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QUANTIFICATION OF SOMATIC SYNAPSE FORMATION IN THE SUPRAOPTIC NUCLEUS OF ADULT RATS FOLLOWING CHRONIC DEHYDRATION

by

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ABSTRACT

QUANTIFICATION OF SOMATIC SYNAPSE FORMATION IN THE SUPRAOPTIC NUCLEUS OF ADULT RATS FOLLOWING CHRONIC DEHYDRATION

By

Barbara Kay Modney

In chronically dehydrated rats, the ultrastructural appearance of the supraoptic nucleus (SON) which contains oxytocinergic and vasopressinergic neurons is markedly different from that of well hydrated animals. Both cell size and the percentage of cells contacted by multiple synapses (i.e. one axon simultaneously synapsing with 2 adjacent neural elements) increase in chronically dehydrated animals. This study combined measures of neuronal surface area with stereological measures of axonal terminals in order to determine the number of single and multiple somatic synapses per neuron in dehydrated and well hydrated rats. Numbers of single synapses/cell were not different between the two groups. However, the number of multiple synapses/cell was significantly greater in dehydrates (mean ± SEM; 22.8 ± 4.5) compared to controls (5.8 ± 1.8). Newly formed multiple synapses are thought to play an important role in the coordinated output of these neurons during extended periods of hormone release.

ACKNOWLEDGMENTS

I think I could more eloquently acknowledge the multitude (there's that word again) of people who have contributed in one way or another to this thesis if only time, energy and space were unlimited. The following will have to suffice as a poor substitute. To Glenn Hatton, who risked sponsoring a student with little preparation but lots of enthusiasm, you have guided me through the mysteries of neuroscience research and taught me, especially through the last year (e.g. MB chapter), the importance of understanding the "big picture". Thanks! To Charlie Tweedle and Isabel Suarez who taught me the things about electron microscopy you don't learn from books, thank you. I look forward to finding more "Tweedle bodies"! To Tony Nunez and Cheryl Sisk, thanks for supporting me throughout the development of this thesis. Both of you have provided encouragement which goes beyond the traditional requirements of committee members. To past and present labmates and friends, thank you. I tried to include all of your names, but this acknowledgement turned into a thesis on friendship. To Gary, I know you hate overemoting, but to not write anything would make people wonder, so thanks. To Mom and Dad, words cannot express (ugh, a greeting card) all you have given me. Research supported by NS 09140.

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LIST OF ABBREVIATIONS

A - Long Feret diameter

B - Short Feret diameter

d - Length of an individual apposition between a terminal and soma

df - degrees of freedom

ε - eccentricity

HNS - hypothalamo-neurohypophysial system

MNC - magnocellular neuroendocrine cell

N - the number of appositions lengths between a terminal and soma

NaCl - Sodium Chloride

Ns - Number of synapses per μm^2

OX - oxytocin

*perf - perforated

psd - postsynaptic density

PVN - paraventricular nucleus

SA - surface area

S.E. - standard error

SON - supraoptic nucleus

Ss - surface density

*t'- t like statistic-Welch's modification for heterogeneous variance

VP - vasopressin

μm - micrometer

Δ - mean terminal contact diameter

*Appendices only

INTRODUCTION

Structural reorganization in the adult nervous system, once thought of as a rare event, has become almost commonplace in neurobiological research. Traditionally, experimental conditions which produced anatomical restructuring were pathological, e.g. lesions. Recent work has shown that the nervous system is capable of striking changes in response to physiological and/or environmental conditions which cannot be considered pathological (e.g. enriched environments, Greenough & Chang, 1985; seasonal reproductive state, Arnold, 1985). One system which undergoes reversible morphological changes under physiological, environmental and pathological conditions is the hypothalamo-neurohypophysial system (HNS). The anatomical features of this system vary with the diurnal cycle, the hydration or reproductive state of the animal, and the behavioral/environmental situation of the animal (see Hatton, 1985 for review and Salm, Kohn & Hatton, 1985). Even as the conditions which produce these anatomical alterations are diverse, so are the changes themselves, ranging from the proliferation of cellular organelles to the changes in the synaptic connectivity of the neurons. The focus of this thesis is on variations which occur in one portion of this system, the supraoptic nucleus (SON) under experimentally induced dehydration.

Features of the Hypothalamo-neurohypophysial System

The SON of the hypothalamus is one of the major nuclei of the HNS. The hypothalamic paraventricular nucleus (PVN) and several smaller accessory nuclei also contain cell bodies which participate in the HNS (see Swanson, 1986 for review). These nuclei contain large magnocellular neuroendocrine cells (MNCs) which synthesize and release the peptide hormones oxytocin (OX) and vasopressin (VP), each hormone being synthesized by separate cell populations. The nuclei are conspicuous due to their large densely packed somata (15-30 μm in diameter) and their locations lateral to the third ventricle (PVN) and at the ventral surface of the brain just lateral to the optic tracts (SON). Electron microscopic observations have shown that even though somata are crowded together, neighboring cells are isolated from each other by thin astrocytic processes. MNCs are richly endowed with organelles that participate in hormone synthesis.

OX and VP, along with their associated neurophysins, are synthesized in separate neurons in both the SON and PVN. Each peptide is synthesized as part of larger precursors on ribosomes attached to endoplasmic reticulum (reviewed by Gainer, 1983). Following packaging into secretory vesicles by the Golgi apparatus, prohormones are axonally transported to their release sites. Postranslational processing of the prohormones to OX and VP and their neurophysins occurs within the vesicles during axonal transport.

MNCs have various projections within the brain and spinal cord (see Swanson, 1986 for review). The projection which forms the efferent portion of the HNS courses through the internal zone of the

median eminence and terminates in the neurohypophysis. Axon terminals which contact the basal lamina surrounding fenestrated capillaries are able to release hormones into the circulation. In general, hormone secretion is related to the electrical activity of MNCs such that action potentials which invade a terminal result in calcium dependent hormone release.

The peripheral effects of OX and VP have been realized for many years. OX promotes contraction of uterine smooth muscle during parturition and is the efferent limb of the milk ejection reflex. VP, or antidiuretic hormone, participates in maintaining fluid homeostasis by causing water reabsorption by the kidneys (see Forsling, 1977 and Roberts, 1977 for reviews of peripheral effects). Typically, experimental manipulations which seek to understand the neurobiology of the HNS use either dehydrated animals which causes both OX and VP to be released, or lactating animals in which there is a rather selective release of oxytocin.

Electrophysiological studies have determined that oxytocinergic and vasopressinergic cells can be distinguished on the basis of their firing characteristics (see Poulain & Wakerley, 1982 for review). In anesthetized lactating rats with suckling pups MNCs may fire in either a "fast continuous" or slow irregular manner. Presumed oxytocinergic cells occasionally exhibit a synchronized high frequency discharge after which a rise in intramammary pressure and milk ejection occur. Vasopressinergic cells do not generally respond to suckling and, in osmotically stimulated rats, they display a phasic firing pattern consisting of alternating periods of silence and bursts of action

potentials.

The Hypothalamo-neurohypophysial System During Dehydration

In its role as a major regulator of fluid homeostasis, the HNS responds rapidly to changes in extracellular fluid volume, osmolality and blood pressure. Alterations in these parameters serve as the major controls for vasopressin release. Oxytocin is also released during osmotic challenges (Jones & Pickering, 1969) although its function in this response is not as well characterized as vasopressin's. While virtually every indicator of HNS activity responds in some way to changes in osmotic pressure, the location and physiology of "osmoreceptors" which transmit this information to MNCs in the hypothalamus remain to be fully described.

The nature and extent of anatomical sites and neurotransmitter systems which relay osmotic information to MNCs is beyond the scope of this thesis. Several postulated osmoreceptive areas appear to be vital for the complete response of the HNS to osmotic challenges (see Sladek & Armstrong, 1985). Portions of the anterior hypothalamus, particularly the organum vasculosum of the lamina terminalis, emerge as sites crucial for a comprehensive response to osmotic challenges.

Although the contribution of afferent input to MNCs during osmotic changes should not be underestimated, neither should the capacity of MNCs to respond directly to changes in extracellular fluid osmolality. Various in vitro experiments have demonstrated that MNCs respond to elevation of osmotic pressure with an increase in their resting membrane potential (i.e. they become slightly depolarized; Mason, 1980). That this depolarization occurs in the absence of synaptic

transmission implies that MNCs are directly osmosensitive. In vitro and in vivo electrophysiological experiments have rather consistently shown that oxytocinergic and vasopressinergic neurons alter their firing characteristics in response to osmotic stimuli (see Poulain & Wakerly, 1982 for review). Oxytocinergic neurons respond by increasing their firing rates in response to both acute (e.g., intraperitoneal injections of NaCl) and chronic stimuli. Acute osmotic challenge results in the transformation of many silent or irregularly firing neurons into phasic neurons. Neurons which display phasic firing under normal osmotic conditions typically respond to increased osmotic pressure by increasing their firing rates within bursts, increasing their burst duration and decreasing their interburst interval. During periods of chronic activation, the proportion of neurons within magnocellular nuclei which fire phasically increases.

These changes in the firing characteristics of both oxytocinergic and vasopressinergic neurons result in more efficient hormone release from the neurohypophysis. Although many factors may modulate hormone release within the neurohypophysis (e. g. possible role of pituicytes- see Hatton, 1985;) there is convincing evidence that the rate and pattern of action potentials enhance the amount of hormone released from the neurohypophysis. Using electrical stimulation of the neural stalk in vitro combined with radioimmunoassay for VP, the precise relationship between various stimulation patterns and VP release can be obtained. Stimulation of the neural stalk in a phasic pattern results in significantly more VP release than continuous stimulation equivalent to an oxytocinergic neuron's firing pattern (Bicknell & Leng, 1981).

Of the many parameters which characterize phasic firing (e.g. firing rate, interspike intervals etc.), VP release per stimulus is best correlated with burst duration (Shaw, Bicknell & Dyball, 1984). A variety of mechanisms appear to participate in generating the stereotypic patterns of activity of SON neurons during activation. It may be that some of the many dramatic anatomical changes that occur in this system during activation contribute to the enhanced activity of SON neurons.

Anatomical studies utilizing diverse techniques have correlated changes within the SON with experimentally increased hormone release. Although the emphasis here is on alterations that occur between MNC somata in the SON, anatomical transformations also occur in the neurohypophysis during dehydration (Tweedle & Hatton, 1980a; 1980b; 1982; 1987). Also, many of these same changes that occur with dehydration have also been shown to occur in female rats during parturition and lactation (Hatton & Tweedle, 1982; Montagnese, Poulain, Vincent, & Theodosis, 1987; Perlmutter, Tweedle & Hatton, 1984; Theodosis, Chapman, Montagnese, Poulain & Morris, 1986; Theodosis & Poulain, 1984, Theodosis, Poulain & Vincent, 1981).

Proliferation of cellular organelles implicated in hormone synthesis and packaging has consistently been reported in research using dehydrated rats (Castel, Gainer & Dellmann, 1984). Some alterations occur very quickly during dehydration. For instance, significant increases in the percentage of neurons which contain multiple nucleoli and dilated endoplasmic reticulum have been found after water deprivation for as little as 12 hours (Hatton & Walters,

1973; Tweedle & Hatton, 1977). It is perhaps because of this proliferation that chronic dehydration results in large increases in cell size (Armstrong, Gregory & Hatton, 1977; Bandaranake, 1974; Ellman & Gan, 1971; Enestrom, 1976, Morris & Dyball, 1974; Hatton & Walters, 1973; Kalimo, 1975).

