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BIOCHEMICAL CHARACTERIZATION OF SYNAPTOSOMAL FUNCTION AND NEURAL REGENERATION IN THE RODENT OLFACTORY PATHWAY

by

SARAH ROCHEL

A dissertation submitted to the Graduate Faculty of Biochemistry in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

at the

CITY UNIVERSITY OF NEW YORK

(1981)

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

7/30/81
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7/30/81
Date

Executive Officer

Supervisory Committee

GENERAL SUMMARY

BIOCHEMICAL CHARACTERIZATION OF SYNAPTOSOMAL FUNCTION AND NEURAL REGENERATION IN THE RODENT OLFACTORY PATHWAY

by

SARAH ROCHEL

Advisor: Professor Frank L. Margolis

The purpose of this project is to study and elucidate biochemical mechanisms of the chemoreceptor neurons in the olfactory pathway. The project consists of two major parts.

The olfactory chemoreceptor neuron reconstitution was studied via the activity of ornithine decarboxylase (ODC) as a monitor of cellular regeneration. ODC activity in olfactory tissue (0.2-0.4 nmol/mg pr/hr) was found to be 10 to 30 times higher than in other cerebral tissues. ODC activity in the olfactory mucosa declined within 3 hours after degeneration of chemoreceptor neurons was induced surgically by nerve section or bulbectomy, or chemically by intranasal irrigation with $ZnSO_4$, Triton X-100 or colchicine. ODC activity recovered when chemoreceptor differentiation from stem cells occurred. This implies that ODC activity is, in part, located in chemoreceptor neurons. Furthermore, change in ODC activity was the first event detected in response to degeneration and regeneration inducing treatments. As such, it may prove useful as a reconstitution marker of chemoreceptor neurons.

A second goal of this study was to provide biochemical evidence that the dipeptide carnosine (β-alanyl L-histidine) acts as a neurotransmitter. Specifically, its release from olfactory nerve endings in response to depolarizing stimuli, has been established and characterized, providing an essential requirement for its classification as a neurotransmitter.

Synaptosomes were prepared from olfactory bulbs of mice by homogenization and differential centrifugation. Subcellular distribution studies indicated that DNA and nuclei were excluded from these fractions, which contained mostly multi (P_1) and mono (P_2) synaptosomal structures. These fractions contained carnosine, enzymes synthesizing the neurotransmitters GABA (GAD) and dopamine (TH), and binding sites for bulbar neurotransmitters.

Membrane potentials ($\Delta\Psi$) of the synaptosomes were characterized using the lipophilic cation tetraphenylphosphonium (TPP^+) as a probe, and the conditions for $\Delta\Psi$ measurements in synaptosomes were established. TPP^+ was found to accumulate in the intrasynaptosomal mitochondria, causing dissipation of their $\Delta\Psi$ in proportion to its concentration. The mitochondial accumulation of TPP^+ was eliminated using the inhibitors of mitochondrial respiration oligomycin/argon. Under these conditions TPP^+ accumulation provides direct evaluation of the plasma membrane $\Delta\Psi$ and its response to drugs.

The membrane potential of olfactory bulb synaptosomes was consequently calculated to be-65 and-77 mV in P_1 and P_2 fractions, respectively. The plasma membrane was depolarized in proportion to increasing external potassium concentrations, indicating $\Delta\Psi$ dependence on the potassium concentration gradient across the membrane, or on

its efflux. Synaptosomal $\Delta\Psi$ was maintained by the Na, K, ATPase-pump, as indicated by the effect of the Na, K, ATPase inhibitor-ouabain-extensive depolarization accompanied by synaptosomal potassium loss, one hour after its application. The synaptosomes maintained an action potential sodium gate, as indicated by veratridine induced depolarization, and its blockade by the sodium gate inhibitor tetrodotoxin.

The release of in vivo synthesized carnosine from synaptosomes was studied in an in vitro superfusion system. The synaptosomal fractions constitute a quite heterogeneous population. However, carnosine is localized solely in the nerve endings subfraction which derive from chemoreceptors. Therefore, its efflux corresponds to the specific properties of these cells.

Carnosine was observed to be relatively immobilized in the synaptosomes, similar to catecholamines and GABA, and unlike nontransmitter amino acids which are characterized by high efflux rates.

Carnosine was released from synaptosomes through two mechanisms. The first, calcium independent spontaneous efflux detected under non-depolarizing conditions. The second, a calcium dependent, depolarization-stimulated release. This latter process was stimulated by 60 mM K^+, or by depolarization induced by veratridine, an action potential sodium gate activator. The sodium gate inhibitor tetrodotoxin reversed the veratridine effect. Cytoplasmic carnosine exchange with external carnosine did not affect the 60 mM K^+induced release, hence it must represent efflux by a mechanism different than the depolarization induced one.

The results of this study are consistent with the model suggesting the packaging of carnosine in synaptic vesicles, and its release by exocytosis upon exposure to depolarizing stimuli.

ACKNOWLEDGEMENTS

I would like to express my thanks and my appreciation to my advisor, Dr. Frank L. Margolis, for his valuable guidance during this research.

I would also like to thank many of the members of Roche Institute of Molecular Biology that have contributed their time and knowledge to the success of this project. Especially I would like to thank Mr. Frank Jenkins and Dr. M. Boublik for the electron microscopic assistance, and the members of my committee, Dr. S. Udenfriend, Dr. Arthur Weissbach, and Dr. Clair Neurath, for their valuable suggestions.

I would like to extend my thanks to Roche Institute of Molecular Biology for the pre-doctoral fellowship awarded to me. Furthermore, this research was conducted in and supported by Roche Institute of Molecular Biology.

Dedicated to the memory of

my father

HANOCH ROCHEL

who instilled in me the

appretiation of learning.

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LIST OF ABBREVIATIONS

ODC - ornithine decarboxylase

CCCP- carbonyl-cyanide-m-chlorophenyl-hydrazone

GAD - glutamate decarboxylase

MDPF - 2 methoxy-2,4-diphenyl-3(2H)-furanone

TPP^+ - tetraphenylphosphonium

TTX - etrodotoxin

$\Delta\Psi$ - membrane potential

TH - tyrosine hydroxylase

GABA - gamma-aminobutyric acid

HEPES - N-2-hydroxy ethyl piperazine-N^1-2-ethane sulphonic acid

EGTA - ethylene glycol-bis (β-amino ethyl ether) N,N'-tetraacetic acid.

Tris - tris hydroxymethyl amino methane

EDTA - ethylene diamine tetraacetic acid

SDS - sodium dodecyl sulfate

NCS - NCS-Tissue solubilizer

NEM - N-ethylmaleimide

LOT - lateral olfactory tract

EPL - external plexiform layer

PG - periglomerular

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CHAPTER 1

I N T R O D U C T I O N

INTRODUCTION

A. *The olfactory bulb as a model of the brain*

The central nervous system is the highest center of integration controlling all life functions. It accumulates information about both external and internal environments via the peripheral sensory system, integrates it and generates a meaningful interpretation, which is then categorized, evaluated and often stored and used to generate appropriate responses.

All these functions are performed by a quite complex network of neurons, in an extremely efficient high capacity computer-like system. People have always been fascinated by the complexity of the brain, and ventures into the understanding of brain function are as old as the history of mankind. Mythological, philosophical, psychological, behavioral and biological or medical analyses are only a few of the approaches taken by various disciplines in the study of the brain.

Neuroscience specifically involves the study of the interconnection of the neuronal network of the brain, its signal form, signal generation and propagation properties, the chemicals involved in signal transmission between neurons at synapses, and the mechanism by which these transmitters modulate neuronal function.

The complexity of the brain rendering it with its unique properties, also makes it quite inaccessible, difficult to manipulate, and complicates the interpretation of the studies involved. Although considerable information has been accumulated about the major functions

of brain structures, its anatomical and functional connections, and a few of the modulating transmitters, as well as the developmental process of cellular organization of a few structures, the detailed understanding of most of the brain units is still not available.

A simple,easy to manipulate system containing the major components of the brain cortex,which can serve as a model is highly desirable. The olfactory pathway is a unique system in which most of these requirements are met. It is one of the most phylogenetically primitive systems of the brain (Ariens et al., 1967), relatively simple, nonetheless it contains all the major cortical layers. Furthermore, it has evolved as an anatomically separate entity from the remainder of the brain, and in rodents is located in the anterior part of the brain, connected to it with only a thin stalk. This provides a system with easy access, amenable to manipulations such as electrical stimulation or surgical lesion without or with only minimal damage to neighboring brain regions. In addition, the cell bodies and dendrites of the primary afferents of the olfactory bulb are extremely accessible through the nares, and therefore can be chemically manipulated via this route without the necessity of performing surgery.

The olfactory bulb is organized in a laminated structure with well defined location of neuron cell bodies. Their ramifications are in part radial and confined within a given layer. This provides a more precise interpretation of electrophysiological, electron microscopic, and transmitter distribution studies.

One of the unique properties of olfactory neurons is that they continuously degenerate and regenerate from undifferentiated progenitor cells all through adulthood (Review by Takagi, 1971; Moulton,

1974). Many of the studies of the olfactory pathway have taken advantage of this property. It provided the investigators with the opportunity to study regeneration of neuronal connections, in addition to the classical degeneration studies following specific lesions. In addition, the reconstitution of olfactory neurons present an interesting and important problem concerning the mechanism by which the growing axons of the new neurons find their way back to the olfactory bulb, how they find the specific glomerulus within the olfactory bulb and the specific post-synaptic site within the glomerulus. The morphological changes occurring during this process were studied in great detail (Mulvaney et al., 1971; Moulton, 1974; Graziadei and Graziadei, 1978). These studies also analyzed thymidine incorporation into DNA by autoradiographic methods, in order to evaluate cell proliferation and differentiation in the olfactory mucosa.

Regeneration was also evaluated biochemically (Harding et al., 1977) and to some extent by electroolfactogram (Takagi, 1971; Simmons and Getchell, 1979) and behaviorally (Oley et al., 1975; Harding et al., 1978). These studies indicate that when the olfactory nerve is lesioned, there is loss of ability to detect food odors and loss of electrical activity accompanied by disappearance of biochemical markers of chemoreceptors and by morphological evidence of retrograde and orthograde degeneration of the chemoreceptor cell population. In addition, a decrease of dopamine and tyrosine hydroxylase in the post-synaptic bulbar neurons was detected (Nadi et al., 1981). The mechanism by which a particular cell is destined to degenerate and a progenitor cell is induced to differentiate and replace it are still obscure. Elucidation of this process is of great interest.

Lastly, the olfactory pathway is associated with detection of odorants in the environment and, therefore, is an important component of an animal's sensory system. It was also implicated in the modulation of certain behavior patterns which are dependent in part on odor communication. These include reproduction (Cain and Paxinos, 1974; Evans et al., 1978; Wang and Hull, 1980) territoriality, food searching (Le Magnen, 1971) and mother recognition (Cooper, 1976; Hofer, 1975). Lately, the olfactory bulb was also implicated in non-olfactory modulation of aggressive behavior (Alberts and Friedman, 1972; Bondler and Chi, 1972; Spector and Hull, 1972; Pradham, 1975). Since olfactory bulbectomized rats show muricidal behavior (Vergens and Kadi, 1963; Ueki and Sugano, 1965) which is selectively inhibited by antidepressants (Kumadaki et al., 1967) which are known to increase the activity of catecholaminergic neurons in the brain, these bulbectomized rats have been used by psychologists as a model for depression.

All the above stated properties render the olfactory bulb quite an attractive model system for the investigation of central nervous system function.

