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# Transplants of Fetal Rat Medial Basal Hypothalamic (MBH) Tissue into MBH-Lesioned Adult Rats

by

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DEDICATION

THIS THESIS IS DEDICATED TO MY SON, JULES, FOR HIS UNFLAGGING ENCOURAGEMENT, UNDERSTANDING AND GENEROUS SACRIFICE OF COUNTLESS HOURS HE WANTED TO SPEND WITH HIS MOM. HIS UNSELFISH DESIRE FOR HIS MOTHER'S HAPPINESS AND FULLFILLMENT IS AN EXAMPLE OF LOVE IN ITS HIGHEST FORM AND DEMONSTRATES A WISDOM FAR GREATER THAN HIS TENDER YEARS.

ABSTRACT

This study was designed to ascertain whether fetal medial basal hypothalamic (MBH) tissue can grow, differentiate and replace MBH functions destroyed by ablating the MBH in a host animal. Fetal cerebellar cortex was implanted as a control tissue. Functional return was evaluated 1) anatomically, using immunocytochemical staining for vasopressin associated neurophysin (VP-NP)and luteinizing hormone releasing hormone (LHRH) within implanted tissue; 2) physiologically, by looking at changes in vaginal cytology, changes in body weight, and blood plasma levels of the pituitary hormones prolactin and growth hormone during nonstressed and stressed conditions; and 3) behaviorally by assessing water intake. Using these parameters it was concluded that although the physiological and behavioral data indicated that the ablation procedure disrupted the MBH functions tested, the MBH implants did not ameliorate the created deficits and that animals implanted with fetal MBH tissue did not respond differently from animals implanted with fetal cerebellar cortex tissue. There was very little immunocytochemical evidence of VP-NP or LHRH production within implanted tissue.

## STATEMENT OF PURPOSE:

Brain transplantation of parts of the brain or other parts of the central nervous system has potential for restoring functional capacity of neural tissue which has been impaired by injury, degeneration, genetic deficiency or surgical procedure. It can also be used to address questions concerning basic neural mechanisms such as plasticity and regeneration.

The feasibility of successful brain transplantation was investigated as early as 1890 when Gilman W. Thompson reported the survival of a piece of tissue excised from the occipital cortex of a cat and transplanted into a similar site in dog brain. In 1917, Elizabeth Hopkins Dunn was the first to demonstrate that the site of transplantation was a critical factor in transplant survival. She found that only transplants which were placed near the choroid plexus of the lateral ventricle survived. Recent investigations confirmed that transplants have an increased survival rate when they are placed near a source of nutrition and that the dispersal of cells before transplantation also increases the rate of survival (Bjorklund and Stenevi, 1984; Brightman, 1983).

Inquiries into the ability of the transplanted tissue to form neural connections with the host's brain tissue gave mixed histological results: some microscopic examinations revealed fibers crossing the borders between the implant and host (Jaeger and Lund, 1980;

Kromer et al., 1979; Lund and Hauschka, 1976); others found a dense glial barrier around the implanted tissue (Azmitia and Whitaker, 1983). The implant's connectivity with the host also has been assessed by physiological measures and changes in behavior (Arendash, 1983; Bjorkland and Stenevi, 1984; Dunnett et al., 1982b; Freed, 1983; Gibson et al., 1984b; Labbe et al., 1983; Luine et al., 1984; Scott et al., 1984). Although physiological and behavioral studies are able to evaluate the functional competence of an implant, they cannot determine the ability of an individual transplanted cell to differentiate and mature.

The present study was designed to ascertain whether fetal medial basal hypothalamic (MBH) tissue can survive, differentiate and replace MBH functions destroyed by ablating the MBH in a host animal. Host-graft integration and functional return were evaluated anatomically, physiologically and behaviorally. Anatomically, we looked for immunocytochemical (ICC) evidence of luteinizing hormone releasing hormone (LHRH) and neurophysin (NP) production within the implanted tissue. Physiologically, assessments were made of changes in 1) body weight, as an index of hypothalamic-regulated food intake, 2) blood plasma levels of the pituitary hormones prolactin (PRL) and growth hormone (GH) which are controlled by the MBH and 3) changes in vaginal cytology as an index of the effects of the lesion and implantation on the estrous cycle. Functional efferent connections from the implant to the host should ameliorate one or more of the deficits

produced by the ablation. If these efferent connections between implant and host rectify plasma levels of GH and PRL then afferent connections to the implant could be assessed by demonstrating a stress response in these systems. Behaviorally, we looked at water intake which also reflects the competence of the animal's VP system. Using these parameters, this thesis addresses the following questions: 1) Do the physiological and behavioral paramenters indicate a successful ablation of the MBH and a return of MBH function by the implanted tissue? 2) Is there neuroanatomical evidence for the production of the hormones NP or LHRH in implanted fetal tissue as shown by immunocytochemistry? 3) Do transplants of fetal MBH tissue differ from transplants of fetal cerebellar cortex tissue? 4) Does the length of time between ablation and implantation affect the viability of the transplant?

The experimental design which follows was carried out two times: The MBH was ablated in ten-week-old female rats and, after 129 days in experiment #1 and 7 days in experiment #2, fetal MBH tissue or fetal cerebellar tissue was implanted into the ablation cyst. Fetal MBH tissue was transplanted to provide a source of cells which might be able to restore host MBH functions. Cerebellar cortex (Cbl Cx) was used as a control tissue. Weight gain, water intake, and blood plasma levels of growth hormone (GH) and prolactin (PRL) were measured at monthly intervals after the ablation and after the implantion of fetal tissue. Each month, vaginal smears were taken on

four consecutive days. Animals were sacrificed and coronal brain sections were stained, using ICC, for LHRH and NP. ICC for somatostatin (SRIF) also was attempted but dropped from the study because of the lack of specificity of staining with the antibodies available. The presence of glial fibrillary acidic protein (GFA) also was assessed, using ICC, to compare the number of reactive astrocytes within and surrounding the implant.

# INTRODUCTION

## NEUROANATOMICAL LOCATION OF PEPTIDES (Figure 1)

Luteinizing Hormone Releasing Hormone (LHRH):

In the rat, LHRH-containing cell bodies have been localized primarily in and surrounding the organum vasculosum of the lamina terminalis (OVLT) in the preoptic area (figure 9). Cell bodies also are found in the anterior hypothalamus as well as in lateral and medial parts of the middle (tuberal) hypothalamus, and fibers from these cell bodies project to a number of hypothalamic, circumventricular and extrahypothalamic structures (King and Anthony, 1984; King et al., 1982).

Within the hypothalamus, LHRH fibers have been reported to be distributed throughout the areas where cell bodies have been noted. In addition, fibers are in contact with the ependymal cells of the third ventricle, the external lamina of the median eminence (figure 8), the medial basal hypothalamus and around periventricular nuclei and the third ventricle. Caudally, LHRH fibers project to the mammillary bodies (Ibata et al., 1983).

It is generally considered that LHRH reaching the median eminence, a circumventricular structure, is released into the portal hypophysial vessels and transported to the anterior pituitary where

it controls the release of pituitary gonadotropins (Guyton, 1981). Fibers projecting to the median eminence are distributed into medial and lateral pathways (Kawano and Daikoku, 1981; King et al.,1982) The medial pathway projects through the periventricular area of the hypothalamus. The lateral pathway is associated with the medial and ventral edges of the medial forebrain bundle. The LHRH fibers in both pathways originate from cell bodies in the medial septal nucleus, the medial preoptic nucleus, and the anterior hypothalamus (King et al., 1982). The median eminence is not the only circumventricular organ in which LHRH-containing axons terminate. Some axons originating from the most anterior LHRH-producing nerve cells in the vicinity of the OVLT terminate on the capillaries within the OVLT (Setalo et al., 1976).

Extrahypothalamic LHRH projections go to a number of structures including the habenular and amygdaloid complexes, hippocampus, (Kozlowski and Hostetter, 1978), lateral septal area, several thalamic nuclei, and brainstem (Ibata et al. 1983, Kozlowski et al. 1978).

<u>Vasopressin</u> (VP),<u>Oxytocin</u> (Oxy),<u>Neurophysins</u>(NP):

As is true of most neuropeptides, the hormones oxytocin and vasopressin are produced, packaged in neurosecretory granules and transported via neurosecretory axons. These hormones are released from axon terminals by exocytosis (Nordmann et al., 1984). Small

proteins with a molecular weight in the range of 10,000 daltons, called NP, are synthesized together with VP and Oxy as carrier proteins from common precursor molecules. They too are packaged in the secretory granules within perikarya and transported to nerve terminals. There are two chemically different NPs in the mammalian hypothalamus; one NP is associated with VP and one with Oxy (Plum and Van Uitert, 1978). Although VP is reported to have behavioral effects (de Wied, 1974; Ettenberg, 1984), it is more well known for its peripheral effects on the collecting ducts in the kidney to increase their permeability to water. The effects on the kidney allow most of the water to be reabsorbed as the tubular fluid passes through the collecting ducts, therefore conserving water in the body (Brownstein, et al., 1980; Doris, 1984; Guyton, 1981). Oxy is associated with contractions of the pregnant uterus, milk ejection during lactation and perhaps uterine contractions during intercourse (Brooks, et al.; 1966; Guyton, 1981).

Classically, it was thought that only magnocellular perikarya located in the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus produced Oxy, VP and their associated NPs and released the hormones Oxy and VP in the posterior pituitary (Swanson and Sawchenko, 1983). The SON consists of magnocellular neurons which project to the posterior pituitary (figure 11). This nucleus is bilaterally located dorsolateral and ventrolateral to the optic chiasm and tract and produces both VP and Oxy in approximately a 1:2

ratio in the rat (Rhodes et al., 1981). The PVN, which lies dorsolateral to the third ventricle in the medial zone of the hypothalamus (figure 11), includes magnocellular nuclei which project to the posterior pituitary as classically described (Swanson and Kuypers, 1980). The PVN also contains parvocellular nuclei and tracing the fibers from these parvocellular subnuclei has greatly expanded the classical view of VP and Oxy projections.

The magnocellular elements of the PVN are organized into three subnuclei designated by Swanson and Sawchenko (1983) as 1) anterior magnocellular part, 2) medial magnocellular part, and 3) posterior magnocellular part. The anterior magnocellular PVN lies ventromedial to the fornix at the level of the medial preoptic area. The medial magnocellular is found embedded in what Krieg (1932) called the anterior periventricular nucleus. The posterior PVN corresponds to the generalized magnocellular nucleus of most authors. Swanson and Sawchenko (1983) and Rhodes et al. (1981) found that the anterior magnocellular subnucleus had a core of VP cells surrounded by Oxy cells, while the medial magnocellular PVN contains primarily oxytocin-containing somata. The posterior PVN contains equal numbers of VP and Oxy cells (Swanson and Sawchenko, 1980, 1983).

The parvocellular elements of the PVN project to the median eminence, brain stem and spinal cord and are organized into five distinct nuclei; 1) the anterior parvocellular (apn); 2) the medial parvocellular, which is divided into the ventromedial (vpn) and dor-

somedial (dpn) parvocellular nuclei; 3) the lateral parvocellular (lpn); 4) the posterior parvocellular (ppn); and 5) the periventricular posterior parvocellular which surrounds the third ventricle (Swanson et al., 1980). The apn projects to the portal capillary plexus in the median eminence (Swanson et al., 1980). The majority of cells in the dpn project to the spinal cord (Swanson and Sawchenko, 1980). Swanson and Kupers (1980) report that vpn and ppn also project to spinal cord and lpn projects to both spinal cord and dorsal medulla.

Recently it has been shown that several accessory magnocellular nuclei are found outside the PVN and SON. These accessory magnocellular nuclei contain Oxy and VP in a 2:1 ratio (Rhodes et al., 1981) and presumably project to the posterior pituitary. Projections from one of these accessory nuclei, the anterior commissural nucleus, have been traced to the posterior pituitary (Armstrong et al., 1980). VP-containing fibers from another accessory nucleus, the nucleus circularis, also apparently project to the posterior pituitary because retrograde degeneration occurs in this nucleus after hypophysectomy (Fisher et al., 1979; Palkovits et al., 1974). Other hypothalamic accessory nuclei have been less well characterized.

The suprachiasmatic nucleus (SCN) constitutes an additional parvocellular nucleus which contains VP and VP-NP, among other neuropeptides. It is located dorsal to the center of the optic chiasm at the base of the third ventricle (Sofroniew and Weindl,

1978). The function of VP in the SCN is not presently understood. The SCN is thought to be an important component of CNS mechanisms participating in the generation of circadian rhythms (Peterson et al., 1980a; Van Den Pol and Powley, 1979), the VP-deficient Brattleboro rat still shows circadian rhythmicity which suggests that there are other neuropeptides in the SCN that may regulate circadian rhythms (Swanson and Sawchenko, 1983). In addition, a circadian variation in VP messenger-RNA is seen in Brattleboro as well as in normal rats (Uhl and Reppert, 1986). SCN isolation and lesioning studies indicate that many VP-containing projections from the SCN may be intranuclear, with a limited distribution external to the SCN. Peterson et al. (1980b) reported that isolating the SCN from the rest of the hypothalamus did not affect VP-containing fibers within the SCN or elsewhere. However, using lesions of the SCN, Hoorneman and Buijs (1982) reported that VP-containing fibers of the SCN project only to the organum vasculosum of the lamina terminalis, the dorsomedial nucleus of the hypothalamus and the periventricular nuclei of the thalamus. Brownfield et al. (in press) described a slightly less restricted but still limited distribution of efferent VP fibers from the SCN to the periventricular nucleus of the thalamus as well as to the dorsomedial nucleus, the periventricular nucleus and the parvocellular portions of the hypothalamus.

