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OLFACTORY EFFERENTS TO THE HYPOTHALAMIC PARAVENTRICULAR AND SUPRAOPTIC NUCLEI: AN ANATOMICAL AND ELECTROPHYSIOLOGICAL ANALYSIS IN THE RAT

by

Kenneth George Smithson II

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ABSTRACT

OLFACTORY EFFERENTS TO THE HYPOTHALAMIC PARAVENTRICULAR AND SUPRAOPTIC NUCLEI: AN ANATOMICAL AND ELECTROPHYSIOLOGICAL ANALYSIS IN THE RAT

by

Kenneth George Smithson II

The morphological and physiological features of a putative connection between the main and accessory olfactory bulbs and the supraoptic (SON) and paraventricular nuclei (PVN) of the rat were studied using a combination of anatomical and electrophysiological techniques. Neurophysin immunocytochemistry revealed the supraoptic nucleus dendritic plexus which coursed anteroposteriorly ventral to supraoptic somata. Additionally, a portion of this plexus also projected ventrolaterally into periamygdaloid areas, a feature of supraoptic nucleus architecture which is not generally appreciated. Injections of the anterogradely transported substances, wheatgerm agglutinin conjugated horseradish peroxidase (WGA-HRP) or *Phaseolus vulgaris* leucoagglutinin into the main accessory bulb and injections of WGA-HRP into the accessory bulb revealed a dense plexus of terminals and fibers ventrolateral

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to the ipsilateral supraoptic nucleus. The pattern of anterogradely labeled fibers and terminals appeared to overlap with the distribution of ventrolaterally projecting neurophysin-containing processes. Since the latter consists of dendritic processes of supraoptic origin, this suggests that both olfactory bulbs project to the SON. No labeled fibers appeared near the PVN. Injections of rhodamine-labeled latex microspheres or Fluoro-Gold® resulted in retrogradely labeled mitral cells throughout the ipsilateral main and accessory bulbs. No mitral cells were retrogradely-labeled after an injection into the PVN. Electrophysiological analysis of this connection using an explant preparation confirmed the existence of a short, variable latency, excitatory response to electrical stimulation of the lateral olfactory tract. These responses were reversibly blocked by the excitatory amino acid receptor antagonist, kynurenic acid. Taken together the anatomical and physiological studies demonstrate a direct monosynaptic connection from the main and accessory bulbs which is excitatory and is mediated through an excitatory amino acid receptor on SON neurons.

To m

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DEDICATION

To my mother Rosemary, who endowed me with an indomitable spirit, without which I would not have reached so high, nor traveled so far, and to my family and friends who have supported me through this long endeavor.

ACKNOWLEDGMENTS

In my ten short years in Glenn Hatton's lab I have made many good friends and colleagues. I have reached the successful culmination of this journey only through your support and love. Thank you all! I wish I could recognize each of you individually, but that would require another volume.

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Glenn Hatton, my mentor, has carefully guided me during this journey in many ways. His approach to research and insight into "our" system have substantially shaped my own science. He has taught me many skills (too many to list), of these, his careful attention to my writing I'll cherish the most. He has generously supported my research providing me with all that I needed to pursue interesting projects. Need I say more. Thank you Glenn. Glenn and his wonderful wife Pat have also cared for me as if I were part of their family. This love and support has nurtured my growth. Thank you both.

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The projects that entail this dissertation took several years and consumed much time, not only of myself but of Kevin Grant, Farshid Marzban, Louise Koran, Inge Smithson, Alisa Zapp, and Doug Hollowell. Thanks you all.

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Several portions of the dissertation were anatomical studies, and in this Dr. M. L. Weiss's contributions have added immeasurably to the quality of the project. Thanks very much Mark, I've learned much from you and I am a better anatomist for it. I also like to thank Drs. L. Smith and B. Spann, fellow anatomists, who offered helpful suggestions on this work.

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Dr. A. Nunez deserves a special thanks. He has become a friend and colleague. His perspectives on research have forced me to defend and often reevaluate my reductionist approach. Perhaps his most important contribution was as my squash partner. This activity has afforded me good physical and mental health during this long task. Thanks Tony!

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Many thanks go to my family, while they had little understanding of this undertaking they unconditionally supported me through this endeavor. A special thanks go to the Sloths Dave and Bob, true friends and confidants. Finally, but certainly not least I like to thank my wife Inge, her love and patience have supported me through these many months. She has also provided much help for the preparation of figures for this documents. Thanks much Wiffo!

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IIn	optic nerve,
3V	third cerebral ventricle;
ACo	anterior cortical amygdaloid nucleus;
AH	anterior hypothalamic area;
AOB	accessory olfactory bulb;
AOD	anterior olfactory nucleus, dorsal subdivision;
AOE	anterior olfactory nucleus ,external portion;
AOL	anterior olfactory nucleus, lateral subdivision;
AON	anterior olfactory nucleus;
AOV	anterior olfactory nucleus, ventral subdivision;
BAOT	bed nucleus of the accessory olfactory tract;
bv	blood vessel;
DAB	3,3' diaminobenzidine tetrahydrochloride
EAA(s)	excitatory amino acid(s);
EPl	external plexiform layer of the main olfactory bulb;
epsp(s)	excitatory postsynaptic potential(s);
Fluoro-Gold	Fluoro-Gold®;
fx	fornix;
Gl	glomerular layer of the main olfactory bulb;
Gr	granule cell layer of main bulb;
GrA	granule cell layer of accessory bulb;
HDB	nucleus of the horizontal limb of the diagonal band;
HNS	hypothalamo-neurohypophysial system;
HRP	horseradish peroxidase;
LH	lateral hypothalamic area;
LOT	lateral olfactory tract;
Me	medial nucleus of amygdala;
Mi	mitral cell layer of the main olfactory bulb;
MiA	mitral cell layer of accessory olfactory bulb;
MOB	main olfactory bulb;
NLOT	nucleus of the lateral olfactory tract;
NAAG	N-acetyl-L-aspartylglutamate
NMDA	N-methyl-D-aspartate;
OC	optic chiasm;
OT	optic tract;
OVLT	organum vasculosum of the lamina terminalis

OX
PBS
Pe
PHA-
Pir
PLCo
PMC
psp(s)
PVN
SCN
SON
SONt
TBS
TT1
Tu
VMH
VP
WGA-

OX	oxytocin;
PBS	phosphate-buffered saline;
Pe	periventricular hypothalamic nucleus;
PHA-L	*Phaseolus vulgaris* leucoagglutinin;
Pir	piriform cortex;
PLCo	posterolateral cortical amygdaloid nucleus;
PMCo	posteromedial cortical amygdaloid nucleus;
psp(s)	postsynaptic potential(s);
PVN	paraventricular nucleus, hypothalamus;
SCN	suprachiasmatic nucleus;
SON	supraoptic nucleus-main (or anterior) portion;
SONt	supraoptic nucleus, tuberal subdivision;
TBS	Tris-buffered saline;
TT_1	ventral tenia tecta
Tu	olfactory tubercle;
VMH	ventral medial nucleus hypothalamus;
VP	vasopressin;
WGA-HRP	wheatgerm agglutinin conjugated to horseradish peroxidase.

1. The Hypothalamic-Neurohypophysial System

The hypothalamo-neurohypophysial system (HNS) is composed of several prominent aggregations of cells located in the anterior hypothalamus. These include the supraoptic (SON), paraventricular (PVN), and anterior commissural nuclei. Several additional smaller cell groups have also been described (Fisher et al.,1979; Peterson,1966), and are collectively referred to as accessory nuclei. A common feature of these cells is their prominent efferent projection to the neurohypophysis (Fig. 1). The major secretory products of the HNS are the neurohormones oxytocin and vasopressin which are released from the terminals located within the posterior pituitary. Unlike many other neuronal systems, the output of this system (i.e. vasopressin and oxytocin) has well defined functional consequences.

The most notable function of vasopressin is to increase reabsorption of water at the collecting duct of the kidney. Oxytocin plays an equally prominent role in the processes of parturition and milk ejection by stimulating contraction of the smooth muscles of the uterus and of the myoepithelia of the breast. Understanding the function of oxytocin and vasopressin has permitted the design of experimental paradigms which manipulate (i. e. stimulate or inhibit) the quantity of these hormones released; an approach which has been profitably employed by many investigators to explore both central and peripheral mechanisms which underlie changing patterns of hormone release. Under this scrutiny, much has been learned about the HNS, which has emerged as a model system for the study of many facets of neurosecretion.

Fig.1. Sch

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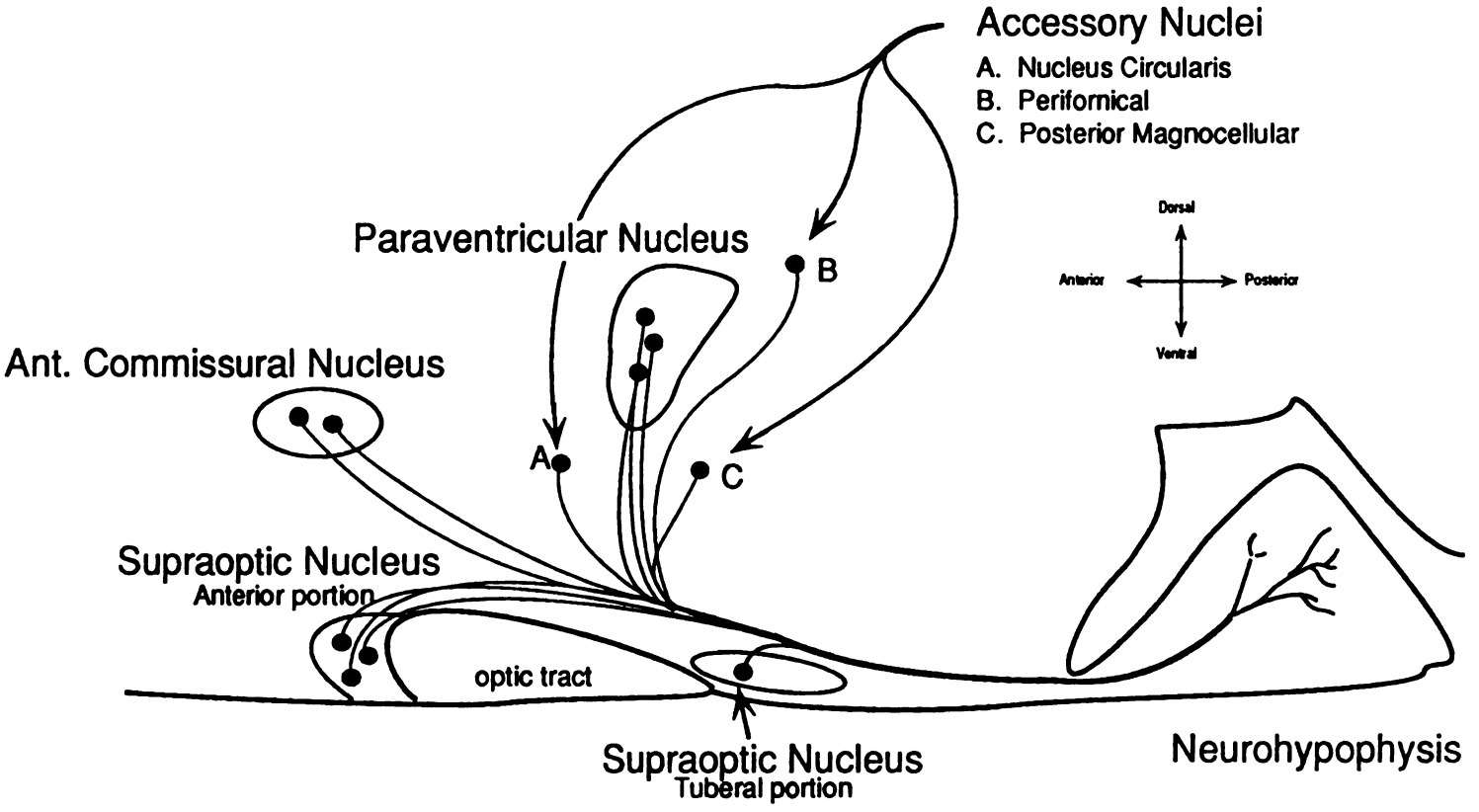
Fig.1. Schematic of the hypothalamo-neurohypophysial system.

Schematic drawing of the HNS in the sagittal plane illustrating the prominent nuclei within this system and their primary efferent to the neurohypophysis.


Accessory Nuclei
A. Nucleus Circularis
B. Perifornical
C. Posterior Magnocellular
Dorsal
Anterior
Posterior
Ventral
Paraventricular Nucleus
B
Ant. Commissural Nucleus
A
C
Supraoptic Nucleus
Anterior portion
optic tract
Supraoptic Nucleus
Tuberal portion
Neurohypophysis

One important aspect in our understanding of the control of oxytocin and vasopressin secretion is the description of afferent input to the HNS. Such information provides insights into the pathways required to transduce and/or relay the changing demands placed upon the animal and may also lead to a clearer understanding of the integrative mechanisms which transform the seemingly stochastic volley of incoming signals into a meaningful physiological response.

1.1. Olfactory Efferents to the HNS?

Suggestions that the main olfactory bulb (main bulb or MOB) may project directly to the region surrounding the SON have appeared several times in the literature over the past decade or so. Using degeneration methods, after main bulb ablation, Scalia and Winans (1975) described terminal fields in the area immediately ventral and lateral to the more posterior portions of the SON. These authors interpreted the projections as terminating in the region bordered medially by the SON and laterally by the anteroventromedial portion of the medial amygdaloid nucleus. A similar pattern of terminal degeneration was found by Heimer, using the same method (Heimer,1978). Precluded by the limitations inherent in the Fink-Heimer method was fine resolution of the terminal distribution in the region. For example, it is difficult to distinguish fine diameter terminals from background and nonspecific staining of the pial membrane. The latter is of particular importance for terminals contacting SON dendrites which run in the ventral glial lamina subjacent to the pial surface. This problem was further exacerbated by the lack of detailed information regarding the SON dendritic architecture.

In a review, Brooks et al.(1980) made the most positive statement concerning the anatomical connections of the olfactory bulb with the SON.

They stated that horseradish peroxidase tracing had shown that "the olfactory bulb seems to project monosynaptically upon SON as seen in the guinea pig...", and then cited "J.R. DeOlmos, personal communication, 1978." No experimental paper has appeared confirming this claim. This statement is intriguing in that such a connection could provide the HNS with direct sensory information. This is a relationship in the hypothalamus which heretofore has been exclusively reserved to the suprachiasmatic nucleus (see, however, Youngstrom et al.,1987).

1.2. Thesis Objectives

The experiments in this thesis are designed to test the hypothesis that the main and/or accessory olfactory bulbs project to nuclei within the HNS and, more specifically, the SON. In order to evaluate this hypothesis, specific experiments were designed to: 1) delineate the somatic and dendritic organization of the SON, 2) determine the distribution of main bulb efferents in relationship to the SON and PVN; 3) similarly investigate accessory bulb efferents to the SON and PVN, 4) ascertain the type and regional distribution of projection neurons contributing to this putative HNS afferent and 5) evaluate some of the neurophysiological and neuropharmacological properties of this putative connection.

Much of the data presented is of special relevance to only the SON, and not the PVN or accessory nuclei of the HNS. Hence, much of what is written will be limited to the SON. Nevertheless, since the SON contains almost twice as many neurosecretory cells as other nuclei within the HNS, modulation of SON activity alone could play a prominent role in altering the profile of oxytocin and vasopressin secretion. Furthermore, the SON lies very close to the subarachnoid space, and nearby the cisterna chiasma; this juxtaposition suggests that the SON may also secrete oxytocin and

vasopressin into the cerebral spinal fluid. This would greatly extend the influence of the SON to include many central targets as well as the well-known peripheral effects.

1.3. Experimental Approach

In the years following these earlier studies, techniques have been developed allowing a much finer resolution of axon terminals at the light microscopic level. Other new methods also permit high resolution retrograde transport studies to be done in confirmation of the data obtained by anterograde methods. Furthermore, with the development of brain slices and explant preparations, much more of the mammalian brain is now accessible to study using intracellular electrophysiological techniques.

In order to characterize the anatomical and physiological properties of a putative connection, a variety of techniques are required. Each method presents a somewhat biased view of the structure under study. Therefore the use of several techniques to illustrate the features of a pathway permits the investigator to identify incongruent findings. This reduces the interpretational errors that may result when only one approach is employed.

Accordingly, the possible existence of direct projections from the main and accessory olfactory bulbs to the SON was investigated using: 1) neurophysin immunocytochemistry to delineate the extent of the SON dendritic zone, 2) anterograde transport of wheatgerm agglutinin-horseradish peroxidase conjugate (WGA-HRP) after injection into either the main or accessory bulb, or *Phaseolus vulgaris* leucoagglutinin (PHA-L) after injection into the main bulb, to label bulb efferents, 3) retrograde transport of rhodamine-labeled latex microspheres (rhodamine beads) or Fluoro-Gold®(Fluoro-Gold) after injection into the SON and PVN to determine projection neurons and, 4) intracellular electrophysiological analysis of

"olfactory"-evoked responses from *in vitro* incubated explants to identify neurophysiological and pharmacological properties of this connection.

1.4. Organization of the Dissertation

The dissertation is organized into 12 sections. Section two provides a brief overview of the anatomy and physiology of the SON. No attempt has been made to completely review this large literature. For this the reader is referred to several recent reviews by Hatton (1990), Morris (1987), Poulain (1982), Silverman (1983), Swanson and Sawchenko (1983), and Wakerley (1987). Rather it provides a framework of essential material for those less familiar with this system. Likewise section three provides a brief introduction to the anatomy of the olfactory system. More complete descriptions may be found in reviews by Scott (1986), Doty (1986), Mair (1986), Wysocki (1986), and Switzer (1985). Sections four to nine contain the experiments comprising this dissertation. Section ten summarizes the results and further discusses the implication of these observations. Section eleven is a group of appendices providing additional details of the materials and methods employed in these experiments.and, finally, section twelve provides the references cited

2. Anatomy of Supraoptico-Neurohypophysial System

The supraoptic nucleus may be subdivided into an anterior portion (SONa or simply SON) and a tuberal (SONt; Bleier et al.,1979) or retrochiasmatic (Peterson,1966) portion. The SONa is located lateral to the optic tract, beginning rostrally around the medial preoptic area and extends caudally to the mid rostrocaudal extent of the anteromedial subdivision of the medial amygdala. This group of cells comprises approximately 41% of the cells within the HNS (Rhodes et al.,1981), the largest percentage of any cell group. The SONt is located medial to the optic tract and posterior to the SONa and contains approximately 7% of the neurons within the HNS (Rhodes et al.,1981).

2.1. Intrinsic Organization of the SON: somatic and dendritic domains

The SONa is primarily composed of large ovoid cells with one to three simply branching dendrites (Dyball and Kemplay,1982; Randle et al.,1986). The ovoid cell bodies are aggregated into a densely-packed "somatic zone" located dorsally within the nucleus. SON somata project their dendritic processes ventrally into a soma-free area immediately superior to the ventral glial limitans. Dendrites within this "dendritic zone" project anteroposteriorly beneath the somatic portion of the nucleus. Individual dendritic processes may extend for over 40% of the rostrocaudal extent of the nucleus within this zone (Randle et al.,1986). A separate dendritic projection has also been described which extends ventrolaterally outside the confines of the "dendritic zone" into the periamygdaloid cortex (Armstrong et al.,1982; Ju et al.,1986; Smithson et al.,1989a).

2.2. Supraoptic Efferents

Axonal processes are formed as either separate processes projecting directly off the cell body, or more frequently as a bifurcation of a dendritic process (Bruni and Perumal,1984; Hatton,1990; Randle et al.,1986). These processes project dorsomedially out of the somatic portion of the nucleus. Once outside the nucleus, axons occasionally bifurcate to form a small caliber collateral processes (Mason et al.,1984; Randle et al.,1986). These collaterals are relatively short, ending within the relatively cell-free perinuclear zone surrounding the dorsolateral borders of the nucleus. The parent axon continues medially into the retrochiasmatic area where axons from the other nuclear groups (e.g. paraventricular and accessory nuclei) join, turn posteriorly to project through the pituitary stalk, eventually terminating within the neurohypophysis.

2.3. Supraoptic Somatic Neurochemistry

It has now been amply illustrated using immunocytochemical techniques that the SON somata contain either oxytocin or vasopressin (Rhodes et al.,1981; Swaab et al.,1975a; Swaab et al.,1975b; Vandesande and Dierickx,1975) along with their respective "carrier proteins" neurophysin I or neurophysin II, respectively (DeMey et al.,1974). It is also clear from these studies and elegantly demonstrated in more recent double-labeling experiments (Hou-Yu et al.,1986) that each neuron produces either vasopressin or oxytocin, but not both. Several authors have described a preferential distribution of oxytocin and vasopressin neurons within the SON (Rhodes et al.,1981; Vandesande and Dierickx,1975). It is unlikely, however, that this organization is maintained within the dendritic zone given the length of the dendritic processes (Randle et al.,1986) and their meandering course within this zone (Hatton,1990; Randle et al.,1986).

Oxytocin and vasopressin are, by far, the most abundant neurosecretory product of SON neurons. However, several other compounds have been co-localized using immunocytochemical techniques within SON somata. These include cholecystokinin (Beinfeld and Palkovits,1981; Vanderhaeghen et al.,1981a; Vanderhaeghen et al.,1981b), dynorphin (Watson et al.,1982), galanin (Gaymann and Martin,1989), angiotensin II (Kilcoyne et al.,1980) glucagon (Tager et al.,1981), and endothelin (Yoshizawa et al.,1990).

2.4. Oxytocin and Vasopressin Production

The preprohormones of oxytocin and vasopressin are synthesized on the rough endoplasmic reticulum of the magnocellular neuroendocrine cells as large pro-hormones (for reviews see Brownstein et al.,1980; Castel et al.,1984; North,1987). These peptides are further processed in the Golgi complex where they are packaged into neurosecretory granules. These immature granules are then transported down the axon by fast axonal transport to eventually reach the terminals within the posterior pituitary. During the transport process, granules undergo a process of maturation whereby the prohormone is cleaved into two products: the active hormone (i.e. vasopressin or oxytocin) and their carrier proteins, neurophysin II, or neurophysin I, respectively. There is no known physiological function as yet for these latter peptides which are also released from the posterior pituitary.

2.5. Stimulus-Secretion Coupling

The process of hormone release is initiated by an action potential from the supraoptic cell body. This action potential is propagated down the unmyelinated axon to the terminal. As the action potential invades the terminal the resulting depolarization results in an influx of Ca^{2+}, from the extracellular environment through voltage-gated Ca^{2+} channels (Cazalis et

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al.,1987; Lemos and Nordmann,1986). Through a cascade of events poorly understood, the elevated intracellular calcium results in the fusion of neurosecretory granules with the terminal membrane and results in the release of oxytocin and vasopressin into the perivascular space of the posterior pituitary. After passing through both the basal lamina and fenestrations in the endothelial membrane the hormones gain access to the general circulation and their various target organs.

2.6. Peripheral Effects of Oxytocin

Oxytocin has two known major peripheral targets: the smooth muscle of the uterus and the myoepithelia of the breast. Two main effects produced by oxytocin on uterine tissue may account for its role in parturition: 1) oxytocin initiates and/or increases the frequency of contractions of the myometrium and 2) oxytocin stimulates prostaglandin synthesis. The effect of oxytocin on myometrial contractions is mediated by receptors located on the sarcolemmal membrane. Similar to its action on the myometrium, oxytocin induces a receptor mediated contraction of the myoepithelial cells of the breast, resulting in the ejection of milk into the lactiferous ducts. It is clear from both rat (Grosvenor et al.,1986) and human (Leake & Fisher, 1985) studies that oxytocin levels rise significantly after the initiation of suckling and are concomitant with the rise in intramammary pressure (Higuchi et al.,1985). In addition to these well-known effects, oxytocin also increases both insulin and glucagon levels in the rat (Dunning et al.,1982; Dunning et al.,1984; Dunning et al.,1985).

2.7. Peripheral Effects of Vasopressin

Vasopressin's most preeminent effect is on the kidney; here this hormone acts through several mechanisms to increase the reabsorption of water at the collecting duct of the nephron (Valtin,1987). The prominent

effects in this process are mediated through a V2 receptor which stimulates a cyclic AMP cascade which eventually increases water permeability of the collecting duct (Muller and Kachadorian,1984). Vasopressin also seems to increase the reabsorption of NaCl in the ascending loop of Henle and increase glomerular filtration rate in juxtamedullar, but not cortical, nephrons. Both these events further increase the ability of the kidney to conserve water and concentrate urine (Valtin,1987).

2.8. Physiology of Oxytocin and Vasopressin Release

Given that oxytocin and vasopressin have effects on different peripheral tissues, and that each serves a different homeostatic mechanism, it is not unreasonable to believe that release of the two hormones could be differentially regulated. Indeed, vasopressin and oxytocin release are largely independent of one another (Kasting,1988). During hypovolemia and hyperosmolality, both vasopressin and oxytocin are released, although the increase in vasopressin release is over three-fold higher than that observed for oxytocin (Kasting,1988; Summy-Long et al.,1984). Parturition, and lactation, on the other hand, "stimulate" a virtually exclusive increase in oxytocin secretion (Grosvenor et al.,1986; Higuchi et al.,1985; Kasting,1988; Summy-Long et al.,1984; Wakerley et al.,1973). Perhaps more interesting than the HNS's ability to "decide" which hormone to release is that the profile of oxytocin and vasopressin release are very different. Oxytocin release occurs in short pulsatile bursts during both milk-ejection (Higuchi et al.,1985) and parturition (Higuchi et al.,1985; O'Byrne et al.,1986), unlike vasopressin which is not released in a pulsatile fashion, rather levels increase/decrease more gradually.

2.9. Electrophysiology of Supraoptic Neurons

Underlying the differential secretion of oxytocin and vasopressin is the ability of the HNS to modulate neuronal activity (i.e. neuronal firing rate) in response to changing demands for these hormones. In particular, lactation and parturition provide an illustrative example of this process. The first evidence that alterations in neuronal activity were directly responsible for changing hormone release came from extracellular recordings of neurons within the supraoptic (Lincoln and Wakerley,1974) and paraventricular (Wakerley and Lincoln,1973) nuclei in actively-lactating rats. Here, while simultaneously recording intramammary pressure (as a bioassay for oxytocin release), several patterns of activity were observed, of which one appeared temporally related to a transient rise in intramammary pressure. In these cells a high frequency burst of action potentials immediately preceded the rise in intramammary pressure, suggesting that the cells were oxytocinergic. These initial observations have subsequently been supported by others in both the rat (Brimble and Dyball,1977 ; Summerlee and Lincoln,1981) and the rabbit (Paisley and Summerlee,1984). The increase in oxytocin secretion during parturition also seemed to be regulated by a similar mechanism. Extracellular recordings during parturition from rat (Summerlee,1981) and rabbit (O'Byrne et al.,1986) revealed high frequency neuronal activity which temporally preceded uterine contractions and the delivery of pups.

The conclusions which may be drawn from the studies mentioned above are that the changing temporal pattern of neuronal firing determines the profile of hormone release. Surges of hormone release in parturition and lactation are the direct result of high frequency firing of the neurons. It has been estimated that most of the oxytocinergic neurons within the SON and PVN are required to fire in relative synchrony to achieve the oxytocin levels

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observed during parturition (Poulain and Wakerley,1982). This requirement suggests that the HNS possesses an additional level of organization which promotes the synchrony of these neurons. Mechanisms proposed to explain this process include rearrangements of neuronal-glial relationships within the SON (Hatton,1988a; Hatton et al.,1984; Hatton and Tweedle,1982; Montagnese et al.,1987; Perlmutter et al.,1984; Salm et al.,1985; Taubitz et al.,1987; Theodosis and Poulain,1984; Theodosis et al.,1981; Theodosis et al.,1988) and neurohypophysis (Hatton,1988b; Hatton,1988c; Tweedle and Hatton,1980; Tweedle and Hatton,1982; Tweedle and Hatton,1987), formation of double synapses (Hatton and Tweedle,1982; Perlmutter et al.,1984; Theodosis et al.,1981), and modulation of direct neuron-neuron communication via gap junctions (Andrew et al.,1981; Cobbett and Hatton,1984; Cobbett et al.,1985; Cobbett et al.,1986; Cobbett et al.,1987). Also afferent input to the SON may possibly participate in these anatomical changes to promote synchrony of neuronal firing.