Changes also occur in intercellular relationships in the SON during dehydration. Dye coupling (an indirect measure of electrotonic coupling) in hypothalamic slices maintained in vitro varies with the in vivo state of the animal (Cobbett & Hatton, 1984; Hatton, Yang & Cobbett, 1987). At the ultrastructural level restructuring of the SON seems to depend in part on glial processes whose position between neural elements varies with the physiological state of the animal. Dehydration is associated with the withdrawal of glial processes from their typical positions between cell somata and dendrites (Chapman, Theodosis, Montagnese, Poulain & Morris, 1986; Perlmutter, Tweedle & Hatton, 1984; Tweedle & Hatton, 1976, 1977). Glial processes are able to withdraw from between adjacent cells quite rapidly, as evidenced by increases in membrane apposition seen after only 12 hours of water deprivation (Tweedle & Hatton, 1977). This glial retraction increases the number of neurons and dendrites directly contacting each other and the percentage of membrane in direct contact.

In addition to the reorganization of neurons and glial cells, plasticity in the SON during chronic dehydration includes the formation of multiple synapses (Chapman, Theodosis, Montagnese, Poulain & Morris, 1986; Tweedle & Hatton, 1984). Multiple synapses consist of one presynaptic terminal simultaneously forming synaptic contacts with two

or more adjacent neurons or dendrites. A significant increase in the percentage of neuronal cell bodies contacted by multiple synapses occurs after 10 days substitution of 2% NaCl for drinking water.

When animals are allowed to rehydrate via free access to drinking water the ultrastructural characteristics of the SON are again similar to normal animals. Following chronic dehydration the percentage of somatic membrane apposed to adjacent cells or dendrites decreases to control levels after 5 days of rehydration while the percentage of cells contacted by multiple synapses does not return to normal levels until the animal has been rehydrated for 14 days.

Statement of the Problem

Given that the percentage of cell somata contacted by multiple synapses increases during chronic dehydration and that somatic size also increases, several intriguing questions have yet to be answered. First, is there an increase in the number of single synapses in the SON which occurs during dehydration, and, if so, how many are formed? Also, does the increase in the percentage of neuronal cell bodies contacted by double synapses represent an increase in the size of existing multiple synapses? If multiple synapses are newly formed, how many are formed and what is their contribution to the total synaptic input to these neurons? Given that the form of synapses has been shown to vary in other systems (i.e. spine synapses, perforated synapses, see Greenough, 1985), do synapses in the SON vary in similar ways? Existing research has not attempted to directly combine information concerning the number of synapses in SON with increases in cell size. Electrophysiological principles which relate to neuron size would

suggest that an increase in cell size leads to a decrease in the cell's input resistance. This would make existing synaptic inputs less efficient. Perhaps the formation of new synapses is a mechanism which compensates for increases in cell size. This experiment was designed to answer the questions listed above with quantitative measures which incorporated increases in cell size.

METHODS

Experimental Groups

Fourteen male Sprague-Dawley rats aged 80-90 days old were equally divided into dehydrates and controls. Dehydrates were given 2% NaCl to drink instead of tap water for 10 days. Control rats were given free access to tap water. All animals were given free access to food and were maintained on a 12:12 light-dark cycle. All animals appeared healthy at the time of sacrifice.

Tissue Preparation

At the end of the ten-day dehydration period, rats were deeply anesthetized with ether and transcardially perfused with a brief saline (0.15 M) rinse followed by 300-400 ml of 2.5% glutaraldehyde, 1.5% paraformaldehyde solution in 0.10 M cacodylate buffer, pH 7.4. Brains were removed from the skulls and immersed in fixative overnight. Following preparation of hypothalamic blocks, 400-500 µm coronal slices were cut on a tissue chopper. The SON was cut out of 3 adjacent slices through the rostral-caudal extent of the nucleus. Following rinses in 0.15 M cacodylate buffer (pH 7.4) tissue slabs were postfixed in a 1:1 mixture of 2% aqueous osmium tetroxide and 1% potassium ferricyanide in 0.2 M cacodylate buffer (pH 7.4). Tissue was *en bloc* stained overnight

in Millipore filtered uranyl acetate, dehydrated in ethanol and embedded in an Epon-Araldite resin mixture (See Appendix B for complete protocol). Each animal was assigned a random two digit code number prior to embedding so that all subsequent sampling was done without knowledge of treatment group. One control animal was discarded from the experiment due to inadequate tissue preservation.

Sections were cut on either a Porter Blum MT-2 or Reichert Ultracut E microtome. Semi-thin (0.5-1.5 μm) sections were heat mounted onto glass slides and stained with methylene blue. Blocks containing the middle third of the SON were used for data collections since this is the largest extent of the nucleus and the ratio of oxytocinergic to vasopressinergic neurons is approximately 1:1. For determination of cell surface area, serial semi-thin sections (0.5 μm) were cut, heat mounted on alcohol cleaned slides and stained on a hot plate for 5-10 minutes with Stevenel's blue (Ridgway, 1986). Serial semi-thin sections were examined with an American Optical microscope fitted with a semi-automatic image analysis system (see below).

For determination of morphometric and stereological data, thin sections (60-90 nm) were cut from one block per animal. In order to remove the compression which occurs during sectioning, sections were exposed to xylene vapors prior to collection on 200 mesh thin-bar copper grids. Serial sections were collected on Butvar coated slot grids. All sections were stained with lead citrate. All electron microscopy was done with a JEOL CX100 microscope at an accelerating voltage of 60 kV.

Serial Sectioning

One block from an animal in each group was selected for serial sectioning in order to determine: a) whether every axonal profile which contacted a magnocellular neuron also formed a synapse with a postsynaptic density and b) whether or not synapses or apposition zones between cell soma and terminals conformed to the stereological assumption of a disk shape. A series of 40 sections was taken from each block. Photomicrographs (final magnification = 9900X) from every 2nd or 3rd section were taken of two SON neurons from each series. Every terminal apposed to the cell soma was examined.

Morphometrics

Magnocellular neurons were randomly sampled from one thin section per animal. In order to assure that a sufficient amount of tissue would be sampled, tissue from a dehydrated animal was sampled extensively at a microscope magnification of 4800X. A progressive mean was calculated for 2 micrographs, 3 micrographs, 4 micrographs etc. It was found through progressive sampling that the number of micrographs which produced less than a 5% change in the mean percentage of axonal contact with somatic membrane was 20. Therefore, 23 to 26 micrographs per animal were taken. Magnification calibration performed with the aid of a diffraction grating replica (Ted Pella) varied less than 1% over the course of the experiment (Mean = 4866X). Prints were enlarged 2.75X so that the final magnification was approximately 13,182X.

On each micrograph the following cellular elements contacting neuronal somata were outlined with overhead projection pens: a) astrocytic processes, b) axonal terminals, c) adjacent dendrites or

cell somata, d) unidentified elements. Only those structures which apposed MNC somata were investigated in this study. Each trace length was measured on a Houston Instruments digitizing pad interfaced with a Zenith 200 computer using software developed in our laboratory designed to give length and area measurements. The total length of somatic membrane was determined by summing the elements listed above. Each terminal was designated as either single (i.e. it apposed only the cell soma) or multiple (it apposed both the cell soma and an adjacent cell or dendrite). "Multiples" were further characterized by the postsynaptic elements they contacted, e.g. soma-dendritic. For each terminal the presence or absence of a postsynaptic density (psd) on the somatic membrane was also noted. Perforations in the psd as well as terminals apposed to somatic spines were also recorded. Data from the individual micrographs were summed for each animal.

The surface density, Ss (notation follows that used by Mayhew, 1979) is defined and calculated as the total length of a cellular element/total postsynaptic membrane. Ss is typically expressed as a percentage and this convention is followed for group comparisons. Ss was determined for each of the following elements contacting cell bodies:

1. Astrocytic processes
2. Soma-somatic or soma-dendritic membrane apposition
3. All axonal terminals
4. Single axonal terminals
5. Multiple axonal terminals
6. Axonal terminals associated with somatic spines

7. Axonal terminals apposed to psds with a perforation

The Ss of single and multiple synapses associated with a postsynaptic density was also calculated.

The total length of all axonal profiles and the length of single and multiple terminals were used to determine the relative contribution of multiples to total axonal contact with the cell. The percentage of axonal profiles which were single, multiple, spine, or perforated was also calculated. The percentage of terminals associated with psds which were single and multiple was also obtained.

Stereological Measures

Although most applications of stereological principles use tissue area (hence volume) as a reference parameter, in this study somatic surface area was used the reference parameter for several reasons. First, it allows statements concerning the number of synapses to be made without reference to tissue volume which probably changes under these experimental conditions. For example, in addition to increases in SON cell area which occur during dehydration, evidence has been obtained that the dendritic region of the SON also expands experimental conditions (Salm, Kohn & Hatton, 1985; Taubitz, Smithson & Hatton, 1987). Second, Mayhew (1981) has reported that measurements based on surface areas reduce the coefficient of variation in a given sample allowing a smaller sample size without a reduction in reliability. Thus, the number of axonal terminals per unit surface area of somatic membrane was calculated for each control and experimental animal.

According to general stereological principles (see Mayhew, 1979 and Kaiserman-Abramof & Peters, 1972), Ss as defined above, also

represents the surface area of axonal contacts (μm^2) per unit surface area of cell membrane. Thus the number of terminals per μm^2 (Ns) can be obtained by dividing Ss by the mean area of contact between the cell and terminal. Ns was determined for single and multiple axonal profiles using the following formula:

$Ns=Ss/s$ where

Ss = surface density as defined above

$s = (\pi/4)\Delta^2$, where Δ equals the axonal contact diameter

In this equation s equals the mean surface area of a contact disk. The calculation Δ was determined using the method of Fullman as described by Williams (1979). This method assumes that the apposition areas between cells and terminals are a polydisperse population of disk-like structures. The formula for calculating the true diameter from which the sectioned lengths arose is:

$\Delta=(\pi/2)(N/\Sigma(1/d))$ where

N = the number of measured lengths (i.e. synapses)

d = the length of axonal contact

Since the accuracy of this method depends on a large number of measurements, was calculated once for controls using 375 measured terminal lengths from control animals and once for dehydrates using 299 measured terminal lengths from dehydrated animals.

Cell parameters

Semi-thin sections from each animal were used to determine various parameters of cell size and shape. Measurements were made using an Olympus C-2 image analysis system consisting of an American-Optical microscope with attached video camera, monitor, optical mouse and IBM

AT computer.

Only cells whose nuclear profile contained a prominent nucleolus were measured since the surface area calculation required that the cell is cut in largest extent. A camera lucida sketch of the SON from one section per animal was made. Twenty cells per animal were randomly selected by placing an acetate sheet containing a numbered grid over the sketch and selecting grid numbers from a random number table. Measured cells were recorded on the camera lucida sketch for further reference. A cell was discarded if its perimeter was not clearly discernable. If twenty cells could not be measured in the first section, adjacent sections were used. The camera lucida sketch assured that cells were not sampled twice.

Selected cells were viewed through the microscope and displayed on the video screen. Each cell's perimeter was manually delineated with the optical mouse, and the following parameters were generated by the computer:

1. Long and short Feret diameters
2. Shape factor - a measure of roundness defined as

$$\frac{4\pi(\text{area})}{\text{perimeter}}$$

3. Aspect ratio - ratio of minimum to maximum Feret diameters

Feret diameters are calculated by the computer as the perpendicular distance between two parallel lines drawn tangent to the perimeter of the cells (Weibel, 1979). In this study the long and short Feret diameters were used as the long and short axes of the cell when calculating surface area (Russ, 1986).

By assuming that cell shape was prolate spheroid the surface area (SA) of each cell was computed as:

$$(\pi/2)(B^2 + AB \text{ arcsine } \varepsilon/\varepsilon)$$

where eccentricity $\varepsilon = \sqrt{(A^2-B^2)}/A$ and A and B equal the maximum and minimum Feret diameters respectively.

Size frequency distributions of all cells in each group were generated in order to compare the distributions of cell size between the two groups. The mean shape factor, aspect ratio and eccentricity from each animal were used to determine whether or not the general shape of the neurons varied with treatment. The mean SA per animal was used to determine the number of synapses per cell body.