VP also has been reported in cell bodies outside of the hypothalamus in colchicine-treated rats. Cell bodies containing VP

have been reported in the bed nucleus of stria terminalis (van Leeuwen and Caffe, 1983) and in the locus coeruleus (Devries and Buijs, 1983). The projections of these putative extrahypothalamic cell bodies is not known.

Glial Fibrillary Acidic Protein (GFA):

The central nervous system (CNS) contains two classes of cells: neurons and glia. In the CNS there are about nine times more glial cells than neurons (Kandel and Schwartz, 1981). Glial cells serve as phagocytic (microglia), myelinating (oligodendrocytes), or supporting (astrocytes) cells for neurons within the CNS. Astrocytes are classified as fibrous or protoplasmic. Protoplasmic astrocytes are found primarily in gray matter and are interspersed between the cell bodies of neurons. They serve as support cells for neurons, and studies by Hatton et. al. (1984) suggest they may aid in the control of VP and Oxy synthesis in the SON and PVN of the hypothalamus. Protoplasmic astrocytes produce little GFA (Bjorklund et al., 1983a). Fibrous astrocytes contain many intracellular filaments, produce GFA and are found as the primary support cell in white matter (Latov et al., 1979). Fibrous astrocytes produce a glial limitans sheath around blood vessels, a subependymal lining around the ventricles and also form an external limiting membrane which surrounds CNS tissue (Bignami and Dahl, 1974b).

It has been shown that protoplasmic astrocytes will produce GFA as a response to injury (Azmitia and Whitaker, 1983; Bignami and

Dahl, 1974a; Bjorklund et al., 1983a; Cavanagh, 1970; Duffy, 1983; Ludwin, 1985; Windle et al., 1952). These GFA producing astrocytes, termed reactive astrocytes, produce gliosis when present in large numbers. Some studies report a dense glial membrane around implanted tissue (Azmitia and Whitaker, 1983; Bjorklund and Stenevi, 1984). Others observe a gliosis that extends from the implanted tissue into the host tissue (Bjorklund et al., 1983a; Duffy, 1983; Ludwin, 1985).

## HYPOTHALAMIC CONTROL OF THE ANTERIOR PITUITARY

### Growth Hormone

GH secretion is increased by hypothalamic GH releasing hormone (GHRH) and decreased by the secretion of two forms of hypothalamic somatostatin (SRIF) (King et al., 1975; Spiess et al., 1983). Cell bodies producing GHRH are located in the arcuate nucleus (ARC), the ventral premammillary nucleus (VPN) and in the area which extends between the ARC and VPN (Merchenthaler et al., 1984). Hypothalamic production of SRIF is primarily located in the periventricular nucleus (Elde and Parsons, 1975).

Stress in the rat results in inhibition of GH secretion (Schalch and Reichlin, 1966). Although the control of this stress response is not clear, it has been demonstrated that antiserum to SRIF prevents stress-induced inhibition of GH secretion in the rat (Arimura et al., 1976; Terry et al., 1976). The source of the SRIF putatively responsible for the stress response is being investigated. This source

probably lies outside the periventricular nucleus since lesions which destroy 85% of the cells in this nucleus do not affect the response of GH to stress (Urman et al., 1985).

Prolactin

The hypothalamic control of PRL is complex, involving several factors. A PRL inhibiting factor (PIF) has been postulated and long regarded to be dopamine secreted into the portal system at the median eminence (Penalva et al., 1984; Draga et al., 1985; Leong et al., 1983). Besides the dopamine PIF, a 56 amino acid fragment of the 96 amino acid protein precursor for LHRH acts as a potent PIF (Nikolics et al., 1985). This protein is termed gonadatropin associated protein (GAP) and has been co-localized in rat brain in the same neurons that make LHRH. Both LHRH and GAP are co-secreted by nerve terminals in the median eminence (Phillips et al., 1985). Gamma aminobutyric acid (GABA) also has been reported to have PIF activity (Leong et al., 1983). In addition to PIF's, PRL releasing factors (PRF) also have been postulated. Many substances found in the median eminence reportedly affect PRL release in the presence of dopamine (Leong et al., 1983).

Under stress conditions, there is an elevation of plasma PRL. The neural control for this response to stress is not understood but is thought to originate in basal telencephalic structures rostral to the MBH (Mioduszewski and Critchlow, 1982; Siegel et al., 1980). Fibers from cell bodies mediating the PRL stress response are thought

to travel through the MBH because complete or anterior hypothalamic deafferentation abolishes the PRL stress response to visual or audiogenic stimuli (Siegal et al., 1980) or to leg-restraint stress (Miodoszewski and Critchlow, 1981).

IMMUNOPRIVILEDGED STATUS OF THE IMPLANTATION

The brain contains a blood-brain barrier which consists of the tight junctions between the endothelial cells of the capillaries (Bacon and Niles, 1983; Copenhauer, 1978). This blood-brain barrier reduces the presence of the host's surveillance cells in brain tissue (Barker and Billingham, 1977; Reif, 1984). As a result, foreign tissue placed in the brain does not activate the immune system to a degree sufficient to elicit the production of the antibody IgG. In addition, fetal tissues have low concentrations of cell surface antigens. The earlier the age of the transplant, the lower its content of antigens (Reif, 1984). These observations are supported by experiments first carried out by Medawar (1948).

Medawar implanted skin homografts in the brain. Homografts (grafts between different individuals of the same species) survived until similar skin homografts were transplanted to peripheral sites on the host's body. Cells of the host's immune system produced antibodies against antigens on the peripherally placed skin homografts. These antibodies crossed the blood-brain barrier of the host and attacked the skin homograft located in the brain. Freed (1983)

repeated this paradigm using fetal brain tissue from 18 to 19 day gestational rat fetuses. He had four experimental groups: 1) syngenic (genetically identical) fetal brain placed in the lateral ventricle of adult rats followed by an allogenic (same species but not genetically identical) skin graft to a peripheral site on the adult rat host; 2) syngenic brain grafts followed by syngenic skin grafts; 3) allogenic brain grafts followed by syngenic skin grafts; and 4) allogenic brain grafts followed by allogenic skin grafts. Freed found that fetal brain tissue survived even after seven months. Implants were not rejected until after an allogenic skin graft transplanted to a peripheral site elicited an immune response, presumably to shared tissue antigens on the fetal brain implant.

ABILITY OF THE IMPLANT TO INTEGRATE WITH THE HOST'S TISSUE

In 1917, Dunn outlined her attempts to transplant cortex tissue from one 10 day old rat to another. She found that the implanted tissue did not interconnect across the cicatricial scar that formed around the implant. She did find, however, that those transplants that broke through the cortex to attach to the area of the lateral ventricle showed a superior rate of viability and growth over those that rested in the depression which remained after a section of host cortex was removed (Dunn, 1917).

In 1976, Lund and Haushka demonstrated synaptic connections between newborn rat brain and superior collicular fragments implanted

from fetal rat and suggested that connections were enhanced because the implanted superior collicular fragments were implanted close to the newborn rat's tectum. Jaeger and Lund (1980) later found that occipital cortex transplanted to tectum also received afferents from the transplant supporting the conclusion that a proper placement of the implant increases afferent sprouting. Jaeger and Lund also found that axons crossed between host and the implant because the interface was uninhibited by scarring. They attributed the lack of scarring to the fact that no lesions or restrictions were made in the host brain before implantation.

In contrast to lesion-induced scarring inhibiting host-implant interconnectivity, it has been suggested that destruction of endogenous neurotransmitters in host tissue may promote pathway regeneration from implant to host. Gage et al. (1984b) found that outgrowth of cholinergic neurons from septal implants grafted into host hippocampal formation was about three-fold greater when the intrinsic cholinergic afferents had been removed previously from the host tissue.

Scanning and transmission EM has been used to look at synapses between host and implant in a study which transplanted the hypothalamus of 17-day-old rat fetuses into the third ventricle of adult Brattleboro rats. Synaptic endings were seen on some of the implanted cells but the origin of the bouton-like processes could not be determined (Gash and Scott, 1980). In another experiment using

Brattleboro rats (Gash et al., 1980), catecholamine histofluorescence showed catecholamine containing fibers crossing the interface between the host tissue and the graft. Few catecholamine containing perikarya were seen in the graft, while many were seen in the host, suggesting connectivity from host to implant. Mahalik et al., (1985), using electron microscopy, also saw host-to-graft and graft-to-host synapses between the caudoputamen of the adult Sprague-Dawley host and the fetal substantia nigra transplants.

## FUNCTIONAL EFFECTS OF INTRACEREBRAL IMPLANTS

The functional activity of implants can be assessed by indirect behavioral and physiological studies. Luine et al. (1984) used lordosis behavior in ovariectomized female rats to assess the effect of fetal raphe cells injected into the third ventricle of adult rats. When serotonin is increased above normal in the ventromedial nucleus of the hypothalamus, lordosis is inhibited. Selective lesioning of serotonin-containing fibers in the hypothalamus by treatment with 5,7-dihydroxy tryptamine results in increased lordotic behavior in ovariectomized female rats following priming with estradiol (Luine et al., 1984). The lordosis response was reduced in animals that received serotonin-rich implants of fetal raphe cells.

In another study which used sexual behavior to assess function, the medial preoptic areas of neonatal male rat brains (containing LHRH) were transplanted into the brains of female litter mates. The

female recipients were later ovariectomized, tested for female sexual behavior, and then implanted with a testosterone-filled capsule and tested for male sexual behavior. Increased male sexual behavior, but also increased female sexual behavior (after minimal estrogen priming), were observed in the female recipients. The neural transplants were identified histologically, via Nissl stain, and Golgi staining was used to identify transplanted neurons. These stains revealed extensive networks of neuronal processes traversing the implant-host interface (Arendash, 1983).

Krieger et al. (1984) also assessed the effectiveness of transplants by examining reproductive activities. Normal fetal preoptic tissue containing LHRH cell bodies was transplanted to the third ventricle of adult female homozygous recessive hpg mice. Homozygous recessive hpg mice are genetically deficient in LHRH resulting in undeveloped gonads and genitalia, low concentrations of pituitary luteinizing and follicle stimulating hormones and an inability of the females to become pregnant. Six of the ten animals implanted with POA tissue mated, became pregnant, and delivered healthy litters. Transplanted tissue was present in the infundibular recess merging with the median eminence. Only one to 16 immunoreactive cells were found per animal yet clearly there was functional production of LHRH from these implants. Control animals implanted with cortex tissue showed no functional recovery (Gibson et al., 1984).

The endocrine function of the VP system was used to assess the success of implants in Brattleboro rats, which are genetically deficient in VP (Scott et al.,1984). Adult Brattleboro rats were implanted with hypothalamic fragments containing VP neurons from normal 17 day old fetuses. After 60 or 90 days, host animals were killed and their brains prepared for EM and ICC. Although EM revealed neurosecretory vesicles within the neurons of the implanted tissue, there was no return of function as measured by urine output, drinking behavior or urine osmolarty. Sladek et al. (1984) implanted normal fetal hypothalamic tissue into Brattleboro rats and into neurohypophysectomized normal male Long Evans rats. Recovery was reported from the inherent or induced diabetes insipidus in each of the two groups. Functional vasopressin grafts exhibited the following features: 1) the VP-producing neurons projected to the median eminence and ramified around portal vessels; 2) magnocellular neurons containing VP migrated to the host-graft interface or even into the host's brain; and 3) the graft was well vascularized especially from vessels which supply the median eminence. Richards and Raisman (1984) observed a transient functional recovery in Brattleboro hosts, but it occurred whether or not the recipient had surviving transplant tissue. They observed as much as a fifty percent decrease in urine volume and a corresponding increase in urine osmolarity. Immunocytochemical staining revealed evidence of VP in the host's magnocellular neurosecretory system, but it was unclear whether this VP

was a result of cells migrating from the implant or from VP production in the host induced by the presence of normal hypothalamic fetal tissue. Richards et al. (1985) later reported an occasional neuron which stained for VP in the magnocellular system of unimplanted homozygous Brattleboro rats.

