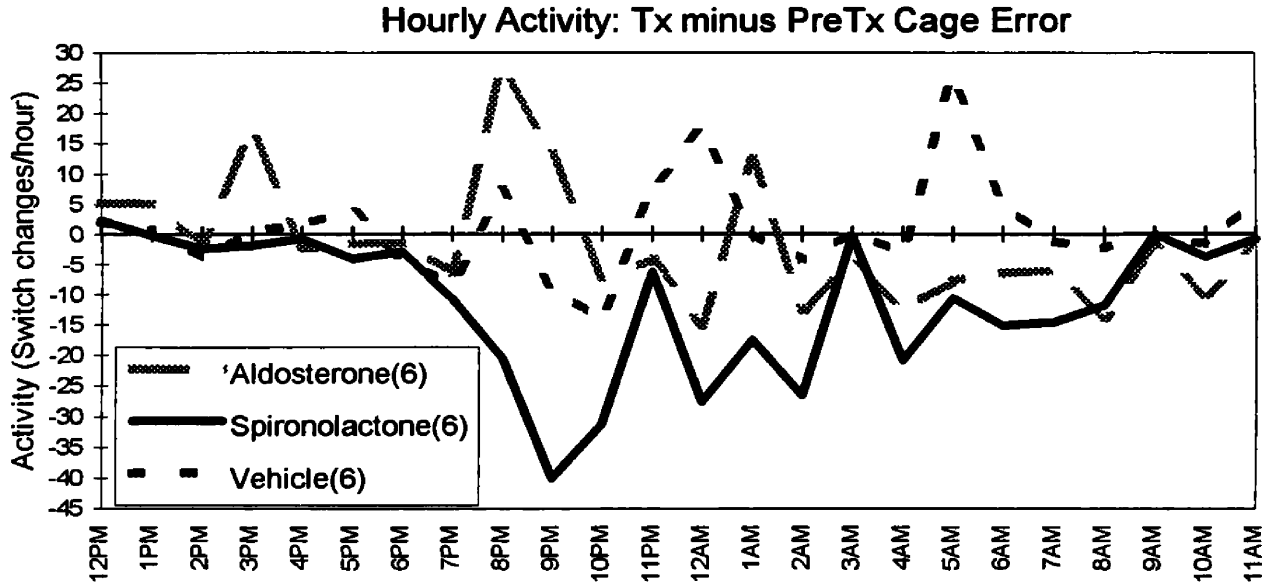



**Figure 2: Hourly Activity:** Net change in activity measured as the difference between pre-treatment and treatment cage movements at hourly intervals over a 24-hour day in n=6 animals per group. Lights are on from 7:00 to 19:00 hour.

**Table 2: Analysis of Variance Hourly Activity: Treatment minus Baseline of Cage Apparatus.** Multiple comparison procedure examined differences between treatment groups at each time in a Bonfonni correction procedure nested on a pooled error term which is rats within treatment groups. #, significance $p<0.05$.

| **DARK Hrs** | **8PM** | **9** | **10** | **11** | **12AM** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spir X Aldo | # | # | # | - | - | # | - | - | - | - | - | - |
| Spir X Veh | # | # | - | - | # | - | # | - | - | # | - | - |
| Aldo X Veh | - | - | - | - | # | - | - | - | - | # | - | - |
| **LIGHT Hrs** | **8AM** | **9** | **10** | **11** | **12PM** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| Spir X Aldo | - | - | - | - | - | - | - | - | - | - | - | - |
| Spir X Veh | - | - | - | - | - | - | - | - | - | - | - | - |
| Aldo X Veh | - | - | - | - | - | - | - | - | - | - | - | - |

*Open Field Test:*

Open field behavior was measured by counting squares entered (inner, outer and total) by each animal in a 10 minute period. Time of initiation of movement out of primary squares was also observed. All of the treatment groups demonstrated a decrease in the total, inner and outer squares entered compared to pre-treatment values, implying icv surgery and recovery caused anxiety in animals. The treatment groups did not significantly differ from each other in the total, inner or outer squares entered.(See Figure 3, 4 and 5, and Table 2) These open field tests were conducted in the morning when no difference in 24-hour activity levels were observed in the cage activity between the treatment groups. It is not known whether analysis of open field behavior in dark (active) period would have provided different results.

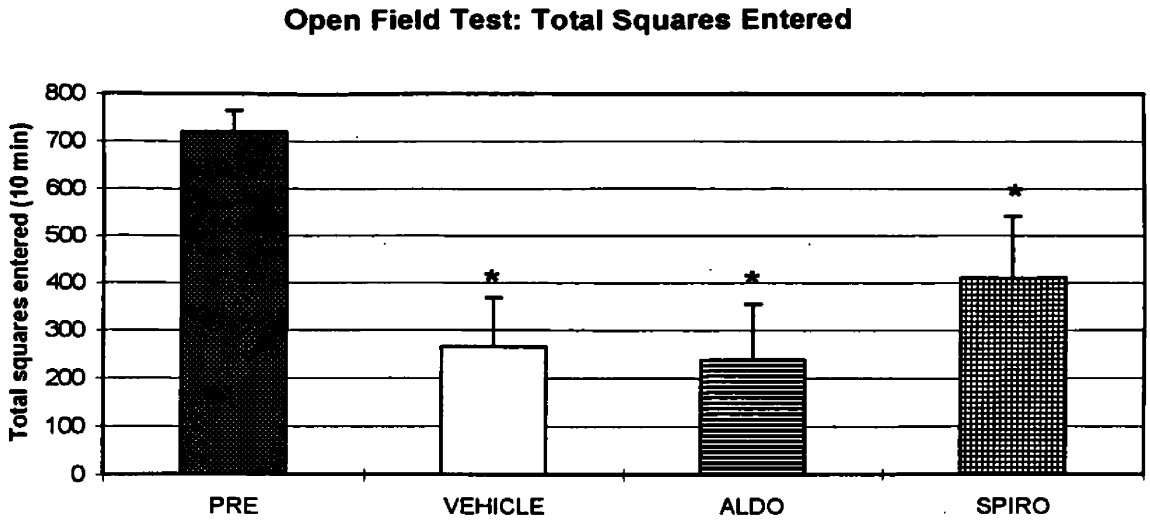



**Figure 3: Open Field Test Total Squares Entered.** Height of each bar represents the mean total squares entered for all rats within each treatment group (ALDO=aldosterone, SPIRO=spironolactone, SHAM=vehicle) and prior to treatment (PRE). The vertical lines represent the SEM. N=6 animals per treatment group, n=18 PRE animals. *, significantly different $p<0.05$ from PRE.