Number of Synapses per Neuronal Cell Body

The number of single and multiple synapses was calculated for each animal by multiplying Ns (i.e. number of synapses per μm^2) by the mean SA of the cells. The total number of synapses per cell body was obtained by the adding the number of single and multiple synapses.

Statistics

All data are expressed as means standard errors. Group comparisons were made using a two-tailed Student's t-test. When extreme heterogeneity of variance was suspected, a F test was used to test homogeneity of variance. Welch's method of correction involving a t-like statistic (t') and approximate degrees of freedom was used in cases of extreme heterogeneity of variance (Gill, 1979). Statistical calculations were made using Statpak Software on an IBM PS/2 Model 50 computer.

RESULTS

Serial Sectioning

A total of 102 axonal terminals were examined through serial thin sections, 54 terminals were from the control tissue and 48 were from the dehydrated tissue. All terminals except for one (found in dehydrated tissue) formed a conventional synapse with a postsynaptic density. As this was the case, all terminals which contacted neurons were considered to form conventional synapses. The number of terminals which formed complex synapses, e.g. synapses with somatic spines, was 12 in the control and 15 in the dehydrate tissue. Multiple synapses appeared to be more frequent in the dehydrate tissue which contained 14 while the control tissue contained two.

Morphometrics

Figure 1 shows the general ultrastructural appearance of MNC's in the SON. The various structures contacting the MNC's surface are indicated. The trace lengths of these structures were used for morphometric analysis. Figure 2 shows the various axonal contact types (single, multiple, spine and perforated) which were investigated.

Figure 3 shows the percentage of somatic membrane contacted by astrocytic processes, axon terminals and soma-somatic or soma-dendritic membrane apposition. No significant differences were found between the two groups in the percent coverage by glial processes (controls, 84.01 ±1.01; dehydrates, 76.70 ± 3.74). A significant increase in the percentage of somatic membrane apposed to MNCs or dendrites was found in dehydrates (7.93 ± 0.78) relative to controls (0.99 ± 0.36, $p < 0.001$) while a decrease in the percentage of somatic membrane covered

Figure 1 - Electron micrograph from a dehydrated animal illustrating the general features of SON ultrastructure. Direct membrane apposition between 3 somata and an adjacent dendrite (den) are delineated with dashed lines. Three terminals (arrows) are contacting the somata. Glial processes are indicated by arrowheads. Bar = 2 μm.

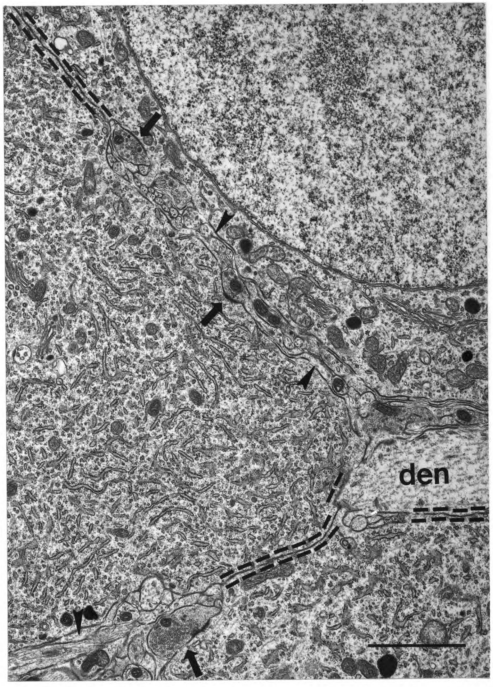

FIGURE 1

Figure 2 - The types of synapses investigated in this study. A: A terminal (*) forming a single synapse with a MNC. B: A terminal (*) which forms a "double" synapse between a cell and adjacent dendrite which are also in direct apposition (dashed line). C: This spine synapse consisted of a terminal (*) which invaginated around the somatic spine. D: A typical perforated synapse, the perforation in the postsynaptic density is indicated by the arrow. (* = terminal) Bars = 1 μm

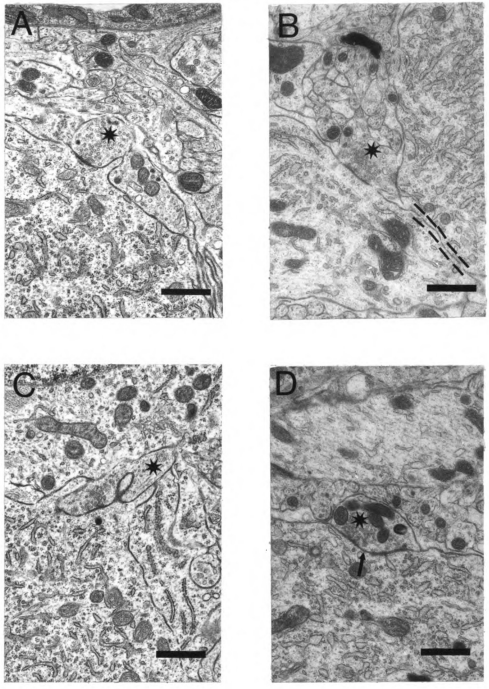

FIGURE 2

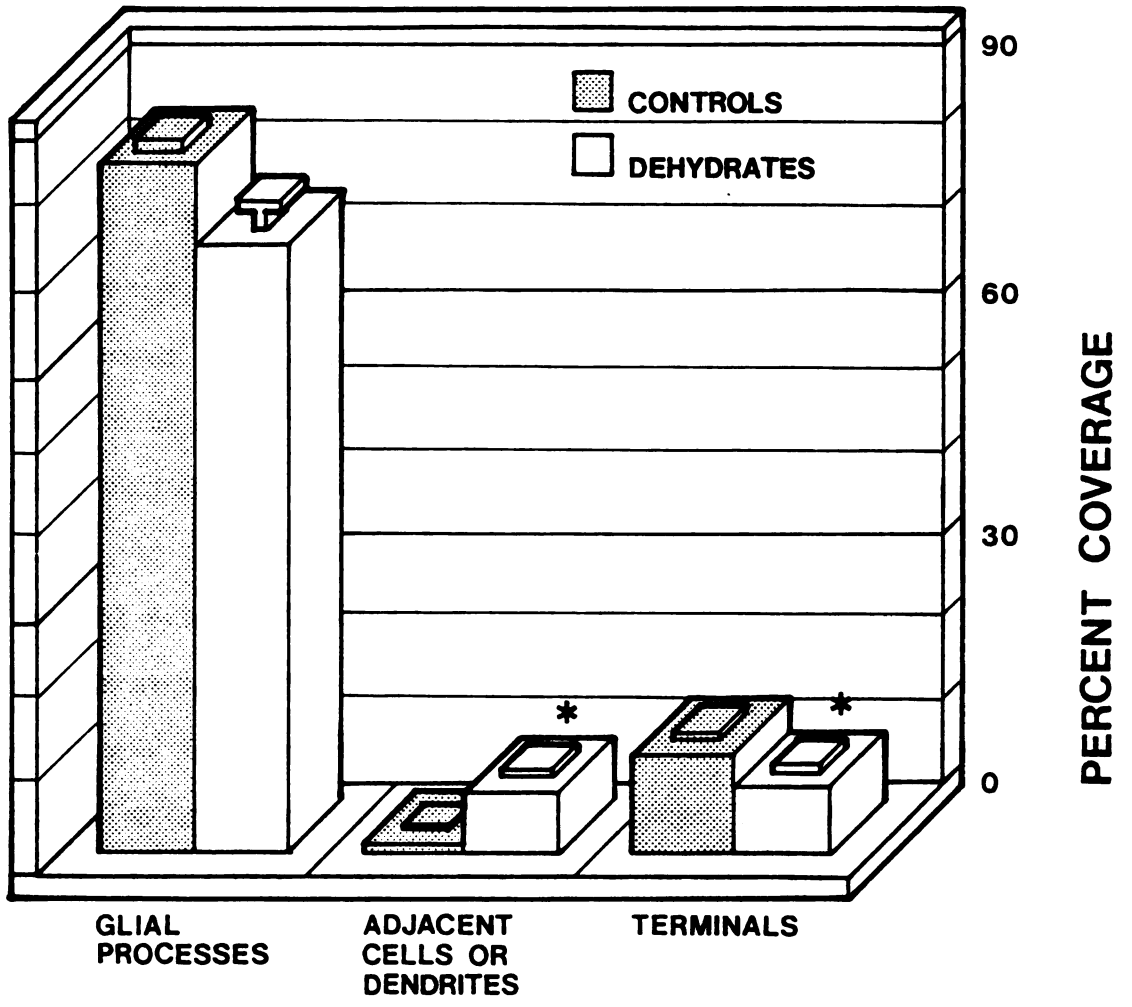

Figure 3 - The percentage of somatic membrane covered by glial processes, adjacent cells or dendrites and terminals. (Mean and S.E., * significantly different.)

by axonal profiles occurred in dehydrates (8.01 ± 0.82) vs controls (12.17 ±0.87, $p < 0.005$).

As illustrated in Figure 4, when terminals were designated as single or multiple, controls had a larger percent coverage by singles (controls, 11.58 ± 1.00; dehydrates 6.79 ± 0.65, $p< 0.001$) while dehydrates had increased coverage by multiples (controls 0.58 ± 0.16; dehydrates, 1.24 ± 0.23, $p< 0.04$). Multiple synapses in control tissue were exclusively "doubles" (i.e. one terminal apposed to two adjacent cells or a cell and a dendrite). Of the 19 multiple synapses in control tissue, 18 were between a soma and an adjacent dendrite. Only one terminal in the control tissue apposed two adjacent cell soma. In contrast, in dehydrated animals multiple synapses were not exclusively "doubles", 4 terminals formed between a cell and 2 additional structures. One "triple" apposed two somata and an adjacent dendrite while 3 terminals formed synapses between 2 dendrites and an adjacent cell. The majority of multiple synapses in dehydrated animals were between a soma and an adjacent dendrite (n=40) while 9 soma-somatic "doubles" were found.

The percent axonal coverage by terminals apposed to somatic spines and somatic membrane with perforated psds is shown in Figure 5. There was no significant difference in the percent coverage on somatic spines (controls, 0.89 ± 0.28; dehydrates, 0.74 ± .018). The percent coverage onto somatic membrane with perforated psds was significantly higher in controls (2.15 ± 0.41) compared to dehydrates (0.97 ± 0.20, $p< 0.04$)

Requiring that axonal profiles be apposed to somatic membrane with a psd reduced the percent coverage by singles and multiples. A