B. *The olfactory pathway - a chemosensory system*

The olfactory system is specifically dedicated to the detection and discrimination of odorant molecules, providing sensory information about the ambient chemical environment. Odorant discrimination in the olfactory pathway of vertebrates is postulated to begin with the interaction of odor molecules with the chemoreceptor cell membrane, which in turn converts the stimulus into coded messages of a train of impulses, propagating along the primary afferent nerve to the olfactory

bulbs. This information is then processed in the olfactory bulbs and further transmitted to deeper structures of the CNS, where it is interpreted as a specific odor and a proper response is generated.

C. *Chemoreceptor - detection and discrimination of odorants*

1. *Odorant discrimination principles*

Recent investigations strongly indicate that the initial events in the olfaction process involve interaction of odorants with specific membrane receptors, possibly proteins. The original studies of receptor site properties, concentrated on the stereochemical properties of the odorant molecules discriminated by the chemoreceptors. These studies used psychophysical measurements in combination with specific anosmia studies. Psychophysical measurements involve presenting individuals with different odorants, at various concentrations, and analyzing their response in terms of sense of odor, quantity (intensity) and similarity to other odors produced by molecules with known stereochemical properties. Specific anosmia (Amoore, 1977) is a genetic deficiency which renders the subject with a reduced ability to detect specific odorants. These studies indicate that odor recognition sites may be a genetically inherited property of the particular chemoreceptor cells (Amoore, 1977). Two functional types of receptor sites have been described: the first having defined dimensions and shape, complementary to specific three-dimensional molecular structure of the specific odorant. The second receptor types are capable of detecting primary odorants according to the electronic properties of their functional groups, and their orientation (Beets, 1970; Amoore, 1970, 1974,

1977; Amoore et al., 1972, 1975a,b, 1976a,b, 1978). Thus, chemoreceptor cells maintain an array of distinct receptor sites capable of recognizing and discriminating between at least ten groups of primary odorants (Amoore, 1977) according to their stereochemical properties.

2. *Receptor molecule nature*

A number of biochemical approaches provided some preliminary evidence for the involvement of membrane proteins in chemosensory recognition via a receptor process (Riddford, 1970; Ash, 1968; Ash and Skogen, 1970; Price, 1978; Cagan and Zeiger, 1978; Fesenko et al., 1979). This hypothesis was supported by binding studies that have indicated proteins containing thiol and amino groups as the receptor sites whose chemoreceptive properties could be altered by group specific reagents (Getchell and Gesteland , 1972; Villet, 1974; Frazier and Heitz, 1975; Menevese et al., 1977, 1978; Delaleu and Holley, 1980). Specifically, n-ethylmaleimide (NEM) and mersalyl block the summed receptor potential (electroolfactogram; Ottoson, 1956; Getchell and Getchell, 1974) in response to odor stimuli. This effect could be prevented by the presence of odorants acting as protecting agents, which compete with the reagents for certain categories of olfactory receptor sites. However, group specific reagents such as NEM may act themselves as odorants. Furthermore, it is possible that the protective power of odorants is achieved by depolarizing the receptor cells, thus changing indirectly the binding sites of the sulphydryl reagents (Delaleu and Holley, 1980). A report that histidine and sulphydryl groups located on the axoplasmic side of the squid axon membrane pro-

duce conduction block when modified (Boumgold et al., 1978) should also be considered.

3. *Receptive field organization*

Based on electrophysiological response of the olfactory mucosa and the secondary response of the olfactory bulb to odorant stimulation, it was postulated that one chemoreceptor cell can interact with one or more primary odorants, and that the interpretation and discrimination of odor stimulus is carried at several levels of the olfactory pathway (Tanabe, 1975). Studies employing the above technique in combination with treatment by group specific protein reagents (Getchell, 1971; Getchell and Getchell, 1974) have led to the conclusion that odorants interact with one or more specific receptor proteins in the olfactory cell membrane, and that specific receptor sites exist for different types of odor molecules.

As summarized by Hornung and Mozell (1977) and Mozell and Jagodowicz (1974), based on recording of spike activity in the olfactory bulb induced in response to specific stimuli, there are two mechanisms at the level of olfactory mucosa which could underlie olfactory discrimination: (a) the receptors are mostly tuned to particular odorants (provides support to Amoore's study (1977)) and (b) the molecules of different odorants are distributed in different space-time patterns across the mucosa according to those physiochemical properties which affect the progress of their migration. These two mechanisms lead to different spatial-time distributions of olfactory input into the olfactory bulb glomeruli.

In conclusion, a general understanding of the functional principles of receptor types and receptor organization providing odorant recognition and discrimination at the olfactory mucosa level is available. However, the biochemical phenomenon of the interaction of specific odorants with chemoreceptor membranes and its conversion into coded signals remains a mystery yet to be solved.

D. *Olfactory Bulb-Odor Stimulus Processing Center*

1. *General Structure and Cell Types*

The axons of the receptor cells enter the olfactory bulb and terminate in rounded structures - the glomeruli, where they establish the first synapses on the second order neuron dendrites. The main structures of the mammalian olfactory pathway are illustrated in Figures 1 and 2.

Mitral cells are the second order neurons constituting the principal output neurons of the olfactory bulb, thus their axons project through the lateral olfactory tract (LOT) to several parts of the CNS. The mitral cell bodies are organized in a thin layer (MBL) below the bulbar surface. Each mitral cell has a primary dendrite which projects to the surface, ramifies and terminates within the glomerulus. Several secondary dendrites branch and terminate within the external plexiform layer (EPL). The axons of the mitral cells project deeper and posterior in the bulb, and then join the lateral olfactory tract. Prior to leaving the bulb, the mitral cell axons extend deep collaterals within the granule layer and recurrent collaterals which branch and terminate in the EPL.

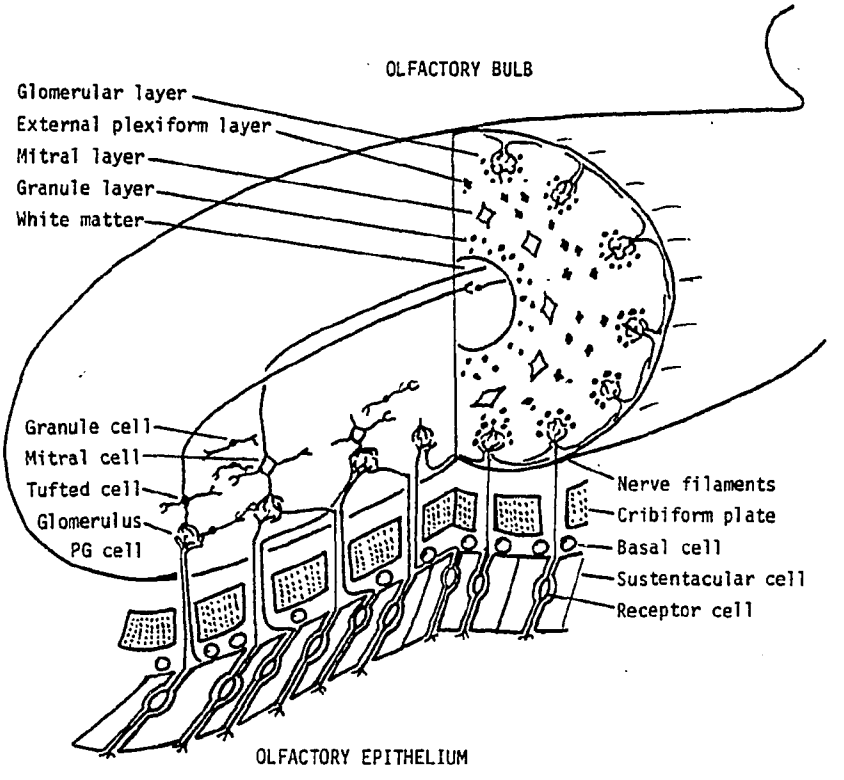

Figure 1. Schematic diagram of olfactory epithelium and bulb. Adapted from Shepherd,1977.

Periglomerular cell bodies surround the glomeruli (Reese and Brightman, 1965, 1970), each sending a dendrite tuft into the glomerulus and axon projecting laterally, which ramify and terminate around the periphery of the neighboring glomerulus. Thus, periglomerular cells are short axon interneurons, likely to be involved in lateral regulation of information flow.

Granule cells - some are embedded among mitral cell bodies, most are clustered in islands in deeper layers. Each cell has a deep process and a peripheral one which ramify and terminate in EPL. The peripheral branches are covered with spines. Thus, granule cells are interneurons with no axons.

Central input of the olfactory bulb is derived from centrifugal fibers (De Olmos et al., 1978) arising from the ipsilateral and contralateral anterior olfactory nucleus (AON), and from cells in the nucleus of the horizontal limb of the diagonal band (Price and Powell, 1970); other fibers arise from raphe and locus coeruleus.

2. *Major functional organization*

The first order neurons - the olfactory receptors - deliver the olfactory information in the glomeruli through excitatory synapses to the mitral cells - the second order neurons. These principal output neurons of the bulb are subjected to multiple neural control in two distinct stages by two different kinds of interneurons; the periglomerular cells at the input stage - in the glomerular layer (Pinching and Powell, 1971a; White, 1972), and the granule cells at the output stage - in the external plexiform layer. Both types of interneurons establish reciprocal inhibitory synapses with mitral cell dendrites (whereas the mitral to P.G. or granule cell connection is excitatory),

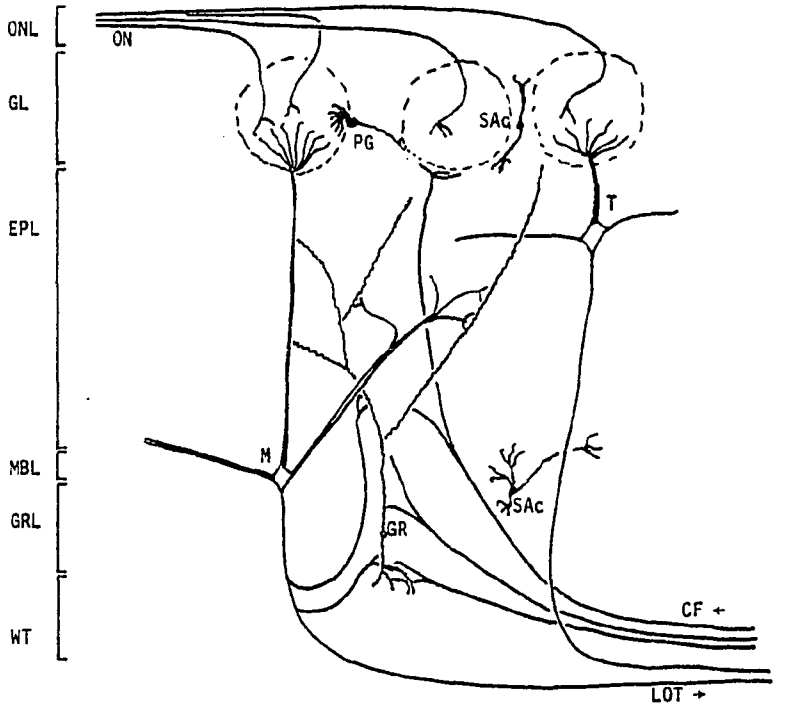

Figure 2. *Olfactory bulb structures in rabbit.* neurons: ON: olfactory nerves; PG: periglomerular cell; SAC: short axon cell; M: mitral cell; T:tufted cell; Gr: granule cell; CF: centrifugal fibers; LOT: lateral olfactory tract; Histological layers are indicated in the left. ONL: olfactory nerve layer; GL: glomerular layer; EPL: external plexiform layer; MBL: mitral body layer; GRL: granule layer; WT: white matter. (adapted from Shepherd)

and receive collaterals from mitral cell axons. Furthermore, P.G. and granule cells receive respectively terminals derived from superficial and deep short axon cells, as well as centrifugal inhibitory and excitatory fibers, mainly through the LOT and the anterior limb of the anterior commisure (AAC). Thus, significant features of the functional organization of the third order level of the olfactory pathway are (1) feed back loops from the third order to the second order neurons, and (2) central modulation of third order neuron activity. The mitral cell axons - the output relay - are terminating in the lateral olfactory cortex, in axodendritic excitatory synapses with superficial pyramidal cells. At this stage a third regulatory control of mitral cells takes place through presynaptic inhibition by small polymorph neurons (GABAergic) (Pickles, 1979; Pickles and Simmonds, 1976).

