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NEUROPLASTICITY IN ADULT HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM: MECHANISMS INVOLVED IN RESPONDING TO A SINGLE SYSTEMIC HYPERTONIC SALINE INJECTION

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D degree in Neuroscience-Psychology

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Date 9 December 1991

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By

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A DISSERTATION

Submitted to Michigan State University as partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Psychology and Neuroscience Program

1991

ABSTRACT

NEUROPLASTICITY IN ADULT HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM: MECHANISMS INVOLVED IN RESPONDING TO A SINGLE SYSTEMIC HYPERTONIC SALINE INJECTION

By

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Neurosecretory neurons of the supraoptic nucleus (SON) which manufacture, transport and secrete neuropeptides are activated by administration of hypertonic NaCl.

Experiment I. Rats were injected with isotonic (0.15M) or hypertonic (1.5M) NaCl solution and sacrificed 5 h later. Electron micrographs were compared to determine resulting morphological differences in SON and posterior pituitary. In SON, in rats injected with 1.5M NaCl, extent of glial contact with magnocellular neuroendocrine cell (MNC) membrane decreased; terminal contact and apposition of MNCs with somata or dendrites increased. Size of nucleoli, cell bodies and amount of Golgi apparatus were larger in animals injected with 1.5M NaCl. Pituitaries of animals receiving 1.5M NaCl showed increased neural contact with basal lamina (BL) and decreased cytoplasmic enclosure of terminals. Large changes induced by 1.5M NaCl suggested it is an effective activator of morphological plasticity in the hypothalmo-neurohypophysial system (HNS).

Experiment II. Rats were injected and sacrificed after 15 min, 1 h or 2.5 h. Resulting changes in SON were smaller; only Golgi apparatus increased over time. In pituitary, terminal apposition with BL for

animals sacrificed 2.5 h after receiving 1.5M NaCl increased above control levels, but below 5 h means from Experiment I. Results suggest limited responses from HNS after short time intervals.

Experiment III: To assess stress-related, versus osmotic effects, rats were anesthetized or adrenal-medullectomized (MDX) prior to injections, and sacrificed 5 h later. Anesthesia caused a decreased response to 1.5M NaCl in all elements measured in SON and neural lobe. Effects of MDX appeared more specific. Changes in measures of glial coverage, produced by hypertonic injections, such as amount of glial versus neural or somatic apposition with MNC membrane and amount of pituicyte apposition with the BL, that were profound in intact rats, were eliminated by MDX. Golgi apparatus, correlating with neuropeptide production, was increased by 1.5M NaCl in MDX rats. Results of Experiment III suggest multiple means of activation of the HNS.

This thesis is dedicated, with gratitude, to my advisor, Dr. Glenn I. Hatton, who gave me the opportunity to return to graduate school, and to my husband Dr. Walter K. Beagley, who put up with me while I did.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Glenn Hatton, and my committee members Dr. Antonio Nunez, Dr. Cheryl Sisk and Dr. Charles Tweedle for their assistance throughout this endeavor. Thanks also go to Louise Koran and Pamela Rusch for their technical help, as well as to Todd Almli for the hours he spent in the darkroom. Without the support of my colleagues Farshid Marzban, Barb Modney, Ken and Inge Smithson, Lee Ann Berglund, Yu Ping Tang, Kris and Mike Kashon, Alan Elliott, Janie Smith and many others, the atmosphere at PRB would never have been conducive to my completion. And finally, I must acknowledge the help from my family, Wally, Nat and especially Douglas and Janet Beagley who added new meaning to the concept of tallying data in truly blind control conditions.

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ABBREVIATIONS

ANOVA -	Analysis of Variance
BL -	Basal Lamina
CRH -	Corticotrophin releasing hormone
HNS -	Hypothalamo-neurohypophysial system
MDX -	Adrenal medullectomized
MNC -	Magnocellular Neuroendocrine Cell
mRNA -	Messenger RNA
NaCl -	Sodium chloride solution
OX -	Oxytocin
PVN -	Paraventricular nucleus
SON -	Supraoptic nucleus
TEM -	Transmission electron microscope
VP -	Vasopressin

INTRODUCTION

The hypothalamo-neurohypophysial system (HNS) is a valuable model with which to study an organism's interaction with its environment as reflected by changes in its central nervous system. Because the physical structure and functions of the HNS are relatively well known, it is possible to identify specific stimuli that activate the HNS and to measure resulting changes in a variety of ways.

The physical structure of the system consists of magnocellular neuroendocrine cells (MNCs), with cell bodies located in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus. These MNCs manufacture and transport neuropeptide hormones down axons which project through the median eminence to the neurohypophysis (Bargmann and Scharrer, 1951). In the posterior pituitary, these axons terminate near the basal lamina (BL)(Bargmann, 1957), a layer of connective tissue which separates the neural compartment from the perivascular space, surrounding fenestrated capillaries (Peters, Palay and Webster,1991). Astrocytic glial cells, called pituicytes, also occupy the BL and surround the MNC terminals. When these terminals are depolarized, they release peptides that enter the blood stream. These peptides have been identified as oxytocin (OX), vasopressin (VP), and dynorphin which is co-localized and co-released

with vasopressin but which may not enter the blood in substantial amounts (For a complete review, see Hatton, 1990).

The HNS has been examined in many ways. Stimulus conditions that increase the manufacture and release of the peptides have been studied, as have peptide synthesis, secretion, and peripheral responses, especially changes in blood plasma levels of the neuropeptides. Electrophysiological investigations have helped to characterize the different types of neurons in the HNS and the distinctive firing patterns of activated MNCs. Finally, with the use of electron microscopic technology, morphological changes in the relationships of the basic cellular elements, i.e. the neurons and glia, have been examined.

Under conditions of increased physiological demand such as lactation, parturition and dehydration, an animal's blood plasma levels of OX and VP are elevated, as a direct result of increased release of the hormones from the posterior pituitary (Jones and Pickering, 1969; Gibbs, 1984; Kasting, 1988). This higher than basal neuropeptide release is accompanied by increased activation of the MNCs. Brimble and Dyball (1977) showed an increase in firing rate and changes to a phasic firing pattern of putative VP MNCs of the SON when a rat was given hypertonic saline injections. Forced ingestion of 2% NaCl was shown by Dyball and Pountney (1973) and by Jones and Pickering (1969) to increase firing rate of MNCs in the SON and to deplete pituitary stores of OX and VP. Poulain and Wakerley (1982) summarized evidence dating back to the 1930s correlating increases in firing levels and changes in firing pattern of these hypothalamic cells with an increase of hormone release from their axon terminals in the pituitary.

Morphological changes, observable with a transmission electron microscope (TEM) occur in the SON MNCs under physiological conditions that increase the physiological demand for OX and VP, such as parturition, lactation, dehydration and forced NaCl ingestion. MNCs show increases in the size of the nucleus and size and number of nucleoli (Armstrong, Gregory and Hatton, 1977), increased cell body area (Dyball and Garten, 1988), and increased activation of Golgi apparatus (Broadwell and Oliver, 1981). There are also changes in apposition between neurons and glial cells (Chapman, Theodosis, Montagnese, Poulain, Morris, 1986), and changes in type and number of synapses onto the MNCs (Hatton and Tweedle, 1982; Theodosis and Poulain, 1984; Modney and Hatton, 1989a, 1989b).

Ultrastructural changes in the neural lobe of the pituitary, as well as the SON, reflect increases in MNC activity. Evidence for increased neuropeptide release into the bloodstream is suggested by changes in the relationship among axonal endings and pituicytes, and their apposition with the BL, in the posterior pituitary (Tweedle and Hatton, 1980; Tweedle and Hatton, 1987; Hatton, 1988a, 1988b). These increases in nerve terminal occupation (and corresponding decreases in pituicyte occupation) of the BL can be produced in the rat neural lobe by a variety of stimuli, including parturition, lactation, water deprivation and several days of 2% saline drinking (Tweedle and Hatton, 1987). Also, in vitro manipulations of osmotic pressure have been found to induce rapid release of engulfed axonal endings by pituicyte cytoplasm (Perlmutter, Hatton and Tweedle, 1984).

In vitro studies (Modney and Hatton, 1989; Perlmutter, Hatton and Tweedle, 1984) have suggested that a strong hyperosmotic stimulus

will rapidly effect changes in the SON and posterior pituitary. Modney and Hatton (1989b), found that changes in glial/soma apposition and formation of double synapses in the SON could occur in vitro, in response to hypertonic artificial cerebrospinal fluid within 5 h. Incubation of pituitaries in media of high osmolalities caused increased neural contact with the BL within 2 h (Perlmutter, et. al., 1984). These findings led to the question as to whether similar rapid changes could occur in the HNS of an intact animal in response to a strong hyperosmotic stimulus.

A strong, hyperosmotic stimulus in the form of an intraperitoneal (IP) injection of 1.5 M NaCl at a dosage of 18 ml/kg has been considered by some researchers to be a stressful stimulus rather than one that is simply acutely dehydrating. Lightman and Young (1987) used 1.5 M NaCl injected IP in doses of 18 ml/kg to create "hypertonic saline stress" in rats, and found corticotrophin releasing hormone (CRH) mRNA and enkephalin mRNA were significantly increased in the SON 4 h later. They found similar increases in CRH and enkephalin mRNA in response to restraint and swim stress. In a subsequent study, the same authors (1988) compared systemic 1.5 M NaCl injections to morphine administration and withdrawal (simple or precipitated by naloxone). They found changes in CRH mRNA to be similar in conditions produced by 1.5 M NaCl injections and naloxone-precipitated withdrawal (but not simple withdrawal) from morphine. This supported their contention that an injection of 18 ml/kg 1.5 M NaCl is a form of "physiological stress". Kasting (1988), measuring the effects of different types of stressors on OX and VP release, found intraperitoneal injections of 1.5 M NaCl potentiated the release of OX

and VP into the bloodstream at higher levels than other forms of stress such as restraint or hypothermia. It seems important then, when considering the effects of an IP injection of 1.5 M NaCl on the posterior pituitary and hypothalamus, to note that some of the observed morphological changes may be due to the stressful, as well as the osmotic, nature of the stimulus.

STATEMENT OF PROBLEM

In a preliminary experiment investigating the effects of this hyperosmotic stimulus (Beagley, Modney and Hatton, 1990) male rats were given intraperitoneal injections of hypertonic (1.5 M) or isotonic (0.15 M) NaCl solution. Rats were sacrificed 5 h after the injections. Pituitaries and supraoptic nuclei of the hypothalamus were prepared for TEM analysis, and were compared to determine morphological differences between the two conditions. In the SON, significant differences between the injection conditions were found in amount of glial contact with MNC membrane, amount of terminal contact with MNC membrane, and amount of apposition with other cell bodies or dendrites and MNC membrane. Both size of nucleoli and over all cell size were significantly larger in the 1.5 M NaCl injected animals. The number of double synapses found along the MNC membrane and the amount of the Golgi apparatus in the MNCs increased significantly in the 1.5 M NaCl treated animal. All these changes were highly significant and occurred within 5 h of injection of 1.5 M NaCl.

Morphometric analysis of electron micrographs of the pituitaries revealed an increase in nerve terminal contact with the BL as a result of the 1.5 M NaCl injection. These data suggested that the relationship of pituicytes to the BL can change rapidly, in accordance with the hypothesis that pituicyte processes withdraw from the BL allowing increased neural contact, thereby facilitating hormone release under

appropriate physiological conditions. There was also a significant decrease in the number of axon terminals engulfed by pituicyte cytoplasm. The findings of this preliminary work were replicated and extended and are described in Experiment I.

The effect of this hypertonic saline stimulus on the HNS raised several questions. The first concerned the nature of the time course of the response. While it has been shown (Pickford, 1969; Brimble and Dyball, 1977; Gibbs, 1984; and Kasting, 1988) that VP levels in the blood plasma increase in response to a hyperosmotic stimulus in about fifteen minutes, and cells in the SON also take about fifteen minutes to change their firing rate and pattern (Brimble and Dyball, 1977), observable morphological changes in response to a thirst inducing stimulus seem to take somewhat longer. Tweedle and Hatton (1987) found that 48 hours of water deprivation or 10 days of forced 2% saline ingestion produce increased apposition of terminals with the BL in the pituitary. Although Perlmutter et. al. (1984) showed rapid increase of terminal apposition with the BL in an in vitro pituitary preparation, and Modney and Hatton (1989b) showed effects of a hyperosmotic media on SON cells in a hypothalamic slice preparation in as little as 5 h, it was not known how this reflects the time course of the response of an intact HNS to the acute injection of 1.5 M NaCl in vivo.

Experiment II was designed to answer this question. SONs and pituitaries were examined from animals perfused 15 min, 1 h and 2.5 h after 1.5 M or 0.15 M NaCl injections. These time intervals allowed for observation of morphological changes in the SON and pituitary which occurred before the end of the 5 h time interval of Experiment I. The

animals were sacrificed and neural tissue was prepared for TEM examination and analyzed in the same manner as in Experiment I.

Another question addressed arose here when considering implications of the fact that the morphological response to other stimuli which induce osmotic changes, such as water deprivation or 2% saline drinking, occurs more slowly than the response of SON cells and pituicytes to this large injection of hypertonic solution. Are the rapid morphological changes observed in Experiment I due to dehydration alone, or are they also influenced by a reaction to a suddenly induced, stressful aversive stimulus?

The role of the HNS as it responds to stressful stimuli has been examined, with varying results, by several different researchers. Gibbs (1984), measured the presence of OX and VP in portal blood in response to restraint, cold, or ether, and found differential release depending on the type of stressor. Kasting (1988) also measured blood plasma levels of OX and VP to a variety of stimuli known to cause neuropeptide release, and found different ratios of increases of the two hormones depending on the release inducing condition. Lightman and coworkers (e.g. Harbuz and Lightman, 1989; Lightman and Young, 1987; Lightman and Young, 1988) have routinely used 18 ml/kg IP injections of 1.5 M NaCl as a stressor to affect levels of messenger RNA for enkephalin or CRH in the PVN, SON and pituitaries. There is evidence, then, that the release of OX and VP into peripheral plasma is increased, perhaps differentially, in response to various types of physiological stressors. Since the changes in pituitary and SON morphology correspond to increased peptide release, there exists the possibility that the large and rapid changes seen in response to our

injections of 1.5 M NaCl may be in part due to the stressful nature of the stimulus. Animals receiving the 1.5 M NaCl injection frequently emit distress vocalizations when injected and appear to be in some discomfort for several hours after the injection.

Experiment III attempted to dissociate, in part at least, the stress-related and osmotically induced components of the observed response to a single 1.5 M NaCl injection. Two manipulations were performed to remove the stress related signal to the HNS, while maintaining the osmotic challenge of 1.5 M NaCl. The first was anesthetizing the animal; the second, removal of the major source of systemic adrenalin, which is normally increased when an animal is stressed (Seyle, 1978).

Presumably a deeply anesthetized animal would not respond to the 1.5 M NaCl injections in the same manner as an unanesthetized animal if the reaction involves perception of pain, stress or discomfort, and the anesthetized animal has been rendered "unconscious" to such perceptions. The HNS, however, should still be capable of responding to the increased osmolality produced by the stimulus. Brimble and Dyball (1977) found that cell bodies in the SON responded to an intracarotid injection of 1.5 M NaCl by an increase in firing rate in the same manner in anesthetized and unanesthetized animals; however they used urethane as an anesthetic and did not look for long term morphological changes.

Animals injected with a surgical dose of equithesin typically remain quiescent for several hours. Pituitaries and SONs of animals injected with 1.5 M or 0.15 M NaCl after being anesthetized for the duration of the painful stimuli resulting from a hypertonic saline injection, were examined for the same changes that occur in the

unanesthetized animal during the 5 h period between injection and perfusion.

There is always a danger that the anesthetic used might suppress cellular activity in a manner other than that which we wish to study, and contaminate the data. Ginsberg and Brown (1957) showed that anesthesia, specifically pentabarbitol, which is one of the major active ingredients in equithesin, might contribute to different OX/VP ratios in the neurohypophysis. Dyball (1975) found increased OX release from neural lobes incubated in media containing urethane, and possible inhibition of OX release from neural lobes incubated with pentobarbitone. Although Ginsberg and Brown did not find any evidence of inhibition of hemorrhage induced neuropeptide release in vivo with either pentobarbitone or urethane, their findings do suggest that the proportions and amounts of OX and VP released were influenced by the type of anesthesia used. For that reason it is especially important to compare anesthetized 1.5 M NaCl animals with the anesthetized control (0.15 M NaCl) animals so that any general effect of the anesthetic on the system will be equal. Measures obtained from anesthetized animals injected with 0.15 M NaCl should also be compared to animals injected with 0.15 M NaCl and not anesthetized, so that changes due only to the anesthesia can be detected. Animals anesthetized for the duration of the 5 h between 1.5 M or 0.15 M NaCl injection and perfusion comprised one of the groups of subjects in Experiment III.

Several researchers (e.g. Leng, Mason, and Dyer,1982; Sladek and Armstrong, 1985) believe that the SON has osmoreceptive properties. Other groups of researchers report evidence for involvement of

different transmitters in neuropeptide hormone release. It has been suggested, for instance, that GABA (Theodosis, Paut, and Tappaz,1986), dopamine (Buijs, Geffard, Pool, and Hoorneman, 1984), norepinephrine (Day and Renaud, 1984), and adrenalin (Pickford, 1969), all are involved in the neurosecretory activity of the hypothalamo-neurohypophysial system. Because so many data are available supporting the role of different factors affecting HNS activity, it seems possible that different aspects of HNS activity may be independently influenced. Of interest to this study, is the possible existence of multiple independent systems separately influencing MNC firing and pituicyte withdrawal.