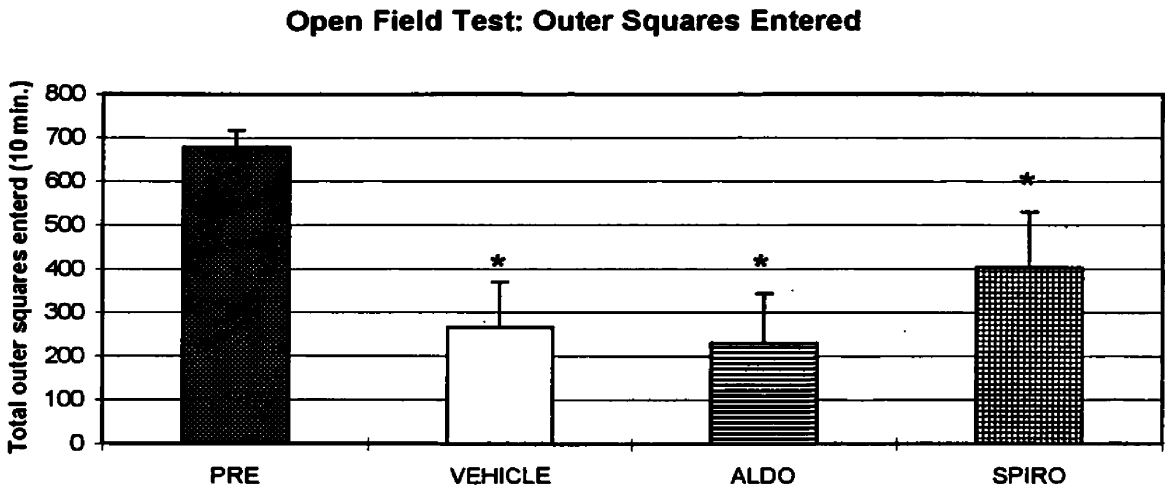



**Figure 4: Open Field Test Outer Squares Entered.** Height of each bar represents the mean outer squares entered for all rats within each treatment group (ALDO=aldosterone, SPIRO=spironolactone, SHAM=vehicle) and prior to treatment (PRE). The vertical lines represent the SEM. n=6 animals per treatment group, n=18 PRE animals, *, $p<0.05$ , significantly different from PRE.

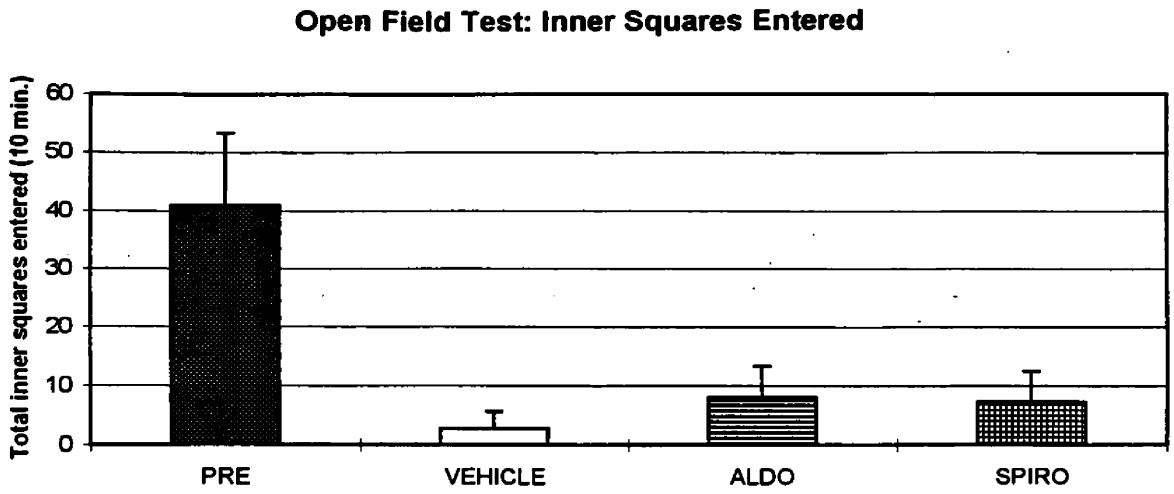



**Figure 5: Open Field Test Inner Squares Entered.** Height of each bar represents the mean inner squares entered for all rats within each treatment group (ALDO=aldosterone, SPIRO=spironolactone) and prior to treatment (PRE).The vertical lines represent the SEM, n=6 animals per treatment group, n=18 PRE animals.

**Table 3: Open Field Test (a) Inner Squares (b) Open Squares and (c) Total Squares.** Dunnett's test for comparison to a control baseline for an approximate analysis between baseline (PRE) and treatment rats was examined. It is approximate because PRE rats are distributed 6 at a time into treatment groups (but a conservative approximation because error was larger than it would be in the fully appropriate analysis). Total and outer squares entered by PRE were significantly different from treatment.

| **(a)Inner Squares Source** | **DF** | **SS** | **MS** | **F** | **p** |
|---|---|---|---|---|---|
| Treatment Groups | 3 | 9877 | 3292 | 2.38 | 0.088 |
| Animals Nested Within Groups | 32 | 44278 | 1384 | | |
| Total | 35 | 54155 | | | |
| **(b)Total Squares Source** | **DF** | **SS** | **MS** | **F** | **p** |
| Treatment Groups | 3 | 1619684 | 539895 | 11.38 | 0.000 |
| Animals Nested Within Groups | 32 | 1518591 | 47456 | | |
| Hours | 35 | 3138274 | | | |

*Plasma Corticosterone Levels:*

Plasma CORT levels demonstrated no differences between treatment groups at any time point examined. The mean of the first bleed (9AM, PRE) of plasma CORT was significantly higher than of the other plasma samples. (Table 5) This may have indicated that basal levels of CORT had not been reached by the time of the first sampling from the change in environment. However, the levels fluctuated greatly between bleed times for an individual rat and between rats bled at the same time within the same group. The mean of minimal CORT value was in the range of 50-100 pg/ml, which also indicates a stress effect. The

level of CORT was elevated in rats even at times expected to show a nadir in CORT secretion (Figure 6 and Table 4 & 5). These results imply that plasma CORT levels were somewhat elevated at all times examined and in all treatment groups. This effect was seen even considering that handling was minimized during the experiment. The fact animals had blood drawn in a room other than their usual housing and animals had less than one day to recover from the intra-jugular venous cannulation surgery may have contributed to elevated CORT levels observed, although previous experiments using this technique have demonstrated a minimal effect of this minor surgery the previous day.[101 102 103 104 105] The lack of any difference of plasma CORT between any of the treatment groups was unexpected as acute studies have demonstrated changes in plasma CORT following acute administration of an icv MR agonist or MR antagonist. However, another chronic study manipulating brain MR with continuous infusion of MR antisense demonstrated no effect on CORT.

It was very difficult to obtain a plasma sample during the nocturnal hours from rats under red light because of the high activity of the rats at this time and the difficulty in seeing the blood. When obtained, often some manipulation of the animals had to be performed introducing a potential for stress. The ability to remove stress free dark hours blood samples would have been beneficial, since CORT plasma levels fluctuate in a circadian rhythm with higher levels occurring during dark hours, and the theory being tested in this research was that MR are involved in the regulation of this circadian rhythm of CORT.