3. *Organization and function of the major relay and control centers*

Organization of the olfactory glomeruli and their afferent input

It has been estimated that about 50 x 10^6 olfactory axons enter the rabbit's olfactory bulb (Allison, 1953). These thin unmyelinated fibers (0.1-0.5 μm diameter) (Andres, 1965, 1970; DeLorenzo, 1968; Gasser, 1956) converge into small fascicles which project to the bulb in larger bundles. A number of such fascicles are surrounded by one Schwann cell (DeLorenzo, 1957; Gasser, 1956). This provides for a possible interaction between axons, although there is no physiological evidence confirming this.

The olfactory nerve bundles intermingle on the surface of the olfactory bulb. However, the olfactory fibers do not branch prior to

their entry into the glomeruli where they form excitatory synapses with the dendrites of P.G., tufted and mostly mitral neurons (Pinching and Powell, 1971; White, 1973). Therefore, the axon of any one chemoreceptor neuron does not terminate in more than one glomerulus, and in turn each glomerulus receives input from a distinct odor receptive field. This was observed through degeneration studies (Land, 1973; Land et al., 1970), and by autoradiographic detection of leucine uptake and transport (Land et al., 1974; Weiss and Holland, 1965). Since there are only about 2000 glomeruli (in rabbit), about 25000 olfactory axons must converge on any one glomerulus (Allison, 1953; Clark, 1957), (within the glomerulus, they terminate, mostly onto 24 mitral cells and 68 tufted cells; Allison, 1953). Furthermore, EM studies (Land and Shepherd, 1974; White, 1972) indicate that axonal branching within the glomerulus are rare. This input organization is likely to be of significance for the nature of processing of olfactory information at the glomeruli.

The bulbar neurons in the glomeruli establish dendrodendritic reciprocal synapses consisting of excitatory mitral to P.G. connections (Hinds, 1970; Pinching and Powell, 1971a,b; White, 1972), and P.G. to mitral connections structurally resembling inhibitory type synapses.

Physiology of glomeruli

Physiological analysis at the glomerular level is hindered by its complex organization. However, unitary activity studies provided some evidence complementary to the anatomical description.

Electric stimulation of an olfactory nerve bundle sets up a volley invading the terminals in the glomeruli and eliciting synaptic

response in the bulbar neurons. Mitral cells are initially excited (to produce one spike) via a monosynaptic relay, and are subsequently suppressed (Shepherd, 1963, 1977; Yamamoto and Iwama, 1962; Yamamoto et al., 1963). P.G. units are activated over two or more synaptic relays (Shepherd, 1963, 1977), and respond with one or more spikes. Analysis of the above and conditioning volley experiments indicated the following (Shepherd, 1971, 1972): The EPSP in a mitral dendritic tuft propagates through the dendrite to generate an impulse in the mitral cell, and also provides local output through triggering of excitatory dendrodendritic synapses onto P.G. cells. The EPSP thus induced in P.G. cell dendrites provides for the generation of impulse in the axon, and for local synaptic output, through activation of inhibitory dendrodendritic synapses onto mitral cells (Fig. 2), providing for self and lateral inhibition of the mitral dendritic tuft.

Organization of external plexiform layer

The EPL is composed of mostly two elements: dendrites of mitral cells and dendrites of granule cells. Both have a primarily radial orientation, and establish numerous reciprocal synaptic connections (Allison, 1953; Hinds, 1969; Hirata, 1964; Price and Powell, 1970; Rall et al., 1966; Reese and Shepherd, 1972; Willey, 1973). According to electron microscopic evaluation, the granule to mitral synapses are inhibitory, whereas the mitral to granule connections are excitatory.

Physiology of the external plexiform layer

Studies using antidromic impulse invasion through shock to the LOT indicated that the pulse is capable of propagating to the mitral

cell bodies, and on to the dendrites. Consequently, the mitral cells undergo a prolonged period of inhibition (Nicoll, 1969, 1971a; Phillips, 1963; Shepherd, 1970; Yamamoto et al., 1963). As analyzed from summed extracellular potentials this possibly results from an inhibitory post-synaptic potential generated by two synaptic relay inhibitory pathway - exhibited via granule cells which were activated by mitral cell dendrites (Rall and Shepherd, 1968; Rall et al., 1966; Shepherd and Haberly, 1979). A direct local dendritic interaction that leads to dendrodendritic inhibition was demonstrated by intra-cellular recording (Jahr, and Nicoll, 1980). As previously indicated, granule cells receive extrinsic input.

Based on specific lesion and subsequent terminal degeneration studies (Price and Powell, 1970a, b), fibers derived from contralateral and ipsilateral AON and from the nucleus of the horizontal limb of the diagonal band, terminate on spines of granule cells, and on gemmules of granule cells in EPL in structurally excitatory synapses. Thus, granule cells serve as the interneuronal inhibitory relay between CNS input and the mitral cells. Physiological evidence supports this conclusion (Ochi, 1963), e.g. in recording of chronic mitral multi-unit activity, a significant influence of centrifugal fibers on this activity was noted (Moulton, 1963). Hunger seems to trigger the removal of centrifugal inhibition, thus facilitating transfer of food odor information (Giachetti et al., 1970). This was indicated by an increase of positive response of mitral multi-unit activity to food odor, whereas hunger had no effect on response to non-food odors. The hunger effect could be duplicated by insulin injection (Pager et al., 1972; Pager, 1977; Cain, 1975).

It is interesting to note that insulin and insulin receptors were reported in the bulb (Havrankova et al., 1978) and therefore may be speculated to interact directly with the olfactory bulb neurons to produce the above effect.

4. *Olfactory bulb transmitters*

As a consequence of the extensive physiological and anatomical studies described above, the olfactory bulb has proven to be favorable for the study of synaptic organization, becoming a model for the study of other brain regions. More extensive biochemical studies, including the identification of transmitters of the different synapses, would lead to a better understanding of the olfactory bulb, and consequently to a more complete model. However, this field of transmitter studies is still in its initial stages. Major approaches and methodologies used in these studies include: microiontophoresis in combination with studies of transmitter and blocking agents effects on electrophysiological activity, histochemical fluorescence, immunochemical and autoradiographic localization, and direct biochemical and enzymatic assays in combination with specific neuronal lesions. The goals of these studies include evaluation of transmitter related enzymes, transmitter content, and distribution of transmitter uptake and binding sites. These studies have provided preliminary evaluations of the distributions of putative transmitters in the different layers, and fiber populations as well as in specific neurons and synaptic sites in the olfactory bulb (Figure 3).

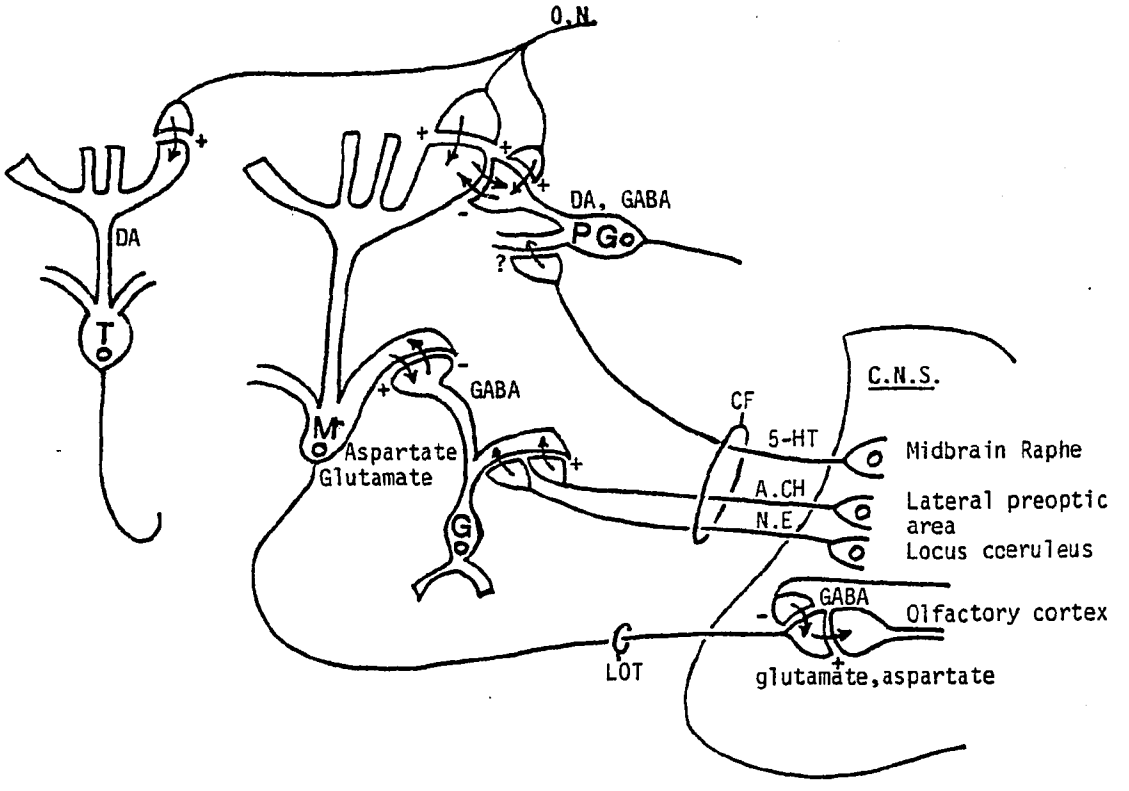

Figure 3. Transmitters of the olfactory bulb, and their cellular localization and function. Thick process = dendrite, thin process = axon, PG = periglomerular cell, M = mitral cell, O.N. = olfactory nerve, T = tufted cell,LOT= lateral olfactory tract, CF = centrifugal fibers, DA = dopamine, GABA = gamma amino butyric acid, A.CH = acetyl choline, N.E. = norepinephrine, 5-HT = serotonin, (+) = excitatory, (-) = inhibitory, arrow = indication of pre to postsynaptic direction. Brain peptides are not indicated, since their function and anatomical distribution are generally unknown.

Intrinsic Transmitters

γ-amino butyric acid (GABA) is implicated as the transmitter in the granule to mitral cell dendrodendritic inhibition, and is located in most periglomerular cells. This was indicated by the ability of GABA to mimic this inhibition, and bicuculline to block it (Nicoll, 1971b; Felix and MacLennan, 1971; MacLennan, 1971). Furthermore, the highest concentration of GABA and its biosynthetic enzyme (GAD) is found in the external plexiform layer (Graham, 1973; Jaffe and Cuello, 1980a; Nadi et al., 1980) as is muscimol binding (Nadi et al., 1980). GAD immunoreactivity was found in the granule cell side of these dendrodendritic synapses (Ribak et al., 1977) and ^{3}H-GABA was found to be taken up by periglomerular and granule cells (Halāsz et al., 1979) specifically by terminals in synaptic contact with mitral cells (Halāsz et al., 1981), or taken up and specifically released by EPL slices (Jaffe and Cuello, 1980b).

