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RAPID FORMATION OF MULTIPLE SYNAPSES IN THE SUPRAOPTIC NUCLEUS OF ADULT RAT HYPOTHALAMIC SLICES

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BARBARA K. MODNEY

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of the requirements for

PhD degree in Psychology/Neuroscience

Glenn J. Hatton
Major professor

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RAPID FORMATION OF MULTIPLE SYNAPSES IN THE SUPRAOPTIC NUCLEUS OF ADULT RAT HYPOTHALAMIC SLICES

By

Barbara K. Modney

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Psychology and Neuroscience Program

1990

ABSTRACT

RAPID FORMATION OF MULTIPLE SYNAPSES IN THE SUPRAOPTIC NUCLEUS OF ADULT RAT HYPOTHALAMIC SLICES

By

Barbara K. Modney

The adult rat supraoptic nucleus (SON) is a known model system for investigations of neuronal plasticity. Previous research has demonstrated that new multiple somatic synapses form in SON during chronic dehydration in vivo. In the present experiments hypothalamic slices containing the SON were maintained in vitro in either high or low osmolality media after which multiple synapses were measured at the electron microscopic level.

In the first experiment slices were maintained in a static bath chamber with constant infusion of dilute medium to compensate for evaporation. The average firing rate of neurons from slices incubated in high osmolality medium was higher than low osmolality slices and a significant increase in multiple synapses was found in the high osmolality slices.

In Experiment II, slices were incubated in low or high osmolality medium in a perifusion chamber which allows for continuous perifusion of fresh medium over the slice. In this experiment there was no detectable osmotic effect on multiple synapses. It could be that multiple synapses were forming in both groups since a significant increase was found in low osmolality slices from this chamber relative to slices from Experiment I.

In Experiment III, the static bath chamber was used again and a time course experiment was designed to determine if the sequence of anatomical events that occurs during multiple synapse formation could be discerned. Slices incubated for 5.5, 2.5 or 1.0 hours in low or high osmolality media. In contrast to the results from Experiment I there was no detectable osmotic effect on any morphometric measure. There was a significant increase in multiple synapses in the 2.5 and 5.5 hour groups relative to the 1.0 hour group.

These results suggest that multiple synapses may form in the SON in vitro (as they do in vivo) in response to deafferentation. This formation is rapid since significant increases were found within 2.5 hours. The SON provides a unique neural system with which to compare and contrast natural and reactive synaptogenesis. The hypothalamic slice preparation may provide a useful tool to investigate a rapid response to deafferentation.

This dissertation is dedicated to my mother

whose strength and capacity for love

was truly remarkable

and to Dad and Gary for their unlimited

unconditional love and understanding.

ACKNOWLEDGEMENTS

I have many people to thank for various things they have done for me since I came to Michigan State. Were I to honestly and faithfully acknowledge you all I would have to write a book.

To Glenn Hatton, who not only provided generous financial support throughout my training, but more importantly provided a role model for what a multidisciplinary neuroscientist should be - Thank You is a gross understatement.

To my dissertation committee members - Drs. Cobbett, Nunez and Tweedle, your willingness to meet with me for many data discussions during these experiments was truly appreciated - your advise and encouragement will never be forgotten.

To A. Salm, K. Smithson, C. Wagner and R. Cole - the distinctions between friends, colleagues and peers simply don't exist, you've been all these and much more. I will consider myself to be truly blessed if I continue to meet people like you who enrich so many aspects of my life.

To the many able technicians who have run the lab and have helped me enormously: K. Grant, L. Koran, P. Rusch, I. Smithson & K. Smithson - Thank you for everything, especially the things that I never realized you did and probably took for granted.

The following people at PRB have been helpful in many ways: G. Beagley, L. Bergland, J. Harper, F. Marzban, C. Sisk, M. Weiss, H. Wieferich, and Q. Yang.

Last but not least, to Ginny, Erik, D'Lynn, Margie, Steve, Pat, Dick, Phyllis, Emy, Jeremy, Jordon, John, Debbie, Katy, Gregor, Betsy, Al, Jen and Annie and Bob, Maribeth, Megan, Mom & Dad-in law and of course Dad and Gary - thanks for constant support, you are the best part of my life.

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ABBREVIATIONS

ANOVA - Analysis of Variance

cv - Coated Vesicle

HNS - Hypothalamo-neurohypophysial system

Hz - Hertz

MNC - Magnocellular neuroendocrine cell

mOsm - Milliosmoles

NL - Neural lobe

Osm - Osmolality

OX - Oxytocin

pr - Polyribosomes

psd - Postsynaptic density

SE - Standard Error

SON - Supraoptic nucleus

VP - Vasopressin

INTRODUCTION

While fulfilling its traditional role as a model system for neurosecretion, the hypothalamo-neurohypophysial system (HNS) has emerged as a unique system with which to investigate adult neuronal plasticity. Associated with changes in the internal as well as external environment of the animal are alterations in many of the morphological characteristics of the HNS. These morphological changes, including the formation of new synapses and changes in neuronal-glial interrelationships, are often clearly related to increased demand for the HNS's secretory products. Few mammalian systems have such a clear and malleable structure-function relationship. By studying this system insight is gained into the ways in which the adult nervous system can respond to required changes in neuronal functioning.

The Hypothalamo-Neurohypophysial System.

Consideration of the HNS as a model for the study of neural plasticity has evolved from its more prominent status as a model for the study of neurosecretion (see Bissett & Chowdrey, 1988; Forsling, 1986; Hatton, 1990 for reviews). One of the most convenient characteristics of this system is the anatomical configuration of the magnocellular neuroendocrine cells (MNCs) and their massive efferent projection to the neural lobe (NL) of the pituitary gland. MNCs are scattered in several nuclei in the anterior hypothalamus (Peterson, 1966). The emphasis here is on the supraoptic nucleus (SON) of the rat, since most of the experimental work discussed

below involves the SON (see Hatton, 1990; Theodosis & Poulain, 1987 for reviews; and see Gregory, Tweedle & Hatton, 1980; Theodosis & Poulain, 1989; and Tweedle & Hatton, 1976; 1977 for relevant work in other magnocellular neuroendocrine cell groups).

The bilateral supraoptic nuclei are located at the ventral surface of the brain just lateral to the optic chiasm and tracts. The nucleus proper can conveniently be divided into a dorsal somatic region and a ventral dendritic region. These regions are not mutually exclusive (i.e. there are dendrites in the somatic region), but rather describe the relative predominance of somata vs. dendrites in the two regions. Neurons are densely packed within the nucleus and are almost exclusively neurosecretory, although the rare non-neurosecretory neuron has been described (Itoh, Iijima & Kowada, 1986). At the ultrastructural level, the dense packing of MNCs is readily apparent and adjacent somata are typically separated by thin astrocytic processes. MNCs are characterized by an abundance of cellular organelles involved in peptide processing (e.g. Golgi apparatus, rough endoplasmic reticulum, ribosomes etc.) as well as the presence of dense core vesicles. MNCs have simple dendritic trees consisting of 1-3 dendrites which project ventrally initially and then turn and course in a rostral-caudal orientation parallel to the ventral surface of the brain (e.g. Armstrong, Schöler, & McNeill, 1982; Dyball & Kemplay, 1982). Many dendrites in this region contain dense core vesicles similar to those seen in SON somata and terminals in the neural lobe (Armstrong et al., 1982; Yulis, Peruzzo & Rodríguez, 1984). Adjacent dendrites are usually separated by thin astrocytic processes in a manner similar to that described between somata in the somatic region (Perlmutter, Tweedle & Hatton, 1984b; Yulis et al., 1984). The most ventral

portion of the SON, adjacent to the pia-arachnoid is the ventral glial limitans which consists of an extensive blanket of interwoven and interconnected astrocytic processes (Armstrong et al., 1982; Yulis et al., 1984). Within this region lie most of the astrocytic cell bodies that send processes dorsally through the nucleus and separate the adjacent dendrites and somata (Salm & Hatton, 1980). The dense packing of MNCs in the SON, the relative segregation of somata and dendrites as well as the homogeneity of cell type within the nucleus (i.e. few interneurons) make a morphometric analysis of these neurons under various experimental conditions relatively straightforward.

MNCs contain several neuropeptides (see Bondy, Whitnal, Brady & Gainer, 1989 for review), the best known and characterized being oxytocin (OX) and vasopressin (VP). These two hormones are manufactured by separate cell populations in the SON that are roughly segregated. That is, oxytocinergic neurons are generally located in the more antero-dorsal regions of the nucleus, while vasopressinergic neurons are located in the more posterior-ventral regions of the nucleus. There is considerable intermingling of the two cell populations so that anatomical identification of the hormone content of a particular cell requires immunocytochemical staining (e.g. Hou-Yu, Lamme, Zimmerman & Silverman, 1986). OX vs. VP containing cells can often be identified on the basis of their electrophysiological characteristics (see Poulain & Wakerley, 1982). Vasopressinergic neurons often display a phasic firing pattern consisting of alternating periods of action potential bursts and silence. In lactating animals with suckling pups a synchronous high frequency burst of action potentials of most oxytocinergic neurons reliably precedes milk ejection.

A major axonal projection from SON neurons is to the NL, although collaterals that terminate in the area lateral to SON (Mason, Ho & Hatton, 1984) and oxytocinergic and vasopressinergic synapses within the nucleus proper have also been described (Choudhury & Ray, 1990; Ray & Choudhury, 1990; Theodosis, 1985). In the NL neurosecretory axons and terminals are intimately associated with pituicytes (astrocytic cells of the NL, Salm, Hatton & Nilaver, 1982; Suess & Pliška, 1981). Pituicytes often surround and/or completely engulf axonal terminals (Tweedle & Hatton, 1980b). Thin pituicyte processes and axonal terminals contact the basal lamina which surrounds the capillaries. Action potentials generated by MNCs invade the terminals and cause calcium dependent hormone release. Hormone that gains access to the fenestrated capillaries enters the pituitary-portal system for distribution to peripheral tissues. Peripheral effects of both hormones have been known for many years (See Amico & Robinson, 1985 and Gash & Boer, 1987 for comprehensive reviews). Oxytocin promotes milk ejection during lactation and uterine contraction during parturition, while VP or anti-diuretic hormone, is best known for its ability to act on the collecting tubules of the kidney to promote water reabsorption.

Even though the conventional characterization of the HNS focuses on hormone release from the NL and hormone action at peripheral tissues, evidence for central projections of MNCs is strong (Buijs, Swaab, Dogterom & van Leeuwen, 1978). Release of these hormones from the axon collaterals that terminate lateral to SON and within the nucleus proper has been demonstrated (Mason, Hatton, Ho, Chapman & Robinson, 1986). Recent evidence suggests that hormone detected within the nucleus may be the result of dendritic release (Pow & Morris, 1989; Tweedle,

Smithson & Hatton, 1988). The functional significance of dendritic hormone release is unclear. Several studies suggest that OX excites MNCs (Freund-Mercier & Richard, 1984; Inenaga & Yamashita, 1986; Yamashita, Okuya, Inenaga, Kasai, Uesugi, Kannan & Kaneko, 1987) and VP may have a similar effect (Inenaga & Yamashita, 1986). It may be that dendritic release of hormone serves to increase MNC activity. A second possibility is that dendritic release of OX participates in the anatomical reorganization of the SON since chronic infusion of OX intracerebroventricularly results in characteristic changes in SON morphology (Montagnese, Poulain & Theodosis, 1990; Theodosis, Montagnese, Rodriguez, Vincent & Poulain, 1986b).

The Reorganized HNS

The most common experimental manipulations used to study anatomical changes in the HNS are dehydration, parturition and lactation. Dehydration, produced by water deprivation or substitution of 2% saline for drinking water, alters virtually every measurable characteristic of both oxytocinergic and vasopressinergic neurons (see Amico & Robinson, 1985; Gash & Boer, 1987 for reviews). Messenger ribonucleic acid (mRNA) levels for OX and VP, as well as several other peptides are elevated by drinking 2% saline. Protein synthesis, post-translational processing of the prohormones, axonal transport of hormones to the NL are all increased during dehydration. Concurrent with this increased synthesis and transport of OX and VP are significant alterations in the electrical activity of MNCs. Oxytocinergic neurons increase their firing rate, while vasopressinergic neurons either evolve a phasic firing pattern or alter existing burst characteristics (e.g. burst duration, firing rate within burst). This generalized increase in the activity of

MNCs during osmotic challenge is probably due, at least in part, to the intrinsic osmosensitivity of MNCs. Ultimately the chronic increased activity of MNCs leads to increased hormone release from the NL and progressive depletion of neurohypophysial hormone. Given that osmotic stimulation is such of robust and powerful stimulus for this system, it is not surprising that the anatomical characteristics of these neurons are also dramatically altered.

Parturition and lactation are most often associated with the activation of oxytocinergic neurons (see Forsling, 1988), although the VP content of the NL is depleted during parturition (Fuchs & Saito, 1977). Also, mapping of metabolic activity of MNCs using the 2-deoxyglucose method has suggested that vasopressinergic neurons are also activated following a bout of suckling (Allen, Stern & Adler, 1984). During parturition oxytocinergic neurons exhibit a generalized increase in firing rate and bursts of action potentials precede expulsion of each fetus. Progressive decreases in neurohypophysial OX and increases in plasma OX occur as parturition progresses. During lactation afferent information produced by suckling pups is relayed through an elusive neural pathway to MNCs in the hypothalamus. Milk ejection follows the synchronized high frequency firing of most of the oxytocinergic neurons in the hypothalamus. This high frequency firing is required for sufficient OX release from the NL to cause milk ejection. As with osmotic stimuli, the activation of oxytocinergic neurons during parturition and lactation is associated with morphological changes in the HNS.

Changes in the activated HNS observed at both the light and electron microscopic level are as ubiquitous as the other indicators of activation. Light microscopic studies have concentrated on changes in cell size, proliferation of Nissl

substance and nucleolar size (Armstrong, Gregory & Hatton, 1977; Bandaranayake, 1974; Ellman & Gan, 1971; Eneström, 1967; Hatton & Walters, 1973; Kalimo, 1965; Morris & Dyball, 1974). Most early electron microscopic studies of the stimulated system concentrated on proliferation and/or depletion of intracellular structures (e.g. rough endoplasmic reticulum, Golgi apparatus, dense core vesicles etc.). More recent studies have focussed on changes in the synaptic and neuronal-glial relationships in the SON and NL following activation.

Two particular anatomical changes which occur in vivo are detected at the electron microscopic level and serve as a starting point for the experiments discussed below. First, the synaptic characteristics of SON of experimentally treated animals are different from control animals. The most obvious and best documented synaptic change in vivo is the appearance of novel multiple synapses. A multiple synapse is defined as a single axonal terminal which simultaneously contacts at least two adjacent post-synaptic structures, i.e. two adjacent somata, two dendrites, or adjacent soma and dendrite. While this type of synapse is relatively rare in normal animals, their frequency is increased in the dendritic region during parturition and lactation (Perlmutter et al., 1984b) and in the somatic region during lactation and dehydration (Chapman, Theodosis, Montagnese, Poulain & Morris, 1986; Hatton & Tweedle, 1982; Montagnese, Poulain, Vincent & Theodosis, 1987; Modney & Hatton, 1989; Theodosis, Chapman, Montagnese, Poulain & Morris, 1986a; Theodosis & Poulain, 1984; Theodosis, Poulain & Vincent, 1981; Tweedle & Hatton, 1984a). That the multiple synapses formed under these conditions are truly related to the physiological demand for hormone release is supported by the fact that they disappear when hormone demand returns to basal levels (e.g. pup weaning

and rehydration). Although this type of synapse was first described using the conventional stimuli of dehydration, parturition and lactation they also form in SON under a number of different experimental conditions including: 1) intracerebroventricular administration of OX for 8 days (Theodosis, et al., 1986b; Montagnese, et al., 1990), 2) transcardial perfusion with hypertonic solution immediately prior to transcardial fixation (Tweedle, Smithson & Hatton, 1989), and 3) following removal of an afferent projection from the subfornical organ to the SON (Weiss, Tweedle, Marzban, Modney & Hatton, 1988).

While the formation of multiple synapses is well-established, very little is known about their precise function. Indeed that they are functional synapses is based only on morphological data. That they often form along with alterations in the electrical activity of these neurons suggests that they participate in the control of the SON during periods of high hormone demand. There is immunocytochemical evidence that some somatic multiple synapses contain GABA (Theodosis, Paut & Tappaz, 1986, see also van den Pol, 1985 Figure 5 and Buijs, van Vulper & Geffard, 1987) and evidence suggests that some may contain dopamine (Buijs, Geffard, Pool & Hoorneman, 1984). There is also evidence that some dendritic multiple synapses are noradrenergic (Tweedle & Hatton, 1984b). Even though the morphological characteristics of the terminals that form somatic multiple synapses are similar following chronic dehydration and lactation it is not known if the same afferent system forms these synapses under the two conditions.

That synaptogenesis occurs in the SON of adult animals is well established. The mechanisms(s) by which these new synapses form has not been determined. There is a suggestion that multiple synapses are formed by the conversion of pre-

existing single synapses (Hatton & Tweedle, 1982; Perlmutter et al., 1984b, 1985; Tweedle & Hatton, 1984a). According to this hypothesis a single terminal may initially contact one SON soma but be separated from another adjacent soma by a thin astrocytic process. Retraction of this process from between the second soma and the terminal would then allow formation of a multiple synapse. This mechanism provides an attractive explanation for the very rapid (20 minutes) formation of multiple synapses that occurs during intravascular perfusion of hypertonic solutions (Tweedle et al., 1989).