A clinically applicable use of implants eventually could be the replacement of transmitters missing in the brains of patients suffering from certain diseases. In Parkinson's disease the connections from the substantia nigra to the striatum provide an inadequate supply of the neurotransmitter, dopamine (Clark, 1982). For a human this results in movement disorders including resting tremor, rigidity due to increased muscle tone and bradykinesia or hypokinesia (Nolte, 1981). In rats, bilateral lesions of the substantia nigra results in a loss of motor function, feeding and drinking abnormalities and a profound behavioral disruption. Unilateral damage results in a rotational behavior which is enhanced by treatment with amphetamines and reversed by the dopamine antagonist, apomorphine (Freed et al., 1984). A number of studies have investigated the efficacy of implanting dopaminergic neurons either from fetal substantia nigra or the host's adrenal tissue into animals exhibiting specific movement disorders and a behaviorial syndrome induced by a unilateral lesion in the nigrostriatal pathway. These studies have shown that anatomical connections between host and implant are made preferentially into areas that are normal targets for the dopamine neurons, but these

neurons show little or no outgrowth when implanted in other areas (Bjorklund and Stenevi, 1984). When nigral implants are placed close to the neostriatum, their normal target, they alleviate both drug induced and spontaneous turning behavior. This behavior returns if the transplant is destroyed (Dunnett and Bjorklund et al., 1982). Autoradiography using [$^{14}$C]2-deoxy-D-glucose showed that dopamine-producing cells within the implant had a metabolic rate equivalent to cells found within intact substantia nigral tissue (Schmidt et al., 1982).

Dunnett et al. (1982b) also experimented with intrahippocampal transplantation of septal grafts as a means of alleviating memory loss related to aging. To test problem-solving ability, they placed rats, one at a time, in a water tank that contained a platform by which the animal could escape. After training, animals learned the platform's placement in the tank. The platform was then removed and the investigators measured the animal's swimming time in the quadrant of the tank where the platform had been. The memory-impaired aged rats that had received a cell suspension graft of fetal cholinergic-rich septal tissue into the hippocampus showed improvement in this spatial learning task over the unimplanted, aged control animals (Gage et al., 1984c and 1984d). In another experiment, the medial frontal cortex was aspirated bilaterally in 21 rats to produce cognitive functional defects. One week later, eight animals were implanted bilaterally with fetal frontal cortex placed within the

aspiration cysts. Six animals were implanted with cerebellar cortex. Animals which received transplants of frontal cortex performed correctly in a spatial alternation test more often than animals which received implants of cerebellar cortex. Anatomical connections from host to implant were demonstrated using the retrograde transport marker horseradish peroxidase ((HRP) Labbe et al., 1983).

These studies demonstrate that transplanted tissue can partly alleviate functional deficits in host animals. Further research is needed to deepen our understanding of the critical factors which affect the anatomical and functional integration of implanted tissue. The present study utilizes several parameters to assess the restoration of function by fetal tissue transplanted into an ablation cyst in adult host animals.

## MATERIALS AND METHODS

### EXPERIMENTAL PARADIGM & ANIMAL PROCEDURES:

Two implant experiments were carried out. Animals in experiment #1 were implanted 129 days after the ablation surgery. Animals in experiment #2 were implanted 7 days after the ablation surgery in order to take advantage of growth factors that were reported to be maximally present within the ablation cyst six days after lesioning (Ebendahl et al., 1983; Gage et al., 1984; Nieto-Sampedro et al., 1982). In all other respects, the experiments used a similar paradigm. Each experiment used a total of thirty ten-week-old female Sprague-Dawley rats from Charles River, weighing 190-230 g. Animals were assigned to experimental groups using a randomized block design. Ten control animals were anesthetized with ether but did not undergo the ablation operation. Of the twenty lesioned animals, ten received implants of fetal MBH and ten received implants of fetal cerebellar cortical tissue.

Ablation of the MBH was performed under ether anesthesia using a modification of the Halasz-Pupp technique. (Halasz and Pupp, 1965) Lesions were placed using a standard stereotaxic apparatus and the DeGroot atlas of the rat brain. Three consecutive cuts were made by rotating a "C" shaped knife, producing a lesion approximately 5mm in length and 3mm in diameter. The ablation target extended from the anterior preoptic area to the caudal extent of the premammillary area

(Figures 2 and 3) and laterally to the columns of the fornix. Multiple hypothalamic nuclei were destroyed. Among these nuclei were: 1) the suprachiasmatic, 2) the periventricular, 3) the arcuate and 4) the ventromedial. After the ablation procedure, many animals became obese because the lateral hypothalamus, which contains the "feeding center", was unopposed by the ventromedial nucleus which has been associated with a satiety mechanism (Anand and Brobeck, 1951; Rolls et al., 1979). The ablation only partially destroyed the dorsal hypothalamus often leaving the paraventricular nucleus partially intact.

Implanted tissue was placed into the ablation cyst just above the median eminence. Four ul of MBH or Cbl Cx tissue from 16-day-old fetuses (taken by caesarean section from a sodium pentobarb-anesthetized rat) were aspirated into the glass tip of a SMI pipette (Scientific Manufacturing Industries, Emmeryville, Ca.). The pipette was then inserted into the stereotaxic apparatus and lowered 8.5 mm from the top of the skull into the ablation cyst.

During the course of the experiment, the rats were housed under conditions of controlled light (0500-1900 h) and temperature ($22 \pm 2^{o}C$). Food pellets and tap water were available ad libitum. During the intervals between monthly measurements of physiological parameters, the rats were housed in group cages (4/cage). Once a month, vaginal cells were sampled daily for 5 consecutive days by gently flushing the vagina with water and then preparing a slide with

this collected wash water. The cellular content was then classified as estrous, proestrous or diestrous. Each month, three days prior to the collection of blood samples, animals were placed in individual cages to reduce stress during sampling. Body weights were measured at this time, a calibrated water bottle was placed in each cage and water intake was measured during a 24 h period. Sixteen hours before the start of the blood collecting proceedure, the animal quarters were locked to minimize inadvertent environmental stimuli. To collect blood for nonstress hormone levels, the rats were individually removed from their cages and taken to an adjacent room where a tail vein blood sample was collected under vacuum (Nerenberg and Zedler, 1975). The blood was obtained without anesthesia, in less than 50 sec after opening the cage. Immediately after blood collection, the hind legs were bound together with a pipe cleaner for 10 min and the rats were again placed in individual cages outside of the animal quarters. Stress was considered to be a composite of stimuli including handling, bleeding, and 10-min. leg restraint. Blood samples were again collected from the tail vein under vacuum at 15min after cage opening, without anesthesia, and used to assess stress levels of GH and PRL.

Before sacrifice, animals were deeply anesthetized with sodium pentobarbital (intraperitoneal dose=50mg/Kg) and then artificially respirated on room air to oxygenate brain tissue until perfusion. Stress conditions were not controlled. The animal was then

transcardially perfused with approximately 200 ml of normal saline at room temperature to flush blood from the brain tissue. The brain was removed from the skull and immerse-fixed for 24 h in neutral buffered formalin (10% formalin in 0.1M sodium phosphate buffer, pH 7.2). The brain was then sectioned into thirds and post-fixed another 48 to 72 h. Other fixatives and times were tried in preliminary experiments. (See appendix A.) Hypothalamic tissue was cut into sections 50u thick on a vibratome in freshly made 0.05 Tris (pH 7.2) with 0.9% NaCl. Sectioned tissue was stored in phosphate-buffered saline until ICC for LHRH and NP was performed.

IMMUNOCYTOCHEMISTRY (ICC)

Technique

The ICC procedure was conducted using the unlabeled antibody enzyme technique of Sternberger (1979). For discussion of this method see appendix B. The ICC reaction was carried out in wells of a microtiter plate (polypropylene). Each well (2ml capacity) held between one and eight tissue sections and received 1.5ml of reac tion agent. Washes and the one-hour incubations took place at room temperature using buffers stored at 4 $^{o}$C. Tissues were agitated during all incubations and washes on a Lab-Line Junior Orbit shaker set at 140 rpm x100.

Endogenous peroxidases that may cause extraneous background staining in the finished product were inactivated by incubating the

tissues for one hour in a 1% solution of hydrogen peroxide mixed in phosphate buffered saline plus gelatin ((PBS+gel) Heitz, 1982). The tissues then were washed 15 min in PBS+gel, followed by three 10-min washes in reaction buffer consisting of PBS+gel enriched with 0.25% triton X and 0.01% filtered normal serum.

The tissue was then incubated with primary antibody in reaction buffer for 24 or 36 h at 4 $^{o}$C. The dilutions of antibody for the 24h incubations were 1:500 for LHRH (Generous gift of G. Kozlowski, U of Texas, Houston) & NP (Chemicon, El Segundo, Calif.)and 1:2000 for GFA (Bioproducts, Brussels, Belgium). When the 36h incubation time was used, LHRH and NP were diluted 1:1000. Somatostatin (Bachem, Torrance, Calif.) was used at both 1:500 and 1:1000.

Tissues were washed three times for 15 min in reaction buffer before a one hour incubation in anti-rabbit gammaglobulin (ARGG) diluted 1:50 in reaction buffer (Cappel Laboratory, Westchester, PA.).

Three 15 min washes in cold reaction buffer followed the ARGG step, then tissues were incubated for one hour in peroxidase anti-peroxidase (PAP) diluted 1:250 in reaction buffer (PAP: Dako Inc., Santa Barbara, California). The tissues were washed three times for 10 min in PBS+gel, then two times for 10 min in 0.05% M Tris, pH 7.5, then stored overnight. On the following day, tissues were given an additional 15 min wash in Tris. After the final Tris wash, the tissues were nickel enhanced (Tsu and Soban, 1982) and then incubated

in 0.5 mg/ml DAB in Tris with 0.003% hydrogen peroxide for 5 min to develop NP or GFA and 10 min to develop LHRH staining. The tissues were washed four times for 10 min in Tris buffer, mounted on an acid washed, gel coated slide, labeled and allowed to dry overnight. Slides were either dehydrated in alcohol or counterstained with thionine, then dehydrated and coverslipped. (Thionine which stains RNA within the cytoplasm of the cell body was found to be useful in visualizing the borders of the implant.)

Controls

Tissue from a normal control animal was processed with each sample of implanted tissue as a control for the ICC procedures.

For each experiment, sections were incubated with unabsorbed primary antibody or with primary antibody preabsorbed with its antigen as a control for staining specificity. Preadsorbed antibody was prepared by adding one mg of antigen to one ml of a 1:100 dilution of antibody in PBS+ gel. This antigen/antibody mixture was incubated at 4 $^{o}$C and spun 24 h later at 2500 rpm for 30 min. The supernatant was diluted to a final antibody dilution of 1:1000 for use in the assay by adding 0.5ml of the supernatant to 4.5 ml of reaction buffer such that it contained 100ug of absorption antigen per ml of final dilution of antibody and was used as the primary antibody in the standard ICC procedure.

Data Analysis

The number of cell bodies that stained for LHRH and NP-VP were

counted in each section from each brain and reported as such.

The number of fibers stained for LHRH or VP-NP were counted in and reported as ratios of the number of fibers divided by the number of sections counted. The number of fibers per section in all MBH implant sections (stained for both LHRH and VP-NP) was compared to all fibers per section found in cerebellar tissue using a t-test (Winer,1971). In exp. #2 one aberrant value was omitted using Grubb's outlier determination (Grubbs, 1950). F tests (Phillips,1978) showed whether data were homogeneous.

The number of GFA-producing cells within the implant and surrounding areas were counted and compared by a Mann-Whitney U test. Cell bodies stained for GFA were counted in three brain areas in ablated animals: 1) the implant, 2) the lateral area of the hypothalamus and 3) the lateral dorsal nucleus of the thalamus. Cell bodies stained for GFA were counted in corresponding areas in intact animals. Counts in the dorsal medial and ventral medial nuclei of the hypothalamus of intact animals were combined as a substitute for the implanted tissue. The data were analyzed with a Mann-Whitney U test when comparing two areas and with a Kruskal-Wallis test (Phillips, 1978) when comparing three areas. The following comparisons were made: 1) medial hypothalamus of intact group vs. implant tissue in Experiment #1; 2) implants in Experiment #1 vs. the implants in Experiment #2; 3) implants in both Experiment #1 and #2 vs. the lateral hypothalamic area of the ablated animals in both ex

periments and the lateral dorsal thalamus of the ablated animals in both experiments.

RADIOIMMUNOASSAY:

Technique

Plasma PRL and GH were assayed using a double antibody technique according to the procedure recommended by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDK), which also provided the immuno-reagents.

Controls

Each plasma sample was serially diluted to assess parallelism with the standard curve. All plasma samples from one collection period were included in a single assay. The standard curves were run in duplicate at the beginning and end of each assay to assess intraassay variation. Interassay variation was monitored with a pooled plasma sample.

For GH the intraassay coefficient of variation was 11.0% for Experiment #1 and 7.2% for Experiment #2 and the interassay coefficient of variation was 2.1% for Experiment #1 and 4.4% for Experiment #2.

The intraassay coefficient of variation for PRL was 8.0% for Experiment #1 and 3.9% for Experiment #2. The interassay coefficient of variation was 9.3% for Experiment #1 and 3.1% for Experiment #2.

GH values are expressed in terms of nanograms per ml of NIADDK-

rGH-RP-1 standard and the assay is sensitive to .244ng/ml. PRL values are expressed in terms of nanograms per ml of NIADDK-rPRL-RP-3 standard and the assay is sensitive to .122ng/ml.

Data Analysis

For each experiment, post-implantation data for body weight and water intake and post-implantation nonstress and stress levels of GH and PRL were assessed for homogeneity of variance using a Tukey test (Steele and Torrie, 1960) and heterogeneous data was transformed, when possible, using square root or logrithmic values of the data. Data was then analyzed using a two-way, repeated measures ANOVA (Winer, 1962) and a Newman-Keuls post hoc test was used to analyze significant differences (Steel and Torrie, 1960).