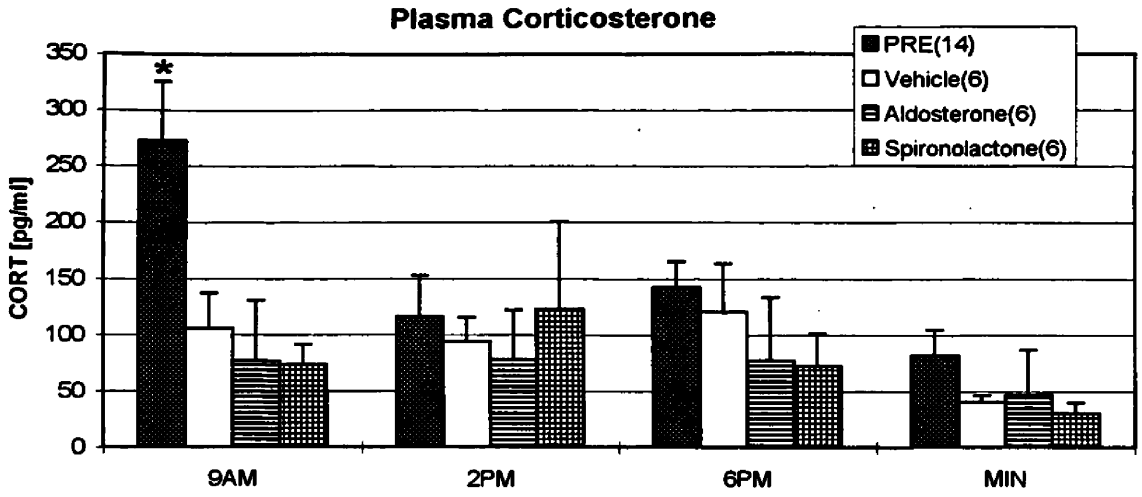



**Figure 6: Plasma Corticosterone Levels at 9:00 (9AM), 14:00 (2PM),18:00 (6PM), and the lowest sample (MIN).** Height of each bar represents the mean plasma CORT [pg/ml] for all rats within each treatment group and prior to treatment (PRE) at 9:00, 14:00 and 18:00 hours; and the lowest value obtained/animal (MIN). The vertical lines represent the SEM. N=6 animals per treatment group, n=12 PRE animals. *, significantly different $p<0.05$.

**Table 4: Analysis of Variance Corticosterone Levels of Treatment and Pre-Treatment Animals.** A Dunnett's test for comparison to a control baseline (pre-treatment) was calculated for an approximate analysis between baseline and treatment animals for each time point and minimum corticosterone sample. It is approximate because the rats in baseline are distributed 6 at a time into the treatment groups and the not all the treatment animals matched up with a pre-treatment measure (n=12) (but it is a conservative approximation because error was larger than it would be in the fully appropriate analysis). There was significantly higher CORT at 9AM of PRE versus treatment groups at 9AM.

| **MIN Plasma CORT, PreTx & Tx** | **DF** | **SS** | **MS** | **F** | **p** |
|---|---|---|---|---|---|
| Treatment and Pre Tx Groups | 3 | 29074 | 9691 | 2.20 | 0.115 |
| Animals Nested Within Groups | 23 | 101212 | 4401 | | |
| Hours | 26 | 130286 | | | |
| **9AM CORT, PreTx & Tx** | **DF** | **SS** | **MS** | **F** | **p** |
| Treatment and Pre Tx Groups | 3 | 89895 | 29965 | 3.39 | 0.035 |
| Animals Nested Within Groups | 23 | 203122 | 8831 | | |
| Hours | 26 | 293016 | | | |
| **2PM Plasma CORT, PreTx & Tx** | **DF** | **SS** | **MS** | **F** | **p** |
| Treatment and Pre Tx Groups | 3 | 20382 | 6794 | 0.31 | 0.818 |
| Animals Nested Within Groups | 23 | 505037 | 21958 | | |
| Hours | 26 | 525420 | | | |
| **6PM Plasma CORT, PreTx & Tx** | **DF** | **SS** | **MS** | **F** | **p** |
| Treatment and Pre Tx Groups | 3 | 52791 | 17597 | 2.14 | 0.123 |
| Animals Nested Within Groups | 23 | 189068 | 8220 | | |
| Hours | 26 | 241859 | | | |

**Table 5: Analysis of Variance of Corticosterone Levels of Treatment Animals.** Between-within split plot analysis of variance examined difference of plasma CORT between treatment groups over time of day, the main effects of

treatment and time of day, as well as an interaction of treatment and time of day. There was no significant difference in plasma CORT depending on the time of day or plasma CORT between treatment groups by time of day or between total CORT of the treatment groups.

| Plasma CORT Source | DF | SS | MS | F | p |
|---|---|---|---|---|---|
| Treatment Groups | 2 | 8568 | 4284 | 0.38 | 0.691 |
| Rats Nested Within Groups | 15 | 169425 | 11295 | | |
| Time of Day | 2 | 1658 | 829 | 0.09 | 0.912 |
| Treatment Groups X Time of Day | 4 | 9918 | 2480 | 0.28 | 0.891 |
| Rats Nested Within Groups X Time | 30 | 269176 | 8973 | | |
| Total | 53 | 458745 | | | |

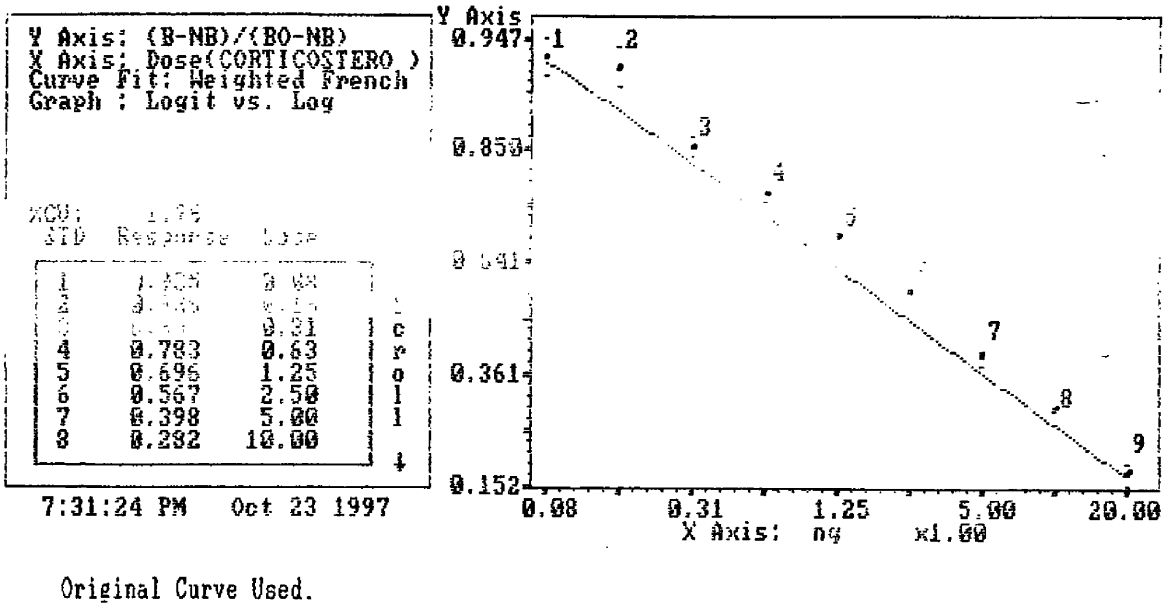



**Figure 7: Standard Curve of Plasma CORT for RIA.**

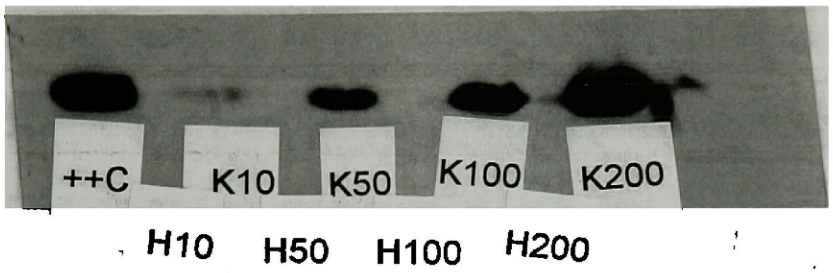
The percentage of gamma counts per minute (cpm) for known quantities of CORT (B) are calculated from the absence of CORT (BO), cpm of non-specific binding of secondary antibody (NB) is subtracted from both B and BO. These are compared in a logit versus log graph against the ng quantity of CORT.