The extent to which multiple synapse formation in the SON conforms to the events that occur during synaptogenesis in developing animals or during reactive synaptogenesis has not yet been investigated. For example, in developing animals synaptogenesis is associated with aggregates of polyribosomes in the postsynaptic element (typically dendritic spines) near the new synapse (Palacios-Prü, Palacios & Mendoza, 1981; Steward & Falk, 1986). Similar aggregates are also seen in dendritic spines of dentate granule cells during the reinnervation that follows removal of the ipsilateral entorhinal cortex (Steward, 1983). A second organelle that has been implicated in synapse formation in developing systems (e.g. Altman, 1971) and during reactive synapse formation (McWilliams & Lynch, 1981) is the coated vesicle. Both of these organelles have been hypothesized to participate in the formation and/or maturation of postsynaptic specializations. Coated vesicles have been shown to contain acetylcholine receptors and could be involved in depositing receptors at the postsynaptic site (Bursztajn, & Fischbach, 1984). Coated vesicles in both pre- and postsynaptic elements may also play a role in cell-cell

recognition during the early stages of synapse formation in developing neural systems (see Vaughn, 1989 for review).

Another anatomical change in the SON described at the electron microscopic level occurs both with dehydration and with parturition and lactation. As mentioned earlier, in the normal animal, thin astrocytic processes typically separate adjacent somata and dendrites. When the HNS is activated adjacent somata/dendrites are not always separated by glial processes, instead extensive portions of somatic/dendritic membrane are seen in direct apposition (Chapman et. al, 1986; Hatton & Tweedle, 1982; Montagnese et. al 1987; Theodosis et. al, 1981; 1986a; Theodosis & Poulain, 1984; Tweedle & Hatton, 1976; 1977). In the dendritic region of SON the increased direct membrane apposition between adjacent dendrites results in the appearance of dendritic bundles (Perlmutter et al., 1984b; Perlmutter, Tweedle & Hatton, 1985), i.e. two or more dendrites having portions of their membrane in direct apposition.

As with multiple synapse formation, glial process retraction has been suggested as the mechanism through which direct neuronal apposition can occur. One study has shown a marked redistribution of immunoreactive staining for glial fibrillary acidic protein in the SON, but not control areas of the hypothalamus, during lactation (Salm, Smithson & Hatton, 1985). A similar study has yet to be done in dehydrated rats. Many functional implications for the absence of glial processes from between adjacent somata and dendrites have been suggested (see Hatton, 1990). Given the ability of astrocytes to buffer extracellular K^+ and to actively uptake various neurotransmitters, several neuronal consequences can be envisioned. For example the accumulation of extracellular K^+ could slightly

depolarize the neurons. Similarly the accumulation of neurotransmitters in the extracellular space could theoretically extend the duration and/or magnitude of their effect.

Accompanying the reorganization of the SON are anatomical changes that occur at the level of the terminals of these neurons. In the NL the frequency of axonal terminals completely surrounded by pituicyte cytoplasm declines in post-partum, lactating and dehydrated animals (Tweedle & Hatton, 1980a; 1982). In addition, the percentage of basal lamina contacted by pituicyte processes decreases (relatively more contact of the basal lamina by axonal terminals) when the HNS is activated in vivo (Tweedle & Hatton, 1987). Similar changes have also been induced in an in vitro NL preparation (Luckman & Bicknell, in press; Perlmutter, Hatton & Tweedle, 1984a; Smithson, Suarez & Hatton, in press). The release of pituicyte engulfed terminals and retraction of processes from the basal lamina allows greater terminal contact at the basal lamina. It is thought that these alterations ultimately permit more hormone direct access to the circulation. Again the active role of the glial elements is thought to participate in the reorganization of the NL during periods of high hormone demand.

STATEMENT OF THE PROBLEM

The experimental work discussed above has used exclusively in vivo models. While these experiments have provided and will continue to provide vital information about SON's plasticity, they do not have the advantages that an in vitro system could provide. Hypothalamic slices and explant preparations maintained in vitro have been invaluable in elucidating many neurophysiological characteristics of MNCs (see Armstrong, Gallagher & Sladek, 1985). These preparations allow investigators to asses the actions of neurotransmitters, the intrinsic membrane properties of MNCs and hormone release in response to experimental manipulations.

In vitro preparations have also been useful in studying the plastic capabilities of the SON and NL. Hypothalamic slices have been used to investigate the extent and modifiability of dye-coupling (an indirect measure of electrotonic coupling) among SON neurons (see Hatton, 1990 for review). For example, the incidence of coupling among SON neurons is considerably higher in slices prepared from lactating animals relative to slices prepared from virgin female animals (Yang & Hatton, 1987). An in vitro neural lobe preparation has been used successfully to investigate dynamic neural-glial changes in response to manipulations of the incubation media (Luckman & Bicknell, 1990; Perlmutter et al., 1984a; Smithson et al., in press).

The success of these in vitro studies provided the impetus to determine if an in vitro hypothalamic slice preparation could be used to investigate anatomical restructuring in the SON. The potential advantages of such an in vitro system include: a) the ability to record the electrical activity of neurons and then examine the tissue anatomically, b) greater knowledge of and control over the external

environment of the tissue relative to in vivo manipulations, c) greater precision in examining the time course of anatomical changes that lead to a reorganized SON. A horizontal hypothalamic slice preparation that has been used extensively for electrophysiological and dye coupling studies (Hatton & Yang, 1989; 1990; Weiss, Yang & Hatton, 1989; Yang & Hatton 1987; 1989) was used to answer the questions and issues concerning plasticity in the SON listed below.

Does an in vitro SON preparation respond to manipulations (e.g. increases in osmolality) of incubation media with anatomical changes that resemble those seen in vivo? Assuming that changes do occur in vitro what is the time course and sequence of events that lead to such changes? If synapse formation does occur in vitro will cytoplasmic organelles associated with this process (e.g. ribosomes) be associated with the increase in synapses?

Figure 1 - Schematic diagram of the methodology used for the present experiments. Briefly horizontally cut hypothalamic slices were incubated in low or high osmolality medium and extracellular single unit activity was monitored. Following fixation the SON was prepared for electron microscopy and morphometric measures were obtained from photomicrographs.

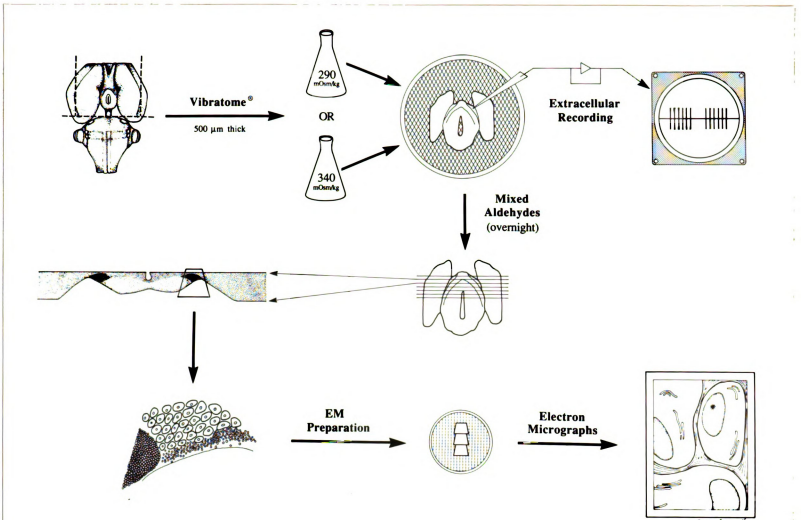
Vibratome®
500 µm thick
290
mOsm/kg
OR
340
mOsm/kg
Extracellular
Recording
Mixed
Aldehydes
(overnight)
EM
Preparation
Electron
Micrographs

Figure 1

GENERAL METHODS

Slice Preparation

A schematic illustration of the methods used in these experiments is shown in Figure 1. Adult male rats (40-60 days old) maintained on ad lib food and water were used. Rats were killed by rapid decapitation without anesthesia within the first five hours after lights on. Following decapitation the brain of each rat was quickly removed and placed in artificial cerebral spinal fluid of low osmolality (290 mOsm/kg) or high osmolality (340 mOsm/kg) containing (mM, low/high osmolality) $NaHCO_3$ (23.0/27.0), KCl (4.6/5.4), NaH_2PO_4 (1.2/1.4), $MgSO_4$ (1.2/1.4) D-glucose (9.4/11.0, NaCl 117.0/138.0, 3-[N-morpholine]propanesulfonic acid (4.6/5.4), $CaCl_2$ (2.2/2.6). For all experiments media were supplied coded so that data were collected without awareness of treatment group membership. The fresh brain was blocked by making two cuts with a razor blade just lateral to the lateral olfactory tract to remove the extreme portions of the cortex, and a single cut just rostral to the pons. The remaining tissue block was mounted dorsal surface down on a Vibratome chuck. A horizontal slice(s) (500-600 μm thick) which included the SON was cut on a Vibratome filled with oxygenated medium maintained at room temperature. These slices were then placed ventral surface down in either of two recording chambers (see specific experiments). Temperature of the bathing medium for both chambers was maintained between 34-36 degrees C.

Neuronal activity was monitored using conventional extracellular recording techniques. Extracellular electrodes were pulled with a Kopf Vertical Pippette Puller (Model 700C) and the tip was manually broken back to 2-3μm. Electrodes were filled with 1M sodium acetate. Spontaneously active cells were recorded on either a

Vetter Model B tape recorder or an Akai Stereo Cassette deck (Model CS-34D). Average firing rates were determined for cells with activity that could be clearly distinguished from noise and that were recorded for at least 2 1/2 minutes. Average firing rates were obtained by playing back the tape and triggering an oscilloscope sweep on the peak of each action potential. The number of sweeps per 10 second interval was recorded by an electronic counter (Hewlett Packard Counter, Model 5512A). The total number of action potentials was divided by total time (seconds) to obtain the average firing rate.

Tissue Processing

Following incubation (see specific experiments for total incubation time) slices were immersed overnight in fixative containing 2.5% glutaraldehyde, 1% paraformaldehyde, 0.5% dimethylsulfoxide in 0.10 M cacodylate buffer, pH 7.4. The following day, coronal sections (300-400 μm) were cut from the horizontal slices. The SON was excised from three adjacent tissue sections and following three buffer (0.15 M cacodylate) rinses the tissue was osmicated for one hour in a 1:1 mixture of 2% osmium and 3% potassium ferricyanide in 0.2M cacodylate buffer, pH 7.4. The tissue was en bloc stained in 4% uranyl acetate overnight, dehydrated in a series of graded alcohols, and embedded in Spurr's resin (see Appendix A for embedding protocol). Semi-thin sections (0.5-1 μm), cut on a Reichert Ultracut E microtome, were heat mounted on glass slides. Sections with adequate tissue preservation were further analyzed at the electron microscopic level. Note that these slices did not necessarily correspond to slices used in the electrophysiological analysis above. Thin sections (60-90 nm) were cut and

collected on 200 mesh thin-bar copper grids, and were stained with lead citrate. A single thin section from each slice was used for data collection.

Morphometrics

Semi-thin sections from slices used in electron microscopic data collection were analyzed using an Olympus C-2 Image Analysis system. Cells were viewed through the microscope and on a video screen. The perimeter of each cell was manually delimited with an optical cursor and the follow parameters were generated by the computer for each cell: 1) Cell area (μm^2), 2) Cell perimeter (μm) and 3) Cell Shape (4πarea)/perimeter). In each slice at least 18 cells which contained a nucleolus were measured.

Length measurements were made from electron micrographs with either a Houston Instruments digitizing table interfaced with a Zenith-200 computer and in-house software (Experiment I - Somatic Region) or a Summagraphics Digitizing table and SigmaScan software. Twenty electron micrographs of the somatic region (12,000X final magnification) per slice were taken. On each micrograph the lengths of the following cellular elements contacting neuronal somatic membrane were measured a) astrocytic processes, b) axonal terminals that contacted only one SON soma (i.e. single synapses), c) axonal terminals that contacted both a soma and an adjacent soma or dendrite (i.e. multiple synapses), d) adjacent soma-somatic/dendritic membrane, e) unidentified elements. (It should be noted that a synapse was inferred each time an axonal terminal apposed a postsynaptic element, regardless of whether or not clearly defined synaptic specialization were present.) The total length of somatic membrane was obtained by summing the length of all the elements listed above. Data from individual micrographs were summed for each animal and the

percentage of somatic membrane contacted by each cellular element was calculated. Additionally, the percentage of synapses that were multiple synapses and the percentage of total axonal contact made by multiple synapses was obtained (total length of axonal contact made by multiple synapses/total length of all axonal contacts * 100). The number of multiple synapses per 100 μm of somatic membrane and the average length (μm) of single and multiple synapses was determined.

Statistical Analysis

Data are presented as the Mean ± Standard Error unless otherwise noted. The hypothesis that neurons from slices incubated in high osmolality medium had a higher firing frequency (Hertz, Hz.) than cells from low osmolality slices was tested using a one-tailed Mann-Whitney U test. A one-tailed test was deemed appropriate since numerous in vivo AND in vitro studies have demonstrated that these neurons respond to increased osmolality

Morphometric data from Experiments I and II were analyzed using a two-tailed t-test for differences between means. Two-tailed tests were used since no previous morphometric measures of hypothalamic slices were available and increases or decreases in many of the measures were conceivable. For example, many of the measurements made are dependent to some extent on cell size which could have increased or decreased in the high osmolality group. Similarly the synapses measures could have decreased based on previous studies of axotomized neurons. Morphometric data from Experiment III which had 6 groups were analyzed using a Two-Factor Analysis of Variance (ANOVA) with medium osmolality (low and high) and incubation time (5.5 hours, 2.5 hours, 1 hour) as factors. All comparisons

between treatment group means were made following a significant F ratio using Tukey's test modified for unequal sample size (Winer, 1962). Correlation coefficients (Pearson's r) between several variables were also calculated. In all cases probability levels of $p < 0.05$ were considered significant.

EXPERIMENT I - OSMOTIC STIMULATION OF HYPOTHALAMIC SLICES

Somatic region

As discussed earlier osmotic challenge is a potent stimulus for activation of the HNS and is associated with numerous changes in the somatic region of SON. Several electrophysiological experiments have demonstrated that MNCs in vitro are capable of responding to osmotic challenges (Bourque, 1989; Bourque & Renaud, 1984; Hatton, Armstrong & Gregory, 1978; Mason, 1980), and anatomical changes similar to those seen in vivo after dehydration occur in the in vitro NL incubated in high osmolality medium (Perlmutter et al., 1984a). A primary goal of this experiment was to determine if slices incubated in high osmolality medium exhibited anatomical changes similar to those seen during osmotic challenge in vivo.

A static bath chamber in which the slice is placed on a nylon net in a medium filled well with constant infusion of dilute media to compensate for evaporation (see Hatton, Doran, Salm & Tweedle, 1980) was used for this experiment. Slices were incubated for between 4-6 hours prior to fixation and tissue processing.

Results - A total of 32 animals were used for this experiment. All slices included for data analysis were alive as determined by the presence of single unit activity. This activity was either spontaneous or elicited by electrode advancement. Slices in which no single unit activity was detected (n=2) were discarded from the study. A total of 21 spontaneously active cells from 14 slices had action potentials that were clearly distinguishable from noise (see Figure 2). All of these cells fired in what could be considered a continuous manner and included 12 cells from slices that incubated in low osmolality media and 9 cells from high osmolality slices. As

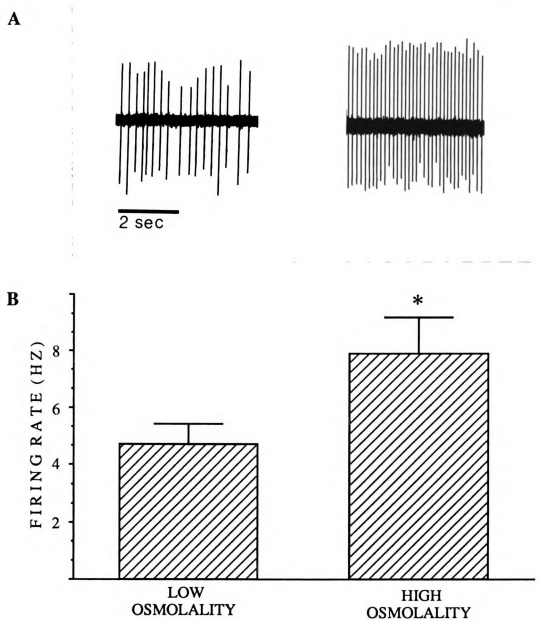

Figure 2 - Single unit activity of neurons from slices incubated in low or high osmolality medium in Experiment I. **A** - Sample traces from neurons incubated in low osmolality (first trace) or high osmolality medium. **B** - A significant increase in average firing rate was obtained in slices incubated in high osmolality medium. * $p < 0.05$

shown in Figure 2 a significant elevation in average firing rate of neurons from slices incubated in the high osmolality medium compared to low osmolality was obtained ($p<0.04$).

Examination of the semi-thin sections at the light microscopic level was used to determine if tissue quality was adequate for electron microscopic evaluation. As shown in Figure 3, the SON is free from most tissue damage due to Vibratome cuts or placement on the chamber net. Slices in which the SON was obviously damaged by the cutting procedure were excluded, as were slices that had extensive vacuolation within the nucleus. On this basis eighteen slices were deemed adequate for electron microscopic analysis. One slice was eliminated based on inadequate membrane preservation which was not detected until electron microscopic evaluation. Nine slices from the 290 group and eight slices from the 340 group were used for morphometric analysis.

At the ultrastructural level the tissue from slices was quite well preserved. A comparison between the characteristic morphology of MNC somata from a conventionally perfused animal and a hypothalamic slice is illustrated in Figure 4. Accurate identification of the cellular elements apposed to SON somatic membrane was readily accomplished in the slice tissue. Multiple synapses (see Figure 5) were more frequent in the high osmolality slices relative to the low osmolality slices. As shown in Table 1, significant increases in the percentage of somatic membrane contacted by multiple synapses ($p < 0.02$), the number of multiple synapses per 100 μm of somatic membrane ($p < 0.05$), the percentage of synapses that were multiple synapses ($p < 0.05$), and the percentage of total axonal contact made by multiple synapses ($p < 0.02$).