2.10. Afferent to the Supraoptic Nucleus

The SON receives numerous afferents from brainstem and forebrain and also hypothalamic origin (Fig. 2) in which a conservative account includes the following structures. A more inclusive listing may be found in Hatton (1990) or Palkovits (1986). Inputs from brainstem sources include ventrolateral medulla (A1), nucleus of the solitary tract (A2 ; Anderson et al.,1989; Tribollet et al.,1985), and locus coeruleus (A6) catecholamine groups (Anderson et al.,1989; Tribollet et al.,1985; Wilkin et al.,1989), lateral parabrachial nucleus (Anderson et al.,1989), and dorsal raphe nucleus (Anderson et al.,1989; Tribollet et al.,1985).

Fig. 2. S

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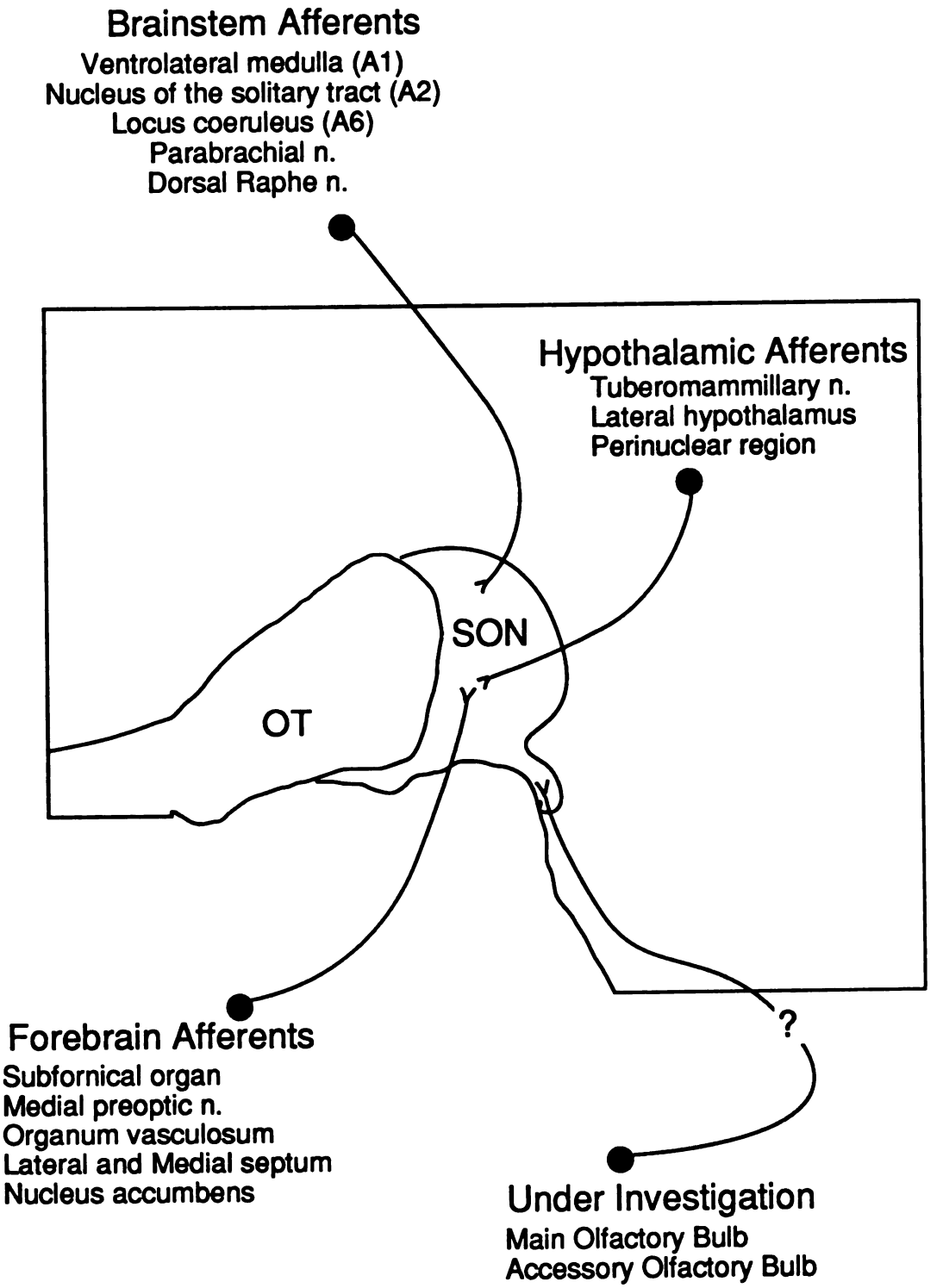
forebrain

Fig. 2. Summary diagram of SON afferents.

Sources of brainstem, forebrain and hypothalamic afferents are illustrated. The present studies seek to determine if the SON receives additional forebrain input from the main and accessory olfactory bulbs.


Brainstem Afferents
Ventrolateral medulla (A1)
Nucleus of the solitary tract (A2)
Locus coeruleus (A6)
Parabrachial n.
Dorsal Raphe n.
Hypothalamic Afferents
Tuberomammillary n.
Lateral hypothalamus
Perinuclear region
SON
OT
?
Forebrain Afferents
Subfornical organ
Medial preoptic n.
Organum vasculosum
Lateral and Medial septum
Nucleus accumbens
Under Investigation
Main Olfactory Bulb
Accessory Olfactory Bulb

Inputs from forebrain structures include the subfornical organ (Anderson et al.,1989; Lind et al.,1982; Miselis,1981; Tribollet et al.,1985; Wilkin et al.,1989), medial preoptic area (and nucleus; Anderson et al.,1989; Tribollet et al.,1985; Wilkin et al.,1989), organum vasculosum of the lamina terminalis (OVLT; Anderson et al.,1989; Tribollet et al.,1985; Wilkin et al.,1989), nucleus accumbens (Anderson et al.,1989) and both lateral and medial portions of the septum (Anderson et al.,1989; Tribollet et al.,1985). Nearby hypothalamic structures, including tuberomammillary nucleus (Weiss et al.,1989), lateral hypothalamus, caudal diagonal band of Broca (Mason et al.,1983; Tribollet et al.,1985), and the perinuclear zone surrounding the nucleus (Mason,1985; Tribollet et al.,1985), also project to the SON.

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3. Introduction to the Olfactory System

In many animals the olfactory system is well-developed and provides essential information for survival and is largely responsible for sensing much of the chemotactic information in the environment. In some animals, such as the albino rat, this is a more essential and informative sensory experience than obtained from the visual system.

In addition to identifying the odors of such animals' environments, the olfactory system plays an essential role in several complex behaviors including maternal behavior (Poindron et al.,1988), gender identification, various aspects of sexual behavior (Wysocki et al.,1986; Wysocki and Meredith,1987), territory marking, and species membership (Doty,1986).

3.1. Receptor Systems in the Olfactory Mucosa

The sensory receptors within the nasal cavities of the rat are not homogenous. Three separate populations of receptors subserve different chemosensory functions. These include the main olfactory neuroepithelium, the septal organ, and the vomeronasal organ (Fig. 3). The nasal cavities are also heavily innervated by free-nerve endings from the ophthalmic and maxillary divisions of the trigeminal nerve and nervus terminalis (Bojsen-Moller,1975).

3.1.1. Main Olfactory Neuroepithelium

Bipolar neurons embedded within the nasal mucosa of the turbinates project exclusively to the main olfactory bulb . These receptors project via the unmyelinated olfactory nerve to terminate within glomeruli of the main bulb in a complex overlapping topographical fashion, i.e. immediately adjacent receptors project to glomeruli which are widely separated (Astic et al.,1987). Because of their sequestered location, receptors within this system

Fig. 3. C

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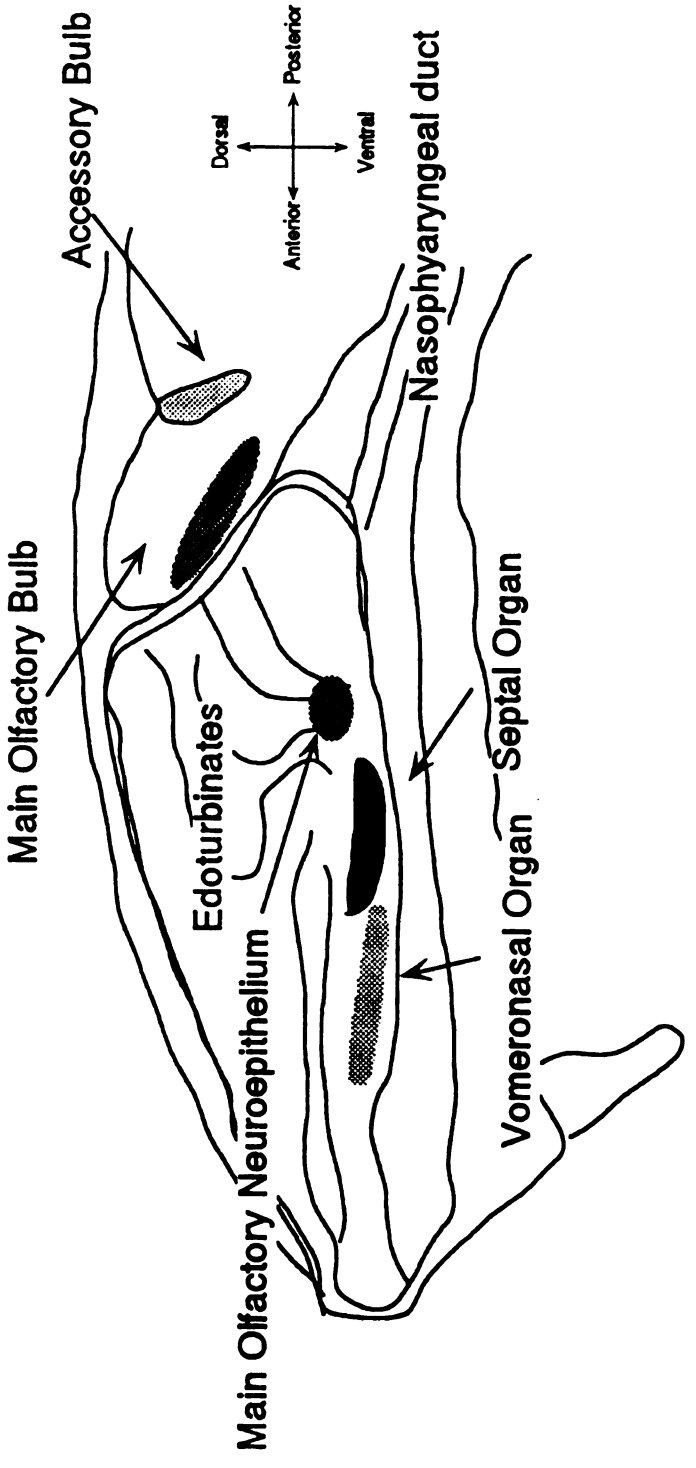
Fig. 3. Chemoreceptor systems in the olfactory mucosa.

Schematic diagram illustrates the position and projection of three types of chemoreceptors in the rat olfactory system: the main olfactory neuroepithelium, vomeronasal organ, and septal organ. Shaded areas of the olfactory mucosa project to similarly shaded areas of the olfactory bulb.


Accessory Bulb
Dorsal
Posterior
Anterior
Ventral
Nasophyaryngeal duct
Main Olfactory Bulb
Edoturbinates
Septal Organ
Main Olfactory Neuroepithelium
Vomeronasal Organ

have access to only volatile chemicals which are freely diffusible in inspired air.

3.1.2. Septal Organ

The septal organ is ventral to the main olfactory receptors at the entrance to the nasopharyngeal passage and isolated from other sensory epithelia by respiratory mucosa (Rodolfo-Masera,1943). Receptors in the septal organ project as a separate fascicle through the cribriform plate to terminate within glomeruli of the main bulb (Bojsen-Moller,1975). Unlike the main olfactory receptors, the septal organ projects to a restricted area within the posteroventral area of the main bulb (Astic and Saucier,1988). The function of the septal organ remains somewhat obscure. Its position at the entrance to the nasopharyngeal passage suggests that it serves an "alerting" function, especially at low tidal volumes (e.g., during quiet respiration) when chemotactic agents may not reach other receptors (Bojsen-Moller,1975; Rodolfo-Masera,1943). This view is consistent with electrofactogram studies of the septal organ which demonstrate that septal organ receptors show greater sensitivity to certain odors than the main olfactory neuroepithelium (Marshall and Maruniak,1986). Also by virtue of its position, the septal organ should have access to both volatile and non-volatile stimuli, suggesting it may play a more comprehensive role in sensing the environment.

3.1.3. Vomeronasal Organ

The vomeronasal organ is a cigar-shaped patch of neuroepithelium located rostrally within the nasal septum and overlies the nasopalatine duct. Thin unmyelinated fibers coalesce to form the vomeronasal nerve which, after passing through the cribriform plate, terminates within the glomeruli of the accessory olfactory bulb. Unlike either the septal organ or the main

olfactory neuroepithelium, the vomeronasal organ innervates only the accessory olfactory bulb (Barber and Raisman,1974; Bojsen-Moller,1975). The vomeronasal organ senses non-volatile compounds which, when dissolved or suspended in saliva (or respiratory mucus), reach these receptors through the nasopalatine duct (Wysocki et al.,1980). The vomeronasal organ is thought to be responsible for guiding many "pre-wired" (Keverne et al.,1986) social behaviors (e.g. maternal behavior, sexual behavior).

3.2. Olfactory bulb organization

The olfactory bulb in the rat is a six-layer laminated structure. It can be subdivided into a rostromedial main olfactory bulb and an accessory olfactory bulb, a crescent-shaped inclusion within dorsolateral margins of the posterior main bulb (Fig. 4). In the main bulb, from superficial to deep, the six layers are: 1) olfactory nerve layer; 2) glomerular; 3) external plexiform; 4) mitral cell; 5) internal plexiform; and 6) granule cell layer.

The accessory olfactory formation is organized similar to the main bulb, but, the mitral cells are more pleomorphic than those of the main bulb. Further, mitral cells are organized into a thick band within the external plexiform layer, hence no real mitral layer exists.

3.3. Olfactory bulb intrinsic organization

The important feature of the intrinsic organization of the bulb (for our purposes) is that the mitral cells, and tufted cells within the external plexiform area, are the output neurons of the bulb. The reader is referred to reviews by Scott (1986), Shepard (1972) and Switzer (1985) for a more detailed discussion. Their axons join within the granule cell layer to form thick fascicles which, at the caudal end of the bulb, converge to form the lateral olfactory tract. Similarly, mitral cells of the accessory bulb also

Fig. 4. Atlas of the olfactory bulb in the sagittal plane.

Sections are 100 μm thick and stained with thionine. A line drawing on the right delineates the various structures within each bulb. Mitral cell layers for each bulb are shaded in the drawings.

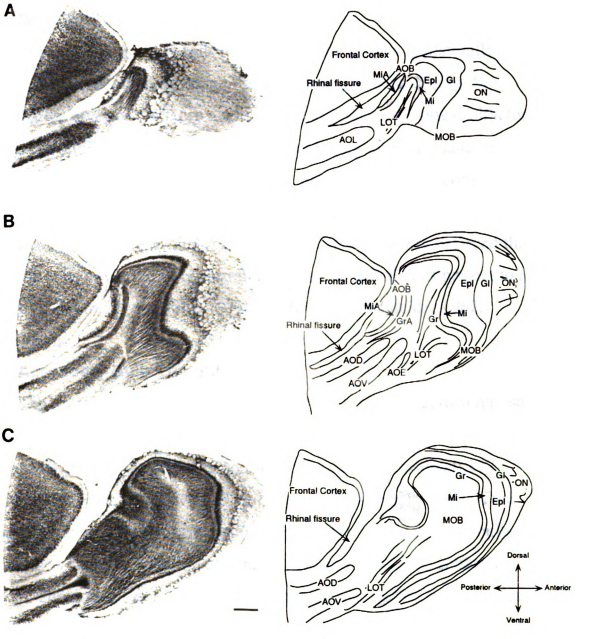
A. The lateral margins of the bulb, here both the main and accessory bulb are visible. In the main bulb the laminated organization is readily apparent. The internal plexiform layer which lies between the mitral cell and granule cell layers has been omitted for the sake of clarity.

B. Sagittal section through the main and accessory bulb 300 μm medial to section in A.

C. Sagittal section through the main olfactory bulb 300 μm medial to B. Here the accessory bulb is no longer visible.

Abbreviations:

AOB	accessory olfactory bulb;
AOD	anterior olfactory nucleus, dorsal subdivision;
AOE	anterior olfactory nucleus, external portion;
AOL	anterior olfactory nucleus, lateral subdivision;
AON	anterior olfactory nucleus;
AOV	anterior olfactory nucleus, ventral subdivision;
EPl	external plexiform layer of the main olfactory bulb;
Gl	glomerular layer of the main olfactory bulb;
Gr	granule cell layer of main bulb;
GrA	granule cell layer of accessory bulb;
LOT	lateral olfactory tract;
Mi	mitral cell layer of the main olfactory bulb;
MiA	mitral cell layer of accessory olfactory bulb;
MOB	main olfactory bulb.


A
Frontal Cortex
AOB
MiA
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Mi
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AOL
MOB
B
Frontal Cortex
AOB
Epl
Gl
ON
MiA
Mi
GrA
Gr
Rhinal fissure
AOD
LOT
MOB
AOV
AOL
C
Frontal Cortex
Gr
Gl
ON
Mi
Epl
Rhinal fissure
MOB
Dorsal
AOD
LOT
Posterior
Anterior
AOV
Ventral

contribute axons to the lateral olfactory tract, joining the tract along its dorsal margins

3.4. Main Bulb Efferents

The mitral and tufted cells of the main bulb project ipsilaterally through the lateral olfactory tract to several caudal structures (Fig. 5), including the anterior olfactory nucleus, primary olfactory cortex, olfactory tubercle, several subnuclei within the amygdala (anterior area, anterior cortical, posterolateral cortical and medial), and the entorhinal cortex (Broadwell,1975; Devour,1976; Heimer,1968; Price and Powell,1971; Scalia and Winans,1975; Shipley and Adamek,1984). It is generally believed that the tufted cells project to only rostral targets while mitral cells project to all target areas (Haberly and Price,1977; Macrides and Schneider,1982; Scott,1981).

3.5. Accessory Bulb Efferents

The mitral cells of the accessory bulb have a somewhat different set of target nuclei (Fig. 6). This relationship, first demonstrated by Scalia and Winans (1975) and subsequently confirmed by others (Broadwell,1975; Devour,1976; Price and Powell,1971; Shipley and Adamek,1984), is the anatomical underpinning of the notion that the accessory and main bulb (hence, the vomeronasal organ and main bulb neuroepithelia) subserve different chemosensory functions. These mitral cells project to the nucleus of the accessory olfactory tract, medial and posteromedial cortical amygdaloid nuclei, and the bed nucleus of the stria terminalis.

Fig.

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Fig. 5 Schematic drawing of the main olfactory bulb efferents.

Arrows represent an input to that structure. As illustrated, axons from mitral cells in the main olfactory bulb project through the lateral olfactory tract to terminate in many caudal targets. Tufted cells which also project to rostral targets are not drawn(e.g., Tu).

Abbreviations:

3V	third cerebral ventricle;
ACo	anterior cortical amygdaloid nucleus;
AON	anterior olfactory nucleus;
LOT	lateral olfactory tract;
Me	medial nucleus of amygdala;
MOB	main olfactory bulb;
Pir	piriform cortex;
PLCo	posterolateral cortical amygdaloid nucleus;
SON	supraoptic nucleus-main (or anterior) portion;
Tu	olfactory tubercle;
NLOT	nucleus of the lateral olfactory tract
IIn	optic nerve.

Main Olfactory Bulb Efferents

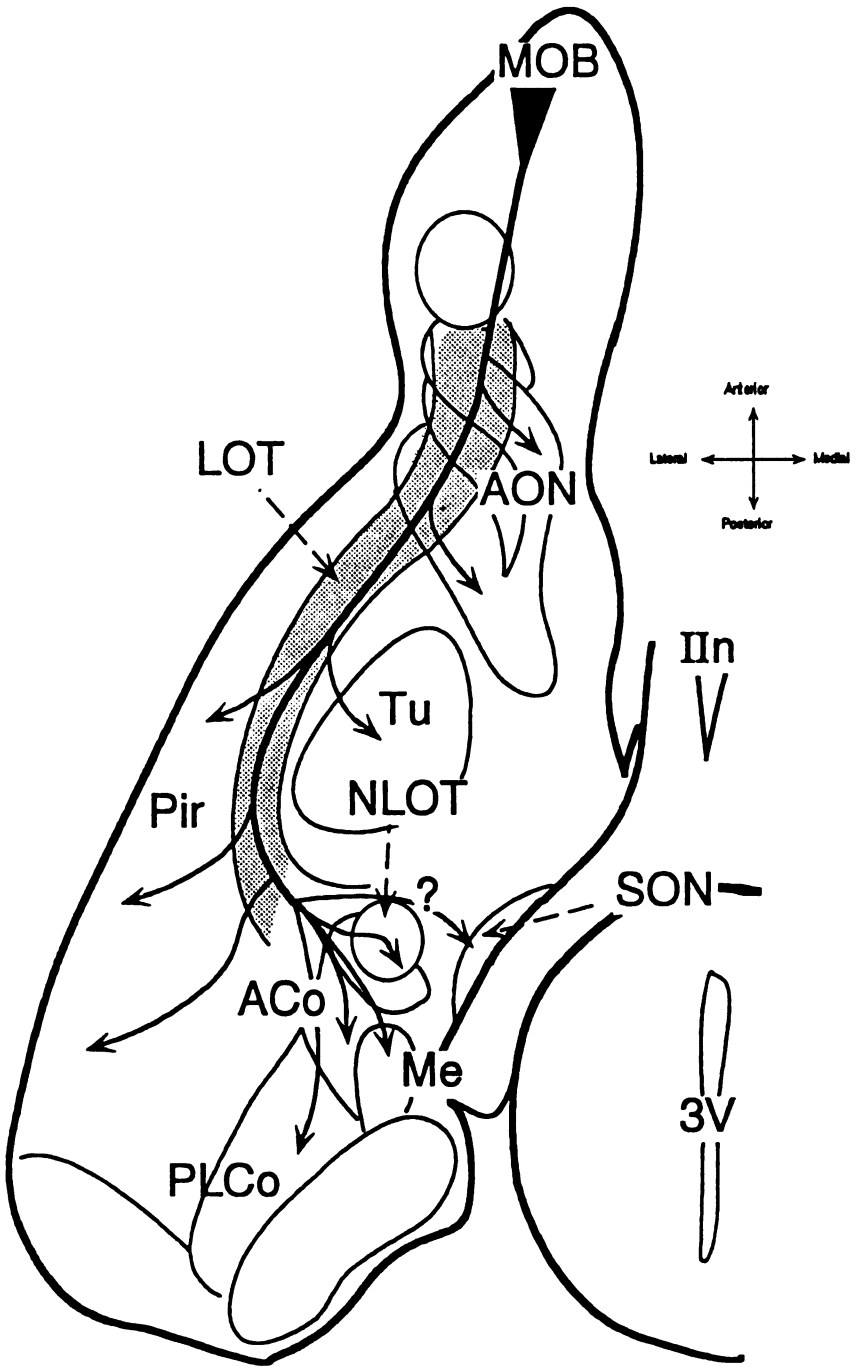


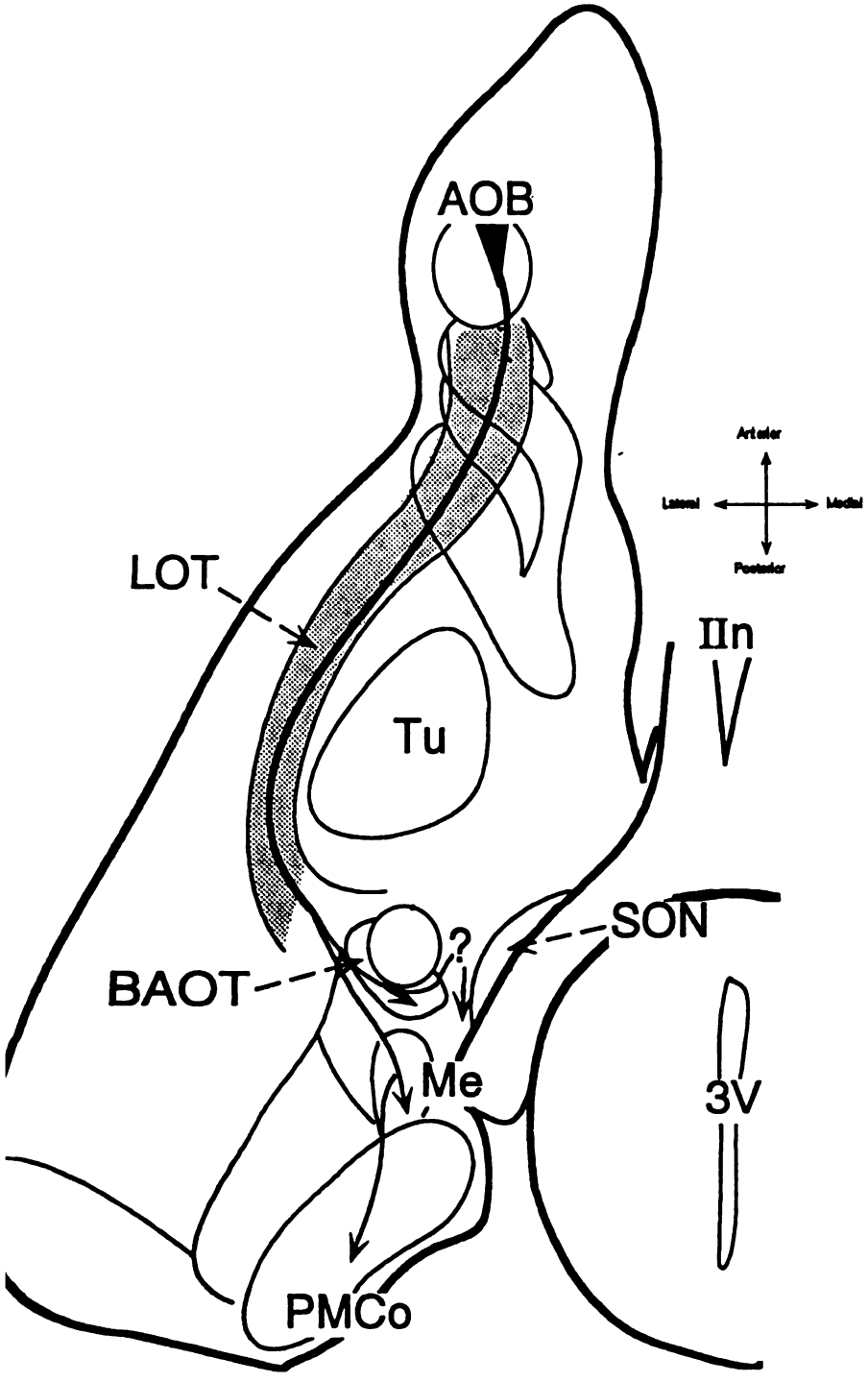
Fig. 6. Schematic drawing of accessory olfactory bulb efferents.

Mitral cells from the accessory olfactory bulb also project through the lateral olfactory tract. There is little overlap between the main and accessory bulb efferents except within the medial amygdala and possibly the SON.

Abbreviations

3V	third cerebral ventricle;
AOB	accessory olfactory bulb;
BAOT	bed nucleus of the accessory olfactory tract;
LOT	lateral olfactory tract;
Me	medial nucleus of amygdala;
PMCo	posteromedial cortical amygdaloid nucleus;
SON	supraoptic nucleus-main (or anterior) portion;
Tu	olfactory tubercle;
IIn	optic nerve.

Accessory Olfactory Bulb Efferents



4. Exp.1. : Cytoarchitectural Organization of the SON Dendritic Zone

4.1. Introduction

An essential first step in an anatomical study of putative projections is to accurately define the extent of the target nuclei. Since both somata and dendrites are post synaptic elements for afferents, their morphology needs to be understood.