*Cannulae Patency:*

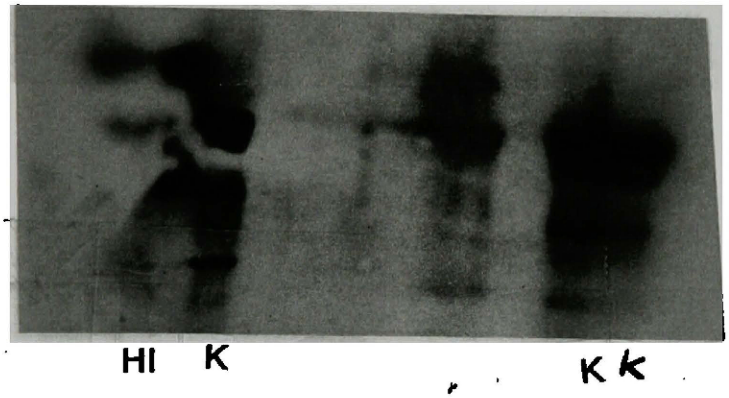
Immediately, after animals were sacrificed, the cannulae were tested for patency and by six days the cannula of several animals were not patent. In studies performed after the experiment it was demonstrated that most cannulae would have remained patent for 3-4 days even with the vehicle and flow parameters used.

*Western Blot of Kidney and Hippocampal MR and GR:*

The positive binding of kidney MR and the lack of antibody binding in the hippocampus could be interpeted that the kidney and hippocampal MR are not identical proteins. The denaturation of Western blots would remove ligands from protecting the MR antibody binding site. Several concentrations and purifications of MR and MR antibody yielded identical results. The positive GR antibody binding in both hippocampal and kidney, demonstrates GR presence in both hippocampal and renal tissues.




a)




b)

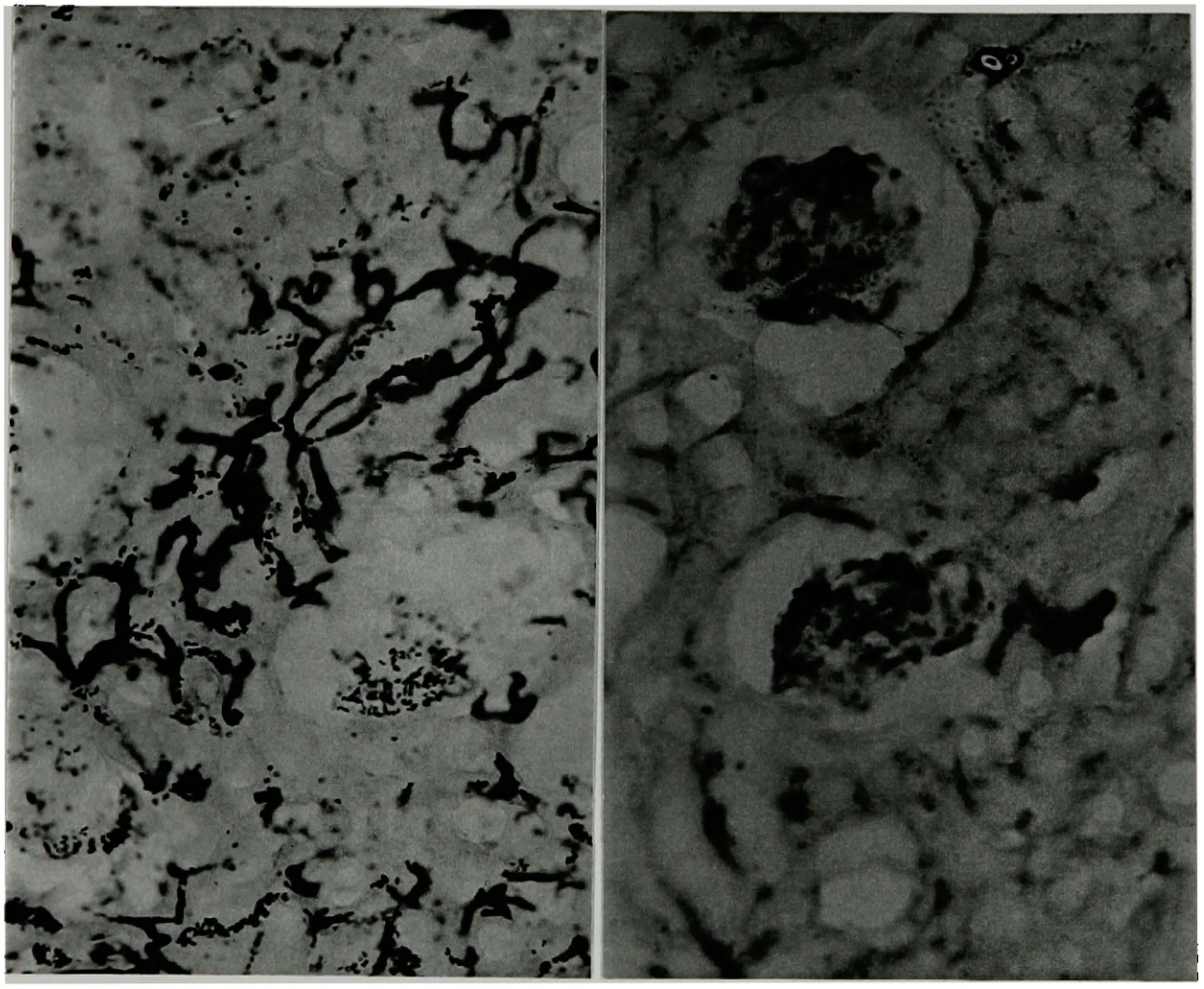
**Figure 8: MR and GR Western Blots of Kidney and Hippocampus.**

a.)The black bands are from the chemoluminescence of MR monoclonal antibody binding of kidney tissues at 200μg (positive control), 10μg, 50μg, 100μg and 200μg total denatured protein/well (+C, K10, K50, K100, K200). To the left of each kidney sample an equal concentration of hippocampal tissue was placed, except for +C (H10, H50, H100, H200).

b.)The black bands are from the chemoluminescence of GR antibody binding of renal (K) and hippocampal (HI) tissues at 200μg total denatured protein/well.

*Immunocytochemistry (ICC) of Kidney and Hippocampal MR and GR:*

The similarity of ICC results with Western blot results further strengthen the observation that the kidney and hippocampal MR appear to be unidentical proteins. However, hippocampal MR are thought to be substantially ligand bound, which could mask the MR antibody binding site.