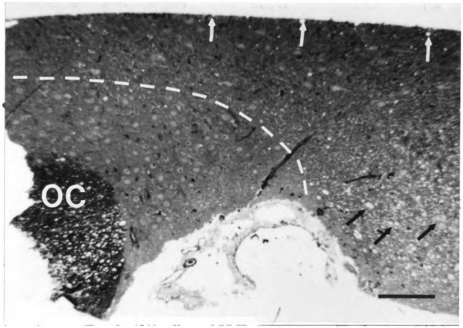

Figure 3 - Photomicrograph of a semi-thin (1 μm) section from a hypothalamic slice. The SON is roughly delimited by the dotted white lines. White arrows point to the dorsal cut surface of the slice where some tissue damage is present. Black arrows point to extensive vacuolization in the area lateral to SON. The nucleus is largely devoid of such damage since is never placed directly on the chamber net. OC - Optic chiasm. Bar = 100 μm

Figure 4 - Electron micrographs showing SON morphology from conventionally prepared tissue (**A**; transcardial perfusion) and a hypothalamic slice (**B**). Portions of SON somata (S) as well as several dendrites (D) are indicated in both panels. As is common in control animals astrocytic processes (arrowheads) separate adjacent postsynaptic elements. Single synapses are indicated by arrows. Comparisons between these two micrographs show that the morphology of slice tissue was often as good as conventionally prepared tissue. Panel **A** is from a normal male rat transcardially perfused with the same fixative used in the present experiments with the exception that dimethylsulfoxide was not included. This tissue was embedded in Epon-Araldite resin and sections were mounted on Butvar coated slot grids. Tissue from panel **B** was prepared according to the protocol listed in the Methods section. Bar = 2 μm

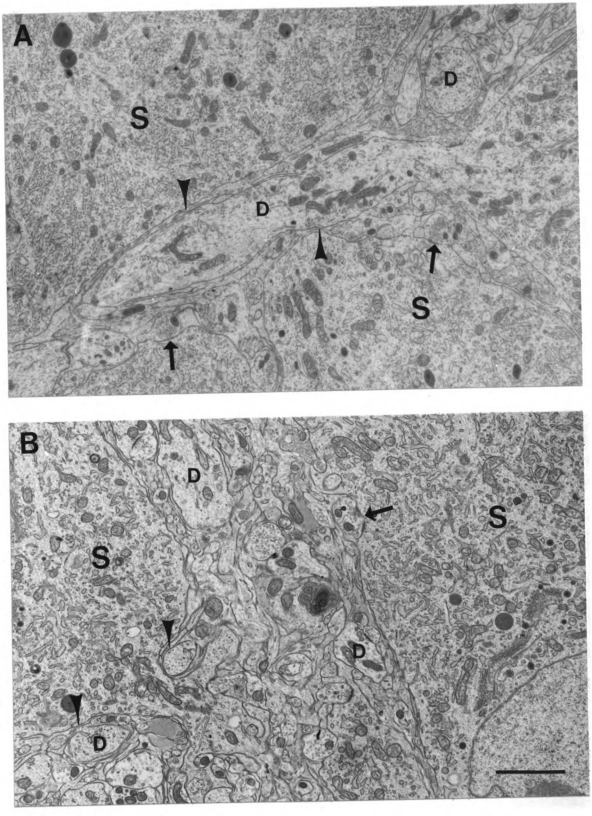

Figure 4

TABLE I - MULTIPLE SYNAPSE MEASURES FROM SLICES INCUBATED IN LOW OR HIGH OSMOLALITY MEDIUM

Group Osmolality	% Somatic Membrane	#/100 µm of Somatic Membrane	% Synapses	% Axonal Contact
Low (n=9)	0.8±0.12	0.78±0.16	8.0±1.1	7.4±0.8
High (n=8)	1.36±0.14*	1.25±0.14#	11.6±1.0#	11.0±1.1*

* $p < 0.02$

# $p < 0.05$

There were no significant differences in any of the following ultrastructural measures (low vs high osmolality): 1) the percentage of somatic membrane contacted by glial processes (82 ± 1.7 vs 77 ± 2.2), 2) the percentage of somatic membrane in direct apposition with adjacent somata and or dendrites (see Figure 6, 2.5 ± 0.7 vs 3.2 ± 1.0), 3) the percentage of somatic membrane contacted by axonal terminals (10.9 ± 1.1 vs 12.4 ± 1.0), 4) the percentage of somatic membrane contacted by unidentified elements (4.5 ± 0.8 vs 6.5 ± 1.1), 5) the mean length (µm) of axonal terminals (1.16 ± 0.04 vs 1.15 ± 0.05). No significant differences between the groups in cell area were detected (μm^2, low vs high osmolality, 431 ± 13 vs 430 ± 23), cell perimeter (µm, 80 ± 1.1 vs 78 ± 1.9) or cell shape (0.84 ± 0.01 vs 0.87 ± 0.01).

Figure 5 - Multiple somatic synapses from hypothalamic slices incubated for at least 4 hours. In **A** a single synapse is formed by an axonal terminal (*) on one of the SON somata (S). A second terminal (**) contacts both SON somata. A small portion of the two somatic membranes is in direct apposition near the multiple synapse (arrowhead). Astrocytic processes (arrows) are interposed between the adjacent soma and the single terminal (*). Bar = 1 μm. In **B** an axonal terminal (**) forms a synapse with both an SON soma (S) and an adjacent dendrite (D). A thin astrocytic process separates a large portion of the terminal membrane (arrows) from the cell body. Direct apposition between the cell body and the dendrite is marked by arrowheads. Bar = 0.5 μm.

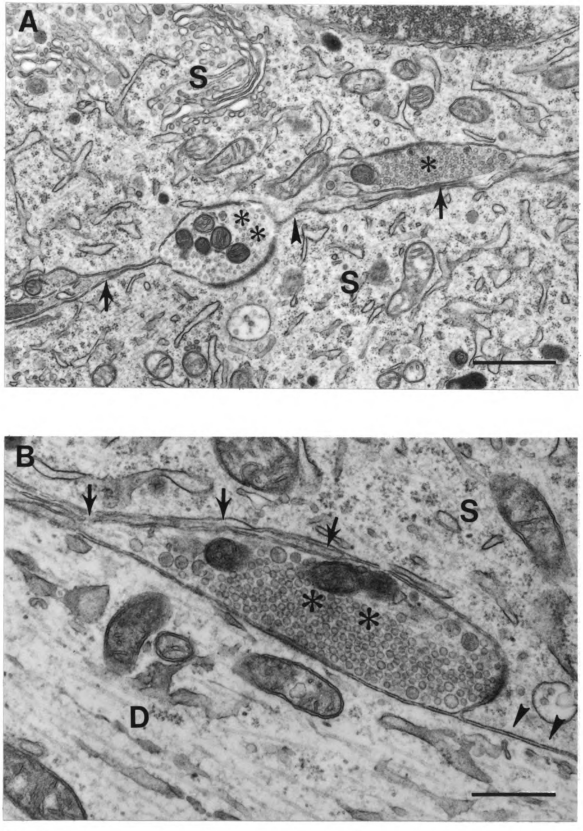

Figure 5

Figure 6 - Photomicrographs showing direct soma-somatic and soma-dendritic membrane appositions. In **A** a large portion of somatic membrane of two adjacent somata (S) is in direct apposition (delimited by arrows). A thin astrocytic process (arrowheads) separates a portion of the two cell membranes. Bar = 1 μm. In **B** a portion of the extensive apposition shown in panel **A** is shown at a higher magnification. The apposition is indicated by arrows while the glial process is indicated by the arrowhead. Bar = 0.2 μm. In **C** the direct apposition between a dendrite (D) and an MNC soma (S) is delimited by arrows. These elements are also contacted by an axonal terminal (*) that forms a multiple synapse. Bar = 0.5 μm.

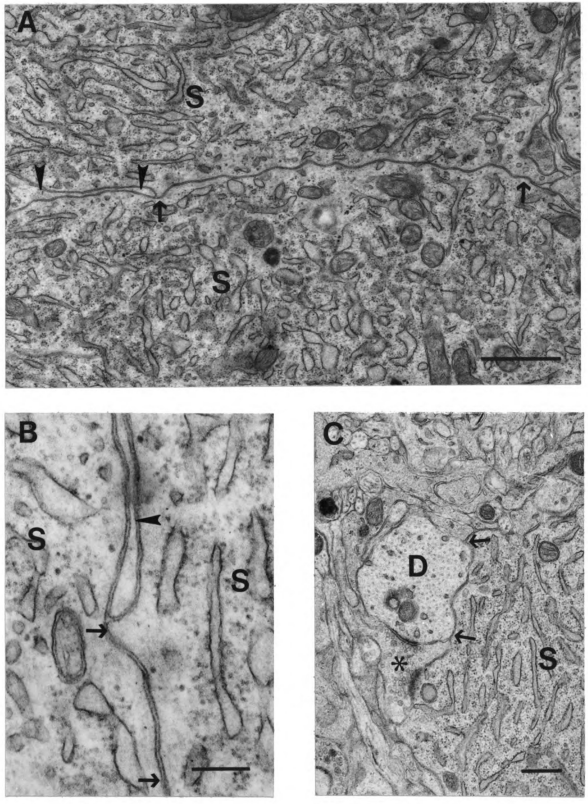

Figure 6

Dendritic region

Given the anatomical changes detected in the somatic region it was of interest to see if dendritic changes were also detectable in osmotically stimulated slices. Dehydration in vivo by substitution of saline for drinking water or water deprivation results in the formation of dendritic bundles, but does not increase dendritic multiple synapses (Perlmutter et al., 1985) Transcardial perfusion with hypertonic solution results in the formation of multiple synapses and dendritic bundles (Tweedle et al., 1989) suggesting that multiple synapses could be forming in the dendritic zone of SON of the slices used for the somatic analysis discussed above. Five slices from the low osmolality group and five slices from the high osmolality group were used for morphometric analysis.

Measurements of dendritic bundling and dendritic multiple synapses were similar to the measurements made in the somatic region. Twenty photomicrographs were taken at a magnification of 5,800X and enlarged 3.5X for a final magnification of 20,300X (these were printed on 11" X 14" photographic paper). The percentage of dendritic membrane contacted by adjacent dendrites, single synapses and multiple synapses was determined using a semi-circular test grid and intersection counting (see Mayhew, 1979). Quantification of dendritic multiple synapses included the percentage of terminals that contacted more than one dendrite as well as the percentage of total axonal contact which was made by such terminals. The mean length (μm) of multiple and single synapses was obtained by tracing the lengths with the Summagraphics Tablet. The percentage of multiple synapses with polyribosomes and/or coated vesicles located near the synapse was also determined.

Figure 7 - Photomicrographs of the SON dendritic region from an animal that was conventionally perfused for electron microscopy (**A**; see Figure 4 for tissue information) and from a hypothalamic slice (**B**). In both panels a few of the dendrites are labeled (D). In **A** a portion of an astrocyte characteristic of this region is shown (nucleus labeled **ast**). Astrocytic processes separate many of the dendrites (arrowheads) and dendrites in direct apposition are also present (arrows). Bars = 2 μm.

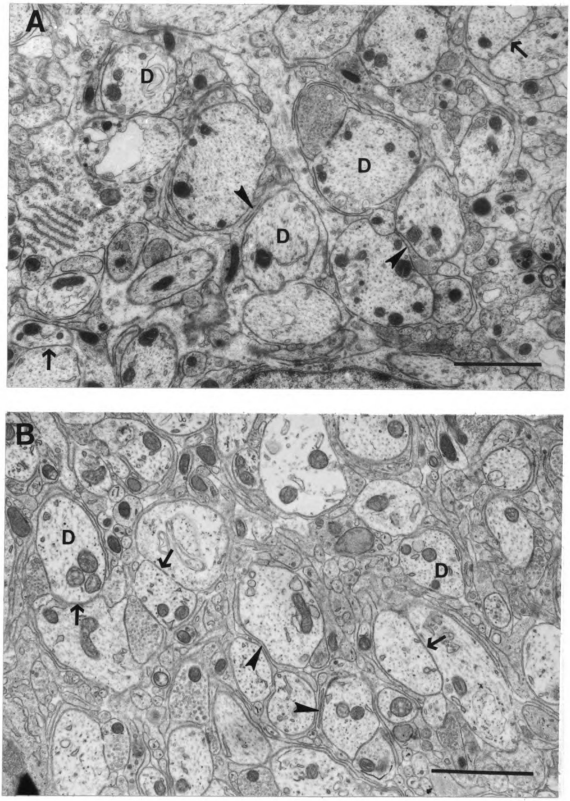

Figure 7

Results - Similar to the somatic region the ultrastructural integrity of the SON dendritic region was quite good. Comparisons between perfused tissue and slice tissue (Figure 7) did not reveal any obvious differences in dendritic, synaptic or astrocytic morphology.

In contrast to the somatic region no significant differences were detected between the two groups (low vs high osmolality) in three multiple synapse measures: 1) percentage of dendritic membrane contacted by multiple synapses (2.39 ± 0.14 vs. 2.98 ± 0.44), 2) percentage of terminals that formed multiple synapses (18.73 ± 1.57 vs 22.52 ± 2.00), 3) percentage of total axonal contact made by multiple synapses (29.54 ± 2.04 vs 32.82 ± 7.10). There was a significant increase in the percentage of multiple synapses that were associated with dendritic polyribosomes (Figure 8B & 8C) in the high osmolality group (18.09 ± 3.10) compared to the low osmolality group (10.21 ± 1.60, $p < 0.05$) while no significant difference was found in the percentage of multiple synapses associated with coated vesicles (Figure 8A, low osmolality 13.41 ± 3.38 vs high osmolality 17.14 ± 3.44). There was no significant difference in the dendritic bundling measure (Figure 9), i.e., the percentage of dendritic membrane apposed to adjacent dendrites (low osmolality 9.72 ± 0.85 vs high osmolality 9.59 ± 0.58). No significant differences were found in the remaining morphometric measures (low vs high osmolality): 1) mean length (μm) of multiple synapses (0.74 ± 0.01 vs 0.72 ± 0.02), 2) mean length (μm) of single synapses (0.87 ± 0.05 vs 0.83 ± 0.02), 3) percentage of dendritic membrane contacted by single synapses (5.74 ± 0.36 vs 6.01 ± 0.35).

Figure 8 - Cellular organelles associated with synapse formation. In **A** an axonal terminal (*) is apposed to an SON soma (S) and a dendrite (D). The long thin arrow points to a coated vesicle that is fused at the dendritic membrane immediately adjacent to the axonal terminal. Portions of the dendritic and somatic membranes are in direct apposition (large arrow). Bar = 0.5 μm. In **B** an axonal terminal (*) is apposed to two dendrites (D). Long arrows point to polyribosomal clusters subjacent to the terminal apposition. Bar = 0.5 μm. In **C** a large cluster of polyribosomes is present immediately subjacent to the axonal terminal (*). The terminal is also apposed to an SON soma (S) that is largely out of the photomicrograph. Bar = 0.5 μm

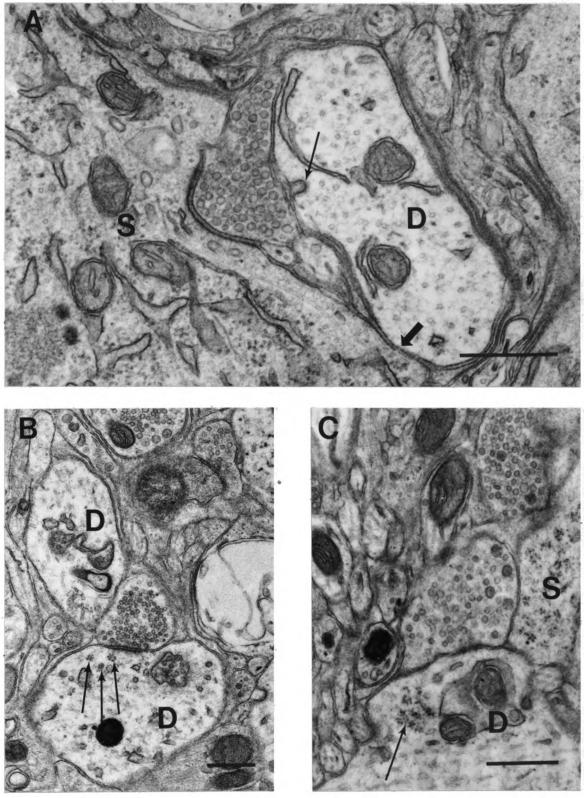

Figure 8

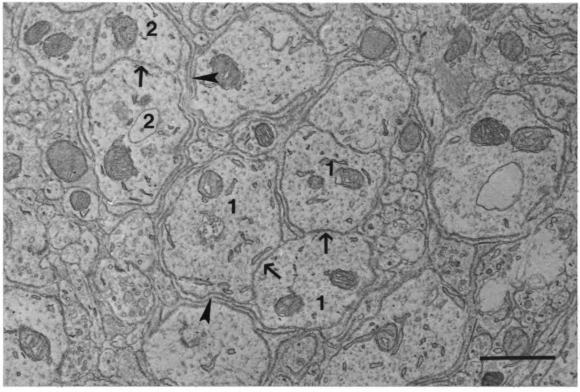

Figure 9 - Dendritic bundles from a slice that incubated 2.5 hours in high osmolality medium. A bundle of 3 dendrites (labeled with 1's) and a bundle of 2 dendrites (labeled with 2's) are indicated. Direct membrane appositions are indicated by the arrows. Thin astrocytic processes (arrowheads) separate adjacent dendrites from the bundles. Bar = 1 μm

Conclusion

In this experiment two changes that are seen in vivo during osmotic challenge, an increase in average firing rate of MNCs and the formation of somatic multiple synapses, were also seen in the slices incubated in high osmolality medium. These results, together with previous in vitro electrophysiological studies, demonstrate that osmotic challenge can be an effective in vitro stimulus and suggest that the in vitro slice preparation can be used to investigate further the mechanisms by which new multiple synapses are formed in the SON during osmotic challenge.