Dopamine is thought to be an intrinsic transmitter and thus unaffected by central fiber lesions (Broadwell and Jacobowitz, 1976; Fallon and Moore, 1978). Its presence was demonstrated in tufted cells and in some periglomerular cells (Halāsz et al., 1977, 1978; Priestley et al., 1979) through uptake of dopamine, and staining for tyrosine hydroxylase (TH) and dopa decarboxylase. This is supported by the uptake of norepinephrine and dopa into cells in glomerular layer (Lichtensteiger, 1966) and by the laminar distribution of dopamine and TH (Jaffe and Cuello, 1980a; Nadi et al., 1981).

Output Trasmitters

Glutamate and aspartate - have been suggested as the transmitters of the major output neurons of the bulb (the mitral cells) at

mitral to olfactory cortex contact, as indicated by selective loss of aspartate and glutamate in olfactory cortex following olfactory bulbectomy (Harvey et al., 1975; Collins, 1979a), and by specific release of aspartate in response to electrical stimulation of LOT (Collins, 1979b), and attenuation of aspartate release following bulbectomy (Collins and Probett, 1981).

Input Transmitters

Acetylcholine - is located almost exclusively in centrifugal fibers arising from the lateral preoptic area, as determined by histochemical mapping and biochemical estimation of acetylcholine esterase and choline acyltransferase after specific lesions of lateral preoptic area and diagonal band (Wenk et al., 1977; Youngs et al., 1979; Godfrey et al., 1977, 1978, 1980), and by anatomical localization (Burd et al., 1977; Carson and Burd, 1980). The acetylcholine fibers are distributed in all layers (Nadi et al., 1980; Godfrey et al., 1980), and acetylcholine is implied to be excitatory to granule cells (Bloom et al., 1964).

Serotonin (5-HT) has been identified in fibers (Dahlstrom et al., 1965; Halāsz et al., 1977, 1978) originating in cell bodies in the raphe. This is supported by anatomical connections revealed through HRP and autoradiographic studies (DeOlmos et al., 1978; Broadwell and Jacobowitz, 1976) and by 5-HT uptake and retrograde transport from the olfactory bulb to the raphe (Araneda et al., 1980a,b). The serotonergic fibers terminate in the glomerular layer (Moore et al., 1978; Halāsz et al., 1977, 1978) possibly onto interneurons.

Norepinephrine (N.E.) fibers originate from locus coeruleus indicated by N.E. disappearance following specific lesions, and terminate in various layers in the bulb (Broadwell, 1977; Fallon and Moore, 1978; Youngs et al., 1979). N.E. was found to be distributed throughout the bulb (Jaffe and Cuello, 1980a; Nadi et al., 1981), while the α and β adrenergic binding sites exhibited different laminar distribution (Nadi et al., 1980).

The dipeptide carnosine (β-alanyl-L-histidine) has been suggested on the basis of biochemical evidence to be the putative neurotransmitter of the olfactory chemoreceptor neurons, presumably acting at their synapses onto the dendrites of the mitral and periglomerular cells in the olfactory bulb glomeruli. This is based on the exclusive localization of carnosine and carnosine synthetase in the chemoreceptor neurons (Margolis, 1974; Margolis et al., 1974; Ferriero and Margolis, 1975; Margolis, 1978), and the presence of carnosine and carnosine binding sites over the glomerular layer, where the first synaptic relay is located (Hirsch et al., 1978; Hirsch and Margolis, 1979; Burd et al., 1980; Nadi et al., 1981). However, no physiological activity has been assigned to carnosine so far, although several attempts in this direction have been made (Tonosaki and Shibuya, 1979; MacLeod and Straughan, 1979; Gonzalès and Freeman, 1979). Therefore, the role of carnosine in the olfactory pathway still remains to be elucidated.

Brain peptides

In recent years various peptides have been reported in the olfactory bulb. However, the physiological role of most of them is unknown, either in the brain or in the bulb. Furthermore, their specific

localization in neurons, including cells of origin, fiber distribution, and terminal field, is largely unknown. Lastly, significant portions of this information were gathered by means of immunohistochemical or radioimmunoassay techniques, both of which are susceptible to the possibility of non-specific staining, depending on the specificity of the anti-sera used. Thus supportive analytical data from a chemical approach is essential. These peptides will be discussed briefly:

Met-enkephalin - the endogenous opioid peptide was detected in the bulb of rat (Yang et al., 1978) and mouse (Lewis and Margolis, unpublished), whereas no enkephalin fibers were localized in the bulb (Wamsley et al., 1980). Opiate binding sites were observed in several bulb layers (Nadi et al., 1980). These were unchanged by deafferentation (Hirsch et al., 1978), but selectively declined in contralateral bulb following unilateral bulbectomy (Hirsch and Margolis, 1980), suggesting their localization in interbulbar neurons. Enkephalin blocks dendrodendritic inhibition of mitral cells at the reciprocal synapses as detected by intracellular recordings (Nicoll et al., 1980). However, a fiber source for this peptide has yet to be identified.

The related peptides β-lipotropin (β-LPH), and β-endorphin have also been detected in the bulb (Przewlocki et al., 1980).

Luteinizing hormone - releasing hormone (LH-RH) - fibers were detected in the bulb (Hoffman et al., 1979). Some originate in the telencephalon and travel through the bulb into the nasal mucosa, intermingled with the olfactory nerve (Jennes et al., 1980; Fukuda and Silverman, 1981). Some fibers terminate in the bulb (Jennes et al., 1980) in granule cell, periglomerular and external plexiform layers (Hoffman et al., 1979). Others originate in LH-RH cell bodies

in the bulb (Jeness et al., 1980). LH-RH may thus be speculated to play a role in olfactory initiated behavior that is severed by lesions of olfactory bulb or LOT (Phillips et al., 1980).

Insulin - and insulin receptors were detected in the bulb (Havrankova et al., 1978) and external plexiform layer is specifically rich in receptors (Young et al., 1980). The precise function of an insulin receptor in central nervous system is unknown.

Cholecystokinin (CCK) - was detected in the bulb (Rehfeld, 1978; Beinfeld et al., 1981), and CCK binding in the bulb is among the highest in the brain (Innis and Snyder, 1980; Saito et al., 1980). The CCK related peptide gastrin is very low in the bulb (Rehfeld, 1978).

Somatostatin content in the bulb (20 ng/g; Brownstein et al., 1975; Brownstein, 1977) is equivalent to the content seen in other brain regions with the exception of the high levels of the hypothalamus.

Angiotensin II - binding sites in the bulb are one of the highest in the brain (Harding et al., 1981), but there is as yet no evidence for the presence of this peptide.

Vasointestinal polypeptide (VIP) - was observed in bulb of rat (Fahrenkrug et al., 1978; Loren et al., 1976; Brownstein, 1980), and pig (Fahrenkrug, 1980). In other brain structures it usually appears in local interneurons.

Vasopressin content is 43 pg/mg dry wt. (Dogterom and Buijs, 1980).

Substance P was detected in the bulb (Paasonen and Vogt, 1956; Brownstein, 1977; Brownstein et al., 1976). Substance P occurs in some juxtaglomerular cells and probably in fibers of centrifugal origin.

In conclusion, in the olfactory bulb, as in many other regions of the nervous system, evidence concerning the presence of transmitter substances and their localization and function is at present suggestive rather than conclusive. However, it is interesting to notice that, with the exception of epinephrine, almost every transmitter or putative transmitter found in the brain, including gastrointestinal and neuroendocrine peptides, are also detected in the olfactory bulb.

These chemical observations coupled with the extensive background of biological information about these neural systems further encourages its exploration as a model for CNS function.

E. *Prospects*

One field in which the olfactory pathway may still prove to be of even greater advantage, and an excellent model, is the study of CNS development. This follows from the unique feature of the chemoreceptor neurons (the primary afferents of the bulb), namely their continuous reconstitution. This property lends itself exceptionally well to the study of relationships between the formation of synapses by the growing axons of the newly formed neurons, to the developmental pattern of transmitter appearance in the post-synaptic neurons of the bulb, and the reverse of these changes when degeneration of synapses is induced by artificial denervation.

A prerequisite for the progress of these studies is better understanding of the regeneration process as well as the identification of the transmitter of these primary synapses. These will, in turn, allow rapid advancement of the study of the interconnection between presynaptic to postsynaptic neuron development in the olfactory bulb.

Preliminary studies of the above aspect have been performed in tissue culture of olfactory mucosa and olfactory bulb, in which the appearance of the biochemical markers carnosine and marker protein with relation to synaptic formation was studied. In a morphological approach (Graziadei et al., 1979) it was observed that even in the absence of appropriate target (after olfactory bulb removal) the olfactory neurons can regenerate, and their newly formed axons induce the formation of glomeruli-like structures and synaptic connections in the forebrain. In a third approach (Nadi et al., 1981) the levels of tyrosine hydroxylase activity and dopamine were found to be transsynaptically regulated by an unknown signal from olfactory neurons, thus olfactory neuron degeneration produced a decline of these biochemical parameters.

F. *General Goals of the Work*

The main objectives of this work were:

a. Studies of the regeneration of the olfactory chemoreceptor neurons, using the cell growth marker enzyme ornithine decarboxylase. In this study degeneration of olfactory neurons was induced surgically or chemically, and the response of ornithine decarboxylase activity was evaluated. Very high levels of ODC were demonstrated in the olfactory pathway compared to other CNS regions. This ODC activity is localized in part in the chemoreceptor neurons, and its response is the first biochemical change occurring when degeneration or regeneration of chemoreceptor neurons are induced.

b. Evaluation of the role of carnosine as a transmitter of the first synapse in the olfactory pathway, using as criterion its depolarization induced release from olfactory nerve endings. Thus, I have

prepared pinched off nerve endings (synaptosomes) from olfactory bulbs, and evaluated them in terms of content of neurotransmitter synthesizing enzyme activities, and transmitter binding sites. I have characterized the synaptosomal membrane potential ($\Delta\Psi$) with the lipophilic cation tetraphenylphosphonium, and studied its response to drugs affecting the neuronal sodium gate, Na^+, K^+-ATPase and mitochrondrial $\Delta\Psi$.

Carnosine efflux by these synaptosomes has been evaluated under normal non-depolarizing conditions and in response to depolarizing stimuli, and its dependence on Ca^{2+} was then determined. These studies suggest that carnosine is packaged and specifically released by synaptic vesicles.

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CHAPTER 2

THE RESPONSE OF ORNITHINE DECARBOXYLASE DURING NEURONAL DEGENERATION AND REGENERATION IN OLFACTORY EPITHELIUM

ABSTRACT

Mature olfactory neurons are continually replaced from a population of progenitor cells. Olfactory nerve section, bulbectomy or treatment with certain chemicals induces degeneration of olfactory neurons followed in some cases by regeneration. Ornithine decarboxylase (ODC) activity was measured in mouse olfactory tissues as an indicator of cellular regeneration. ODC activity in olfactory tissue (0.2-0.4 nmole/mg protein/h) is 10-30 times higher than in a variety of other cerebral tissues. Within 3 hours after unilateral olfactory nerve section, ODC activity in the epithelium declines to 50% of control followed by a slow return to basal activity by 6 days. In the same animals, ODC activity increases several fold in bulb (1 day) with a gradual decline to normal (9 days). Except for an early transient increase, the effects of unilateral bulbectomy on epithelial ODC activity are similar to those seen after nerve section. The changes in ODC activity following intranasal irrigation with 10 mM colchicine also closely mimic those seen after nerve section. The effects of intranasal irrigation on ODC activity with 0.5% Triton X-100 or 0.17 M $ZnSO_4$ are more complex. Thus, when the mature neuronal population is degenerating after surgery or chemical treatments, ODC activity decreases in the epithelium. The subsequent increase of ODC activity prior to reconstitution of the mature neuronal population probably reflects the regeneration mechanism of the olfactory epithelium. The increase of ODC activity in the olfactory bulb after nerve section is best interpreted as a cellular injury response. These alterations in ODC activity in olfactory tissues after chemical and surgical treatments constitute

the earliest biochemical events observed in these tissues in response to cellular damage.