The second part of Experiment III was designed to investigate the possibility that one of the influences on the activity of the HNS might be a signal from systemic adrenalin. Work by Harbuz and Lightman (1988) suggests that systemic adrenalin may play a role in stimulating or monitoring the release of VP/OX from the pituitary. Their study found mRNA in SON cells associated with the neuropeptide release, decreased in adrenalectomized animals; therefore, adrenalin might be a blood borne factor responsible for a component of the HNS's response to the 1.5 M NaCl stimulus.

There is additional research supporting the involvement of catecholamines in pituicyte withdrawal in response to a hyperosmotic stimulus. Retrograde tracing with horseradish peroxidase injected into the neural lobe combined with immunohistochemical detection of tyrosine hydroxylase, as done by Garten, Sofroniew and Dyball (1989) shows a direct noradrenergic (NA) projection from the A2 region of the brainstem to the neurohypophysis. Smithson, Suarez and Hatton

(1990) stimulated beta-adrenergic receptors on isolated neurointermediate lobes by incubating them in low concentrations of isoproterenol. This resulted in alteration of pituicyte morphology in a manner comparable to that produced in vivo by dehydration, parturition or lactation. A systematic comparison of similarity of salt loading induced pituitary changes produced in vivo, with changes induced in the isolated neural lobe incubated with the beta-adrenergic agonist, was conducted by Luckman and Bicknell (1990). Luckman and Bicknell also determined that the decrease of glial coverage of the BL could be blocked by a beta-adrenergic, but not by an alpha adrenergic antagonist. Finally, Hatton, Luckman and Bicknell (1991) working with cultured pituicytes induced a change from amorphous to stellate morphology in response to adrenalin. This change in shape in the cultured pituicyte, similar to that seen in the animal in response to osmotic challenge and in the isolated neural lobe incubated in hyperosmotic or beta-agonist containing medium, was blocked by a beta-2 but not a beta-1, antagonist. The cultured pituicytes showed a significantly greater change in response to adrenalin, which stimulates beta-2 receptors more strongly than noradrenalin.

The neural lobe is located outside of the blood brain barrier and might respond to catecholaminergic signals originating from the adrenal gland and carried via the blood stream. There is long standing evidence that the peptides released from the neural lobe act upon the adrenal glands (Gaunt, Lloyd and Chant, 1957) and it is equally possible that signals from the adrenal gland act directly or indirectly on the HNS.

Elimination of the peripheral source of adrenalin by bilateral removal of the adrenal medulla, might decrease the rapidity or magnitude of the pituicytes' response to the 1.5 M NaCl injection as seen in Experiment I.

The SON is in direct contact with the pial surface of the brain. The glial cells in the SON are in contact with the blood vessels and although the blood brain barrier is more difficult to penetrate in this area of the brain than in the neural lobe, it is easier for some substances to pass through the barrier in the SON than in less vascularized areas such as the ventromedial hypothalamus (Gross, Sposito, Pettersen, and Fenstermacher, 1986). Furthermore, perfusion of the brain with hyperosmolar solutions has been shown to increase barrier permeability (Rapopart, 1970). A large systemic injection of 1.5 M NaCl might be the equivalent of hyperosmotic perfusion, allowing a blood borne signal such as adrenalin to affect glial cells in the SON.

Therefore, in Experiment III, a group of animals were bilaterally adrenal-medullectomized, maintained with access to both a 1% NaCl solution and tap water for one week, then injected with 1.5 or 0.15 M NaCl solutions, and perfused 5 h after the injection. If rapid increase of adrenalin in response to the stimulus is necessary to cause the morphometric changes in the pituitary and/or SON, the adrenalectomized animals should not show the effect, or may show changes of lesser magnitude. It must be noted however, that there are direct NA projections to the pituitary from the A2 region of the brainstem (Garten, et. al., 1989) and also NA projections to the SON from areas A1 and A6 (Wilkin, Mitchell, Ganten and Johnson, 1989)

which, if activated, might compensate in part for lack of adrenalin coming from adrenal medullae.

In addition to comparing rats given 1.5 M NaCl with an equal number of rats given 0.15 M NaCl for every experimental condition, some additional control procedures were necessary in order to be certain that the hypertonic saline activation of the MNCs is not a result of an overall toxic or general activational effect on the brain. From 8 rats (4 receiving 1.5 M NaCl; 4, 0.15 M NaCl) sections were examined from the hippocampus (suggested by Dyball and Garten,1988) and the suprachiasmatic nucleus (SCN) (suggested by Nunez, personal communication) in the same rostral caudal plane from which the corresponding SON sections were taken. The hippocampus has been shown to be involved in endocrine reaction to stressful stimuli (Joels and de Kloet, 1989) and the SCN is the anatomical substrate for a major circadian clock in mammals, but seems to be insensitive to major homeostatic challenges. Neither the hippocampus nor the SCN is known to play a role in responding to osmotic stimuli; but have large cells comparable to the SON.

Micrographs were also examined, and quantified morphometrically, of pituitaries and SONs of rats who received no NaCl treatment, to obtain control values for any influence that non-experimental factors (e.g. colony conditions, handling, perfusion) might have had on the morphology of the elements measured in the SON and posterior pituitary.

Individual methods, results and a discussion of data for each specific experiment are presented below. A general conclusion is then

drawn about the nature of the response of the HNS to this rapidly acting hyperosmotic stimulus.

EXPERIMENT I

Experiment I was designed to compare the effects of large systemic doses of hypertonic (1.5 M) and isotonic (0.15 M) NaCl solutions on the morphology of the HNS. Ultrastructural differences in the SON and posterior pituitary resulting from acute or chronic dehydration had been observed in vivo only after several days (e.g. Tweedle and Hatton, 1984; Tweedle and Hatton, 1987). In vitro studies, however, (e.g. Modney, and Hatton, 1989b; Perlmutter, Hatton, and Tweedle, 1984) have shown morphological changes can occur in the posterior pituitary and SON slices incubated in hypertonic artificial CSF within 5 h. In experiment I, we attempted to produce an in vivo paradigm that would produce large ultrastructural changes after time intervals comparable to those observed in the in vitro studies. It was predicted that animals would respond within 5 h to the large systemic injection of 1.5 M NaCl with decreases in glial and corresponding increases in terminal and somatic apposition with MNCs, increases in cell body size, and increases in the number of multiple synapses as seen in other instances of physiological challenge. Rats were injected, therefore, with large doses of hypertonic (1.5 M) or isotonic (0.15 M) NaCl solution and sacrificed 5 h after the injection to determine if morphological changes would occur within this time in response to this stimulus.

Methods:

Eight male Sprague-Dawley rats 80 to 100 days old on a 12:12 reversed light cycle were given IP injections of 18 ml/kg 1.5 M NaCl solution 1 h after lights on. Eight were injected with the same volume of isotonic (0.15 M) NaCl solution. After 5 h they were deeply anesthetized with equithesin and perfused by a brief transcardial rinse of 0.15 M NaCl followed by a fixative of 2.5% glutaraldehyde and 1% formaldehyde in a 0.1 M cacodylate buffer. For perfusion, a Masterflex variable speed pump using tubing with 0.06 inch inner diameter was adjusted so the flow rate was approximately 5 ml/min, a flow rate that preserves pituitary ultrastructure. Brains and pituitaries were removed from the skulls and left in fix overnight. Coronal slices 500 mm thick were cut with a tissue chopper through the anterior-posterior extent of the SONs. SONs were recovered from three adjacent slices. Neural lobes were dissected free of anterior and intermediate lobe tissue, oriented in resin blocks and trimmed so that sections could be taken from the center of the broadest part of the neural lobe. This area includes terminal projections from both the SON and the PVN (Alonso and Assenmacher, 1981). SONs and neural lobes were removed and postfixed in 1% osmium tetroxide for 1 h, en bloc stained with uranyl acetate overnight, dehydrated in alcohol, and embedded in Spurr's resin. Blocks containing the middle third (rostrocaudally) of the SON were used for data collection. Thin sections (silver-gold interference color) were examined with a Philips 201 C or a Japan Electron Optics Laboratory 100 CX electron microscope.

Each animal's score on each of the various measurements was based on a mean of 10-15 micrographs for both areas, analyzed at a print magnification of 13,500 X enlargement. Point intersection analysis (Williams, 1981) was used to determine glial, somatic or axon terminal membrane apposition for the soma in the SON and to compute somatic, nuclear and nucleolar size, and amount of Golgi apparatus in cross sectional profiles of MNCs in the SON. Different cellular elements were traced with different color markers. Apposition measurements were taken by placing a line grid over the micrograph and counting point intersections of the soma membrane and of the different structures contacting the membrane, at the places where they contacted the membrane. Ratios were computed for the number of intersections of a particular type of structure in apposition with the membrane, to the number of intersections with the total membrane in each micrograph. For instance, if a given micrograph contained a MNC soma whose membrane was intersected by the line grid at 100 points, and glial cells were abuting the membrane at 48 of these intersection points, the ratio of glia to total membrane would be 48/100, or 48%. Size (for soma, nucleus, nucleoli) and Golgi apparatus measurements were computed by putting a cross grid over the micrograph and counting the number of points of intersection across the surface area of the cell body, nucleus or nucleoli. These areas could then be compared for the different treatment conditions. Intersections with Golgi apparatus were also counted with this type of grid, and a ratio of the Golgi apparatus to the total cell surface are were computed and compared. Counts were also made of single and multiple synapses (defined as a synapse contacting the somata of two or more

MNCs, or the soma of an MNC and a dendrite) in the 1.5 M NaCl injected and 0.15 M NaCl injected conditions. A Sigma Scan Digitizing program for an IBM PC was used to measure the extent of terminal contact with the MNCs.

Point intersection morphometric analysis (Williams, 1981) was also done on the pituitary micrographs to determine the percentage of BL contacted by nerve terminals versus pituicyte processes. After pituicyte processes and terminals had been traced with colored marker, a line grid was laid over the micrograph and points of intersection of the grid with the different colored pituitary and terminal outlines were counted. A tally was made of total intersections with neurons and glia along the BL, and a ratio of terminal intersections to the total BL intersections was computed. The number of axon profiles completely engulfed by each pituicyte (identified by separate pituicytes in a micrograph each having a nucleus) was counted.

For each dependent variable, the two groups were compared using unpaired two-tailed t-tests. For statistical purposes, n was the number of animals, not number of micrographs used for each measure. Relationships among the various elements were examined by an X-Matrix Correlation. A STAT VIEW II program for the Macintosh IICX computer was used to perform all statistics.

Results

Figures 1 and 2 show examples of SON tissue under the two NaCl treatment conditions. Figures 1a and 1b and 1c are electron micrographs of the SON from a rat injected with 1.5 M NaCl. Figure 2 is from a rat injected with 0.15 M NaCl. A comparison of these serves

to illustrate the morphological changes induced by a single hypertonic saline injection.

Data from electron micrographs of the SON of 14 rats (8 receiving injections of 1.5 M NaCl; 6,0.15 M NaCl) were compared to determine morphological differences in the MNC somata in the two conditions. In the SON, significant differences between the injection conditions were found in: (a) amount of glial contact with MNC membrane, (b) amount of terminal contact with MNC membrane, and (c) amount of apposition with other cell bodies or dendrites and MNC membrane. See Table 1.

Table 2 presents a comparison of mean length of contact of the synapsing terminals with the MNC membrane, and the number of synapses per 100 mm of MNC somatic membrane for the two saline conditions. Also shown for each group are the number of multiple synapses per 100 μm of membrane and percent of synapses on a MNC soma that were multiple synapses. Although the mean number of synapses per 100 μm of somatic membrane did not differ for the two conditions, the size of the individual terminals and the number of multiple synapses was larger for the 1.5 M NaCl treated animals, as was the percentage of MNC cell bodies contacted by multiple synapses.

Cross sectional profiles of MNCs were measured to determine cell body size and amount of nucleolar material and Golgi apparatus. The mean cell body size of MNCs in the 1.5 M NaCl condition was 14% larger than in the 0.15 M NaCl condition (30.44 $\mu m^2 \pm 2.6\ \mu m^2$ and 26.07 $\mu m^2 \pm 4.6\ \mu m^2$, $p < .025$). Although we did not find a difference in number of nucleoli in the two conditions, there was a difference in size of nucleoli. Amounts of nucleolar material and Golgi apparatus

were computed as surface area covering a selection of cross sectional profiles of the MNCs. The mean area of nucleolar material in rats injected with 1.5 M NaCl was significantly greater than that for rats injected with 0.15 M NaCl (0.88 $\mu m^2 \pm 0.16$ μm^2 and 0.58 $\mu m^2 \pm 0.11$ μm^2, $p < .01$). In the 1.5 M NaCl condition, Golgi apparatus covered 2.8% ± 0.4 % of the surface area of cross sectional profiles of MNCs as opposed to 1.6% ± 0.2% for animals receiving 0.15 M NaCl ($p < .01$).

Table 3 shows how the various elements measured in the SON relate to each other as they changed during the experimental condition. The various elements in apposition with the MNC membrane are, of course, negatively correlated with each other, and relative sizes of cell bodies and nuclei increase together, but there appears to be relatively high correlation between other measures. Most notably, cell body size is negatively correlated with amount of glial apposition; and Golgi activation is highly correlated with the number of multiple synapses contacting the MNC.

Figure 3 illustrates the changes in the posterior pituitary induced by 1.5 M NaCl. Figure 3a is an electron micrograph of a section of the posterior pituitary from a rat injected with 1.5 M NaCl and Figure 3b is from a rat injected with 0.15 M NaCl. Morphometric analysis of electron micrographs of the pituitaries revealed an increase in nerve terminal contact with the BL as a result of the 1.5 M NaCl injection. Six animals receiving an injection of 1.5 M NaCl showed a mean of 67.3 % ± 2.2% terminal contact with the BL, five animals receiving of injections of 0.15 M NaCl showed a mean of 36.4 % ± 2.7 % neural contact. These differences were significant ($p < .0001$). There was also a significant decrease in the number of axon terminals engulfed by

Figure 1. Electron Micrographs of Magnocellular Supraoptic Neurons from a Rat Injected with 1.5 M NaCl. (A. Two somata which have portions of their membrane in direct apposition (between dashed lines). B. Two somata with appositions and large synapses including multiple synapse (indicated by arrows). C. Enlarged multiple synapse. N = nucleus of MNC. GA = Golgi apparatus. Bars = 1 μm.)

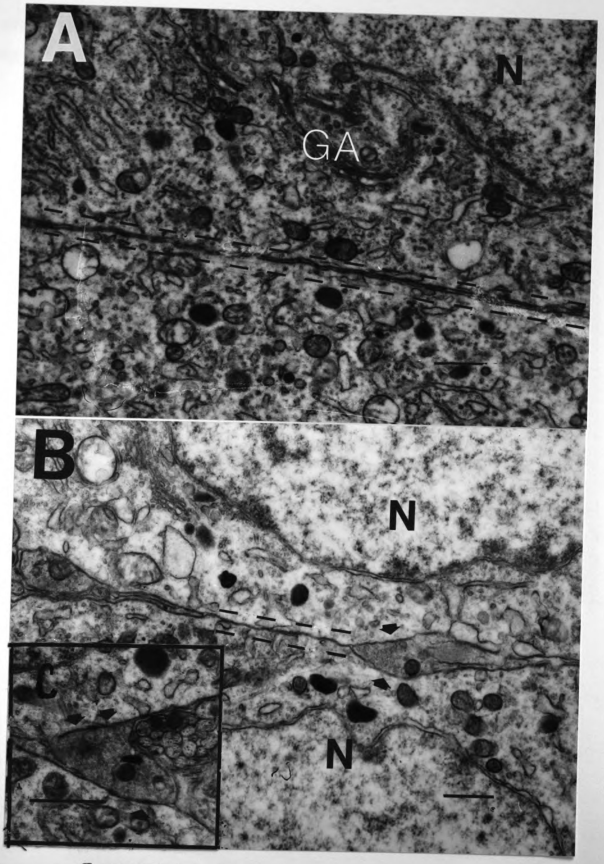


FIGURE 1.

Figure 2. Electron Micrograph of Magnocellular Supraoptic Neurons from a Rat with a Control 0.15 M NaCl Injection. (N = nucleus of MNC. Bar = 1 μm.)

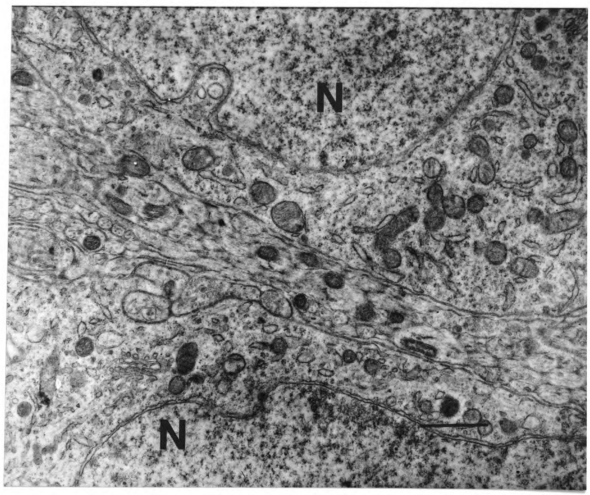


FIGURE 2.