EXPERIMENT II - SLICES IN THE PERIFUSION CHAMBER

An important advantage of using an in vitro preparation is the ability to control precisely the duration of the experimental treatment, in this case exposure to the high osmolality medium. A time course experiment utilizing the slice preparation was designed to determine if the sequence of anatomical events in somatic multiple synapse formation could be elucidated. A constant perifusion used for electrophysiological studies (e.g. Yang & Hatton, 1989) was used for this experiment. The slice is placed ventral surface down on a small piece of filter paper that is embedded in bone wax. Small strands of cotton gauze are laid on top of the slice to facilitate fluid movement over the slice and fresh oxygenated medium is constantly perifused over and around the slice for the entire incubation period. One advantage of this chamber is that solutions can be changed rapidly. In this experiment slices equilibrated in the low osmolality medium for 1.5 hours in an attempt to establish a common baseline for all the slices. Approximately half of the slices had their medium switched to high osmolality for four hours while the remaining slices were constantly perifused with low osmolality medium.

In order to determine whether data obtained in this chamber would replicate the results from Experiment I counts of multiple synapses were made while the tissue was viewed on the electron microscope. The percentage of terminals that formed multiple somatic synapses was calculated by examining terminals apposed to SON somata. As in Experiment I, when a terminal also apposed an adjacent soma or dendrite a multiple synapse was recorded. For each slice 100 terminals were classified as single or multiple synapses.

Results

A total of 16 animals were used for this study. All slices incubated for 5.5 hours. Two slices were eliminated since no electrophysiological responses were obtained. The remaining 14 slices had action potentials which were either spontaneous or elicited in response to electrode advancement. Relative to slices incubated in the static bath chamber, slices incubated in the perifusion chamber seemed to yield less stable recordings, although this is difficult to assess quantitatively since twice as many slices were prepared for the static bath in Experiment I. Only four cells were spontaneously active for at least 2 1/2 minutes, two in the low osmolality medium (12.5 and 7.7 Hz.) and two in the high osmolality medium (2.7 and 3.7 Hz).

Examination of semi-thin sections showed that eight slices (n=3 low osmolality, n=5 high osmolality) had adequate tissue morphology for electron microscopic evaluation. In general, and based in part on slices not included in the present experiments, excellent tissue quality was more difficult to obtain in the perifusion chamber relative to the static bath. This was most obvious at the ventral surface of SON which was often extensively vacuolated. It is likely that some of this damage was due to placement of gauze on the dorsal surface of the slice (near but not on SON) which added additional pressure to the ventral surface of the tissue. Nonetheless, as shown in Figure 10, adequate tissue preservation for morphometric analysis was possible with the perifusion chamber.

In contrast to the results from Experiment I, there was not a detectable increase in the percentage of synapses that were multiple synapses between the two groups (low osmolality, 14.3 ± 1.2 vs. high osmolality 13.6 ± 1.2, $p > 0.05$). An average

of 7.9 ± 1.1 of the synapses from slices incubated in low osmolality medium were multiple synapses in Experiment I. This is significantly lower than the percentage obtained in this experiment ($p < 0.01$). No difference between the chambers was detected in the slices incubated in high osmolality medium ($p > 0.05$).

Conclusion

Two methodological differences existed between Experiments I and II. First and obviously was the different chamber and second was the pre-incubation of all slices in low osmolality medium for 1.5 hours prior to incubation in high osmolality medium. The significant increase in multiple synapses in slices from the perifusion chamber relative to those from the static bath suggests that synapses formed in both groups in the perifusion chamber regardless of osmolality. Since the results from the static bath chamber more closely followed the in vivo situation, i.e. an osmotic induction of multiple synapses, the static bath chamber was used for the time course experiment.

Figure 10 - Photomicrographs of hypothalamic slices incubated in the perifusion chamber. In **A** the general characteristics of the somatic region of SON are shown. Portions of two somata (S, one nucleus is labeled) are shown with several dendrites (D) in the center of the field. Axonal terminals form single somatic synapses (*) and two terminals form dendro-dendritic (**) multiple synapses. Bar = 2.0 μm. In **B** dendrites (D) from the dendritic region are shown. Two dendritic bundles of 2 dendrites (labeled 1 and 2) as well as a bundle of 4 (labeled with 3's) are in the field. Axonal terminals forming single (*) and multiple synapses (**) are indicated. Bar = 1 μm

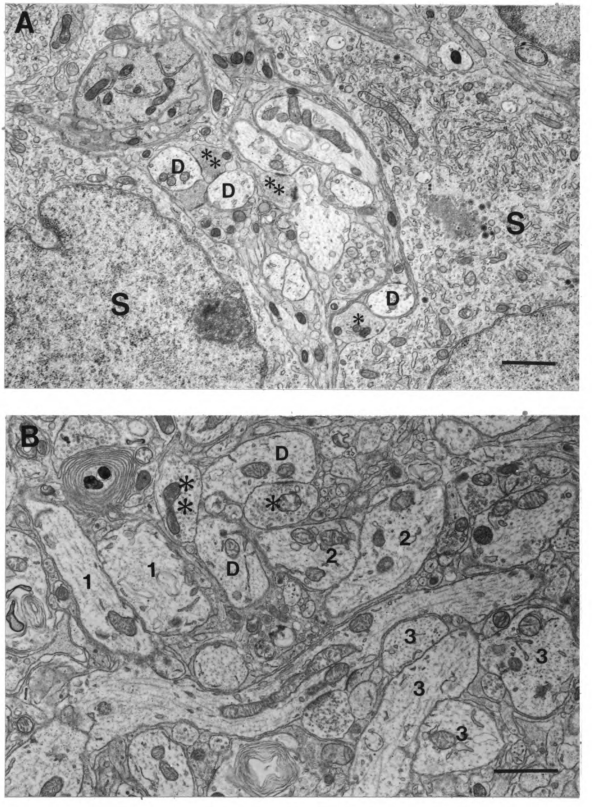

Figure 10

EXPERIMENT III - TIME COURSE OF SYNAPSE FORMATION

The methodological procedure for this experiment was essentially identical to that used for Experiment I, with the exception that a new chamber with two inner wells was built. This allowed slices from two animals to incubate simultaneously in two separate media. The animals used on a given day were usually siblings. Each day both low and high osmolality medium were provided coded. Slices incubated continuously for 5.5 hours, 2.5 hours or 1 hour in either the low or high osmolality medium. Single unit recordings were obtained from the 5.5 hour and 2.5 hour slices only. For all but two slices medium samples were taken from the incubation chamber at the end of the experiment. Samples were frozen until the osmotic pressure of the solution was determined at a later date.

In addition to the morphometric measures discussed in the General Methods section the following measures were also made: 1) the average length (μm) and number of direct soma-somatic/dendritic appositions per 100 μm of somatic membrane, 2) the percentage of soma-dendritic multiple synapses associated with dendritic polyribosomes and, 3) the percentage of multiple synapses with coated vesicles in the post-synaptic element.

Results

A total of 80 animals used in this experiment were equally divided into low and high osmolality groups (32 slices incubated for 5.5 hours, 28 slices for 2.5 hours and 10 slices for 1 hour). Four slices were eliminated from the 5.5 hour group due to technical difficulties during the incubation time.

All slices in the 5.5 hour and 2.5 hour groups had single unit activity, either spontaneous or elicited by electrode advancement. Of the 17 cells recorded, six

were recorded from high osmolality slices, three in each time period. The remaining 11 cells were recorded from slices in the low osmolality group (five from the 5.5 hour group and six from the 2.5 hour group). There was no detectable increase in the average firing rates (Hz) of cells from slices incubated in the high osmolality medium (6.6 ± 0.6) compared to cells from the low osmolality medium (7.6 ± 1.1).

A total of 38 slices were used for electron microscopy which included 14 slices from the 5.5 hour group (low osmolality, n = 8; high osmolality n = 6), 12 slices in the 2.5 hour and 12 slices in the 1 hour incubation groups were equally distributed between low and high osmolality groups (i.e. 6 each). Two slices, one from the 2.5 hour high osmolality group and one from the 1.0 hour low osmolality group were not included in any of the analyses since they were statistical outliers (Dixon & Massey, 1969) on the multiple synapse measures, although their values on the other morphometric measures were not extreme. The high osmolality, 2.5 hour slice had no multiple synapses; a situation not encountered in any of the slices included in these experiments and also never seen in perfused control animals analyzed with an identical sampling and morphometric method (data from Modney & Hatton, 1989). The 1.0 hour slice had an enormous number of multiple synapses (28%), a value not seen even in chronically dehydrated animals in vivo (high value from Modney & Hatton, 1989 was 22%).

Analysis of the samples taken from the incubation chambers revealed that the osmotic pressure of the chamber media was not necessarily 290 mOsm/kg or 340 mOsm/kg. In the 5.5 hour group, the average osmotic pressure was 272.8 ± 12.9 in the low osmolality slices and 308.3 ± 18.8 in the high osmolality slices, values that

TABLE 2 - CORRELATION COEFFEICIENTS BETWEEN MORPHOMETRIC MEASURES AND INCUBATION MEDIA OSMOTIC PRESSURE

MEASURE	INCUBATION TIME			ALL SLICES
	1 HOUR	2.5 HOUR	5.5 HOUR	
MULTIPLE SYNAPSES				
% somatic membrane	-0.26	-0.42	0.28	-0.11
#/100 μm of somatic membrane	-0.33	-0.47	0.34	-0.12
% synapses	-0.24	-0.41	0.31	0.07
% axonal contact	-0.16	-0.32	0.14	-0.10
DIRECT SOMA-SOMATIC SOMA-DENDRITIC APPOSITION	0.38	-0.27	-0.12	-0.02

were not significantly different. In the 2.5 and 1.0 hour groups the average osmotic pressure of low osmolality slices was 299.1 ± 4.1 and 292.4 ± 5.3 respectively and 337.7 ± 5.7 (2.5 hour) and 334.3 ± 8.0 (1 hour) for the high osmolality slices. In the 2.5 hour and the 1 hour groups the high osmolality group media osmotic pressures were significantly higher than the 1 hour group.

Given the variability in the osmotic pressure of the incubation medium samples, particularly in the 5.5 hour groups, it was important to know if the osmotic pressure at the end of the incubation time was significantly correlated with the major dependent variables (i.e. multiple synapse measures, percentage of somatic membrane contacted by adjacent somatic membrane). This was especially true since the results of Experiment I suggested that an increase in multiple synapses occurs with increased osmolality. The four multiple synapse measures and the percentage of somatic membrane contacted by adjacent somata or dendrites were not significantly correlated with the osmotic pressure of the incubation media (see Table 2). This was true if all 36 slices were used to calculate the correlation coefficient or if the correlations were calculated with only the slices from a single time period.

The ANOVAs did not reveal a main effect of osmolality on any of the dependent variables, nor were there any significant interactions between incubation time and osmolality. A complete summary of the ANOVAs can be found in Table 3 and group means for each measure are listed in Table 4.

As shown in Figures 11 and 12 three of the four multiple synapse measures increased over time, the exception being the percentage of somatic membrane contacted by multiple synapses ($p < 0.07$). Increases in these measures were detectable by 2.5 hours with no further increases exhibited at the 5.5 hour time period. Significant decreases in cell area and cell perimeter (Figure 13) occurred

TABLE - 3 ANOVA TABLES EXPERIMENT III

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY GLIAL PROCESSES

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.55	0.73	0.788
Time	2	28.43	3.77	0.034
Interaction	2	17.34	2.30	0.117
Error	30	7.53		

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY ADJACENT DENDRITES OR MNC SOMA

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.10	0.13	0.909
Time	2	5.45	0.70	0.503
Interaction	2	12.81	1.65	0.209
Error	30	7.76		

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY SINGLE SYNAPSES

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.66	0.17	0.683
Time	2	2.04	0.53	0.597
Interaction	2	1.20	0.31	0.635
Error	30	8.44		

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY MULTIPLE SYNAPSES

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	3.29E-3	8.2E-3	0.928
Time	2	1.12	2.85	0.074
Interaction	2	1.16	1.47	0.244
Error	30	0.39		

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY UNIDENTIFIED ELEMENTS

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.32	0.95	0.761
Time	2	3.12	9.11	8.0E-4
Interaction	2	0.27	0.79	0.42
Error	30	0.34		

TABLE 3 (Cont'd.)

NUMBER OF SINGLE SYNAPSES PER 100 μm OF SOMATIC MEMBRANE

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.31	0.12	0.725
Time	2	2.07	0.84	0.439
Interaction	2	0.48	0.20	0.822
Error	30	2.45		

NUMBER OF MULTIPLE SYNAPSES PER 100 μm OF SOMATIC MEMBRANE

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	3.1E-4	1.3E-3	0.971
Time	2	1.14	4.98	0.013
Interaction	2	0.29	1.26	0.298
Error	30	0.23		

NUMBER OF SOMA-SOMATIC/DENDRITIC MEMBRANE APPOSITIONS PER 100 μm OF SOMATIC MEMBRANE

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.60	0.41	0.528
Time	2	2.71	1.83	0.178
Interaction	2	2.63	1.77	0.187
Error	30	1.48		

PERCENTAGE OF AXONAL CONTACT MADE BY MULTIPLE SYNAPSES

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.62	0.02	0.884
Time	2	103.89	3.61	0.039
Interaction	2	46.60	1.62	0.214
Error	30	28.74		

PERCENTAGE OF SYNAPSES THAT WERE MULTIPLE

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.15	6.7E-3	0.935
Time	2	118.40	5.25	0.011
Interaction	2	33.64	1.49	0.241
Error	30	22.56		

TABLE 3 (Cont'd.)

MEAN LENGTH (μm) OF SINGLE SYNAPSES

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	9.0E-4	0.07	0.791
Time	2	0.01	1.09	0.349
Interaction	2	3.6E-3	0.28	0.745
Error	30	0.01		

MEAN LENGTH (μm) OF MULTIPLE SYNAPSES

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.01	0.19	0.664
Time	2	6.3E-4	0.01	0.988
Interaction	2	0.14	2.58	0.093
Error	30	0.05		

MEAN LENGTH (μm) OF SOMA-SOMATIC/DENDRITIC APPOSITIONS

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	0.12	0.67	0.420
Time	2	0.18	1.02	0.374
Interaction	2	0.29	1.61	0.216
Error	30	0.18		

CELL PERIMETER (μm)

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	4.86	0.48	0.495
Time	2	49.33	4.85	0.015
Interaction	2	0.15	0.02	0.985
Error	30	10.17		

CELL AREA (μm^2)

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	25.58	0.05	0.830
Time	2	9031.71	16.51	1.0E-4
Interaction	2	325.61	0.59	0.558
Error	30	547.02		

CELL SHAPE

SOURCE	df	MEAN SQUARE	F-test	p
Osmolality	1	1.4E-3	1.13	0.296
Time	2	5.6E-6	4.54	0.019
Interaction	2	2.0E-2	1.63	0.212
Error	30	1.2E-3		

TABLE 4 - GROUP MEANS AND STANDARD ERRORS FOR EXPERIMENT III

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY GLIAL PROCESSES

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	86.37 ± 0.22	82.99 ± 1.50	84.02 ± 0.60	84.31 ± 0.55
HIGH	85.50 ± 1.30	85.41 ± 0.94	81.72 ± 1.10	84.14 ± 0.77
TOTAL	85.89 ± 0.87	84.09 ± 0.96	83.04 ± 0.75	84.23 ± 0.50

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY ADJACENT DENDRITES OR MNC SOMATA

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	3.73 ± 0.50	6.52 ± 1.65	4.75 ± 0.67	5.04 ± 0.63
HIGH	4.58 ± 1.15	3.99 ± 0.47	6.09 ± 1.60	4.94 ± 0.70
TOTAL	4.19 ± 0.65	5.37 ± 0.97	5.32 ± 0.77	4.99 ± 0.46

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY SINGLE SYNAPSES

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	9.07 ± 0.99	8.25 ± 0.75	8.69 ± 0.71	8.65 ± 0.41
HIGH	8.76 ± 1.10	8.45 ± 0.71	9.62 ± 0.66	8.97 ± 0.48
TOTAL	8.90 ± 0.71	8.34 ± 0.50	9.09 ± 0.45	8.80 ± 0.31

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY MULTIPLE SYNAPSES

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	0.74 ± 0.25	1.74 ± 0.34	1.35 ± 0.20	1.31 ± 0.17
HIGH	1.10 ± 0.94	1.23 ± 0.12	1.55 ± 0.25	1.30 ± 0.11
TOTAL	0.94 ± 0.20	1.51 ± 0.20	1.44 ± 0.15	1.31 ± 0.11

TABLE 4 (Cont'd.)