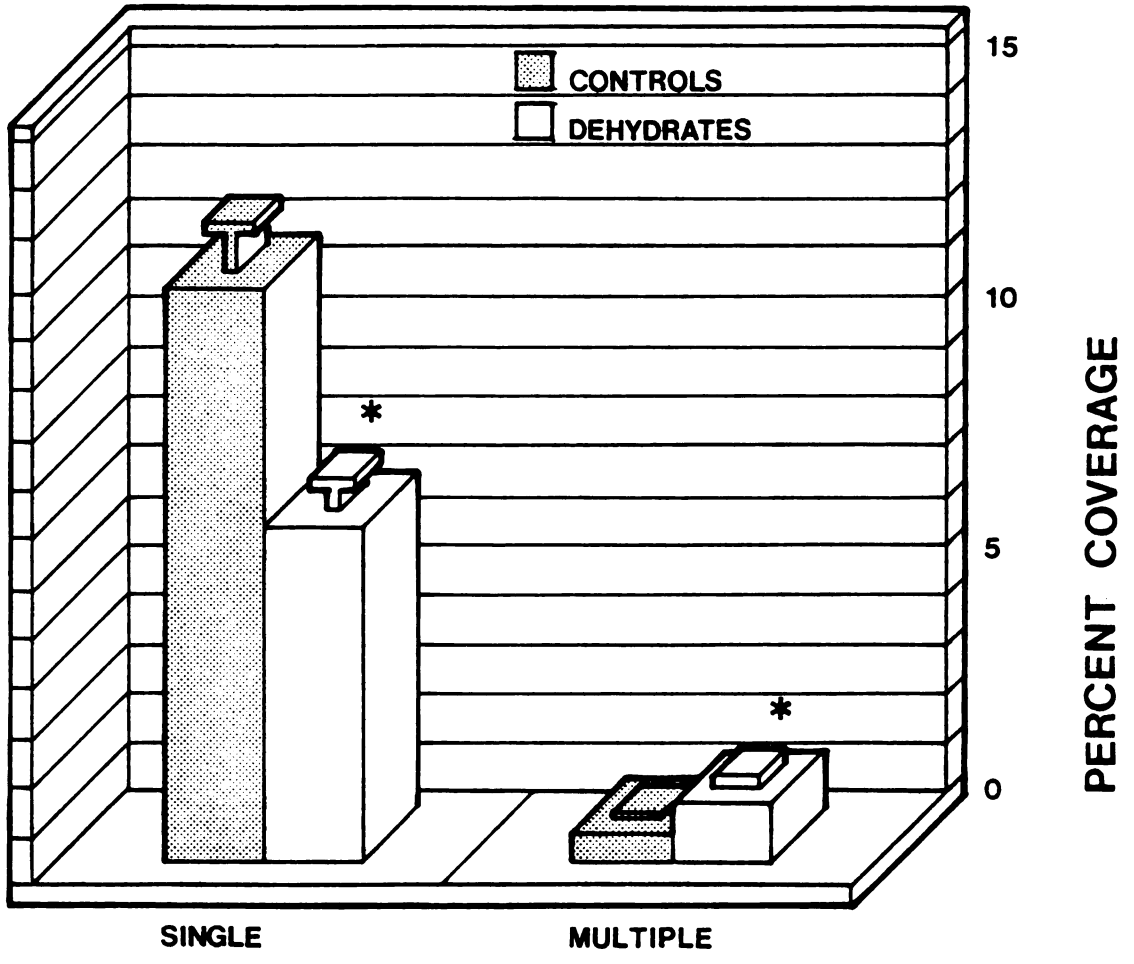

Figure 4 - The percentage of somatic membrane contacted by terminals which formed single and multiple synapses. (Means and S.E., * significantly different)

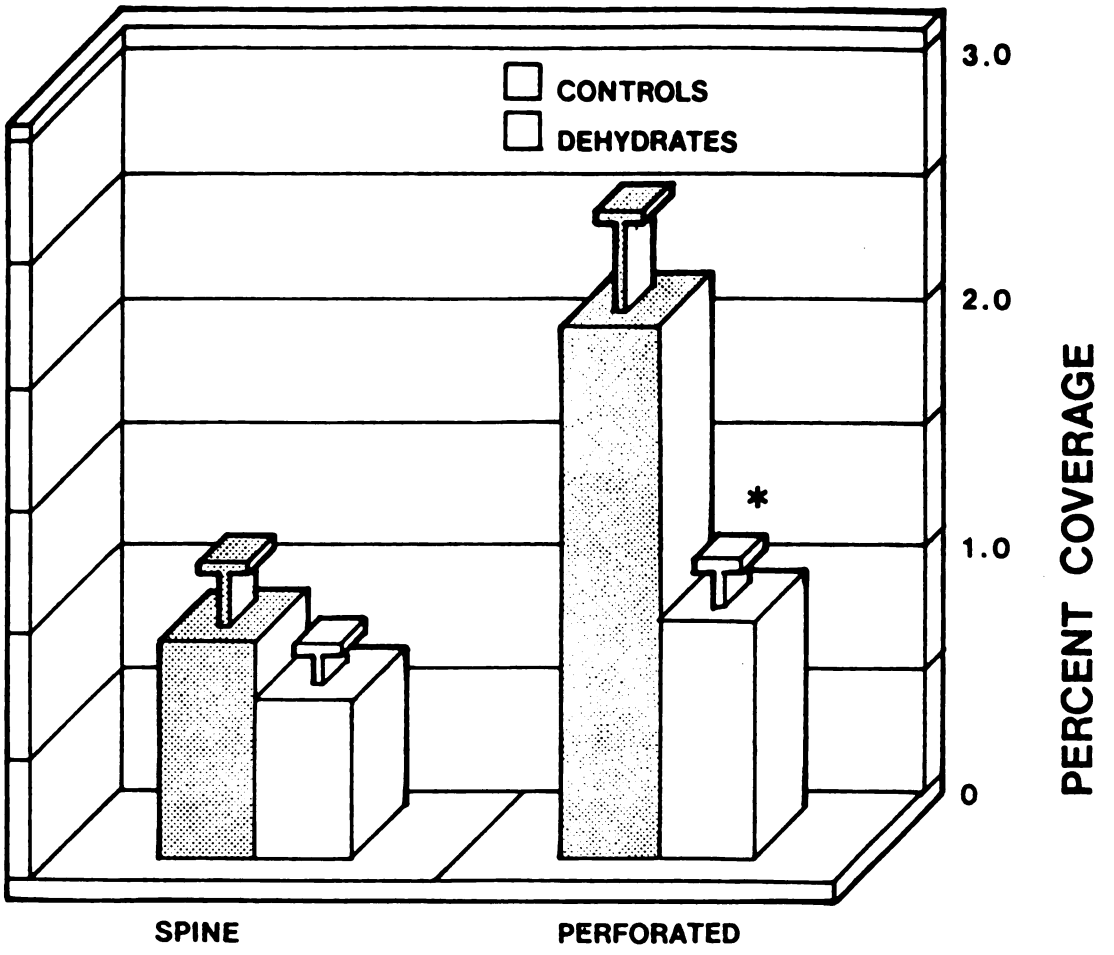

Figure 5 - The percentage of somatic membrane contacted by terminals associated with somatic spines and perforated postsynaptic densities (Mean and S.E., * significantly different.)

significant difference in coverage by single terminals was still apparent (controls, 8.23 ± 0.95; dehydrates 4.57 ± 0.47, $p < 0.01$). When multiples were compared there was no significant difference between the two groups (controls, .48 ± 0.11; dehydrates 0.65 ± 0.16, $p > 0.05$). When singles and multiples apposed to somatic membrane with psds were expressed as a percentage of all psd associated terminals a significant increase in the proportion which were multiples was found in dehydrates (14.7 ± 2.2) compared to controls (6.03 ± 1.3, $p < 0.007$).

Figure 6 illustrates the relative contribution of singles and multiples to the total axonal contact with MNC somata. Dehydrates had a significantly higher percentage of their total axonal coverage made by terminals which formed multiple synapses compared to controls (dehydrates, 15.15 ± 1.79; controls, 5.34 ± 1.79, $p < 0.0027$) with a corresponding decrease in percentage made by single terminals. The percentage of terminals which were multiple, spine and perforated is shown in Figure 7. Although no significant differences were found in spine (controls, 5.88 ± 1.47, dehydrates 7.27 ± 1.89) or perforated synapses (control 12.00 ± 1.89, dehydrates 7.33 ± 1.70) a significant increase in the percentage of terminals which formed multiple synapses was seen in dehydrates (17.37 ± 2.02) compared to controls (5.36 ± 1.66, $p < 0.001$).

Stereological Measures

The calculated mean disk diameter (Δ) for controls was 1.43 μm and 1.36 μm for dehydrates. Thus the mean surface area of an individual contact between a terminal and cell soma for controls was 1.60 μm^2 for

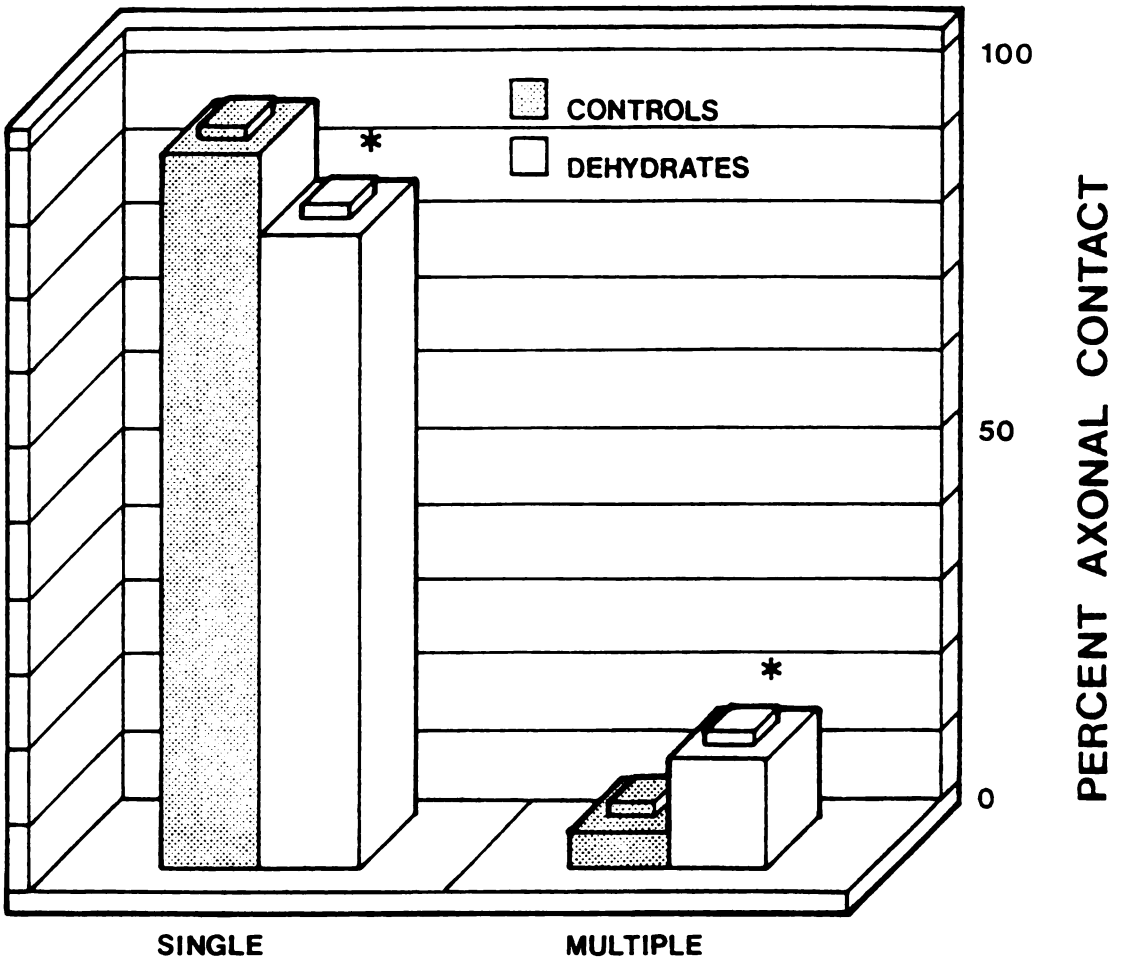

Figure 6 - The percentage of total axonal contact with made by terminals which formed single and multiples synapses. (Mean and S.E., * significantly different.)