INTRODUCTION

The chemoreceptor neurons of the vertebrate olfactory pathway are unusual in exhibiting continual turnover throughout life. These neurons differentiate, mature, die and are replaced by new neurons derived from stem cells in the basal layer of the olfactory epithelium. This phenomenon, perhaps uniquely expressed by these neurons, has been documented in a large number of species by morphological (Mulvaney et al., 1971; Moulton, 1974; Graziadei and Monti-Graziadei, 1978), biochemical (Harding et al., 1977; Margolis, 1978), behavioral (Oley et al., 1975; Harding et al., 1978) and neurophysiological (Takagi, 1971; Tanabe et al., 1975; Simmons and Getchell, 1979) techniques.

In addition to this continual neuronal turnover under normal conditions, it has been observed that surgical or chemical lesion of this pathway causes rapid retrograde degeneration of the chemoreceptor neuronal perikarya in the olfactory epithelium with attendant orthograde degeneration of their synaptic terminals in the bulb (Graziadei and Monti-Graziadei, 1978; Graziadei and Okano, 1979). These degenerative changes are then followed by stem cell differentiation and maturation and ultimately by reconstitution of the normal chemoreceptor neuron population in the olfactory epithelium and reestablishment of functional connections in the bulb presumably via the reformation of synapses with dendrites of mitral, tufted and periglomerular cells in the olfactory bulb glomeruli. The morphological changes (Graziadei and Monti-Graziadei, 1978) are accompanied by concomitant biochemical (Harding et al., 1977), neurophysiological (Takagi, 1971; Tanabe et al., 1975; Kiyohara and Tucker, 1978) and behavioral changes (Oley et al., 1975; Harding et al., 1978), which reach minima within the first

week after lesion and then return to normal 4-6 weeks in homeotherms (Harding et al., 1978) and more slowly in poikilotherms (Simmons and Getchell, 1979). Clearly, this is an unusual neural tissue which is characterized by exhibiting extensive and continual cellular and synaptic remodeling, and it is important to try to understand the underlying molecular mechanisms of these events. If there is a signal molecule involved in this process which triggers the stem cell population, then identification of it or of the earliest response to it is critical. One approach to the latter aspect would be to evaluate responses of ornithine decarboxylase (ODC) activity in this system.

ODC activity (EC 4.1.1.17, L-ornithine decarboxylase) is typically high in rapidly growing tissues, as in embryos (Snyder et al., 1979), and in rapidly growing tumors (Weber et al., 1972; Heby and Russell, 1973). This enzyme activity is very responsive to changes in the physiological state of cells and tissues as evidenced by the rapid increase in ODC activity in tissues treated with specific growth factors (Jänne et al., 1968; Stastny and Cohen, 1970; MacDonnell et al., 1977; Lewis et al., 1978), hormones (Jänne et al., 1978) or surgical lesions (Russell, 1973). In contrast, conditions which block cellular growth or proliferation result in the fall of ODC activity (Heby and Russell, 1973), while inhibitors of ODC are reported to inhibit proliferation of certain cell lines (Mamont et al., 1976). As the rate limiting enzyme in polyamine biosynthesis (Tabor et al., 1964; Pegg et al., 1968), ODC plays a potentially important role in regulating cellular growth and proliferation (Russell, 1973).

In view of its ubiquitous responsiveness, ODC would be expected to be a useful probe in a system exhibiting cellular turnover and

synaptic remodeling such as the vertebrate olfactory pathway. In this paper, we demonstrate that very high levels of ODC activity are present in olfactory bulb and epithelium and that this activity is very responsive to a variety of chemical and surgical lesions.

MATERIALS AND METHODS

Materials

L-[1-^{14}C]ornithine mono-hydrochloride (59 mCi/mmol) and NCS tissue solubilizer were purchased from Amersham-Searle Corporation (Arlington Heights, IL). Glycylglycine and pyridoxal-5'-phosphate were obtained from Sigma Chemical Co. (St. Louis, MO); the anesthetic xylocaine (2% Lidocaine HCl) from Astra Pharmaceutical Products Inc. (Worcester, MA), and dithiothreitol from Calbiochem (San Diego, CA). Chloropent was purchased from Fort Dodge Laboratories (Fort Dodge, IA). All other materials were purchased from Sigma or Calbiochem and were the highest quality available. Top stoppers and center wells were from Kontes Glass Company (Vineland, NJ). Mice of the CD-1 strain were purchased from Charles River Breeding Laboratories (Wilmington, MA). Rats of the WKY Strain were from a barrier maintained, closed breeding colony at Hoffmann-La Roche, Inc. (Nutley, NJ).

Tissue preparation

Three-to four-month old virgin female CD-1 mice were used unless otherwise indicated and were killed by CO_2 asphyxiation followed by exsanguination. Olfactory bulbs and epithelium were rapidly dissected onto dry ice, weighed and homogenized with 10-20 volumes of 25 mM Tris-HCl (pH 7.5) containing 5 mM dithiothreitol and 0.1 mM EDTA. After centrifugation at 48,000 x g for 60 min at 4°C the supernatant was used as the enzyme source for the ODC assay.

ODC assay

ODC activity was measured as the release of $^{14}CO_2$ from L-[1-^{14}C] ornithine by a modification of the method of Jänne and Williams-Ashman (1971). The standard incubation mixture contained in final volume of 0.5 ml: 0.1 mM Na_2CO_3, 100 mM glycylglycine (pH 7.1), 5 mM dithiothreitol, 0.4 mM pyridoxal-5'-phosphate, 0.1 mM non-radioactive L-ornithine, 300 µl tissue extract (0.4-0.6 mg protein) and 0.5 µCi L-[1-^{14}C] ornithine (59 mCi/mmol). After 15 min preincubation at 37°C, the reaction was initiated by adding the radioactive ornithine and incubating for 60 min more. The reaction was terminated by the addition of 0.5 ml of 40% trichloroacetic acid and followed by an additional 60 min to allow the release of all of the $^{14}CO_2$ formed. The $^{14}CO_2$ was trapped with 200 µl of NCS on glass fiber filters held in center wells suspended in the stoppered incubation tubes. At the end of the incubation, the center wells were transferred to counting vials containing 10 ml toluene-scintillation fluid and radioactivity was measured in a Beckman liquid scintillation spectrometer. Protein was determined by the method of Sedmak and Grossberg (1977), using the dye Coomassie brilliant blue G-250 and bovine serum albumin as standard.

ODC activity measured under these conditions was found to increase linearly with time for at least 2 h and was also proportional to the quantity of extract protein. The release of $^{14}CO_2$ was stoichiometric with putrescine formation (determined by the method of Kremzner et al., 1970). ODC activity exhibited an absolute requirement for pyridoxal-5'-phosphate and was enhanced by 5 mM dithiothreitol (Jänne and Williams-Ashman, 1971). The apparent K_m for ornithine was about

0.1 mM at pH 7.1 and was inhibited by 1 mM semi-carbazide (97%), 100 mM ammonium chloride (28%) and 1 mM putresine (28%). There was no effect of the analogs; citrulline, α-ketoglutarate, γ-aminobutyric acid, arginine, lysine or 1,5-diaminopentane at 1 mM.

Olfactory nerve section

Unilateral olfactory nerve section was performed (Harding et al., 1977) on Chloropent anesthetized mice. A small cut was made on the midline between the eyes and the skin was retracted, exposing the skull. A small hole was drilled above the front end of the bulb, using a dentist's drill. The bulb was then pushed slightly backward with a humidified air stream to expose the olfactory nerve and to prevent damage to the bulb while sectioning the nerve. A small knife was inserted into the hole and the nerve was sectioned by several side-to-side movements of the knife along the cribiform plate. The hole was covered by Gelfoam (Upjohn) and the skin closed by wound clips. The mice were allowed to recover from the anesthetic on an electric heating pad. Sham-operated mice were identically treated except that the skull was not fully penetrated during drilling.

Chemical nerve section

Chemicals were applied to intranasal irrigation with 100 µl solution (Margolis and Grillo, 1978) at the desired concentrations in aqueous medium: $ZnSO_4$ [0.17 M]; Triton X-100 [0.5% in saline] and colchicine [10 mM in saline].

Olfactory bulb ablation

Unilateral bulbectomy was performed on anesthetized mice. A small cut was made on the midline between the eyes and the skin was retracted, exposing the skull. A small hole was drilled above the bulb, using a dentist's drill and the bulb was removed by aspiration. The hole was covered with Gelfoam and the skin was closed with wound clips, and recovery was as for nerve section. Sham-operated mice were identically treated except that the skull was not fully penetrated during the drilling and aspiration was omitted.

In vitro incubation

Nasal turbinates containing the olfactory epithelium were incubated in vitro in vials containing 1.5 ml of HEPES medium (Neidle et al., 1973): 119 mM NaCl; 5 mM KCl; 0.75 mM $CaCl_2$; 1.2 mM $MgSO_4$; 1.0 mM NaH_2PO_4; 1.0 mM $NaHCO_3$; 25 mM HEPES; 12 mM NaOH; 10 mM glucose, 10 U/ml penicillin and 10 µg/ml streptomycin (pH 7.4) at 37°C for 4 hr under oxygen. The incubation was stopped by cooling on ice and aspiration of the medium. The tissue was then frozen and ODC activity was measured as above.

RESULTS

ODC activity in the developing nervous system

The developmental pattern of ODC activity was measured in soluble protein extracts of olfactory epithelium, bulb, cerebellum and hippocampus from male WKY rats (30 to 115 days of age).

The ODC activity in olfactory tissue exceeded that in cerebellum and hippocampus at all ages tested, and at 4 months of age was ten-fold than that in the cerebellum and three-fold than that in hippocampus (Fig 1.). There was a progressive decline in ODC activity with age, reaching the adult level earlier in cerebellum and hippocampus than in olfactory bulb and epithelium.

Since the activity of ODC had reached a plateau within 90 days in all tissues measured, animals selected for further studies were 90 to 100 days of age to avoid problems associated with measuring surgically related changes against a declining baseline.

Tissue distribution of ODC activity in mice

The developmental study demonstrated that ODC activity is higher in olfactory epithelium and bulb than in other neural tissues. In order to test the generality of this observation, a study of the tissue distribution of ODC activity was undertaken.