PERCENTAGE OF SOMATIC MEMBRANE CONTACTED BY UNIDENTIFIED ELEMENTS

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	0.09 ± 0.09	0.51 ± 0.25	1.18 ± 0.27	0.68 ± 0.17
HIGH	0.06 ± 0.06	0.91 ± 0.34	0.99 ± 0.25	0.64 ± 0.16
TOTAL	0.08 ± 0.05	0.69 ± 0.20*	1.10 ± 0.18*	0.66 ± 0.12

* $p < 0.05$ compared to 1 hour

NUMBER OF SINGLE SYNAPSES PER 100 μm OF SOMATIC MEMBRANE

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	7.38 ± 0.80	6.87 ± 0.47	7.55 ± 0.46	7.29 ± 0.31
HIGH	7.11 ± 0.93	7.18 ± 0.47	8.07 ± 0.63	7.47 ± 0.41
TOTAL	7.23 ± 0.60	7.01 ± 0.32	7.78 ± 0.37	7.38 ± 0.25

NUMBER OF MULTIPLE SYNAPSES PER 100 μm OF SOMATIC MEMBRANE

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	0.72 ± 0.21	1.51 ± 0.21	1.26 ± 0.18	1.20 ± 0.13
HIGH	0.89 ± 0.24	1.15 ± 0.06	1.47 ± 0.18	1.17 ± 0.12
TOTAL	0.81 ± 0.16	1.34 ± 0.12*	1.35 ± 0.12*	1.18 ± 0.12

$p < 0.05$ compared to 1.0 hour

NUMBER OF DIRECT SOMA-SOMATIC/DENDRITIC APPOSITIONS PER 100 μm OF SOMATIC MEMBRANE

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	2.55 ± 0.37	3.93 ± 0.74	2.76 ± 0.37	3.07 ± 0.31
HIGH	2.25 ± 0.33	2.76 ± 0.25	3.45 ± 0.66	2.80 ± 0.29
TOTAL	2.40 ± 0.24	3.40 ± 0.44	3.06 ± 0.31	2.95 ± 0.21

TABLE 4 (Cont'd.)

PERCENTAGE OF AXONAL CONTACT MADE BY MULTIPLE SYNAPSES

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	7.35 ± 1.92	17.29 ± 2.74	13.85 ± 2.01	13.23 ± 1.53
HIGH	11.10 ± 2.68	12.79 ± 0.89	13.79 ± 1.85	12.55 ± 1.14
TOTAL	9.40 ± 1.75	15.25 ± 1.65*	13.82 ± 1.36	12.91 ± 0.96

*$p < 0.05$ compared to 1 hour

PERCENTAGE OF SYNAPSES THAT WERE MULTIPLE

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	8.62 ± 1.87	17.98 ± 2.04	14.43 ± 1.82	14.02 ± 1.35
HIGH	11.15 ± 2.60	13.91 ± 0.87	15.57 ± 1.60	13.52 ± 1.14
TOTAL	10.00 ± 1.62	16.13 ± 1.30*	14.92 ± 1.22*	13.79 ± 0.88

*$p < 0.05$ compared to 1 hour

MEAN LENGTH (μm) OF SINGLE SYNAPSES

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	1.24 ± 0.08	1.19 ± 0.04	1.15 ± 0.03	1.19 ± 0.03
HIGH	1.24 ± 0.03	1.17 ± 0.05	1.20 ± 0.04	1.21 ± 0.02
TOTAL	1.24 ± 0.04	1.18 ± 0.03	1.17 ± 0.03	1.20 ± 0.02

MEAN LENGTH (μm) OF MULTIPLE SYNAPSES

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	0.95 ± 0.14	1.12 ± 0.04	1.16 ± 0.12	1.09 ± 0.06
HIGH	1.23 ± 0.05	1.07 ± 0.06	1.04 ± 0.07	1.11 ± 0.04
TOTAL	1.10 ± 0.04	1.09 ± 0.05	1.11 ± 0.07	1.10 ± 0.04

TABLE 4 (Cont'd.)

MEAN LENGTH (μm) OF SOMA-SOMATIC/DENDRITIC MEMBRANE APPOSITIONS

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	1.48 ± 0.12	1.55 ± 0.13	1.75 ± 0.18	1.61 ± 0.09
HIGH	1.97 ± 0.24	1.44 ± 0.14	1.70 ± 0.15	1.72 ± 0.11
TOTAL	1.75 ± 0.16	1.50 ± 0.09	1.72 ± 0.12	1.66 ± 0.07

CELL PERIMETER (μm)

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	68.0 ± 1.6	65.8 ± 1.3	63.9 ± 0.9	65.6 ± 0.77
HIGH	68.8 ± 1.0	66.3 ± 1.1	64.8 ± 1.8	66.7 ± 0.86
TOTAL	68.4 ± 0.09	66.1 ± 0.8	64.3 ± 1.0*	66.1 ± 0.57

*p < 0.05 compared to 1 hour

CELL AREA (μm^2)

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	316.0 ± 11.3	294.1 ± 13.5	271.9 ± 7.0	290.5 ± 7.0
HIGH	327.4 ± 10.5	296.8 ± 9.8	262.8 ± 4.1	295.3 ± 8.2
TOTAL	322.2 ± 7.5	295.6 ± 8.1*	268.0 ± 4.4*#	292.9 ± 5.3

*p < 0.05 compared to 1 hour
#p < 0.05 compared to 2.5 hour

CELL SHAPE

	INCUBATION TIME			
OSMO	1.0 HR	2.5 HR	5.5 HR	TOTAL
LOW	0.849 ± 0.013	0.850 ± 0.011	0.834 ± 0.011	0.843 ± .006
HIGH	0.860 ± 0.009	0.840 ± 0.003	0.794 ± 0.026	0.831 ± .012
TOTAL	0.855 ± 0.008	0.846 ± 0.006	0.817 ± 0.013	0.837 ± .006

with increased incubation time. Cell perimeter did not decrease significantly until 5.5 hours while cell area was decreased by 2.5 hour and a further significant decrease occurred by 5.5 hours. A significant decrease in the cell shape factor was found between the 1 hour and the 5.5 hour groups suggesting that cells were not shrinking in a uniform manner. The percentage of somatic membrane contacted by glial processes decreased between the 1 hour and the 5.5 hour groups and an increase in the percentage of membrane contacted by unidentified elements occurred by 2.5 hours.

Correlation coefficients were calculated to determine the relationships among several dependent variables. Given that cell size can alter the interpretation of morphometric data based on the somatic membrane (e.g. # of multiple synapses per 100 μm of somatic membrane) and that cell size decreased over time in this experiment, correlation coefficients were determined between cell perimeter and the multiple synapse measures based on somatic membrane. Cell perimeter was used as the most meaningful variable in this instance since synapses are measured along the perimeter of the cell. Contrary to the expected negative correlation between cell size and multiple synapses, there was no significant correlation between the percentage of somatic membrane contacted by multiple synapses and cell perimeter ($r = - 0.08$, $p > 0.05$) or the number of multiple synapses per 100 μm of somatic membrane and cell perimeter ($r = - 0.18$, $p > 0.05$). The second relationship of interest, between direct soma-somatic/dendritic apposition and multiple synapses, did not correlate significantly ($r = 0.15$, $p > 0.05$).

Data collected on the percentage of multiple synapses with dendritic polyribosomes and coated vesicles were not appropriate for statistical analysis due to

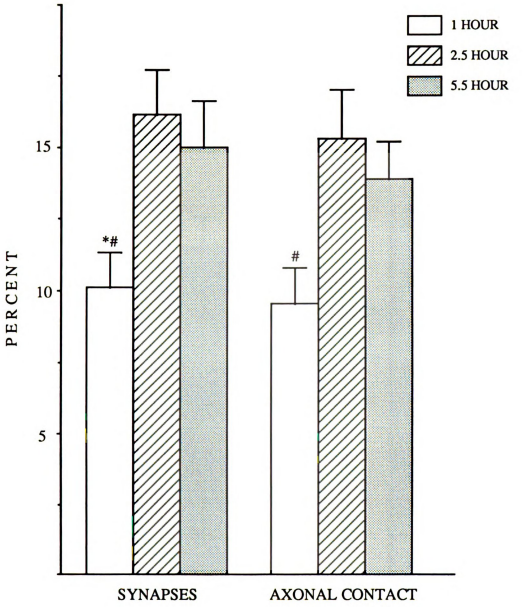

Figure 11 - Comparisons of the percentage of synapses that were multiple and the percentage of total axonal contact made by multiple synapses. Significant increases over time were found on both measures.

* $p < 0.05$ compared to 5.5 hour
# $p < 0.05$ compared to 2.5 hour

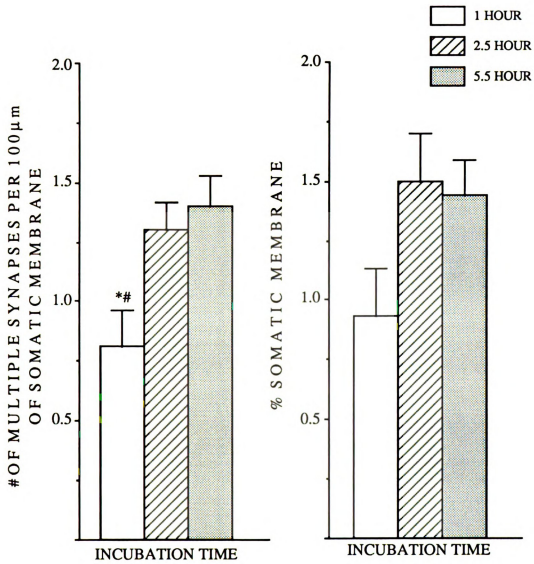

Figure 12 - Multiple synapse measures for the three incubation times. The percentage of somatic membrane contacted by multiple synapses did not reach the $p < 0.05$ significance level ($p < 0.07$). In contrast, the number of multiple synapses per 100 µm of somatic membrane did increase signficantly.

* $p < 0.05$ compared to 5.5 hour

# $p < 0.05$ compared to 2.5 hour

the presence of numerous 0's and extreme variability. This is likely to be a sampling problem since the number of multiple synapses from a given animal is fairly small. Further partitioning into groups of this small number results in the extreme variability. These data are presented in Figure 14 which is based on all the multiple synapses within each time period, not the group means.

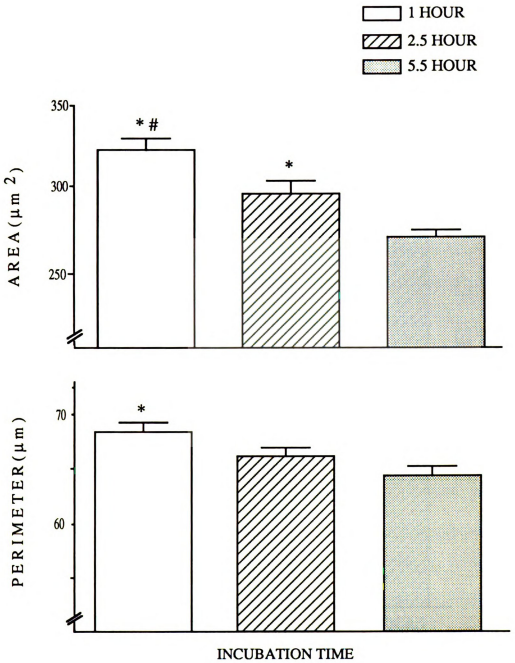

Figure 13 - Comparisons of cell area and perimeter over the three incubation times. Both measures decreased over time.

* $P < 0.05$ compared to 5.5 hour
# $p < 0.05$ compared to 2.5 hour

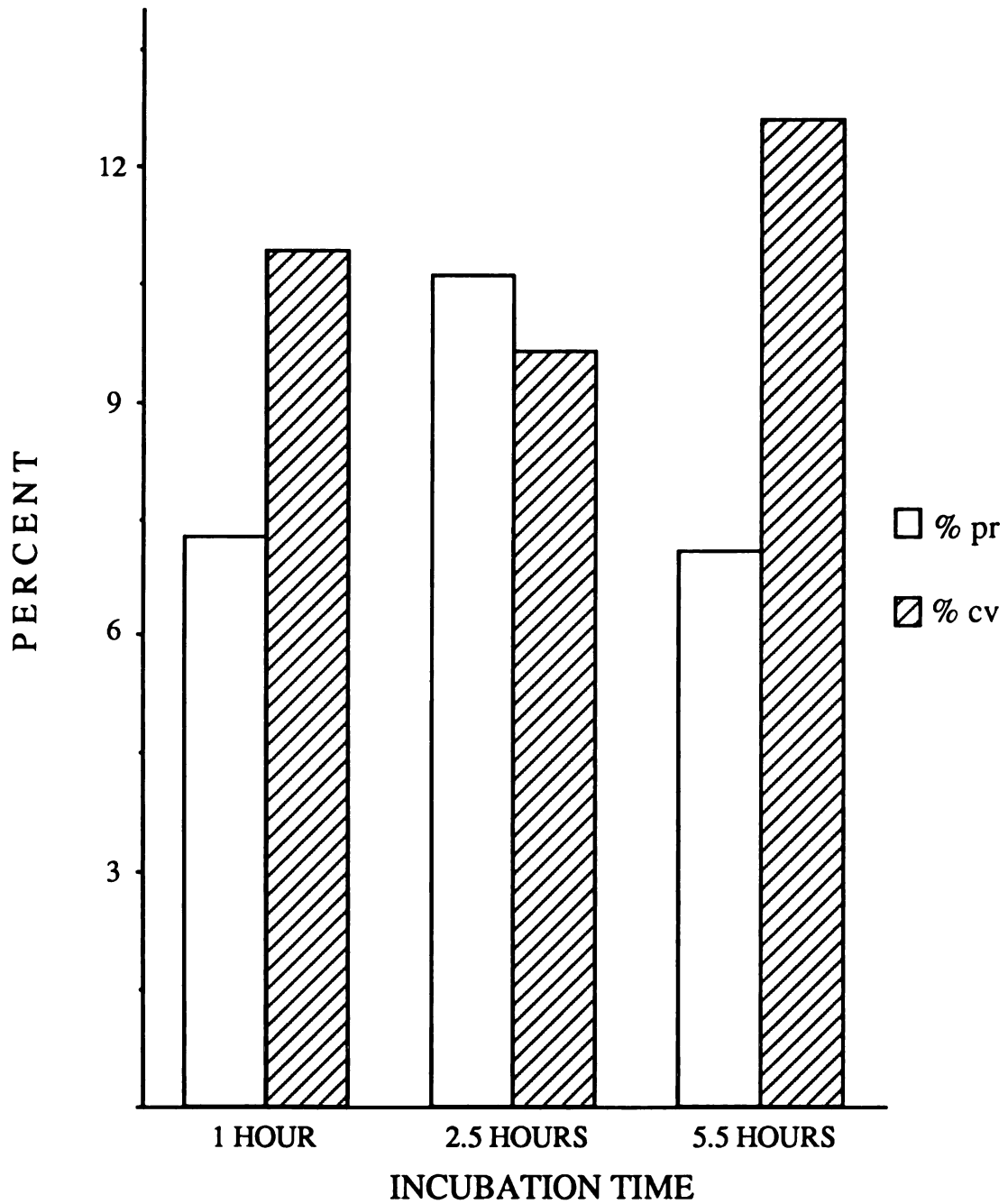

Figure 14 - The percentage of multiple synapses associated with dendritic polyribosomes (pr) and coated vesicles (cv). No obvious or consistent differences were seen over the incubation times, perhaps due to extreme variability between animals.

DISCUSSION

An enduring question for neurobiologists concerns the mechanisms by which specific neural connections are formed and modified over time. Among the multitude of issues raised by this broad query are how, and under what conditions synapses form? As with most questions in neuroscience, a variety of techniques and approaches have been used to further our understanding of synaptogenesis. The electron microscopic evaluation of neural tissue from cultures, developing and adult animals has provided a wealth of information concerning the characteristics of, and conditions under which new synapses form. An important general finding of the present work is that hypothalamic slices are capable of initiating synapse formation in a relatively short period of time. As with previous in vivo work, synaptogenesis in the SON in vitro appears to be a specific response since multiple synapses increase without concurrent increases in the single synapse morphometric measures.

In Experiment I the increased osmolality of the medium was clearly associated with changes in MNCs that are similar to those seen with dehydration in vivo. Than MNCs alter their electrophysiological characteristics during in vitro osmotic manipulations is well documented (Bourque, 1989; Bourque & Renaud, 1984; Hatton et al., 1978; Mason, 1980). In the present study the 50% increase in firing rate supports the contention that these cells can respond to osmotic challenges administered in vitro. This contention is further supported by the obtained increase in somatic multiple synapses in high osmolality slices. The formation of multiple synapses in response to osmotic challenges administered in vivo is well-established (Chapman, et al., 1986; Modney & Hatton, 1989; Tweedle & Hatton, 1984a), this experiment is the first report of synapses forming in vitro in response to osmotic

stimulation. In contrast to data obtained from the somatic region there was no detectable increase in multiple synapses in the dendritic region of SON. This is in contrast to the results of Tweedle et al. (1989) who found significant increases in dendritic multiple synapses following transcardial perfusion with hypertonic solutions. However, chronic dehydration produced by 2% saline drinking for 10 days does not result in the formation of dendritic multiple synapses (Perlmutter et al., 1985). Thus the relationship between dendritic multiple synapses and osmotic activation of MNCs is unclear. With regard to the present in vitro data, it is possible that synapse formation requires an intact afferent input, i.e., the terminals that form multiple synapses may have to remain connected to their cell bodies. In this respect, input to the dendritic region may come from afferent systems that are no longer intact in the slice, while input to the somatic region, which could come from closer perinuclear areas, would remain intact. Until more information is obtained concerning the precise termination of particular afferent systems, as well as information concerning the ability of distal severed axons to form synapses, this contention remains a hypothesis.

In Experiment II and III, there was no detectable relationship between the medium osmolality and the various dependent variables. In Experiment III there was a significant increase over time in several of the multiple synapses measures, with no corresponding increase in the single synapse measures. These data support a previous in vivo study (see below) that demonstrated that multiple synapses form rapidly (within 24 hours) in response to deafferentation.

Methodological Considerations

Morphometric Measures - Since the primary goal of these experiments was to analyze quantitatively the morphological characteristics of MNCs from slices, several assumptions and issues should be considered when interpreting these data. Synapses were inferred any time an axonal terminal apposed a post-synaptic structure regardless of whether synaptic specializations were present. Serial section analyses of SON synapses in vivo (Modney & Hatton, 1989; Smithson & Hatton, 1990) have revealed that 90% of the terminals that contact a post-synaptic structure form a conventional synapse. It is possible that a similar situation exists in vitro. The data collected here show an increase in the number of terminals apposed to more than one post-synaptic element. This has been interpreted as supporting the statement that multiple synapses have formed. A more extensive sampling procedure, and/or serial sections would be necessary to make this statement definitive.