To assess reproductive cyclicity, the number of diestrous days were counted in three consecutive months after implantation. In exp. #1 the animals were 9, 10 and 11 months old. In exp. #2 the animals were 8, 9 and 10 months old. In each experiment, the mean number of diestrous days in the ablated groups was compared to the mean number of diestrous days in the intact group using a t-test.

# RESULTS

## PHYSIOLOGICAL DATA

### Vaginal smears (Table VI)

A t-test indicated that in both experiments the animals that had undergone ablation and subsequent implantation had significantly more diestrous days than the intacts (exp. #1: $t=-5.4$, $p<.05$; exp. #2: $t=0.6$, $p<.05$). The mean number of diestrous days was 13.4 in the MBH-implanted group and 13.1 in the Cbl cx implanted group in exp. #2.

### Body Weight

Experiment #1 (Figure 4A) - The ANOVA revealed that the three experimental groups differed in post-ablation, post-implantation body weight. ($F=3.53$, $p<.05$) Results of the post hoc test showed that the MBH-implanted group was heavier than the intact group ($p<.05$) but did not differ significantly from the cerebellar-implanted group. There were no significant differences between the three groups at the time of ablation or between the two ablated groups at time of implantation.

Experiment #2 (Figure 4B)- In this experiment, the data were heterogeneous, and transformation did not resolve the problem. The ANOVA, performed on untransformed data, indicated no significant differences between the three groups. This lack of significance

probably resulted from the large variances within each group.

Water Intake - Water intake is expressed as ml water consumed in 24h/g body weight.

Experiment #1 (Figure 5A) - Water intake after implantation was found to differ significantly between groups ($F=11.39$ $p<.01$). The post hoc test showed that the lesioned animals had higher water intakes ($p<.05$) than the intact controls but did not differ significantly from each other.

Experiment #2 (Figure 5B) - In this experiment, the variances were heterogeneous ($F = 10.96$, $p < .005$), and transformation did not reduce the heterogeneity. Analysis of untransformed data showed that the groups differed significantly ($F=10.57$, $p<.01$), and that the lesioned animals had higher water intakes ($p<.05$) than did the intact controls but did not differ significantly from each other.

Plasma GH Concentrations (Figure 6A&B, Table I)

Experiments #1 and #2 - In the two experiments, a comparison of post-implantation data for nonstress and stress GH levels in the three experimental groups differed significantly (exp.#1/nonstress $F=44.14$, $p<.05$;stress $F=25.43$, $p<.05$: exp.#2/nonstress $F=14.82$, $p<.05$;stress $F=5.46$, $p<.05$). In each case, values in the ablated, implanted groups were lower ($p<.05$) than in the intact group but did not differ significantly from each other.

Plasma PRL Concentrations (Figure 7A&B, Table II)

Experiment #1 - Analysis showed heterogeneity of variance in

nonstress PRL data in exp. #1 that was not removed by transformation. Analysis without transformation indicated that there were no significant differences between the groups. The stress data were homogeneous and analyss showed that PRL levels after stress were similar between groups.

Experiment #2 - Nonstress levels of PRL showed significant differences ($F=29.66$, $p<.05$) and post hoc analysis showed higher values in the ablated, implanted groups than the intact animals but the ablated, implanted groups did not differ significantly from each other. There were no significant differences between the groups for stress levels of PRL.

IMMUNOCYTOCHEMISTRY DATA

Implant Survival

In both experiments, all implants survived, became vascularized (figure 17) and contained cells which appeared to be neurons.

Cell Bodies Within the Implant

There were many cell bodies that stained for both LHRH and NP in undamaged host tissue (Figures 9&12). NP fibers and cell bodies adjacent to implant tissue frequently ended abruptly and did not enter the implant (Figure 13). Although Nissl staining demonstrated neuronal growth throughout the implants, only implanted tissue from the second experiment had cell bodies within the implant which showed ICC staining for NP or LHRH. Two NP cell bodies were seen within

cerebellar-implanted tissue in one animal (Figure 16), and one LHRH cell body was observed in MBH tissue.

Fibers Within the Implant (Tables IV and V)

Experiment #1 - There were five MBH and six cerebellar implants which were large enough to section and search for ICC- stained fibers. Because implants of different sizes had unequal number of sections, the data are expressed as numbers of fibers per section. One MBH implant contained a tangle of NP-stained fibers. Using Grubb's outlier determination, this tissue was eliminated from the calculation of the results. The mean number of fibers per section stained for both NP and LHRH did not differ significantly between MBH and cerebellar implants (Table IV) according to the t-test.

Experiment #2 - There were five surviving animals with MBH implants and six surviving animals with cerebellar implants. The number of fibers within MBH implanted tissue that were stained for both LHRH and NP was compared with the number of fibers stained for both hormones within the cerebellar implants (Table V). A t-test did not reveal significant differences in the number of fibers per section. In one brain, many LHRH fibers were observed streaming between the subfornical organ in the host's tissue and the adjacent implant (Figure 10A) and were clearly localized within the implanted tissue (Figure 10B).

GFA Immunocytochemistry

No significant differences were found in the number of GFA-producing cells between lesioned and nonlesioned rats in any of the areas compared. Histological examination revealed little or no subependymal glial limiting membrane around the implants except where the implant was in direct contact with CSF within the ablation cyst. No glial border was found between the implant and the host when they were in direct contact (Figure 17).

# DISCUSSION

The results of this study showed that fetal tissue, either MBH or Cbl Cx, survived and grew when transplanted into a cyst produced around the third ventricle of adult animals, but that the transplants did not compensate for neuroendocrine abnormalities created by the ablation procedure. The conclusions that the lesion created functional deficits and that these deficits were not ameliorated by implantation are based on the physiological data on body weight, water intake, blood plasma levels of GH and PRL and by anatomical observations using ICC.

In exp. #1, the body weights of animals in the MBH implanted group were above those in the intact group but not significantly different from the Cbl Cx-implanted group. This result suggested that the lesion disrupted the functional competence of an area regulating satiety reportedly located within the MBH (Anand and Grobeck, 1951; Urman et al., 1985), and that the function of this brain area was not replaced by implanted MBH tissue. The three groups did not differ significantly in body weight in exp. #2, probably due to the large variances around the means which may reflect differences in the location of the lesions.

The water intake in both experiments was greater for the implanted groups than for the intact control animals. This result, along with the lack of differences between the two implanted groups, suggests that the lesion produced symptoms of DI, and that implanted

MBH tissue did not reverse the deficit. (The lesioned animals in both experiments showed the symptoms of DI although many VP-NP staining cells remained in undestroyed portions of the SO and magnocellular PVN.) In exp. #2, there was a transient reduction in water intake nine weeks after implantation which is difficult to interpret. The intact control group also showed decreased water intake during the ninth week, but their intake remained at the lower level, while the implanted groups showed a decrease only at nine weeks. It is possible that the implanted groups showed a transient release or production of VP. Transient release of VP in normal fetal tissue transplanted into Brattleboro rats has been reported by Richards and Raisman (1984). Richards and Raisman found a 50% decrease in urine volume and a corresponding increase in urine osmolarity for the first two weeks after implantation. The recovery occurred whether or not the transplant contained surviving hypothalamic tissue. Future experiments might examine peptide production within the implant at regular intervals after implantation to assess the possibility of transient hormone production. The cell bodies staining for VP-NP in Cbl Cx tissue in exp. #2 suggests that cerebellar tissue implanted into hypothalamus may be capable of neuroendocrine plasticity. Plasticity has been described for motilin-containing Purkinje cells transplanted into hypothalamus (Nilaver et al., 1984; Perlow et al., 1984).

Nonstress and stress GH levels in implanted animals in both ex-

periments were lower than GH levels in the intact group, suggesting that the ablation disrupted hypothalamic neurons which contained GHRH (Merchenthaler et al., 1984). There was no significant difference in post-implantation nonstress GH levels between the MBH and Cbl Cx groups, indicating that the MBH tissue did not restore GHRH function. Hypothalamic motilin also has been shown to have an effect on growth hormone similar to GHRH (Samson et al., 1984) and because motilin staining has been shown in Cbl purkinje cells (Nilaver et al., 1982), it is possible that there was some return of function in the MBH tissue which was masked by a similar increase in the Cbl Cx-implanted group. However, this putative recovery would be only partial because nonstress GH levels in both transplanted groups were significantly depressed below nonstress GH levels in the intact controls. Stress levels of GH in implanted animals, in both experiments, were significantly lower than stress GH levels in the intact animals which may indicate that without the positive influence of GHRH the inhibiting influence of SRIF outside the periventricular area may exert a greater effect. Given this assumption, the lack of differences between the two implanted groups supports the lack of functional GHRH replacement in the MBH-implanted animals.

The elevated nonstress PRL levels in exp. #2 in the ablated animals were probably due to the destruction of terminals releasing PIFs such as dopamine, GAP, and GABA (Leong et al., 1983; Nikolics et al. 1985). Again, implantation did not effect a recovery and there

were no significant differences between the two implanted groups. The lack of significant differences in the nonstress and stress levels in exp. #1 and stress levels in exp. #2 are probably due to the large variation in the data. PRL stress response levels in exp. #2 in the ablated groups were similar to values found in the intact group. These stress levels may be due to a removal of the PIF's normally inhibiting PRL release which may leave putative PRF's unopposed.

The increased number of diestrous days in the ablated groups in both experiments indicates a disruption of cyclicity unrelieved by implantation.

The lack of physiological recovery, suggests a lack of functional competence of the implanted tissue. The transplants grew, but the paucity (as shown by ICC) of the peptides VP-NP and LHRH are consistent with the physiological data and, taken together, these data suggest that there was no functional recovery.

Anatomical evidence for neuroendocrine replacement by fetal transplants was minimal. Only three cell bodies were seen in these experiments; one cell body stained for LHRH in MBH transplanted tissue and two cell bodies stained for VP-NP within a cerebellar implant. These three cell bodies were found in the experiment in which fetal tissue was transplanted 7 days after lesioning. A comparison of the number of fibers staining for LHRH and VP-NP in the MBH-implanted animals showed no significant differences from those in

Cbl Cx implanted animals in either experiment. It was not possible to determine whether these fibers came from cell bodies within the implant or from the host tissue. The small number of peptidergic cell bodies found in implanted tissue suggests that the fibers probably originated in the host tissue, which implies that the implant acted as a fiber bridge, as seen in studies of hippocampal transplants (Bjorklund and Stenevi, 1984).

The growth of transplanted tissue accompanied by an abscence of anatomical or physiological evidence of peptide production in cells within the transplant was unexpected. Several explanations could account for lack of neuroendocrine peptide production and function within the implant: 1. It is possible that the implants were not from the MBH; 2. The cells which produce peptides might have been crowded out or anatomically restricted by fibrous astrocytes; 3. Productive cells may have grown or differentiated and then degenerated or dedifferentiated over time; 4. Endogenous factors in the host may have inhibited peptide production or neuronal differentiation in the implanted tissue.

It is difficult to show conclusively whether the implanted tissue came from fetal MBH because histological examination does not clearly resolve the source of the implanted tissue. Two implants identified as MBH show a lamellar pattern more indicative of hippocampal structures but the majority of MBH implants do not have distinguishing features. In this study, tissue from the fetal animal

was removed by the suction of a glass SMI pipette tip. In future experiments, fetal tissue might be dissected from a coronal block which includes the area intended for implantation. Such a techniqued has been used successfully by a number of investigators (Gibson et al., 1984; Scott and Sherman, 1984).

ICC directed against GFA was used to assess the relative number of fibrous astrocytes. If the protoplasmic astrocytes normally found in the fetal tissue received an "injury message " which transformed them to reactive (fibrous) astrocytes (Azmitia and Whitaker, 1983; Bignami and Dahl, 1974a), they could have crowded neuronal cells or formed a glial scar preventing the formation of connections between the host and implant. However, no significant differences were found in the relative number of GFA-producing cells between lesioned and nonlesioned rats in any of the areas compared, suggesting that glial transformation was not a significant factor in causing the lack of neuroendocrine development of the transplants.

It is difficult to assess whether the transplants contained cells which produced peptides, but which eventually degenerated. A subsequent experiment could be planned to look at implant growth and productivity over more points in time to decide whether immunoreactive, peptidergic cells show a peak and then a loss of productivity. However, after 8 months of implantation, histological examination revealed vascularization within the central portions of even the largest implants and histological observation suggested that

degeneration was not a general feature of the implants.