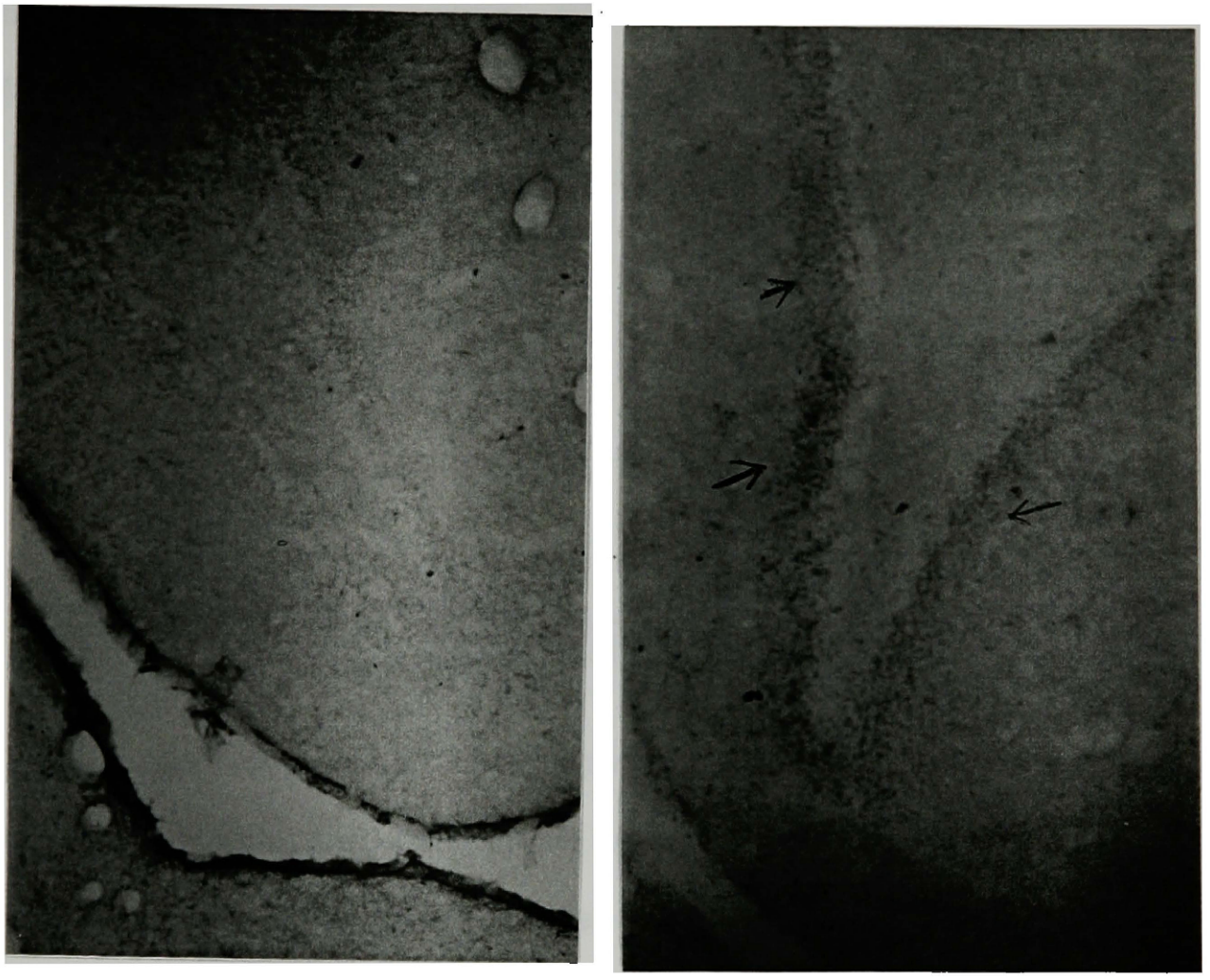


a.)MR in kidney b.)GR in kidney

**Figure 9: MR and GR ICC of Renal Tissue (20x Magnification).**

**a.)** The stained cells are MR monoclonal antibody binding MR in kidney tissue.

**b.)** The stained cells demonstrate GR antibody binding in kidney.



a.)MR in hippocampus b.)GR in hippocampus

**Figure 10: MR and GR ICC of Hippocampal Tissue (20x Magnification).**

**a.)** This figure shows a lack of MR antibody binding in hippocampal dentate gyrus tissue.

**b.)** The arrows in this figure point to stained cells in hippocampal dentate gyrus tissue binding GR.

*1995 Survey of Montana MedicAid Antidepressant Recipients:*

There were three to four times more women recipients of antidepressants than men for any adult age group. Women in their child bearing years were by far the largest number of recipients of antidepressant drugs.

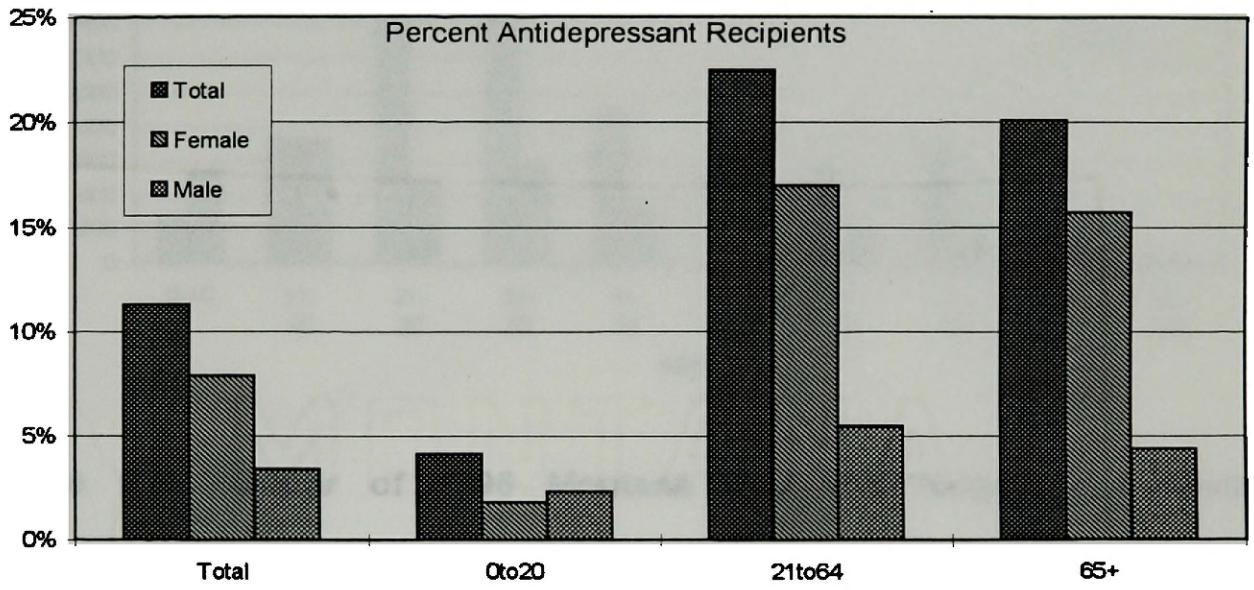



**Figure 11: Percent of 1996 Montana MedicAid recipients receiving antidepressant drugs, categorized by gender and age.** Height of each bar represents the percentage of total, male and female patients receiving antidepressant prescriptions in three age categories from the sample population of people eligible for Montana MedicAid. The data demonstrate expected differences in gender specific antidepressant therapy based on previous literature data demonstrating a higher percentage of women with clinically diagnosed depression than men. However, although literature predicts older patients will be clinically diagnosed with depression at a higher rate than younger individuals, the present results demonstrate that women and men in the

21-64 age group show as high an incidence of antidepressant prescriptions as the ages 65+.