Several of the measures made are sensitive to changes in the size of the measured cellular element. With regard to terminals that form multiple synapses, their size can alter their apparent frequency (larger terminals would appear more often in random sections). In the present experiments no detectable changes in the apposition length between terminals and somata for either single or multiple synapses was found. Thus, the increased number of multiple synapses is not due to changes in their size. A similar principle applies to cell size which could influence the number and/or percentage of somatic membrane contacted by synapses. The present results support the statement that multiple synapses are actually being formed in vitro regardless of decreases in cell size (which could lead to more

synapses/100 μm without actually increasing the number of synapses). Three arguments support this conclusion: 1) in Experiment I there was not a detectable change in cell size; 2) if cell size was influencing the number of synapses or the percentage of somatic membrane contacted by synapses this should also be detected in the single synapse measures. No changes were seen in these measures in Experiments I and III. 3) In Experiment III there was no correlation between cell size and the multiple synapse measures.

Experiment I vs Experiment III - In Experiment I multiple synapses formed in response to an osmotic manipulation while in Experiments II and III no osmotic effect was detected. This discrepancy between the experiments is difficult to explain. Methodologically experiments I and III appear identical, although there were minor changes in the actual preparation and location of the Experiments. Experiment I was done in an electrophysiology set-up in the basement of the building while Experiments II and III used an electrophysiology set-up on the second floor. In terms of the most of the experimental equipment (except the Vibratome, see below) there were no significant variations between these two electrophysiology setups. There may have been slight variations in the ambient temperature and humidity between these locations, however, it is difficult to see how these relatively minor changes could alter the pattern of results obtained. A second difference between the experiments was the Vibratome that was used to cut the slices. In Experiment I an old Vibratome that was often "finicky" was used. While the slice thickness was nominally 500-600 μm the control for thickness did not always raise the stage so that slices were often cut by visual approximation. Slices in Experiment II and III were cut with a new Vibratome that cut and

advanced quite consistently (nominally 550 μm). Any difference between the two groups in slice thickness could alter the extent and/or pattern of deafferentation between slices in the different experiments. At this point there is no way to determine which, if any, of these possibilities resulted in the discrepancy among the three experiments.

It is possible that the lack of an osmotic effect in Experiment III was due to technical problems with the incubation medium which was clearly not accurate in the 5.5 hour group. In general the measured osmolality of the medium for the 5.5 hour groups was lower than anticipated, however this was not universally true since some measures were quite reasonable (e.g. 341 mOsm/kg). It may be that had the medium really been 340 mOsm/kg for 5.5 hours multiple synapses would have increased more than the low osmolality slices. While this is a distinct possibility, the values from both the 2.5 hour groups are already quite high (percent synapses multiple = 16.1) relative to values from Experiment I (high osmolality = 11.6) and values from chronically dehydrated rats in vivo (17.4, data from Modney & Hatton, 1989). Evidence from in vivo studies suggest that there is a ceiling for the number of multiple synapses that form with prolonged lactation (Montagnese et al., 1987), it is not known if a similar ceiling exists in vitro. Since no significant increase in multiple synapses occurred between the 2.5 hour and the 5.5 hour groups this ceiling may have been reached by 2.5 hour in vitro. The lack of significant correlations between osmotic pressure and the multiple synapse measures also argues against an osmotically induced increase in these synapses in Experiment III. It seem likely that whatever induced synapse formation in Experiment III, most likely a reactive process, prevented the detection of an osmotic effect.

In addition to the multiple synapse measures the cell size measure were quite a bit lower in Experiment III compared to Experiment I and in vivo values (5.5 hour cell area (μm^2) 268 ± 4.0; Experiment I low osmolality = 431 ± 13; in vivo control males = 380 ± 21). Since the size of post-synaptic elements in some neural systems is clearly dependent on intact afferent input (e.g. Rubel, Hyson & Durham, 1990) the relatively smaller cells in Experiment III could mean that these cells underwent more deafferentation that the slices from Experiment I.

Electron Microscopic Evaluation of Brain Slices

While ultrastructural studies of brain slices are relatively rare compared to their use for electrophysiological studies they have provided some important information concerning the slice preparation. The extent to which these preparations correspond to the in vivo situation can only be determined by continued investigations using both in vivo and in vitro approaches. In the case of the SON the present experiments demonstrated that synapse formation can occur very quickly in vitro. The notion that in vitro preparations may be a useful tool for combining anatomical and electrophysiological studies is certainly not new.

Most electron microscopic investigations of slices have emphasized the general ultrastructural integrity of the tissue over varying incubation times and its similarities to perfused tissue of the same brain region (see Table 5). Several studies have attempted to correlate electrophysiological and/or biochemical measures with the morphological characteristics of the slice. In general electrical activity is associated with good ultrastructural preservation (Bak, Misgeld, Weiler & Morgan, 1980; Frotscher, Misgeld & Nitsch, 1980; Yamamoto, Bak, & Kurokawa, 1970). Similarly, a good correlation between tissue preservation and stimulated cGMP

TABLE 5 - ELECTRON MICROSCOPIC INVESTIGATIONS OF BRAIN SLICES

Slice Preparation	Incubation Time	Source
Cerebellum	2 hours	Garthwaite, Woodhams, Collins & Balazs, 1979
Cortical Areas	5 hours	Bak, Misgeld, Weiler & Morgan, 1980
	5 hours	Yamamoto, Bak & Kurokawa, 1970
	2 hours	Pitkänen, Korpi & Oja, 1985
Lateral Geniculate	10 hours	Crunelli, Leresch, Hyde, Patel & Parnaveles, 1987
Medulla/Pons	5 hours	Hinrichsen, 1980
Hippocampus	10 hours	Chang & Greenough, 1984
	8 hours	Frotscher & Misgeld, 1989
	4 hours	Frotscher, Misgeld & Nitsch, 1981
	1.5 hours	Jensen & Harris, 1989
	2.5 hours*	Lee, Oliver, Schottler & Lynch, 1981
	2.0 hours*	Meshul & Hopkins, 1990
	8.0 hours	Misgeld & Frotscher, 1982
	4.0 hours*	Petukhov & Popov, 1986
	5.5 hours	Reid, Schurr, Tseng & Edmonds, 1984
	12 hours	Schurr, Reid, Tseng & Edmonds, 1984
Hypothalamus	9 hours	Hatton, Doran, Salm & Tweedle, 1980
	0.5 hours	Pow & Morris, 1990

* not explicitly given

production was found in cerebellar slices (Garthwaite, Woodhams, Collins & Balaz, 1979).

Two general findings of some of the investigations listed in Table 5 were also observed in the present study and deserve comment. First, qualitatively, there is a general decline in the ultrastructural integrity of slices with increased incubation time. In the studies presented here this is most clearly demonstrated in Experiment III where the percentage of slices used for electron microscopic analysis increased with decreasing incubation time, particularly between the 1 hour and 5.5 hour groups. This does not necessarily imply that the best preservation was in the shortest incubation time, since excellent tissue preservation was obtained with a long incubation time (e.g Figure 5A which is from a slice that were incubated for 5.5 hours). Perhaps the longer incubation times provide more time for things to go wrong.

The second general conclusion reached by most slice studies is that tissue quality is not uniform throughout the slice. When viewed in cross-section two outer zones generally have poor tissue quality and an inner zone is well preserved. In most cases these outer layers correspond to the cut edges of the tissue block, one of which lies on the chamber floor while the other is exposed to air. In this study a similar pattern was observed. The ventral surface of the brain, which was placed on the net in the static bath or on filter paper in the perifusion chamber, consistently contained vacuolated and/or swollen processes and cells. The orientation of SON in the slice generally prevented the ventral surface of SON from being placed directly on the chamber floor. Often, portions of the pia-arachnoid remained attached ventral to SON and perhaps this prevented extensive damage to

the dendritic zone and glial limitans. Several studies suggest that hypoxia may be responsible for extensive vacuolation in hippocampal slices (Misgeld & Frotscher, 1982; Reid, Schurr, Tseng & Edmonds, 1984), although controlled studies using slices from other brain regions have not been performed. The second outer zone, which corresponds to a cut surface which is usually exposed to air also exhibits poor preservation, but it is not as extensive as the ventral or downward placed tissue. This damage is presumably due to the cutting procedure itself and varies somewhat from slice to slice. In this study the best slices used for morphometric evaluation had Vibratome cuts that were dorsal to the SON and so the nucleus proper was not included in the damaged region. Occasionally the most dorsal cells in the nucleus were slightly damaged but this was not preferentially distributed among groups nor were there detectable changes in the morphometric measures. The third zone of the slice is the inner zone which exhibits the best tissue morphology and corresponds to virtually the entire SON when horizontal slices are prepared.

In contrast to the general ultrastructural characterization of brain slices few studies have quantitatively investigated changes in morphology with varying experimental procedures. Using slices of cerebral cortex Pitkänen, Korpi & Oja (1985) have demonstrated a significant depletion of synaptic vesicles in axonal terminals when slices are incubated in Na^+ free medium (choline was substituted for Na^+ so that medium was isotonic) compared to medium with Na^+. Interestingly, a significant but not as extensive depletion was also observed in slices that incubated in the normal Na^+ containing medium relative to perfused tissue or tissue that was immersion fixed immediately after decapitation. This suggests that morphometric

measures obtained from slices incubated in "normal" slice medium are not necessarily identical to those observed in vivo.

Several studies have investigated quantitatively morphological changes in hippocampal slices following the induction of long-term potentiation (LTP). Two studies (Chang & Greenough, 1984; Lee, Oliver, Schottler & Lynch, 1981) have provided evidence that LTP induced by stimulation of the lateral-commissural projection from hippocampal field CA3 to field CA1 results in synapse formation. The increase in the number of synapses was detectable within 15 minutes of stimulation, lasted for at least 8 hours, and was not present in control stimulated slices (Chang & Greenough, 1984). Recently Meshul & Hopkins (1990) have used this same system in vitro (i.e. CA3-CA1) to demonstrate ultrastructural changes in the synaptic vesicle population in terminals, presumably at the potentiated synapses. In contrast, LTP induced by stimulating the mossy fiber projection from the dentate to CA3 does not lead to the formation of new synapses but alters the morphological characteristics (i.e. psd length) of existing synapses (Petukhov & Popov, 1986).

The results of the hippocampal slice studies together with the present experiments support the view that in vitro slice preparations can be used to study rapid synaptogenesis. In addition the present experiments suggest that in vitro preparations may be more dynamic than previously thought in terms of their ability to reorganize in relatively short periods of time.

Reactive and Rapid Synapse Formation

As argued earlier the results of the present experiments demonstrate that somatic multiple synapses form rapidly in the SON in vitro. Such rapid formation of multiple synapses in SON is not unprecedented since dendritic multiple synapses

form within 20 minutes following intense osmotic activation "in vivo" (Tweedle et al., 1989). In Experiment I osmotic stimulation resulted in somatic but not dendritic multiple synapses. There was an increase in the percentage of dendritic multiple synapses associated with polyribosomes in slices incubated in high osmolality medium. The significance of this is not clear since there was no detectable increase in the dendritic multiple synapse measures. While the majority of the literature supports the association between polyribosomes and synapse formation, there is evidence that they may also be associated with modified existing synapses (Hwang & Greenough, 1986). Perhaps dendritic multiple synapses were being modified by the high osmolality medium.

In Experiment III multiple synapses increased over time. It seems likely that in this experiment synapses were forming in response to deafferentation. A rapid response to deafferentation in SON has been demonstrated in vivo. Removal of the afferent projection from the subfornical organ to the SON by knife cuts or lesions results in multiple synapse formation within 24 hours, the earliest time point studied (Weiss et al., 1988). The results of the present experiments suggest that deafferentation can result in reactive synapse formation even earlier than this 24 hour period, perhaps as early as 1 hour, certainly within 2.5 hours.

Reactive synaptogenesis in neural systems is ubiquitous (see Cotman, Nieto-Sampedro & Harris, 1981). It is but one part of a complex compensatory mechanism that is initiated following deafferentation. Synapse replacement, while a general process, varies in its time course quite a bit from system to system. The rate at which new synapses form may depend on a number of factors. The extent of the lesion, the length of the severed distal axons and the rate at which the

degenerating axons are cleared by glia are thought to influence the rate of synapse replacement. In most systems reactive synaptogenesis is much slower than the time course demonstrated here (e.g. 5-7 days). Cotman et al. (1981) postulate that a major rate limiting step in reactive synaptogenesis is the time it takes to clear away degenerating terminals. In several systems however new synapses form prior to complete removal of the damaged fibers, or even before evidence of degeneration is observed.

Chen and Hillman (1982) examined the time course for synapse replacement following partial deafferentation of Purkinje cells in the cerebellum. As early as 10 hours after deafferentation Purkinje cell spines were often dually innervated, i.e. a single spine was contacted by two terminal boutons, one of which was apparently degenerating. Since dual innervation is extremely rare in control animals the authors conclude that new synapses had formed within 10 hours of the lesion. Dual innervation of spines was not seen at later time points suggesting that the second terminal remains after the degenerating terminal is removed. Murray and Goldberger (1986) report that reactive synaptogenesis after partial deafferentation of Lamina II in the cat spinal cord was partially complete at the first time point investigated (2.5 days). No evidence of degeneration was seen at this time suggesting that it was cleared away rapidly. In a system that is remarkably similar to the SON in terms of morphological changes Goshgarian and colleagues (Goshgarian & Yu, 1990; Goshgarian, Yu & Rafols, 1989) have described the rapid formation of multiple synapses (and dendritic bundles) in the phrenic nucleus. Within 4 hours of spinal cord hemisection (the first time point studied) multiple synapses were significantly elevated relative to control animals (Goshgarian et al.,

1989). In this study multiple synapses reached their maximum number by four hours since no further increase for up to four days post-hemisection was detected.

Rapid synapse formation is not only a response to deafferentation since new synapses can form rapidly in response to other manipulations. As mentioned earlier synapse formation in hippocampal slices occurs within 15 minutes and depends on the patterned electrical stimulation of LTP. Rapid formation of synapses in vivo occurs following induction of LTP by stimulation of the Schaffer collaterals to CA1 pyramidal cells (Lee, Schottler, Oliver & Lynch, 1980). (Note: synapse formation apparently does not occur in the dentate gyrus of the hippocampus following induction of LTP by stimulation of the entorhinal cortex, see Desmond & Levy, 1990.) New synapses also form in the hippocampal CA1 pyramidal cell layer within 15-30 minutes following activation with kainic acid (Petit, LeBoutillier, Markus & Milgram, 1989). Interestingly but unfortunately not quantitative, Hinrichsen (1980) has reported the presence of growth cones in slices of the medulla/pons (5 hour incubation time) suggesting that perhaps new connections are beginning to form in this preparation. Such growth cones were not seen in conventionally prepared perfused tissue from normal animals.

The literature reviewed above suggests that synapse formation in adult animals can occur quite rapidly. The extent to which this statement applies to the nervous system in general awaits further exploration. It could be that rapid synapse formation is common but investigators have simply not "looked" for evidence of synapse formation early enough. Rapid multiple synapse formation in SON has been relatively easy to detect since somatic multiple synapses are relatively rare in normal animals.

The precise stimulus for rapid synapse formation is not known. Clearly synaptogenesis during LTP is dependent on patterned neuronal activity. Based in part on the massive literature from the neuromuscular junction, Cotman et al. (1981) consider neuronal inactivity as one of the major postulates governing axonal sprouting following deafferentation.

In SON, multiple synapse formation during dehydration and parturition/lactation is clearly associated with increases in MNC activity. A strong case can be made that intravascular perfusion of hypertonic solutions increased MNC activity which resulted in very rapid multiple synapse formation (Tweedle et al., 1989). Multiple synapses also form during intracerebroventricular injections of OX (Theodosis et al., 1986) a manipulation that may have increased MNC activity since OX excites oxytocinergic neurons (e.g. Yamashita et al., 1987). In Experiment I multiple synapses formed along with a detectable increase in the average firing rate of MNCs.

The interrelationships between MNC inactivity, deafferentation and multiple synapse formation are less clear. One study has shown that multiple synapses form following deafferentation (Weiss et al., 1988). Given that the afferent projection removed in this experiment (subfornical organ) is most likely excitatory (Jhamandas, Lind & Renaud, 1989) it may be that MNC activity decreased. Synapse formation in SON could conceivably occur in response to both increased and decreased MNC activity.

Multiple Synapse Formation

Multiple synapses as defined here are not unique to the SON. Certainly the complex synaptic glomeruli characteristic of many brain regions (e.g. olfactory bulb,

cerebellum, and other, see Peters, Palay & Webster, 1976) qualify according to the definition "a single axonal terminal that simultaneously contacts 2 or more postsynaptic elements". What makes multiple synapses in SON interesting and worthy of special attention is their specific formation and disappearance in adult animals. Although no one has yet demonstrated the exquisite matching of true physiological condition and multiple synapse formation as occurs in the SON during lactation, multiple synapses are present and malleable in several neural systems. A selective survey of multiple synapse formation in other neural systems may provide some clues as to how and when these synapses are formed.

Multiple synapses in developing systems. Synaptogenesis in developing animals is an extremely complex process that varies somewhat according to the neural system being investigated (see Burry, Kniss & Scribner, 1984 and Vaughn, 1989 for reviews). In several systems the development of adult patterns of innervation seems to involve both the formation and elimination of synapses. The extent to which synaptogenesis in adult animals mimics the process that occurs during development is unknown, but clearly some aspects are similar (e.g. accumulations of polyribosomes and coated vesicles). The intent here is not to review the developmental process extensively but rather to glean some insight into the nature of multiple synapse formation as it occurs during development.