Negative feedback from endogenous peptidergic cells in host tissue might inhibit neuronal differentiation and/or production of peptides in implanted fetal tissue. ICC for LHRH and VP-NP in this study revealed many LHRH and NP-VP productive cells that were undestroyed in the host brains. Such a negative feedback mechanism may explain the lack of success in experiments that attempt to replace VP in homozygous Brattleboro rats genetically deficient in VP (Scott et al., 1984; Sladek et al., 1984). In normal animals, the VP gene contains 3 exons coding for the prohormone propressophysin (Russell et al., 1980) which is proteolytically cleaved to produce VP (coded on exon A), a NP (largely coded on exon B), and, at the carboxy terminus, a glycosylated peptide coded on exon C (Richards et al., 1985). The deficiency of VP in the homozygous Brattleboro mutant is attributed to a single base deletion in exon B (Schmale and Richter, 1984). The propressin made in the Brattleboro is identical to the normal one in the amino acid sequence from the N-terminus to the deletion site, but the base deletion results in a mRNA lacking the glycosylation site, the five cysteine residues which may affect the secondary folding of molecule and the signal which normally terminates protein synthesis (stop codon) (Richter and Schmale, 1984). Yet, despite these changes, it has recently been reported that the magnocellular neurons of the Brattleboro rat contain VP in very small amounts (1 cell in 583) and this small number of cells can be

detected by ICC (Richards and Raisman, 1984; Richards et al., 1985). Less frequently, Herring bodies are found in the posterior pituitary of Brattleboro rats. The lack of success in replacing VP production by implanting Brattleboro rats with normal fetal tissue might be explained by the fact that the small fraction of endogenously produced VP may interfere with the production of VP in transplanted tissue or the propressin produced might inhibit VP production without its tertiary structure.

The success of transplantation in restoring function in the genetically LHRH deficient hpg mice might be due to the absence of a negative feedback system in the hpg mouse. The gene for hypothalamic LHRH has been isolated (Adelman et al., 1986), and more recently it was found that in the hpg mouse there is a 33 kilobase deletion in the DNA coding for the precursor protein for LHRH (Adelman et al., in press). The magnitude of this deletion would probably result in a foreign protein that would be degraded before it reached the cell cytoplasm (John P. Adelman; personal communication). A future experiment to test this hypothesis might be to implant magnocellular PVN and SON cells from fetal mice into the hpg mouse and implant POA cells from these same donor fetuses. A lack of ICC staining for VP-NP in the implant in these test animals combined with normal development of LHRH-containing cells might suggest the existence of negative feedback mechanisms that control neuronal differentiation. Further studies could correlate the DNA, RNA and proteins of these

transplanted VP cells to determine whether the genetic message is present but not expressed as a protein.

In conclusion, measures of physiological and behavioral paramenters indicated that the ablation procedure disrupted the MBH functions tested, that MBH implants did not ameliorate the created deficits and that animals implanted with fetal Cbl Cx tissue did not respond differently from animals implanted with MBH tissue. In addition, despite the increased size of those MBH implants implanted 7 days after lesioning over those implanted 129 days after lesioning, ICC revealed little or no evidence of VP-NP or LHRH production in these tissues. This study demonstrates the difficulty of simultaneously correcting a number of deficits. The study is further complicated by the presence of a surgically created cyst and the presence in the host of those substances which are being supplemented by the implanted tissue. Further experiments are needed to define the factors critical for transplant success.

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## APPENDIX A - TISSUE PREPARATION

Tissues were fixed in a variety of ways to determine the fixation method for ICC which was most compatible with the antibodies being used and with the vibratome sectioning technique. The fixatives compared included 3% paraformaldehyde/2% glutaraldehyde, 5% acrolein, 4% paraformaldehyde, Zamboni's Fixative and 10% neutral buffered formalin (King et al., 1983; Pearse, 1980; Piekut, 1983). Results of these preliminary experiments showed that the most effective procedure for the ICC technique in use is fixation by immersion in neutral buffered formalin. The ABC method (Vectastain, Vector Laboratories) was compared to the PAP method (Sternberger, 1979). Within the conditions of this experiment the PAP method gave superior results.

## APPENDIX B - DISCUSSION OF ICC TECHNIQUE

Our laboratory uses the unlabeled antibody enzyme method developed by Sternberger (1979). This technique visualizes the antigen of interest by utilizing a sandwich of antibodies. The first antibody, or primary antibody, is an IgG made against the antigen of interest in a species other than the test animal. Rabbits were used to make IgG against the rat hypothalamic peptide hormones. Our GFA antibody was also made in rabbits. The second antibody, or sandwich antibody, is made against the constant region (Fc fragment) of rabbit IgG. The constant region of an IgG antibody is the same for all IgG

molecules made in one species. IgG antibody molecules are Y shaped with the constant region occupying the tail of the Y and the distal ends of the forked portion contain a reactive variable region (Fab fragments). There are two identical variable regions to each antibody molecule and it is in this region of the molecule that the antigen is recognized.

When primary antibody is applied to the tissue, its Fab fragments attach to the antigens of interest and the Fc fragment remains unattached and exposed. Since the second antibody has been made to the rabbit's IgG, the second antibody's Fab portion recognizes the Fc portion of the first antibody and attaches to it. However, all Fab sites are not taken up and some will remain exposed after the application of the second antibody. In the next step, a third antibody is made in the same animal as the first. In our case, a rabbit is used. This third antibody is raised against the peroxidase molecule and its Fab fragments are flooded with and taken up by peroxidase molecules before it is applied to the test tissue. The Fc fragment of this third antibody (otherwise known as the PAP complex) remains free and can react with the unoccupied Fab portions of the second antibody already bound to the tissue (Heitz, 1982; Sternberger, 1979).

The final reaction in our immunocytochemical process is the peroxidase reaction of Graham and Karnovsky (1966). Tissues are incubated in diaminobenzedine (DAB) and hydrogen peroxide. DAB serves as the electron donor to the HRP-hydrogen peroxide complex which un-

dergoes oxidative polymerization and cyclization during the reaction to form the phenazine polymer or final reaction product. The phenazine has a golden brown color. Pretreatment with nickel ammonium sulfate produces a black colored product.

Non-specific staining can be a problem with this ICC technique. Non-specific staining can be reduced by using high affinity antiserum. An antibody with a high affinity for its antigen is less likely to bind at undesired sites. Purified polyclonal or monoclonal antibodies can be used to decrease the problem. Background can also be reduced by blocking reactive sites on tissue sections by using normal serum in the reaction buffer. The normal serum should be from the same species as the one used to produce the second antibody. The normal serum may contain antibodies which can bind nonspecifically to sites found endogenously in the tissue. Since these antibodies are of low affinity and have a low avidity for their binding sites, they can be removed during the final wash processes before incubating the tissues with DAB. Non-specific staining was not a problem in our experiments.

TABLE I: F and p values and degrees of freedom (df) for ANOVA comparing levels of plasma growth hormone between the three experimental groups on untransformed post-implant data.

| | Experiment #1 | | Experiment #2 | |
|---|---|---|---|---|
| | Nonstress | Stress | Nonstress | Stress |
| F | 44.14 | 25.43 | 14.82 | 5.46 |
| p | <.05 | <.05 | <.05 | <.05 |
| df | 2 | 2 | 2 | 2 |

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TABLE II: F and p values and degrees of freedom (df) for ANOVA comparing levels of plasma prolactin between the three experimental groups on untransformed post-implant data.

| | Experiment #1 | | Experiment #2 | |
|---|---|---|---|---|
| | Nonstress | Stress | Nonstress | Stress |
| F | 1.28 | 2.66 | 29.66 | 0.38 |
| p | not sig. | not sig. | <.005 | not sig. |
| df | 2 | 2 | 2 | 2 |

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TABLE III: SIZE OF IMPLANT AT TIME OF PERFUSION (p<.05)

| | | N | $\overline{X}$ | S.D. |
|---|---|---|---|---|
| MBH | exp.#1 | 8 | 2.6mm$^2$ | $\pm$2.4 |
| | exp.#2 | 5 | 7.6mm$^2$ | $\pm$4.1 |
| CBL CX | exp.#1 | 6 | 2.8mm$^2$ | $\pm$1.9 |
| | exp.#2 | 6 | 3.8mm$^2$ | $\pm$2.4 |

TABLE IV: FIBERS PER SECTION IN EXPERIMENT #1

| | N | $\overline{X}$ | S.D. |
|---|---|---|---|
| MBH (LHRH & NP) | 9 | 1.18 | ±0.83 |
| Cerebellar (LHRH & NP) | 12 | 0.53 | ±0.86 |
| LHRH (MBH & Cerebellar) | 11 | 0.69 | ±0.65 |
| NP (MBH & Cerebellar) | 10 | 0.94 | ±1.12 |

TABLE V: FIBERS PER SECTION IN EXPERIMENT #2

| | N | $\overline{X}$ | S.D. |
|---|---|---|---|
| MBH (LHRH & NP) | 12 | 2.26 | ±2.25 |
| Cerebellar (LHRH & NP) | 10 | 2.38 | ±2.32 |
| LHRH (MBH & Cerebellar) | 11 | 2.40 | ±2.24 |
| NP (MBH & Cerebellar) | 11 | 2.23 | ±2.37 |

TABLE VI: The number of diestrous days counted in three estrous cycles in experiment #1 and in four estrous cycles in experiment #2. The number of diestrous days were compared between ablated and intact animals in each experiment.

| Experiment #1 | N | $\overline{X}$ | S.D. |
|---|---|---|---|
| Ablated | 16 | 11.7 | $\pm 3.8$ |
| Intact | 9 | 4.4 | $\pm 3.8$ |
| **Experiment #2** | | | |
| Ablated | 11 | 13.3 | $\pm 2.2$ |
| Intact | 8 | 7.6 | $\pm 2.3$ |

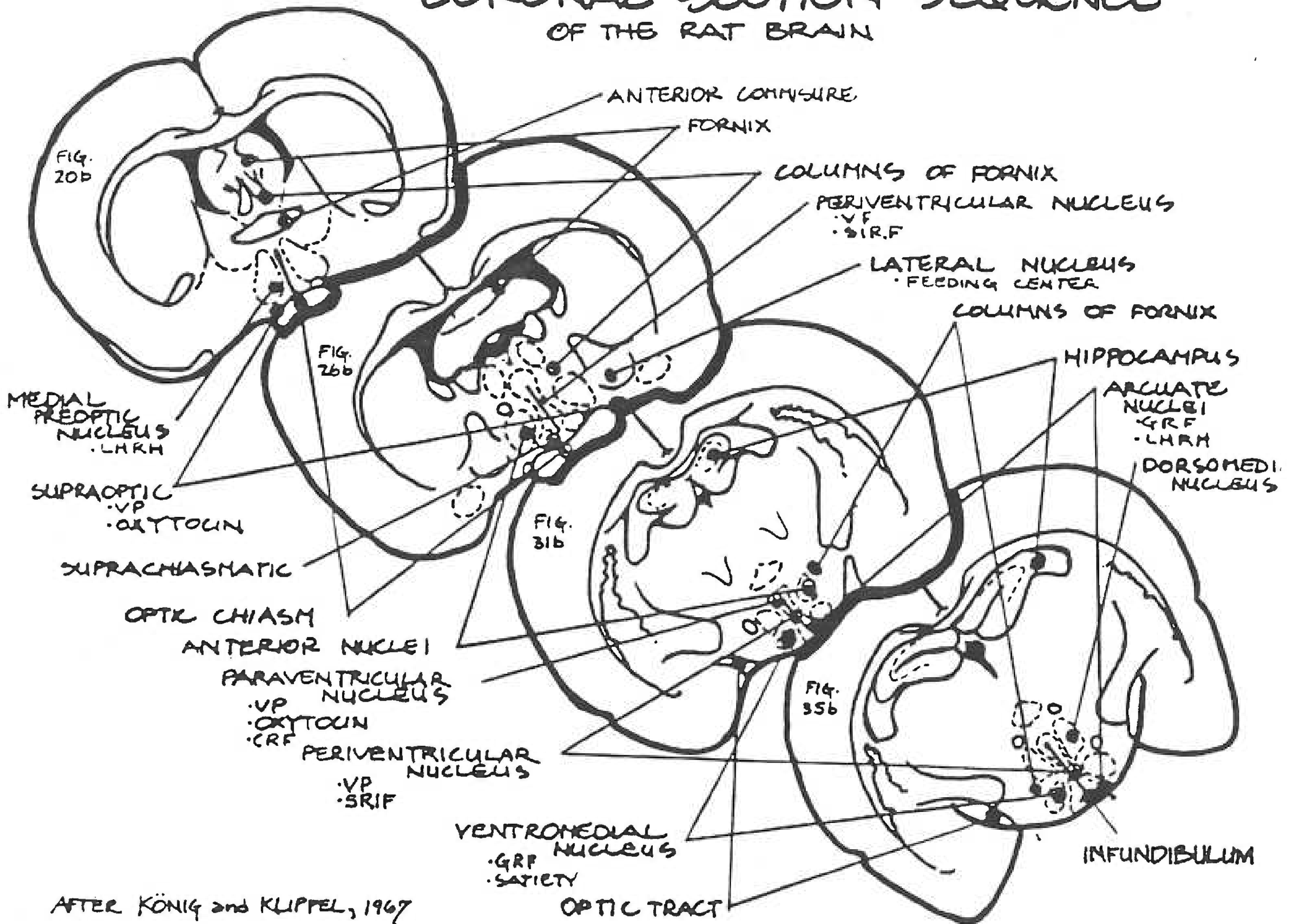



Figure 1. The location of LHRH, VP, Oxytocin, GRF and SRIF in rat brain diagrammed in representative sections from the preoptic area to the median eminence.