Table 1

Mean and S.E.M. Percentages of Magnocellular Secretory Cell Membrane in Apposition With Each Element:

Condition	Glia	Terminals	Soma and/or Dendrites
1.5 M NaCl (n=8)	57.1 ± 2.3	13.1 ± 1.1	22.6 ± 1.7
0.15 M NaCl (n=6)	73.2 ±1.1**	9.8 ±0.9*	7.1 ± 1.5**

*$p < .02$

**$p < .0001$

Table 2

Relations Between Synaptic Elements and Magnocellular Neuroendocrine Cell Membrane

Condition	Total contact Terminal/ MNC (mm)	Synapses per 100 mm	# multiples per 100 mm	% multiples per soma	% soma w/multiple
1.5 M NaCl (n=8)	11.6 ± 0.26	0.068 ±.014	0.02±001	24 ± 1.2	58 ± 3.8
0.15 M NaCl (n=6)	8.6±0.32**	0.078±.005	0.01 ±.001*	12 ± 0.4**	34 ± 3.6*

* $p<.001$

** $p<.0001$

Table 3

Correlation Matrix

for Measured SON Elements in Experiment I

	glial. app	term app	som app	cb size	nucli size	golgi	mult syn	syn size
glial apposition	1							
terminal apposition	-.819	1						
somatic apposition	-.933	.594	1					
cell body size	-.802	.574	.695	1				
nucleoli size	-.625	.477	.568	.605	1			
golgi	-.962	.832	.866	.721	.572	1		
# multiple synapses	-.877	.68	.886	.501	.318	.912	1	
synapse size	-.904	.704	.897	.602	.759	.882	.8	1

Figure 3 - Micrographs of the Posterior Pituitaries of Rats Injected With 1.5 M NaCl and 0.15 M NaCl (A. Neural lobe of a rat injected with 1.5 M NaCl. Pituicyte cytoplasm (P) is retracted and most terminals (t) abut the basal lamina (filled arrows). B. Neural lobe of a rat injected with 0.15 M NaCl. Axons (ax) are enclosed by pituicyte cytoplasm and the pituicyte is interposed between axons and the basal lamina and fenestrated capillary (indicated by open arrows). N = pituicyte nucleus. Bar = 1 μm.)

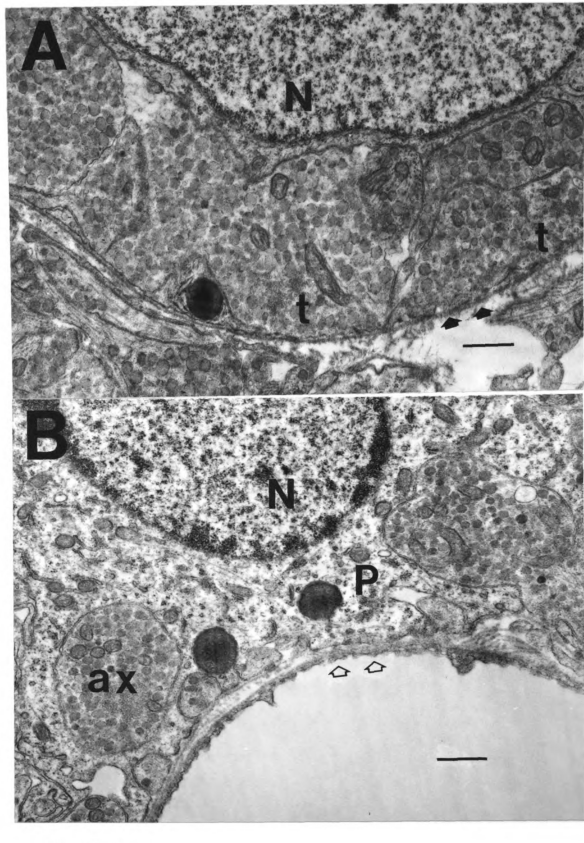


FIGURE 3.

pituicyte cytoplasm. For animals injected with 1.5 M NaCl, the mean number of axons enclosed per pituicyte was 0.96 ± 0.27; as opposed to 1.56 ± 0.15 for the animals injected with 0.15 M NaCl. ($p < .05$). Correlation between amount of pituicyte coverage of the BL in the pituitary and the glial apposition of MNCs in the SON is extremely high, with a correlation coefficient of +.906.

Discussion

Results of Experiment I indicate that 1.5 M NaCl is an extremely effective stimulus for eliciting rapid structural changes in the SON and posterior pituitary known to be correlated with neuropeptide release. In the MNCs of the SON, several morphometric changes indicated acute activation of the system. The size of the MNCs, the amount of material in the nucleoli, and length and number of the Golgi apparatus all increased with injections of 1.5 M NaCl. Armstrong, Gregory, and Hatton (1977), have suggested that the increase in nucleolar material is linked to an increase in production of ribosomal RNA which is used in the manufacture of the additional neuropeptides required when the system has been stimulated with a 1.5 M NaCl injection. Golgi saccules hypertrophy and become more elongated in shape during secretory granule formation (Broadwell and Oliver, 1981) and this is reflected in this study by the increase in percentage of Golgi apparatus in the cytoplasm. These changes, therefore, are consistent with an increase in hormone manufacture.

The high correlation between the elements measured suggests they are all good indicators of a system highly activated for increased release of neuropeptides. The large correlations among cell body size,

synapse size and number of multiple synapses, and their high negative correlation with glial apposition, is consistent with the idea that the withdrawal of glial processes from between MNCs allows for increased soma-somatic apposition and for synapses to make contact with membranes of MNCs that were previously occupied by glial membrane.

The increase in somatic apposition suggests withdrawal of the glial processes normally interposed between the MNC soma, possibly to increase communication between the cells. The increase in the size of synapses contacting MNCs, and the increase in the number of multiple synapses suggests glia withdraw in the SON to facilitate increased interaction among the MNCs. Early work by Tweedle and Hatton (1980) and more recent studies by Smithson, Suarez and Hatton (1990) and Luckman and Bicknell (1990) have provided evidence that glial cells in the neural lobe can actively retract under some conditions by withdrawing their astrocytic projections.

Although withdrawal of glial projections in the SON has not yet been conclusively demonstrated, the changes in numbers of multiple synapses that we observed argue against the possibility that the increased soma size leads to merely passive exclusion of the glial cells. Increased amount of terminal contact with the MNCs was not due to an increased number of terminals in the 1.5 M NaCl condition, but to an increase in the length of individual terminals due to increased size or a change in shape of each terminal contacting the somata. This too could be the result of glial retraction; as glia retract, increased contact of terminals with somata could occur. Modney and Hatton (1989) suggested that the increase in terminal size occurs to maintain a

constant synaptic density on the larger MNC soma. Of the synapses making contact with the MNCs in animals that received 1.5 M NaCl, a larger percentage was the type making multiple contacts. Also, of the MNCs sampled for these animals, a larger percentage of cells was contacted by terminals making multiple synapses than in animals who received injections of 0.15 M NaCl. This increase in number of multiple synapses and larger synapses correlates with the increased overall excitability of these neurons during periods of increased hormone release. Also, the presence of more synapses that contact multiple somata and the increase in apposition between somata in the animals receiving 1.5 M NaCl, suggests that this stimulus condition may lead to coordination of excitabilities and/or activity among MNCs in the SON.

Our data also suggest that when the system is challenged the relationship of pituicytes to the BL can change rapidly, thus supporting the hypothesis that pituicyte processes withdraw from the BL to allow increased neural contact and facilitate hormone release (Tweedle and Hatton, 1980). The neural apposition with the BL in the posterior pituitary was significantly increased and the number of axons enclosed by pituicytes decreased. These changes suggest glial cells retract and allow the neurosecretory terminals increased access to the BL to release more hormone into the bloodstream as the stimulus condition demands.

All of these changes which are induced by hypertonic saline injections have been seen in response to other challenges such as acute and chronic dehydration, lactation and parturition. Thus, an IP injection of 1.5 M NaCl is an effective stimulus for inducing

morphological changes shown to be consistent with activation of the hypothalamo-neurohypophysial system. That such dramatic reorganization occurs in the pituitary and SON within 5 h shows that the system can respond rapidly in vivo to a stimulus of this nature, more rapidly, in fact, than it does to acute or chronic dehydration by water deprivation or saline drinking.

ADDITIONAL CONTROL PROCEDURES

Control procedures were employed in order to be certain that the morphological changes we observed in the SON in response to hypertonic saline activation were not a result of an overall toxic or general activational effect. Comparisons were made, therefore, between elements in apposition with large cells in non HNS areas of the brain for animals receiving either 1.5 M NaCl or 0.15 M NaCl injections. Sections were examined from both the hippocampus and the SCN in the same rostral caudal plane from which the corresponding SON sections were taken. If large cells in the hippocampi or SCNs of animals receiving 1.5 M NaCl injections showed decreased glial and increased terminal or somatic apposition compared to animals receiving 0.15 M NaCl injections, we would be forced to consider the possibility of an effect of hypertonic saline stimulus on glial cells that was not specific to the HNS..

Methods

1. Sections were taken and prepared for TEM from the hippocampus and the SCN from 8 rats (4 receiving 1.5 M NaCl; 4, 0.15 M NaCl) in the same rostral caudal plane from which the corresponding SON sections were taken. Ten to fifteen micrographs were taken of CA1 cells from the hippocampus and of large cells from the SCN of each rat, and the specimens from the two NaCl injection conditions were compared for glia, soma and dendrite apposition to

determine if the hyperosmotic stimulus has any effect in these areas of the brain. In both cases, for each dependent variable, the two groups were compared using unpaired two-tailed t-tests.

2. Two untreated rats were taken directly from the colony room, anesthetized and perfused. Tissue from their SONs and neural lobes was examined in the same manner as rats in Experiment I.

Results

1. Hippocampus and SCN showed no effect of different injection conditions. Hippocampal cells typically showed a relatively large level of somatic apposition (between 12% and 14%) and a smaller area of membrane contacted by synapses (around 7%). Figure 4 shows hippocampal cells from rats injected with 1.5 M NaCl and 0.15 M NaCl.

Cells from the SCN, conversely showed very little somatic apposition with each other (around 5%) and a higher portion of membrane contacted by synapses (13%). There was no difference between cells in either area due to the saline injection conditions. Figure 5 shows SCN cells from rats from the two injection conditions.

2. All measures of SON and neural lobe elements for the two animals perfused without treatment were within the range of scores for animals injected with 0.15 M NaCl with no additional manipulations. This was true in both the SON and Neural Lobe, as can be seen in Tables 4 and 5.

Figure 4 - Electron Micrographs of Hippocampal Cells from Rats Injected with 1.5 M NaCl((A) and 0.15 M NaCl (B). N = cell nucleus. Bar = 1 μm.)

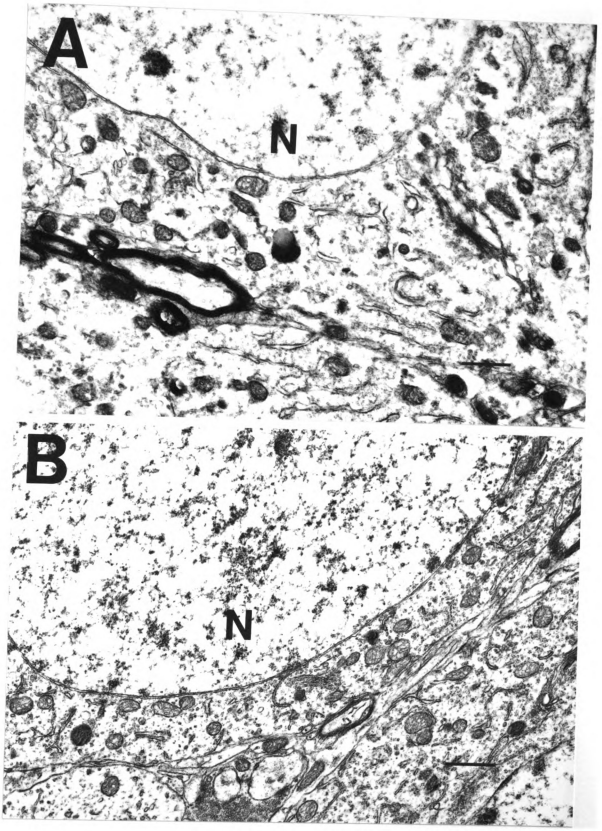


FIGURE 4.

Figure 5 - Micrographs of Suprachiasmatic Nucleus Cells from Rats Injected with 1.5 M NaCl ((A) and 0.15 M NaCl (B). N= cell nucleus. Bar = 1 μm.)

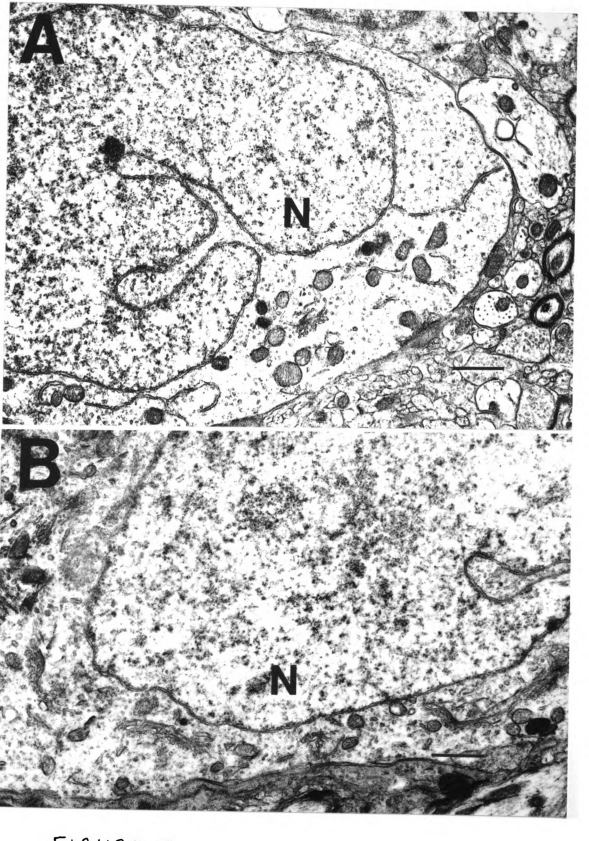


FIGURE 5.

Table 4

A Comparison of SON Elements for Rats Receiving Injections of 1.5 M NaCl and 0.15 M NaCl with Untreated Rats.

	Elements in Apposition			Size in μm^2		
Condition	Glial	Terminal	Soma and/or Dendrites	CellBody	Nucleus	Nucleoli
1.5 M NaCl	63 ± 1	13 ± 1.5	18 ± 1	29.3 ± .9	10.4 ± .6	.76 ± .06
.15 M NaCl	79 ± 1	8 ± .7	8 ± 1	24.5 ± 1.9	8.6 ± .3	.73 ± .08
Untreated Rat A	77	8	8	24.9	8.1	.64
Untreated Rat B	80	7	7	23.3	8.8	.56

Table 5

Measures in Neural Lobe for Untreated Rats Compared to Measures for Rats Injected with 1.5 M NaCl and 0.15 M NaCl.

Condition	% Neural Apposition with BL	% Pituicyte Apposition with BL	Axons Enclosed by Cytoplasm
1.5 M NaCl	59 ± 2	40 ± 2	.92 ± .19
0.15 M NaCl	35 ± 3	64 ± 3	1.84 ±.32
Untreated Rat A	38	62	2.16
Untreated Rat B	35	65	1.5

Discussion

The lack of differences in response to an injection of 1.5 M NaCl versus an injection of 0.15 M NaCl in either hippocampal MNCs or SCN MNCs suggests that response to a stimulus of this nature is specific to the HNS. The effects seen in the SON and neural lobe do not appear to be the result of an overall activation or toxicity of brain tissue caused by the hypertonic saline, but a plastic response to enable the organism to deal with a hyperosmotic stimulus from its environment.

The lack of difference between measures for rats anesthetized with 0.15 M NaCl and rats perfused immediately after removal from the colony room, lends support to the former as an adequate control condition for observing the effects of the 1.5 M NaCl injection in this system.

EXPERIMENT II

In Experiment I, morphological changes in the HNS were shown to occur within 5 h after a large systemic injection of hypertonic NaCl solution. Despite evidence that electrical activity in the SON (e.g. Brimble and Dyball, 1977) and neuropeptide level in the plasma (Gibbs, 1984; Kasting, 1988) show measurable increases within 15 min after the onset of a hypertonic saline stimulus stimulus this had not been correlated with ultrastructural changes at the EM level. Previous ultrastructural examinations of the HNS had described changes in response to 2% NaCl ingestion as occurring within 10 da and H_2O deprivation as occurring within 48 h. The purpose of Experiment II, therefore, was to examine time courses of the changes seen in Experiment I in the SON and posterior pituitary after injection with hypertonic saline. The time intervals after stimulus onset selected for examination were 15 min (when the first changes in the HNS have been reported to occur) 1 h (reported as time of maximal electrical activity or neuropeptide release) and 2.5 h (halfway between stimulus onset and the time we observed a large effect in Experiment I). We wished to determine at what point after stimulus onset the earliest morphometric changes occurred and which changes, if any correlated with the reported times for hormone release and electrical activity of the MNCs in the SON.