The activity of ODC in olfactory bulbs of 3-month old mice is 0.16 nmoles $^{14}CO_2$/mg protein/h (Table 1), which is slightly exceeded by that in olfactory epithelium. The ODC activity in cerebral cortex, hippocampus, colliculus and cerebellum are quite low and range between 3.4-13%

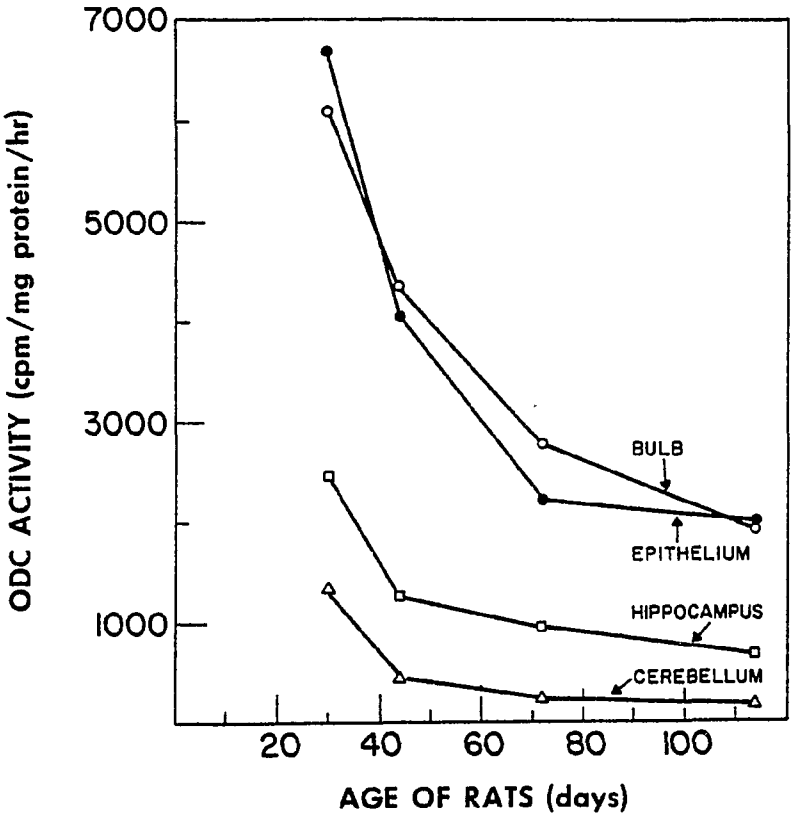

Figure 1. *ODC activity during development of rat.* ODC activity was measured as the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine by soluble tissue extracts from rats. Each point represents the average from two groups of two rats (each assayed in duplicate).
Ages: 30, 44, 72 and 114 days. Specific activity: 16,970 cpm/nmole.
Olfactory epithelium (●), olfactory bulb (o), cerebellum (Δ) and hippocampus (□).

TABLE 1. *Tissue distribution of ODC activity in mouse tissue*

Tissue	nmole $^{14}CO_2$/ mg protein/h	% Olfactory epithelium
Olfactory epithelium	0.21	100
Olfactory bulb	0.16	75
Cerebellum	0.02	13
Cortex	0.007	3.4
Hippocampus	0.023	11
Colliculus	0.035	12
Heart	0.031	15
Kidney	0.37	176
Liver	0.007	3.3
Lung	0.03	14
Spleen	0.042	20

ODC was measured as the generation of $^{14}CO_2$ from L-[1-^{14}]ornithine by tissue extracts. Each value represents the average of six mice assayed in duplicate, age 90 days (16,230 cpm/nmole).

of the ODC activity in olfactory epithelium. The activity of the non-neural tissues tested was also very low in comparison to the olfactory epithelium with the exception of the kidney. Similar results were obtained in rats (Data not shown.)

This high ODC activity in olfactory tissue is unusual for neural tissue, but is consistent with the continual reconstitution phenomenon of the receptor neurons and suggests that ODC could be used as a probe to study this neural turnover. One approach is to monitor the response of ODC to olfactory bulb ablation or olfactory nerve section.

Changes in ODC after unilateral olfactory nerve section in mice

Surgical nerve section was used to induce receptor neuron degeneration and regeneration. Mice were sacrificed at different times after surgery, and ODC activity was measured independently in both bulbs and in both sides of the olfactory epithelium in nerve-sectioned mice, in unilaterally sham-operated mice and in untreated mice. Changes in ODC activity occurred in both bulb and epithelium with different vectorial characteristics (Table 2 and Fig. 2).

ODC activity in the epithelium from the operated side exhibited a two-fold decrease within 3 h after treatment and then increased gradually between 1-3 days after treatment. It is of interest that ODC activity in the unoperated epithelium exhibited a transient and less dramatic decline even though the nerve on this side was not damaged. The reduction of ODC on the unoperated side was smaller in magnitude (minimum at 63% of control on the first day) and of shorter duration, returning to control levels by the second day.

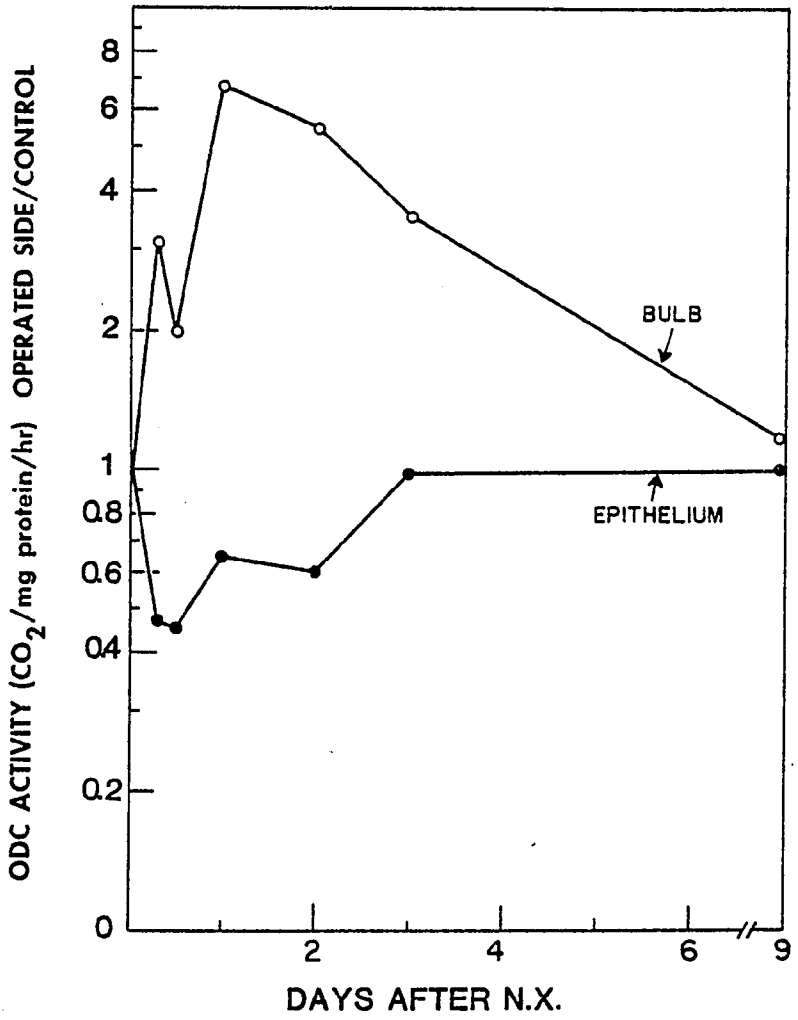

Figure 2. *Effect of unilateral nerve section on ODC activity in olfactory epithelium and bulb.* Ordinate: The ratio of ODC activity (nmoles $^{14}CO_2$/mg protein/h) in operated side to control. Abscissa: Days after surgery. The values represent the average of two groups of two mice assayed in duplicate. Epithelium (●) and bulb (o).

TABLE 2. *Effect of unilateral nerve section on ODC activity in olfactory epithelium*

Treatment	Tissue	Percent of ODC activity in control mice. Time after operation.					
		3 h	5 h	1 day	2 days	3 days	9 days
Nerve Section:							
operated	Epithelium	47	45	65	60	98	100
unoperated	Epithelium	88	68	63	96	110	101
ratio[a]:		0.56	0.65	1	0.62	0.87	0.99
operated	Bulb	310	202	670	545	350	133
unoperated	Bulb	266	124	163	143	122	105
ratio[a]:		1.19	1.57	4.1	3.8	2.8	1.3
Sham:							
operated	Epithelium	186	148	134	106	150	129
unoperated	Epithelium	152	140	130	112	133	129
ratio[a]:		1.25	1.03	1.03	0.93	1.12	1.00
operated	Bulb	300	147	269	183	224	119
unoperated	Bulb	405	139	162	128	195	123
ratio[a]:		0.84	1.11	1.76	1.42	1.17	1.17

These data are from a representative experiment and the values represent the average of two groups of two mice (each assayed in duplicate). ODC activity was calculated relative to the activity values obtained for untreated control mice taken as 100%.

[a]These values represent the ratio of ODC activity in the operated side to that in the unoperated side of unilaterally nerve-sectioned mice.

Although the effect of unilateral surgery on ODC activity was of a bilateral nature, one can use the ratio of operated side to unoperated side to compensate for non-specific effects. This approach is frequently, but not always, valid due to the possible influence of other neural interactions between the two sides. Therefore, the results were also compared to sham and unoperated mice.

In contrast to the relative constancy of the values of ODC in the epithelium of the control- and sham-operated mice, the values in the bulb were much more susceptible to change. Nevertheless, when evaluated either as within animal (i.e. operated versus unoperated side) or relative either to sham or to control untreated mice, the response of ODC in the bulb, which was surgically treated, was delayed relative to the rapid fall in the epithelium. Here, however, the response was an increase to six-fold control levels, which slowly returned to normal over several days. Thus, both the rate of response and the rate of return were slower in the bulb than in the epithelium. Although a slight increase in ODC activity was seen on the unoperated side, it was not significantly different from the transient response seen in the sham-treated mice.

Mixing experiments

Mixing experiments were carried out in order to further evaluate the changes that occurred in ODC activity in the olfactory bulb (increase) and epithelium (decrease)as a result of nerve section.

Extracts from nerve-sectioned mice were mixed with extracts from control mice and ODC activity was measured. It is assumed that the presence of a soluble activator or inhibitor in the extracts from

TABLE 3. *Influence of mixing extracts from control and nerve-sectioned olfactory tissue on ODC activity.*

	Tissue	cpm			
		Control	NX	Mixture of Control + NX	Calculated Sum of Control + NX
I.	Epithelium	558	248	918	836
II.	Epithelium	632	343	1110	975
III.	Bulb	178	760	972	938

The ODC activity of high-speed supernatants of olfactory tissue homogenates were measured as described in "Methods". The mixed activity was measured in 1:1 (volume) mixture of supernatants from two sources. Nerve section was performed 1 day before the mixing experiment. The results are expressed as the counts in $^{14}CO_2$ from separate extracts and from the mixture of the extracts. Each value represents the results of three groups of three mice assayed in duplicate.

nerve-sectioned mice would stimulate or inhibit, respectively, the activity of ODC from control mice.

The results of these measurements (Table 3) indicate that no inhibitory activity is present in the extract of epithelium, and no stimulatory activity is present in that of bulbs from nerve-sectioned mice.

Chemical nerve section

A second method (in addition to nerve section) for causing olfactory epithelium degeneration involved the intranasal irrigation of mice with certain compounds. Depending on the compound and its concentration, this procedure can cause permanent or temporary anosmia accompanied by specific morphological, biochemical and electrophysiological changes (Takagi, 1971; Margolis and Grillo, 1978 and unpublished observations; Harding and Wright, 1979).

The first agent used was $ZnSO_4$ (0.17 M), which was previously studied by a number of investigators (Takagi, 1971; Alberts, 1974; Harding et al., 1978). It was found to cause extensive damage to the olfactory epithelium accompanied by development of long-term anosmia (Harding et al., 1978). Morphologically, sloughing of the olfactory epithelium is observed accompanied by loss of electrophysiological response to odorant stimulation (Takagi, 1971; Alberts, 1974) and decreases in the biochemical markers of the receptor neurons in the olfactory epithelium and bulb (Harding and Margolis, 1976; Margolis and Grillo, 1977; Harding et al., 1978).