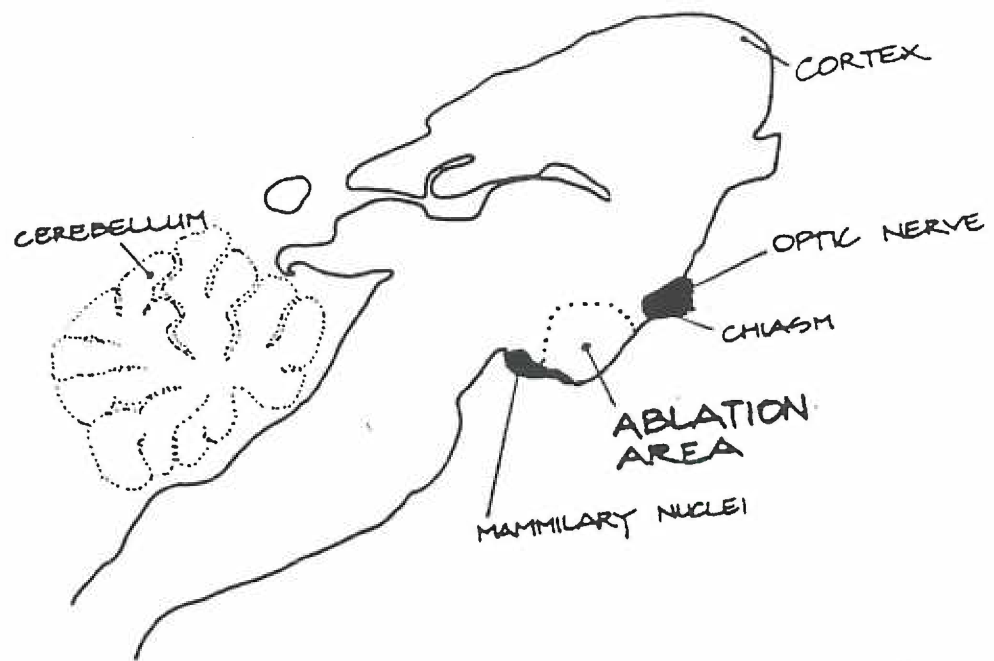



Figure 2. Drawing of a sagittal section of rat brain which illustrates the rostral and caudal borders of the ablation area.

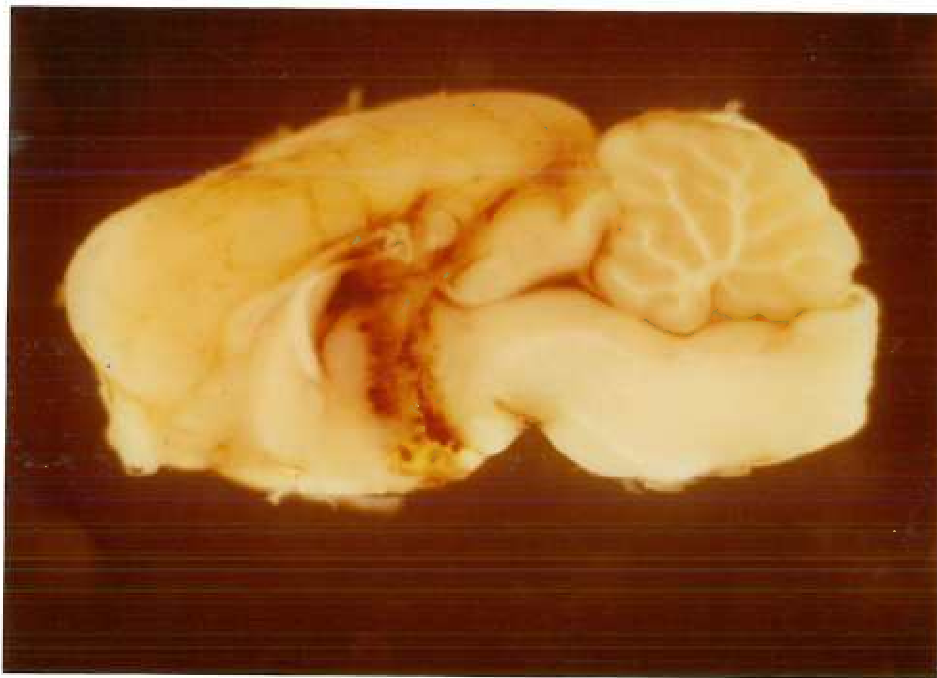
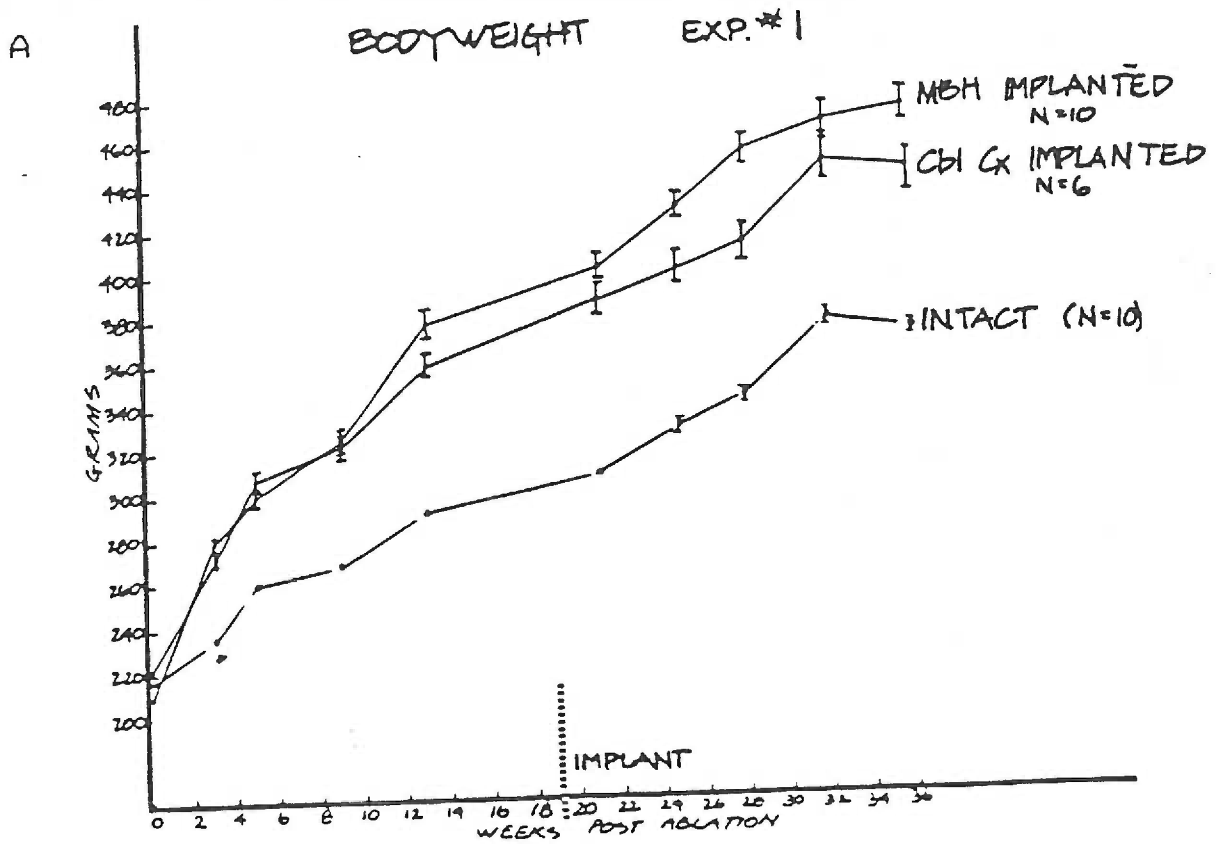


Figure 3. Sagittal section of rat brain which reveals the ablation track through the cortex and thalamus. The ablation cyst extending from the preoptic area caudally to the caudal extent of the premammilary area is highlighted within the hypothalamus.



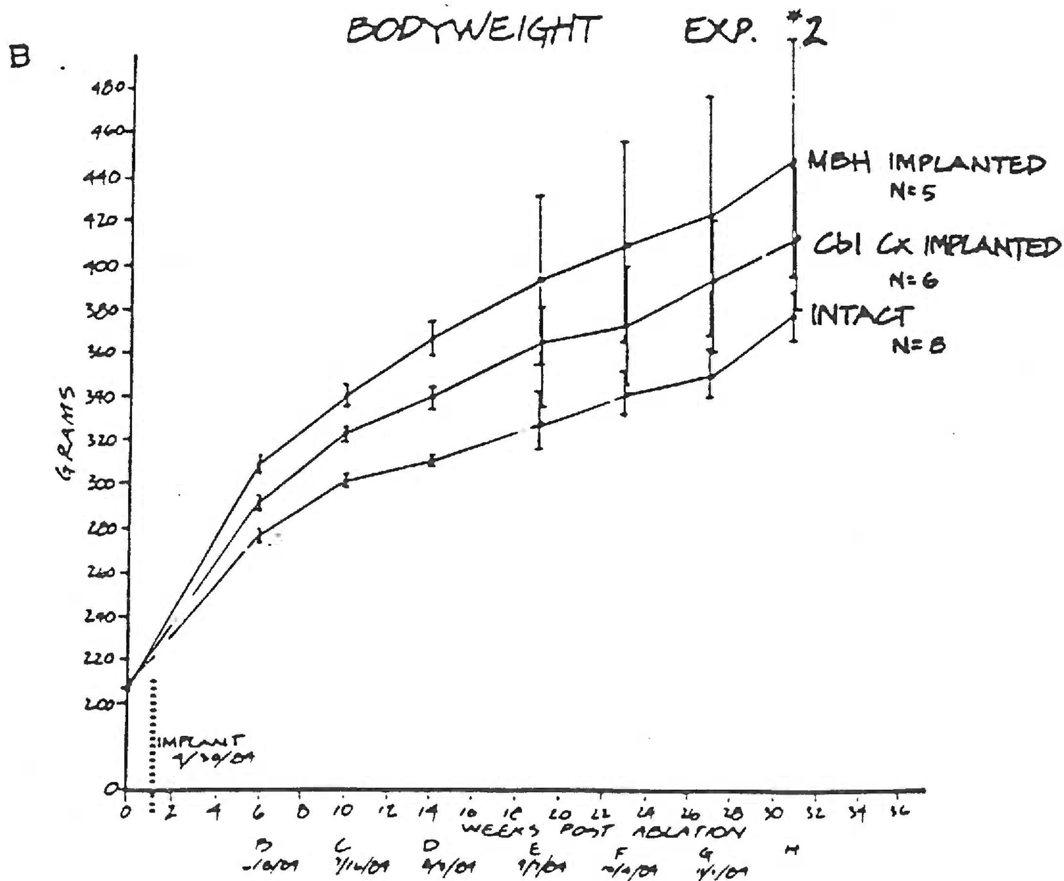
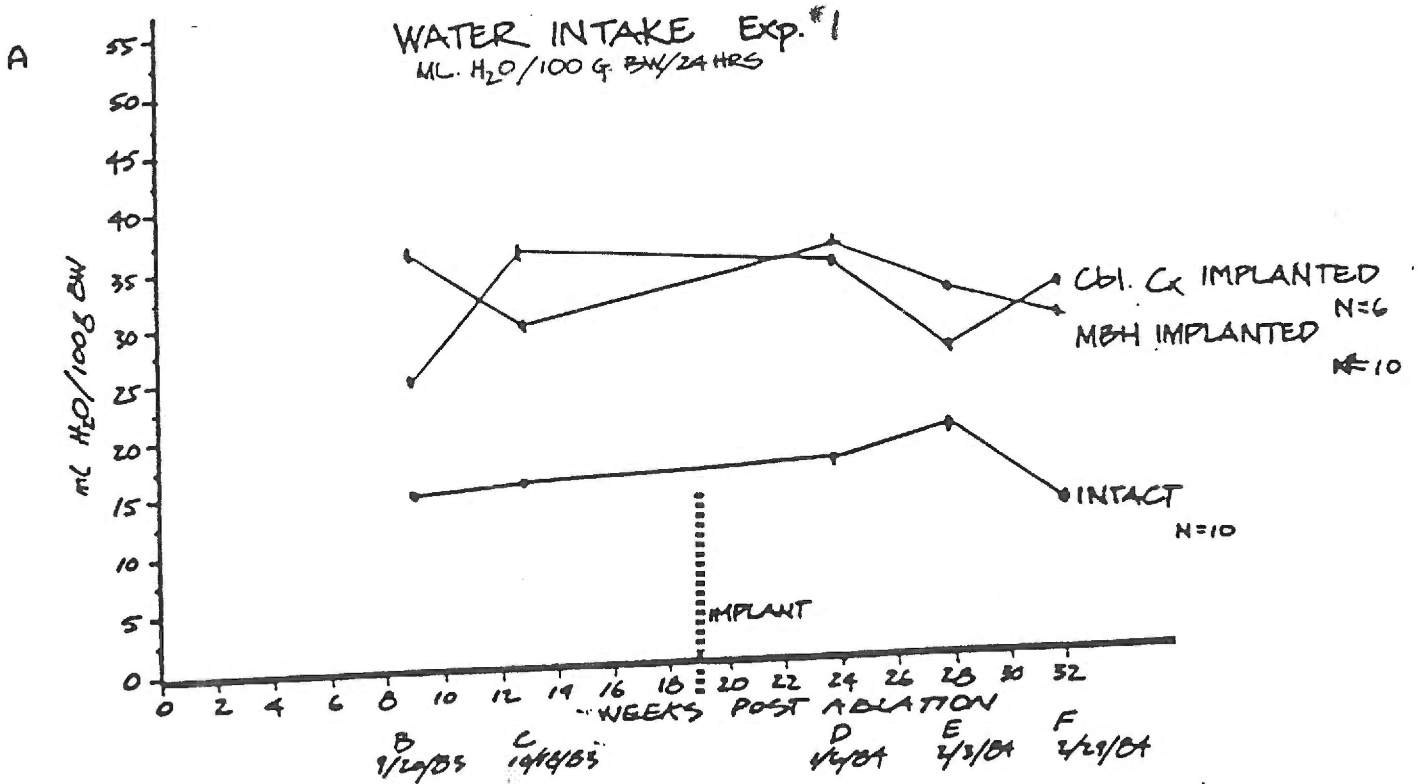




Figure 4. Line graphs depicting the average body weights for each experimental group at different time points. Verticle lines at each point represent the standard error of the mean. In some cases the line representing the standard error does not extend beyond the drawn point.
A. Experiment #1. B. Experiment #2.