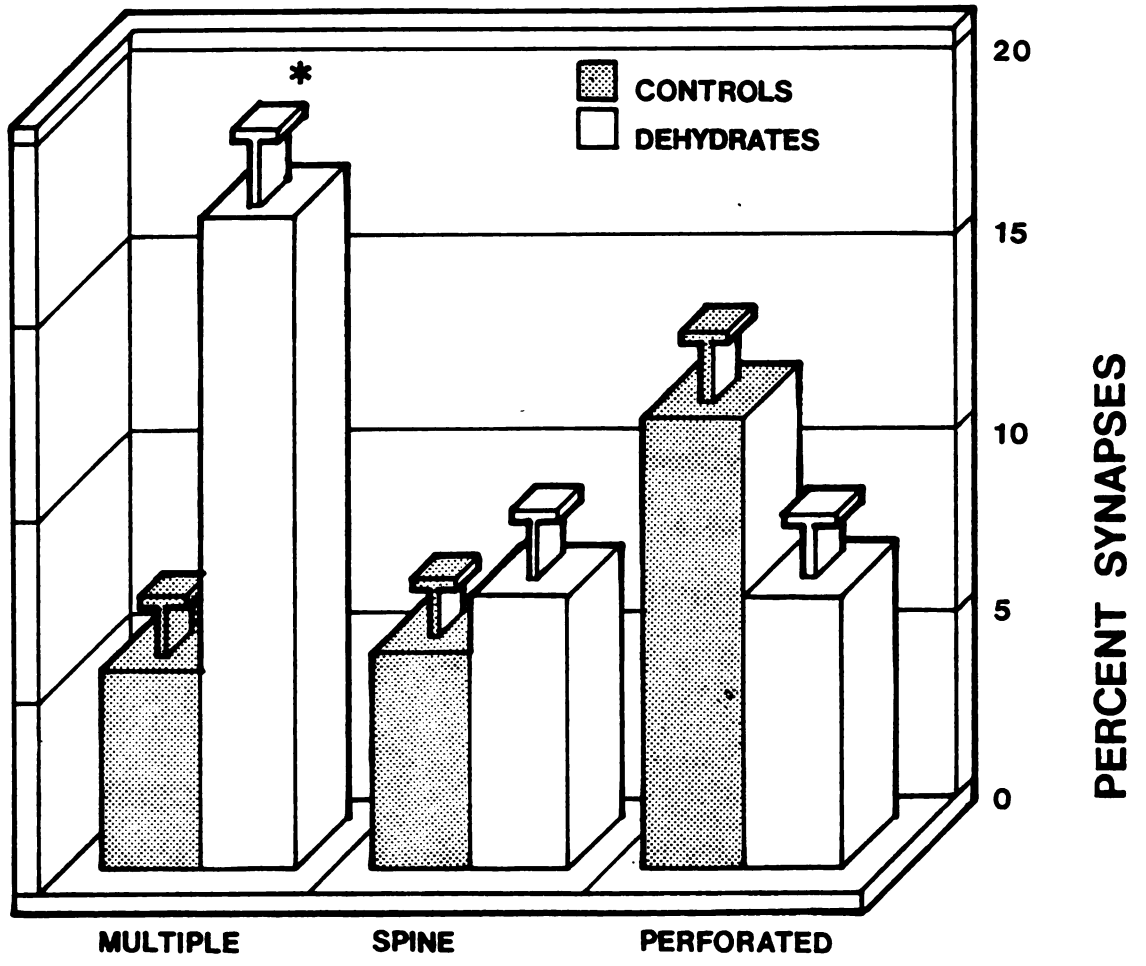

Figure 7 - Percentage of synapses which were multiple, spine and perforated. (Mean and S.E., * significantly different.)

controls and 1.46 μm^2 for dehydrated animals. It appears that the contact area from dehydrated animals is smaller than controls, however, a statistical comparison requires that a mean diameter be calculated for each animal from approximately 300 synapse lengths. The sampling in this study was not that extensive.

The number of terminals which formed single synapses per 100 μm^2 of somatic surface area was significantly higher in control animals (7.19 ± 0.63) compared to dehydrates (4.63 ± .44, $p < 0.006$) while the number of terminals which formed multiple synapses per 100 μm^2 was significantly lower in controls (0.36 ± 0.10) compared to dehydrates (0.85 ± 0.16, $p < .03$).

Cell Parameters

Table 1 lists the groups means and standard errors for each of the shape measures obtained. There were no significant differences between controls and dehydrates in any of these parameters. Figure 8 shows the size frequency distribution of cell surface area (μm^2) for all of the measured cells. The distribution of cell surface areas for dehydrated animals shifted to the right. As shown in Figure 9 the mean surface area of cells in dehydrated animals (2694 ± 116) was significantly larger than controls (1570 ± 83, $p < 0.0001$).

TABLE 1 - CELL SHAPE PARAMETERS

GROUP	ASPECT RATIO	SHAPE FACTOR	ECCEN-TRICITY
Control			
Mean	0.6785	0.7965	0.7143
S.E.	0.0063	0.0105	0.0058
Dehydrate			
Mean	0.6869	0.7882	0.7073
S.E.	0.0193	0.0145	0.0194

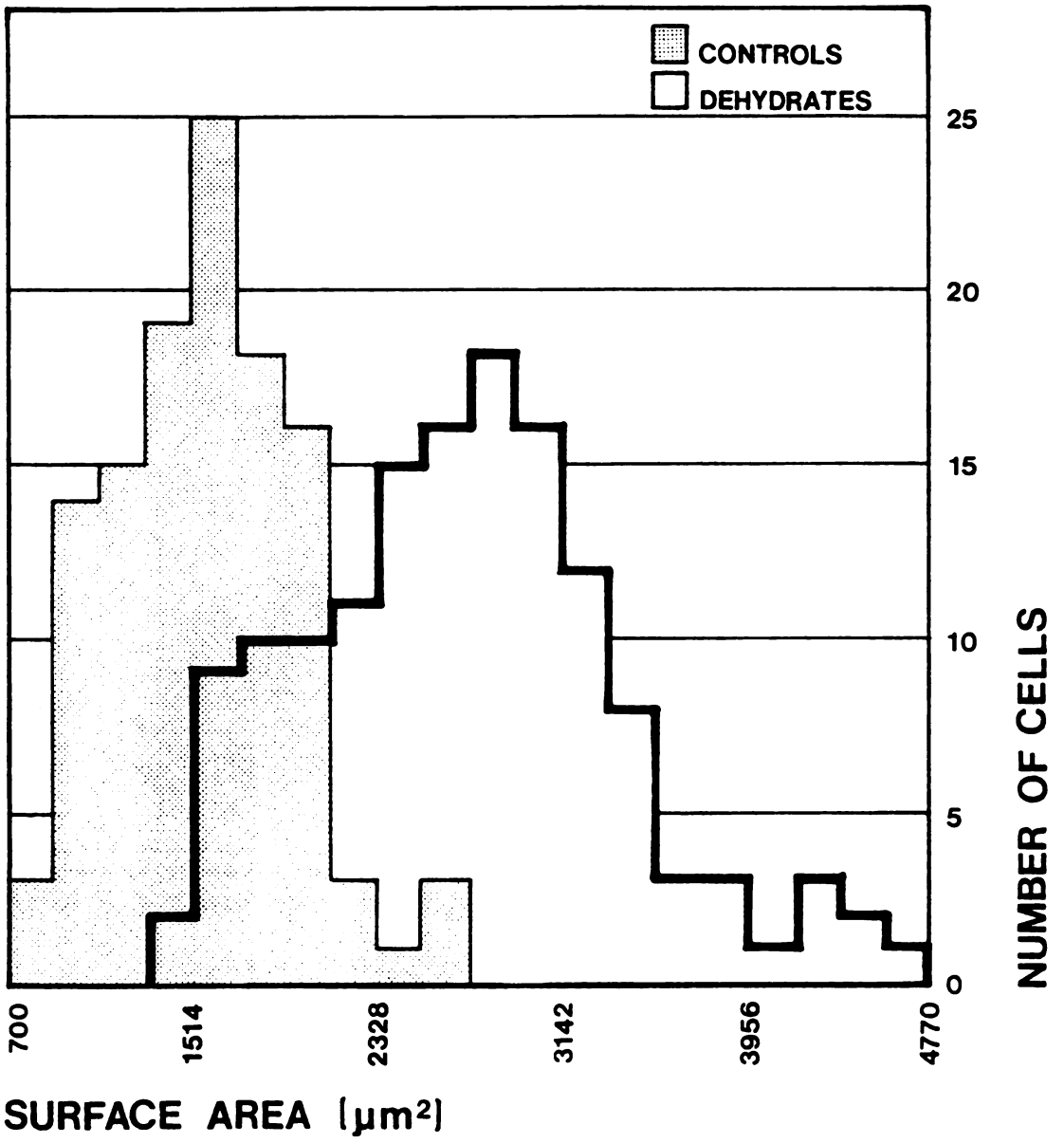

Figure 8 - Size frequency distribution of MNC somatic surface area for control and dehydrate animals.

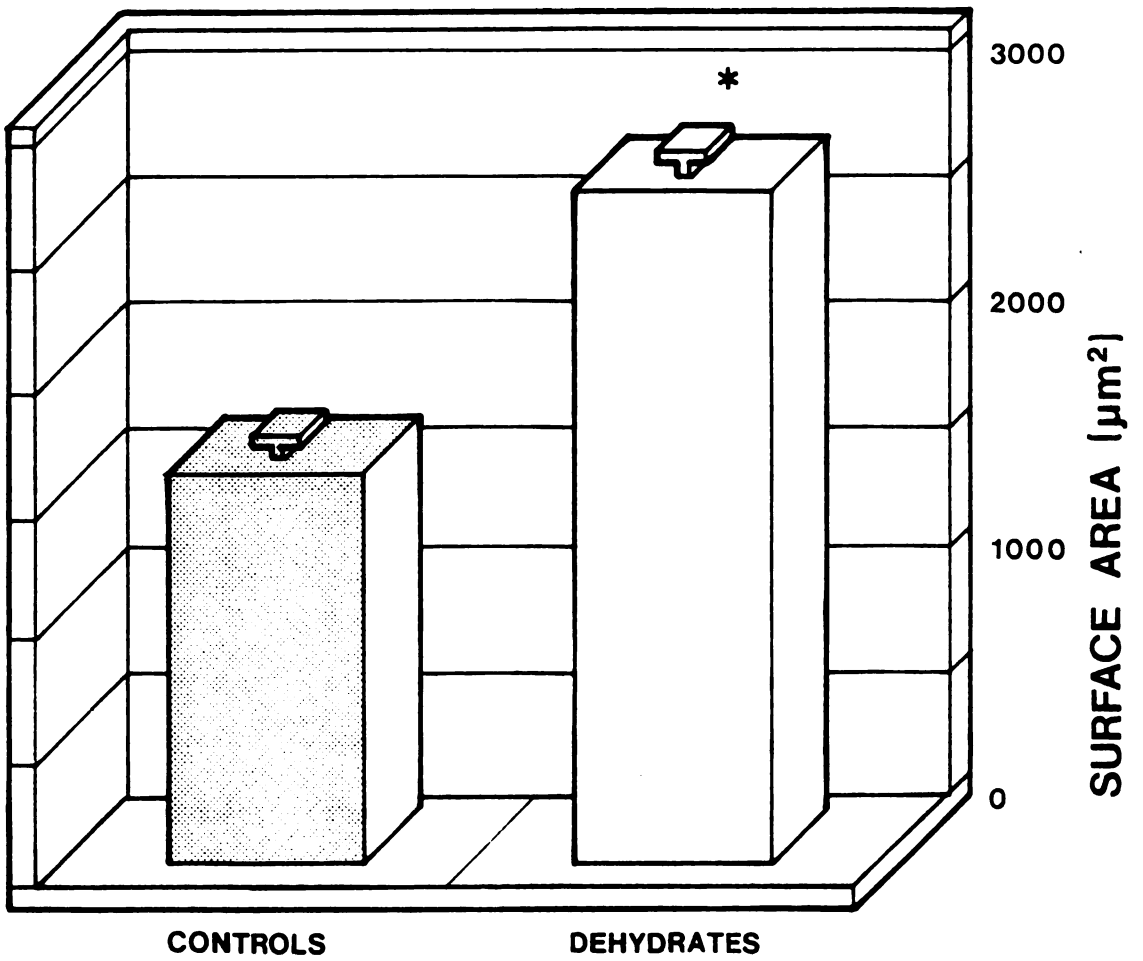

Figure 9 - The mean surface area of neurons within each experimental group (Mean and S.E., * significantly different).