Methods

Thirty rats were injected in the same manner as those in Experiment I, 1.5 M NaCl (n=15), 0.15 M NaCl (n=15) . Groups of five rats from each injection condition were anesthetized and perfused 15 min, 1 h, or 2.5 h after saline injection. Rats (on a 12:12 reversed light cycle) were typically injected between 1 and 3 h after lights on and perfused after the appropriate time intervals. In an attempt to minimize any circadian effect on the response to the hypertonic injection, all 1.5 M NaCl injected animals were paired for injection and perfusion times with a 0.15 M NaCl injected animal. In this way, the differences in time of injection and perfusion were equal for the two injection conditions. SONs and posterior pituitaries were removed, prepared for and examined by TEM, and micrographs were analysized in the same manner as in Experiment I, with one exception. Values for percent of Golgi apparatus in the MNCs in Experiment I were computed on the basis of a ratio of Golgi apparatus to the total cell surface area (Beagley and Hatton,1992, in press). Because Golgi apparatus was never found in the MNC nucleus, and because size of nucleus varied for different experimental conditions, in Experiments II and III ratios were computed on the basis of amount of Golgi apparatus to the area of MNC cytoplasm in the micrograph (cell body minus nucleus). This resulted in slightly larger, but more accurate estimates of amount of Golgi apparatus in the MNC. Statistical analysis for each anatomical site were performed by a two by three way analysis of variance (ANOVA) (saline condition across time intervals) and followed by Tukey test post hoc comparisons when appropriate.

Results

Among the the groups sacrificed at the three different time intervals after the injection of 1.5 M or 0.15 M NaCl, in the SON there was no significant main effect of saline treatment (F's =.003 to 2.8; p<0.98 to 0.11) or time of sacrifice (F's = 1 to 1.9; p<0.38 to .18) for apposition with glia, terminals, somata or size of cell bodies or nuclei. Also, no significant interaction effects were present (F's =.35 to 1.3; p<.71 to .29). The means for these measures are shown in Table 6.

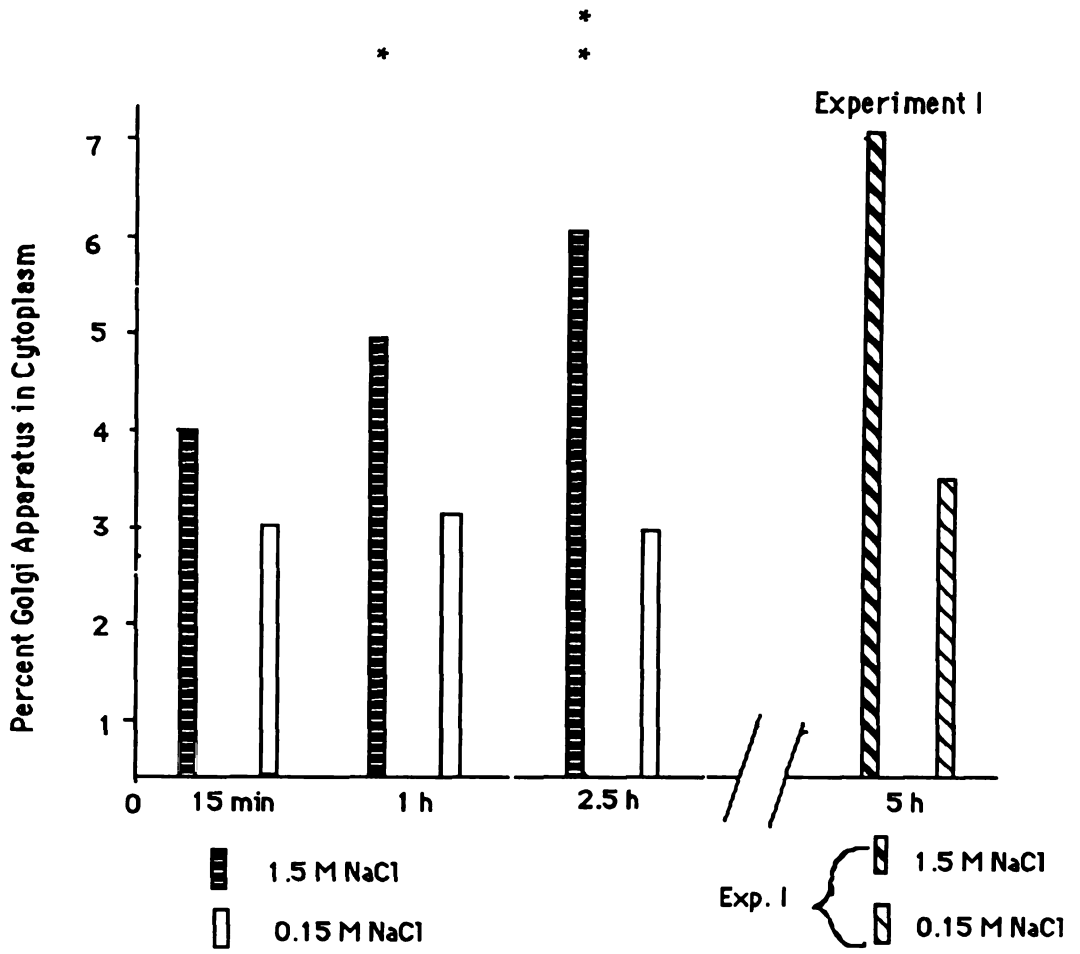
There was a highly significant effect of saline treatment on the percentage of Golgi apparatus in the cytoplasm (F = 44.2, p<.001) as well as a significant interaction effect of saline treatment X time of sacrifice (F = 4.8, p <.02). The relationship among the groups is shown in the graph, Figure 6. The mean amounts of Golgi apparatus in MNC cytoplasm for animals injected with 1.5 M NaCl and perfused at at 1 h and 2.5 h, but not at 15 min after injection are significantly above the amounts for animals injected with 0.15 M NaCl at those times (1 h: p < .05; 2.5 h: p < .01). Bars representing the adjusted means for animals in Experiment I (computed as described in the method section of Experiment II) are included in Figure 6 for comparison with the shorter time intervals.

Table 7 shows the relations between synaptic elements and MNC membrane for all animals in Experiment II. There were no significant effects of saline treatment (Fs= .38 to 4.1; ps < .58 to .06) , time of sacrifice (Fs =1.02 to 1.74; ps <.38 to .2) or saline treatment X time of sacrifice interaction (Fs = .19 to 3.2; ps <.82 to .06) on mean amount of total terminal contact per MNC membrane, mean number of synapses

Table 6

Effects Time Interval Between Injection and Perfusion on Apposition of MNCs and Cell Body and Nucleus Size in SON of Rats Receiving Injections of 1.5 M NaCl or 0.15 M NaCl.

Condition	% Glial Apposition	%Terminal Apposition	%Somatic and or Dendritic Apposition	Cell Body Size μm^2	Nucleus Size μm^2
1.5 M NaCl, 15 min (n=5)	73 ± 2	6 ± 1	11 ± 2	27.6 ± 1.6	9.1 ± .9
0.15 M NaCl 15 min (n=4)	74 ± 2	8 ± 2	6 ± 1	28.1 ± .8	9.7 ± .7
1.5 M NaCl 1 h(n=4)	74 ± 2	5 ± 1	9 ± 2	25.3 ± 1.1	8.8 ± 1.1
0.15 M NaCl 1 h (n=4)	79 ± 3	6 ± 2	6 ± 1	25.8 ± 1.1	8.4 ± .8
1.5 M NaCl 2.5 h (n=4)	73 ± 2	8 ± 2	10 ± 1	29.0 ± 2.4	9.8 ± .8
0.15 M NaCl 2.5 h (n=4)	75 ± 3	8 ± .6	7 ± 2	28.2 ± .7	10.3 ± .2



* $p < .05$

**$p < .01$

Figure 6: Changes Over Time in Golgi Apparatus as a Percentage of Cytoplasm. (Means: 1.5 M NaCl at 15 min = 4.1 %± .2%; 0.15 M NaCl at 15 min = 3 %± .2%; 1.5 M NaCl at 1 h =4.9% ± .1%, 0.15 M NaCl at 1 h = 3.1% ± .4%; 1.5 M NaCl at 2.5 h =5.9 % ±.8% , 0.15 M NaCl at 2.5 h = 2.8 % ±.5%; Adjusted 1.5 M NaCl at 5 h = 7.1% ± .2%, adjusted 0.15 M NaCl at 5 h = 3.5%± .9%.)

Table 7

Relations Between Synaptic Elements and MNC Membrane for Rats Sacrificed at Different Time Intervals After Injections

Condition	Term/MNC contact µm	# Syn/100µm membrane	#Mult/100µm membrane	%MNCsomata w /multiples *
1.5 M NaCl 15 min (n=5)	3.4 ± .43	.113 ± .012	.021 ± .003	47 ± 7
0.15 M NaCl 15 min (n=4)	3.82 ± .15	.128 ± .019	.022 ± .005	48 ± 9
1.5 M NaCl 1 h	3.8 ± .53	.113 ± .026	.018 ± .005	44 ± 11
0.15 M NaCl 1 h (n=4)	2.8 ± .27	.108 ± .018	.013 ± .007	24 ± 8
1.5 M NaCl 2.5 h (n=4)	4.5 ± .25	.093 ± .003	.026 ± .001	57 ± 4
0.15 M NaCl 2.5 h (n=4)	3.4 ± .14	.103 ± .012	.014 ± .003	32 ± .05

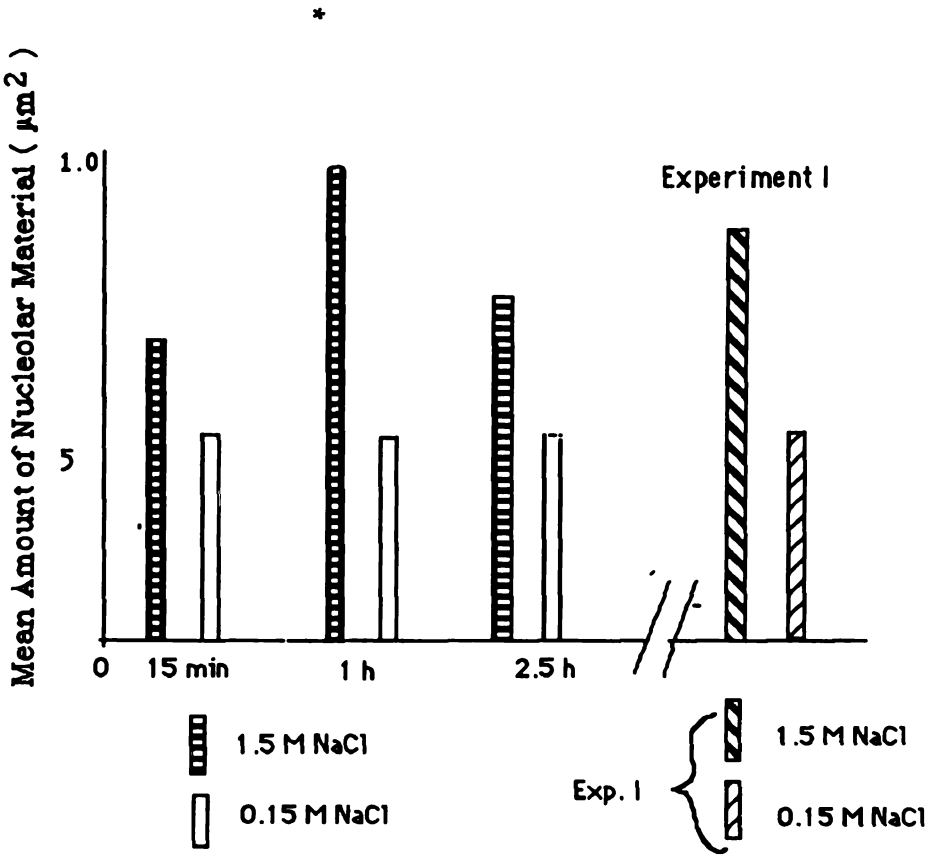
* $F = 5.7$, $p < .02$ for Saline main effect only.

per 100 μm of membrane, or the mean number of multiple synapses per 100 μm of membrane among the different groups receiving the different NaCl treatments and sacrificed at different times after the injection(F. The effect of saline treatment on percentage of MNCs contacted by multiple synapses is significant ($F= 5.7$; $p <.02$) with no significant time ($F=1.7$; $p<.20$) or saline treatment X time of sacrifice interaction ($F=1.7$,; $p<.19$). However, in the post hoc Tukey test, none of the individual mean values for the 1.5 M NaCl injection was found to be significantly greater than the corresponding time interval individual means for 0.15 M NaCl injections.

At one hour, animals receiving 1.5 M NaCl showed a large increase in mean amount of nucleolar material. Saline treatment was found to have a significant ($F=8.7$, $p < .008$) effect on amount of nucleolar material which was increased by 1.5 M NaCl injections. There was no significant time ($F=.13$, $p <.87$) or saline treatment X time of sacrifice interaction ($F=.95$, $p < .40$) effects. The effect of saline was mainly due to the large amount of nucleolar material for rats injected with 1.5 M NaCl and sacrificed 1 h post injection. In post hoc comparison tests, rats injected with 1.5 M and sacrificed after 1 h had significantly more nucleolar material than rats injected with 0.15 M NaCl and sacrificed 1 h after the injection ($p <.05$). There was no significant difference between saline conditions at 15 min or 2.5 h.

Neither saline treatment, nor time of sacrifice had a significant main effect on neural/glial apposition changes in the neural lobe. There is, however a significant saline treatment X time of sacrifice interaction effect ($F= 5.6$, $p<.01$) The animals show a small but

significant increase in neural apposition with the BL in the neural lobe 2.5 hours ($p < .05$) but not 15 min or 1 h after a 1.5 M NaCl injection compared to animals receiving 0.15 M NaCl injections. As shown in the bar graph in Figure 8, this change is smaller than that which has been shown to occur 5 hours after a 1.5 M NaCl injection but is larger than the mean terminal apposition with the BL for animals sacrificed 15 min and 1 h after receiving an injection of 1.5 M NaCl. There was no difference in the number of axons enclosed by the pituicytes among any of the conditions in Experiment II.



$p < .05$

Figure 7: Changes in Mean Amount of Nucleolar Material Over Time. (Means: 15 min: 1.5 M NaCl = .74 $\mu m^2 \pm .059$, 0.15 M NaCl = .58 $\mu m^2 \pm$ .057; 1 h: 1.5 M NaCl = 0.93 $\mu m^2 \pm .17$, 0.15 M NaCl = .50 $\mu m^2 \pm .04$; 2.5 h: 1.5 M NaCl= 0.77 $\mu m^2 \pm .09$, 0.15 M NaCl = .50 $\mu m^2 \pm .15$. From Experiment I, 5 h: 1.5 M NaCl = 0.88 $\mu m^2 \pm 0.16$ μm^2 ; 0.15 M NaCl = 0.58 $\mu m^2 \pm 0.11$ μm^2.)

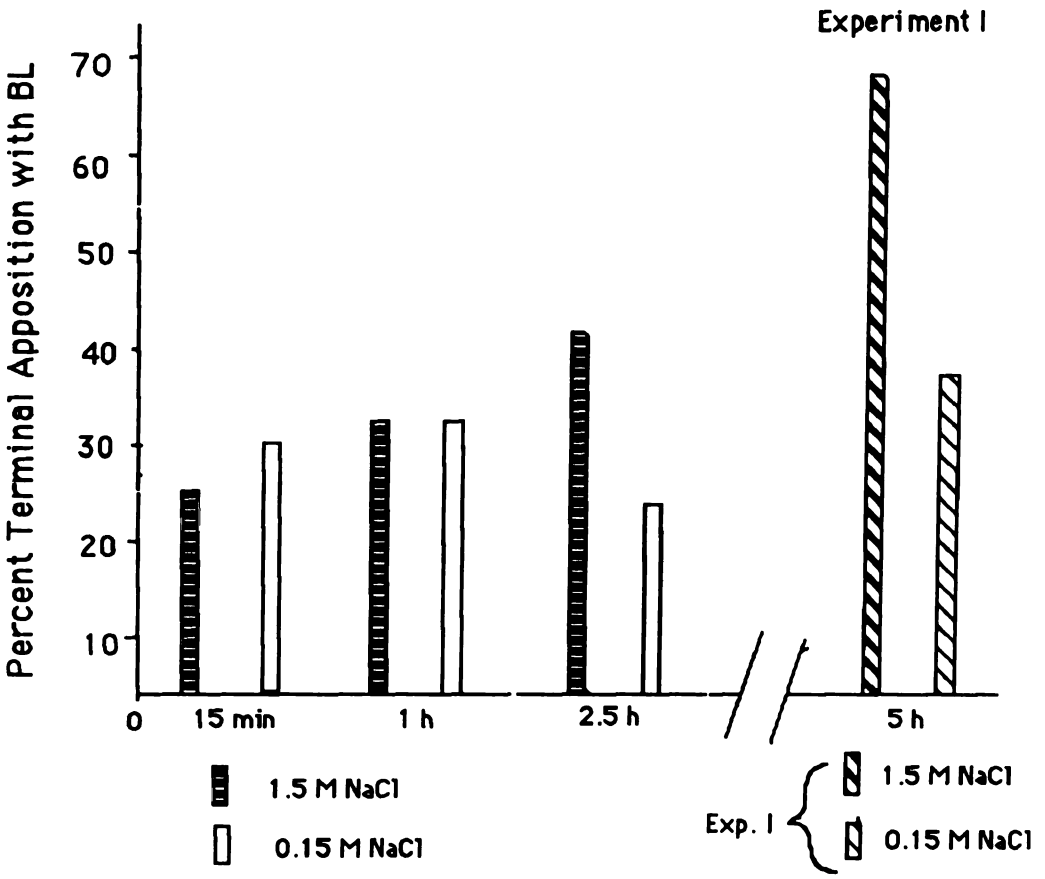


Figure 8. Percent Neural Apposition for Animals Sacrificed at Different Time Intervals after 1.5 M or 0.15 M NaCl Injections. (Mean percent neural apposition for 15 min: 1.5 M NaCl = 26% ± 2; 0.15 M NaCl = 31% ± 2; for 1 h: 1.5 M NaCl = 33% ± 4; 0.15 M NaCl = 33% ± 3; 2 h: 1.5 M NaCl = 42% ± 4; 0.15 M NaCl = 28% ± 1 Means for % neural apposition after 5 h, from Experiment I: 1.5 M NaCl = 67.3% ± 2.2 ; 0. 15 M NaCl = 36.4% ± 2.7.)