The use of Nissl stains amply illustrates the cell bodies within many neuronal systems. Indeed, much of the present nomenclature and organization of cell aggregates into nuclei are based on these stains. The distribution of PVN and SON somata has likewise been described for many years (Bleier et al.,1979). There is little ambiguity as to their organization.

The morphology and distribution of dendritic processes in many systems, however, are less well understood. In some areas (e.g. cerebellum, cerebral cortex) Golgi methods have revealed much of the detail of these dendritic trees. Unfortunately, the SON and PVN do not stain well with this technique. The SON and/or PVN dendritic architectures have now been analyzed using immunocytochemistry (Armstrong et al.,1982; Sofroniew and Glasmann,1981), Golgi (Armstrong et al.,1982; Dyball and Kemplay,1982), retrogradely transported HRP (Ju et al.,1986) and intracellular injection of Lucifer Yellow CH (Hatton,1990; Randle et al.,1986). Unfortunately, these techniques have different sensitivities, consequently disagreements have arisen.

4.2. Experimental Question

One such disagreement particularly pertinent to these studies is the issue of whether SON dendrites project into periamygdaloid areas.

The neurophysins, oxytocin- and vasopressin-associated neurophysins are found in the soma, dendrites, and axons of oxytocin- and vasopressin-producing cells in the PVN and SON and other neurons which produce these peptides. Since neurophysins are widely distributed within these neurons they may be used as markers to study the morphology of these cells.

4.3. Methods Exps. 1-4: General Methods for Anatomical Studies

Male and female Sprague-Dawley rats 90-150 days old either obtained from the Holtzman Co. or raised in our colony were used. Surgeries were performed on animals which were deeply anesthetized with equithesin. Micropipettes containing one of several tracers were placed, with the aid of a stereotaxic holder, either by visual guidance or with coordinates from the atlas of Paxinos and Watson (1986). Glass micropipettes used for injections were manufactured on a Kopf vertical electrode puller from microfilament glass (1.0 mm O.D., 0.58 mm I. D.) after which a final tip diameter of 5-15 μm for the iontophoretic injection or 25-50 μm for pressure injections was obtained by breaking the tip with a Kimwipe®. After appropriate survival times, animals were transcardially perfused, the brains removed from the skull and stored in buffer prior to further histological processing. Unless otherwise noted, all reagents were diluted in one of two buffers: 1) 0.05 M Tris, 0.15 M NaCl, pH 7.4 (TBS) or 2) 0.01 M Na phosphate, 0.14 M NaCl, pH 7.4 (PBS). Sections were examined with a Zeiss standard microscope using brightfield, polarized darkfield, and/or epifluorescence (50 W Hg) illumination. The epifluorescence was configured with a "green" filter set consisting of a BP 546 supplemental exciter, FT 580 dichroic mirror, or a LP 590 barrier; and a "UV" set consisting of a G 365 supplemental exciter, FT 395 mirror, and LP 420 barrier.

4.4. Methods Exp. 1: Neurophysin Immunocytochemistry

Male rats were fixed with Bouin's fixative, brains were removed and stored in the same fixative at 5°C for several days. In some cases, 24 h prior to fixation the animal was given an intracerebral ventricular injection of colchicine (0.1 μg/ kg). Brains were sectioned in either the coronal, sagittal, or horizontal plane with a Vibratome® at a thickness of 100 μm. Tissue sections were rinsed in TBS over a period of several days to completely remove the fixative. Neurophysin-containing processes were detected by an immunocytochemical method (Hsu et al.,1981). This was accomplished by the successive application of the following reagents: rabbit anti-neurophysin (48 h at 4°C; Chemicon), biotinylated goat anti-rabbit (24 h at 4°C), and then avidin-biotinylated HRP complex (24 h at 4°C; Vector). The tissue was rinsed for 2 h with TBS at room temperature after each reagent incubation. The HRP was visualized with a glucose oxidase, imidazole, diaminobenzidine solution (Smithson et al.,1984). Labeled perikarya and their processes were identified by their dark brown coloration, as seen in brightfield illumination.

4.5. Results Exp. 1: Perikaryal labeling with Neurophysin Immunocytochemistry

Dense immunocytochemical labeling around the SON revealed three distinct patterns; these corresponded to somatic, axonal, and dendritic labeling of SON neurons as previously reported (Armstrong et al.,1982). Features of neurophysin labeled material are illustrated in the coronal (Fig. 7), sagittal (Fig. 8) and horizontal planes (Fig. 9).

Fig. 7. Distribution of neurophysin immunoreactivity in 100 μm thick coronal sections.

Panels A-I, covering 3 consecutive plates, illustrates the course of SON dendrites in the periamygdaloid cortex. In each panel the photomicrograph on the right illustrates the SON dendritic zone at higher magnification. The immunocytochemical reaction product appears dark gray to black while the unstained tissue is light gray. In all panels the SON dendrites are delineated with arrowheads. Bar= 375 & 250 μm for the photomicrographs on the left and right respectively.

A-C. A dark black aggregation of immunoreactive cell bodies lateral to the optic tract is the somatic portion of the SON. The wavy arrow (in A) denotes the prominent efferent to the neurohypophysis. Arrows in the higher power photomicrographs illustrate axons projecting into the perinuclear zone/lateral hypothalamus. In each plate note the SON dendrites which project into the periamygdaloid cortex.

Abbreviations:

SON supraoptic nucleus-main (or anterior) portion;
OT optic tract.

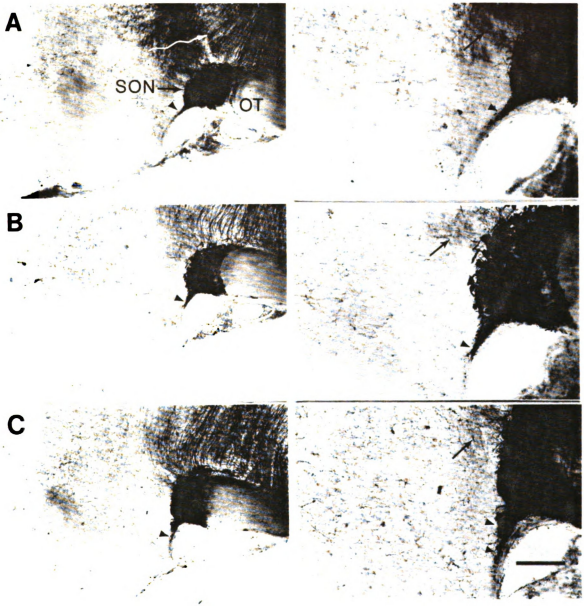

A
SON
OT
B
C

Fig. 7 (cont.): Plate 2

D-E. Note the long dendritic projections (arrowheads) in D that extent several hundred microns into the periamygdaloid cortex. Also note the neurophysin-immunoreactive fibers (arrows) which appear juxtaposed to the nearby lateral olfactory tract.

Abbreviations:

SON supraoptic nucleus-main (or anterior) portion;
OT optic tract.

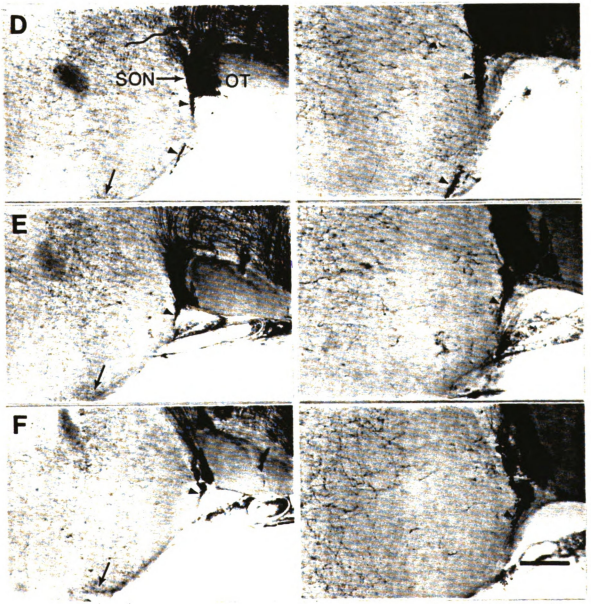

D
SON
OT
E
F

Fig. 7 (cont.): Plate 3

G-I. Note that at these posterior levels of the nucleus, few SON cell bodies remain. However, SON dendrites are still observed in the cortex.

Abbreviations:

SON supraoptic nucleus-main (or anterior) portion;
OT optic tract.

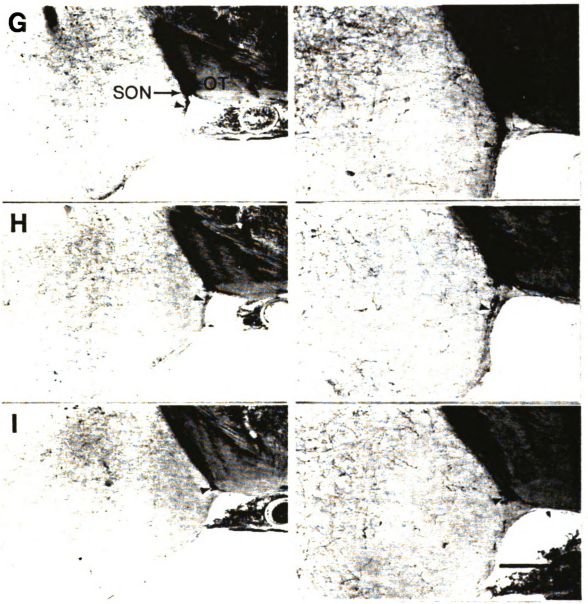

G
SON
OT
H
I

Fig. 8. Distribution of neurophysin immunoreactivity in 100 μm thick sagittal sections.

Panels A-F proceed from lateral to medial to further illustrate the course of SON dendrites. Anterior is positioned left, while dorsal is up. SON dendrites are delineated with arrowheads. Bar = 375 & 150 μm for photomicrographs on the left and right, respectively.

A. SON somata at the posterolateral margins of the nucleus are positioned dorsal to the medial amygdala (Me in A).

B & C. More medially, the region subjacent to the optic tract is filled with SON dendrites running in an anteroposterior direction. Also seen at these lateral levels of the SON are efferents which project dorsally (arrows) into the lateral hypothalamus.

Abbreviations:

Me	medial nucleus of amygdala;
OT	optic tract;
SON	supraoptic nucleus-main (or anterior) portion.

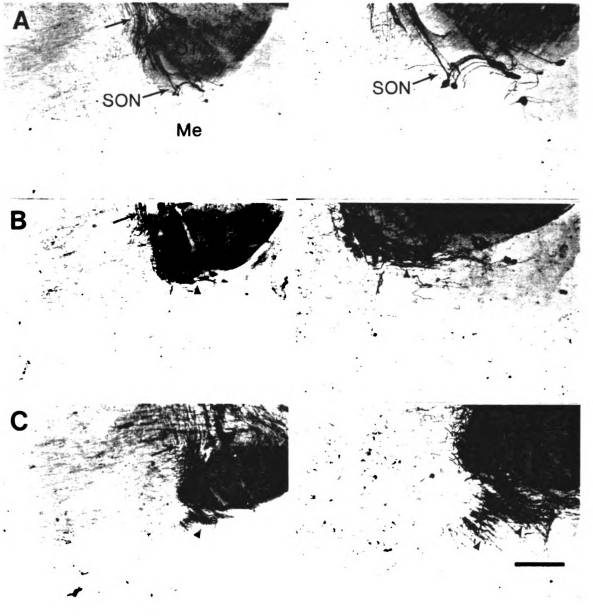

A
SON
Me
SON
B
C

Fig.

D-F
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(arro
Abb

Fig. 8. (cont.) Plate 2

D-F. Progressing medially the periamygdaloid cortex disappears (see E), after which SON dendrites lie immediately subjacent to SON somata. An efferent to the lateral hypothalamus (upper arrow in D) and another projecting anteriorly towards the olfactory tubercle/lateral preoptic area (arrows in E & F) are observed at these levels.

Abbreviations:

OT	optic tract;
SON	supraoptic nucleus-main (or anterior) portion.

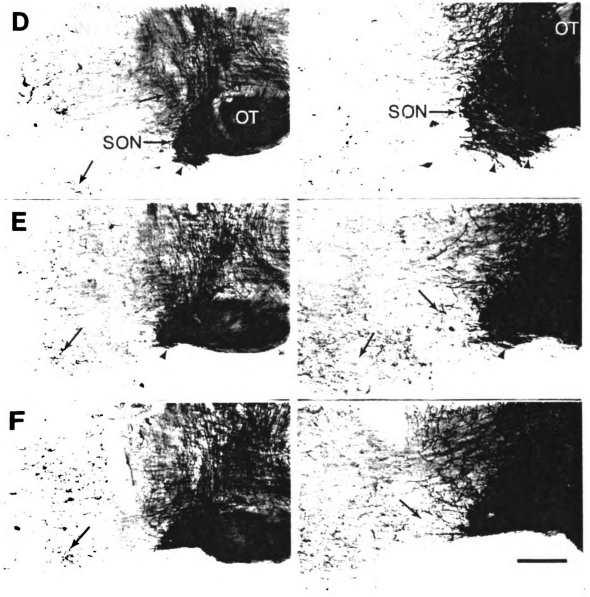

D
OT
SON
SON
OT
E
F

Fig. 9. Distribution of neurophysin immunoreactivity in 100 μm thick sections in the horizontal plane.

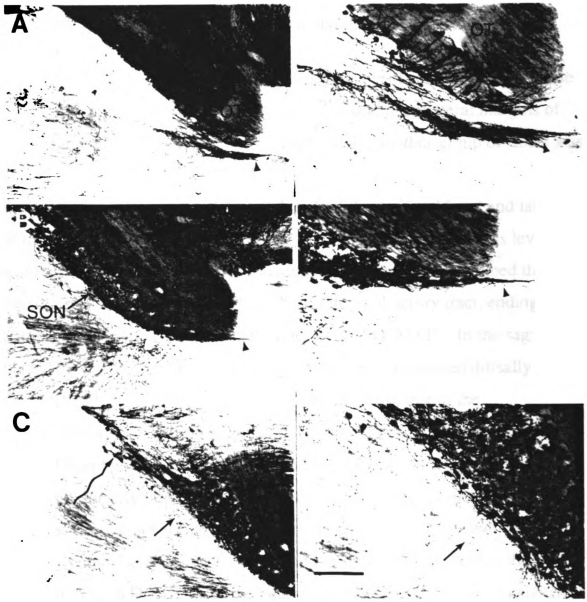
Panels A-C are arranged ventral to dorsal, anterior is left and medial is up. As before, SON dendrites are marked with arrowheads. Bar = 375 & 150 μm for panels on the left and right, respectively.

A-B. Note the thick plexus of SON dendrites at the caudal margins of the SON extending beyond the confines of the nucleus.

C. At more dorsal levels neurophysin-immunoreactive somata (wavy arrow) in the preoptic area appear as a rostral extension of the SON. Many thin neurophysin processes (arrow) were also observed in the perinuclear zone of the SON.

Abbreviations:

OT optic tract;
SON supraoptic nucleus-main (or anterior) portion.


A
B
SON
C

4.5.1. Somatic Labeling

SON somata appeared as an aggregation of densely stained ovoid cells located lateral to the optic tract (Figs 7) and dorsal to a plexus of thick well-stained processes (i.e. SON dendrites). The distribution of SON somata is well documented and deserves little further comment. One important observation, however, was that the SONa extends further rostrally than generally appreciated as is readily apparent in the horizontal plane (Fig.9 C). Here SON somata appear at levels as rostral as the medial preoptic area and are positioned ventromedial to the anteroventral preoptic nucleus.

4.5.2. Axonal Labeling

Axonal processes were observed as a plexus of small caliber varicose fibers radiating dorsomedially from the dorsal and dorsolateral margins of the somatic portion of the nucleus. Additionally, another group of fibers was also observed at this level of the SON. These fibers projected laterally into the adjacent nucleus of the horizontal limb of the diagonal band and lateral SON perinuclear zone (Fig. 7 B & C). Within the amygdala at this level were also thin fascicles of discontinuous fibers which circumscribed the dorsolateral margins of the nucleus of the lateral olfactory tract, ending abruptly within the lateral olfactory tract itself (Fig.7 D-F). In the sagittal plane (Figs. 9) a dense projection was observed which arched dorsally over the optic tract representing the well-known SON efferent to the neurohypophysis. Additionally, at the lateral margins of the nucleus two other efferent pathways appeared to project from the nuclear region. One projected dorsally into the lateral hypothalamus and horizontal limb of the diagonal band as several groups of fibers (Fig. 8 A-D). The other projected anteriorly into the olfactory tubercle and ramified diffusely within this structure (Fig. 8 D-F). Unlike the neurohypophysial and lateral hypothalamic

projections in which fibers could be traced from the nuclear borders for a considerable distance, this olfactory tubercle projection appeared as a collection of discontinuous fibers. Consequently their origin is undetermined.

4.5.3. Dendritic Labeling

The dendritic labeling was observed as a dense collection of thick varicose processes immediately ventral to SON somata, including a fascicle of processes which also projected ventrolaterally into periamygdaloid regions along the ventromedial surface of the brain. In the coronal plane, the periamygdaloid projecting dendrites were first observed at the mid rostrocaudal levels of the nucleus (Fig. 7 A). Here the dendrites appeared to project directly ventral, extending more than several hundred micrometers (Fig. 7 D) into the cortex. Caudally, the number of ventrally projecting dendrites diminished rapidly. At these levels, only punctate neurophysin labeling (i.e. SON dendrites cut in cross-section) was observed within the cortex (Fig. 7 F-I). That this labeling was of dendritic processes was more easily observed in the sagittal plane (Fig. 8) at the posterolateral boundaries of the SON. Here many thick processes were observed coursing in an anteroposterior orientation ventral to the optic tract within the periamygdaloid cortex (Fig. 8 B-E). Progressing medially this cortical region diminished rapidly along with this group of SON dendrites. It also appeared that dendrites extended beyond the caudal boundaries of the somatic region, as observed in the horizontal plane (Fig. 9 A & B).

4.6. Discussion Exp. 1.

4.6.1. Somatic Labeling

The distribution of neurophysin-labeled somata are consistent with previous reports employing immunocytochemical techniques (Armstrong et

al.,1982; Sofroniew and Glasmann,1981; Yulis et al.,1984). However, only a passing mention (Rhodes et al.,1981; Sofroniew,1985; Yulis et al.,1984) has been made of the rostral extension of SON somata into the medial preoptic area. Immunocytochemical analysis of the distribution of oxytocin and vasopressin cells (Rhodes et al.,1981) suggest these cells may be oxytocinergic. The medial preoptic area has been implicated in the control of maternal behavior (Numan,1990). Furthermore, oxytocin seems to play an important role in promoting this behavior (Insel,1990; Pedersen,1982). Whether the nearby SON sends collateral branches of axons into the medial preoptic area is an important question awaiting further experimental evaluation.

4.6.2. Dendritic Ramifications

That SON dendrites do ramify ventrolaterally into periamygdaloid regions is not generally appreciated. Rather they are believed to traverse ventral to the cell bodies within the mediolateral confines of the nucleus (Dyball and Kemplay,1982; Felten and Cashner,1979; Randle et al.,1986). However, immunocytochemical studies (Armstrong et al.,1982, and the present study) as well as an analysis of retrogradely-filled SON neurons from posterior pituitary injections (Ju et al.,1986) clearly illustrate that many SON dendrites do traverse out of the confines of the nucleus. This fact is easily appreciated in the coronal plane where several prominent fascicles of dendrites project ventrally into the periamygdaloid cortex. However, since the predominant orientation of this dendritic projection is anteroposterior, the prominence of this projection disappears rapidly in this plane of section. It is replaced with small punctate profiles of dendrites cut in cross section, an appearance which may easily be mistaken for non-specific staining. Consequently, while the coronal plane clearly illustrates this dendritic

projection, it also obfuscates the magnitude of the projection. The frequent and almost exclusive use of the coronal plane by many investigators has, in all likelihood, perpetuated this misunderstanding.

5. Exp. 2: Anterograde Tracing of Main Olfactory Bulb Efferents to the SON

5.1. Introduction

It seems clear after evaluation of the SON dendritic zone in the previous experiment and reviewing published line drawings of anterograde labeling of main bulb efferents from the rat (Heimer,1968; Scalia and Winans,1975) and rabbit (Broadwell,1975; Scalia and Winans,1975) that the main bulb probably projects to the SON. Yet no specific comment on this putative connection was made in these papers, and has only since appeared in a review by Heimer (1978).

5.2. Experimental Question

Does the main bulb project to the SON? If so, what are the distribution of inputs within the SON? Does the main bulb also send projections to other nuclei within the HNS? Armed with more sensitive and specific anatomical tracing tools, this reevaluation of main bulb efferents should provide a more definitive answer to these questions.

5.3. Methods Exp. 2A: Anterograde studies with WGA-HRP

Pressure injections (6 bilateral and 12 unilateral) of 100-150 nl of 0.5 - 0.7% WGA-HRP were stereotaxically placed into the main bulb of 18 female rats. The animals were allowed to survive 24-72 h after which they were perfusion-fixed with 500ml of 1% paraformaldehyde, 2% glutaraldehyde in 0.1 M Na phosphate, pH 7.4, followed by equal volumes of 10% sucrose in PBS. The brains were stored in this solution for up to 3 days after which they were frozen sectioned at 40-50 μm in either the sagittal, horizontal or coronal plane. After sectioning, the tissue was processed immediately or stored overnight at 4°C in PBS. The WGA-HRP was visualized with tetramethyl benzidine (Mesulam,1982). In order to

facilitate precise localization of staining, tissue sections were arranged into three adjacent sets consisting of every third section. Two sets were counterstained with bisbenzimide (Schmued et al.,1982) while the third was counterstained with thionine. All slides were coverslipped with DPX, then stored at 4°C to prevent deterioration of the labeling. Labeled fibers and terminals were identified by their bright granular appearance in darkfield illumination.

5.4. Methods Exp. 2B: Anterograde studies with PHA-L

The PHA-L method of Gerfen and Sawchenko (Gerfen and Sawchenko,1984) was employed with some modifications in the histological processing to permit thinner tissue sections. Briefly, bilateral iontophoretic injections of PHA-L (2.0 mg/ml solution; Vector Labs) were placed into the main bulbs of 22 female rats and 2 male rats. After survival times ranging from 7-21 days, animals were fixed with 4% paraformaldehyde in a 0.1M Na acetate pH 6.5, followed by 4% paraformaldehyde, 0.5% glutaraldehyde in a 0.1M Na borate, pH 9.5 (Berod et al.,1981). The brains were removed and stored in TBS for 1-3 days, then embedded in polyethylene glycol (Smithson et al.,1983) for sectioning in the horizontal, sagittal or coronal plane. Ribbons of tissue sections of various thicknesses between 10-25 μm were cut on a rotary microtome and collected onto an acetate film strip as they came off the blade. Sections were then rinsed in TBS to remove the polyethylene glycol and stored in TBS for further processing. The PHA-L was detected by an immunocytochemical method similar to that previously described. This was accomplished by the successive application of the following reagents: goat anti-phaseolus (24-48 h at 4°C; Vector), biotinylated rabbit anti-goat (1-2 h at room temperature), and then avidin-biotinylated HRP complex (1-2 h at room temperature). After rinsing, the

HRP was visualized with a glucose oxidase, imidazole, diaminobenzidine solution (Smithson et al.,1984). After mounting, tissue sections were counterstained in either thionine or bisbenzimide. Labeled processes and terminals were readily identified in brightfield illumination by their dark brown coloration which appeared bright in darkfield and polarized-darkfield illumination.

5.5. Results Exp. 2A: WGA-HRP labeling

5.5.1. Injection Sites

All injections of WGA-HRP were confined to the main bulb with no involvement of the accessory bulb or anterior olfactory nuclei, except in one case in which there was limited involvement with both these structures.

5.5.2. Anterograde Labeling

The WGA-HRP technique consistently revealed more anterograde labeling around SON than the PHA-L technique. Anterograde labeling with WGA-HRP, however, did not reveal the same quality of morphology as did PHA-L, in that individual terminal morphology could not be readily discerned at the light microscopic level. In the coronal plane, dense labeling was observed throughout the ipsilateral lateral olfactory tract. Immediately anterior to the nucleus of the lateral olfactory tract, anterograde label was observed outside of this tract along the ventromedial surface of the brain. Further caudally, at the mid-rostrocaudal extent of the SON, punctate labeling was observed along the ventromedial edge of the brain spreading dorsally to the ventrolateral margins of the SON (Fig. 10 C & D). In sagittally cut material dense anterograde label was seen throughout the lateral olfactory tract, while a less dense more punctate pattern of labeling was observed deeper within the 1a layer of the piriform cortex (Fig. 11). Caudal to the nucleus of the lateral olfactory tract, and at the lateral borders

Fig. 10. Overlapping distributions of neurophysin stained processes and WGA-HRP labeling around the SON in the coronal plane.

A & B. Neurophysin staining in a 100 μm thick section midway through the rostrocaudal extent of the SON.

A. Immunocytochemistry illustrating a dense somatic region immediately lateral to the OT, a prominent efferent projection (closed arrow) dorsally, and a ventrally located dendritic projection which courses outside the confines of the nucleus (open arrow).

B. At higher power, the efferent projections are illustrated by a dense plexus of varicose fibers projecting dorsally, and dorsolaterally (closed arrows). The dendritic region of the nucleus lies immediately ventral (open white arrows) to SON cell bodies and also send a prominent projection ventrolaterally (black open arrows) into periamygdaloid regions. A blood vessel interrupts the dendritic projection in this section (clear space).

C & D. Distribution of labeled terminals and fibers in the coronal plane after an injection of Wheatgerm-HRP into the main bulb, as seen in polarized darkfield. This injection encroached marginally on the accessory bulb and rostral anterior olfactory nucleus. These micrographs are taken at approximately the same rostral-caudal level of the SON as those shown A & B.

C. The WGA-HRP labeling is seen in darkfield as a bright punctate stippling on the dark background of surrounding unlabeled tissue. In this section a prominent aggregation of labeled terminal (arrows) appears immediately ventrolateral to the somatic portion of SON. The OT is located medial to the nucleus and appears bright in polarized darkfield. A few retrogradely labeled cells are also seen scattered throughout the adjacent neuropil.

D. At higher power the darkfield illumination was combined with epifluorescence to reveal bisbenzimide counterstained somata; conspicuous is the aggregation of cells in the SON (arrow). The WGA-HRP label (open arrows) is seen ventrolateral to the somatic region of SON within the field of the ventrolaterally projecting dendrites. Bar = 178 μm in A and C and, 100 μm in B and D.

Abbreviations:

OT	optic tract;
SON	supraoptic nucleus-main (or anterior) portion.