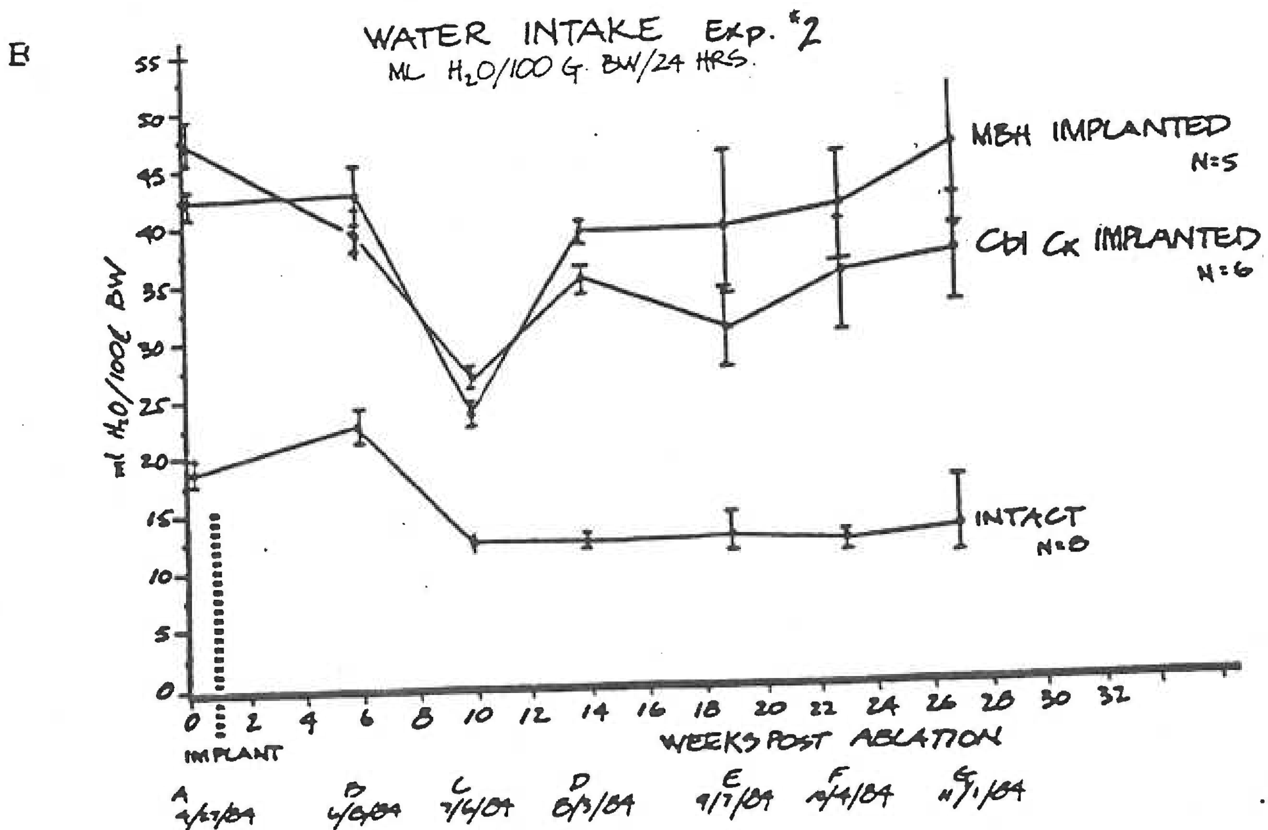
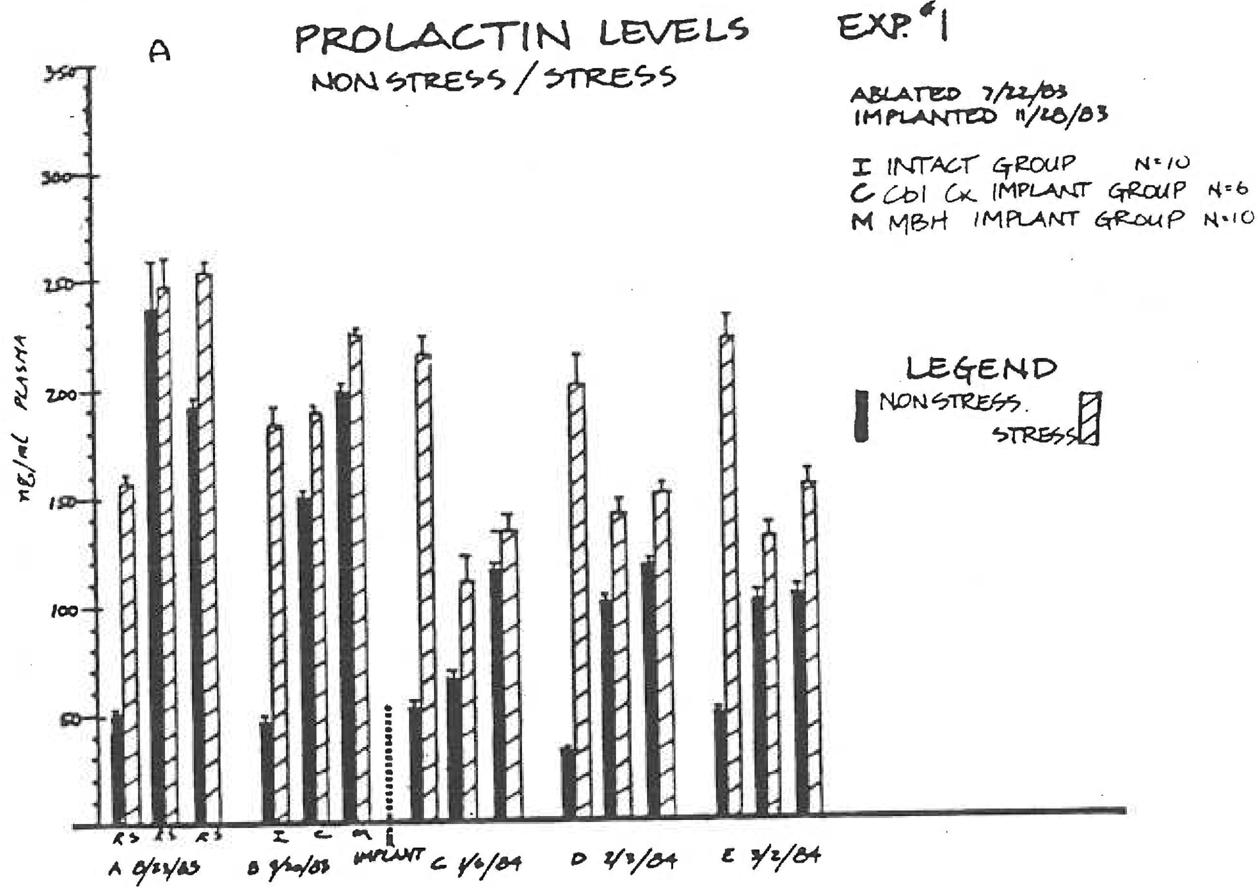




Figure 5. Water intake expressed as ml water per 100 grams body weight: Group mean ± standard error of the mean.
A. Experiment #1. B. Experiment #2.

Figure 6. Blood plasma levels of growth hormone ± standard error of the mean. A. Experiment #1. B. Experiment #2.



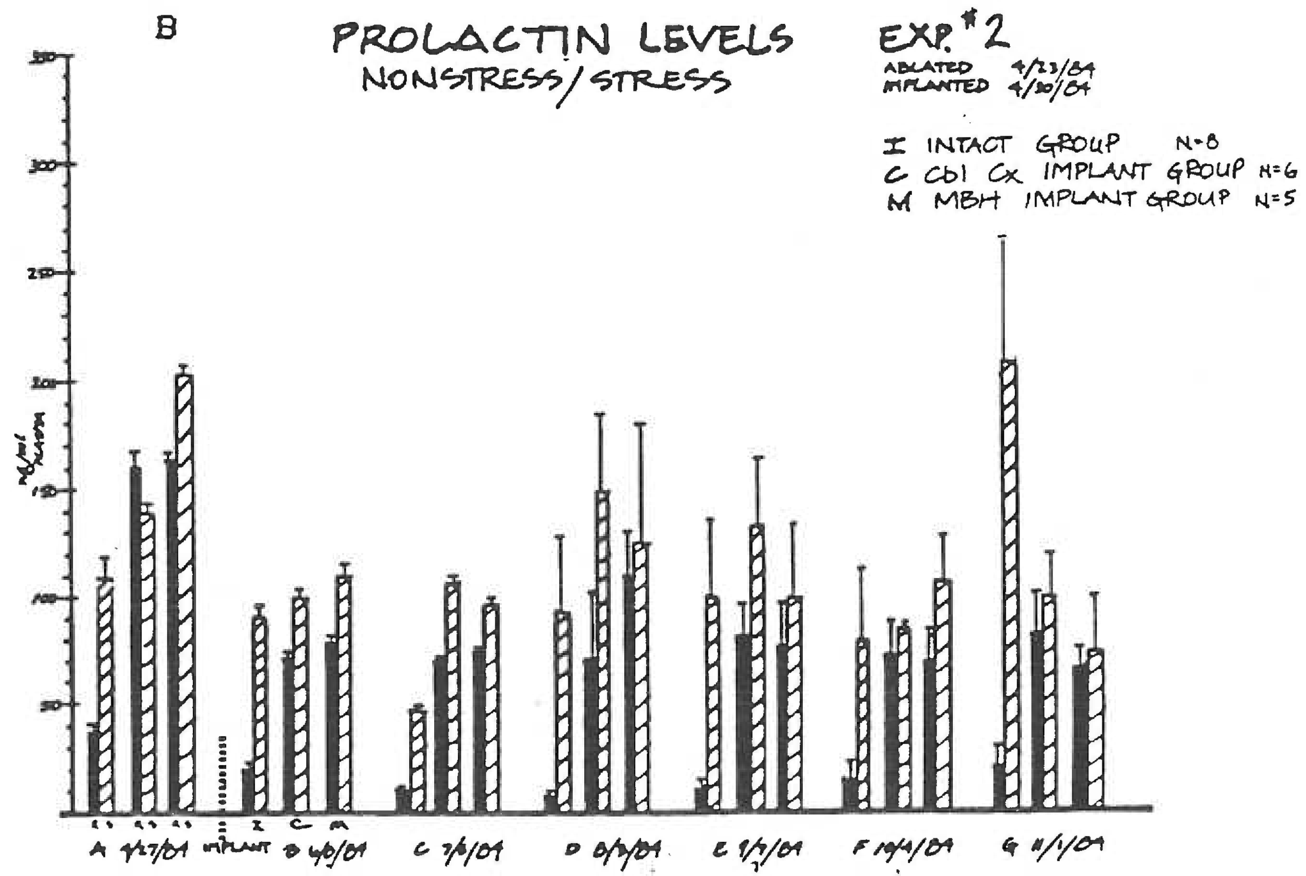




Figure 7. Blood plasma levels of prolactin ± standard error of the mean. A. Experiment #1. B. Experiment #2.

Figure 8. Vibratomed section of an uncolchicined, normal, female rat brain immunocytochemically stained with neurophysin. Portions of the paraventricular nucleus around the third ventricle, and portions of the supraoptic nucleus are shown.

Figure 9. Vibratomed section of the median eminence in an uncolchicined, normal, female rat brain immunocytochemically stained for LHRH. Numerous LHRH-containing fibers are seen in the zona externa.