Discussion

In Experiment II, in animals sacrificed 15 min after injection of hypertonic saline, had no significant changes in the ultrastructure of the HNS detectable at the level of EM analysis used in this study that correspond to the increases in electrical activity in the SON, and in levels of plasma neuropeptides reported by other researchers at this time (Brimble and Dyball, 1977; Dyball and Pountney ,1973; Jones and Pickering , 1969). Nor were there many changes in response to the 1.5 M NaCl solution in animals sacrificed 1 h after the injection, the time that electrical activity and plasma levels of neuropeptides have been reported to reach a maximum level (Kasting, 1988; Gibbs, 1984). The data from Experiment II suggest, in fact, that for about 2.5 hours after an injection of 1.5 M NaCl, the response of the HNS as measured by changes in the ultrastructure, is limited. And at 2.5 h, there are only a few measurable morphometric changes, and these changes are smaller than those found at 5 h.

In the SON, at 15 min, 1 h and 2.5 h, there was no difference in apposition of glial cells, terminals or soma with MNCs among any of the groups. Nor was there a significant difference in size of MNC cell bodies or nuclei, and the number of multiple synapses did not increase. This suggests that significant retraction of glial projections from between the MNCs has not yet occurred at 2.5 hours after the hypertonic saline injection.

Amount of Golgi apparatus in MNC cytoplasm increased in groups that received 1.5 M NaCl injections and were sacrificed 1 or

2.5 h after the injection. The increase in percentage of Golgi apparatus in the MNC cytoplasm continued as the time between injection and sacrifice increased. The percentage of Golgi apparatus in the cytoplasm 5 h after the hypertonic saline injection (as shown in Experiment I), is even larger than at 2.5 h. This suggests the MNCs respond rapidly and continuously to the hypertonic stimulus with an increase in production of neuropeptide. Furthermore, the increase in Golgi apparatus appears to independent of any changes in glial apposition.

The hypertonic saline appeared to increase the number of MNCs contacted by multiple synapses in animals sacrificed 1 h and 2.5 h after injections of 1.5 M NaCl. Although this increase in cell bodies contacted by multiple synapses is not coupled with a corresponding increase in number of multiple synapses per 100 mm of membrane, increase in terminal apposition or in synapse size, it may be the first indication that some interaction is occurring between MNCs in the SON in response to the hypertonic stimulus.

The amount of nucleolar material was greatest in the group sacrificed 1 h after a 1.5 M NaCl injection; this may indicate the first response of the system. The expression of a large amount of nucleolar material at 1 h after an injection of 1.5 M NaCl is consistent with the finding by Sharp, Sagar, Hicks, Lowenstein and Hisanaga, (1991) of maximal c-fos expression (which reflects increased nuclear transcriptional activity) at 1 h after a large systemic injection of hypertonic saline. The increased nucleoli size may reflect increased transcriptional activity and increased production of ribosomal RNA prior to manufacture of the additional neuropeptides (Armstrong, Gregory, and Hatton, 1977) which are required when the system has

been stimulated with a 1.5 M NaCl injection, and may proceed other changes in response to the stimuli. The level of nucleolar material remains high at 2.5 h after injection of 1.5 M NaCl, but is not as high as at 1 h. The mean amount of nucleolar material found 5 h after a 1.5 M NaCl injection (in Experiment I) is elevated above that found at 2.5 h, but not as high as at 1 h. This suggests that after the initial large increase, the level of transcriptional activity remains high for an extended period of time, perhaps to allow for continued neuropeptide production.

A small but significant change in neural/glial organization has occurred in the neural lobe of the posterior pituitary 2.5 h after the hypertonic saline injection. The amount of terminal apposition with the BL is less than that which has been shown to occur 5 h after an injection of 1.5 M NaCl (Experiment I) but it is greater than that measured at earlier time intervals (15 min and 1 h) after the hypertonic injection or at any time after the isotonic injection. The fact that the pituicytes respond by retracting from the BL before the glial cells in the SON change their apposition with MNCs could suggest they are both under the influence of a blood borne factor such as adrenalin. Because of their proximity to, and contact with the peripheral circulatory system, and the low blood brain barrier of the neural lobe, pituicytes would receive a stronger signal prior to glial cells in the SON.

The results from Experiment II, added to other findings reported in the literature suggest that when initially challenged with a large systemic injection of hypertonic saline, the HNS responds with a rapidly observable (15 min) change in electrical activity in the SON. This is accompanied by release of neuropeptides into the blood plasma.

Within 1 h, both the electrical activity and the plasma release have reached an assymptotically high level which can remain elevated for several hours. With a sustained electrical activity and neuropeptide release comes an increased demand for neuropeptide production. This is reflected morpholgically by the significant increase in amount of nucleolar material and Golgi apparatus in the cytoplasm after 1 h. Within 2.5 h, glial retraction from between MNCs in the SON and away from the BL in the posterior pituitary starts to occur. This early retraction is indicated most significantly by changes in neural/glial apposition in the posterior pituitary, but is also reflected in the SON by the increase in number of MNCs with multiple synapses. Formation of multiple synapses appears to precede other changes due to glial retraction in the SON. By 5 h, there is a significant decrease in glial apposition with the MNCs, and a corresponding increase in terminal and somatic apposition, as well as an increase in cell body size. Measures that indicate an increase in terminal size and an increase in number of multiple synapses along the MNC membrane are also larger.

EXPERIMENT III

The nature of the stimulus signalling HNS activation was further investigated in Experiment III. Hatton et. al. (1991) showed that the addition of adrenalin to cultured pituicytes caused shape changes consistent with retraction from the BL. This suggested to us that adrenalin might be a systemic signal causing glial projections to retract to allow increased terminal apposition with the BL in the posterior pituitary, and increased apposition between MNCs in the SON.

Adrenalin release is triggered from the adrenal medulla by the sympathetic nervous system in response to a variety of stressful stimuli, such as pain, cold, insulin, electrical shock, or stimuli that produce the emotional states of fear or rage. It is released into the blood stream and acts on a variety of targets, e.g. skeletal muscles, blood vessels, heart lungs to enable the animal to engage in the fight or flight response as described by Cannon (1912, cited by Tepperman, 1968). Researchers such as Lightman and Young (1987,1988) and Kasting (1988) have used large doses of hypertonic saline as a stressor to produce changes in the HNS. Since large systemic injections of hypertonic saline have been shown to be stressful, it would be expected, therefore that they might cause a sympathetic release of adrenalin.

In Experiment III, we attempted to remove the adrenalin signal by two methods to determine the effect its absence would have on the response of the HNS to a hypertonic saline injection. Animals were

anesthetized to reduce the "perception" of the 1.5 M NaCl stimulus and subsequent stress-induced release of adrenalin. Other animals underwent bilateral removal of the adrenal medullae in order to remove the source of adrenalin that we hypothesized to be a systemic signal activating the HNS. Neither manipulation interfered with the osmotic nature of the stimulus so we could determine which (if any) of our measures responded primarily to the osmotic challenge.

Methods

In Experiment III, animals received IP injections of 18 ml/kg 1.5 M or 0.15 M NaCl, and also one of three possible treatment conditions.

1. Baseline condition: Ten animals were injected in the same manner as animals in Experiment I (half received 1.5 M NaCl; half 0.15 NaCl) and were sacrificed 5 h post injection with no additional treatment . They were perfused and SON and posterior pituitary tissue was prepared for TEM analysis in the same manner as in Experiments I and II.

2. Anesthetized condition: Ten animals were anesthetized with 3.3 mg/kg equithesin prior to injections of either 1.5 M or 0.15 M NaCl. After receiving equithesin doses of this level, animals became immobile and did not respond to an ear or tail pinch. They remained quiescent for the five hours between saline injection and re-anesthetization and perfusion. SON and posterior pituitary tissue was prepared and analyzed as described in Experiment I.

3. Medullectomized condition: Ten rats were bilaterally adrenal-medullectomized and allowed to recover from surgery. In order to

reduce the mortality rate caused by the trauma of acute bilateral medullectomies, the adrenal medulla on one side was removed, the animal was allowed to recovery from surgery for five days, then the remaining medulla was removed. A small group of animals received sham medullectomies, with the surgery performed in an identical manner, except the adrenal medulla was briefly exposed but not removed. To compensate for any possible damage to the adrenal cortex, rats were given a drinking bottle containing 1% saline as well as a bottle of the usual tap water. One week after recovery from the second surgery, the animals were subjected to the same injection conditions (half received 1.5 M NaCl; half, 0.15 M NaCl) as the animals in Experiment I. These animals were anesthetized and perfused five hours after the injection; pituitaries and SON nuclei were examined in the same manner as in Experiment I.

Means for SON and posterior pituitary measures in the baseline and anesthetized animals were compared using a 2 X 2 Factorial ANOVA with Tukey post hoc tests when indicated; values of $p < .05$ were considered significant. The SON and posterior pituitary measures for the two NaCl conditions of MDX animals were compared using a Student t-test.

Results

A. Anesthetized Animals

The effects of anesthesia on the HNS of animals receiving 1.5 M or 0.15 M NaCl injections were evaluated with a 2 X 2 Factorial ANOVA; F and p values for SON measures are shown in Table 8. There was a significant saline treatment X anesthesia interaction effect on the values of all the SON elements measured except amount of nucleolar material and number of multiple synapses contacting the MNC membrane. Post hoc comparisons showed that there were significant differences in the SON between animals who received injections of 1.5 M NaCl and 0.15 M NaCl in the non-anesthetized condition for all elements in apposition with MNC membrane, cell body and nucleus size, amount of nucleolar material, percent of MNCs contacted by multiple synapses and amount of Golgi apparatus in the MNC cytoplasm. Differences between elements in the SON between 1.5 M NaCl and 0.15 M NaCl injected animals in the anesthetized condition were significant only for total number of terminals contacting the MNC membrane and amount of Golgi apparatus in the cytoplasm; for all other SON elements measured, the anesthesia suppressed the changes usually induced by hypertonic saline. For measures of total number of terminals contacting the MNC membrane and amount of Golgi apparatus in the cytoplasm, as well as for glial and somatic apposition, and percent of MNCs contacted by multiple synapses, the 1.5 M NaCl injected unanesthetized animals measured significantly above the 1.5 M NaCl injected anesthetized animal; thus the anesthesia was shown to significantly modulate the effect of the

hypertonic saline injection for these measures. There were no other significant differences between SON elements in the two saline injection conditions in anesthetized animals.

Mean amount of nucleolar material and number of multiple synapses contacting the MNC membrane were both significantly affected by the saline condition only (and not by anesthesia or saline X anesthesia interaction), suggesting that for these elements, the anesthetized animals respond to the injections of 1.5 M NaCl in a manner similar to the unanesthetized animals. The mean values for SON elements for each group are presented in Tables 9 and 10.

In the posterior pituitary, saline treatment, anesthesia and saline treatment X anesthesia interaction were all found to have significant effects on terminal and pituicyte apposition with the BL. Post hoc tests in both cases showed the only significant differences occurred between the two unanesthetized saline conditions and the unanesthetized and anesthetized animals that received 1.5 M NaCl injections. Saline treatment alone was found to have a significant effect on number of axons enclosed by pituicytes. Table 11 shows the means and the F and p values for these pituitary measures.

Table 8
Significance Levels Comparing Effects of Saline Treatment and Anesthesia on SON Measures

	Effect of Factors		
Element Measured	Saline	Anesthesia	Saline X Anesthesia
Glial apposition *	F = 43.4, p <.001	F = 4.8, p <.04	F = 17.4, p <.001
Somatic Apposition *	F = 10.8, p <.006	F = 6.9, p <.02	F = 8.4, p <.01
Terminal contact *	F = 20.6, p <.005	F = 6, p <.03	F = 10.1, p <.007
Terminal Apposition**	F = 31.07, p <.001	F = .03, p <.87	F = 4.67, p <.05
Cell body size **	F = 5.5, p <.03	F = 1.6, p <.22	F = 4.6, p <.05
Golgi **	F = 65.9, p <.001	F = 4.2, p <.06	F = 6.4, p <.02
%MNC w/ mult syn**	F = 4.5, p <.05	F = 2.6, p <.13	F = 6.8, p <.02
Nucleoli size ***	F = 7.9, p <.02	F = 1.7, p <.21	F = .71, p <.41
#Mult.syn/100 µm membrane ***	F = 10.8, p <.006	F = .012, p <.9	F = .97, p <.34

* Saline treatment, Anesthesia, and Saline treatment X Anesthesia Interaction are significant.

** Saline treatment and Saline treatment X Anesthesia Interaction are significant.

***Saline treatment effect is significant.

Table 9

Comparison of the Effect of Anesthesia on Apposition of MNCs and Cell Body, Nucleus and Nucleoli Size in SON of Rats Receiving Injections of 1.5 M NaCl or 0.15 M NaCl.

	Percent Apposition w/MNC			Sizes (in μm^2)		
Condition	Glial	Terminal	Somatic and/or Dendritic	Cell Body	Nucleus	Nucleoli
1.5 M NaCl (n=5)	63 ± 1 * #	13 ± 1.5**	18 ±1* #	29.3± .9**	10.4 ± .6**	.86 ± .06 (saline only)
0.15 M NaCl (n=5)	79 ± 1	8 ± .7	8 ± 1	24.5 ± 1.9	8.6 ± .3	.73 ± .08
1.5 M NaCl +anesthesia (n=4)	72 ± 2	10 ± .9	10 ±1	25.9 ± 1.1	9.1 ± .8	.80 ± .09
0.15 M NaCl +anesthesia (n=4)	75 ± .5	9 ± .8	10 ± .5	23 ± 2.3	8.9 ± .7	.53 ± .06

* greater than 0.15 M NaCl, $p < .01$

** greater than 0.15 M NaCl, $p < .05$

greater than 1.5 M NaCl anesthetized, $p < .05$

Table 10

Comparison of the Effect of Anesthesia on Relations Between Synaptic Elements and Magnocellular Secretory Cell Membrane in SON of Rats Receiving Injections of 1.5 M NaCl or 0.15 M NaCl.

Condition	Term/MNC contact µm	#synapse /100 µm	#mult/100 µm	% MNCs w/multiples	% Golgi Apparatus
1.5 M NaCl (n=5)	8.8 ± .54 ** ##	.082 ± .012	.028 ±.004	69 ± 4 * #	8.4 ± .4 ** #
0.15 M NaCl (n=5)	5.0 ± .18	.083 ± .004	.018 ± .003	24 ± 8	4 ± .01
1.5 M NaCl Anesthesia (n=4)	5.9 ± .67 @	.12 ± .004	.023 ± .005	48 ± 11	6.5 ± .5@
0.15M NaCl Anesthesia (n=4)	5.1 ± .44	.12 ± .007	.015 ± .002	49 ± 2	4.3 ± .4

* greater than 0.15 M NaCl, $p < .01$

** greater than 0.15 M NaCl, $p < .05$

greater than 1.5 M NaCl anesthetized, $p < .05$

greater than 1.5 M NaCl anesthetized, $p < .01$

@ greater than 0.15 M NaCl anesthetized, $p < .05$

Table 11. Pituicyte Measures for Anesthetized and Non Anesthetized Animals Injected with 1.5 M or 0.15 M NaCl

Condition	%Terminal Apposition	%Pituicyte Apposition	Axons enclosed by pituicyte (saline effect only)
1.5 M NaCl baseline n=6	59 ± 2* ##	39 ± 2* #	.92 ± .19
0.15 M NaCl baseline n=6	35 ± 3	64 ± 3	1.8 ± .32
1.5 M NaCl anesthetized n=4	39 ± 4	61 ± 4	.74 ± .36
0.15 M NaCl anesthetized n=4	38 ± 4	61 ± 4	2.2 ± .44
Main Interaction Effects	Saline: F=15.7, p <.001	Saline: F=16.7, p <.0008	Saline: F=17.3, p <.0007
	Anesthesia: F=6.95, p <.017	Anesthesia: F=8.7, p <.009	Anesthesia: F=.093, p <.76
	Saline X Anesthesia: F=14.4, p <.001	Saline X Anesthesia: F=16.76, p <.0008	Saline X Anesthesia: F=.84, p <.37

* greater than 0.15 M NaCl, p <.01

greater than 1.5 M NaCl anesthetized, p <.01

greater than 1.5 M NaCl anesthetized, p <.05

B. MDX Animals

After removal of their adrenal medullae, animals were given the option of drinking 1% NaCl solution or tap water. However, no MDX animal drank a measurable quantity of the 1% NaCl solution; all consumed the tap water as usual. Post perfusion examination of MDX animals confirmed that adrenal cortices were intact, and adrenal medullae were absent with little or no visible regeneration of medullary tissue. Measures for SON and posterior pituitary elements of sham operated animals to the stimuli were within the range of values of animals who had received no surgery (baseline condition). Tables 12 and 13 present the SON elements measured for MDX , sham MDX and baseline condition animals receiving either 1.5 M or 0.15 M NaCl injections.