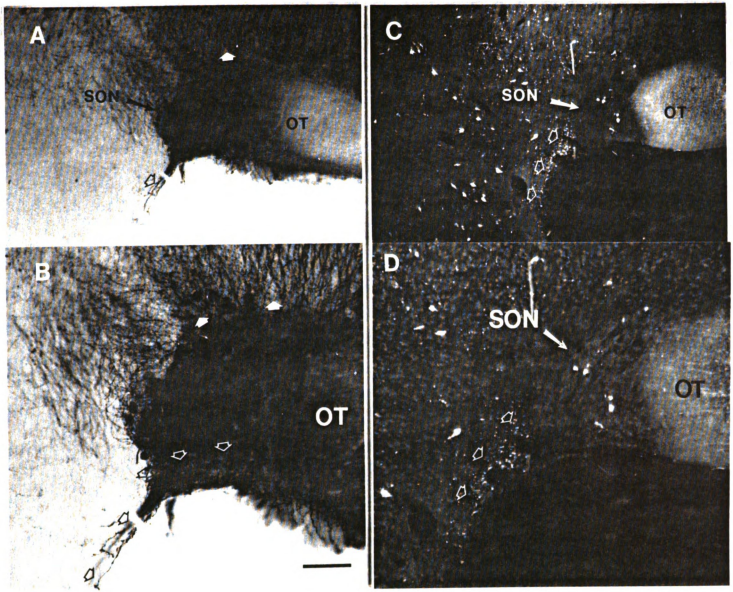
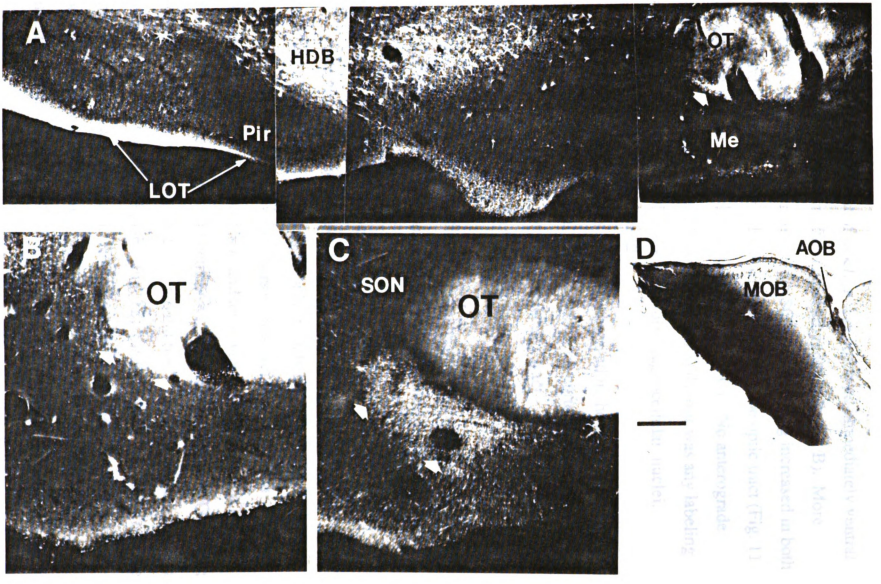

A
SON
OT
C
SON
OT
B
OT
D
SON
OT

Fig. 11. The distribution of labeled cell bodies, fibers, and terminals, after an injection of WGA-HRP into the main bulb.

A. A montage of low power darkfield photomicrographs in a parasagittal plane (50 μm thick) through the lateral margins of SON, just medial to nucleus of the lateral olfactory tract. Rostrally (left), the anterogradely-labeled fibers present as a thin band within the LOT tract (arrows), while deeper within the 1a layer of the Pir punctate terminal labeling is observed. Many retrogradely-labeled cell bodies are also seen within HDB. At the level of the OT, another band of terminals (closed arrowhead) circumscribes the ventral margins of the OT, dorsal to the Me.
B. At higher power, the labeled fibers and terminals (closed arrows) are immediately ventral to the OT. This area encompasses the perinuclear area of the SON as well as the lateral aspects of the SON dendritic zone.
C. Another section, 150 μm medial to that seen in B illustrates many terminals/axons (closed arrows) lateral and ventral to SON. At this level SON cell bodies are positioned along the anterior margins of the OT, while the dendritic field is distributed ventrally within the area of labeling.
D. Site of injection within the main bulb at its largest extent; note that the accessory bulb remains unstained. Bar = 190 μm in A, 100 μm in B and C, and 400 μm in D.

Abbreviations:

AOB	accessory olfactory bulb;
LOT	lateral olfactory tract;
OT	optic tract;
MOB	main olfactory bulb;
HDB	nucleus of the horizontal limb of the diagonal band;
Me	medial nucleus of amygdala;
Pir	piriform cortex;
NLOT	nucleus of the lateral olfactory tract;
SON	supraoptic nucleus-main (or anterior) portion.


A
Pir
LOT
HDB
OT
Me
B
OT
C
SON
OT
D
AOB
MOB

of the SON, a small crescent of labeling was observed immediately ventral to the optic tract and dorsal to the medial amygdala (Fig. 11 B). More medially, within the lateral margins the SON, this labeling increased in both density and size to virtually fill the area subjacent to the optic tract (Fig. 11 C compare with neurophysin labeling in Fig. 8 A-C) No anterograde labeling was observed around the contralateral SON, nor was any labeling seen around the SONt, PVN or the accessory magnocellular nuclei.

5.5.3. Retrograde Labeling

Retrograde labeling was routinely observed in the nucleus of the horizontal limb of the diagonal band and piriform cortex ipsilaterally, and bilaterally in the nucleus of the lateral olfactory tract.

5.6. Results Exp 2B: PHA-L labeling

5.6.1. Injections sites

All injections were confined to relatively small regions of the main bulb (Fig. 12C) with no involvement of either the anterior olfactory nucleus or the accessory bulb.

5.6.2. Anterograde Labeling

The number of labeled cell bodies within the main bulb varied considerably among injections (here only the labeled cells are those which contribute to the PHA-L-labeled fibers). However, each injection consistently revealed labeled fibers and terminals adjacent to the SON. PHA-L labeling was in good agreement with that observed with WGA-HRP; however, the density of labeled fibers around the SON was considerably less with PHA-L. In the sagittal plane (Fig. 12), the full course of fibers from the bulb to the SON was observed. Rostrally at the main bulb, separate fiber bundles within the bulb converged to form a single thick fascicle at the anterior margin of the lateral olfactory tract. Proceeding caudally, the

number of labeled fibers within the tract diminished progressively. Caudal to the piriform cortex, only a few fibers remained in a thin fascicle on the ventral surface of the brain. Posterior to the nucleus of the lateral olfactory tract, another aggregation of fibers appeared immediately anterior and ventral to the SON. The labeled axons (Fig. 12 B) had many varicosities, and a few appeared to have terminal-like endings. Similar to that observed in the WGA-HRP studies, labeled fibers and terminals were first observed lateral to the SON and superior and medial to the margins of the medial amygdala. Progressing medially toward the lateral margins of the SON, the amount of labeling increased dramatically. Reconstruction of this projection from adjacent sections (Fig. 13 B) revealed that it approached the SON anteriorly along its ventrolateral margin as a sheet of fibers approximately 250 μm thick. Furthermore, this distribution of axons seemed to overlap considerably with the arrangement of neurophysin stained processes (i.e. dendrites; Fig. 13 A) as observed along the lateral margins of the nucleus. In material prepared in the horizontal plane, many fibers gave rise to small collateral branches whose bulbous terminal-like structures ended along the posterolateral margins of the nucleus. Additionally, fibers and terminals were also observed more anteriorly in the perinuclear zone of the nucleus (Fig. 14). While not a prominent feature of this projection, terminals were occasionally found within the somatic portion of the SON.

5.7. Discussion Exp 2

The pattern of anterograde labeling observed here from injections limited to the main bulb are in agreement with previous studies of main bulb efferents in the rat (Heimer,1968; Scalia and Winans,1975), and those from other mammals (Broadwell,1975; Davis and Macrides,1981; Devour,1976; Scalia and Winans,1975; Shipley and Adamek,1984). Retrograde labeling,

Fig. 12. The distribution of labeled axons and terminals, after an injection of PHA-L into the main bulb.

A. A montage of low magnification darkfield photomicrographs through a parasagittal plane (15 μm thick) along the lateral margins of the SON. Rostrally (left) many thick axonal process may be seen within the LOT. Throughout the full extent of the Pir the stained axons remained tightly organized within the LOT. Immediately anterior to the SON an aggregation of processes (delineated with lines) fans out immediately anterior to the SON.

B. This area (delineated at lower power in A) contains many fibers (two of which are indicated by open arrows) with varicosities, and terminal profiles.

C. Injection site at its largest extent through a parasagittal plane of the main bulb illustrates that the PHA-L was completely confined to the anterior portions of the main bulb. Many mitral cells (open arrow) appeared darkly stained by the DAB chromogen. Bar = 253 μm in A, 138 μm in B and 800 μm in C.

Abbreviations:

DAB	3,3' diaminobenzidine
LOT	lateral olfactory tract;
MOB	main olfactory bulb;
OT	optic tract;
Pir	piriform cortex;
SON	supraoptic nucleus-main (or anterior) portion.

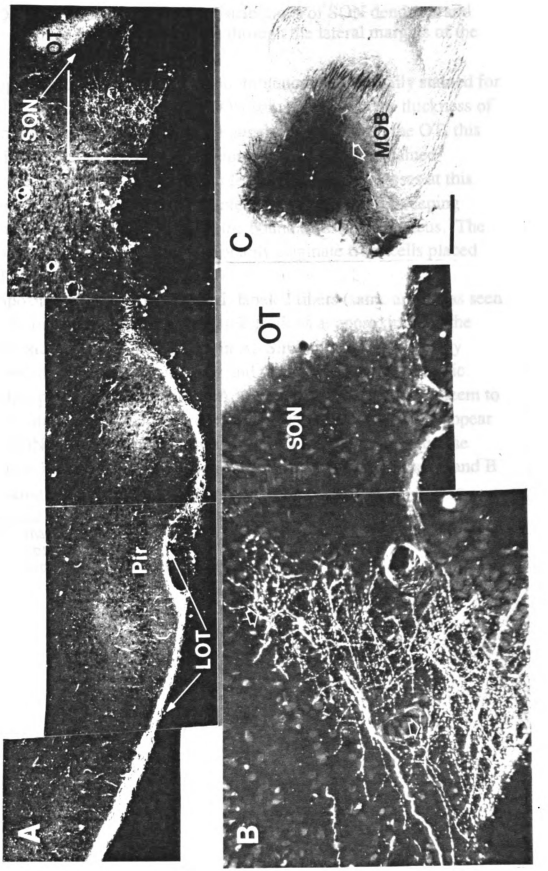

A
Plr
LOT
SON
OT
B
OT
SON
C
MOB

Fig. 13. Diagram of the overlapping distributions of SON dendrites and main bulb axons in a parasagittal plane through the lateral margins of the SON.

A. Tracing from a 100 μm thick section immunocytochemically stained for neurophysin. Open circles represent SON somata. Due to the thickness of the section it appears as if SON somata are embedded within the OT, this however, is not the case. Thick lines represent neurophysin stained processes (i.e. dendrites). A prominent feature of these processes at this mediolateral level is that they extend anterior to the nucleus, sweeping ventrolaterally and posteriorly outside the boundaries of the nucleus. The most anteriorly extending processes probably originate from cells placed more medially within the nucleus.

B. A composite line drawing of PHA-L labeled fibers (same animal as seen in Fig. 3) from four alternate 15 μm thick sections at approximately the same mediolateral level as that drawn in A. Similar to Fig. 12 B many PHA-L-labeled fibers are seen anterior and ventrolateral to the somatic (open circles) portion of the nucleus. Additionally, these processes seem to sweep from lateral to medial towards the nucleus. A few processes appear overlying SON somata; occasionally a process was observed within the nucleus. This however, is not a general feature of these afferents. A and B are at the same magnification.

Abbreviations:

MOB main olfactory bulb;
OT optic tract;
SON supraoptic nucleus-main (or anterior) portion.

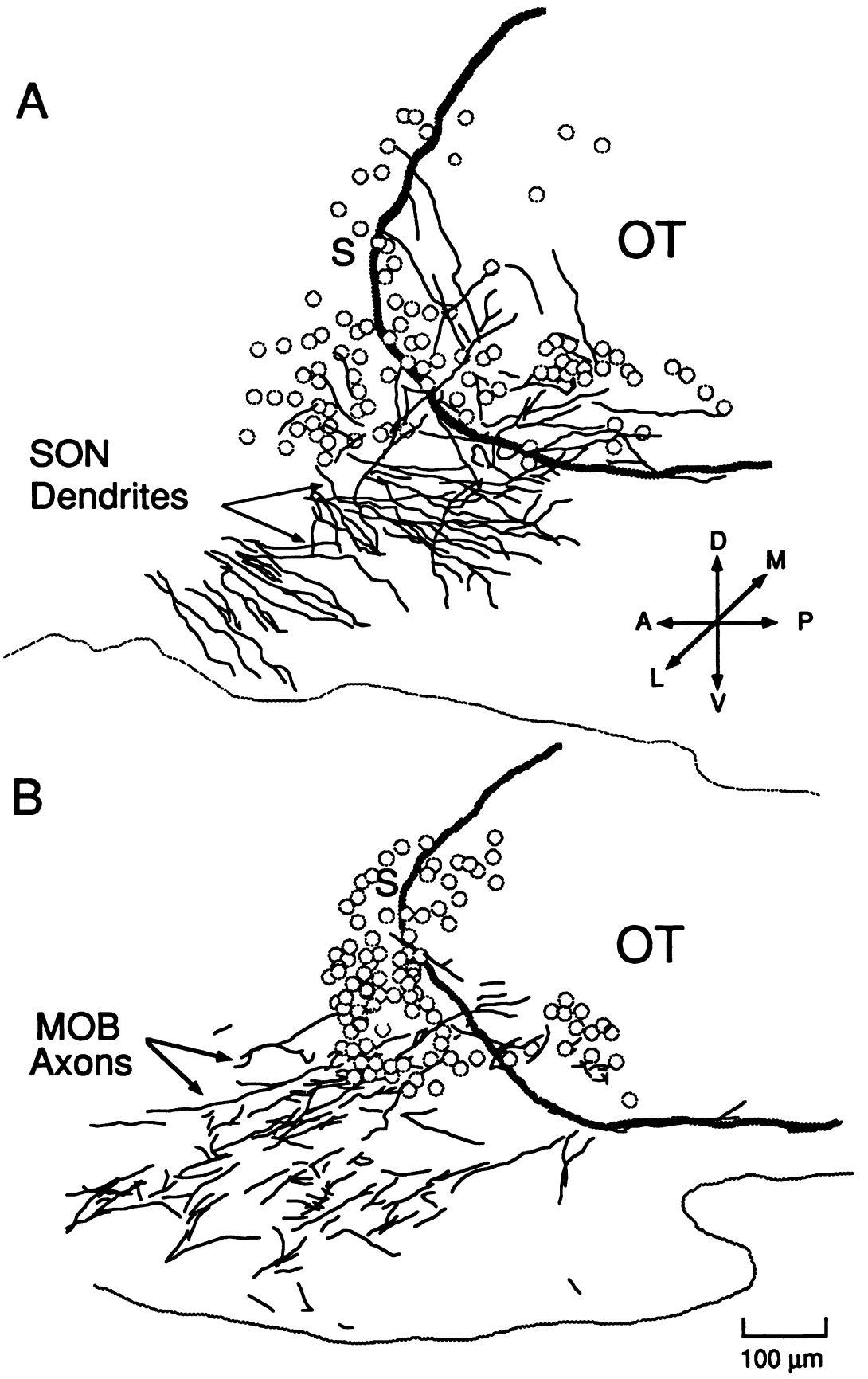
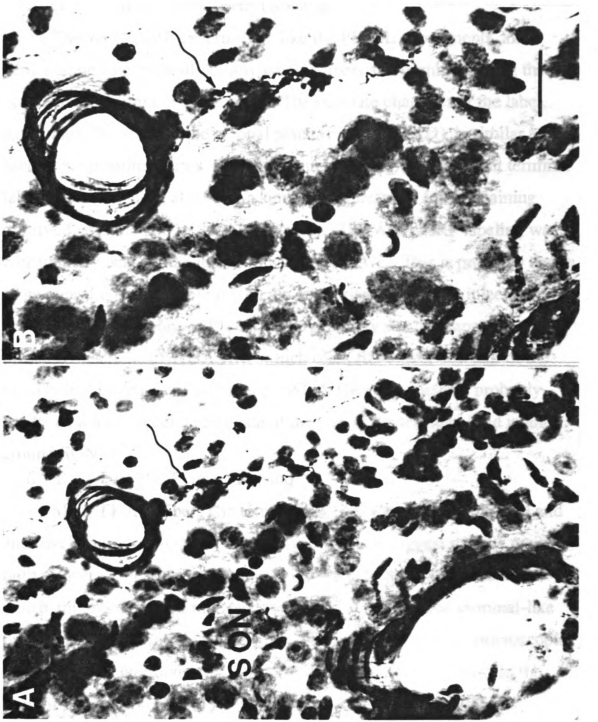

A
S
OT
SON
Dendrites
D
M
A
P
L
V
B
S
OT
MOB
Axons
100 µm

Fig. 14. PHA-L labeled fibers in the perinuclear zone of the SON.

A. A photomicrograph illustrating the approach of fibers to the supraoptic nucleus (SON) in the horizontal plane. Rostral is positioned up and medial is to the left. Fibers are seen running along the lateral border of the SON. In some cases these fibers (arrow) give off bulbous-like terminal profiles. A thionine counterstain appears light gray in cell bodies unstained by the immunocytochemical procedure.

B. Features of several bulbous profiles (arrow) at higher power. Bar= 75 μm and 30 μm in A & B, respectively.


A
SON
B

while not the focus of this study, was also in agreement with that previously reported (DeOlmos et al.,1978; Shipley and Adamek,1984).

5.7.1. Exp 2A: WGA-HRP Labeling

The WGA-HRP experiments, like the PHA-L experiments, also demonstrated a pattern of densely labeled fibers and terminals along the ventrolateral margin of the nucleus. The punctate character of the label, particularly that seen in the coronal plane (Fig. 10 C & D), is similar to that seen in the piriform cortex. Since the latter is thought to represent terminal labeling (DeOlmos et al.,1978), a similar interpretation for the staining observed ventrolateral to SON is plausible. The WGA-HRP labeling was considerably denser than that seen with the PHA-L. This is probably due in large measure to the differences in the respective sizes of the injection sites. Iontophoretic injections of PHA-L were generally small compared to the pressure injections of WGA-HRP which often filled over one-half of the main bulb. Given these differences, WGA-HRP experiments probably produced a more accurate estimate of the size of the terminal field in and around SON.

5.7.2. Exp 2B: PHA-L Labeling

Many PHA-L fibers and terminal profiles were observed lateral and anteroventral to the SON. This was interpreted to suggest that the main bulb projects, at least in part, monosynaptically onto SON cell dendrites which are present in this area (see section 4.5.3). That these terminal-like profiles are indeed terminals is given credence by the electron microscopic analysis by Wouterlood et al. (1985) of PHA-L labeled material. In the sagittal plane, approaching fibers had less terminal profiles than that observed in the horizontal plane. The reasons for this difference are unclear

but may represent a mediolateral orientation of terminals as they ramify over SON dendrites.

Many labeled fibers had a varicose appearance, suggesting a possible *en passant* synaptic arrangement.

Results from the PHA-L experiments also illustrate fibers and terminals in the perinuclear region of the SON. Cells in this area around the SON are known to project into the nucleus (Hatton et al.,1985; Hatton et al.,1983; Mason et al.,1984; Tribollet et al.,1985) and have been postulated to function as intermediaries in modulating a divergent set of efferents (e.g. from locus coeruleus, parabrachial, raphe, subiculum) which terminate within this region (Tribollet et al.,1985). These data are consistent with the view that the main bulb may also be connected to SON in a polysynaptic manner.

6. Exp. 3: Anterograde tracing of Accessory Olfactory Bulb Efferents to SON

6.1. Introduction

Similar to our observations concerning main bulb efferents to the SON, it also seems clear after evaluation of the SON dendritic zone and reviewing published line drawings of anterograde labeling of accessory bulb efferents from the rat (Heimer,1968; Scalia and Winans,1975) and rabbit (Broadwell,1975; Scalia and Winans,1975) that the accessory bulb also probably projects to the SON. No other reports or inferences of such a connection have been published.

6.2. Experimental Question

Simply stated, does the accessory olfactory bulb also project to SON? It is clear from the previous experiments that the PHA-L labeling while revealing the morphology of the bulb efferents greatly underestimates input to the SON. The WGA-HRP labeling on the other hand better represents the magnitude of the input to the SON. Since the accessory bulb efferent is likely less robust because of the fewer mitral cells involved, WGA-HRP labeling is more likely to reveal a connection if one exists.

6.3. Methods Exp. 3: Anterograde studies with WGA-HRP

Pressure injections of 60-160 nl of 0.3% WGA-HRP were stereotaxically placed, under visual guidance, unilaterally into the accessory bulbs of 3 female and 2 male rats. Iontophoretic injections of 0.7 % WGA-HRP were made in a similar fashion in the accessory bulbs of 20 additional rats (3 male and 17 female). Two of these injections were into both accessory bulbs. All iontophoretic injections were made at an angle approximately 23° from the vertical and roughly parallel to the long axis of the accessory bulb mitral cell layer.

Animals were allowed to survive 24-72 h; then they were perfusion-fixed with one of two perfusion protocols: 1) 500 ml of 1% paraformaldehyde, 2% glutaraldehyde in 0.1 M Na phosphate—pH 7.4, followed by equal volumes of 10% sucrose in 0.1 M Na phosphate, 0.14 M NaCl, pH 7.4 or 2) 500 ml of 0.75% paraformaldehyde, 1.5% glutaraldehyde in 0.05 M Na phosphate—pH 7.4, followed by equal volumes of 20% sucrose in 0.05 M Na phosphate, 0.14 M NaCl, pH 7.4. The brains were then stored in their final perfusion solution at 4° C for up to 3 days, after which they were frozen sectioned at 50 μm in either the coronal or sagittal planes. After sectioning, the tissue was processed immediately or stored overnight at 4°C in PBS. The WGA-HRP was visualized with tetramethyl benzidine (Mesulam,1982). In order to facilitate precise localization of staining, tissue sections were arranged into 3 sets of alternate sections. Two sets were counterstained with bisbenzimide (Schmued et al.,1982) while the third was counterstained with thionine. All slides were coverslipped with DPX, and stored at 4°C to prevent deterioration of the anterograde labeling. Labeled fibers and terminals were identified by their bright granular appearance in darkfield illumination.

6.4. Results Exp. 3: WGA-HRP labeling

6.4.1. Injection Sites

In previous work in the main bulb (Smithson et al.,1989b) injections of anterograde tracers were easily confined to this structure. The accessory bulb, however, is much smaller. Consequently, these injections required closer scrutiny. We have taken the dense core of the tetramethyl benzidine reaction product, which surrounds the injection site, to be the effective site of uptake as suggested by Mesulam (Mesulam,1982). On this basis, injections were classified into five groups (Fig. 15). These injection sites

Fig. 15. Accessory olfactory bulb injection sites.

A. *Line* drawing of the main and accessory bulbs. Injections sites included the following structures.

B. The AOB only, N=2.

C. The AOB and rostral AOD, N=4.

D. The AOD only, N=1.

E. The frontal cortex, N=8, or the rostral AOB and MOB, N=1.

F. The AOB and the MOB, N=11.

Abbreviations:

AOB	accessory olfactory bulb
AOD	anterior olfactory nucleus, dorsal subdivision;
AOE	anterior olfactory nucleus, external portion;
AOV	anterior olfactory nucleus, ventral subdivision;
EPl	external plexiform layer of the main olfactory bulb;
Gl	glomerular layer of the main olfactory bulb;
Gr	granule cell layer of main bulb;
GrA	granule cell layer of accessory bulb;
LOT	lateral olfactory tract;
Mi	mitral cell layer of the main olfactory bulb;
MiA	mitral cell layer of accessory olfactory bulb;
MOB	main olfactory bulb.

included those structures involving portions of: the accessory bulb only (2), the accessory bulb and a small portion of the dorsal subdivision of the anterior olfactory nuclei, the rostral anterior olfactory nucleus (4), the accessory bulb and a small portion of the main bulb (1), the overlying frontal cortex (8) and injections which encroached extensively into the adjacent main bulb (11).

6.4.2. Anterograde labeling

No anterograde labeling was observed within the lateral olfactory tract after an injection into the anterior olfactory nucleus (i.e.AOD and AOL). Labeled fibers were observed within the anterior commisure, an observation consistent with previously demonstrated projections of the anterior olfactory nucleus to the contralateral main bulb (Davis and Macrides,1981, see also Switzer et al.,1985). Injections limited to the accessory bulb and those that also encroached upon the anterior olfactory nucleus had similar patterns of anterograde labeling within the lateral olfactory tract and amygdaloid areas. Illustrated and described is anterograde labeling from a unilateral injection into the caudal accessory bulb which spread slightly into the anterior olfactory nucleus (i.e. Fig. 15 C). Anterograde labeling appeared at rostral levels within the dorsomedial margins of the lateral olfactory tract, and remained in this position until the caudal termination of the tract near the nucleus of the lateral olfactory tract. Ventral to this nucleus, the projection turned dorsally while remaining within the superficial layers of the ventromedial surface of the amygdala. Caudally, posterior to the nucleus (i.e. NLOT), retrogradely labeled cells within the bed nucleus of the accessory olfactory tract appeared, capping the anterogradely labeled fibers in their dorsal projection (Fig. 16). As the projection progressed posteriorly (Fig. 16 A-D), both anterogradely labeled fibers and retrogradely labeled

Fig. 16. Distribution of labeled cell bodies and terminals after an injection of WGA-HRP into the ipsilateral accessory bulb.

Polarized darkfield photomicrographs of coronal sections, proceeding from rostral (A) to most caudal (E) illustrating retrogradely labeled cell bodies and anterogradely labeled axons/terminals. Sections are 50 μm thick and 100 μm apart. These levels of the SON correspond approximately to those levels of the SON illustrated in Fig. 7. D-I. Bar = 150 μm & 75 for photomicrographs, left & right, respectively

A. At this rostrocaudal level, retrogradely labeled cells in the BAOT and anterogradely labeled fibers ventrolateral to these cells are observed. Photomicrograph on th right illustrates feature of the labeled BAOT neurons and fibers/terminals at higher power.

B. More caudally, labeled cell bodies and fibers (arrows) are immediately subjacent to SON. At higher power terminals decorate the ventrolateral margins of SON.

Abbreviations:

BAOT bed nucleus of the accessory olfactory tract
OT optic tract;
SON supraoptic nucleus-main (or anterior) portion.

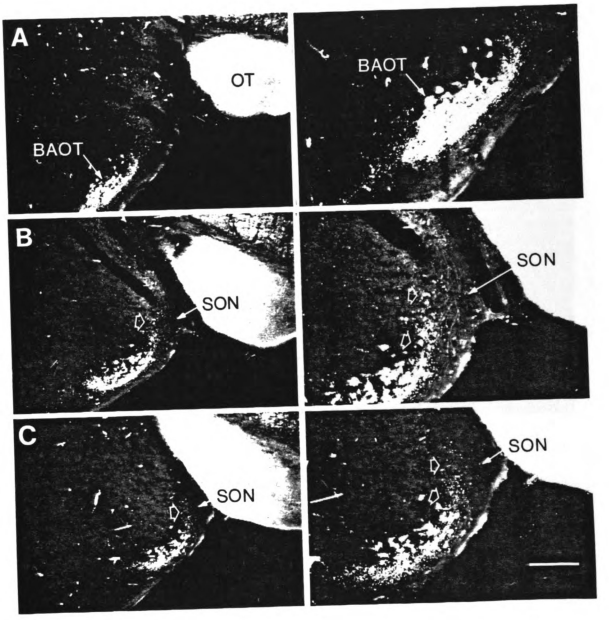
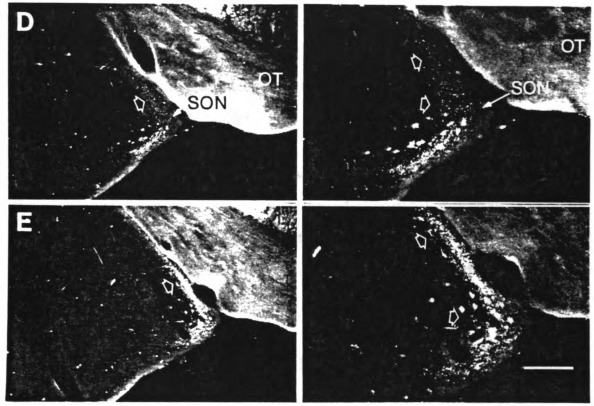

A
OT
BAOT
BAOT
B
SON
SON
C
SON
SON

Fig. 16 (cont.) Plate 2

D-E. Anterograde labeling at more caudal levels of the SON after an accessory bulb injection. Here, anterogradely labeled terminals continue to interdigitate with the SON dendritic zone.


D
OT
SON
OT
SON
E

neurons within the nucleus of the accessory olfactory tract migrated dorsally towards the ventral margins of the SON. Here, at these posterior levels of the SON, anterograde labeling was distributed within the region occupied by ventrolaterally projecting SON dendrites (Fig. 7 D-I). The accessory bulb projection continued posteriorly in its dorsal path along the optic tract sweeping adjacent to the medial amygdala and posteromedial cortical amygdaloid nucleus.