Few studies have addressed the issue of multiple synapses in developing animals. As mentioned earlier glomeruli fit the definition of a multiple synapse and their development is probably at the extreme end of multiple synapse formation in terms of complexity. Methodologically the formation of multiple synapses during development is difficult to assess since often increases in terminal number occur at

the same time that multiple contacts are being formed. This makes it impossible to know whether pre-existing terminals are forming additional contacts or if the new terminals are forming multiple contacts during their growth stage. Studies of glomeruli formation have suggested that both of these processes occur depending on the neural system involved.

There is evidence that the mossy fiber system in the developing cerebellum establishes multiple synapses quite early since growth cones are seen forming several contacts (Hamori & Somogyi, 1983; Mason & Gregory, 1984). In contrast, studies of the mossy fiber system in the hippocampus (Amaral & Dent, 1981), multiple synapses in the superior colliculus (Lund & Lund, 1972) and retino-geniculate glomeruli (Mason, 1982; Brunso-Bechtold & Vinsant, 1988) suggest that multiple synapses begin as relatively simple single synapses that develop over time into more complex multiple synapses. This progression from single to multiple synapses in the lateral geniculate requires action potentials since eye injections of tetrodotoxin for 8 weeks postnatally prevents glomeruli formation. These inactive terminals retain their single synapse configuration (Kalil, Dubin, Scott, & Stark, 1986).

While there is no evidence to suggest that glial process withdrawal participates in the formation of multiple synapses during development the data support the concept of a conversion from single to multiple synapses. Mason (1982) has suggested that glial encapsulation of glomeruli acts to prevent terminals from forming additional synaptic contacts. This is based on the observation that synapse formation is complete at around the same time that glial processes

completely surround the glomerulus. It is the opposite of this, glial withdrawal, that is thought to promote/permit multiple synapse formation in the SON.

Multiple synapses in adult animals - Perhaps the first report of alterations in multiple synapses in adult animals was given by Raisman (1969) who utilized the now common multi-synaptic index ("the number of multiple contacts per 100 single contacts"). Raisman has described in meticulous detail the reinnervation of the septal nuclei by remaining intact fibers. Part of this reinnervation is accounted for by an increase in the number of terminals that form multiple synapses (Raisman, 1969; Raisman & Field, 1973). Increases in multiple synapses following deafferentation have also been reported in the spinal cord (Bernstein & Bernstein, 1977; Goshgarian et al., 1989), the hippocampus (Cotman, Gentry & Steward, 1977; Matthews, Cotman & Lynch, 1976; Steward, Vinsant & Davis, 1988) and in the dorsal lateral geniculate nucleus (Kalil & Behan, 1987). Interestingly, in some areas of the spinal cord, deafferentation appears to result in a loss of multiple synapses followed by synapse replacement by smaller terminals that make single contacts (Murray & Goldberger, 1986).

Multiple synapses have been shown to respond to manipulations other than deafferentation in areas other than SON. In the spinal nucleus of the bulbocavernosus muscle testosterone maintains synaptic input to the sexually dimorphic motor neurons (Matsumoto, Micevych & Arnold, 1988). The response of these neurons to castration is the opposite of what occurs in SON with activation. That is, multiple synapses and direct appositions between adjacent somata and dendrites decrease. These decreases are prevented by testosterone replacement at the time of castration. There is evidence for environmental regulation of multiple

synapses in the cat visual system. In this case, placement of cats in an enriched environment is associated with significant decreases in the number of GABAergic multiple synapses (Beaulieu & Collonnier, 1988).

A few general conclusions can be reached concerning multiple synapses. They are common and malleable in many neural systems (in fact they may be more common than previously thought since not all studies of plasticity measure this type of synapse). Their formation and disappearance can be influenced by several different experimental manipulations. Following deafferentation multiple synapses are often a unique synapse type that is not present or is rare in normal animals.

Few investigators have postulated a mechanism by which a single terminal eventually contacts two postsynaptic elements. An exception is the mechanism originally proposed for SON (Hatton & Tweedle, 1982) and postulated by Goshgarian (1989) for multiple synapse formation, namely retraction of glial processes from between adjacent neural elements. In the case of deafferented systems it is often implied that part of the reactive process involves existing single synapses converting to multiple synapses (e.g. Raisman, 1969), however in many cases terminal proliferation occurs at the same time as multiple synapse formation (Steward et al., 1988). In these systems strong statements regarding a conversion process from single to multiple synapses are more difficult to make.

Whether multiple synapses serve a function that is unique to their anatomical configuration, i.e. two postsynaptic elements that simultaneously receive an excitatory or inhibitory signal, is not clear. In the case of deafferentation multiple synapse formation is seen as a mechanism for maintaining synapse numbers and the functional significance of multiple vs single synapses has been largely ignored. In

the phrenic nucleus the appearance of multiple synapses occurs concurrent with the unmasking of a previously ineffective neural pathway (Goshgarian et al., 1989). That is, a neural pathway which in normal animals does not evoke a postsynaptic response is able to activate the postsynaptic cells following neural injury. It is not known if this now effective pathway is the one that forms multiple synapses nor is it clear if the anatomical configuration is significant.

In SON multiple synapses have often been postulated as one of several potential mechanisms that serves to coordinate and/or synchronize MNC activity (e.g. Hatton & Tweedle, 1982; Theodosis et al., 1981). Whether this is their function in the deafferented SON is speculative. It may be that the conversion of single to multiple synapses is simply a very efficient and rapid way to maintain the synaptic input to MNCs following deafferentation.

Direct Appositions and Multiple Synapses

Previous in vivo studies have consistently found increases in direct postsynaptic membrane apposition in both the somatic and dendritic regions of SON during osmotic challenges. No increases in direct apposition were detected the any of the present experiments. In addition, multiple synapses have always been associated with increases in direct membrane appositions. The reverse is not necessarily true since direct appositions can occur without increases in multiple synapses, e.g. during acute dehydration. The present results show that multiple synapses can increase without detectable changes in direct appositions. The lack of a significant correlation between multiple synapses and direct appositions in Experiment III suggest that the two changes occur independently. It may be that very subtle changes in stimulus intensity (i.e. magnitude of osmotic increase) as well

as duration (number of days/hours of osmotic stimulation) produce different patterns of these two morphological changes.

In the phrenic nucleus following spinal cord hemisection a similar dissociation of membrane appositions and multiple synapses have been described. Multiple synapses reach their maximum number by four hours while only the length of dendrodendritic appositions increased at this time. At later time point (2 days) the number of appositions and the dendritic bundling measures increased (Goshgarian et al., 1989). In this system dendrodendritic appositions have also been shown to increase without concurrent increases in multiple synapses following two days of chronic hypoxia (Goshgarian & Yu, 1990).

While glial retraction has been postulated as the mechanism by which morphological changes in SON and the phrenic nucleus occur, direct evidence for this mechanism remains elusive. One of the few studies that has provided direct evidence for glial changes (Salm et al., 1985) demonstrated a redistribution of immunoreactive glial fibrillary acidic protein (GFAP) during lactation which is consistent with glial retraction. Similar direct evidence for glial retraction has been difficult to obtain at the electron microscopic level. Since glial processes cover the vast majority of MNC somatic membrane, the small decreases in glial contact that would occur with the addition of relatively small multiple synapses do not significantly change this measure. Data collected on glial contact from chronically dehydrated rats is complicated by the fact that cell size is increased dramatically in these animals (Modney & Hatton, 1989). (In the phrenic nucleus glial contact has not been measured.) In the present Experiment III there was a significant decrease in the percentage of somatic membrane contacted by glial processes, with no change

in the extent of direct soma/somatic membrane apposition. This decrease can be accounted for by the significant increase in coverage by unidentified elements. It is likely that the increased coverage by unidentified elements is related to the general decline in tissue morphology associated with increased incubation time. A cogent argument can, and has been made (see Hatton, 1990) that direct appositions between neural elements are most likely due to glial retraction since these changes occur without concurrent changes in soma or dendrite size. This is still however, indirect evidence for the postulated mechanism. Future morphological studies that continue to measure glial elements may be able to provide more direct evidence for glial retraction.

SUMMARY

It is clear from the present experiments that neuronal connections can be modified rapidly in vitro. What remains unclear is the precise stimulus for this in vitro reorganization. The results of Experiment I demonstrated that these synapses could form in response to a well-characterized stimulus for SON neurons, namely increased osmolality. In Experiment III there was no significant effect of osmolality, however multiple synapses formed in both low and high osmolality slices over time. The results of the third experiment support a previous in vivo experiment that demonstrated reactive synapse formation in SON following removal of an afferent input to MNCs. While reactive synapse formation in SON is not as well established as synaptogenesis in response to osmotic stimulation, the data presented here suggest that reactive synaptogenesis can be a rapid and robust phenomena.

Given the discrepancy between these experiments it is clear that further in vivo and in vitro studies must be done to reach a firm conclusion about the what stimulates synapse formation in SON. The SON provides neurobiologists with a model system to investigate how synapses in an adult mammalian system form. Since synaptogenesis occurs in response to both physiological (e.g. dehydration) and pathological (e.g. deafferentation) manipulations, a rare opportunity exists to compare and contrast this process during these two different conditions.

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APPENDICES

APPENDIX A - TISSUE EMBEDDING PROTOCOL

APPENDIX A

TISSUE EMBEDDING PROTOCOL

General Notes

1) Spurr's resin was obtained from Electron Microscopy Sciences (Low Viscosity Embedding Kit). Follow manufacturer's recipe when mixing the resin components.
2) Osmicated tissue was typically stored in cacodylate buffer until the end of the week and all the slices from a given week were embedded together. Be sure that before staining with uranyl acetate that tissue is rinsed well with distilled water.

DAY ONE

Slice Preparation - Fix by immersion overnight.

DAY TWO

Core and osmicate tissue. If embedding immediately stain overnight in uranyl acetate, or store in buffer.

DAY THREE

1) Three water rinses (10 minutes each)
2) Stain tissue with saturated uranyl acetate (4%), filtered with a 0.22 μm Millipore filter, store overnight in refrigerator.

DAY FOUR - Infiltration

1) Water rinses (3 @ 15 minutes each)

APPENDIX A (Cont'd.)

2) 50%, 75%, 80%, and 95% alcohol (1 rinse each dilution for 10 minutes)

3) 100% alcohol (4 rinses @ 15 minutes each - OPEN A FRESH BOTTLE)

4) 2:1 mixture of alcohol to resin (3 hours)

5) 1:2 mixture of alcohol to resin (3 hours)

6) 100% resin mixture (overnight)

DAY FIVE - Embed

1) Embed tissue (molds or flat embed on release agent coated slides)

2) Polymerize overnight in an oven @ 65-70° C.

APPENDIX B - SUPPLIES & EQUIPMENT

APPENDIX B

SUPPLIES AND EQUIPMENT

EQUIPMENT

Advantage Computer	
SummaSketch Tablet	465.00
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
Jandel Scientific	
Sigma-Scan Software	465.00
JEOL (Approximate)	
JEOL CX-100 Electron Microscope	300,000.00
Kopf Vertical Pippette Puller	2,910.00
LKB Knifemaker II	4,875.00
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

APPENDIX B (cont'd)

SUPPLIES & EQUIPMENT (cont'd)

MSU Computer Center (1985)	
Zenith 150 Computer w/monochrome monitor	1,499.00
Olympus (1986)	
C-2 Image Analysis System (excluding microscope)	8,000.00
Polysciences - Vibratome	3,750.00
Technical Manufacturing Corp. (1987)	
Micro-g air table	2,340.00
Ted Pella, Inc (1987)	
Infiltron (approximate equivalent)	577.00
Sodium Vapor Safelight	398.00
Tektronix Oscilliscope	9,865.00
Thomas Scientific (1987)	
Mettler Balance	2,795.00
Blue M Gravity Oven	669.00
Fiber Optic Illumination System	2,795.00
Sage Syringe Pump (approx replacement - 2 channel)	1,550.00
Refrigerator/Freezer	1375.00
YSI Temperature probe & controller (replacement)	1005.00
VWR Scientific (1987)	
Stereozoom microscope (approx. replacement)	1,336.00
Wild Microscope	5,000.00

SUPPLIES

Electron Microscopy Sciences (1987)	
Copper Grids (200 mesh-thin bar: 100/vial)	13.50
Forceps	
3C	8.50
anticappillary, self-closing	19.50
Electron Microscopy Sciences (cont'd.)	
Glassine Envelopes for negatives (1000)	34.50
Glutaraldehyde (Biological Grade) 500 ml	6.50
Grid Boxes (12)	32.00
Uranyl Acetate (25 g)	12.00

APPENDIX B (cont'd)

SUPPLIES & EQUIPMENT (cont'd)

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
Desiccator (small glass)	74.23
Electron Microscopy Film (box w/100 sheets)	35.70
Kodabromide F4 Paper (8" x 10", 250 sheets)	97.50
Polycontrast Paper (11" x 14", 50 sheets)'	37.11
Sigma Chemical Co. (1987)	
Cacodylate Acid (500 g)	182.00
Paraformaldehyde (1 kg)	12.75
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
Wescor Osmolality Standard	37.25

APPENDIX C - DATA TABLES

EXPERIMENT I

APPENDIX C

DATA TABLES - EXPERIMENT I

TABLE 6 - CELLULAR ELEMENTS CONTACTING MNC SOMATIC MEMBRANE

	Glial Processes		Axonal Terminals		Direct Apposition*		Not Identified		Total
	µm	%	µm	%	µm	%	µm	%	µm
290 mOsm									
Slice #									
35	550.6	80.6	75.9	11.1	13.3	1.9	43.2	6.3	683.0
37	599.8	85.8	65.9	11.0	10.2	1.4	12.9	1.8	699.8
48	572.6	74.8	90.6	11.8	32.4	4.2	69.9	9.1	765.5
52	560.9	88.9	45.2	7.2	1.5	0.2	22.7	3.5	630.3
53	629.8	87.2	58.5	8.1	26.6	3.5	8.2	1.1	722.3
59	433.5	77.4	88.9	15.5	4.0	0.7	36.4	6.3	572.7
63	379.3	75.9	81.9	16.4	8.6	1.7	29.7	5.9	499.4
65	497.6	82.7	42.3	7.0	40.3	6.7	21.3	3.5	601.6
70	525.9	84.4	64.8	10.4	13.7	2.2	18.4	3.0	622.9
Mean	527.5	82.0	67.0	10.9	16.7	2.5	29.2	4.5	644.1
SE	26.5	1.7	6.1	1.1	4.4	0.7	6.3	0.8	27.4
340 mOsm									
Slice #									
38	488.1	82.7	60.5	10.2	15.7	2.6	25.9	4.4	590.3
43	517.4	73.7	117.6	16.7	11.3	1.6	55.4	7.9	701.6
46	398.1	67.9	100.3	17.1	13.6	2.3	73.5	12.5	585.5
56	560.1	81.7	79.4	11.6	12.5	1.8	33.1	4.8	685.1
58	549.6	71.2	94.7	12.2	74.0	9.6	52.6	6.8	771.0
66	474.2	84.0	62.2	11.0	11.4	2.0	16.6	2.9	564.5
67	501.0	83.4	61.3	10.2	11.8	2.0	26.4	4.4	600.5
69	467.3	77.3	65.5	10.5	23.3	3.8	50.0	8.2	604.1
Mean	494.5	77.0	80.5	12.4	21.7	3.2	37.9	6.5	637.8
SE	18.1	2.2	7.7	1.0	7.6	1.0	8.36	1.1	25.7
t =		1.53		-1.0		-0.62		-1.45	
p =		0.14		0.33		0.55		0.16	

* Direct apposition between adjacent somata and or dendrites

APPENDIX C (cont'd.)

TABLE 7 - MULTIPLE SYNAPSE MEASURES

	% Somatic Membrane	#/100 µm of Somatic Membrane	% Synapses	% Axonal Contact
290 mOsm/kg Slice #				
35	1.31	1.46	14.5	11.76
37	0.85	0.86	8.95	7.70
48	0.55	0.39	4.22	4.69
52	0.46	0.32	5.61	6.46
53	0.45	0.28	3.92	5.98
59	0.67	0.70	5.80	4.31
63	1.50	1.60	11.10	9.16
65	0.65	0.66	8.89	9.27
70	0.76	0.80	8.77	7.34
MEAN	0.80	0.78	8.0	7.4
SE	0.12	0.16	1.1	0.8
340 mOsm/kg Slice #				
38	1.41	1.19	11.86	13.77
43	1.62	1.28	9.57	9.72
46	1.82	1.54	11.69	10.64
56	1.33	1.02	11.86	11.45
58	1.72	1.95	15.96	14.00
66	0.84	0.87	9.80	7.64
67	1.48	1.50	15.00	14.56
69	0.66	0.66	6.90	6.30
MEAN	1.36	1.25	11.6	11.0
SE	0.14	0.14	1.0	1.1
t =	-2.97	-2.16	-2.30	-2.73
p =	0.01	0.05	0.04	0.01

APPENDIX C (cont'd.)

TABLE 8 - MEAN LENGTH (μm) OF APPOSITION BETWEEN AXON TERMINALS AND MNC SOMATIC MEMBRANE

290 mOsm/kg		**340 mOsm/kg**	
Slice #	Length	Slice #	Length
35	1.10	38	1.02
37	1.14	43	1.25
48	1.27	46	1.30
52	1.28	56	1.34
53	1.14	58	1.01
59	1.28	66	1.22
63	1.13	67	1.02
65	0.96	69	1.09
70	1.14		
MEAN	1.16		1.15
SE	0.04		0.05
t =	0.13		
p =	0.90		

APPENDIX C (cont'd.)