SON elements of MDX animals that received injections of 1.5 M NaCl were compared to SON elements of MDX animals that received 0.15 M NaCl. Amount of Golgi apparatus in the cytoplasm of MDX animals injected with 1.5 M NaCl was significantly greater than in MDX animals injected with 0.15 NaCl ($p < .002$, Student t-test). This was the only SON element to have significantly different values in the two MDX saline injection conditions. Percent of Golgi apparatus in the cytoplasm of MDX animals receiving 1.5 M NaCl appeared similar to that of intact animals who had received injections of 1.5 M NaCl; percent of Golgi apparatus in the cytoplasm of MDX animals receiving 0.15 M NaCl appeared similar to that of intact animals who had received injections of 0.15 M NaCl. As shown in Tables 12 and 13, for most other SON of elements, values obtained from MDX animals injected with 1.5 M or 0.15 M NaCl did not appear to differ from those

obtained from intact animals receiving 0.15 M NaCl injections. Size of nucleus, amount of nucleolar material, and total MNC membrane contacted by terminals are exceptions to this. Both 1.5 M NaCl injected and 0.15 M NaCl injected MDX animals had very large nuclei and nucleoli. MDX animals in both injection conditions also appeared to have less terminal contact with the MNC membrane than baseline or anesthetized animals in either injection condition.

The percentage of MNCs contacted by multiple synapses is not significantly different between MDX animals in the two saline injection conditions. As numbers in Table 13 indicate, however, there does appear to be a higher percentage of MNCs contacted by multiples in the MDX than in the baseline 0.15 M NaCl injection condition. This number is not as high as the mean for animals in the baseline 1.5 M NaCl condition.

In the posterior pituitary, there were no differences between the MDX animals in the two injection conditions for neural or pituicyte apposition with the BL, or number of terminals enclosed by pituicytes (Mean percent neural apposition with BL: 1.5 M NaCl = 33% ± 3, 0.15 M NaCl = 36% ± 2, $p < .44$; mean percent pituicyte apposition with BL: 1.5 M NaCl = 66% ± 3, 0.15 M NaCl = 63% ± 2, $p < .47$; mean number axons enclosed by pituicyte cytoplasm: 1.5 M NaCl = 1.2 ± .3, 0.15 M NaCl = 1.8 ± .3, $p > .24$).

Table 12

Comparison of the Effect of Adrenal-medullectomy on Apposition of MNCs and Cell Body, Nucleus and Nucleoli Size in SON of Rats Receiving Injections of 1.5 M NaCl or 0.15 M NaCl.

	Percent Apposition w/MNC			Sizes (in μm^2)		
Condition	Glial	Terminal	Somatic and/or Dendritic	CellBody	Nucleus	Nucleoli
1.5 M NaCl baseline (n=5)	63 ± 1	13 ± 1.5	18 ±1	29.3± .9	10.4 ± .6	.86 ± .06
0.15 M NaCl baseline (n=5)	79 ± 1	8 ± .7	8 ± 1	24.5 ± 1.9	8.6 ± .3	.73 ± .08
1.5 M NaCl						
Sham A	60	17	20	29.2	10.3	.88
Sham B	64	11	21	32	12.4	.85
0.15 M NaCl						
Sham C	79	6	10	20.7	7.9	.74
Sham D	75	8	8	22.6	8.3	.66
1.5 M NaCl+ MDX (n=5)	73 ± 2	8 ± .4	12 ± 2	25.3 ± 1.3	11.6 ± .9	.93 ± .1
0.15 M NaCl + MDX (n=5)	74 ± 2	6 ± 1	12 ± 2	24.8 ± .9	10.7 ± .5	.82 ± .09

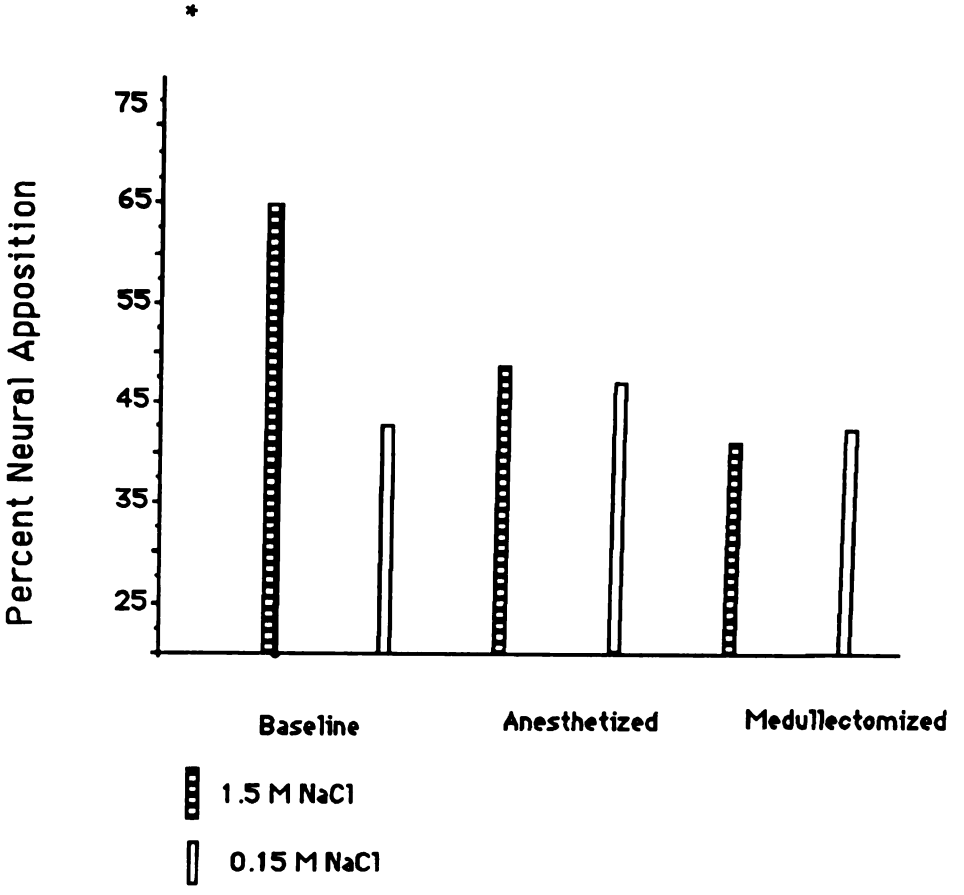
Table 13

Comparison of the Effect of Adrenal-medullectomy on Relations Between Synaptic Elements and Magnocellular Secretory Cell Membrane in SON of Rats Receiving Injections of 1.5 M NaCl or 0.15 M NaCl.

Condition	Terminal/MNC contact mm	#synapse/ 100 mm	#mult/100 mm	% MNCs w/ multiples	% Golgi Apparatus
1.5 M NaCl baseline (n=5)	8.8 ± .54	.082 ± .012	.028 ±.004	69 ± 4	8.4 ± .4
0.15 M NaCl baseline (n=5)	5.0 ± .18	.083 ± .004	.018 ± .003	24 ± 8	4 ± .01
1.5 M NaCl					
Sham A	7.9	.07	.021	57	9
Sham B	9.1	.08	.030	64	8
0.15 M NaCl					
Sham C	4.9	.08	.016	29	4
Sham D	5.2	.09	.012	25	4
1.5 M NaCl+ MDX (n=5)	2.8 ± .3	.08 ± .01	.022 ± .003	49 ± 7	8.4 ± .7 *
0.15 M NaCl + MDX (n=5)	3.1 ± .23	.10 ± .01	013 ± .002	35 ± 5	3.8 ± .7

Both anesthesia and adrenal medullectomy interfered with increased terminal apposition with the BL normally seen in an intact, awake animal in response to an injection of 1.5 M NaCl. Animals who received 1.5 M NaCl injections and no further treatment (baseline condition) were the only group to show a significant increase in terminal (and decrease in pituicyte) apposition with the BL. Figure 8 is a bar graph comparing mean neural apposition for baseline, anesthetized, and medullectomized animals receiving 1.5 M and 0.15 M NaCl injections.

Figure 10 is a micrograph illustrating the neural lobe in the condition normally produced by an IP injection of 1.5 M NaCl . The stellate shape is typical of an activated pituicyte and contrasts with the amorphous shape of the pituicytes in Figures 11 and 12. Figure 11 is a micrograph of the neural lobe of an animal medullectomized prior to receiving the 1.5 M NaCl injection and Figure 12 is a micrograph from the neural lobe of an animal which received an injection of 0.15 M NaCl. The shape of pituicyte and amount of cytoplasmic apposition with the BL is similar for these conditions.



p <.001

Figure 9: Neural Apposition in the Posterior Pituitary in Anesthetized and Medullectomized Animals. (Means: Baseline 1.5 M NaCl = 59 %± 2%, 0.15 M NaCl = 35% ± 2%; anesthetized 1.5 M NaCl = 38% ± 4%, 0.15 M NaCl = 38% ± 4%; MDX 1.5 M NaCl = 33% ± 3%, 0.15 M NaCl = 36% ± 4 %. p <.001, Student t-test.)

Figure 10. Electron Micrograph of a Neural Lobe of an Intact Rat Injected With 1.5 M NaCl. (The pituicytes acquire a characteristic stellate form. Pituicyte cytoplasm (p) is retracted and most terminals (t) abut the basal lamina (BL). N = pituicyte nucleus; l = lipid. Bar = 1 μm.)

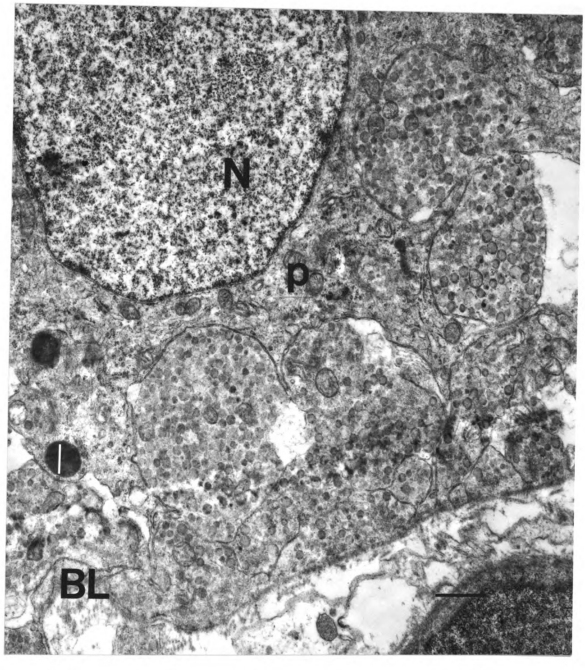


FIGURE 10.

Figure 11. Electron Micrograph of Neural Lobe of Medullectomized Rat Injected with 1.5 M NaCl (Axons (ax) are enclosed by pituicyte cytoplasm (p) and the pituicyte is interposed between axons and the basal lamina (BL) and fenestrated capillary (f c). N = pituicyte nucleus; l= lipid. Bar = 1 μm.)

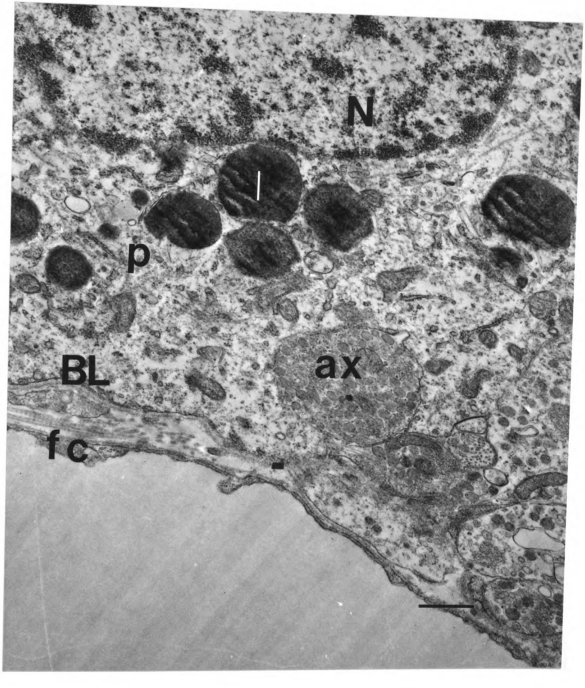


FIGURE 11.

Figure 12. Electron Micrograph of Neural Lobes of Rat Injected with 0.15 M NaCl. (Axons (ax) are enclosed by pituicyte cytoplasm (p) and the pituicyte is interposed between axons and the basal lamina BL. N = pituicyte nucleus; t = terminal. Bar = 1 μm.)

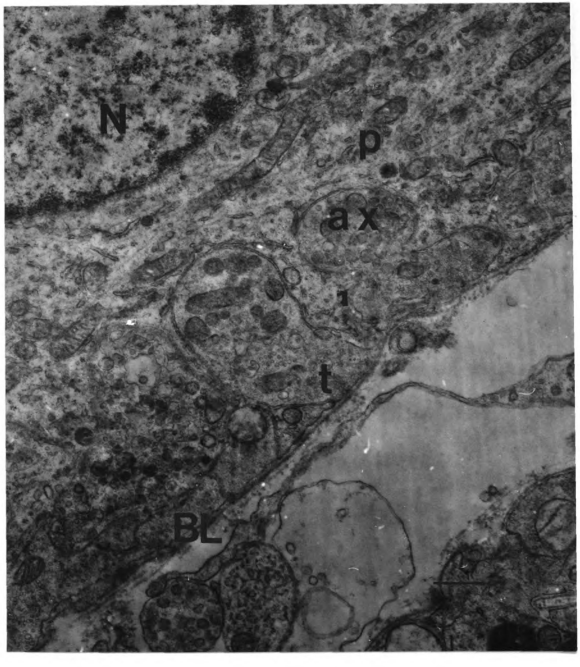


FIGURE 12.

Discussion

Animals in the baseline conditions replicated the results from Experiment I. There were significant differences between animals receiving hypertonic and isotonic saline injection for all SON and posterior pituitary elements measured. Anesthetizing the animal prior to the large IP injection of hypertonic saline removed the changes in most SON elements produced by the hypertonic saline injections (such as changes in elements in apposition with the MNC membrane, increases in cell body size and amount of nucleolar material, and number of multiple synapses) and modulated the increases in total number of terminals contacting the MNC membrane and amount of Golgi apparatus in the cytoplasm. Anesthesia also removed changes in neural/glial apposition with the BL in the posterior pituitary, but did not change the decrease in axons enclosed by pituicyte cytoplasm sown to be caused by the hypertonic saline injection in an awake animal. Adrenal medullectomy removed all SON and posterior pituitary differences attributed to the large hypertonic saline injection, except the increase in Golgi apparatus in the MNC cytoplasm.

Results from Experiment III suggest that adrenalin plays an important role in signalling some types of activity in the HNS. Both anesthetizing the animal for the 5 h interval between injection of 1.5 M NaCl and perfusion, and removal of the adrenal medullae interfered with the response of the SON to the hypertonic stimulus. Many of responses interfered with by anesthesia or medullectomy could be the result of reduced glial retraction from between the MNC somata. For animals who were anesthetized or adrenal-medullectomized prior to the 1.5 M NaCl injection, there was little or

no difference in any of the apposition measures which typically characterize an animal injected with hypertonic saline after 5 h, when compared to animals receiving isotonic saline injections, such as increase in synaptic terminal contact or contact with other MNCs or their dendrites. Nor is there a large increase in cell body size, which could be related to glial retraction (Modney and Hatton, 1989a). All these findings suggest lack of retraction of glial cells in the SON in response to a 1.5 M NaCl injection in animals who have been anesthetized or who are without systemic adrenalin.

For some SON elements, hypertonic saline did effect a change on the ultrastructure in the anesthetized animals, but the magnitude of these effects were smaller than in the non-anesthetized animals. Anesthesia significantly reduced or eliminated the differences normally seen when comparing the effects of hypertonic saline injections to isotonic saline injections in the baseline condition. In the anesthetized animal, only amount of nucleolar material and number of multiple synapses contacting the MNC membrane were increased by the hypertonic saline injection without significant interference from the anesthesia. These responses, therefore may respond only to osmotic stimuli and not systemic adrenalin. Findings from Experiment II suggest that amount of nucleolar material increases before glia retract from the MNC membrane, suggesting this may be an early and very sensitive response to the hypertonic NaCl injection stimulus. The increase in number of multiple synapses suggests that this response to the hypertonic stimulus may be capable of occurring in conditions of minimal glial retraction.