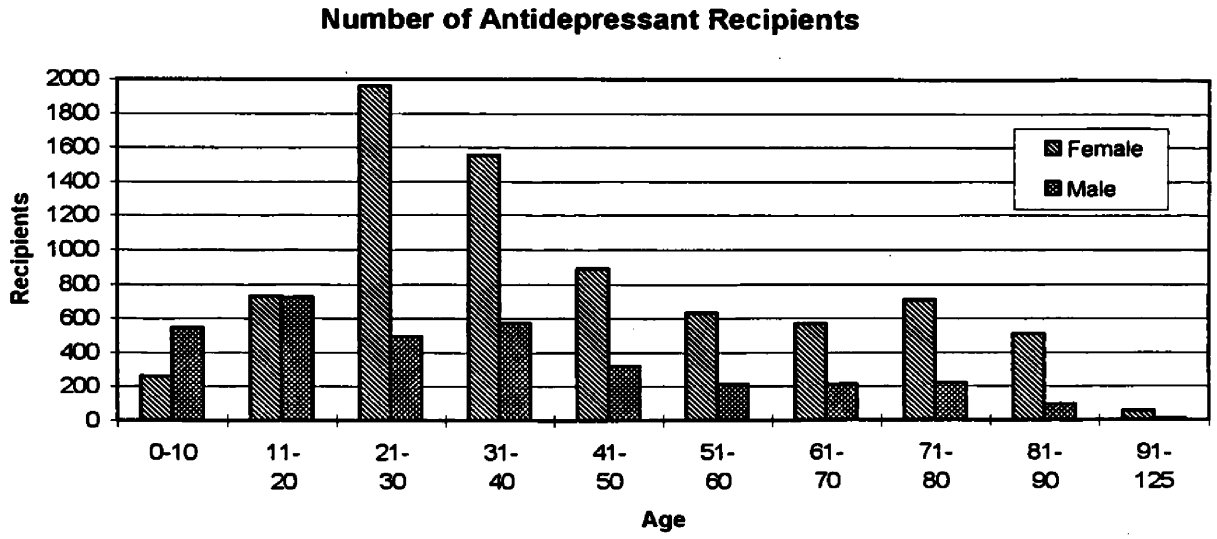



**Figure 12: Number of 1996 Montana MedicAid recipients receiving antidepressant drugs, categorized by gender and age.** Height of each bar represents the number of total, male and female antidepressants recipients of Montana MedicAid further segregated by age.

This breakdown demonstrates that the largest number of people receiving antidepressant prescriptions in this survey population is associated with women in child-bearing years (21-40).

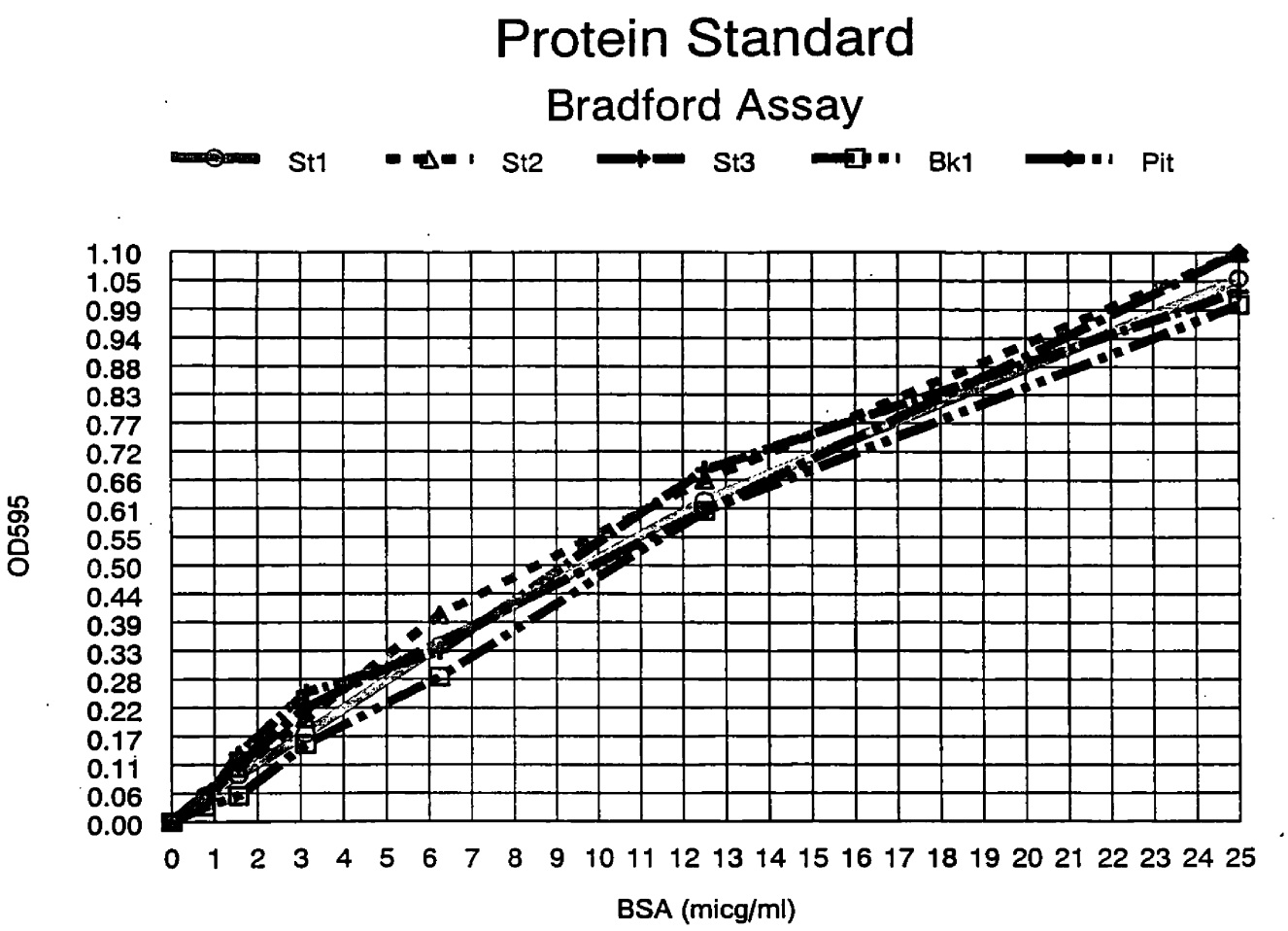



**Figure 13: Standard Curve of pure protein concentration created with BSA.** The lines are representative of the reading on a spectrometer at OD 595 wavelength with various known amounts of BSA in a Bradford dye.

# Chapter 4

## Discussion

*Osmotic Pump Experiment*

Chronic spironolactone reduced rat total 24-hour locomotor activity, most noticibly during the active hours, which for rats is during the dark phase (19:00-7:00 hours). This provides evidence that 1) MR are linked to locomotor activity and potentially to depression and 2) are important when CORT levels, and subsequently GR activation are believed to be highest. The manner by which this linkage is manifested was unexpected from the action of brain MR proposed in the literature.