Number of Synapses per Neuronal Cell Body

Figure 10 illustrates that the number of terminals which formed single synapses per cell body was not significantly different between the two groups (controls 111 ± 9, dehydrates 125 ± 14). The number of multiple synapses per cell body was significantly higher in dehydrates (22.82 ± 4.5) than controls (5.8 ± 1.8, $p < 0.009$). The total number of synapses per cell body did not differ between the two groups (controls 120.0 ± 8), dehydrates (148 ± 17).

DISCUSSION

Quantitative Methods

Since the goal of this study was to obtain measures of the synaptic input to MNCs, several assumptions made in the quantitative analysis deserve comment. The morphological criteria used to measure synapses varies considerably in morphometric literature. The strict morphological definition of a synapse requires that a presynaptic terminal with vesicles is apposed to postsynaptic membrane with a well defined postsynaptic density. Many investigators assume that any apposition between a terminal and postsynaptic membrane eventually forms a conventional synapse and thus use the apposition between a terminal and postsynaptic membrane, regardless of psds, as an indicator of synaptic input. This study followed this model and measured the trace lengths of all terminals which contacted MNC somata. These lengths were then used to calculate the number of synapses per 100 μm^2 (Ns) and the number of synapses per soma. Although the possibility exists that the above measures were overestimated since all contact lengths were used (i.e., perhaps not all terminals form conventional

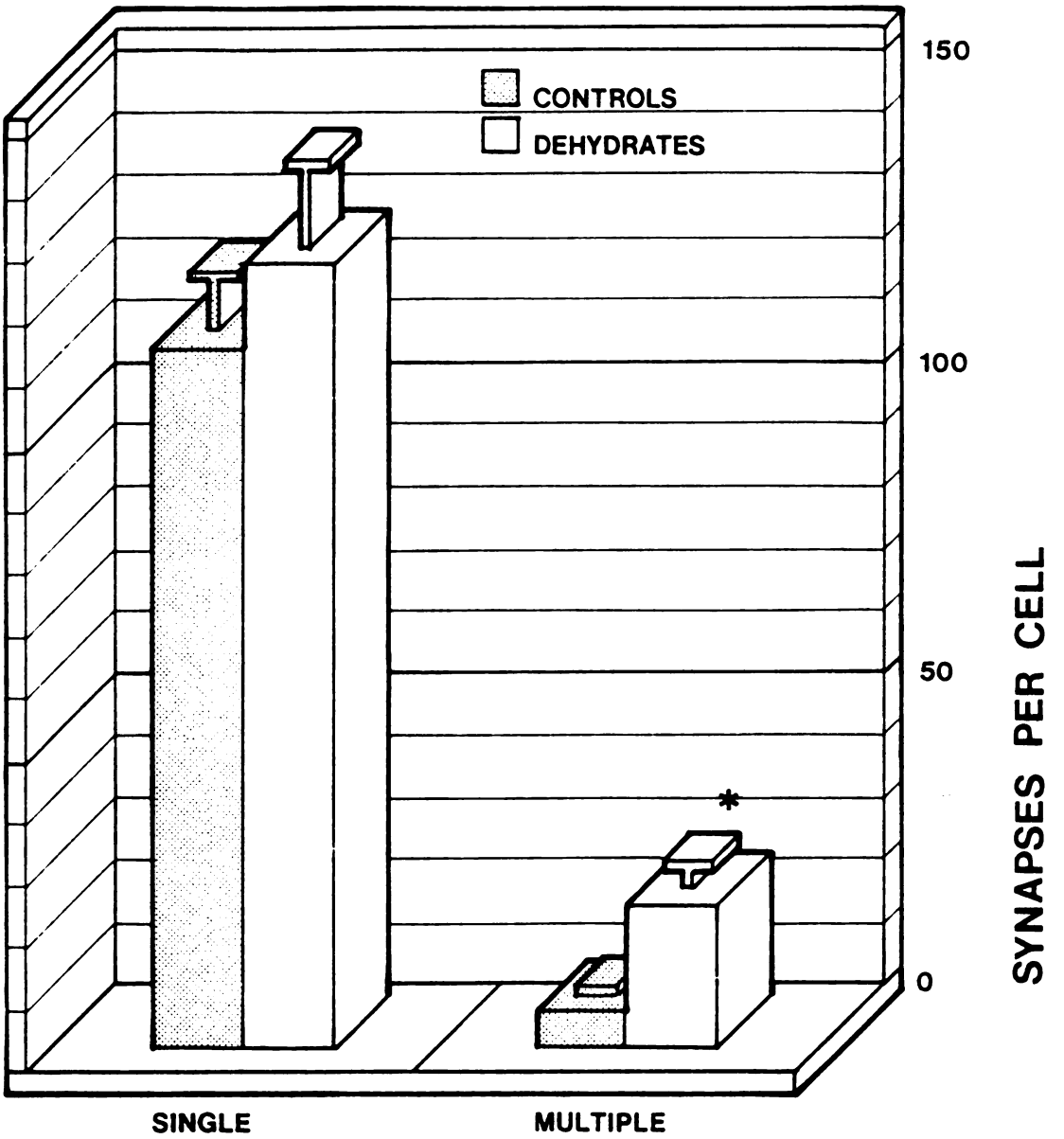

Figure 10 - The number of single and multiple somatic synapses per cell (Mean and S.E., * significantly different).

synapses with psds), the extent the overestimation is probably quite small since over 97% of the terminals followed in serial section formed synapses.

Not all the terminals investigated in this study conformed to the assumption that appositions zones between terminals and cell somata were disks. The percentage of terminals which were associated with somatic spines in serial sections analysis was 22% in controls and 31% in dehydrated animals. These percentages are much higher than those obtained in single sections where the percentages ranged from 1.5% to 11.1% in control animals and 0 to 13% in dehydrated animals. Although shape assumptions do not influence the surface density (Ss) measures, they could affect the calculation of number of synapses per μm^2 (Ns) and the number of synapses per soma. The extent to which this shape assumption influences these calculations is unknown, although the extent of the bias probably remains constant for both groups. Methods for determining the number of synapses per unit volume of tissue are available which do not require shape assumptions (see de Groot & Bierman, 1986), however whether or not these procedures are also applicable to the number of contacts per unit surface area is not known. As mentioned earlier, tissue volume was not used in this study since differential changes in tissue volume are likely under the present experimental conditions.

Typically estimates of cell surface area assume that neurons are either shaped like spheres (e.g. Case & Matthews, 1985) or prolate spheroids (e.g. Kaiserman-Abramoff & Peters, 1972). Since previous research showing increases in SON cell size have used a prolate

spheroid as a model shape for neurons (e.g Ito, Iijima & Kowada, 1986; Kalimo, 1975) and since the measured shape parameters did not suggest that MNCs were spheres, the surface area of MNCs was calculated using the formula for a prolate spheroid. The sampling technique used to select cells (i.e. only cells with a prominent nucleolus) may have biased the measured surface area of MNCs since multiple nucleoli are not uncommon, and nucleoli are often eccentrically placed. Thus some of the cells may not have been sectioned through their largest extent and their surface area would have been underestimated. This is particularly true of the dehydrated animals where multiple nucleoli are more common than in well hydrated animals (Hatton & Walters, 1973). The extent of the underestimation is not known, although calculation of a progressive mean for long and short Feret diameters from 20 cells from a control animal and 20 cells from a dehydrate animal resulted in less than 10% variation in these means.

Multiple Synapses and MNC Somata

In the first report of synapse formation in the SON after chronic dehydration, Tweedle and Hatton (1984) reported an increase in the percentage of magnocellular neurons contacted by multiple synapses from 0% in well hydrated animals to 20% in chronically dehydrated animals. These percentages are probably underestimates since the morphological criteria of a synapse included the presence of a synaptic thickenings, and measures were made on single thin sections. The present has further documented multiple synapse formation in the SON. Virtually every measure pertaining to these multiple synapses substantiates this claim. The increase is reflected not only in the number of multiple

synapses per cell body, but also in the substantial increase in the relative number of terminals which formed multiple contacts as well as the relative contribution of multiple terminals to the total axonal contact with MNCs. That the percentage of somatic membrane covered by multiples with an associated psd was not higher in dehydrated animals does not necessarily contradict this conclusion. Given that a large increase in cell surface area occurs, an equal increase in a given structure must occur to maintain equal coverage of the somatic membrane. This is clearly demonstrated in the significant decrease in the percentage of somatic membrane covered by single terminals in dehydrated animals. This result does not reflect a decrease in the number of single terminals but rather an increase in the reference surface. Thus, knowing that the surface increases and that the percent membrane coverage by psd-associated multiple synapses did not significantly decrease, one could infer that these contacts did, in fact, increase. The significant increase in the percentage of psd-associated terminals which were multiple synapses in dehydrated animals provides further evidence for this conclusion.

The lack of a significant difference in the total number of terminals per soma is surprising given the significant increase in the number of multiple synapses per soma and no change in the number of single synapses per neuron. There are two very different but perhaps not exclusive explanations for this paradox. First, the increase in the number of multiple synapses per cell, although significant may be "lost" in the variability in the total number of somatic synapses. Second, the formation of multiple synapses is thought to be due in part

to the conversion of singles into multiples. If a single terminal which contacts a cell soma becomes a multiple by forming a new contact with adjacent cell somata or dendrites then the number of total terminals per soma would not change. Although this offers a partial explanation for not detecting an increase in the total number of terminals per neuron, the data presented here do not support the conclusion that terminals which form multiple contacts are constructed from existing somatic singles. If that were the case, a decrease in the number of single synapses would be expected in the dehydrated group. Since there was no significant difference in the number of singles per neuron, there is probably some proportion of synaptic input to MNCs which is newly formed.

It should be noted that the number of somatic synapses per neuron derived in this study is much larger than those reported in the literature (111, controls; 125, dehydrates). An average of 57 somatic synapses per neuron has been calculated based on a volumetric study (Leranth, Zaborszky, Marton & Palkovits, 1975). This method derived the number of synapses based on a morphological criteria which required postsynaptic densities, not terminals, which may partially account for the observed difference. Itoh, Iijima & Kowada (1986) using a morphometric method similar to the one used in this study reported 49 somatic synapses per neuron. The average surface area of MNCs and the percentage of somatic membrane contacted by terminals reported by this group was approximately the same as those measured in this study. The discrepancy between the number of synapses per cell is directly attributable to a different mean surface area of individual terminal

contacts which was 1.60 μm^2 for controls and 1.46 μm^2 for dehydrates in this study compared to 2.56 μm^2 reported by Itoh et al. (1986).

The data pertaining to perforated contacts and contacts onto somatic spines must be regarded as preliminary since they were collected using a sample size which was optimized for all axonal terminals not for spine or perforated synapses. As might be expected there was a great deal of variability within the groups in these measures. One could infer that the lack of a decrease in the percent MNC somatic membrane covered by axonal terminals associated with somatic spines reflects an increase in these terminals or their size. However, given that the percent coverage by spine associated terminals is low in both controls and dehydrated animals and the apparent discrepancy in their percentage found between the serial sectioning and morphometric data, this conclusion awaits a more comprehensive investigation.

The experimental evidence accumulated to date indicates that the synaptic characteristics of the SON are altered in very specific ways under conditions of increased hormone demand. In this study the specificity of SON's response to activation is reflected in the observation that only the number of multiple synapses per cell body increased in chronically dehydrated animals. Further evidence for specificity in SON's plastic responses to activation has accumulated from ultrastructural studies of the dendritic region (located ventral to the cell bodies) and electrophysiological experiments. Multiple synapses in the dendritic region have also been found to increase in frequency but only immediately following parturition, not during

lactation or chronic dehydration (Perlmutter, Tweedle & Hatton, 1984; 1985). Perhaps the formation of multiple synapses is related to an increase in the activity of specific afferent inputs to this system which vary under the different experimental conditions. This hypothesis is supported by recent in vitro electrophysiological experiments which reveal that dye coupling among SON neurons increases after electrical stimulation of the lateral olfactory tract, but only under rather specific experimental conditions. Lateral olfactory tract stimulation results in increased dye coupling in lactating animals and virgin females which have been induced to behave maternally by exposure to rat pups (Modney, Yang and Hatton, 1987; Yang & Hatton, 1987). Such stimulation does not affect dye coupling in male or untreated virgin female rats. Thus, the experimental literature related to plasticity in the SON reveals that this nucleus is indeed capable of remarkable changes during increases in hormone demand, but that the nature and extent of its reorganization are dependent on both physiological and environmental conditions.