Large systemic injections of hypertonic saline produced no change in most SON elements in MDX animals. There were no differences between hypertonic and isotonic saline groups for elements in apposition with the MNC membrane, cell body and nucleus size, amount of nucleolar material and numbers of multiple synapses contacting MNC membranes. When compared to intact animals, HNS elements for MDX animals in both saline conditions appear very similar to those of the baseline animals injected with isotonic saline.

There is evidence, however, that animals without their adrenal medulla are capable of responding to the hypertonic stimulus, as the amount of Golgi apparatus in the MNC cytoplasm of animals receiving 1.5 M NaCl is elevated to the level of the intact animal receiving 1.5 M NaCl. Increased amount of Golgi apparatus reflects increased production of secretory vesicles for release (Broadwell and Oliver, 1981). This suggests that the MNCs are responding to the 1.5 M NaCl stimulus in a manner independent of glial retraction. Glial retraction does not appear to be necessary for increased hormone synthesis.

Removing the adrenal medullae appears to affect the nucleus of the SON cells, as animals in both conditions show enlarged nuclei and nucleoli. Any further changes occurring to the MNC nucleus as a result of the hyperosmotic injection cannot be detected. Whether this is due to a lack of response to the hypertonic stimulus or a reflection of the nucleus having reached a maximal asymptotic size cannot be determined. Also, MDX rats showed a significantly smaller mean total terminal contact with MNC membrane than intact rats. As there was no difference in number of synapses per 100 μm of MNC membrane among the groups, the difference in total terminal contact could be

attributed to smaller sized individual terminal contacts with the MNC. The injection condition (1.5 M NaCl versus 0.15 M NaCl) did not appear to affect this measure; the difference in terminal contact too, would have to be attributed to the adrenal medullectomy.

The evidence of interference with glial retraction in the SON in animals which have sustained removal of the source of their systemic adrenalin could be explained by the fact that the SON is in direct contact with the pial surface of the brain. This puts SON glial cells in close proximity with many blood vessels which could allow for an effective blood borne signal. The stimulus of hypertonic saline could increase the penetrability of the blood brain barrier, allowing systemic adrenalin to signal retraction in the glial cells in the SON in the intact animal. Without the adrenal medullae, the animal is incapable of producing sufficient quantities of adrenalin to stimulate glial retraction in the SON in the 5 h time interval between injection and sacrifice.

In the neural lobe both anesthesia and adrenal medullectomy manipulations inhibited changes in neural BL apposition that occur within 5 h in response to a hypertonic injection in intact animals. Pituicyte coverage of the BL remained at levels found for animals receiving isotonic injections in both cases. This suggests anesthesia either inhibits the retraction of the glial cells directly, or suppresses a peripheral response (such as a stimulus-induced increased adrenalin output from the adrenal medullae) that would normally activate the system in the presence of the 1.5 M NaCl injection. Barbiturates, including pentabarbitol, which is the main active ingredient in equithesin, have been shown to selectively depress transmission in the sympathetic nervous system ganglia and reduce levels of circulating

epinephrin (Stoelting, 1987). Since removal of systemic adrenalin also cancels the response of the neural lobe to the 1.5 M NaCl injection, it is tempting to speculate that blood borne adrenalin is the agent responsible for acting on the pituicytes causing their withdrawal.

However, the response of the HNS to 1.5 M NaCl for all the elements measured in the anesthetized animal appear to be somewhat suppressed, suggesting a more general inhibition of activity by the anesthesia. Dyball (1965) found that pentobarbitone decreased OX release from incubated neural lobes by interfering with calcium uptake from the incubation media. Direct interference with calcium uptake might explain the inhibitory effect of the anesthesia on the HNS. Alternatively, if an animal is anesthetized, it may be insufficiently stressed to mobilize increased release of adrenalin from the adrenal medulla, and the same absence of adrenalin may be responsible for lack of glial retraction (and neuropeptide release) from the neural lobe. The two methods of action may not be incompatible; as Hatton, Mason, and Bicknell (1990) have suggested mobilization of calcium ions may be involved in the response of the glial cells.

Hatton et. al. (1991) confirmed that adrenalin, a potent β 2 agonist, transformed cultured pituicytes from rounded to stellate shapes. These studies support the idea that adrenalin is involved in neurohypophysial plasticity. Although many studies (Day and Renaud, 1984; Day, Randle, and Renaud, 1985; Randle, Mazurek, Kneifel, Dufresne, and Renaud, 1986) have examined the effects of catecholamines on VP and OX release from the SON, the increase in neuropeptide release has generally been attributed to increased activity of the MNCs mediated by α1 receptors on these cells. However, the

method used in these studies of perfusing NA through hypothalamic explants or onto the exposed base of an animal's brain would not adequately distinguish neural from glial activity in this area. The relative contribution of systemic adrenalin to the response of the SON requires further investigation. Nevertheless, removal of the adrenal medulla in the present study does eliminate the response produced by 1.5 M NaCl in the HNS in intact animals.

GENERAL CONCLUSION

The basic findings of the three experiments comprising this study are: 1) A large systemic injection of hypertonic saline is capable of causing significant morphometric changes in the ultrastructure of the SON and posterior pituitary within 5 h. These changes are consistent with increased neuropeptide manufacture and release. 2) Ultrastructural changes in the system at times earlier than 5 h are limited. The earliest indication of glial retraction occurs in the posterior pituitary at 2.5 h, at that time, significant glial retraction in the SON has not yet occurred. 3) Anesthetizing the animal or eliminating systemic adrenalin by removing its adrenal medullae interferes with the response of the HNS to the hypertonic saline stimulus.

The results of the above experiments suggest that multiple systems are activated in the response of the HNS to a stimulus such as a large IP injection of 1.5 M NaCl. Systems which function to signal the MNCs to increase manufacture of neuropeptides to be released into the blood stream under conditions of increased demand respond relatively rapidly. The activation of such system(s) causes increase in electrical activity in the SON (Brimble and Dyball,1977) and increase of levels of neuropeptides in the blood plasma (Gibbs,1984; Kasting,1988) within 15 min of stimulus onset. This activity could be mediated by a reflexive response of the sympathetic nervous system, as the 15 min response

time is too rapid to allow for increased osmotic pressure to reach the pituitary via the blood stream or SON via CSF. It could also be signalled by systemic baroreceptors firing in response to lowered fluid volume as cellular and interstitial fluid are drawn into the gut on the osmotic gradient. Whatever the signal, the increased electrical activity and neuropeptide release reported in the literature occur independently of the ultrastructural changes measured in this study.

The increased demand for neuropeptides is first reflected in our morphological measures at 1 h by the increase of Golgi apparatus in the MNC cytoplasm. Changes in nucleolar material would be expected with the increased demand for neuropeptide production, and interestingly, the largest difference in nucleolar material is not at the times of maximal Golgi activation but also at 1 h after the 1.5 M NaCl injection, which precedes maximal Golgi apparatus levels found in the cytoplasm. One hour was also determined to be the time for maximal expression of c-fos in VP MNCs after an injection of hypertonic saline (Sharp et. al.,1991). This increase in nucleolar mass, then, could reflect an increase in nucleolar activity (neuropeptide producing mRNA?) preparatory to sending information to the cytoplasm to increase neuropeptide production.

Other responses of the HNS, which are reflected by the ultrastructural changes we observed in the SON and posterior pituitary 5 h after stimulus onset may be mediated by a blood borne signals, specifically the osmotic nature of the stimulus and/or adrenalin.

Sladek and Armstrong (1985) reviewed several studies examining the possible location of osmoreceptors and/or sodium

receptors that result in VP release. They suggest CNS signals leading to VP release may originate in the anterior hypothalamus, specifically the anterior ventral third ventricle (AV3V), the subfornical organ (SFO) and the *organum vasuclosum* of the *lamina terminalus* (OVLT) (where there is evidence for a more penetrable blood brain barrier) as well as the SON. Because of the large osmolality changes required to elicit significant changes in neuronal firing in vitro studies, they conclude it is unlikely that osmotic depolarization is the sole mechanism responsible for somatic regulation of VP release in vivo. The osmoreceptor role of the VP neuron may be to maintain responsiveness of the system to chronic stimulation. Our findings are consistent with this theory. While we cannot discount the importance of the osmotic character of the stimulus, the decreased response in anesthetized and MDX animals suggests an additional factor is signalling activity in the HNS. Under normal environmental conditions an osmotic challenge would also be stressful; this is exacerbated with out large systemic injection of hypertonic saline. Animals deprived of systemic adrenalin did not respond to the hypertonic saline stimulus in the same manner as awake, intact animals.

Activity of the HNS in response to a systemic adrenalin signal involves glial retraction from between MNCs in the SON to allow for increased communication between the cell bodies, and pituicyte retraction from the basal lamina to facilitate release of neuropeptides from the neurohypophysis. That the signalling agent may be adrenalin, is borne out by the failure of the system to respond in animals who have had the medulla portion of their adrenal glands

removed. This system responds more slowly, as one would expect of a hormonal signal. Most effects do not even start to occur until 2.5 h after the onset of the stimulus, and even at that time interval after injection the pituitary response is small and the SON shows no significant glial retraction.

The findings of this study initially appear to contradict in vitro findings by Modney and Hatton (1989b) and Perlmutter et. al. (1984). In both studies the HNS appeared to respond to incubation in hypertonic CSF with changes in ultrastructure. The preparations involved only isolated neural tissue and no adrenalin was added to the media. If adrenalin is necessary for glial retraction, why did changes consistent with glial retraction occur in vitro? In the SON, there exists the possibility that the abundant NE terminals found in the area liberated their contents into the perfusate. This would provide some, if not optimal, catecholaminergic stimulation. This could not be true in the neural lobe, where there are no NE terminals and no intrinsic source of adrenalin, however. It is more likely, in both cases, that the isolated preparation is more exposed to and therefore more likely to respond to the highly osmotic stimulus. If we postulate that multiple systems affect activity in the HNS, in the slice preparation, one type of stimulus might be sufficient. The buffers and barriers normally present in an intact animal would not be present in the slice preparation, and simple hyperosmolality might be adequate to provoke the glial retraction.

As suggested by work of Lightman and Young (1987,1988), a 1.5 M NaCl injection of this size can be considered stressful. Lightman found changes in mRNA for CRH and enkephalin in the SON and PVN in response to a 1.5 M NaCl injection to be similar to those

produced in these nuclei during naloxone precipitated withdrawal from opiates. This would suggest similarity in the organism's responses to the two conditions, and would support the contention that the 1.5 M NaCl injection is stressful and that the HNS is involved in responses to an acute challenge such as stress. If 1.5 M NaCl is a type of physiological stressor of the magnitude of naloxone precipitated opiate withdrawal, this stress might also be partly responsible for the large and rapid morphological changes in the SON and posterior pituitary found in Experiment I.

Sympathetic-induced HNS activity may play a special role in an organisms response to stress. Kasting (1988) showed that different stressors produced different patterns of VP and OX release. The neuropeptides could be released simultaneously, independently and in different ratios depending on the stimulus. Gibbs (1984) measured VP and OX with CRH in the median eminence (ME) and determined these posterior pituitary neuropeptides are involved release of ACTH from the anterior pituitary. He believed they potentiated CRH activity. By potentiating ACTH release, the HNS interacts with the hypothalamo-pituitary-axis (HPA) and the subsequent release of corticosteroids, and gives a high degree of flexibility to the stress response system as OX and VP have been shown to vary qualitatively and quantitatively in response to different types of stressors. The HNS may be involved in rapid potentiation of ACTH release, but most studies suggest it is important in long term control of ACTH secretion. For instance, Antoni, Kovacs, Dohanits, Makara, Holmes and Mazurek, 1988) found that posterior PVN lesions lead to long term (6 week) increases in OX and VP in the ME. This suggests the HNS can has the capacity for long

term structural and functional changes which allow neuropeptide levels to increase and act on the HPA as demand arises.

The HNS increases release of neuropeptides in response to many of the same signals such as pain, fever, nausea, and a variety of other stressors (Cunningham and Sawchenko, 1991) that increase release of adrenalin by the sympathetic nervous system. Furthermore, in addition to maintaining perfusion pressure and intravascular volume by retaining fluid and NaCl from the kidneys, VP has been shown to be a vasoconstrictor and to increase hepatic glycogenolysis. OX can cause constriction of arteriolar smooth muscle and potentiate the effects of VP on the kidneys (Cunningham and Sawchenko, 1991). These actions are similar to those of adrenalin in an aroused animal; this suggests the HNS neuropeptides may potentiate or maintain the effects of adrenalin when the organism is required to undergo stress for a long period of time.

Adrenalin release from the adrenal medulla and its reaction on various targets in the periphery have long been established as a characteristic response to stress. The findings of this study suggest that adrenalin plays a crucial role in the HNS by acting on astrocytic glia and causing it to retract to increase communication among MNCs in the SON and facilitate neuropeptide release in the posterior pituitary. The neuropeptides, in turn, act in the periphery to enable the organism to better deal with a long lasting stressful stimulus.

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APPENDIX A

TISSUE EMBEDDING PROTOCOL

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TISSUE EMBEDDING PROTOCOL

Day 1: Experimental treatment and perfusion of rat. Remove brains and neural lobes from skull, postfix in buffered aldehydes overnight. Tissue can be stored in cacodylate buffer up to an additional 48 hours.

Day 2: Core and osmicate tissue for one hour. If embedding immediately, stain overnight in uranyl acetate, or store in buffer.

Day 3. a. Rinse in distilled water, changing water three times ten minutes each.
b. Stain tissue with saturated uranyl acetate (4%) filtered with a 0.22 mm Millipore filter, store overnight in refrigerator.

Day 4: Infiltration
a. Rinse in distilled water, changing water three times, fifteen minutes each.
b. Rinse in 50%, 75%, 80%, 95% alcohol, each dilution for ten minutes.
c. Rinse in filtered 100% alcohol (four rinses, 15 minutes each) Open a fresh bottle at beginning of day.
d. Rotate in 2:1 mixture of alcohol to Spurr's Resin (obtained from Electron Microscopy Sciences, Low Viscosity Embedding Kit, follow manufacturer's directions for preparation) for three hours.
e. Rotate in 1:2 mixture of alcohol to resin, for three hours.
f. Rotate in 100% resin overnight.

Day 5: Embedding
a. Embed tissue (molds or flat embedded on release agent coated slides)
b. Polymerize from 24 to 48 hors in an oven at 65-70° C.

APPENDIX B

SUPPLIES AND EQUIPMENT

APPENDIX B

SUPPLIES AND EQUIPMENT

A. Equipment used courtesy of Dr. Hatton and/or Michigan State University

Advantage Computer Sigma Scan Tablet - 465.00
Jandel Scientific Sigma Scan Software - 465.00
Japan Electron Optics Laboratory CX-100 Electron Microscope - 300,000.00
LKB Knifemaker II - 4875.00
Mager Scientific Reichert Ultracut E. ultramicrotome - 23160.00
Masterflex variable speed pump - 800.00
Philips 201 Electron Microscope - 100,000.00
Technical Manufacturing Corp. Micro-g air table - 2340.00
Thomas Scientific Mettler Balance - 2795.00
Thomas Scientific Blue M. Gravity Oven - 669.00

B. Tissue preparation and histology supplies

Dr. Spurr's Embedding media kit - 23.00
Flat embedding molds 9.50 each (5) - 47.50
Glutaraldehyde 25% solution, biological grade 1 gal - 40.00
Grid storage box - 100 grid capacity - 5.00 each, 54.00 dozen
Liquid release agent 100 ml. - 12.00
Osmium Tetroxide - Crystalline 1 g. - 36.50
Paraformaldehyde -powder 500 g - 5.00
Potassium Ferricyanide 100 g. 11.00
Sodium Cacodylate-trihydrate 100 g. - 40.00
Specimen Grids 200 mesh, copper 8.00 vial of 100 (3) - 24.00
Toluidine Blue 50 g. - 30.00
Uranyl Acetate 25 g. - 16.50

Misc. includes
Slides, coverslips, syringes, razor blades, passthrough pipettes, petri dishes, crazy glue, wooden sticks, cotton tipped applicators, slide boxes 250$

C. Photographic supplies

Electron Microscopy film 31/4x4 47.00 pk; 893 case
Glassine negative envelopes 1000 pk. 56.00
D-19 developer 20 pk 79.00
Dektol case of 20 - 85.00
Fixer case 20 66.00
Stop bath case 20 50.00
Paper 8x10 250 sheets @ 100.00 box (4)
Misc. 50.00

APPENDIX C

RAW DATA EXPERIMENT I

APPENDIX C

RAW DATA EXPERIMENT I

Table 14: Mean SON Scores For Animals Injected with 1.5 M NaCl, Experiment I

	A. Elements in Apposition with MNC Membrane:			B. Sizes of Cell Bodies and Nucleoli	
Percent:	Glial	Neural	Somatic	Cell Body mm^2	Nucleoli mm^2
RAT 1	63	17	17	31.03	.785
Rat 2	57	17	23	33.84	1.022
Rat 3	56	14	30	29.77	.792
Rat 4	57	13	27	33.54	1.20
Rat 5	64	16	16	28.05	.814
Rat 6	43	23	27	30.44	.888
Rat 7	62	15	20	29.60	.785
Rat 8	55	19	21	29.98	.822

C. Synapse characteristics:

	total term size	#synapses/10 0 µm memb	mult/ 100 µm membr.	% somata w/ mults
Rat 1	11.2	.097	.023	54
Rat 2	12.3	.077	.014	62
Rat 3	11.1	.078	.019	71
Rat 4	11.9	.071	.016	47
Rat 5	10.9	.075	.019	64
Rat 6	12.5	.07	.021	50
Rat 7	11.5	.081	.018	58
Rat 8	11.7	.069	.019	58

Table 15: Mean SON Scores for Animals Injected with 0.15 M NaCl, Experiment I

A. Elements in Apposition with MNC Membrane: Percent:	Glial	Neural	Somatic	B. Sizes of Cell Bodies and Nucleoli: Cell Body μm^2	Nucleoli μm^2
RAT 9	71	12	12	27.14	.518
Rat 10	73	13	10	21.71	.692
Rat 11	71	12	7	28.30	.444
Rat 12	71	12	2	30.6	.548
Rat 13	76	17	4	19.4	.585
Rat 14	77	10	8	28.22	.748

C. Synapse characteristics:

	total term size	#synapses/100µm mem	#mult/100µm mem	% soma w/mults.
Rat 9	9.3	.069	.008	28
Rat 10	8.4	.078	.011	43
Rat 11	8.3	.062	.007	36
Rat 12	9.4	.092	.011	42
Rat 13	7.3	.094	.012	20
Rat 14	9.0	. 071	.009	36

Table 16: Mean Percent Neural Apposition and Mean Number Enclosed Axons In the Neural Lobe of Rats in Experiment I.