Meijer and de Kloet proposed that a continuous decrease in limbic GR and a high ratio of activated MR to GR in the hippocampus are involved with depression by reducing brain serotonin, hippocampal serotonin's 1A receptor (5-$HT_{1A}$), and attenuating 5-$HT_{1A}$ neuronal hypopolarization.[106] The effect of stress to increase in the activity of the rate limiting enzyme of serotonin synthesis, tryptophan hydroxylase is blocked by central administration of a GR antagonist.[107] [108] [109] GR activation and circulating CORT appear to both enhance tryptophan hydroxylase expression and neuronal serotonin release (capacity).[110] [111] [112] Therefore, decrease in brain GR could decrease serotonin synaptic release, which has been theorized to be associated with depression.[54]

5-$HT_{1A}$ agonists have many central effects and some 5-$HT_{1A}$ agonists are used as antidepressants and anxiolytics, such as buspirone, ipsapirone and flesinoxan.[113] This G-protein coupled serotonin receptor, 5-$HT_{1A}$, is physiologically influenced by MR in at least two manners. MR activation decreases the protein expression of 5-$HT_{1A}$.[114 115 116 117 118] In electrophysiological experiments of hippocampal neurons which co-express MR, GR and 5-$HT_{1A}$, an MR agonist attenuates the neuronal response (hypopolarization) to serotonin and 8-OH DPAT (a specific 5-$HT_{1A}$ agonist).[119 120] The MR agonist effect is removed by co-administration of a GR agonist. Therefore, serotonin's effects on these 5-$HT_{1A}$ receptors would be most influential during physiological states of high CORT and least influential at low CORT or low GR activation, but not complete CORT removal. Electrophysiological effects have been observed to be decreased with both short-term and long-term administration of low CORT to the hippocampal neurons.[121]

In the Meijer and de Kloet model, low GR would result in a decrease in serotonin production by the raphe nuclei and dexamethasone would fail to suppress CORT by binding to hypothalamic GR. The high hippocampal MR/GR ratio would attenuate 5-$HT_{1A}$ hypopolarization of neurons and decrease 5-$HT_{1A}$ expression. These effects, they theorize, would contribute to depression.[122]

The present results, that an MR antagonist decreases locomotor activity, do not contradict that low GR could be involved in depression, but they provide evidence that low MR might also be involved. Continuous administration of

spironolactone into the brain would have been expected to decrease the MR to GR ratio, increasing 5-$HT_{1A}$ protein expression and hypopolarization effect. These predicted effects were thought not to induce depression-like behavior, such as low total 24-hour locomotor activity.[122] However, spironolactone lowered activity during the dark (active) hours, when GR activation should be high and thus, high hippocampal GR/MR ratio. The neurochemical systems involved in this lowering of locomotor activity are unknown, but, hippocampal 5-$HT_{1A}$ is an unlikely candidate. Although, 5-$HT_{1A}$ has effects on locomotor activity most literature reviewed (from a Medline search of abstracts) implied this effect is from the 5-$HT_{1A}$ in the suprachiasmatic nucleus and the raphe nuclei, while serotonin 5-$HT_{1B}$ receptors, catecholaminergic receptors and other receptors are involved in locomotor activity at the hippocampus.[123]

The depression-like effects of both MR and GR provides for an alternative hypothesis to be proposed. Both the activated MR and the activated GR bind to the same sequences of DNA response elements (GRE) and to individual response elements, MRE and GRE. [15 16 17] Activated hippocampal MR or GR are found co-localized in some nuclei compartments and not co-localized in other nuclear compartments.[124] The dimerization of steroid receptors is thought to be necessary for steroid/transcription interactions to occur. This dimerization occurs after one activated receptor has already bound to the proper GRE.[125] Both homodimers and heterodimers can occur with MR and GR respectively.[126] It is quite feasible, that DNA response elements could exist for GR+GR, MR+MR

and MR+GR dimers or that one receptor of the dimer could be replaced by another, making a homodimer into a heterodimer. In normal physiological conditions CORT binding GR fluctuates from 5% to 90% with the circadian level of available CORT, while almost all MR are thought to be bound and activated. In this condition, the binding and activation of GR would be the limiting factor for the MR+GR heterodimer. By pharmacologically lowering MR binding by interactions with spironolactone, the MR+GR heterodimer would also be decreased like it is in circumstances of low CORT and low GR activation, except now the availability of MR is the limiting factor. Therefore, the MR+GR heterodimer would be decreased by low MR or low GR. Perhaps, this MR+GR heterodimer is involved in clinical depression.

Another possible explanation might be that MR+MR homodimer alters the expression of a protein and this alteratrion of the protein would be necessary in order for GR+GR alterations of a separate protein(s) to occur. Therefore, either low MR or low GR activation would be involved in depression. Similar to what was observed in low activated brain MR in rats, adrenalectomized (ADX) rats (when neither MR nor GR are activated) demonstrated a decrease in total 24-hour wheel running activity, but no change in the circadian rhythm of activity.[127]

Explorative behavior of treatment animals (including vehicle) all decreased from before treatment values, but there was no difference between treatment groups. In addition, the animals did not appear sickly nor was there difference in body weight in any of the treatment groups (data not shown).

Therefore, it is unlikely the differences in total 24-hour locomotor activity are from an effect other than spironolactone treatment.

If both MR and GR are necessary for GR to have effects on the serotonergic system, then the half of the hypothesis described by Meijer and de Kloet would be correct. Low GR activation results in low serotonin synthesis and release contributing to depression. However, there may need to be MR activation in order for GR to exert a definitively full effect on locomotor activity and on moods.

| Activated dimer | low or no MR+GR | mid MR+GR | high MR+GR |
|---|---|---|---|
| Serotonin | low | mid | high |
| Locomotor Activity | low or none | mid | high |
| CORT regulation | poor | average | ? |
| Mood | depressed/sleep | mid | manic |

**Table 6: Proposed relationship between activated MR+GR level, locomotor activity, serotonergic activity, CORT regulation and mood.**

This change in activity and possibly serotonin levels, indicates that both the MR and GR might be involved in the etiology of depression. Animals receiving acute brain injections of MR antagonists exhibit "depression-like" behaviors.[128] [129] [130] Activated hippocampal MR directly and independent of GR suppress 5-$HT_{1A}$ expression. [118] [131] A high number of 5-$HT_{1A}$ (implying low MR) are seen in patients who suffer from depressive disorders and in people who

have committed suicide by non-violent means.[132] These high levels of 5-$HT_{1A}$ could have resulted from low MR (hippocampal 5-$HT_{1A}$). Brain MR are thought to be involved in the regulation of plasma CORT.[25 29 30 32 33] Low brain MR would cause a lack of CORT regulation in a similar manner to that seen in depression. Antidepressants cause an increase in hippocampal MR in a time course similar to their therapeutic effects.[63 64 65 66 67 68 69 70] Both GR and MR are reduced with chronic immobilization (learned helplessness) and similar chronic stresses, while 5-$HT_{1A}$ receptors increase.[49 133] Thus, low brain MR activation may well be involved in the etiology of certain types of depression.

However, only one patient case has been reported of spironolactone being associated with depression.[134] Of note, lethargy (decreased locomotor activity) is a side effect of spironolactone diuretic therapy.[135] Interestingly, spironolactone has been successful as a substitute of lithium in bipolar disorder patients.[136 137] However, the pharmacological dose administered directly into the brain to rats in this experiment were at a much greater concentration in the brain than a pharmacotherapeutic dose administered orally to people. This might explain, the lack of complaints of depression in people taking spironolactone. Another explanation is that MR antagonists do produce lethargy, but not lethargy associated with depression.