Both colchicine (10 mM) and Triton X-100 (0.5%) were found by Margolis and Grillo (1978) to induce temporary anosmia, accompanied by

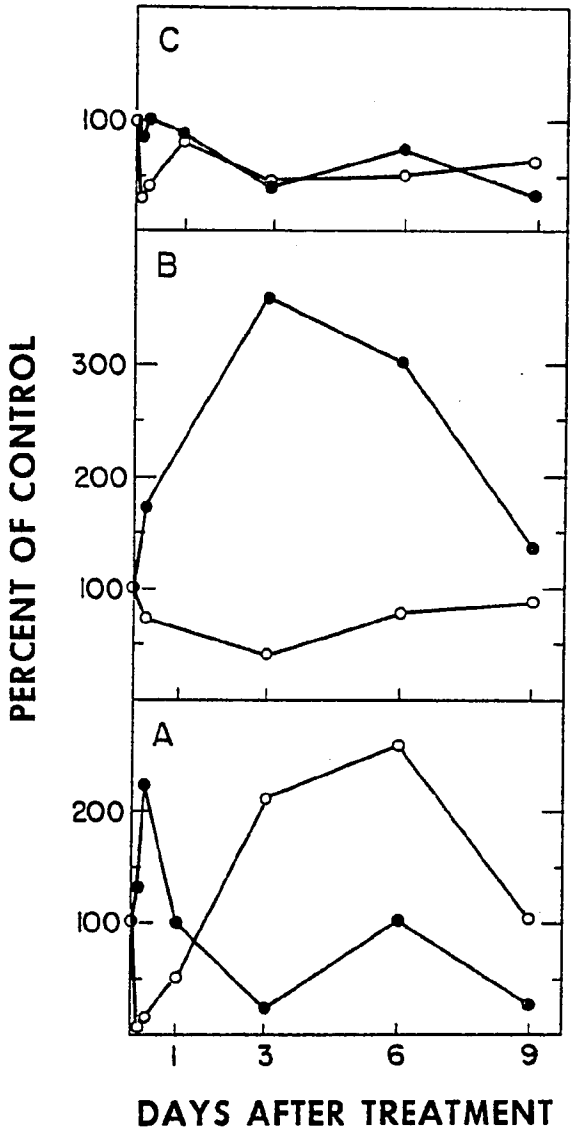

Figure 3. *Effect of chemical nerve section on ODC activity.* One-hundred µl of 0.17 M $ZnSO_4$ (a), 10 mM colchicine (b) or 0.5% Triton X-100 (c) were administered by intranasal irrigation. Mice were sacrificed at 3 h and 1, 3, 6 and 9 days after treatment, and ODC activity in olfactory epithelium and bulb was measured. Values represent the average from two groups of two mice, each assayed in duplicate. Control levels of ODC activity were: Epithelium, 0.2-0.4 nmoles/mg protein/h and bulb, 0.1-0.2 nmoles/mg protein/h. Ordinate: Percent of change in ODC activity after treatment. Abscissa: Days after treatment. Epithelium (o) and bulb (●).

morphological changes in the receptor cells and loss of specific biochemical markers as above. All of these markers returned to control levels after 2 months.

Although the chemical agents chosen for this study induce reduction of biochemical markers and cause anosmia, each has a different mechanism of action due to its different interaction with the tissue. This was expressed by differences in time courses of the biochemical, behavioral and morphological events in the olfactory epithelium. Study of the effects of the chemical treatments of olfactory epithelium on ODC activity is valuable for the understanding of their mechanisms of action.

Zinc sulfate treatment caused an immediate drop of ODC in the epithelium (Fig. 3a), reaching practically zero 3 h after irrigation, followed by an increase reaching 50% 1 day after treatment, 200% of control after 3 days, 260% after 6 days and returning to control after 9 days. The initial rapid decline of ODC is probably due to the $ZnSO_4$-induced coagulation of proteins and the death of all the cell types constituting the olfactory epithelium down to the basal lamina. Presumably, the increase of ODC activity following the initial decline represents increased cellular activity associated with tissue repair processes. This is accompanied by the appearance of scavenging cells which arrive at the epithelium, cleaning and removing the huge mass of degenerating cells and cell debris (Burd, 1979), suggesting that the resulting ODC activity is located in those cells.

In the bulb, ODC activity increases 3 and 6 h after intranasal $ZnSO_4$ treatment, reaching 240% of control, followed by a decline to 100% at 1 day and to 20% at 3 days. ODC activity remained low 9 days

after irrigation with $ZnSO_4$, and was still very low (20% of control) 54 days after $ZnSO_4$ treatment.

Colchicine causes disaggregation of microtubules; therefore, it can interfere with mitotic cells or with mature nerve cells which contain large numbers of microtubules (Borisy and Taylor, 1967; Wilson et al., 1974). Irrigation of the olfactory epithelium with 10 mM colchicine caused a decrease of ODC activity in epithelium at 6 h and 3 days after treatment (to 70% and 40% of control, respectively) [Fig. 3b], followed by a progressive increase to 85% of control at 9 days. An increase of ODC activity was detected in the olfactory bulb at 6 h and 3 days after colchicine application (170% and 350%, respectively), followed by a decline to 135% at 9 days.

Colchicine-induced changes in ODC activity in the olfactory epithelium and bulb resemble the changes that were detected after nerve section but their development is slower. While ODC activity in epithelium had reached a minimum at 3 h after nerve section and increased toward normal levels at 3 days, ODC activity reached a minimum only 3 days after colchicine treatment and approached its original levels at 6-9 days. This delayed effect of colchicine on ODC activity in comparison to nerve section correlates with the delayed return of biochemical markers and reversal of anosmia detected after colchicine treatment (Margolis and Grillo, 1978).

Triton X-100 is a detergent which presumably interacts with the cell membranes. The morphological damage seen after intranasal application of 0.5% Triton X-100 is less dramatic than after the colchicine or $ZnSO_4$ treatments (F.L. Margolis, unpublished observations). The recovery of the biochemical and behavioral parameters is faster after

Triton X-100 application than after colchicine or $ZnSO_4$ treatments (the latter is essentially irreversible).

The overall effect of Triton X-100 treatment on the levels of ODC activity (Fig. 3c) has a different pattern than those seen after colchicine or $ZnSO_4$ applications. ODC activity in the epithelium declines very rapidly, identical to the declines detected after nerve section, colchicine or $ZnSO_4$ and then returns to control level very slowly, reaching about 60% of control after 9 days. The activity of ODC in the olfactory bulbs did not exhibit the dramatic increase that was detected after other nerve section treatments. Instead of the expected increase, a slow decrease of ODC in the bulb was detected, reaching 40% of control at the 3rd day after treatment and remaining below control for up to 9 days.

This effect is consistent with the assumption that the bulk of the increase of ODC activity in the bulb after surgical nerve section is due to glial cell proliferation. A minimal loss of olfactory bulb weight occurs after Triton X-100 treatment, compared to that seen after $ZnSO_4$ (Margolis and Grillo, 1978), suggesting that the extent of the damage and resultant tissue repair processes are less extensive, as expressed by the lack of increase of ODC activity in the bulb after Triton X-100 treatment.

Olfactory bulbectomy

A third way to induce degeneration of the receptor neurons in the olfactory epithelium is olfactory bulbectomy (surgical removal of the olfactory bulb), thus it was important to study the effect of bulbectomy on ODC activity in the olfactory pathway.

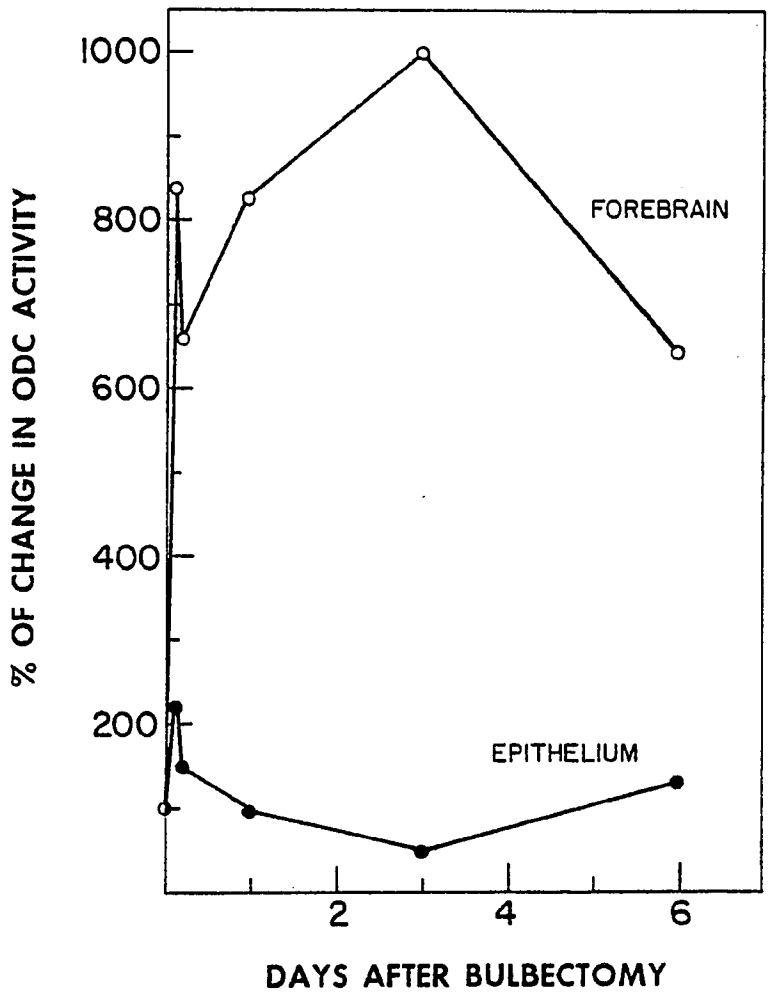

Figure 4. *The effect of unilateral bulbectomy on the activity of ODC in the epithelium and forebrain from the operated side.* Mice were unilaterally bulbectomized on the right side, and ODC activity in the olfactory epithelium (●) and in a 2-3 mm coronal section of rostral forebrain (o) was measured at 3 and 5 h and 1, 3 and 6 days after bulbectomy. Ordinate: Percent of change in ODC activity from operated side. Abscissa: Time after bulbectomy. Values are from a representative experiment of nine mice in each group assayed in duplicate.

TABLE 4. *Effect of bulbectomy on ODC activity in olfactory epithelium, bulb, forebrain and cerebellum*

Treatment	Tissue	Percent of ODC activity in control mice. Time after operation.				
		3 h	5 h	1 day	3 days	6 days
Bulbectomy:						
operated	Epithelium	220	150	95	50	132
unoperated	Epithelium	110	170	63	60	80
ratio[a]:		2	0.89	1.53	0.73	1.6
	Bulb	399	385	96	134	85
operated	Forebrain	838	661	829	1065	646
unoperated	Forebrain	581	961	207	734	117
ratio[a]:		1.44	0.69	4	1.45	5.5
	Cerebellum	227	241	56	128	105
Sham:						
operated	Epithelium	167	136	108	111	113
unoperated	Epithelium	128	122	112	105	105
ratio[a]:		1.3	1.1	0.96	1.1	1.1
operated	Bulb	220	220	215	92	104
unoperated	Bulb	204	157	130	89	81
ratio[a]:		1.1	1.6	1.65	1	1.3
operated	Forebrain	250	286		115	136
unoperated	Forebrain	230	290		93	97
ratio[a]:		1.1	0.99		1.2	1.4
	Cerebellum	220	250		122	120

Mice were unilaterally bulbectomized on the right side, and ODC measured at 3 and 5 h and 1, 3 and 6 days after bulbectomy. Each value represents the average of duplicate assays from groups of nine mice.