Condition	% Neural Apposition with BL	Mean # Enclosed Axons
1.5 M NaCl	76	1.2
1.5 M NaCl	65	1.0
1.5 M NaCl	71	.7
1.5 M NaCl	57	.4
1.5 M NaCl	65	.8
1.5 M NaCl	70	.
0.15 M NaCl	41	1.1
0.15 M NaCl	35	1.8
0.15 M NaCl	34	.8
0.15 M NaCl	30	1.8

APPENDIX D

MEANS FOR ANIMALS IN EXPERIMENT II

APPENDIX D

MEANS FOR ANIMALS IN EXPERIMENT II

Table 17 : Mean SON Element Measures for Rats Sacrificed 15 Minutes Post Injection

	Percent Apposition			Size Measures (mm^2)			
	glial	terminal	soma	cellbody	nucleus	nucleoli	% Golgi
1.5 M NaCl	67	6	16	22.45	6.56	.954	.036
1.5 M NaCl	75	6	8	29.00	11.35	.679	.04
1.5 M NaCl	75	10	5	24.94	7.486	.700	.036
1.5 M NaCl	72	6	4	30.21	9.182	.611	.038
1.5 M NaCl	77	2	1	31.23	11.03	.786	.046
0.15 M NaCl	74	11	4	28.73	9.35	.702	.037
0.15 M NaCl	71	10	6	28.02	8.27	.458	.02 6.
0.15 M NaCl	81	04	5	25.78	9.563	.656	.032
0.15 M NaCl	73	09	8	29.753	11.72	.519	.03

Table 18 : Mean SON Element Measures for Rats Sacrificed 1 h Post Injection

	% Apposition			Size Measures (mm^2)			
	glial	terminal	soma	cellbody	nucleus	nucleoli	%Golgi
1.5 M NaCl	72	6	13	27.469	10.388	.916	.06
1.5 M NaCl	79	4	5	19.63	5.69	.834	.046
1.5 M NaCl	75	1	8	28.706	10.54	.916	.055
1.5 M NaCl	71	10	8	25.552	8.73	1.35	.035
0.15 M NaCl	84	2	5	26.125	10.00	.611	.038
0.15 M NaCl	83	4	4	23.222	6.26	.458	.037
0.15 M NaCl	75	10	5	25.17	8.25	.427	.02
0.15 M NaCl	75	7	10	28.584	9.434	.519	.03

Table 19: Mean SON Element Measures for Rats Sacrificed 2.5 h Post Injection

	%Apposition			Size Measures (mm^2)			
	glial	terminal	soma	cellbody	nucleus	nucleoli	% Golgi
1.5 M NaCl	75	5	10	30.937	9.67	935	.05
1.5 M NaCl	70	6	11	35.11	12.16	756	.058
1.5 M NaCl	68	11	11	24.337	7.89	.735	.06
1.5 M NaCl	72	11	8	25.758	9.54	.618	.069
0.15 M NaCl	76	8	7	29.52	10.67	.229	.034
0.15 M NaCl	68	10	8	27.38	9.95	.947	.03
0.15 M NaCl	74	9	7	29.218	10.57	.443	.025
0.15 M NaCl	82	10	3	26.56	10.16	.718	.023

Table 20. Multiple Synapses For Rats Sacrificed at Different Post Injection Time Intervals

condition	% cell bodies w/ mult	#mult/100 µm MNC mem	MNC contacted by term	syn/100 µm MNC memb
1.5 M NaCl 15 min	60	.021	3.54	.139
1.5 M NaCl 15 min	55	.024	4.2	.113
1.5 M NaCl 15 min	50	.029	2.8	.132
1.5 M NaCl 15 min	22	.015	4.6	.11
1.5 M NaCl 15 min	46	.017	2.1	.072
0.15 M NaCl 15 min	25	.009	4.1	.156
0.15 M NaCl 15 min	50	.033	3.8	.161
0.15 M NaCl15 min	50	.023	4.3	.11
0.15 M NaCl15 min	66	.024	3.4	.084
1.5 M NaCl 1 h	66	.031	4.6	.093
1.5 M NaCl 1 h	60	.021	3.5	.11
1.5 M NaCl 1 h	21	.007	2.8	.081
1.5 M NaCl 1 h	30	.015	4.3	.164
0.15 M NaCl 1 h	0	0	2.2	.066
0.15 M NaCl 1 h	27	.01	3.1	.09
0.15 M NaCl 1 h	27	.01	3.4	.138
0.15 M NaCl 1 h	40	.033	2.6	.14
1.5 M NaCl 2.5 h	45	.025	5.8	.086
1.5 M NaCl 2.5 h	58	.031	4.9	.099
1.5 M NaCl 2.5 h	66	.025	4.8	.099
1.5 M NaCl 2.5 h	60	.025	4.5	.09
0.15 M NaCl 2.5 h	33	.011	3.5	.098
0.15 M NaCl 2.5 h	25	.013	3.0	.087
0.15 M NaCl 2.5 h	46	.022	3.6	.141
0.15 M NaCl 2.5 h	25	.009	3.5	.092

Table 21: Basal Lamina Apposition and Enclosed Axons; Time Interval Pituicyte

Condition	Percent apposition BL terminal	pituictye	enclosed axons	lipids
15 min 1.5 M NaCl	27	73	1.2	5.2
15 min 1.5 M NaCl	31	68	.75	1.08
15 min 1.5 M NaCl	25	75	1	3.58
15 min 1.5 M NaCl	20	79	.75	7.68
15 min 0.15 M NaCl	25	74	.214	1.35
15 min 0.15 M NaCl	31	69	.93	2.2
15 min 0.15 M NaCl	31	69	.75	.41
15 min 0.15 M NaCl	36	64	1.27	2.09
1 h 1.5 M NaCl	25	75	.25	.72
1 h 1.5 M NaCl	28	72	.16	.75
1 h 1.5 M NaCl	35	65	.73	1.5
1 h 1.5 M NaCl	37	63	1	1.8
1 h 1.5 M NaCl	41	58	1.5	1.36
1 h 0.15 M NaCl	28	72	1.46	1.76
1 h 0.15 M NaCl	38	61	.23	1.38
1 h 0.15 M NaCl	24	76	1.66	.46
1 h 0.15 M NaCl	43	56	.25	2.5
2.5 h 1.5 M NaCl	45	55	.86	.66
2.5 h 1.5 M NaCl	50	49	.92	3.38
2.5 h 1.5 M NaCl	41	59	.36	1.909
2.5 h 1.5 M NaCl	33	66	.25	2.16
2.5 h 0.15 M NaCl	28	68	.5	2
2.5 h 0.15 M NaCl	27	71	.58	1.5
2.5 h 0.15 M NaCl	28	71	.28	1.28
2.5 h 0.15 M NaCl	29	69	.909	1.45

APPENDIX E

MEANS FOR ANIMALS IN EXPERIMENT III

APPENDIX E

MEANS FOR ANIMALS IN EXPERIMENT III

Table 22: Scores for Elements Measured in SON in Experiment III

CONDITION	% MNC membrane Apposition			Sizes (mm2)			
	Glial	Term	Soma	CB	Nuc	Nucli	%Golgi
1.5 M NaCl	66	13	17	30.5	9.3	.63	.07
1.5 M NaCl	65	15	14	29.9	9.4	.67	.09
1.5 M NaCl	63	15	19	29.9	9.9	•	.09
1.5 M NaCl	59	17	21	29.2	10.3	.88	.09
1.5 M NaCl	64	09	21	32	12.4	.85	.08
0.15 M NaCl	78	6	11	22.6	7.8	.74	.04
0.15 M NaCl	81	8	07	20.7	8.6	.66	.04
0.15 M NaCl	76	9	8	29.3	9.1	.95	.04
0.15 M NaCl	79	9	6	25.4	8.9	.58	.04
1.5MNaCl ans	67	15	13	29.2	11.3	.7	.06
1.5MNaCl ans	75	12	08	24.3	7.8	.88	.06
1.5MNaCl ans	72	11	7	25.6	8	.61	.08
1.5MNaCl ans	75	13	10	24.6	9.3	1	.06
0.15MNaClans	74	8	9	18.6	9.6	.46	.05
0.15MNaClans	74	11	10	23.3	8.8	.7	.05
0.15MNaClans	76	9	11	29.2	10.2	.41	.03
0.15MNaClans	75	11	9	20.9	7.1	.56	.04
1.5MNaClMDX	72	8	10	27.7	14.3	1.29	.07
1.5MNaClMDX	72	7	16	24.6	9.5	.72	.07
1.5MNaClMDX	71	7	8	27.2	10.3	.78	.11
1.5MNaClMDX	79	8	9	26.2	10.8	.89	.09
1.5MNaClMDX	70	9	17	20.6	13.3	.97	.08
0.15MNaCMDX	71	8	12	27.6	11.8	1.1	.05
0.15MNaCMDX	79	5	9	23.5	10.1	.54	.05
0.15MNaCMDX	80	4	9	22.7	9	.75	.04
0.15MNaCMDX	73	4	10	26.5	10.6	.92	.01
0.15MNaCMDX	67	9	19	23.8	11.7	.82	.04

Table 23 - Mean Synaptic Elements for SONs Experiment III

Condition	# multiples / 100µm membr.	% multiples / MNC	Total MNC contact by term	syn/100µm
1.5 M NaCl	.025	58	8.79	.0
1.5 M NaCl	.036	72	8.86	.11
1.5 M NaCl	.041	82	10.25	.11
1.5 M NaCl	.02	67	6.90	.07
1.5 M NaCl	.018	64	9.11	.05
0.15 M NaCl	.016	29	4.9	.08
0.15 M NaCl	.01	40	5.13	.09
0.15 M NaCl	0	0	3.48	.07
0.15 M NaCl	.004	27	5.3	.09
1.5 M NaCl anesth	.04	80	7.73	.12
1.5 M NaCl anesth	.015	43	5.7	.12
1.5 M NaCl anesth	.018	46	5.7	.11
1.5 M NaCl anesth	.019	25	4.5	.13
0.15 M NaCl anesth	.011	53	4.3	.13
0.15 M NaCl anesth	.02	46	5.6	.11
0.15 M NaCl anesth	.012	50	4.6	.14
0.15 M NaCl anesth	.016	46	6.2	.11
1.5 M NaCl MDX	.019	33	2.0	.07
1.5 M NaCl MDX	.016	60	3.9	.07
1.5 M NaCl MDX	.025	47	2.5	.1
1.5 M NaCl MDX	.036	35	3.0	.07
1.5 M NaCl MDX	.014	69	2.5	.12
0.15 M NaCl MDX	.011	33	2.3	.07
0.15 M NaCl MDX	.014	27	3.2	.1
0.15 M NaCl MDX	.013	33	3.0	.09
0.15 M NaCl MDX	.007	27	3.7	.13
0.15 M NaCl MDX	.02	54	3.2	.12

Table 24 - Neural Lobe Mean Scores for Experiment III

	Percent BL Coverage			
Condition	terminal	pituicyte	enclosed axons	lipids
1.5 M NaCl	57	41	1.05	3.466
1.5 M NaCl	65	34	.55	1.46
1.5 M NaCl	54	45	1.21	2
1.5 M NaCl	53	46	.05	2.07
1.5 M NaCl	55	41	1.07	2.43
1.5 M NaCl	62	36	.86	2.6
1.5 M NaCl	64	34	1.65	3.9
0.15 M NaCl	35	62	2.608	3.6
0.15 M NaCl	38	62	1.8	1
0.15 M NaCl	43	56	.355	3.4
0.15 M NaCl	39	60	1.8	1.6
0.15 M NaCl	24	75	2.15	1.6
0.15 M NaCl	29	70	2.3	2.3
1.5 M NaCl Anest	34	65	1.85	2.14
1.5 M NaCl Anest	49	51	1.69	2.38
1.5 M NaCl Anest	42	58	2.27	.36
1.5 M NaCl Anest	30	69	1.78	3.2
0.15 M NaCl Anest	47	52	2.1	2.5
0.15 M NaCl Anest	29	70	2.09	4
0.15 M NaCl Anest	42	58	1.93	.428
0.15 M NaCl Anest	35	63	2.6	.46
1.5 MNaCl MDX	27	73	.3	.5
1.5 MNaCl MDX	35	64	2	2.8
1.5 MNaCl MDX	36	62	.9	1.2
1.5 MNaCl MDX	28	71	1.9	1.8
1.5 MNaCl MDX	41	58	1	2.8
0.15 MNaCl MDX	34	65	2.4	2.3
0.15 MNaCl MDX	38	62	2.6	.5
0.15 MNaCl MDX	35	64	1.2	2.9
0.15 MNaCl MDX	42	57	1.6	.5
0.15 MNaCl MDX	31	68	1.1	1.8

APPENDIX F

HIPPOCAMPAL AND SCN APPOSITION SCORES

APPENDIX F

HIPPOCAMPAL AND SCN APPOSITION SCORES

Table 25 - Hippocampal MNCs Apposition Means

Condition	Percent Apposition glial	neural	somatic
1.5 M NaCl	60	9	31
1.5 M NaCl	80	5	15
1.5 M NaCl	81	11	06
1.5 M NaCl	81	13	06
0.15 M NaCl	72	7	20
0.15 M NaCl	75	12	12
0.15 M NaCl	81	8	10
0.15 M NaCl	84	5	10

Table 26 - SCN MNCs Apposition Means

Condition	Percent Apposition glial	neural	somatic
1.5 M NaCl	77	13	8
1.5 M NaCl	83	08	8
1.5 M NaCl	83	11	6
1.5 M NaCl	77	16	6
0.15 M NaCl	77	18	7
0.15 M NaCl	74	14	15
0.15 M NaCl	84	10	6
0.15 M NaCl	77	16	7

APPENDIX G

DATA FOR UNTREATED RATS

APPENDIX G

DATA FOR UNTREATED RATS

Table 27 : Scores for Untreated Rats

A. SON:

	Apposition			Sizes (μm^2)			
	Glial	Neural	Somatic	Cell Body	Nucleus	Nucleoli	Golgi
Rat 166	77	8	8	24.9	8.1	.64	.038
Rat 167	80	7	7	23.3	8.8	.56	.042

B. Pituitaries:

	Apposition BL				
	Terminal	Pituicyte	Axons Enclosed		Lipids
Rat 166	38	62	2.16	2.08	
Rat 167	35	65	1.5	.83	

APPENDIX H
DATA FOR SHAM MEDULLECTOMIES

APPENDIX H

DATA SHAM MEDULLECTOMIES

Table 28: SON Values For Sham Medullectomies

	Percent Apposition w/MNC			Sizes (in μm^2)		
	glial	term.	soma	cell body	nucleus	nucleoli
1.5 M NaCl	60	17	20	29.2	10.3	.88
1.5 M NaCl	64	11	21	32	12.4	.85
0.15 M NaCl	79	6	10	20.7	7.9	.74
0.15 M NaCl	80	8	08	22.6	8.3	.66

Condition	mult/ 100 mm	% MNCs w/mult	Term/MNC contact µm	syn/ 100µm	% Golgi Apparatus	
1.5 M NaCl	.021	57	7.9	.07		.09
1.5 M NaCl	.030	64	9.1	.08		.08
0.15 M NaCl	.016	29	4.9	.08		.04
0.15 M NaCl	.012	25	5.2	.09		.04

Table 29: Pituitary Scores for Sham Medullectomies

	Percent BL Coverage			
Condition	terminal	pituicyte	enclosed axons	lipids
1.5 M NaCl	59	38	.86	2.6
1.5 M NaCl	63	35	.75	2.0
0.15 M NaCl	35	62	2.4	3.6
0.15 M NaCl	33	63	1.8	1