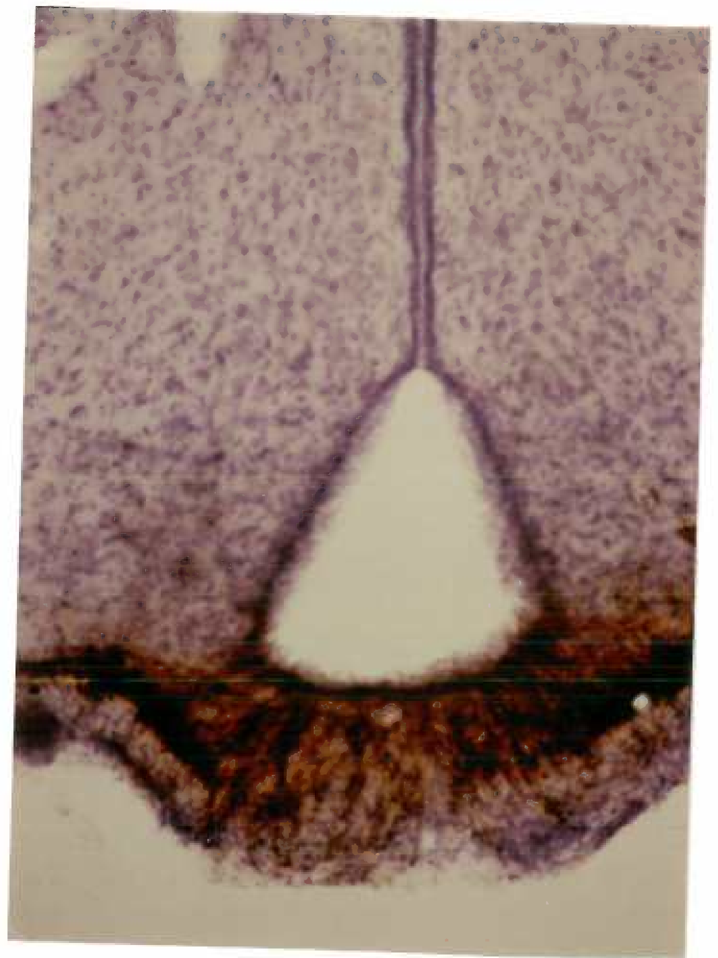


Figure 14. Photograph of implanted cerebellar tissue which has been immunocytochemically stained for NP. The implanted tissue has seperated into two pieces. The ventral piece has a laminar structure associated with normal cerebellar cortex and the dorsal piece shows a more homogenous structure which could be associated with deeper cerebellar tissue.

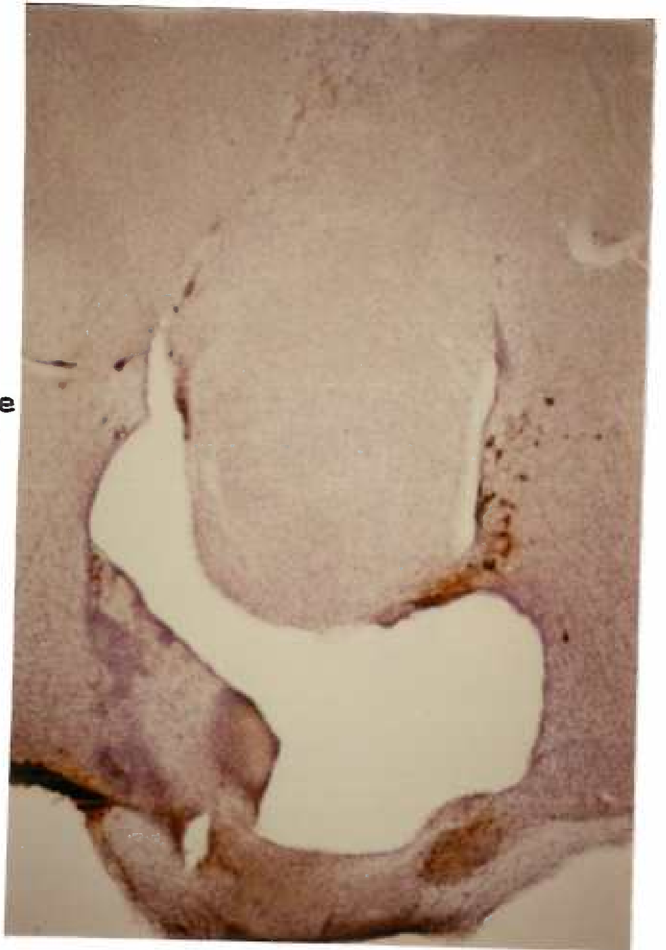
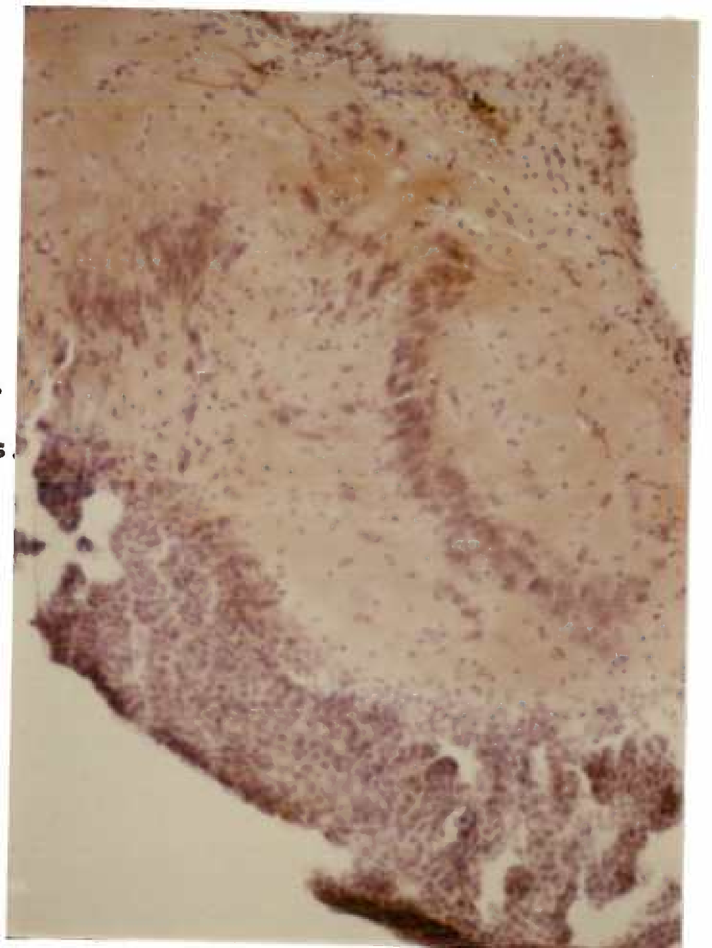


Figure 15. Photograph of implanted cerebellar tissue which has been immunocytochemically stained for NP. Note normal histological appearance of transplanted fetal choroid plexus.



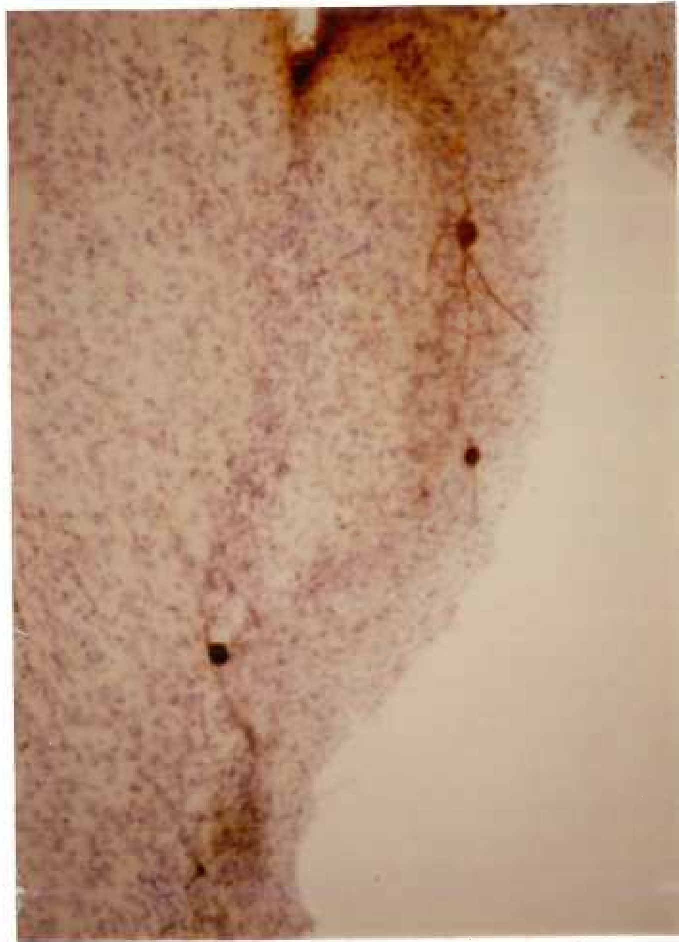


Figure 16. Photograph of cerebellar cortex-implanted tissue in rat brain which has grown into but not filled the ablation cyst around the third ventricle. Note ependymal-shaped cells lining the fetal tissue which is in contact with cerebral spinal fluid. Two cell bodies stained for NP are seen within the implanted tissue (counterstained with thionine and nickel enhanced).

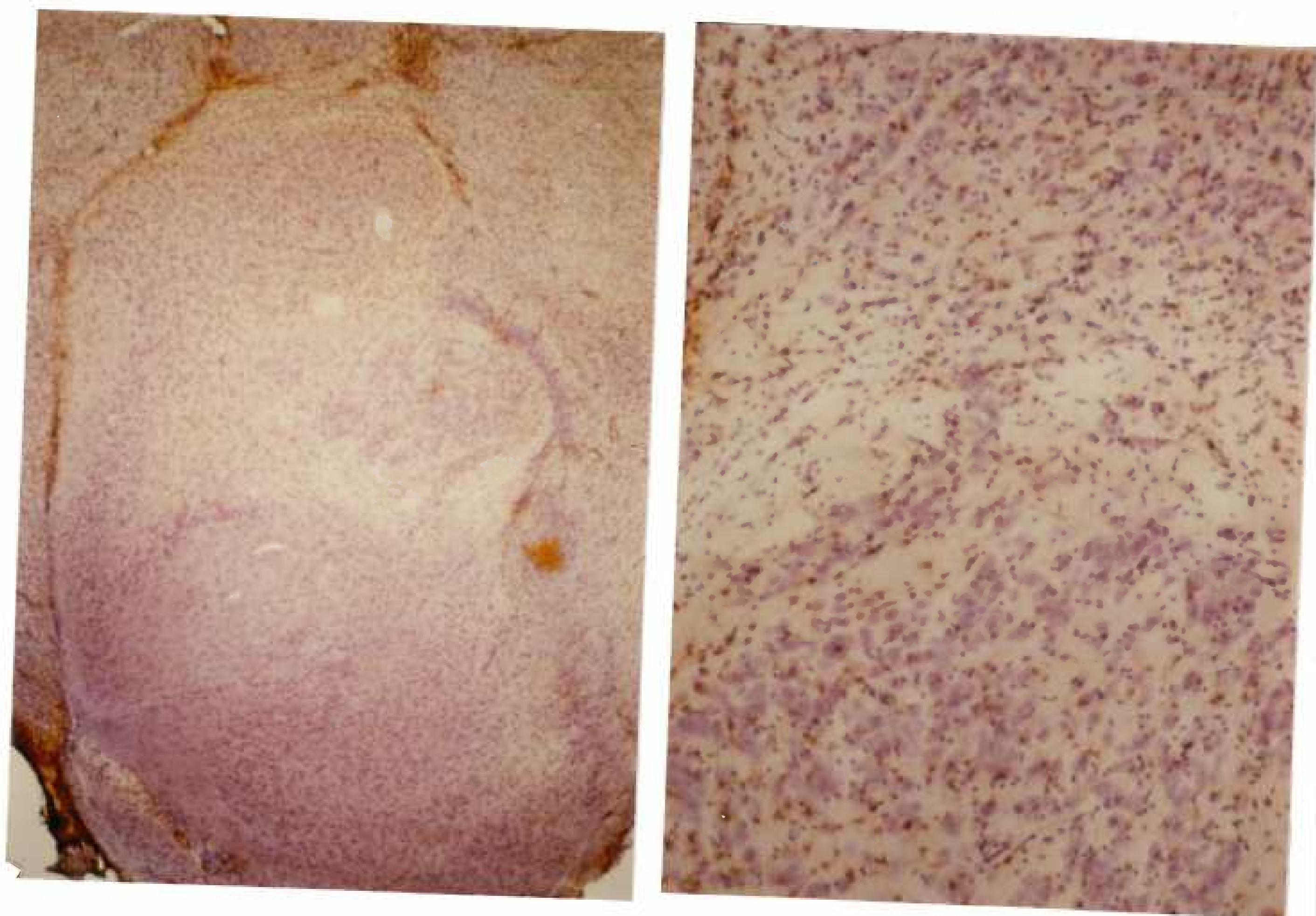


Figure 17. A. Fetal MBH-implanted tissue which has been stained for GFA. A stained, discontinuous glial border can been seen on the left, between host and implant but is not present one the right. Note the vascularization within the implant (arrows) and the minimal presence of gliosis. B. Higher magnification of fetal MBH-implanted tissue stained for GFA. Again, note the lack of heavy gliosis.