TABLE 9 - SOMATIC SIZE AND SHAPE MEASURES

	Area (μm^2)	Perimeter (μm)	Shape
290 mOsm			
Slice #			
35	360.1	72.2	0.86
37	413.0	77.6	0.85
48	384.7	80.0	0.75
52	384.7	80.6	0.88
53	448.0	82.6	0.83
59	427.6	80.2	0.84
63	441.1	80.1	0.86
65	486.8	83.7	0.87
70	461.0	82.2	0.84
Mean	431.2	79.9	0.84
SE	13.3	1.1	0.03
340 mOsm			
Slice #			
38	522.3	88.9	0.87
43	386.9	74.7	0.88
46	396.0	76.3	0.85
56	389.1	73.4	0.90
58	405.8	77.7	0.84
66	499.0	83.2	0.89
67	367.5	73.1	0.86
69	442.4	79.0	0.88
Mean	429.9	78.2	0.87
SE	22.8	1.9	0.01
t =	0.05	0.76	-1.92
p =	0.47	0.46	0.07

APPENDIX C (Cont'd.)

TABLE 10 - AVERAGE FIRING RATES

290 mOsm/kg		**340 mOsm/kg**	
Cell #	Rate (Hz)	Cell #	Rate (Hz)
8	2.7	1	5.5
11	3.4	2	3.4
15	7.6	5	9.1
18	5.0	6	6.9
20	5.2	7	11.9
21	7.9	27	7.7
22	5.8	29	2.7
23	3.3	30	5.5
24	3.3		
33	8.7		
34	2.7		
35	3.4		
Mean	4.70		7.49
SE	0.71		1.30

APPENDIX C (cont'd.)

TABLE 11 - DENDRITIC SYNAPSES

290 mOsm/kg Slice #	Total # Terminals	Terminals Apposed to >1 Dendrite	Multiple Synapses w/pr*	Multiple Synapses w/cv#
35	238	43	3	5
52	328	43	7	9
53	231	46	4	4
59	307	79	7	3
65	279	56	5	12
340 mOsm/kg Slice #				
38	241	47	11	9
46	338	92	24	12
56	486	90	12	9
58	358	71	13	21
66	312	86	8	12

*pr - polyribosomes

#cv - coated vesicles

APPENDIX C (cont'd.)

TABLE 12 - DENDRITIC MULTIPLE SYNAPSES

290 mOsm/kg Slice #	% Terminals Apposed to >1 Dendrite	% Multiple Synapses w/pr	% Multiple Synapses w/cv
35	18.07	6.98	11.63
52	13.11	16.28	20.93
53	19.91	8.70	8.70
59	22.48	10.14	4.35
65	10.07	8.93	21.43
MEAN	18.73	10.20	13.41
SE	1.57	1.60	3.38
340 mOsm/kg Slice #			
38	19.50	23.40	19.15
46	27.22	26.09	13.04
56	18.52	13.33	10.00
58	19.83	18.31	29.58
66	27.56	9.30	13.95
MEAN	22.53	18.09	17.14
SE	2.00	3.10	3.34
t =	-1.49	-2.26	-0.77
p =	0.17	0.05	0.47

APPENDIX C (cont'd)

TABLE 13 - PERCENTAGE OF DENDRITIC MEMBRANE CONTACTED BY CELLULAR ELEMENTS

290 mOsm/kg Slice #	Single Synapses	Multiple Synapses	Adjacent Dendrites
35	5.87	2.36	9.11
52	6.93	1.96	7.91
53	5.90	2.86	10.00
59	4.68	2.35	8.73
65	5.44	2.44	12.84
MEAN	5.74	2.39	9.72
SE	0.36	0.14	0.85
340 mOsm/kg Slice #			
38	5.73	2.42	8.07
46	5.45	4.48	10.78
56	7.23	2.50	8.32
58	5.31	3.50	10.11
66	6.32	2.03	10.69
MEAN	6.01	2.98	9.59
SE	0.35	0.44	0.58
t =	0.48	-1.27	0.12
p =	0.64	0.23	0.90

APPENDIX C (cont'd)

TABLE 14 - AVERAGE LENGTH (μm) OF DENDRITIC SYNAPSES AND PERCENTAGE OF DENDRITIC AXONAL CONTACT MADE BY MULTIPLE SYNAPSES

	Single Synapses	Multiple Synapses	% Axonal Contact made By Multiples
290 mOsm/kg			
Slice #			
35	0.86	0.74	28.67
52	0.89	0.78	22.05
53	0.92	0.71	32.57
59	0.90	0.71	33.43
65	0.80	0.75	30.96
MEAN	0.87	0.74	29.54
SE	0.05	0.01	2.04
340 mOsm/kg			
Slice #			
38	0.85	0.71	26.56
46	0.87	0.80	56.63
56	0.79	0.69	25.00
58	0.85	0.66	40.09
66	0.79	0.67	15.81
MEAN	0.83	0.72	32.82
SE	0.02	0.02	7.10
t =	1.60	1.16	-0.44
p =	0.14	0.28	0.66

APPENDIX D - DATA TABLE

EXPERIMENT II

APPENDIX D

DATA TABLE - EXPERIMENT II

TABLE 15 - PERCENTAGE OF SYNAPSES THAT WERE MULTIPLE SYNAPSES

290 mOsm/kg		**340 mOsm/kg**	
Slice #	% Multiple Synapses	Slice #	% Multiple Synapses
115	15	107	15
120	15	110	17
121	12	113	12
		119	14
		122	10
MEAN	14.33		13.60
SE	1.20		1.20
t =	0.40		
p =	0.70		

APPENDIX E - DATA TABLES

EXPERIMENT III

APPENDIX E

DATA TABLES - EXPERIMENT III

TABLE 16 - STRUCTURES CONTACTING MNC SOMATIC MEMBRANE*

1 HOUR INCUBATION TIME

	Glial Processes		Single Synapses		Multiple Synapses		Direct Apposition#		Not Identified		Total (μm)
290 mOsm/kg Slice #	(μm)	%	(μm)	%	(μm)	%	(μm)	%	(μm)	%	
189	665	89.9	39.9	5.39	3.7	0.50	28.5	3.85	3.1	0.47	740
193	644	85.8	72.0	9.59	0.8	0.11	33.5	4.46	0.0	0.00	751
194	639	88.0	64.1	8.84	4.2	0.58	18.4	2.54	0.0	0.00	726
207	575	85.5	73.0	10.87	6.2	0.92	17.9	2.67	0.0	0.00	672
209	609	82.6	78.7	10.66	11.8	1.60	37.8	5.13	0.0	0.00	737
340 mOsm/kg Slice #											
190	603	80.5	67.0	8.94	5.1	0.68	73.4	9.81	0.0	0.00	749
192	735	87.7	52.2	6.23	2.3	0.28	48.4	5.77	0.0	0.00	838
195	613	84.5	88.0	12.14	5.0	0.69	19.0	2.62	0.0	0.00	725
197	594	84.4	76.0	10.80	17.2	2.45	16.7	2.37	0.0	0.00	703
206	616	85.9	67.4	9.39	9.4	1.31	24.3	3.38	0.0	0.00	717
208	706	89.9	39.5	5.04	9.4	1.19	27.7	3.53	2.8	0.36	785

*See Tables 3 & 4 for Anova Results and Group Means #Direct Soma-somatic/dendritic membrane apposition

APPENDIX E (cont'd.)

TABLE 16 (cont'd.)

2.5 HOUR INCUBATION TIME

290 mOsm/kg Slice #	Glial Processes (μm)	%	Single Synapses (μm)	%	Multiple Synapses (μm)	%	Direct Apposition (μm)	%	Not Identified (μm)	%	Total (μm)
160	657	78.5	86.1	10.29	26.0	3.11	67.7	8.09	0.0	0.00	837
161	766	84.7	86.6	9.58	16.2	1.79	27.6	3.06	8.1	0.90	905
170	793	86.0	57.6	6.25	10.3	1.12	55.8	6.05	5.1	0.55	922
176	657	87.0	64.0	8.48	6.6	0.88	15.9	2.11	11.2	1.49	755
184	722	83.1	79.1	9.09	11.6	1.34	55.3	6.36	1.2	0.14	870
185	711	78.6	52.4	5.79	19.7	2.18	121.5	13.44	0.0	0.00	904
340 mOsm/kg Slice #											
159	803	86.8	67.6	7.32	11.6	1.26	38.3	4.14	4.2	0.46	925
162	666	82.3	88.1	10.89	10.4	1.28	36.9	4.56	7.8	0.97	809
169	709	86.4	66.9	8.15	9.3	1.14	17.7	2.15	18.1	2.20	820
181	667	84.2	71.4	9.03	12.6	1.60	37.5	4.74	3.2	0.40	791
188	716	87.3	56.4	6.87	7.3	0.89	35.9	4.37	4.4	0.54	821

APPENDIX E (cont'd.)

TABLE 16(cont'd.)

5.5 HOUR INCUBATION TIME

290 mOsm/kg Slice #	Glial Processes (μm)	%	Single Synapses (μm)	%	Multiple Synapses (μm)	%	Direct Apposition (μm)	%	Not Identified (μm)	%	Total (μm)
125	815	84.4	84.1	8.71	6.4	0.66	84.1	6.23	0.0	0.00	965
130	655	86.7	50.6	9.34	11.1	1.47	11.4	1.51	7.5	0.99	755
132	726	83.0	70.3	8.03	16.0	1.82	56.5	6.46	6.4	0.73	875
138	765	83.2	64.7	7.04	13.9	1.51	63.9	6.89	12.6	1.38	920
139	661	81.4	100.9	12.43	3.8	0.47	34.5	4.25	11.5	1.42	812
141	619	83.8	55.1	7.47	12.1	1.63	39.3	5.32	12.8	1.73	738
146	723	83.6	78.4	9.06	18.0	2.08	23.3	2.69	22.1	2.56	865
149	719	86.1	62.4	7.48	9.8	1.17	38.5	4.61	5.2	0.63	834
340 mOsm/kg Slice #											
124	656	76.9	61.3	7.19	5.8	0.89	116.8	13.70	11.1	1.30	852
129	666	82.3	77.3	9.54	11.1	1.37	44.1	5.41	10.4	1.28	810
134	673	84.6	65.9	8.29	15.8	1.98	34.3	4.31	6.3	0.79	795
137	589	81.8	80.6	11.19	9.7	1.35	38.0	5.28	2.5	0.35	720
143	706	80.5	96.7	11.02	10.0	1.14	48.2	5.50	16.2	1.85	877
150	673	84.1	84.2	10.52	20.6	2.58	19.9	2.37	3.2	0.40	800

APPENDIX E (cont'd.)

TABLE 17 - PERCENTAGE OF SYNAPSES THAT ARE MULTIPLE AND PERCENTAGE OF TOTAL AXONAL CONTACT MADE BY MULTIPLE SYNAPSES

	290 mOsm/kg			**340 mOsm/kg**	
1 HOUR	%	% Axonal		%	% Axonal
Slice #	Synapses	Contact	Slice #	Synapses	Contact
189	9.09	8.55	190	7.93	7.09
193	3.57	1.17	192	4.35	4.25
194	8.33	6.14	195	5.26	5.40
207	7.04	7.84	197	18.18	18.50
209	15.07	13.07	206	12.28	12.27
			208	18.92	19.04
2.5 HOUR					
Slice #					
160	21.83	23.26	159	17.14	14.65
161	18.07	15.73	162	12.00	10.52
170	20.00	15.17	169	13.85	12.25
176	10.17	9.38	181	12.99	15.05
184	14.29	12.82	188	13.56	11.48
185	23.53	27.38			
5.5 HOUR					
Slice #					
125	9.86	7.05	124	15.62	11.01
130	14.28	13.60	129	16.42	12.60
132	15.66	18.52	124	19.12	19.32
138	15.15	17.70	137	13.33	10.75
139	4.60	3.68	143	9.18	9.40
141	18.33	17.96	150	19.78	19.68
146	21.59	18.69			
149	15.94	13.57			

APPENDIX E (cont'd.)

TABLE 18 - AVERAGE LENGTH (μm) OF SINGLE AND MULTIPLE SYNAPSES

	290 mOsm/kg			**340 mOsm/kg**	
1 HOUR					
Slice #	Single Synapses	Multiple Synapses	Slice #	Single Synapses	Multiple Synapses
189	0.998	0.934	190	1.154	1.02
193	1.333	0.428	192	1.187	1.17
194	1.482	1.049	195	1.222	1.25
207	1.106	1.242	197	1.207	1.23
209	1.269	1.075	206	1.348	1.35
			208	1.318	1.34
2.5 HOUR					
Slice #					
160	1.266	1.370	159	1.166	0.968
161	1.274	1.079	162	1.335	1.152
170	1.201	0.859	169	1.194	1.037
176	1.208	1.104	181	1.066	1.266
184	1.199	1.058	188	1.106	0.914
185	1.007	1.234			
5.5 HOUR					
Slice #					
125	1.314	0.911	124	1.135	0.759
130	1.176	1.111	129	1.380	1.013
132	1.005	1.229	134	1.198	1.214
138	1.160	1.391	137	1.240	0.971
139	1.220	0.962	143	1.087	1.114
141	1.125	1.870	150	1.153	1.146
146	1.136	0.948			
149	1.076	0.891			

APPENDIX E (cont'd)

TABLE 19 - NUMBER OF SINGLE AND MULTIPLE SYNAPSES PER 100 μm OF SOMATIC MEMBRANE

	290 mOsm/kg			340 mOsm/kg	
1 HOUR					
Slice #	Single Synapses	Multiple Synapses	Slice #	Single Synapses	Multiple Synapses
189	5.41	0.54	190	7.75	0.67
193	7.19	0.27	192	5.25	0.24
194	6.06	0.55	195	9.93	0.55
207	9.82	0.74	197	8.95	1.99
209	8.41	1.49	206	6.97	0.98
			208	3.82	0.89
2.5 HOUR					
Slice #					
160	8.13	2.27	159	6.27	1.30
161	7.52	1.66	162	8.15	1.11
170	5.21	1.30	169	6.82	1.10
176	7.02	0.79	181	8.46	1.26
184	7.59	1.26	188	6.21	0.97
185	5.75	1.77			
5.5 HOUR					
125	6.63	0.72	124	6.33	1.17
130	7.94	1.32	129	6.91	1.36
132	7.99	1.48	134	6.92	1.64
138	6.09	1.09	137	9.02	1.39
139	10.23	0.49	143	10.14	1.03
141	6.64	1.49	150	9.12	2.25
146	7.98	2.20			
149	6.95	1.32			

APPENDIX E (cont'd)

TABLE 20 - NUMBER OF DIRECT SOMA-SOMATIC/DENDRITIC MEMBRANE APPOSITIONS PER 100 μm OF SOMATIC MEMBRANE AND THEIR AVERAGE LENGTH (μm)

	290 mOsm/kg			**340 mOsm/kg**	
1 HOUR					
Slice #	Length	#/100μm	Slice #	Length	#/100 μm
189	1.78	2.16	190	2.93	3.34
193	1.67	2.66	192	1.86	3.10
194	1.15	2.20	195	1.90	1.38
207	1.49	1.79	197	1.28	1.85
209	1.30	3.93	206	1.52	2.23
			208	2.31	1.53
2.5 HOUR					
Slice #					
160	1.65	4.90	159	1.47	2.81
161	1.31	2.32	162	1.37	3.34
170	1.80	3.36	169	1.04	2.07
176	1.06	1.99	181	1.44	3.28
184	1.54	4.14	188	1.89	2.32
185	1.95	6.86			
5.5 HOUR					
Slice #					
125	1.67	3.73	124	2.12	6.45
130	1.04	1.46	129	1.52	3.58
132	2.26	2.85	134	2.14	2.01
138	1.47	4.67	137	1.52	3.47
139	2.46	1.72	143	1.72	3.19
141	1.87	2.84	150	1.92	2.00
146	1.11	2.43			
149	1.18	2.40			

APPENDIX E (cont'd)

TABLE 21 - SOMATIC SIZE AND SHAPE MEASURES

1 HOUR	290 mOsm/kg				340 mOsm/kg		
Slice #	Area (μm^2)	Perimeter (μm)	Shape	Slice #	Area (μm^2)	Perimeter (μm)	Shape
189	327	71.0	0.802	190	345	69.8	0.871
193	328	70.3	0.838	192	365	73.0	0.851
194	297	65.1	0.863	195	312	66.9	0.870
207	344	70.3	0.866	197	313	66.6	0.872
209	283	63.3	0.875	206	295	67.2	0.818
				208	335	69.1	0.879
2.5 HOUR							
Slice #							
160	343	71.0	0.735	159	300	66.0	0.675
161	324	68.7	0.731	162	333	70.7	0.689
170	281	65.3	0.720	169	285	65.1	0.669
176	274	63.1	0.739	181	288	65.7	0.731
184	257	73.2	0.737	188	277	64.2	0.697
185	286	63.2	0.768				
5.5 HOUR							
Slice #							
125	249	60.1	0.859	124	262	65.1	0.784
130	269	64.0	0.822	129	261	63.0	0.826
132	294	67.9	0.803	134	273	72.2	0.674
138	247	62.4	0.790	137	244	58.9	0.877
139	262	61.7	0.858	143	269	66.8	0.794
141	274	65.4	0.809	150	266	63.0	0.842
146	273	63.3	0.852				
149	304	66.2	0.878				

APPENDIX E (cont'd.)

TABLE 22 - MEASURED OSMOLALITY OF CHAMBER MEDIUM

5.5 HOUR				2.5 HOUR				1.0 HOUR			
290 mOsm/kg		340 mOsm/kg		290 mOsm/kg		340 mOsm/kg		290 mOsm/kg		340 mOsm/kg	
Slice #	Osm	Slice #	Osm	Slice #	Osm	Slice #	Osm	Slice #	Osm	Slice #	Osm
125	323	124	?	160	290	159	341	189	277	190	347
130	295	129	350	161	283	162	322	193	301	192	353
132	254	134	259	170	307	169	347	194	302	195	340
138	279	137	273	176	300	181	351	207	300	197	342
139	201	143	310	184	307	188	326	209	282	208	325
141	254	150	248	185	307						
146	285										
149	291										

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