Given that multiple somatic synapses increase during chronic dehydration, the question still remains as to the mechanism through which this occurs. As mentioned earlier, previous research has suggested that multiple synapses in the SON are formed through the conversion of single contacts into multiples. The observation that only multiple synapses increase in frequency certainly supports this hypothesis. Since there was not a concomitant decrease in the number of single synapses per soma, the formation of multiples must not occur exclusively through the conversion of somatic singles into multiples.

Of course it is entirely possible that existing dendritic singles are converted into soma-dendritic multiples. This hypothesis has not yet been tested. Also, the possibility that axonal sprouting participates in the reorganization of the synaptic characteristics of SON, through either the formation of multiple synapses or in maintaining the number of single synapses, cannot be eliminated.

Previous ultrastructural studies of the SON have demonstrated that increases in soma-somatic and soma dendritic direct membrane apposition occur during dehydration (Chapman, Theodosis, Montagnese, Poulain & Morris, 1986; Perlmutter, Tweedle & Hatton, 1984; Tweedle & Hatton, 1976, 1977). A similar significant increase in direct membrane apposition was found in this study. That increases in membrane apposition occur before significant increases in cell size occur led to the conclusion that astrocytic processes, which are normally interposed between adjacent neural elements, retract from this position and allow these appositions to occur (Tweedle & Hatton, 1977). Glial retraction has also been postulated to participate in the formation of multiple contacts by allowing axonal terminals access to cell somata and dendrites. In this study, the percentage of cell membrane contacted by glial processes was not significantly different in control and dehydrated animals indicating that perhaps glial contact per soma increased after 10 days of dehydration. A similar finding has been reported by Chapman et al. (1986). Indeed there is report that astrocytes proliferate during dehydration in young animals (Patterson and LeBlond, 1977). The combination of results from acute and chronic dehydration suggest that the role of astrocytic processes throughout

dehydration is more complex than a permanent retraction of glial processes. Perhaps an initial glial retraction allows for increased neural contact (between neurons, dendrites and terminals) and as cells increase in size glial processes cover the "new" portion of the somatic membrane. That a decrease in glial processes is associated with the initial stages of synaptogenesis has recently been demonstrated in the ventral posterior nucleus of the rat thalamus (Wells & Tripp, 1987a; 1987b). In this nucleus, lesions of the dorsal column nuclei result in reactive synaptogenesis which does not begin until 30 days post-lesion. A study of the time course of this reactive process revealed that the initial stages of synapse formation were associated with a decrease in the area of neuropil occupied by glial processes compared to nonlesioned controls. As synapses were replaced, glial process area again increased such that at the completion of synaptogenesis there was no difference in the area occupied by glial processes between lesioned animals and controls. A similar process could be envisioned in the SON where the initial stages of synapse formation are associated with a decrease in glial contact. After chronic dehydration (i.e. 10 days of saline drinking) perhaps synapse formation has been completed and glial processes again cover an equivalent proportion of the somatic surface. Since there is currently no information about when multiple synapses form during chronic dehydration this hypothesis remains to be tested with a time course study that incorporates all of the various parameters which are affected by dehydration, i.e. cell size, glial coverage and number of contacts.

The precise functional significance of multiple contacts in the SON is, at present unknown. Little is known about the neurotransmitters contained in the terminals which form multiples or the peptide content of the cells they contact. Immunocytochemical electron microscopic studies have shown that terminals which form multiple contacts may contain dopamine (Buijs, Geffard, Pool & Hoorneman, 1984) or GABA (Theodosis, Paut & Tappaz, 1986) which suggests that the formation of multiples may not be transmitter specific. There is some evidence that the alterations which occur during chronic dehydration may effect primarily oxytocinergic neurons, (Chapman, et. al, 1986) but this result has not been replicated. That multiple synapses contribute to the overall excitability and serve to coordinate the activity of the groups of SON during periods of increased hormone demand seems likely, especially considering the alterations in the electrophysiological characteristics of MNCs during activation of the system. Perhaps future studies will better delineate the time course for the appearance of these synapses, and the nature of their contribution to the excitability of SON neurons during periods of chronic hormone release.

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APPENDICES

APPENDIX A

SUPPLIES AND EQUIPMENT

EQUIPMENT

Diatome, Inc. (1987)		
Diamond Knife (2.4mm)	$	2,500.00
EQS Systems Inc (1984)		
Houston Instruments Hipad Digitizing Tablet		828.00
Gilson Medical Elec Inc (1985)		
Peristaltic Pump		1,136.00
Kodak (1982)		
Kodak Ektamatic Print Processor		780.00
JEOL (Approximate)		
JEOL CX-100 Electron Microscope		300,000.00
Logitech (1985)		
Modula 2 compiler		560.00
Mouse (3-button)		119.00
Mager Scientific (1987)		
Reichard Ultracut E ultramicrotome		23,160.00
" " section counter		657.00
Nikon Alphaphot microscope		1000.00
Markson, Inc (1987)		
Markson pH meter		495.00
pH electrode		89.00
Temperature probe		59.00
Millipore, Inc. (1987)		
Milli-Ro 4 (Water purification system)		1,482.00
Milli-Q (" ")		1,947.00
Cartridges for above system		793.00
MSU Computer Center (1985)		
Zenith 150 Computer w/monochrome moniter		1,499.00
Olympus (1986)		
C-2 Image Analysis System (excluding microscope)		18,000.00
Procomp Computer Products (1985)		
Epson RX-85 Printer		339.00

APPENDIX A (cont'd.)

Sigma Chemical Co. (1987)	
Cacodylate Acid (500 g)	182.00
Paraformaldehyde (1 kg)	12.75
Technical Manufacturing Corp. (1987)	
Micro-g air table	2,340.00
Ted Pella, Inc (1987)	
Arkay Print dryer	720.00
Graflab memory timer (for enlarger)	248.35
Infiltron (approximate equivalent)	577.00
Print washer	436.00
Sodium Vapor Safelight	398.00
Thomas Scientific (1987)	
Mettler Balance	2,795.00
Blue M Gravity Oven	669.00
VWR Scientific (1987)	
Stereozoom microscope (approx. replacement)	1,336.00

SUPPLIES

Electron Microscopy Sciences (1987)	
Araldite-Embed (kit)	24.00
Butvar B-98 (30ml)	5.00
Copper Grids (200 mesh-thin bar: 100/vial)	13.50
Copper Slot Grids (100/vial)	11.00
Diffraction Grating	20.00
Forceps	
3C	8.50
anticappillary, self-closing	19.50
Glassine Envelopes for negative (1000)	34.50
Glutaraldehyde (Biological Grade) 500 ml	6.50
Grid Boxes (12)	32.00
Propylene Oxide (4 qts)	16.00
Uranyl Acetate (25 g)	12.00
Millipore, Inc. (1987)	
GSTF filters (.22μm)	15.00
MSU Stores (1987)	
Assorted glassware (beakers, volumetrics, etc)	100.00
Assorted disposable plastics (syringes, etc)	60.00
D-19 developer	2.89
Dessicator (small glass)	74.23
Ektamatic SC Paper (500 sheets)	146.59
Ektamatic Activator	3.34
Ektamatic Stabilizer	5.01

APPENDIX A (cont'd.)

MSU Stores (cont'd)	
Electron Microscopy Film (box w/100 sheets)	35.70
Rapid Fix (used for negatives & prints)	4.96
Stevenson Metal Supply (1987)	
Osmium tetroxide (1 g)	35.00
Vets Ace Hardware (1987)	
Red Devil Razor Blades (100)	9.40
VWR Scientific (1987)	
Microscope Slides (case)	110.62

APPENDIX B

Tissue Embedding Protocol

Epon-Araldite Resin from Electron Microscopy Sciences - follow manufacturer's "recipe" for mixing resin

DAY ONE - Perfusion (see Methods section for details)

DAY TWO

1. 0.15 M Cacodylate Buffer rinses (3 rinses - 15 min each)
2. Osmicate for 1 hour (1:1 ratio of 2% aqueous osmium tetroxide and $K_3(CN)_6Fe$ in 0.2 M cacodylate buffer)
3. Water rinses (3 @ 15 min each)
4. En bloc stain with 4% aqueous uranyl acetate filtered with 0.22 μm Millipore filter, store in refrigerator overnight

DAY THREE - Tissue in vials should be agitated throughout infiltration

1. Water rinses (3 @ 15 min each)
2. 50% ETOH (10 min)
3. 70% ETOH (10 min)
4. 80% ETOH (10 min)
5. 95% ETOH (10 min)
6. 100% ETOH (4 @ 15 min each; use unopened bottle of ETOH)
7. Propylene Oxide (4 @ 15 min each)
8. 1:1 ratio of Propylene Oxide to resin mixture (4 hours)
9. 1:2 ratio of Propylene Oxide to resin mixture (4 hours)
10. 100% resin mixture (overnight)

DAY FOUR

1. Place resin into embedding molds with tissue
2. Polymerize in oven at 65-70° for 2-3 days

APPENDIX C

DATA TABLES

APPENDIX C

TABLE 2 - RAW DATA: STRUCTURES CONTACTING MNC SOMATA (μm)

ANIMAL	GLIAL PROCESS	AXONAL CONTACTS	UNIDENTIFIED	ADJACENT CELLS OR DENDRITES	TOTAL
CONTROL					
C29	432.37	57.21	13.35	0.00	502.93
C52	585.77	85.76	28.37	5.33	705.23
C65	597.85	94.40	22.74	6.43	721.42
C69	470.39	51.85	11.95	2.37	536.56
C89	479.66	62.50	22.39	7.55	572.10
C99	332.60	65.28	3.82	10.62	412.32
DEHYDRATE					
D12	320.88	73.44	132.21	54.24	580.77
D17	641.47	55.24	51.64	94.65	843.00
D47	470.68	49.73	31.09	29.40	580.90
D54	482.75	41.37	31.18	53.80	609.10
D56	351.71	27.30	16.09	32.60	427.70
D83	434.03	39.64	5.23	31.10	510.00
B95	468.40	44.84	43.56	43.95	600.75

APPENDIX C (Cont'd.)

TABLE 3 - RAW DATA: TERMINAL TYPE

ANIMAL	SINGLE	SINGLE	MULTIPLE	MULTIPLE	SPINE	SPINE	PERF
PERF (#)	(µm)	(#)	(µm)	(#)	(µm)	(#)	(µm)
CONTROL							
C29	54.98	53	2.23	1	10.32	6	10.68 6
C52	83.96	74	1.80	3	4.30	3	12.27 7
C65	90.32	72	4.08	3	8.96	5	23.8015
C69	45.12	41	6.73	5	5.05	4	7.92 5
C89	57.65	54	4.85	6	2.26	2	5.15 4
C99	64.62	62	0.66	1	0.51	1	14.00 9
DEHYDRATE							
D12	60.18	54	13.26	14	0.00	0	8.31 5
D17	46.58	41	8.66	9	10.40	6	3.52 2
D47	38.77	37	10.96	9	3.26	2	5.53 4
D54	34.57	29	6.80	8	7.67	5	2.21 1
D56	23.02	21	4.28	6	1.41	2	2.28 1
D83	36.47	30	3.17	3	1.52	1	7.37 3
D95	40.29	34	4.55	4	4.98	4	10.10 6

APPENDIX C (Cont'd.)