No anterograde labeling was observed near the contralateral SONa. Furthermore no anterograde labeling was observed near the SONt, PVN or accessory magnocellular nuclei.

6.4.3. Retrograde labeling

Many retrogradely-labeled cells within the ipsilateral bed nucleus of the accessory olfactory tract and posteromedial cortical amygdaloid nucleus were consistently observed. A few retrogradely cells were also observed within the medial amygdala, the bed nucleus of the stria medularis, and infrequently, the caudal portion of the horizontal limb of the diagonal band.

6.5. Discussion

The pattern of anterograde labeling observed here, from injections limited to the accessory bulb as well as those which encroached slightly upon the rostral anterior olfactory nucleus is in agreement with previous reports of accessory bulb efferents in the rat (Heimer,1968; Scalia and Winans,1975), and those from other mammals (Broadwell,1975; Davis and Macrides,1981; Devour,1976; Scalia and Winans,1975; Shipley and Adamek,1984). Retrograde labeling, while not the focus of this study, was also in agreement with that previously reported (DeOlmos et al.,1978; Shipley and Adamek,1984).

Similar to those reported in previous experiments of main bulb efferents (in this thesis , and Smithson et al.,1989b), accessory bulb efferents approach the ventrolateral margins of the SON, terminating within the area occupied by SON dendrites. The accessory bulb projection approaches the nucleus more posteriorly than main bulb efferents, terminating near the caudal borders of the nucleus. While this was not a quantitative study, the magnitude of the terminal labeling after an accessory bulb injection appeared smaller than after main bulb injections. In part, this is because the accessory bulb projection approaches the nucleus more caudally where only a small portion of the SON somata and dendritic region remain.

7. Exp. 4: Retrograde tracing of Bulb efferents

7.1. Introduction

The literature suggests that there is an approximate rostrocaudal topography of main bulb projection neurons to their target nuclei (i.e. anterior positioned mitral cells project to more anterior targets; Haberly and Price,1977). This notion, however, was recently disputed (Ojima et al.,1984). Another important feature of the main bulb is that it contains two types of projection neurons, mitral cells and tufted cells. Mitral cells project to all target nuclei. The efferents of tufted cells, on the other hand, are limited to primarily rostral targets (Haberly and Price,1977). The accessory bulb seems to have a different organization. Projection neurons are exclusively mitral cells with little topography described for its projections.

7.2. Experimental Question

The previous experiment employing anterograde tracers suggests strongly that both the accessory and main bulb project to the SON. This conclusion could be strengthened considerably by a complementary retrograde tracing study. The prediction from the anterograde studies is that mitral (or tufted) cells would be retrogradely labeled after confined injections into the SON. Furthermore, since these projections are virtually unknown, no other published information is available, consequently this experiment could also determine the projection neuron involved (i.e whether mitral or tufted), as well as any preferential distribution within bulbar structures.

7.3. Methods: Exp. 4: Retrograde studies with fluorescent tracers.

Pressure injections of 20-70 nl of Fluoro-Gold (Schmued and Fallon,1986; 1-2% in distilled water; courtesy of L. Schmued) or 70-150 nl of rhodamine-label latex microspheres (rhodamine beads; LUMA-FLUOR

Inc.) were stereotaxically placed into the SON of 17 male rats. Injections were aimed at either the SON or tuberal portion of the supraoptic nucleus (SONt). In most cases, injections were also placed in the ipsilateral PVN, with the tracer that was not injected into the SON or SONt (e.g. Fluoro-Gold into PVN and rhodamine beads into SON). After a 3-7 day survival time, the animals were fixed with neutral buffered paraformaldehyde (4%) or formalin (10%). The brains were blocked into diencephalic and telencephalic portions, left in fixative overnight, then transferred to a solution containing 10-30% sucrose, 2% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3-7.4) and stored at 5°C prior to sectioning. Blocks were frozen sectioned at 50 μm in the coronal plane through the preoptic/ diencephalic block and sagittally through the olfactory bulb/ telencephalic block. Sections were collected into either PBS or TBS and arranged into three adjacent sets consisting of every third section and stored refrigerated until they were mounted. After mounting, two sets were often counterstained with either ethidium bromide (Schmued et al.,1982) or bisbenzimide (for rhodamine bead injections) and the remaining set with thionine, after which they were coverslipped in DPX or Entellan. Cells were identified by their bright fluorescence when exposed to the appropriate filter combinations. We only considered an area to definitely project to the SON if retrogradely labeled cells were observed with both tracers.

7.4. Results Exp. 4

7.4.1. Injection Sites

Fluoro-Gold injection sites have been described (Schmued and Fallon,1986) as containing essentially three zones: a) a central zone which usually shows some necrosis, b) a zone of brilliant fluorescence where all tissue elements are stained, and c) a region of very diffuse fluorescence in

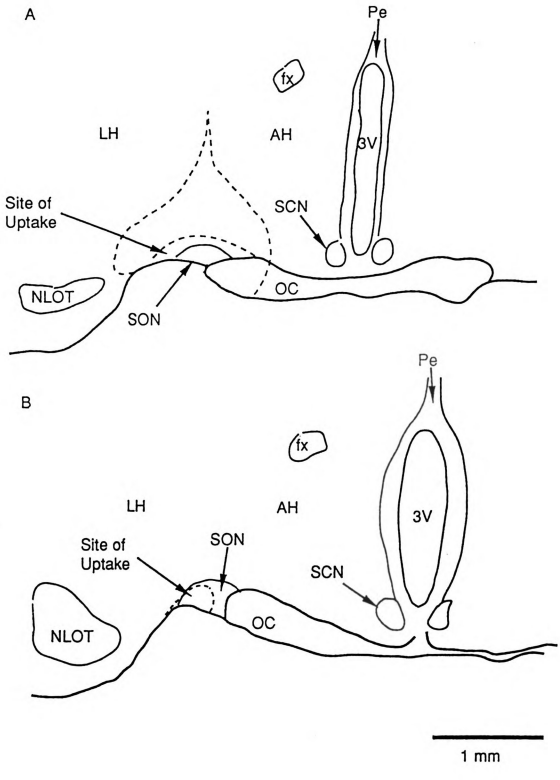
Fig. 17. Line drawings illustrating injections sites of A) Fluoro-Gold and, B) rhodamine-labeled microspheres, into the SON.

A. The effective site of uptake from this Fluoro-Gold injection is demarcated by the inner dashed line, and includes the SON and some of the perinuclear zone surrounding the nucleus. This region did not, however, extend laterally into amygdaloid areas. Retrogradely labeled cells within the main bulb from this injection are presented in Fig. 18. As with all our Fluoro-Gold injections diffuse perikaryal staining (outer dashed line) could be observed surrounding the site of tracer uptake.

B. Injections of rhodamine-labeled microspheres were typically more confined (dashed line), limited to the somatic and dendritic portions of the SON, however, not completely filling its anteroposterior dimensions. Retrogradely labeled cells within the accessory bulb from this injection are shown in Fig. 19. A and B are at the same magnification.

Abbreviations

3V	third cerebral ventricle;
AH	anterior hypothalamic area;
fx	fornix;
LH	lateral hypothalamus
NLOT	nucleus of the lateral olfactory tract;
OC	optic chiasm;
Pe	periventricular hypothalamic nucleus;
SCN	suprachiasmatic nucleus;
SON	supraoptic nucleus-main (or anterior) portion.


A
Pe
fx
LH
AH
3V
Site of
Uptake
SCN
NLOT
OC
SON
B
Pe
fx
LH
AH
3V
Site of
Uptake
SON
SCN
NLOT
OC
1 mm

which occasional cellular elements are stained. The injection sites here also showed a similar organization. Accordingly, we too have considered the site of uptake for the Fluoro-Gold as encompassing only the first two zones of the injection site. The Fluoro-Gold injections used in the present study were largely limited to the SON, nearly filling the complete anteroposterior dimension of the SON. In most cases the region of brilliant fluorescence was confined to the SON and surrounding perinuclear area (Fig. 17 A). These injections were further subdivided into those which spread dorsally into the perinuclear area or lateral hypothalamus, and those spreading ventrally along the ventromedial surface of the periamygdaloid cortex. In the latter case, the adjacent medial amygdala was never involved. In all cases the third region (the area of diffuse fluorescence) spread beyond the confines of the SON into the lateral hypothalamus and occasionally ventrally into the periamygdaloid region. Uptake from Fluoro-Gold injections into the PVN frequently extended beyond the confines of the nucleus into adjacent areas including the zona incerta, perifornical region, and the lateral hypothalamus. In the case of the rhodamine bead injections, the uptake site of the tracer was very localized and taken to be the area of intense fluorescence surrounding the end of the injection tract. Rhodamine beads were virtually limited to either the SON (Fig. 17 B) or SONt and adjacent optic tract. SON injections of rhodamine beads did not fill the complete anteroposterior dimensions of the nucleus, but were centered in its posterior portions.

7.4.2. Distribution of retrogradely labeled cells

Qualitatively, the two fluorescent tracers yielded the same results with respect to retrograde filling of mitral cells within the main and accessory bulbs. Unilateral injections into the SON revealed only ipsilateral labeling within the main (Fig. 18) and accessory bulbs (Fig. 19). Retrograde labeling

Fig. 18. Distribution of labeled cells in the main bulb after an injection of Fluoro-Gold into the SON.

A. A montage of low power epifluorescence micrographs through a parasagittal plane of the main bulb. Many labeled cells within the mitral cell layer of the main bulb are observed. No retrogradely labeled cells were seen in the EPl or Gl of the main bulb and no regional distribution of labeled cells was noted. Open arrow points to a reference blood vessel.
B. At higher power (arrow points to the same blood vessel) many large and small mitral cells are retrogradely labeled through the full thickness of the mitral cell layer.
C. Brightfield micrograph of a section adjacent to that seen in A through the main bulb illustrating the relationship of the Mi, EPl and Gl in the sagittal plane. Bar= 190 μm in A, 45 μm in B and 600 μm in C.
Abbreviations

Mi	mitral cell layer of the main olfactory bulb;
EPl	external plexiform layer of the main olfactory bulb;
Gl	glomerular layer of the main olfactory bulb.

A
Mi
EPI
GI
B
C
Mi
EPI
GI

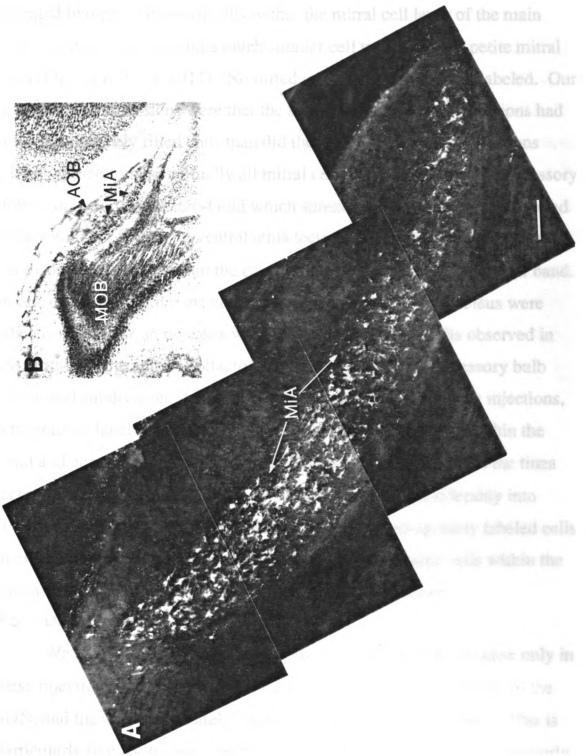
Fig. 19. The distribution of labeled cells in the accessory bulb after an injection of rhodamine-labeled microspheres into the SON.

A. Montage of epifluorescence micrographs in a parasagittal plane through the MOB and AOB illustrating many labeled cells within the mitral cell layer of the accessory bulb.

B. Low power bright field micrograph of a thionine stained section adjacent to that seen in A. Bar= 100 μm in A and 500 μm in B.

Abbreviations

AOB accessory olfactory bulb;
MiA mitral cell layer of accessory olfactory bulb;
MOB main olfactory bulb.


A
MIA
B
AOB
MIA
MOB

of mitral cells throughout the main and accessory bulbs was observed without apparent regional distribution. Additionally, Fluoro-Gold injections revealed two populations of cells within the mitral cell layer of the main bulb: large mitral cells, and a much smaller cell type, possibly petite mitral cells (Fig. 18 B; Cajal,1911). No tufted cells were retrogradely labeled. Our quantitative impressions were that the small rhodamine bead injections had fewer retrogradely filled cells than did the larger Fluoro-Gold injections which seemed to label virtually all mitral cells within the main and accessory bulbs. Injections of Fluoro-Gold which spread dorsally consistently labeled cells within the ipsilateral ventral tenia tecta (i.e. TT_1, or medial transition zone), and bilaterally within the caudal horizontal limb of the diagonal band. In one case, cells within the contralateral anterior olfactory nucleus were labeled. However, in no cases were retrogradely labeled cells observed in the portion of the anterior olfactory nucleus bordering the accessory bulb (i.e. dorsal subdivision; AOD; see Fig. 4) In ventrally spreading injections, retrogradely-labeled cells were virtually limited to mitral cells within the main and accessory bulbs, with a rare retrogradely-labeled cell in the tinea tecta. Injections into the PVN (even those that spread considerably into surrounding areas) or the SONt failed to reveal any retrogradely labeled cells in either the main or accessory bulbs. Retrogradely labeled cells within the frontal cortex were routinely observed from these injections.

7.5. Discussion

We have presented data from a Fluoro-Gold injection, because only in these injections did we fill the complete anteroposterior dimensions of the SON, and thus more accurately represents the input to the nucleus. This is particularly true for the main bulb efferents which terminate more anteriorly in the SON. That leakage of Fluoro-Gold into the subarachnoid space may

in part account for this labeling is highly unlikely. First, in those cases in which we have missed the SON and injected this space (data not presented) mitral cells were never labeled. Secondly if Fluoro-Gold were to be distributed in this space by diffusion one would expect that mitral cells would be labeled bilaterally, this was also never observed.

The rhodamine bead injections, on the other hand, were smaller and confined to the more posterior portions of the SON, and thus underestimates the main bulb projection. While these injections labeled fewer mitral cells in the main bulb, all mitral cells within the accessory bulb were labeled by this injection. This finding is congruent with the fact that the main bulb efferent terminates more anteriorly than accessory bulb efferents.

8. Discussion of Anatomical Experiments

8.1. Summary of Anatomical Results

Immunocytochemical staining for neurophysin revealed immunoreactive processes (i.e. dendrites) of probable SON origin extending ventrolaterally outside the boundaries of the nucleus into periamygdaloid areas. Injections of WGA-HRP into the main or accessory bulbs or injections of PHA-L into the main bulb revealed anterogradely-labeled fibers and terminals ventrolateral to ipsilateral SON somata; the same area occupied by ventrolaterally projecting SON processes. Injections of rhodamine beads or Fluoro-Gold into the SON resulted in many retrogradely labeled mitral cells throughout the main and accessory bulbs.

We have approached the demonstration of this pathway through the use of several different techniques in order that their individual biases may be overcome. That the interpretation of the observations from the, PHA-L, WGA-HRP, Fluoro-Gold, and rhodamine beads are in good agreement with each other argues strongly against the possibility of artifactual staining in each case. Furthermore, many problems associated with interpreting "tract tracing" experiments center around two chief issues; a) uptake of the tracers by damaged fibers of passage, and b) the size of the effective site of uptake. The location and relatively large size of the main bulb within the rat brain, permits injection in such a fashion as to virtually eliminate these problems for the WGA-HRP and PHA-L experiments. Injections into SON with Fluoro-Gold or rhodamine beads, however, must be more closely scrutinized because of these problems. This is especially true because the nearby amygdala is known to receive main bulb efferents. Some of the Fluoro-Gold injections did spread into the lateral hypothalamus and possibly into the amygdaloid areas making our interpretation more tentative. However, the

rhodamine bead injections which were strictly confined to the SON also resulted in retrogradely labeled mitral cells throughout the main and accessory bulbs, thus supporting our interpretation of these results. Likewise injections into the accessory bulb in many cases also spread to the adjacent anterior olfactory nucleus. However, several facts argue against this nucleus, rather than the accessory bulb, as the source of afferent to the SON: 1) no retrogradely labeled cells were observed in this portion of the anterior olfactory nucleus (i.e. adjacent to the accessory bulb) after injections of either Fluoro-Gold or rhodamine beads into the SON, 2) the pattern of anterograde labeling within the lateral olfactory tract and caudal targets was similar to injections which were confined to the accessory bulb, and 3) the pattern of anterograde labeling after injections into only the anterior olfactory nucleus was limited to very rostral structures and did not involve the lateral olfactory tract.

Our data suggest that the SON receives a relatively large input from the main bulb and a somewhat smaller input from the accessory bulb. This interpretation is supported by both, the dense terminal labeling from WGA-HRP injections into the main and accessory bulbs, and the extensive mitral cell labeling after injections of Fluoro-Gold into the SON. The implications of these findings are that mitral cells of the main bulb which presumably project to the anterior olfactory nucleus and piriform cortex also project to the SON. That individual mitral cells may project to both these structures is supported by electrophysiological (Scott,1981) as well as anatomical evidence (Luskin and Price,1982; Ojima et al.,1984). While these authors have not investigated these relationships in the caudal piriform cortex or the SON, Ojima (Ojima et al.,1984) noted that the trajectory of HRP-stained fibers within the lateral olfactory tract suggested that mitral cells could

project to the anterior olfactory nucleus and anterior as well as the caudal piriform cortex. It appears from our work that the main bulb projection to the SON is a continuation of that to the caudal piriform cortex, and is thus likely that individual mitral cells may project to the anterior olfactory nucleus, divergent areas of the piriform cortex, and the SON.

Additional strong support for a monosynaptic projection comes from the observations of retrogradely filled mitral cells after injections of Fluoro-Gold or rhodamine beads into the SON. In particular, the small injections of rhodamine beads which were completely confined to SON somata and dendrites (e.g. Fig. 17 B) consistently revealed labeled mitral cells within the main and accessory bulbs, while injections into the SONt or PVN never labeled mitral cells.

Many anatomical studies have described the efferent projection from the main and accessory bulbs (for a concise description see Switzer et al. 1985) and other mammals. Indeed, the patterns of anterograde labeling and retrogradely labeled cell are in agreement with these previous reports. Nonetheless, no anatomical study to date has described the connection between either the main or accessory bulb and the SON. This is probably in large part because the dendritic projections of SON neurons have only recently begun to be understood (Armstrong et al.,1982; Ju et al.,1986, and the present study). In all likelihood, some labeling formerly interpreted as evidence for a medial amygdaloid afferent was in reality a SON afferent.

Many have also looked at the afferents to the SON, (Iijima and Ogawa,1981; Tribollet et al.,1985; Wilkin et al.,1989) but again no mention was made of retrogradely filled cells in either the main or accessory bulbs. Data from both the anterograde and retrograde studies fail to show any apparent monosynaptic bulbar connections with the PVN. This distinguishes

these efferents to the SON by the fact that virtually all other known major inputs to the SON also go to the PVN (Tribollet et al.,1985). The functional significance of this difference is presently unclear. Retrogradely-labeled cells were consistently observed within the ventral tenia tecta suggesting that this area may also project to the SON.

9. Exp 5. :Electrophysiological analysis of connection

9.1. Introduction

The mitral cell, the major output neuron of the main and accessory olfactory bulbs, provides excitatory input to the pyramidal cells of the piriform cortex. Extracellular analysis of diencephalic neurons also reveals a predominantly excitatory response to olfactory stimuli (Kogure and Onoda,1983; Komisaruk and Beyer,1972; Scott and Leonard,1971; Scott and Pfaffmann,1967). Indeed, a recent anatomical study employing intracellular injection of HRP demonstrates that mitral cells send collateral projections to divergent areas within the piriform cortex (Ojima et al.,1984). It is not unlikely that these diencephalic connections are collaterals of the same mitral cells rather than a special subset of pyramidal neurons.

The previous anatomical experiments (section 4-7) suggest that both the main and accessory bulbs project to the SON monosynaptically, and possibly polysynaptically. Intracellular analysis of this newly-discovered connection in the *in vitro* brain slice demonstrated a predominantly short-latency excitatory response to stimulation of the lateral olfactory tract (Hatton and Yang,1989). This finding supports the hypothesis that the main and accessory bulb are monosynaptically connected to the SON. This study also offers further evidence that mitral cells provide direct excitatory input to diencephalic neurons.

The identity of the chemical(s) which mediate mitral cell neurotransmission is controversial. Several lines of evidence suggest that the transmitter may be glutamate (Bradford and Richards,1976; Yamamoto and Matsui,1976), aspartate (Collins,1979; Collins et al.,1981; Collins and Probett,1981), or the related dipeptide N-acetyl-L-aspartyl-L-glutamate (NAAG; Anderson et al.,1986; Blakely et al.,1987; ffrench-Mullen et

al.,1985; Zollinger et al.,1988). Regardless of this disagreement, it is clear that the excitatory postsynaptic effects observed are mediated through excitatory amino acid (EAA) receptors systems (see Mayer and Westbrook,1987 for a review of these receptor systems) because the excitatory responses are blocked by known antagonists of these receptors (Collins,1982; Collins and Howlett,1988; ffrench-Mullen et al.,1985; Hori et al.,1982).

Recently, Gribkoff and Dudek (1988; 1990) have demonstrated electrically-evoked excitatory postsynaptic potentials (epsp) in SON neurons which were reversibly blocked by the EAA antagonist, kynurenic acid (Cotman et al.,1986; Mayer and Westbrook,1987; Perkins and Stone,1982; Watkins and Evans,1981). This demonstration of an EAA input to the SON provides the first evidence that these compounds may mediate fast excitatory responses in this system. Unfortunately, the afferent(s) mediating these SON responses are unknown. Two likely candidates are the main and accessory bulb efferents to the SON (Smithson et al.,1989b; Smithson et al.,1988) which, when electrically stimulated, elicit a fast, short-latency excitatory response (Hatton and Yang,1989).

9.2. Experimental Question

Here the possibility that these short-latency responses were mediated through an EAA receptor was investigated in an *in vitro*-incubated explant which contain virtually the complete olfactory projection from the rostral end of the lateral olfactory tract to the SON.

9.3. Methods Exp. 5: Methods for Electrophysiology experiments

Male and female Sprague-Dawley rats 36-58 days old were used. All animals were maintained in a 12:12 h light-dark cycle and given food and water *ad libitum*. Artificial cerebral spinal fluid used to prepare and

maintain the explant contained the following ingredients (in mM) NaCl (126), NaH_2PO_4 (1.3), $NaHCO_3$ (26), KCl (5), $CaCl_2$ (2.4), $MgSO_4$ (1.3), glucose (10) and MOPS (3-[N-Morpholino]propanesulfonic acid) (5), pH 7.4.

9.3.1. Explant Preparation

Animals, while freely exploring a guillotine, were quickly decapitated. The parietal and frontal bones were removed thus exposing the cortex and the olfactory bulbs. The lateral olfactory tracts were carefully transected immediately caudal to the olfactory bulbs. The brain was then gently coaxed from the skull while cutting the neurohypophysial stalk and the optic tracts, and then immediately placed into medium (5 °C) which had been gassed with 95% O_2, 5% CO_2. Explants were hand-cut with a razor blade by first blocking the tissue behind the infundibulum, then by cutting the tissue 2 mm dorsal to an imaginary line between the SON and the most rostral portion of the lateral olfactory tract. In older animals with larger brains, the piriform cortex lateral to the tract was also removed. The SON-containing tissue block was then transferred to a ramp-type recording chamber (Haas et al.,1979) and laid ventral-side up on a bed of filter paper affixed to the ramp floor with bone wax (Ethicon). The chamber was maintained at 32-35°C by a heated water-jacket. Strands of surgical gauze were carefully draped over the tissue to direct the flow of medium as it was superfused over the explant. The gassed medium was delivered to the chamber by a 4-channel peristaltic pump (Gilson) at 1.0 - 1.5 ml/ min, and warmed to chamber temperature as it passed through tubing within the water jacket. The dura and blood vessels overlying the SON were carefully dissected away with Dumont #5 forceps and iris scissors (Robaz), under microscopic observation with direct

illumination. The explant was allowed to incubate for two or more hours prior to intracellular recording.

9.3.2. Electrical Stimulation

A stimulating electrode was placed in the most rostral portion of the lateral olfactory tract, approximately 6 mm anterior to the SON (Fig. 20). In some cases, a stimulating electrode was also placed in the cut end of the pituitary stalk to antidromically activate SON neurons. In a few cases, a third electrode was also placed in the olfactory tubercle about midway between the SON and the stimulating electrode in the lateral olfactory tract. Either concentric or twisted bipolar electrodes were used. In all cases when multiple electrodes were employed, they were of the same design. Constant current stimulus pulses, 0.10 -0.15 ms duration, at 2-4 Hz with currents between 2.0 -100 μA were typically employed to evoke responses.

9.3.3. Electrophysiology

Glass microelectrodes, manufactured on a Brown and Flaming-type puller (Sutter Instruments) and filled with 3 M K^+ acetate (80-150 mΩ), were used for these recordings. Electrodes, visually guided under microscopic observation and direct illumination, were positioned in the SON which was easily located by its position in relation to the optic tract (Fig. 20 B). Impalements were obtained by advancing the electrode in 4-8 μm increments using a microdrive (Burleigh Instruments) while concomitantly ejecting brief pulses of positive or alternating current. Signals were amplified with a conventional intracellular preamplifier (Neurodata). After further amplification with an oscilloscope, signals were recorded onto magnetic tape (Hewlett Packard) for later analysis.

Fig. 20. Photomicrographs of the explant preparation.

A. Low power photomicrograph of the explant illustrating the uncut ventral surface of the brain. Two stimulating electrode (S) as well as an intracellular recording electrode (R) have been placed in the tissue.
B. At higher magnification the position of the stimulating electrodes in the rostral lateral olfactory tract (LOT), and the pituitary stalk (PS) is observed. The intracellular recording electrode is positioned in the SON.

A
B
S
B
PS
OC
SON
LOT

9.3.4. Application of EAA antagonist kynurenic acid

The focus of the present study was to determine if the short-latency excitatory responses from lateral olfactory stimulation, previously reported (Hatton and Yang,1989), are mediated through an EAA receptor. This was accomplished, after obtaining a stable impalement in a SON neuron, by first determining the nature (i.e. whether excitatory or inhibitory) and threshold of the lateral olfactory tract evoked response, then exchanging the superfusion medium with medium containing 1 mM kynurenic acid (Sigma) while monitoring the effects of this EAA antagonist on the evoked responses. Finally, impalement permitting, the explant was returned to control medium.

9.4. Results

Recordings were obtained from a total of 66 neurons from 22 animals. These neurons all had overshooting action potentials initially following impalement, with a mean transmembrane potential of -57 mV ± 1 mV (mean ± standard error) and input resistances which ranged from 70-217 MΩ (108 ± 5 MΩ).

9.4.1. Synaptic Responses

In fifty of the recorded neurons, stimulation of the lateral olfactory tract resulted in only excitatory responses. No inhibitory responses were observed. The remaining neurons were unresponsive to lateral olfactory tract stimulation. These responses displayed variable latencies (Fig. 21) and, at threshold, often revealed small depolarizing postsynaptic potentials (psp; see Fig. 23 A), or action potentials preceded by a small epsps . In contrast, antidromic activation via stalk stimulation, when successful, produced a short latency response with a virtually constant latency (Fig. 21 C). Stimulation of the olfactory tubercle never resulted in an evoked

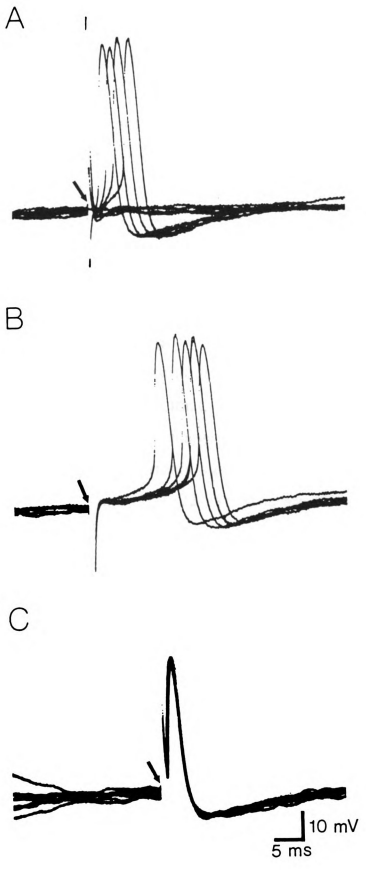
Fig. 21. Oscilloscope traces of evoked responses in SON neurons to electrical stimulation of the lateral olfactory tract (A & B) and neurohypophysial stalk (C)

In all cases arrows denote onset of stimulation.