That the MR agonist, aldosterone, did not alter locomotor activity at all, is not a surprise, because a high percentage of MR are always bound, so adding

an exogenous ligand to an already at least 80% activated receptor might have little net effect.

Hippocampal MR were predicted to be involved with regulation of CORT levels, for it had been shown that acute administration of MR agonists decreased CORT and MR antagonists increased CORT.[25 29 30 32 33] However, in the present study there was no change in plasma CORT between any treatment groups. The effects of the drug treatments could have been masked by stress of being in a new environment, resulting in high CORT levels. However, several precautions were taken to minimize this possibility. Animals were brought up in their home cages several hours before the experimentation. Cannula were connected to collecting tubes at least one hour prior to removal of the first blood sample, and many precautions were taken to assure stable, quiet lab conditions during the course of the experiments. Another possibility is that the brain adjusted to the chronic drug treatments and thereby normalized CORT levels. A similar chronic study of manipulating brain MR by continuous infusion of MR antisense (MRAS) also had no effect on CORT levels.[82]

The ability to remove stress free blood samples during dark hours would have been beneficial, since CORT plasma levels fluctuate in a circadian rhythm with higher levels occurring during dark hours. This is especially true since, spironolactone effects on locomotor activity were noticeable only during the dark hours. However, it was very difficult to obtain a plasma sample during the nocturnal hours from rats under red light. When obtained, often some

manipulation of the animals had to be performed introducing a potential for stress and altered CORT levels.

In the future, a study should be conducted on CORT levels after a dexamethasone bolus to see if CORT regulation is altered by the low activated MR in the brain.

*1995 Survey of Montana MedicAid Antidepressant Recipients*

The 1995 survey of Montana MedicAid patients showed at least three times more women received anti-depressant therapy versus men and that women in their child bearing years represented by far the largest antidepressant prescription recipients. The higher incidence of women diagnosed with depression compared to men has been previously reported and might be influenced by estrogen and/or progesterone levels. A large change in plasma estrogen or progesterone concentrations has been associated with clinical depression; including the dramatic decrease which occurs in estrogen and progesterone after child birth or menopause, or the increase in estrogen and progesterone which can result from drug contraceptive therapy.[6 7 8] It is possible that the highest frequency of recipients of an antidepressant prescription (women between the ages 21-40) corresponds also with the highest use of drug contraceptives. The analysis utilized could not show recipients of concurrent prescriptions, such as oral contraceptives and antidepressants, which would have been interesting considering the highest number of prescriptions for drug

contraceptives occur in the age group of the highest number of antidepressant prescriptions.

Interactions between gonadal hormones and CORT and CORT receptors do occur.[138] [139] [140] [141] Females (both human and animal) are known to have higher basal CORT, as well as larger responses to stress and immune challenges than males.[142] [143] [144] [145] The level of CORT and CORT sensitivity to various stressors fluctuates with the menstrual cycle.[146] Progesterone is a very strong MR antagonist *in vitro*, with a higher affinity than spironolactone and *in vivo* (but estrogen appears to need to be present for this effect, implying progesterone *in vivo* effects on MR may not be directly at the MR binding domain).[147] Either spironolactone or progesterone have been successful prophylactic therapeutics of pre-menstrual syndrome (PMS), this suggests that hippocampal MR activation or MR+GR activation might be too high in PMS.

One should keep in mind that the survey includes primarily people with low income. Certainly, some of the women in child bearing years are single mothers. Since a large percentage of these women are probably receiving birth control, perhaps the oral contraceptives are a factor in the depression. Another possibility is that antidepressant drugs are the latest "*Mother's Little Helper*", a Brave New World *soma*, which removes emotions to help mothers with the stress of raising children. In this sense, the antidepressants may also be overprescribed in these age groups. This brings up some serious social issues about Montana, if many of our mothers are on antidepressants.

*Monoclonal MR Antibody Specific to the Ligand-Binding Site Experiment*

The monoclonal antibody specifically raised to the ligand-binding site of the peripheral MR in the kidney, did not bind to hippocampal MR in the present study, either by western blot or immunocytochemistry (ICC) analysis. The ICC detection of hippocampal MR could have been potentially masked by the ligand, because most MR are thought to be ligand bound. However, the tissue samples in the western blot analysis were boiled for two to three minutes, incubated with a powerful detergent and moved through a gel by a high power electrical circuit. By definition and from these conditions, all the proteins in a western blot are thought to be denatured. It is unlikely, that such a denatured protein would still be masked by a ligand. In order, to make sure that the lack of MR binding was not from low protein concentration, up to 300 μg total protein of a tissue sample had been placed in a well. The hippocampal tissue GR was detected in an identical procedure, using a polyclonal antibody to GR. Currently, no such polyclonal antibody commercially exists for brain MR analysis.

Therefore, the most likely conclusion from the present MR results is that ligand binding site of MR in the hippocampus is not the same amino acid composition as the ligand binding site of MR in the kidney. The difference in the ligand-binding domain of MR could represent a difference in active versus inactive MR, or it could truly represent a difference in hippocampal versus kidney MR. Splice variants of MR have been found in the hippocampus.[148]

Certain fragments of activated, but not inactivated MR are resistant to proteolysis.[149] [150] This indicates that a structural change occurs in MR upon ligand activation. Earlier studies have suggested that there is a slight variation between the hippocampal and kidney MR, which might allow for the ligand specificity of the tissues.[151] [152] Non-peptide post-translational modifications could also explain the various observed difference between kidney and hippocampal MR, such as sugar or lipid additions or deletions.

In the future, a study should be conducted on the binding of the monoclonal antibody in chronically adenalectomized rats. This would allow the hippocampal MR to assume an unbound inactive form. If there still is no binding by the monoclonal antibody to these MR, then the only remaining possibility is that brain MR are distinct from peripheral MR.

Since, spironolactone has been successfully used in the treatment of bi-polar disorder and pre-menstrual syndrome and that lithium has MR antagonist-like qualities, then high MR+MR homodimer or high MR+GR heterodimer or both, might be involved with manic-like moods. The low brain GR theory of depression, coupled with the decrease of locomotor activity in these experiments suggest that low MR+GR heterodimer might be involved in depression. Novel drugs which enhance activated MR+GR in a time course faster than present therapeutics may represent future antidepressants. The lack of a monoclonal antibody bound to the ligand binding site of hippocampal tissue, but functional binding to MR in renal tissue, suggests the ligand-binding site might be different.

Since, spironolactone has successfully been used as a mood stabilizer, perhaps to high MR+GR are involved with bi-polar disorder. Future mood stabilizers might make use of the binding-site difference to specifically block brain MR.

## Summary

1. The chronic brain administration of a MR antagonist lowered total 24-hour locomotor activity suggesting that the brain MR may be involved in the etiology of behaviors associated with depression.

2. A survey of people eligible for Montana MedicAid in 1995 found three times more women received antidepressants than men and the largest group of recipients was women between the ages 20 to 40 years. Whether this was correlated to oral contraceptives or other gonadal hormone factors was not discernible in the present analysis.

3. Hippocampal MR were not detectable with a monoclonal antibody specific to the peripheral ligand binding site, while kidney MR were detected. This was true for both western blot and ICC analysis. The data suggest that two different MR proteins exist, either from post-activation alterations or pre-activation expression/ alteration.

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