TABLE 4: RAW DATA - TERMINALS APPOSED TO SOMATIC MEMBRANE WITH POSTSYNAPTIC DENSITIES

ANIMAL	SINGLE (µm)	SINGLE (#)	MULTIPLE (µm)	MULTIPLE (#)	TOTAL (µm)	TOTAL (#)
CONTROL						
C29	40.07	33	2.23	1	42.30	34
C52	63.70	49	1.80	3	65.50	52
C65	54.66	42	4.08	3	58.74	45
C69	27.88	24	5.28	3	33.16	27
C89	41.93	37	2.77	3	44.70	40
C99	50.64	45	0.66	1	51.30	4 6
DEHYDRATE						
D12	39.73	30	8.68	10	48.41	40
D17	34.53	26	5.70	5	40.23	31
D47	25.03	22	4.50	4	29.53	26
D54	19.29	16	2.60	2	21.89	18
D56	16.30	14	2.74	3	19.04	17
D83	28.45	21	1.45	2	29.90	23
D95	25.30	20	1.70	2	27.00	22

APPENDIX C (Cont'd.)

TABLE 5 - CELL PARAMETERS: Means and standard errors from 20 cells per animal

ANIMAL	LONG DIAMETER (µm)	SHORT DIAMETER (µm)	ASPECT RATIO	SHAPE FACTOR	ECCEN-TRICITY	SURFACE AREA (µm2)
CONTROL						
C29	29.76	20.18	0.6909	0.7640	0.7025	1713
SE	1.06	0.66	0.0289	0.0372	0.0284	89
C52	28.34	19.98	0.6780	0.8153	0.7159	1529
SE	0.79	0.61	0.0270	0.0213	0.0278	68
C65	28.58	19.33	0.6812	0.7666	0.7064	1571
SE	1.01	0.52	0.0280	0.0199	0.0284	77
C69	31.42	20.85	0.6776	0.8241	0.7088	1854
SE	1.01	0.65	0.0304	0.0152	0.0331	78
C89	25.10	17.31	0.6931	0.8115	0.7101	1252
SE	0.72	0.61	0.0207	0.0139	0.0199	76
C99	28.54	18.47	0.6500	0.7974	0.7422	1500
SE	1.02	0.69	0.0245	0.0150	0.0232	98
Group Mean	28.62	19.35	0.6785	0.7965	0.7143	1570
SE	0.85	0.53	0.0063	0.0105	0.0058	83

APPENDIX C (Cont'd.)

Table 5 (cont'd.)

DEHYDRATE

ANIMAL	LONG DIAMETER (μm)	SHORT DIAMETER (μm)	ASPECT RATIO	SHAPE FACTOR	ECCEN-TRICITY	SURFACE AREA (μm2)
D12	35.90	26.50	0.7455	0.8516	0.6492	2808
SE	1.40	1.03	0.0224	0.0155	0.0266	202
D17	41.49	26.65	0.6498	0.7261	0.7438	3118
SE	1.12	0.89	0.0262	0.0180	0.0245	149
D47	35.09	25.44	0.7408	0.8006	0.6453	2602
SE	1.51	0.82	0.0283	0.0198	0.0323	155
D54	35.35	22.58	0.6501	0.7792	0.7456	2241
SE	1.07	0.70	0.0264	0.0369	0.0212	97
D56	37.29	25.58	0.6938	0.7853	0.7027	2720
SE	1.06	0.73	0.0250	0.0158	0.0252	106
D83	42.24	25.30	0.6120	0.7684	0.7799	2970
SE	1.53	0.66	0.0237	0.0152	0.0188	137
D95	34.17	24.30	0.7165	0.8060	0.6845	2402
SE	1.21	0.67	0.0184	0.0164	0.0192	128
Group mean	37.36	25.19	0.6869	0.7882	0.7073	2694
SE	1.22	0.53	0.0193	0.0145	0.0194	116
t-tests	t=5.68	t=7.79	t'=0.41	t=0.45	t'=0.35	t=7.60
p values	p<0.001	p<0.001	p>0.05	p>0.05	p>0.05	p<0.0001
			df=7		df=6	

APPENDIX C (Cont'd.)

TABLE 6 - PERCENTAGE OF MNC SOMATIC MEMBRANE COVERED BY VARIOUS CELLULAR ELEMENTS

ANIMAL	GLIA	AXONAL	MNC OR DENDRITE	NOT IDENTIFIED
CONTROL				
C29	85.97	11.38	0.00	2.65
C52	83.06	12.16	0.76	4.02
C65	82.87	13.09	0.89	3.15
C69	87.67	9.66	0.44	2.27
C89	83.84	10.92	1.32	3.91
C99	80.67	15.83	2.58	0.93
Mean	84.01	12.17	0.99	2.82
SE	1.01	0.87	0.36	1.16
DEHYDRATE				
D12	55.25	12.65	9.34	22.76
D17	76.09	6.55	11.23	6.48
D47	81.03	8.56	5.06	5.35
D54	79.26	6.79	8.83	5.12
D56	82.23	6.38	7.62	3.76
D83	85.10	7.73	6.10	1.03
D95	77.97	7.47	7.32	7.23
Mean	76.70	8.02	7.93	7.40
SE	3.74	0.82	0.78	2.67

t-tests

Glia $t' = 1.88$ $p > 0.0500$ $df=6$

Axonal Contacts $t = 3.45$ $p < 0.0050$

MNC or Dendrites $t = 7.58$ $p < 0.0001$

Unidentified $t' = 1.55$ $p > 0.0500$ $df=6$

APPENDIX C (Cont'd.)

TABLE 7 - PERCENTAGE OF MNC SOMATA COVERED BY TERMINALS (BY TYPE)

ANIMAL	SINGLE	MULTIPLE	SPINE	PERF
CONTROL				
C29	10.93	0.44	2.05	2.12
C52	11.90	0.30	0.61	1.74
C65	12.50	0.56	1.24	3.30
C69	8.40	1.25	0.94	1.48
C89	10.10	0.80	0.40	0.90
C99	15.67	0.16	0.12	3.40
Mean	11.58	0.58	0.89	2.16
SE	1.01	0.16	0.28	0.10
DEHYDRATE				
D12	10.36	2.30	0.00	1.43
D17	5.50	1.00	1.23	0.42
D47	6.67	1.89	0.56	0.95
D54	5.70	1.11	1.26	0.36
D56	5.38	1.00	0.33	0.52
D83	7.20	0.62	0.30	1.45
D95	6.70	0.76	0.83	1.68
Mean	6.79	1.24	0.64	0.97
SE	0.65	0.23	0.18	0.21

t-tests

Single	$t = 4.12$	$p < 0.002$
Multiple	$t = 2.23$	$p < 0.047$
Spine	$t = 0.76$	$p > 0.050$
Perforated	$t = 2.69$	$p < 0.020$

APPENDIX C (Cont'd.)

TABLE 8 - PERCENTAGE OF TOTAL AXONAL COVERAGE MADE BY SINGLES & MULTIPLES

CONTROL			DEHYDRATE		
ANIMAL	SINGLE	MULTIPLE	ANIMAL	SINGLE	MULTIPLE
C29	96.10	3.90	D12	81.99	18.07
C52	96.86	2.01	D17	84.32	15.68
C65	95.68	4.32	D47	78.01	22.05
C69	87.02	12.98	D54	83.50	16.43
C89	92.24	7.76	D56	84.32	15.68
C99	98.96	1.01	D83	92.10	8.01
			D95	89.93	10.16
Mean	94.64	5.34		84.88	15.15
SE	1.79	1.79		1.78	1.78

t-tests

Single $t = 3.822$ $p < 0.0028$

Multiple $t = 3.347$ $p < 0.0027$

APPENDIX C (Cont'd.)

TABLE 9 - PERCENTAGE OF SYNAPSES FORMING SINGLE, MULTIPLE, SPINE AND PERFORATED CONTACTS

ANIMAL	SINGLE	MULTIPLE	SPINE	PERF
CONTROL				
C29	98.15	1.85	11.11	11.11
C52	96.10	3.90	3.90	9.09
C65	96.00	4.00	6.67	20.00
C69	89.13	10.87	8.70	10.87
C89	90.00	10.00	3.33	6.67
C99	98.41	1.59	1.59	14.29
Mean	94.63	5.37	5.89	12.00
SE	1.66	1.66	1.47	1.90
DEHYDRATE				
B12	79.41	20.59	0.00	7.35
B17	82.00	18.00	12.00	4.00
B47	80.43	19.57	4.35	8.70
B54	78.38	21.62	13.51	2.70
B56	77.78	22.22	7.41	3.70
B83	90.91	9.09	3.03	9.09
B95	89.47	10.53	10.53	15.79
Mean	83.62	17.23	7.26	7.33
SE	2.03	2.03	1.90	1.70

t-tests

Single	$t = 4.48$	$p < 0.009$
Multiple	$t = 4.48$	$p < 0.009$
Spine	$t = 0.55$	$p > 0.050$
Perforated	$t = 1.83$	$p > 0.050$

APPENDIX C (Cont'd.)

TABLE 10 - PERCENT COVERAGE ON MNC SOMATA WITH POSTSYNAPTIC DENSITIES

CONTROL	Singles	Multiples	Total
C29	7.97	0.44	8.41
C52	9.03	0.26	9.29
C65	7.58	0.57	8.15
C69	5.20	0.98	6.18
C89	7.33	0.48	7.81
C99	12.28	0.16	12.42
Mean	8.23	0.48	8.71
SE	0.96	0.12	0.85
DEHYDRATE			
D12	6.84	1.49	8.33
D17	4.10	0.68	4.78
D47	4.31	0.77	5.08
D54	3.17	0.43	3.60
D56	3.81	0.64	4.45
D83	5.58	0.28	5.86
D95	4.21	0.28	4.49
Mean	4.57	0.65	5.22
SE	0.47	0.18	0.58

t-tests

Single	$t = 3.598$	$p < 0.0042$
Multiple	$t = 0.8487$	$p > 0.0500$
Total	$t = 3.469$	$p < 0.0052$

APPENDIX C (Cont'd.)

TABLE 11 - NUMBER OF SINGLE AND MULTIPLE SYNAPSES PER 100 μm2 OF MNC SOMATIC MEMBRANE

CONTROL			DEHYDRATE		
ANIMAL	SINGLE	MULTIPLE	ANIMAL	SINGLE	MULTIPLE
C29	6.80	0.27	D12	7.08	1.57
C52	7.40	0.19	D17	3.76	0.68
C65	7.78	0.35	D47	4.56	1.29
C69	5.23	0.78	D54	3.90	0.76
C89	6.22	0.50	D56	3.68	0.68
C99	9.75	0.10	D83	4.92	0.42
			D95	4.58	0.52
Mean	7.19	0.36		4.64	0.95
SE	0.63	0.10		0.44	0.16

t-tests

Single $t = 3.39$, $p < 0.006$

Multiple $t = 2.46$, $p < 0.031$

APPENDIX C (Cont'd.)

TABLE 12 - NUMBER OF SYNAPSES PER MNC SOMATA

CONTROL				DEHYDRATE			
ANIMAL	SINGLE	MULTIPLE	TOTAL	ANIMAL	SINGLE	MULTIPLE	TOTAL
C29	116.48	4.69	121.17	D12	198.84	44.14	242.99
C52	113.20	2.85	116.05	D17	117.24	21.32	138.56
C65	122.19	5.47	127.66	D47	118.63	33.61	152.24
C69	96.87	14.41	111.28	D54	87.34	17.01	104.34
C89	77.89	6.23	84.13	D56	100.04	18.59	118.63
C99	146.19	1.49	147.69	D83	146.19	12.59	158.78
				D95	110.02	12.48	122.50
Mean	112.14	5.96	118.00		125.47	22.82	148.29
SE	9.46	1.85	8.53		14.03	4.46	17.36

t-tests

Singles $t = 0.77$ $p > 0.050$

Multiples $t = 3.29$ $p < 0.007$

Total $t = 1.48$ $p > 0.050$