A. Four consecutive traces illustrating a variable latency response to 3 μA of + polarity current at 2 Hz. The shortest latency response observed is spontaneous activity of the cell.

B. Variable latency responses of the same cell as in A to 6 μA of - polarity current; five consecutive traces are shown.

C. Seven consecutive traces illustrating a constant latency response of another cell to antidromic activation following stalk stimulation.


A
B
C
10 mV
5 ms

response.The current required to elicit a response varied with the stimulus polarity employed, as well as between preparations. That is, response thresholds varied less within a single explant than between preparations. The median threshold current for positive polarity stimulation was 9.5 μA and, when employed, negative polarity stimulation required approximately twice as much current to evoke a synaptic response. Synaptic latencies also varied with stimulus polarity. The mean synaptic latency was 3 ms and 10 ms for positive and negative polarity stimulation, respectively (Fig. 22). In either case, synaptic latencies decreased as the stimulation current was increased above that which produced a threshold response.

9.4.2. Kynurenate Blocking

Out of the 50 cells; demonstrating synaptic responses from electrical stimulation of the lateral olfactory tract, long-term stable impalements were maintained during the medium exchange in 15 neurons. In each case, the evoked response at threshold currents was blocked in kynurenate-containing (1 mM kynurenate) medium (Figs. 22-24). This was observed approximately 3-4 min into the exchange. Higher currents, however, often produced an evoked response (Fig. 23 D). Further superfusion of the explant with kynurenate-containing medium resulted in blocking the evoked response at increasingly higher currents. After approximately 7 min in kynurenate-containing medium, currents 50% larger than threshold currents failed to evoke a response. Indeed in most cases, currents several times threshold also failed to evoke a response. Throughout the superfusion in kynurenate-containing medium, action potentials could be evoked by current injection through the intracellular amplifier or, when previously successful, through antidromic activation via the pituitary stalk (Fig. 22). The kynurenate-containing medium had no apparent effect on either the spike

Fig. 22. Effects of bath application of 1 mM kynurenic acid on responses evoked in SON neurons by electrical stimulation of the lateral olfactory tract.

A. Variable latency responses evoked by electrical stimulation of the lateral olfactory tract.

B. This evoked response is no longer observed after approximately 3 min in kynurenate-containing medium.

C. Action potentials, however, may be elicited with current injection (0.1 nA).

D. Action potential may also be produced by antidromic activation via stalk stimulation. Arrows indicate onset of stimulus, or visible portion of the stimulus artifact in all panels.

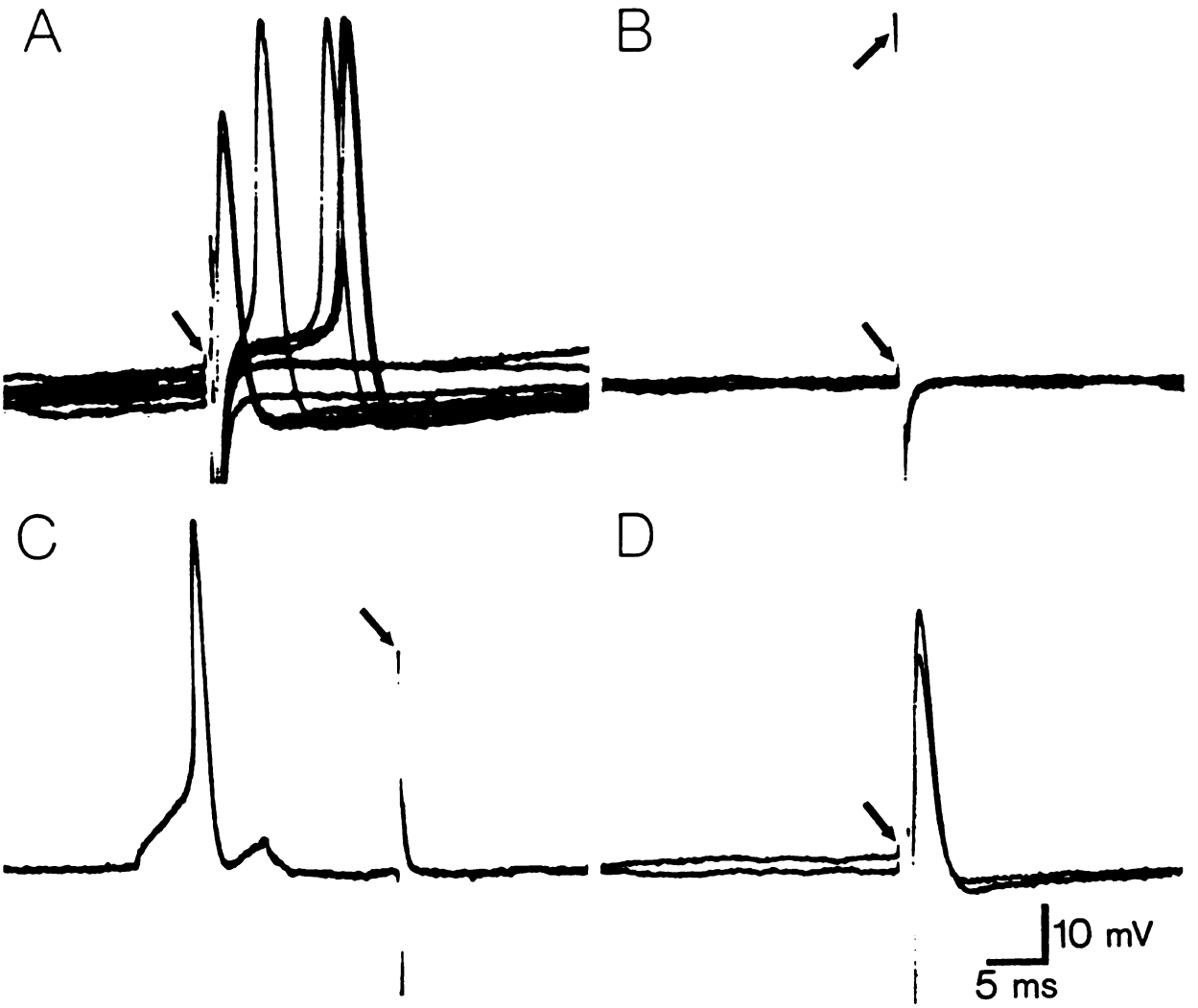

A
B
C
D
10 mV
5 ms

Fig. 23. Effects of kynurenic acid on the threshold of the evoked responses.

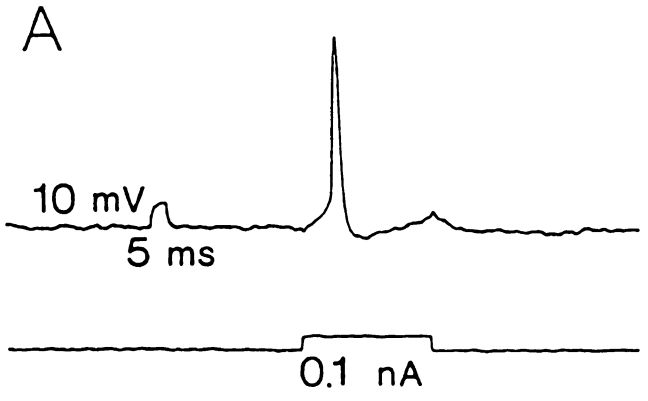
A & B. Oscilloscope traces of multiple consecutive records of synaptic responses in SON neurons to stimulation of the lateral olfactory tract at threshold currents, 10 μA positive polarity and 20 μA negative polarity, A & B respectively. Note the small depolarizing event in A (arrowhead) and the variable latencies of the responses in both records.

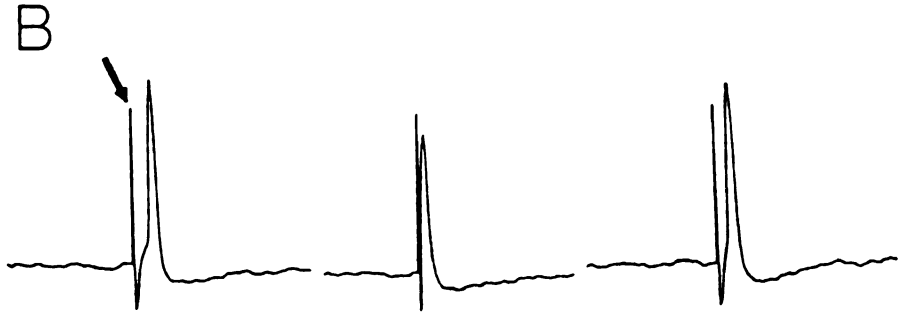
C. After 3 min in kynurenate-containing medium electrically evoked responses are blocked at 30 μA (-polarity), but as before may be elicited by current injection through the intracellular amplifier.

D. However, immediately following trace in C responses from lateral olfactory stimulation may be evoked with 60 μA of current. This response failed altogether with longer treatment in this medium. Arrows as in figures 1 & 2.

Fig. 24. Reversiblity of kynurenate blockade of the excitatory responses in a SON neuron to lateral olfactory stimulation.

A. SON action potential evoked by intracellular current injection.
B. Responses to lateral olfactory stimulation with 50 μA of positive polarity current.
C. After 3 minutes in kynurenate-containing medium stimulation of the lateral olfactory tract with up to 150 μA no longer produced an effect.
D. After a 4 min wash-out in control medium a lateral olfactory response is once again observed with 50 μA of current. In B-D 3 consecutive responses are illustrated, here the stimulus artifact is indicated by an arrow in only the first trace.


A
10 mV
5 ms
0.1 nA


B


C

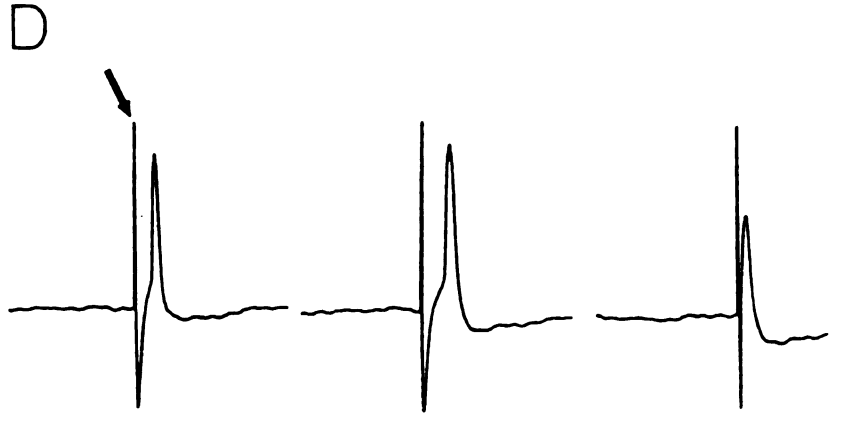
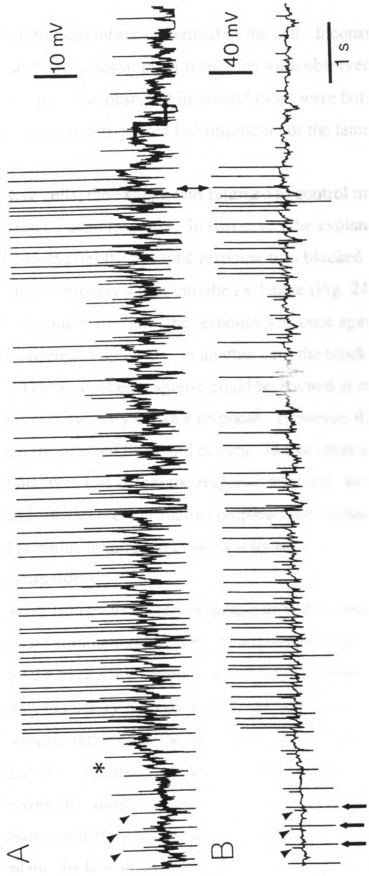

D

Fig. 25. Long depolarizations in response to lateral olfactory stimulation.

A. Shown at increased amplification to that in B. action potentials in A are truncated. Stimulation of the lateral olfactory tract in this SON neuron appeared to result in a prolonged depolarization (onset of depolarization marked with an asterisk), which upon cessation of the stimulation (double-headed arrow) resulted in repolarization of the cell's membrane. Arrows with bar denote the resultant hyperpolarization. A few calibration pulses (10 mV, 5 ms) are labeled with arrowheads at the beginning of each trace.


10 mV
40 mV
1 s
A
B
*

threshold or transmembrane potential of the cell. Inconsistently, both increases and decreases in input resistance were observed in the presence of kynurenic acid. Also observed in several cases were bursts of action potentials apparently triggered by stimulation of the lateral olfactory tract (Fig. 25).

In five cells, the explant was returned to control medium in an attempt to restore the blocked response. In two cases, the explant was returned to control medium after the synaptic response was blocked at threshold currents, approximately 4 min into the exchange (Fig. 24 C). Following a 4 min wash in control medium, the response was once again observed at threshold currents (Fig. 24 D). In another case the blockage was partially reversed. That is, evoked response could be elicited at currents below that which did not previously evoke a response. However, these currents were higher than the original threshold current. In two other cases, the impalements were lost before the response returned. In these last three cases the initial blockades were virtually complete with currents 4-10 X above the threshold stimulus required to evoke a response.

9.5. Discussion

A short latency excitatory response in SON neurons to lateral olfactory tract stimulation has been demonstrated which was reversibly blocked by the excitatory amino acid (EAA) antagonist kynurenic acid. The short latency excitatory responses observed here in the explant are similar to those previously reported in the brain slice (Hatton and Yang,1989; Hatton and Yang,1990). The range of stimulus currents required to elicit threshold responses was also similar. Synaptic latencies, while marginally longer here, probably reflect the differences in placement of stimulating electrodes. In the explant, the lateral olfactory tract is intact rostrally to the anterior

olfactory nucleus, i.e. approximately 4-6 mm anterior to the SON. In the slice this tract is intact for approximately 2-3 mm. In this regard, the explant has an advantage over the slice in that it is easier to resolve these fast events. The similarities observed between the two, different, preparations further strengthen the conclusion that the SON receives an excitatory input from the olfactory bulbs. These findings are also consistent with previous anatomical data (presented in this thesis) which suggest that the SON receives monosynaptic efferents for both the main (Smithson et al.,1989b) and accessory (Smithson et al.,1988) bulbs. An additional finding in the present electrophysiological studies is that these excitatory responses are blocked by kynurenic acid, a result in agreement with previous anatomical (Anderson et al.,1986; Blakely et al.,1987), biochemical (Collins,1984) and electrophysiological (Collins,1982; Collins and Howlett,1988; ffrench-Mullen et al.,1985; Hori et al.,1982)studies, suggesting that mitral cell neurotransmission is mediated, at least in part, via EAAs.

Kynurenate blocked the evoked responses without noticeably altering either the cells' input resistance, spike threshold, or membrane potential; features of kynurenate pharmacology which have been previously reported for SON neurons (Gribkoff and Dudek,1990). Taken together, this evidence suggests that the kynurenate blockage of these evoked responses was not due to the indiscriminate alterations of the cells' membrane properties which might have rendered it unresponsive.

Recently, Gribkoff and Dudek (1990), employing a SON-containing hypothalamic brain slice demonstrated spontaneous and electrically-evoked epsps which were kynurenate sensitive. A plausible interpretation of this experiment is that kynurenic acid is acting postsynaptically on SON EAA receptor similar to that observed in other systems (Cotman et al.,1986).

Using the same concentration of kynurenic acid, we have confirmed and extended these findings: one source of the EAA input is from the olfactory bulbs. Interestingly, our time-course of antagonist blockade was very similar to that reported by Gribkoff and Dudek (1990), with complete inhibition of the evoked events occurring around 7 min. Infrequently, a stimulus dependent slow depolarization, which resulted in bursts of action potentials was observed. The prominent feature of this afferent under our experimental conditions were fast epsps which often resulted in action potentials. This suggests that this input was activating either a quisqualate or kainate receptor (for reviews on these different receptors see Cotman and Iversen,1987; Johnson and Koerner,1988; Mayer and Westbrook,1987). Indeed, the fast epsps observed by Gribkoff and Dudek (1990) were not diminished by DL-2-amino-5-phosphonopentanoic acid, an antagonist of the N-methyl-D-aspartate (NMDA) EAA receptor, suggesting these events were not mediated through this receptor. SON neurons, however, are likely to have NMDA receptors since application of NMDA does produce effects which are reversible and blocked by appropriate antagonists (Gribkoff and Dudek,1990). Furthermore, since Mg^{2+}, a known (non-competitive) antagonist of the NMDA receptor was included in the medium these experimental conditions probably artificially masked the interaction of this receptor with incoming olfactory information.

In fact, it is quite plausible that during conditions in which hormone release is enhanced (e.g. during dehydration, parturition, or lactation) this receptor becomes more efficacious. In these activated states, the SON dendritic zone undergoes a dramatic reorganization of neuronal-glial relationships (see Hatton (1988a; 1990) for a review) resulting in increased dendritic bundling; that is increased direct apposition of dendritic

membranes with each other. This could lead to decreased spatial buffering of $[K^+]_o$ by glia in the area of increased apposition resulting in activity dependent increase in $[K^+]_o$, subsequent depolarization of the membrane, and release of the Mg^{2+} block of the NMDA channel.

In conclusion, electrical stimulation of the lateral olfactory tract produced a short, variable latency excitatory response in the SON. On the basis of previous anatomical investigations in this thesis, this represents the electrophysiological properties of a monosynaptic projection from the main and accessory bulbs to SON dendrites. These excitatory responses are mediated through an EAA receptor which is blocked by kynurenic acid. Further investigations with more specific antagonists should reveal which receptor sub-type is involved in these responses.

10. General Conclusions

10.1 Functional Significance

The functional significance of a connection from the main bulb or accessory bulb to the SON is presently unclear. Unfortunately, there are few well-designed behavior studies which offer any insight into the function of this connection. The few studies that have been published suggest that this connection may participate in fluid homeostasis. Studies in the rat (Novakova and Dlouha,1960) and in the sheep (Bell et al.,1979) demonstrate changes in fluid balance resulting in an increase in urine volume after bilateral bulb ablation. These changes could reasonably be caused by a reduction in circulating vasopressin. That such is the case, is supported by observations in the rat, that bulb ablation decreases circulating vasopressin (as judged by a plasma bioassay; Novakova and Dlouha,1960) Together these observations suggest that the olfactory bulbs play an excitatory role in vasopressin secretion. This view is consistent with the previous report that this connection is excitatory to vasopressin neurons (Hatton and Yang,1989). Additionally, a recent report has demonstrated that olfactory bulbs have receptors for atrial natriuretic peptide (Gibson et al.,1988), further implicating a role in fluid homeostasis for this connection.

These olfactory efferents also provides an excitatory input to oxytocin cells (Hatton and Yang,1989) suggesting a function other than fluid homeostasis for this pathway. Indeed, in two recent reports it was found that electrical stimulation of the lateral olfactory tract in brain slices from either lactating, or maternally-behaving, animals dramatically increased the amount of dye-coupling that was observed (Hatton and Yang,1990; Modney et al.,1990). This observation suggests that in animals which presumably have increased dendritic bundling, that is dendritic membranes directly

apposed with one another without an intervening glial process, (Perlmutter et al.,1984; Salm et al.,1988; Taubitz et al.,1987) this input can selectively increases cell-cell communication, presumably to promote release of oxytocin. It is likely that increases in dye-coupling will also occur in other "activated" states (i.e. dehydration). That activation of a connection can specifically alter junctional conductances between cells is an important observation that deserves further experimentation.

10.2 Summary

In summary results from anterograde tracing studies with PHA-L and WGA-HRP as well as retrograde tracing experiments, provide strong evidence that the main and accessory bulbs are connected to the SON in a monosynaptic manner. The anatomical data also suggests that this connection may be polysynaptic as well. Additionally, the use of an *in vitro* explant preparation, has confirmed one finding in earlier reports (Hatton and Yang,1989; Hatton and Yang,1990; Modney et al.,1990); that this connection is excitatory. Furthermore, results from these electrophysiological studies suggest that this excitatory response is mediated through (an) excitatory amino acid receptor on SON neurons (Fig. 26).

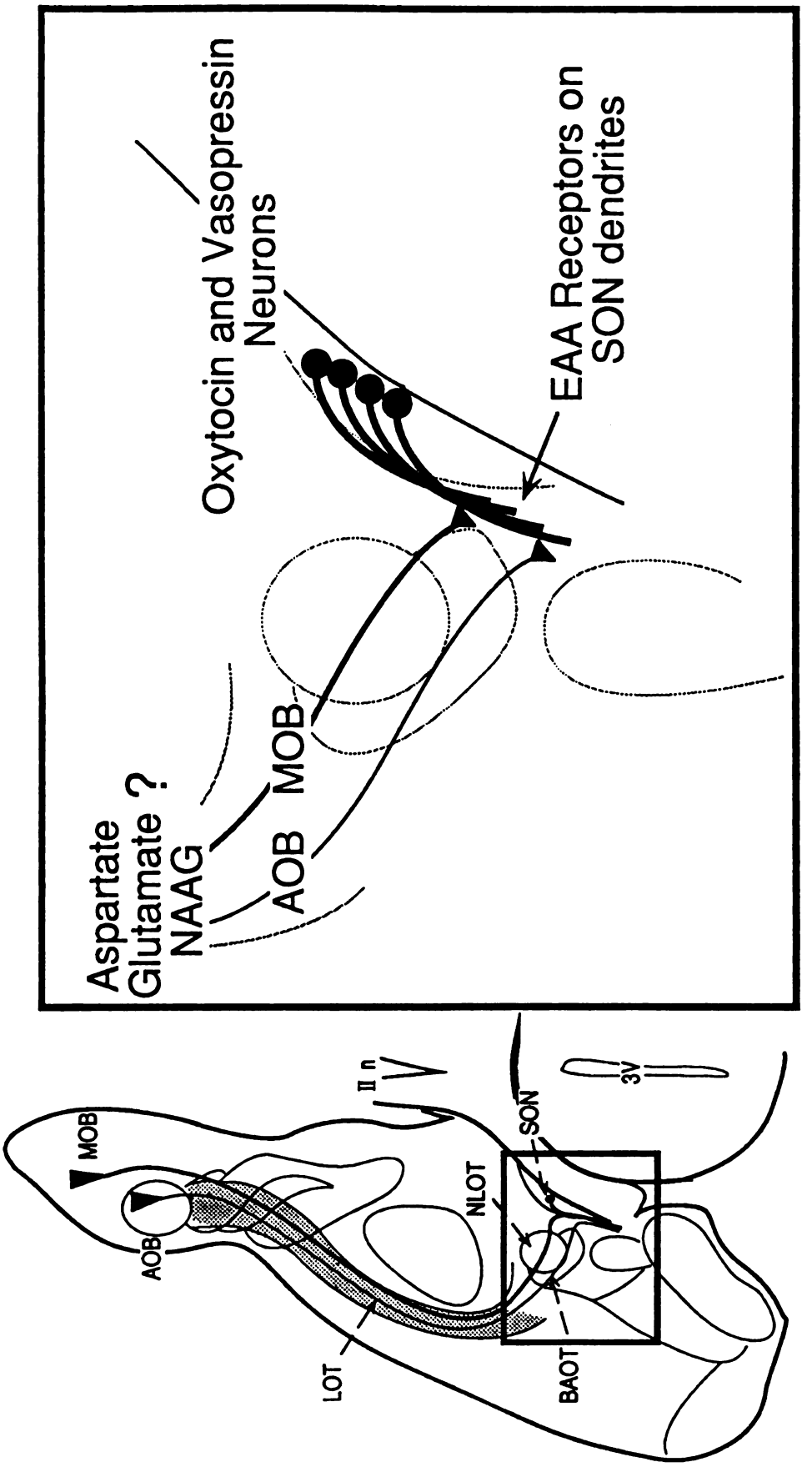
Fig. 26 Schematic of olfactory connections with the SON.

Diagram on left illustrates full course of olfactory projection from mitral cells in the MOB and AOB through the LOT. Inset is drawn at higher magnification on the right .

Inset: MOB and AOB efferents project to dendrites of SON neurons which lie outside the nucleus proper. Electrophysiological evidence is consistent with and EAA as a transmitter (e.g. aspartate, glutamate, or NAAG), and a non-NMDA EAA receptor on these SON dendrites.

Abbreviations

IIn optic nerve.
3V third cerebral ventricle;
AOB accessory olfactory bulb;
BAOT bed nucleus of the accessory olfactory tract;
EAA(s) excitatory amino acid(s);
LOT lateral olfactory tract;
MOB main olfactory bulb
NAAG N-acetyl-L-aspartylglutamate
NLOT nucleus of the lateral olfactory tract;
SON supraoptic nucleus-main (or anterior) portion.


Aspartate
Glutamate ?
NAAG
AOB
MOB
Oxytocin and Vasopressin
Neurons
EAA Receptors on
SON dendrites
MOB
AOB
LOT
II n
NLOT
SON
BAOT
3V

11. Appendices

11.1 Buffers

0.05M Phosphate w/ 20% sucrose pH 7.4

200 g	sucrose
2.749 g	Dibasic sodium phosphate (Na2HPO4)
1.311 g	Monobasic sodium phosphate (NaH2PO4)

Directions
adjust pH to 7.4
Q.S. to 1 liter

0.1 M NaCl in 0.05M Phosphate pH 7.4

5.844 g	NaCl
2.749 g	Dibasic sodium phosphate (Na2HPO4)
1.311 g	Monobasic sodium phosphate (NaH2PO4)

Directions
adjust pH to 7.4
Q.S. to 1 liter

0.2 M Acetate pH 3.3

16.40 g	Na Acetate (anhydrous) OR
27.22 g	Na Acetate . 2H2O
16 ml	12N HCl

adjust pH to 3.3
Q.S. to 1 liter

0.2 M Na phosphate buffer

22.996 g	Dibasic sodium phosphate (Na2HPO4)
5.244 g	Monobasic sodium phosphate (NaH2PO4)

Directions
adjust pH to 7.4
Q.S. to 1 liter

10 mM Na phosphate buffer pH 8.0

Used to reconstitute the PHA-L lectin

1.348 g	Dibasic sodium phosphate (Na2HPO4)
0.069 g	Monobasic sodium phosphate (NaH2PO4)

Directions
pH 8.0
QS to 1 liter

Immunocytochemistry

1.48 g	Dibasic sodium phosphate (Na2HPO4.2H2O)
0.43 g	Potassium monobasic phosphate (KH2PO4)
7.0 g	NaCl
5.0 g	Tris base
0.2 g	Sodium Azide (NaN3)

adjust pH to 7.8
Q.S. to 1 liter

PBS 10X Conc. (Phosphate buffered saline)

This PBS solution was used in conjunction with HRP histochemistry, for the purposes of storing tissue and rinsing the brain prior to fixation with aldehydes.

3.170 g — Monobasic sodium phosphate (NaH2PO4)
10.93 g — Dibasic sodium phosphate (Na2HPO4)
81.82 g — NaCl
Directions
QS to 1 liter
This is 10X the normal concentration.
To use the typical proportions are:
100 ml Conc. PBS
Bring water to around 750 ml
Adjust pH to 7.4
QS to one liter

PBS w/ 10% sucrose

100 ml — PBS 10X conc.
100 g — sucrose
adjust pH to 7.4
Q.S. to 1 liter

11.2 Chromogens/Substrates

TMB for HRP histochemistry

Solution A
555 ml water
30 ml 0.2M acetate buffer pH 3.3
0.3 g sodium nitroprusside

Solution B
15 ml — 100% EtOH
0.015 g — TMB (tetramethy benzidine)
May be dissolved by immersing the vessel in the sonicator
Directions
Prepare solutions immediately prior to use.
Before use they should be stored in the dark.
Mix solution B into solution A with mixing just prior to incubation.

3,3' diaminobenzidine for Immunocytochemistry

The chromogen mixture is made from two solutions, a substrate and an enzyme solution, both of which are made well in advance of the immunocytochemical procedure.
The substrate mixture contains:
100 mg% — 3,3'-diaminobenzidine.4HC1 (DAB; Sigma, type II)
200 mg% — ß-D-glucose,
40 mg% — NH4Cl
8 mg% — imidazole
0.15 M Tris buffer pH 7.0 at RT
Store frozen.
The enzyme solution contains:
28 mg% — glucose oxidase (GOD; Sigma type IX 30,000U/g activity),
2 mg% — thimerosal buffered
in 0.15 M Tris pH 7.0,

Store at 5 °C for up to several months.
Prior to performing the histochemical reaction the frozen substrate solution is thawed and filtered (Gelman GA-3; 1.2 µm pore), to which 30-50 µl of the enzyme solution per milliliter of substrate solution is added.
Since the substrate solution and the chromogen mixture contain DAB, which is a possible carcinogen, proper handling is advised. Glassware, lab surfaces, and equipment which have been contaminated with DAB or either of these solutions may be decontaminated in a 10% solution of household bleach (sodium hypochlorite and water).

11.3 Fixatives

0.75% paraformaldehyde, 1.50% glutaraldehyde in 0.05M phosphate pH 7.4

75.0 ml	paraformaldehyde 10% aq..
60.0 ml	glutaraldehyde 25% aq
2.749 g	Dibasic sodium phosphate (Na2HPO4)
1.311 g	Monobasic sodium phosphate (NaH2PO4)

adjust pH to 7.4
Q.S. to 1 liter

1% parformaldehyde, 1.25% glutaraldehyde in 0.1M Phosphate pH 7.4

100.0 ml	paraformaldehyde 10% aq.
50.0 ml	glutaraldehyde 25% aq.
11.498 g	Dibasic sodium phosphate (Na2HPO4)
2.622 g	Monobasic sodium phosphate (NaH2PO4)

adjust pH to 7.4
Q.S. to 1 liter

2.5% Glut, 1.0% para in 0.1M cacodylate with 0.5% DMSO

10.25 g	cacodylate with 2.5 waters of hydration
50.0 ml	paraformaldehyde 10% aq..
50.0 ml	glutaraldehyde 25% aq
2.5 ml	DMSO

adjust pH to 7.4
Q.S. to 500 ml

4% paraformaldehyde in .1 M sodium acetate pH 6.5

400.0 ml	paraformaldehyde 10% aq.
8.203 g	sodium acetate anhydrous

Directions
In 300 ml water dissolve the sodium acetate first.
Then add the paraformaldehyde
Adjust pH to 6.5 with concentrated HCl
Transfer to 1 liter volumetric with 3 rinses and then Q.S. to the line.

4% paraformaldehyde in 0.1M PO4 pH 7.4

400.0 ml	paraformaldehyde 10% aq.
11.498 g	Dibasic sodium phosphate (Na2HPO4-anhy)
2.622 g	Monobasic sodium phosphate (NaH2PO4.1 H2O)

adjust pH to 7.4
Q.S. to 1 liter

4% paraformaldehyde, .05% glutaraldehyde in .05 M sodium borate pH 9.5

400.0 ml	paraformaldehyde 10% aq.
2.0 ml	glutaraldehyde 25% aq.
19.1 g	sodium borate .10 H2O

Directions
In 300 ml water dissolve the sodium borate first.
Then add the para and glut
Adjust pH to 9.5 with concentrated NaOH or HCl
Transfer to 1 liter volumetric with 3 rinses and then Q.S. to the line.

10% parformaldehyde

300 ml	DD water
50 g	paraformaldehyde
10	pellets of NaOH

Heat to 60°C w/ stirring
Transfer to 500 ml volumetric
QS to 500 ml
Filter w/ Whatman #4

11.4 Slice Medium

Concentrated brain slice medium (4X working dilution)

Chemical	2 liter	1 liter	500 ml	250 ml
NaHCO3	17.440 g	8.720 g	4.360 g	2.180 g
KCl	2.960	1.480	0.740	0.370
NaH2PO4*H20	1.379	0.689	0.345	0.172
MgSO4	1.250	0.625	0.312	0.156
D-glucose	14.400	7.200	3.600	1.800
NaCl	58.000	29.000	14.500	7.250

SM 310 with MOPS (working dilution of slice medium)

Chemical	2 liter	1 liter
Conc. A2 solution	508.4 ml	254.2 ml
MOPS	2.093 g	1.046 g
CaCl2*2H20	0.712 g	0.356 g

11.5 Protocols

Immunocytochemistry of 100 micron sections

Bouin's fixative 150:50:10 (picric acid, formaldehyde, acetic acid
Primary 24-72 H @4°C incubation but as long as 7 days
Rinse 2 hours 6-8 changes in buffer
Secondary 24 H @4°C
Rinse 2 hours 6-8 changes in buffer
ABC 24 H @4°C
Rinse 2-3 H 10-12 changes in buffer
Sera solutions diluted with buffer containing 2.0% Triton-X-100

HRP histochemistry with TMB

Injections

typical injection volume 100-150nl of WGA-HRP

Survival time
24-72 hours

Fixation

Rinse briefly with PBS about 5 minutes
Perfuse with 500 ml fixative
Total time of perfusion should be 30 min. Fixative should flow fast at first and then be slowed down so that total fixation time is 30 minutes.
Perfuse with 500 ml PBS w/ 10% sucrose
Same rate and volume as fixative

Storage

May be stored at 4°C for up to 7 days in the PBS-sucrose mixture

Sectioning

Brains are sectioned on freezing microtome into cold PBS.
Rinse in one change of cold PBS and either react or store for 1-2 days. Some say they may be stored for 7 days at this point

Tissue histochemistry.

Rinse tissue in 6- 30 second rinses of distilled water
Pre-incubate tissue in chromogen mixture (without hydrogen peroxide) for 20 min. on rocker table.
All sections should be covered with chromogen solution.
Prepare chromogen solution, as before, with the addition of hydrogen peroxide.
Solution should contain 1.0 to 5.0 ml of hydrogen peroxide per 100 ml of chromogen solution. Typical examples are closer to 1.0-1.5 ml per 100 ml solution.
Dump out pre-incubation solution, and replace with chromogen solution with hydrogen peroxide
Incubate for 20 min.
No evidence that reaction has to be done in the dark
All sections should be covered with chromogen solution.
Rinse 6 times with 0.01 M Acetate buffer for total 30 minutes
Tissue may be store for 4 H at 4°C
Mount sections from acetate solution air dry
Sections may be left to air dry for 7 days at room temperature without loss of reaction product

Dehydration and stabilization

In order to ensure that reaction product remains permanent section must be dehydrated (at least briefly)
Acetone has less deleterious effects on staining then does ethanol, methanol or isopropyl alcohols

Short schedule for unstained sections:

2 x 10s in distilled water
70% EtOH (10s)
95% EtOH (10s)
2 x 100% (10s)

Long schedule for counterstained tissue

2 x 10s in distilled water
70% EtOH Acetate buffered to pH 3.3 (1-2 min cold)
95% EtOH Acetate buffered to pH 3.3 (differentiate cold)
2 x 100% Acetate buffered to pH 3.3 (1-2 min cold)

Clearing and coverslipping

After appropriate dehydration tissue is cleared in several changes (3 x 10 min) of xylene
Dehydration by one of the protocol above is essential to insure the stability of reaction product
Exposure to xylene to as much as 72 H has no effect on the reaction product

Thionine Staining

100% EtOH	5 min.
Xylene #3	15 min.
d. H2O	5 min.
100% EtOH	5 min.
95% EtOH	2 min.
70% EtOH	2 min.
d. H2O	5 min.
Thionine stain	
d. H2O	1 min.
70% EtOH	30-60 s
95% EtOH	differentiate
100% EtOH	3 min.
Xylene 1	5 min.
Xylene 2	10 min.
Coverslip	

11.6 Equipment Sources

COMPANY	ADDRESS		PHONE
A-M Systems, Inc.	1220 75th Street S.W.	Everett, WA 98203	1-800-426-1306
Activational Systems Inc.	24580 Forterra Dr.	Warren, MI 48089	313-756-1222
Advantage Computer Systems	405 Grand	Lansing, MI	371-1085
Aldrich Chemical Co., Inc.	940 West Saint Paul Ave.	Milwaukee, WI 53233	1-800-558-9160
Antibodies Inc.	P.O. Box 1560	Davis, Ca. 95617	800-824-8540
Apothecary Surgical Supply	737 N. Grand Ave.	Lansing, MI 48906	482-0882
Bachem, Inc.	3132 Kashiwa St.	Torrance, CA 90505	213-539-4171, 213-530-1571
Baxter Scientific	30500 Cypress	Romulus, MI 48174	800-482-3740
Beckman Instruments	3311 N. Kennicott Ave.	Arlington, Heights, IL 60004	800-742-2345
Bergon Brunswic Drug Co.	2450 Oak Industrial Dr. N.E.	Grand Rapids, MI 49505	800-632-5690
Biomedical Research Instruments	12264 Wilkins Ave.	Rockville, Maryland 20852	800-327-9498
Biomedical Research Instruments	12264 Wilkins Ave.	Rockville, Maryland 20852	800-327-9498
Boehringer Mannheim Biochemicals	7941 Castleway dr.	Indianapolis, In. 46250	800-428-5433
Brain Research Laboratories	P.O. Box 88	Waban, Mass. 02168	617-965-5544
Braintree Scientific	P.O. Box 361	Braintree, Ma. 02184	

Burleigh Instruments, Inc.	Burleigh Park, P.O. Box E	Fishers, NY 144539988	
Burrows Co.	3422 Lousma	Wyoming, MI 49509	800-632-2460
Business Resources Inc.	P.O. Box 8079	Ann Arbor, Mi. 48107	517-482-4647
C.L. Sturkey	646 Kulp Rd., Box 182	Perkiomenville, PA 18074	(215) 754-7296
Calibochem Corp.			
Cambridge Research Biochemicals, Inc.	Suite 202, 10 East Merrick Rd.	Valley Stream, NY 11580	800-327-0125
Castle Photo	501 E. Michigan Ave.	Lansing, MI	484-5230
Chemicon International, Inc.			
Cole Palmer Instrument Co.	7425 N. Oak Park Ave.	Chicago, IL 60648	800-323-4340
Comtel Instruments	21223 Hilltop Street	Southfield, MI 48307	313-358-2505
Cramer Industries, Inc. (AKA Cramer Inc.)	625 Adams	Kansas City, Kansas 66105	913-621-6700
David Kopf Instruments	7324 Elmos Street, P.O. Box 636	Tujunga, CA 91042	818-352-3274
Deknatel	600 Airport Rd., P.O. Box 2980	Fall River, MA 02722	800-843-8600 (Customer Service)
Diatome	P.O. Box 125	Fort Washington, Pa. 19034	215-646-1478
Direct Safety Company	P.O. Box 50050	Phoenix, Arizona 85076-0050	800-528-7405
E-Y Laboratories, Inc.	P.O. Box 1787	San Mateo, CA 94401	1-800-821-0044
Edmund Scientific	101 E. Gloucester Pike	Barrington, NJ 08007-1380	609-573-6250 or 609-547-3488
Electron Microscopy Sciences	Box 251	Fort Washington, PA 19034	800-523-5874
Ellsworth Adhesive Systems	8310 W. Parkland Ct., P.O. Box 23961	Milwaukee, WI 53223	
Emmitt Instrument Service, Inc.	31036 Grand River Ave., P.O. Box 32	Farmington, MI 48024	(313) 477-4070
Energy Beam Sciences	11 Bowles Road, P.O. Box 468	Agawam, MA 01001	800-992-9037
EQS Systems	8588 Mayfield Rd.	Cesterland, Oh. 44026	216-729-2222
Fine Science Tools, Inc.	321-B Mou;ntain Highway	North Vancouver, B.C., Canada V7J 2K7	604-980-6127
Fisher Scientific	32231 Schoolcraft Dr., Suite 201	Livonia, MI 48150	313-261-3320

Fluka Chemical Corp.	980 S. Second Street	Ronkonkoma, NJ 11779	1-800-fluka-us
Fulton Radio	1208 Greenwood Ave., P.O. Box 480	Jackson, MI 49204	(517) 784-6106
Gaylord Bros., Inc.	P.O. Box 4901	Syracuse, NY 13221	800-448-6160
George Tiemann & Co.	84 Newton Plaza	Plainview, N.Y. 11803	516-694-6283
George Worthington Co.	P.O. Box 13037, 1611 N. Grand River	Lansing, MI 48901	
Gilson Medical Electronics, Inc.	Box 27, 3000 W. Beltline Hwy	Middleton, WI 53562	608-836-1551
Glass Co. of America, Inc.	Oakland and Ridge Ave.	Bargaintown, NJ 08232	(609) 927-8784
Global Computer Supplies	45 S. Service Rd.	Plainview, NY. 11803	800-845-6225
Gould Inc. Instruments Division	3631 Perkins Ave.	Cleveland, Oh. 44114	216-361-3315
Gould Instruments	32307 Malley Road	Madison Heights, MI 48071	
Hamilton Co.	P.O. Box 10030	Reno, Nevada 89520-0012	1-800-648-5950
Hasselbring Clark	5858 S. Aurelius Rd.	Lansing, MI 48911	393-6210
Imagination	2032 43rd Ave. E. #8	Seattle, Washington 98112	
Inacomp Computer Center	2848 E. Grand River	E. Lansing, MI 48823	351-1777
Inmagic	2067 Massachusetts Ave	Cambridge, Ma. 02140-1338	617-661-8124
J & H Berge Inc.	4111 S. Clinton Ave.	S. Plainfield, NJ 07080	
Jandel Scientific	65 Kock Rd.	Corte Madera, CA 94925	800-874-1888
Janssen Life Science Products	5325 Fulton	Atlanta, Georgia 30336	800-624-0137
JDR Microdevices	110 Knowles Dr,	Los Gatos, Ca. 95030	800-538-5000
John Wiley and Sons	Dept. 8-6241 P. O. Box 6792	Somerset, New Jersey 08875-9976	
John Bell Engineering Inc.	400 Oxford Way	Belmont, Ca. 94002	415-392-0411
Lab Safety Equipment	P.O. Box 1368	Janeville, Wi.53547-1368	800-356-0783
Laboratory Supplies Co.	29 Jefry Lane	Hicksville, NY 11801	(516) 681-7711
Ladd Research Industries	P.O. Box 1005	Burlington, Vermont 05402	800-451-3406

LKB Instruments, Inc.	9319 Gaither Rd.	Gaithersburg, Maryland 20877	800-638-6692
Logitech	805 Veterans Blvd.	Redwood City, Ca. 94063	415-365-9852
Luma Fluor Inc.	50 New Valley Rd.	New City, N.Y. 10956	914 638-6719
M & M Repair Service	Rural Route 5, Box 259	Warsaw, Ind. 46580	(219) 858-9352
Mager Scientific	P.O. Box 160	Dexter, Mi. 48130	800-521-8768
Management Information Source	1107 N.W. 14th Ave.	Portland, Or 97209	800-manuals
Mc Master- Carr	P.O. Box 4355	Chicago, Il. 60680	312-833-0300
Meer Dental Supply Co.	3016 Vine Street	Lansing, MI	351-7774
Mettler Instrument Corp.	Princeton-Hightstown Rd., Box 71	Hightstown, NJ 08520	609-448-3000
Michigan Brass and Electric	1901 W. Saginaw Street, P.O. Box 15068	Lansing, MI 48901	489-3232
Michigan Office Supply	3950 N. Grand River	Lansing, Mi. 48906	517-323-3527
Millapore Corp.		Bedford, MA 01730	1-800-225-1380
Misco	One MISCO plaza	Holmdel, N.J. 07733	800-631-2227
Morgan Instruments	808 N. Rochester Rd.	Clawson, Mi. 48017	
National Diagnostic	198 Rt. 206South	Somerville, NJ	
Neuro Data Instruments Corp.	35 Waterside Plaza	New York, NY 10010	212-685-7580
New Line Software	P.O. Box 289	Triverton, RI 02878	401-624-3322
Newark Electronics	500 N. Pulaski Rd.	Chicago, IL 60624	312-784-5100
Norman Camera Company	3602 S. Westnedge	Kalamazoo, MI 49008	616-343-0460
Panamax	150 MitchellnBlvd.	San Rafail, Ca. 94903	
Patterson Dental Co.	1238 Anderson Rd.	Clawson, MI 48017	313-435-2424
Polaroid	784 Memorial Dr.	Cambridge, Mass 02139	800-343-4846
Polysciences, Inc.	400 Valley Road	Warringaton, PA 18976-2590	800-523-2575
Precision Systems, Inc.	16 Tech Circle	Natick, MA 01760	508-655-7010
Priority One Computers	21622 Plu;mmer St.	Chatsworth, CA 91311	800-423-5922
Procomp Computer Products Inc.	27631 John	P.O. Box 71187 Madison, Mi.	
Randop Surgical Supplies	31742 Enterprise Dr.	Livonia, Mi. 48105	313-427-4810

Research Biochemical Inc.	9 Erie Dr.	Natick, MA 01760-1312	508-651-8151 (FAX 508-655-1359)
Rhodes Medical Instruments	21044 Ventura Blvd.	Woodland Hills, Ca. 91346	818-347-3577
Sargent-Welch	4717 Hinchley Industrial Parkway	Cleveland, Ohio, 44109	800-321-8620
Scientific Instrument Services, Inc.	R.D. #3, Box 593	Ringoes, N.J. 08551-9675	201-788-5550
Second Source	P.O. Box 337	Buarte, CA 91010-9974	800-772-3924
Setler's			800-426-3938
Seton Name Plate Corp.	P.O. Drawer FH 1331	New Haven CT 06505	800-243-6624
Sigma Chemical Co.	P.O. Box 14508	VSt. Louis, MO 63178	800-325-3010
Sitler's Supplies Inc.	702 E. Washington	Washington, Iowa 52353	319-653-2123, 800-426-3938
Sitler's Supplies, Inc.			800-426-3938
Southeastern Software	7743 Briarwood Dr.	New Orleans, La. 70128	504-246-8438
SPI Supplies	P.O. Box 342	West Chester, PA 19380	1-800-2424-SPI
Squire Cogswell Co.	22545 Heslip Drive	Novi, MI 48050	(313) 348-3700
Stevenson Metal Supply	239 East 79th St.	New York, NY 10021	(212) 794-8927
Superior Business Form, Inc.	927 Beech St.	Lansing, MI 48912	(517) 487-8754
Technical Manufacturing Corp.	15 Centennial Dr.	Peabody, Ma. 01960	508-532-6330
Ted Pela, Inc.	P.O. Box 2318	Redding, California 96099	800-237-3526
Tektronix, Inc.	24155 Drake Rd.	Farmingtonn, MI 48024	(313) 478-5200
Thomas Scientific	99 High Rd. Hill at Interstate 295 P. O. Box 99	Swedesboro, N.J. 08085-0099	609-467-2000
Unimetrics	501 Earl Road	Shorewood, IL 60436	800-854-6931
Vector Laboratories, Inc.	30 Ingold Rd.	Bulingame, CA 94010	(415) 697-3600
VWR Scientific	P.O. Box 66929, O'Hare AMF	Chicago, IL 60666	800-932-5000
VWR Scientific	3140 Grand River Ave.	Detroit, MI 48208	(313) 588-8500
W.A. Butler		Brighton, MI	800-482-5920
Wedemeyer Electronics	800 Merrill St.	Lansing, Mi.	484-9009
Wescor, Inc.	459 South Main St.	Logan, Utah 84321	800-453-2725

Whatman Lab Sales	5285 N.E. Elam Young Parkway, Suite A-400	Hillsboro, Oregon 97124-9981	800-942-8626
White and White	19 Lagrave	Grand Rapids, MI 49503	800-632-4683
Word Perfect Corp.	1555 N. Technology Way	Orem, UT 84057	
World Precision Instruments	375 Quinnipac Ave.	New Haven, Ct. 06513	203-469-8281
Zenith Marketing Services Dept.	Hilltop Rd.	St. Joseph, Mi. 49085	
Zymed Laboratories	52 South Linden Ave.	South San Francisco, CA 94080	800-874-4494

11.7 List of Equipment

ITEM	CATALOG #	PRICE	COMPANY
(+-) CPP	C-104	50.00/5 g	Research Biochemical Inc.
(-)-Bicuculline methoidide	B-6889	11.70/25 mg	Sigma Chemical Co.
(DBP) Dibutyl phthalate	13100	3.00	Electron Microscopy Sciences
(l)-2-amino-4-phosphonobutyric acid	A-102	19.00/100 mg	Research Biochemical Inc.
25% glutaraldehyde solution, biological grade	16400	7.00/500 ml bottle	Electron Microscopy Sciences
1-5 µl Finnpipetter	R-6247-60	99.00	Cole Parmer
1 ethyl-3-(3-dimethyl aminopropyl) carbodiimide	E-7750	$63.55/25g	Sigma Chemical
1 ul microsyringe with 2303D needle	5071R	35.00	Unimetrics
2.4 diamond knife for ultra thin sectioning		2250.00	Diatome
3/8 in. circle micro point spatula 12 inch, Prolene 9-0	1795G		Burrows
3/8 in. taper point silk, BV1 6-0	K802H		Burrows Co.
4-Button Cursor for DT-114	DT-11-186	100.00	EQS Systems
4x5" cut film holders for camera		8.92	Norman Camera

5A18N amplifier with 768V modification	5A18N, with #768V modification	845.00+125.00(mod.) (6/88)	Tektronix.
5A22N amplifier	5A22N	1365.00	Tektronix
5B12N time base	5B12N	1490.00	Tektronix
6V 15W 2 pin bayonet light bulb for microscope illuminator	8018	10.60	Mager Scientific Inc.
10 ul microsyringe with 2040D needle	TP5010R	25.50	Unimetrics
12 inch ion exchange filters	CDMB 012 02	104.00/pack	Millapore Corp.
17-beta estradiol-3-sulfate	E-9505	16.20/25 mg	Sigma Chemical Co.
17-beta-estradiol, 3-sulfate	E-9505	15.15/25 mg	Sigma Chemcial Co.
17 alpha-estradiol	E-8750	11.55/100 mg	Sigma Chemical Co.
17 beta-estradiol	E-8875	5.90/250 mg	Sigma Chemcial Co.
22mm Square Pell A Way Molds	18646A	19.15	Polysciences
22x40mm Rectangular Mold peel Away	18646C	20.20	Polysciences
25 compartment net, coarse	2512	49.00	Brain Research Laboratories
27 guage 1/2" disposable hypodermic needles	5109	16.00/box	Apothecary Surgical Supply
30 Megabyte Seagate Hard Drive		399.00	JDR Microdevices
32 oz. (1 liter) glass bottles	150-1525	9.21/case of 12 bottles	General Stores
40-200 µl Finnpipetter	R6247	145.00	Cole Parmer
50 µl microsyringe with 2040D needle	TP5050R	27.00	Unimetrics
100 watt mercury bulb for fluorescent burner	HBO-100W2	116.00	Setler's
300 Mesh nickel grids	G300-Ni	10.00/vial	Electron Microscopy Sciences
750 Tini-Jax	41	0.80 each	Fulton Radio Supply Co.
6522 Parallel Interface	79-295A	69.95	John Bell Engineering, Inc.
6522 Parallel Interface for Apple IIe	79-295A	69.95	John Bell Engineering Inc.

7101 1 microliter syringe, special needle (tapered), KH	86200	39.00	Hamilton Co.
7101 syringe barrel assembly	17720	20.50 each	Hamilton Co.
Accuchart recording Paper (no lines)	11-2923-38	10.00	Gould Inc. Instruments Division
Accuchart recording paper	11-2923-38	9.00	Gould Instruments
Acetone, glass distilled	10016	16.00/box of 4 bottles, 100 ml each	Electron Microscopy Sciences
Acid-free openbak pamphlet files (green journal boxes)	QM4912	3.65 each for 3 thru 11; 3.30 each for 12 thru 35	Gaylord Bros.
Acid-free openbak pamphlet files (green journal boxes)	QM4710	3.25 each:11 or less, 2.90 each:12 to 35	Gaylord Bros., Inc.
"Advanced Black and White Photography"	KW-19	9.90	Castle Photo
Albumin, bovine	A-7638	80.55/5 g	Sigma Chemical Co.
Albumin, bovine, fraction V	A 6793	11.80	Sigma Chemical Co.
Allis tissue forceps	160-799	39.90	George Tiemann & Co.
ALM micro dessecting retractor	13-1090	76.00 each	Biomedical Research Instruments
Alpha 1 Computer Operators Chair	E4-A-1-BK	153.00	Michigan Office Supply
Aluminium Sliding Door Track Assembly	1720A24	10.22	Mc Master- Carr
Anti-cholera toxin, beta subunit	227040	45.00/1 ml	Calibochem Corp.
Anti-Phaseolus vulgaris agglutinin (E+L)	AS-2224	55.00/1 mg	Vector Laboratories, Inc.
Anti-wheat germ agglutinin	AS-2024	55.00/1 mg	Vector Laboratories, Inc.
Antimouse IgG F(ab')2-peroxidase	60530	54.00	Boehringer Mannheim Biochemicals
Appleworks Manual		14.95	Management Information Source

APV; DL-2-amino-5a-phosphovaleric acid	PA7020B	65.00/50 mg	Cambridge Research Biochemicals, Inc.
Araldite / Embed 812 resin kit	13940	26.00/kit	Electron Microscopy Sciences
Auroprobe EM protein A G5	23.709.41	198.00	Janssen Life Science Products
Avidin GCP (gold colloidal particles), 1 ml	GA-02	85.00/ 1 ml	E-Y Laboratories, Inc.
Bear oil lubricant, microtome blade oil	12-624	56.00	Fisher Scientific
Beetrode	MEPH1	95.00	World Precision Instruments
Beta-estradiol, 3-benzoate	E-9000	4.50/200 mg	Sigma Chemcial Co.
Biocytin	B-4261	$83.25/250mg	Sigma Chemical
Biotinylated anti-goat I;gG (H + L)	BA-5000	50.00/1.5 mg	Vector Labs
"Black and White Darkroom Techniques"	KW-15	9.95	Castle Photo
Bone wax	W31G	34.42/box	Burrows Co.
Buchner filter funnel	5214-D13	19.39/pack of 6	Thomas Scientific
Buchner funnels	S35620-A	17.45/pack of 6	Sargent-Welch
Bulb, Bausch & Lomb replacement for microscope slide projector	42-42-86	20.00 (6/88)	Baxter Scientific
Bulkhead fitting for milli RO filter System		19.00	Millipore Inc.
Burnishing tool with fine tip	SLC-B/F	4.75 each	Energy Beam Sciences
Burnishing tool with standard tip	SLC-B/S	4.75 each	Energy Beam Sciences
Butane Laboratory burner	BK290	19.95	Laboratory Supplies Co.
Butvar B-98 solution	11860	6.00/100 ml	Electron Microscopy Sciences
Cable release	1619	5.60	Norman Camera
Cacodylc acid, sodium salt	C-0250	232.00/500 g	Sigma Chemical Co.
Can Opener	c2403	6.00	Sigma
Cannon Fascimile Machine	model # FAX-270	1900.00	Hasselbring Clark
Capillary tubing, standard glass	6270		A-M Systems, Inc.

Carbide Burrs	160-1297	2.00 each	George Teimann co.
Centronics (male/male) computer cable	C301-10	69.90	Inacomp Computer
Ceramic Ring Slide	12-568-30	4.20	Fisher
Cetylpyridinium chloride	C-9002	5.80/25 g	Sigma Chemcial Co.
Chien one-hole grids 100/vial	9GC20H	14.95	Ted Pella
Chloroauric acid	C 4776	33.05/1 g	Sigma Chemical
Cholera toxin B	C 7771	144.5 for 2 mg	Sigma chemical
Cholesterol	C-1145	9.70/250 mg	Sigma Chemical Co.
Cibachrome print developing kit, 1 liter size	--	20.00/ kit	Castle Photo
Cimetidine	C-4522	$12.95/5g	Sigma Chemical
Circular Stand for Pipettes	R-6217-10	8.75 each	Cole Parmer
Compu- Gard Surge Protector	C4070	89.00	Global Computers
Computer Stand	E4-6102	89.95	Michigan Office supply
Computer turntable	E4-318TT-WT	48.00	Business Resources Inc.
Computer Zenith with 1 512 K ram floppy disk drive. (1)20MB Hard drive	ZF 241-82	1,799.00	Zenith Marketing Services Dept.
Continuous feed index cards, detached size: 3 x 5 inch	QR8863-1	29.95/box of 4000 cards	Misco
Contrad 70 cleaner	2901-C30	34.00 each	Thomas Scientific
Control reference standards for osmommeters	2202	20.00/pack, 12 ammpules/pack	Precision Systems, Inc.
Corning glass coverslips, no.1, 24 x 60 mm	6672-A48	51.20/ case	Fischer
Cushions for electrophys. chair			Cramer Industries, Inc. (AKA Cramer Inc.)
Cyalume Lightsticks	G2467&	16.65	Lab Safety Equipment
DAB; 3, 3-diaminobenzidine, tetrahydrochloride	D-5637	7.25/1 g	Sigma
Data Switch computer printer swithbox, AABB parallel	156257	89.95	Inacomp Computer Center

Deknatel black, braided, silk suture, size: 3/0	160-1226	29.40/100 yard spool	George Tiemann & Co.
Deknatel silk suture, black braided, size 6/0	160-1215	6.50/spool	George Teimann & Co.
Deknatel silk suture, black braided, size 6/0	160-1226	21.80	George Tiemann & Co.
Deknatel silk suture, black braided, size 9/0	160-1225	42.90/spool	George Tiemann & Co.
Delicate retractor	160-560	58.30	George Teimann co.
Dental engine belts			Meer Dental Supply Co.
Dental Mirror	6830-f75	4.42	Thomas
Diamond tip pencil	08-675	13.75/ 1 pencil	Fischer Scientific
Diethyl pyrocarbonate	D-5758	8.00/5 g	Sigma Chemical
Dip Miser	70510	26.00	Electron Microscopy Sciences
Disposable heat pen	8417	17.65 each	Polysciences, Inc.
Disposable multi-purpose respirator mask	L 03-110	10.25 each	Direct Safety Company
DNQX	D123	30.00/25 mg	Research Biochemicals Incorporated
Double palm gloves	A 07-487	4.00/pr	Direct Safety Co.
Dow Corning Medical Adhesive Type A	891	20.00	Randop Surgical Supplies
DPX Mounting Media	44581	19.50/500 ml bottle	Fluka Chemical Corp.
DPX mounting Media	44581	19.50/500 ml bottle	Fluka Chemical Corp.
Dual channel intracellular recording amplifier	IR-283	2970.00	Neuro Data Instruments Corp.
Dumont #5 tweezer	10-1425	18.00 each	Biomedical Research Instruments
Dumont anticapillary tweezers, style #4	510-4	27.00	Ted Pella
Dumont E2a epoxy covered forcep	11220-21	7.95 each	Fine Science Tools Inc.
Dumont forceps, 5/45 mc	11253-25	22.95 each	Fine Science Tools, Inc.

Dumont Tweezers, nonmagnetic, style #5	505NM	17.65	Ted Pella
Dumont tweezers, #4	504NM	16.15	Ted Pella
Duracell 3 V Mercury Battery	0	4.00	Wedemeyer Electronics
Ektachrome film developing kit, 1 gal. size	--	40.00/ kit	Castle Photo
Electron Microscope Film 4489	144-2772	42.39/50 sheets	MSU General Stores
Embed-araldite resin embedding kit	13940	26.00/kit	Electron Microscopy Sciences
Eye gaurds for Reichert stereo star zoom microscope	41441-164	5.00/pr.	VWR
Face Shield	11-409-5	29.61 each	Fisher Scientific
Fast setting epoxy Kit	TW 8778-00	43.80	Cole-Palmer Instrument Co.
Filter adapter set	4584-T05	17.65/set of six	Thomas Scientific
Fischer automatic slide dispenser	12-558-30	21.00	Fischer Scientific
Five barrel micropipettes	P-5	175.00/box of 50 pipettes	Activational Systems Inc.
Flat Specimen Holder FT	9701762	63.00	Mager Scientific
Flip-up binocular magnifiers	N70,697	22.95	Edmund Scientific
Fluorescien labeled beads (Fluorescent latex microscpheres)		80.00/100 ul	Luma Fluor Inc.
Fluoromount-G slide mounting medium	OB1100-38	20.00/25 ml bottle	Fisher Scientific
Formvar solution (0.50%) in ethylene chloride	15820	5.50/100 ml	Electron Microscopy Sciences
FX Camera Cone	79406	210.00	Mager Scientific
Fx-85 Printer (epson)		338.00	Procomp Computer Products Inc.
FX35 `Manual Film Advance camera back	79408	210.00	Mager Scientific
FX35 Camera Back 35mm for existing Nikon Automatic Photomicro	79408	210.00	Mager Scientific
Gas dispersion tube	11-138B	19.83 each	Fisher Scientific
Gasket for vacuum pump	AA34	.30	Squire Cogswell Co.

Gel foam	--	17.35/bottle	Meer Dental Supply Co.
Gillette Super Blue razor blades	216648 (this is a bergon Brunswic cat. #)	26.76	Bergon Brunswic Drug Co.
Glass Knife Trufs Disposable 100 per box	1716	24.50	Polysciences
Glass knife storage box	16659	13.10 each	Polysciences, Inc.
Glass strips for use with knife maker 30 pc/box	7890-04	99.00	LKB Instruments Inc.
Glassine negative holders	74054	40.00/box of 1000 holders	Electron Microscopy Sciences
Glow in dark tape	#GL 1	38.90	Seton Name Plate Corp.
Glucose Oxidase	G-2133	$28.00	Sigma Chemical
Glucose oxidase	G-6500	6.25/1000 units	Sigma Chemical Co.
Glucose oxidase, type V	G-6500	16.75/10,000 units	Sigma Chemical Co.
Goat anti-rabbit IgG (H & L) gold, 20 nm gold particle	17548	85.50/1 ml	Polysciences, Inc.
Goat anti-rabbit IgG gold conjugate	49-6135	46.00/0.25 ml vial	Zymed Laboratories
Gold Seal cover glass	6672-A48	51.20/10 oz. case	Thomas Scieentific
Gralab darkroom timer, model 300	2081	89.20/ 1 timer	SPI Supplies
Grating Replica Waffle 2160/mm.	607	19.00	Ted Pella
Grid Storage Boxes	71156 (in catalog supplement 88-89)	68.00/dozen	Electron Microscopy Sciences
GSTF filters, 0.22 um, 25 mm diameter	GSTF 025 00	34.40/pkg	Millapore Corp.
Halogen bulb for Zeiss scope	FCR	4.64 each	Sitler's Supplies, Inc.
Hastings 7x magnifying Lens	11500	65.00	Ladd Research Ind.
Heparin sodium injection 1000 USP units/ml	0641-2440-45	14.70/ 1 pkg	Bergen Brunswig Drug Co.
Hiraoka Staining Dish	7344	13.70	Polysciences, Inc.
Hiraoka Staining Kit	4635	52.10/kit	Polysciences, Inc.
Histamine dihydrochloride	H-7250	$8.20/5g	Sigma Chemical

Histamine, R(-)-alpha-methyl, oxalate salt	H-111	120.00/100 mg	Research Biochemicals
Histo mount	HS103	15.00	National Diagnostic
Histo-clear solvent	HS-200	16.50	National Diagnostic
Histo-Prep disposable base molds	15-182-501C	45.00/ case	Fischer Scientific
Histostain peroxidase kit	95-6143-P (SAMPLE kit)	free sample	Zymed Laboratories, Inc.
HVHP filters	HVHP 025 00	44.60/pack of 100 filters	Millapore Corp.
Hydrogen Tartrate salt(-) Nicotine di-(+) tartrate	n5260	12.96	Sigma
Hypodermic Needles, 3.5 inch	N730 (90080)	20.00/ pk of 6	Hamilton Co.
Immunocytochemistry Third edition	1-86721-7	55.00	John Wiley and Sons
Inchworm translator with motor controller and motor controller key pad	IW 701-01 inchworm, 25 mm travel with lateral stability option	1431.00	Burleigh Instruments, Inc.
Index cards, continuous feed, 4000 7x4 white	74-1-6x4	11.76	Superior Business Form, Inc.
Ink, black, cartridge of, for model 220 Brush Recorder	11-2730-15902	38.25/ 1 cartridge	Gould, Inc.
Inmagic extended upgrade		25.00	Inmagic
IntenSE M silver enhancement kit for light microscopy	30.115.45	57.75/kit	Janssen Life Sciences Products
Intramedic Polyethane Tubing (100t)	9565-S30	62.24	Thomas Scientific
Iris scissors	161	13.49	Fine Science Tools, Inc.
Isoproterinol-HCl	I 6504	17.20/500 mg	Sigma Chemical
Jeol SEM Aluminum Mounts, Pkg. 50	75350	10.00	Electron Microscopy Sciences
Kainic acid hydrobromide	G-20	12.50/100 mg	Research Biochemical Inc.
Ketamine	BRL 01210	4.06/3 vials	W.A. Butler
Kynurenic acid	K-3375	10.86/1 g	Sigma Chemcial Co.
L-Phosphatidylcholine	P3644	6.85	Sigma
L-glutamic acid diethyl ester HCl	C-101	12.50/5 mg	Research Biochemical Inc.

L-lysine, acetate, crystalline	L 1884	8.10	Sigma Chemical Co.
L-Phenylephrine	P-6126	$32.40/25g	Sigma Chemical
L-Phospphatidic Acid	P9511	7.00	Sigma
Lab Hot plate model D	BK436-D	34.15	Laboratory Supplies
Lactated Ringer's injection	153-7176	8.03/case	General Stores
Lactic acid, silver salt, 5g	L-6266	20.00/5 g	Sigma
Leather palm work gloves	A 07-311	2.95/pr	Direct Safety Co.
Liquid release agent	70880	5.00/100 ml bottle	Electron Microscopy Sciences
LKB Knife Maker II	LKB 2178-001	4875.00	LKB Instruments, Inc.
Logimouse c7		99.00	Logitech Inc.
Logitech C7 Mouse w/ plus Software		119.00	Logitech
Low viscosity Spurr's embedding kit	14300	23.00/kit	Electron Microscopy Sciences
Mac Transfer and Mac Transfer PC		90.00	Southeastern Software
Master Flex variable speed pump, size 14	J-7553-20	327.50	Cole Palmer Instrument Co.
Max 6 Surge Protector		99.00	Panamax
Mayo-Hegar needle holder, 8" length	160-1028	46.55 each	George Tiemann & Co.
MCE-100x25mm electrode	MCE	25.00	Rhodes Medical Instruments
Mercury lamp for Zeiss microscope	HBO-50W-L2	127.00	Second Source
Mercury lamp, 100 watt	DC 100W OSRAM (HBO 100W/2)	127.00/bulb	Second Source
"Mere 2" reference electrode		50.00	World Precision Instruments, Inc.
Methyl Salicylate.	M-6752	10.00	Sigma Chemical
Meuller cadmuim-plated steel alligator clips	60	.16	Fulton Radio Supply Co.
Micro blade	160-339	21.00/pack of 6 blades	George Tiemann Co.
Micro dissecting scissors	11-2210	32.00 each	Biomedical Research Instruments
Micro steele Burrs	160-1293	1.25 each	George Teimann co.
Micro steele Burrs	160-1294	.80 each	George Teimann co.

Micro-g Isolation Table with 30 by 60 in.plastic laminate top	63-552-01	2340.00	Technical Manufacturing Corp.
Micro-point round hook	10064-14	43.55 each	Fine Science Tools, Inc.
Microfilament capillary glass, single barrel	6010	27.00/pkg when 5 or more ordered	A-M Systems, Inc.
Microscope dust cover	77332	19.00/ cover	Mager Scientific, Inc.
Microscope, focusing unit, eyepieces	41846-053, 41850-050, 41848-059, respectively	835.00, 150.00, 150.00, respectively	VWR Scientific
Microtome blade resharpening	none	20.00 (125 mm); 25.00 (185 mm); 45.00 (250 mm) as of 5/88	C.L. Sturkey
Mini-bone rongeur	46-1030	136.00 each	Biomedical Research Instruments
Minipuls pump tubing-PVC manifold tubes	F117936	15.00/pack	Gilson Medical Electronics, Inc.
Model 39 DP Deltaphase isothermal Pad		48.50	Braintree Scientific
Modula-2/86 Professional software Package		560.00	Logitech
Molecular Seives	M-6141	20.40/500 g	Sigma Chemcial Co.
Molecular Sieves	M-170?	25.00	Sigma Chemical
Molecular Sieves 8-12 mesh heads w/ indicator (BEADS)	M2635	25.30	Sigma
Molecular Sieves, 1/8 pellet	M2260	6.60	Sigma
Mueller cadium-plated steel micro-gator clip	34	.19	Fulton Radio Supply Co.
n-propyl gallate	P 3130	13.30/100 g	Sigma
NAAG; N-acetyl-L-aspartyl-glutamic acid	GACE 110	90.00/100 mg	Bachem, Inc.
Nalgene Bucket with cover	173-08-044	14.70 each	VWR Scientific
Nalgene carboy spigot	029637	12.70	Fisher Scientific
Nalgene two-piece Buchner filter	10-362D	7.52 each	Fisher Scientific
NAPCO Vacumn Oven 0.013 m3	13-262-1	654.50	Fisher

Needle cleaning kit	10206	18.00	Unimetrics
Nicholas Illuminator 6V intensity	BL 31-33-05-28	106.00	Fischer Scientific
NMDA; N-methyl-D-aspartic acid	PA 7040 C	27.00/100 mg	Cambridge Researcch Biochemicals, Ltd.
No-filament micropipette blank	1211?	15.00	Glass Company of America, Inc.
Normal Goat Serum	1410	30.00	Antibodies Inc.
Olsen-Hegar needle nolder combined with suture scissors	40-1050	73.00 each	Biomedical Research Instruments
Omega dot capillary tubing	1011		Glass Co. of America, Inc.
Osmium tetraoxide	none		Stevenson Metal Supply
Osmolality reference standards, vial pack	OV-029	37.75/12 vial pack	Wescor, Inc.
Osmolality reference standards, vial pack	OV-100	37.75/12 vial pack	Wescor, Inc.
Osmotic Standard Control 290	12-827-25B	15.60	Fisher Scientific Company
Osram long life bulb 6Volt 10 watt 2000hr.	64410	14.10	Mager Scientific
P1351 Toshiba Matrix Printer	P1351	1,293.00	Procomp Computer Products Inc.
Paraformaldehyde	P-6148	14.45/kg	Sigma Chemcial Co.
PC system DESK	C5335W	485.00	Global Computer Supplies
Pelco anti-capillary self-closing tweezer	510-5	27.00/each	Ted Pella
Pelco Mica Sheets 25mm by 75mm 20/package	53	4.40	Ted Pella
Perma Wash	---		Castle Photo
Permount	SP-15-500	13.70	Fisher
Peroxidase from horseradish (HRP), type VI	P 8375	293.00	Sigma Chemical CO>
Peroxidase from horseradish, type VI	P 8375	293.85/100,000 units	Sigma
Peroxidase Vectastain ABC kit (rabbit IgG)	PK-4001	155.00/ kit	Vector Laboratories, Inc.
Peroxidase Vectastain ABC Kit, Goat IgG	PK-4005	155.00/kit	Vector Laboratories, Inc.

Phaseolus Vulgaris Leucoagglutinin (PHA-L) Biotinylated	B-1115	80.00/5 mg vial	Vector Laboratories, Inc.
Phaseolus vulgaris leucoagglutinin (PHA-L), unconjugated		50.00/5 mg	Vector Laboratories, Inc.
Pin vise	XA7322	3.55 each	Scientific Instrument Services, Inc.
Pipette pump	53502-233	10.90 each	VWR Scientific
PL 2.5x Projection Lens type CF	79410	126.00	Nikon Inc.
Polaroid 405 film holder	77076	90.00	Mager Scientific
Polyethylene glycol (PEG), MW 1450	P-5402	13.30/1 kg bottle	Sigma Chemical Co.
Polyethylene glycol, Mol. wt.: 1000	P 3515	12.30/kg	Sigma
Polyethylene negative storage sheets	74014	7.50/pack of 25 sheets	Electron Microscopy Sciences
Polyvinylpyrroliodone	PVP-40	8.10/50 grams	Sigma Chemical Co.
Pomona banana plug to pin stack-up patch cord	1126-24	3.50	Fulton Radio Supply Co.
Pomona bananaplug to pin stack-up patch cord, red	1126-24	3.50 each	Fulton Radio Supply Co.
Pomona BNC receptacle to 3/4" double banana plug	1269	4.46 each	Fulton Radio Supply Co.
Pomona stack-up solderless banana plug, black	1325	10.50/pkg.	Fulton Radio Supply Co.
Pomona stack-up solderless banana plug, red	1325	10.50/pkg.	Fulton Radio Supply Co.
Print drying rack, wooden	none	24.95	Imag/ination
Print file archival negative preservers	45-4B	17.00/box of 100 sheets	Castle Photo
Professional Package 1.1 Programing Language upgrade for IBM	BLS 8087	75.00	Logitech
Progesterone	P 0130	4.40/1 g	Sigma Chemmcial Co.
Promethazine	P-4651	$11.45/25g	Sigma Chemical

Protein A, soluble	P 6650	67.05/5 mg	Sigma
Protein A, soluble	P 6650	22.75/1 mg	Sigma Chemical CO.
Protein G	P 9659	25.00 (7/88)	Sigma Chemical Co
Pyrilamine (malente salt)	P-5514	$5.00/5g	Sigma Chemical
Quisqualic acid	PC 7055 A	24.00/5 mg	Cambridge Research Biochemicals, Ltd.
Rabbit anti-neurophysin	AB 948	185.00/1 ml	Chemicon International, Inc.
Red Devil razor blades	none	3.77/box (6/88)	George Worthington Co.
Red safelight filter, #1A	152-1517	25.05	Castle Photo
Red Sealing Wax	15-530	6.00	Fisher
Refill fuel container	BK291	5.00	Laboratory Supplies Co.
Regular surgeons' needles	160-1170	$6.10/doz.	George Tiemann & Co.
Reichert Jung Standard knife holder for model2040 microtone	09236	240.00	VWR
Reichert Stereostar microscope with zoom power, mag. 0.7 - 4.2X	41846-053	835.00	VWR Scientific
Reichert Ultracut E 43 Ultramicrotome with component	99701 69	23,160.00	Mager Scientific
Reichert-Jung Multi Purpose 2040 Autocut Rotary Microtome		4,402.17	VWR
Replacement cover for vacuum type desiccator (250 mm)	3750-M60	101.57 each	Thomas Scientific
Replacement dark slide for Polaroid film back	776776A	10.40/slide	Polaroid
Replacement wicks for alcohol burner	04-236A	7.50/pack of 12 wicks	Fisher Scientific
Reverse osmotic filter, 1/pack	CDRO 012 SO	534.00	Millapore Corp.
Rhodamine Avidin D	A2002	50.00/5 mg	Vector Labs
Rhodamine Filter Set	487715	548.00	Morgan Instruments
Rhodamine labeled beads (fluorescent latex microspheres)		105.00/100 ul	Luma Fluor Inc.

Right angle hemostat, 5 1/2	160-790	44.30	George Tiemann & Co.
Safety Goggles	G5526	4.50	Lab Safety Supply
Scissor sharpening		$4.75/pr.	M & M Repair Service
Scoopula spatula	14-357	7.00/pack of 6	Fisher Scientific
Scotch brand (3M) audio recording tapes	807-1/4-1700	8.00/tape	Comtel Instruments
Screw Driver set	B242-A	3.00	Laboratory supplies
Section Counter and Advaced totalizer	9652401	657.00	Mager Scientific
Selector II Tacking Iron	1288	52.99 each	Silver Lead Paint Co.
Self-retaining retractor	160-550	35.20	George Tiemann Co.
Serum Biomailer	03-531	7.00	Fisher
Service on 2 microscopes	--	(labor) 260.00, (travel) 40.00	Emmitt Instrument Service, Inc.
Servicing of Mettler analytical balance	--		Mettler Instrument Corp.
Sigma-scan software digitizing program	3013 (product #)	495.00	Jandel Scientific
Sign Designer (for IBM)	1010	49.95	Channelmark Cor.
Silastic sealer	?	?	Baxter
Silastic Tubing I.D. .057"	T5715-s6	25.07	Baxter
Silastic tubing	602-155	26.00/50 ft. roll	White and White
Silastic tubing	602-175	26.00/50 ft roll	White and White
Silicone gasket for the oven	13-262-3	15.00	Fisher
Silicone tubing for Mark's peristaltic pump	J6411-14	23.00/25 ft.	Cole-Palmer Instrument company
Silicotungstic acid	3424	16.55/25 g	Polysciences, Inc.
Silver tape for glass knife boats	114-12	12.20	Ted Pella
Simon Slide Viewing System	SVS-5822	199.00	Scientific Instruments Services
Single Channel syringe pump	P8537-1	750.00	Baxter Scientific
Single channel intracellular recording amplifier	Model # IR-183	2200.00	Neuro Data Instruments Corp.
Single slot (oval) copper grids	1020-CU	14.50/vial	Electron Microscopy Sciences
Slide mounts, glass	--	4.24/box	Norman Camera Company
Small, stacking bins	317A0102	6.00/pkg of 6 bins	Whatman Lab Sales

Snap Caps	242612	9.40	Fisher
Socket wrench set	B242-C	5.90	Laboratory supplies
Sodium m-peroidate	S 1878	7.75/25 g	Sigma Chemical
Softlogic Solutions Disk optimizer (software package)	187.401	36.00	New Line Software
Souten knife blade (reinforced)	122	55.00	David Kopf Instruments
Spacers, brass unthreaded round cadmium plated for electrophys. electrode	(Fulton's #) B 1556-1/2-11 (Waldom)	10.20/100 spacers as of 5/88	Fulton Radio
SPC technology rack mount power outlet box model 1902-BF	60N2493	52.62	Newark Electronics
SPC technology rack mounted power outlet box	60N2493	52.62 each	Newark
Spring scissors, curved	15001-08	137.95 each	Fine Science Tools, Inc.
Spurr's low viscocity embedding kit	14300	23.00/kit	Electron Microscopy Sciences
Square mech grids, nickel	0300-Ni	9.75/vial	Electron Microscopy Sciences
Stainless steele Lab Cart (400lb.) cap.	BK94-B	135.25	Laboratory Supplies
Stak Rack Kit		31.95	Imagination
Standard flat embedding mold for electron microscopy	4-21775	6.00 each	Ladd Research Industries
Standard Knife Holder for model 2040 Microtone	09236	385.00	VWR
Standard square nickel grids, 300 mesh	G300-Ni	10.00/vial	Electron Microscopy Sciences
Sterile Speciman Containers	14-375-141	33.00 1case	Fisher
Stinging Methods for Sectioned Material (book)	24921	25.50	Ted Pella
Storage oscilloscope, rack mounted dual beam	R5113	5005.00	Tektronix
Stylus for Hi Pad "dt-114"	D11-109	95.00	EQS Systems

Summasketch II, 12 x 12 inch digitizing tablet	none	465.00	Advantage Computer Systems
Surgical suture silk, size 3-0	110-S	17.00/spool	Deknatel
Surgical suture silk, size 8-0	102-S	27.00/spool	Deknatel
Sylgard elastomer	none		Ellsworth Adhesive Systems
Sylvania projection lamps	none	29.79	Michigan Brass and Electric
SYS ZF 151 52 Zenith PC		1,499	Zenith/L.E. Lighthart
T Max 400 film	none	26.00/100 ft roll	Castle Photo
T Max concentrated developer	---	6.70/bottle	Castle Photo
T Max P3200 film	none	5.40/roll	Castle Photo
Tape, silver	114-12	12.20/roll	Ted Pella, Inc.
Temperature probe (replacement) for pH meter	117FO114	69.00/ 1 probe	Whatman Lab Sales, Inc.
Testosterone propionate	T-1875	13.40/5 grams	Sigma Chemical Co.
Tetraethylammonium chloride hydrate, 25 g	11,304-2	9.40/ 25 g	Aldrich Chemical Co., Inc.
Tetrodotoxin (TTX), 1 mg	T-6254	90.55/ 1 mg	Sigma
Thin-walled microfilament capillary glass	6150	29.00/pack of 250	A-M Systems, Inc.
Thioglycolic acid	T-3758	9.15/100 ml	Sigma Chemical Co.
Thionin	T-3387	42.70/25 g	Sigma Chemical Co.
Thomas DUG Super Safelight (sodium vapor safelight)	TH72882-10	270.00	VWR Scientific
Three channel timer	m457718	31.95	J & H Berge Inc.
Tico Rachet retractor	160-555	42.90	George Tiemann Co.
Tini-plug miniature phone plug, black	740	1.05 each	Fulton Radio Supply Co.
Tissue-Tek OCT compound	4583	4.53/4 fluid oz. bottle	American Scientific Products
TMB; 3,3',5,5'-tetramethylbenzidine	T 2885	16.45/gram	Sigma
Tracing Board 18x 24(4lamp)	BK249-E2	169.50	Laboratory Supplies
Trimaide, safety aid razor blade holder	72550	4.00	Electron Microscopy Sciences

Tris hydrochloride	T-3253	44.95/500 g	Sigma Chemcial Co.
Triticum Vulgaris	L 9640	32.4	Sigma Chemical Co.
Trufs, blue plastic glass knife boats	2208-100	99.00	LKB Instruments, Inc.
Tubing, Dialysis, MWCO 12000-14000	3787-D10	39.80/box	Thomas Scientific
Tubing, Silicone, 25 ft.	J6411-14	23.00/25 ft.	Cole-Palmer Instrument Company
Tungsten-carbide scriber	5680-F10	13.70 each	Thomas Scientaific
Turbo Pascal Translator for Modular II		49.00	Logitech
UFX -11 Photomicrographic camera system	79450	3976.00	Mager Scientific
Ultra-clear centrifuge tubes	344058	78.00/pack of 50 tubes	Beckman Instruments
Ultrastructure size calculator	7328	5.00	Polysciences
Vacuum desicator, 171 mm diameter	08-594-15A	28.00	Fisher Scientific
Vane for vacuum pump	AA345	1.30	Squire Cogswell Co.
Vanna dissecting scissors	11-1160	151.00	Biomedical Research Instruments
Variable Speed Rotator	R4140	560.36	American Scientific Products
Vector Red Alkaline Phosphate Reagent	SK5100	45.00	Vector
Vibratome series 1000	101	3495.00	Ted Pela, Inc.
Viton Gloves size 10-12 mill	210-847-2	30.50	Aldrich
Volumetric flask, 2 liter	29610-207	37.85	VWR Scientific
VWR brand lens paper	52846-000	11.00/1 pack of 12 books	VWR Scientific
VWR microscope slides	48312-002	137.50/10 gross	VWR Scientific
Wagner Scissors	14068-12	23.99	Fine Science Tools, Inc.
Wall Lenk butane burner head	709-4618	19.50 each	Patterson Dental Co.
Wall Lenk butane laboratory burner	709-4691	5.50/can	Patterson Dental Co.
Wall mounted eyewash station	S1350-1	19.25 each	Baxter Scientific

Water Filters for darkroom	TR 70259-50	136.45	VWR
Watson bulk film loader	Watson-100	17.90	Castle Photo
Wheat Germ Agglutinin	L-1020	60.00/25 mg	Vector Laboratories, Inc.
Wheaton alcohol burner	2077-G15	3.61 each	Thomas Scientific
Wheaton Sample Bottles w/plastic snap cap	225532	65.08	Fisher
Word Perfect program, version 5.0 for 5 1/4" drive		135.00	Word Perfect Corp.
Yellow Springs Instruments temperature probe, YSI 402	61249-002	48.00 each	VWR Scientific
z99Contrad 70, cleaner	2901-C30	34.00/5 liter	Thomas Scientific
Zipper + Gold Internal Modem with Software	ROPRIZM24H	119.00 each	Priority One Computers

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