[a]These values represent the ratio of ODC activity in the operated side to that in the unoperated side of unilaterally bulbectomized mice.

At several times after unilateral bulbectomy ODC activity was measured in the epithelium on the control and surgically treated sides, as well as in the remaining bulb and in the forebrain and cerebellum. The activity of ODC in the olfactory epithelium on the operated side increased sharply after bulbectomy (Table 4 and Fig. 4) and was 250% of control at 3 h. Thereafter, the activity of ODC in olfactory epithelium slowly declined to 50% of control by 3 days. On the 6th day, ODC activity in the epithelium had returned to control values. This was clearly a different pattern from that seen after olfactory nerve section (Fig. 2).

ODC activity in the contralateral olfactory bulb was 400% of control at 3 and 5 h after bulbectomy. This was followed by a rapid decline to control values for the balance of the experiment.

Removal of the olfactory bulb results de facto in a certain amount of damage or lesion to the forebrain, to which it is connected both neurally and mechanically. Therefore, we also studied the effect of bulbectomy on ODC activity in this brain region. ODC was measured in the rostral forebrain and its activity was found to increase dramatically to 800% of control 3 h after bulbectomy, reaching a maximum of 1000% after 3 days and remaining in excess of 600% at 6 days after treatment. This pattern resembles the increase of ODC activity that was induced in the olfactory bulb after surgical nerve section and presumably is an "injury response" related to gliosis and repair. To test whether this response was exhibited throughout the CNS, ODC activity was measured in the cerebellum after bulbectomy and in the olfactory bulb, forebrain and cerebellum of sham-operated mice. The changes in ODC activity in these brain regions are of the magnitude of a two-fold bilateral

increase at 3 and 5 h after treatment, with no significant difference between the operated side and the unoperated side. Therefore, a change of this magnitude in ODC activity of bulbectomized mice was not considered to be a significant effect of bulbectomy. The response of ODC activity in the remaining olfactory bulb is due either to unintended trauma or is a response to the disruption of anatomical pathways which pass between the two bulbs via the anterior commissure.

Effect of asparagine on ODC activity

Asparagine was reported by Chen and Canellakis (1977) to cause an increase of growth rate of neural cell cultures that was preceded by induction of ODC activity in those cells. Several other studies described the use of enzyme asparaginase (summarized by Calabresi and Parks, 1975) as a carcinostatic agent. These findings led us to study the effect of asparagine on ODC as a possible growth signal or growth regulator of the reconstituting olfactory epithelium.

Olfactory epithelium was incubated in vitro in the presence and absence of asparagine. At the end of the incubation period, ODC activity in the tissue was measured. The incubation of the olfactory epithelium in vitro in the presence of asparagine (10 mM for 4 h at 37°C) resulted in a two-fold increase in ODC activity compared to control epithelium that was incubated without asparagine. ODC activity in the incubated control was elevated as compared to the non-incubated epithelium. Incubation in the presence of both asparagine and 1.7% goat serum resulted in a three-fold increase in ODC activity (Fig. 5a). However, when the effect of asparagine was measured in epithelium after bulbectomy, the induction of ODC by asparagine was reduced and amounted

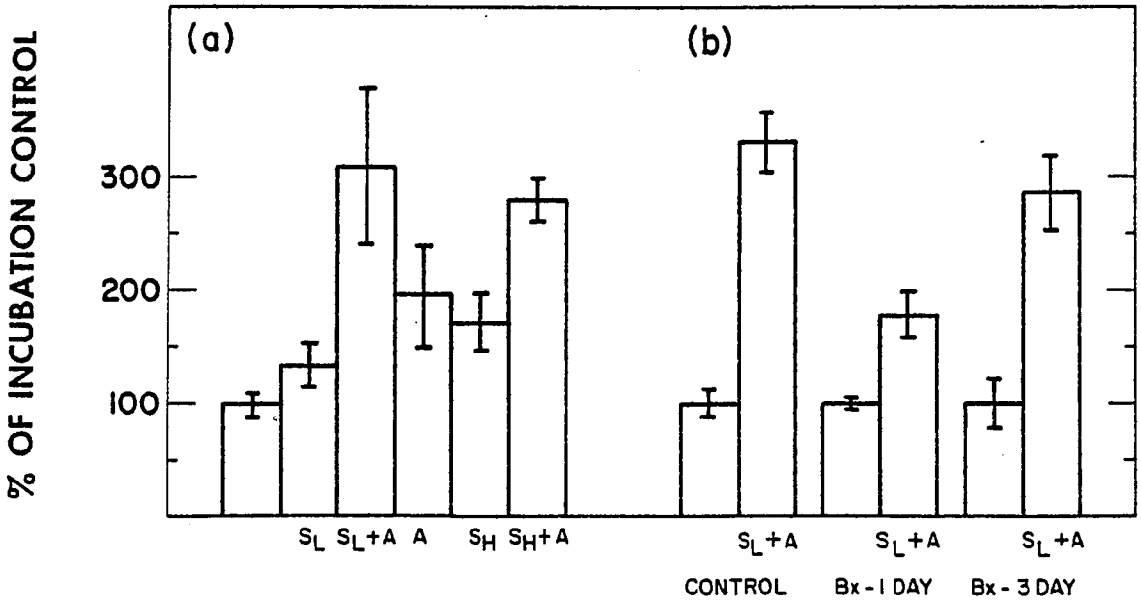

Figure 5. *Effect of asparagine and serum on ODC activity in epithelium and its relation to bulbectomy.* (a) Mouse olfactory epithelium was incubated in the presence or absence of 10 mM asparagine (A), 1.7% goat serum (S_L) or 7% goat serum (S_H) and their combinations (S_L + A and S_H + A) at 37°C for 4 hr and ODC activity measured in duplicate (n = 3). All treatments were statistically different from control, with $p < 0.01$ for serum and asparagine and $p < 0.001$ for other treatments. (b) Bulbectomized (Bx) mice compared to control mice incubated at 37°C for 4 h in the presence and absence of 1.7% serum and 10 mM asparagine (S_L + A). Epithelium from bulbectomized mice was from the operated side.

to only 177% 1 day after bulbectomy as compared to 333% in control. The effect of asparagine was not significantly different from control (a 286% increase) 3 days after bulbectomy (Fig. 5b). This suggests the existence of two populations of asparagine sensitive ODC activity, one of which is preferentially affected at short times after bulbectomy. Whether this is due to differential induction or differential stabilization is unknown (Chen and Canellakis, 1977).

DISCUSSION

ODC activity has been studied in mice after treatments inducing chemoreceptor neuron degeneration followed by regeneration in the olfactory epithelium and accompanying remodeling in the olfactory bulb. The effect of asparagine on ODC was investigated in order to reach a better understanding of the biochemical events occurring in the olfactory system during regeneration.

ODC activity in the olfactory bulb and epithelium is much higher than is seen in other neural tissues at all ages tested. Although a similar pattern of post weaning decline is seen in several rat tissues, the olfactory tissues plateau to much higher final levels and at later ages than do either cerebellum or hippocampus, two regions which also exhibit postnatal cellular multiplication (Altman, 1969; Kaplan and Hinds, 1977).

The olfactory epithelium, which is a unique neural tissue undergoing continuous neuronal regeneration, was expected to exhibit high levels of ODC activity based on the findings in many other regenerating systems (Snyder et al., 1970; Russell, 1973). The CNS, on the other hand, is a non-regenerating system and therefore was expected to have very low ODC activity in comparison to olfactory epithelium. Our observation that ODC in mouse olfactory epithelium is 10-20 times higher than those of all CNS regions tested is consistent with this view, although the similarly high activity in the olfactory bulb was unexpected.

The distribution of ODC activity among stem cells, differentiating cells and mature cells in proliferating or regenerating tissues is still an unresolved problem. Our observations in the olfactory epi-

thelium show that ODC activity declined whenever degeneration of mature receptor neurons was induced either by nerve section, bulbectomy or by chemical treatments.

ODC is considered to be a very sensitive marker of growth rate, having a half life of 11-15 min (Russell, 1973) and responding very rapidly to changes in cellular environment. Therefore, the fall of ODC activity after the above treatments, but prior to the appearance of any degenerative changes in the receptor cells, suggests that about 50% of the ODC activity in the olfactory epithelium is compartmented within the mature or maturing receptor neurons. This unexpected association of ODC with mature cells of rapidly proliferating epithelium was also observed in the small intestinal mucosa, where ODC activity increased as cells migrated out of the crypt (Baylin et al., 1978). Additional support for this hypothesis is derived from the mixing experiments, where no inhibitor activity could be detected in the epithelium after nerve section. Therefore, it is probable that the loss of ODC activity did not result from the release of inhibitor by one type of cells (mature neurons), which inhibits the ODC activity in the other (stem cells), but rather resulted from loss of the enzyme activity itself from receptor cells destined to degenerate. Our studies of asparagine effects on ODC activity are consistent with the existence of two populations of ODC activities. One which may be in the mature neuron is more sensitive to asparagine and is lost after bulbectomy, while the second which is less asparagine sensitive and remains after bulbectomy may be in the basal cells. This hypothesis must be substantiated through additional studies using immunochemical methods to determine the cellular localization and level of enzyme protein.

Our results in the olfactory pathway are in contrast to the effects of regeneration-inducing treatments in other tissues (liver, thyroid, uterus and adrenals [Russell, 1973]), which are reported to cause a five- to five hundred-fold increase of ODC activity. This difference in response of ODC activity to induction of regeneration may be related to the fact that the olfactory epithelium is a continually regenerating tissue (Moulton, 1974), which exhibits very high baseline levels of ODC activity, as compared to the tissues noted above. This implies that the level of ODC activity in the stem cells is sufficient to support the cell replacement occurring after nerve section, as it supports the normal continual regeneration process in the olfactory epithelium. Therefore, there may not be a need for special induction of ODC in this tissue when experimental regeneration is induced. Graziadei and Monti-Graziadei (1978), Monti-Graziadei and Graziadei (1979) and Graziadei and Okano (1979), in contrast, report a large increase in mitotic activity after nerve section in the frog, mouse and pigeon. Our data imply that normal mechanisms of stem cell proliferation and differentiation are being used to replace the degenerated neurons, and that elevation of ODC activity beyond normal is not required. Further substantiation of this theory would be dependent upon immunohistochemical localization of ODC in olfactory epithelium, permitting direct monitoring of cells which contain ODC and of the cellular response of ODC cells.

An interesting phenomena that has been observed in the olfactory pathway is the high activity of ODC in olfactory bulb and the elevation of ODC activity in the olfactory bulb after nerve section or colchicine administration and at short times after $ZnSO_4$ treatment. Similarly,

there is a massive increase in forebrain ODC activity following bulbectomy. The high activity of ODC in normal olfactory bulb cannot be related to rapid cell turnover, since only very slow cellular proliferation was detected in the glomerular layer (Kaplan and Hinds, 1977). However, this activity may be related to the restructuring of dendrites of mitral, tufted and short axon periglomerular cells that are continuously losing old inputs from epithelium and receiving new inputs.

The several-fold increase of ODC activity in the bulb of the operated side after nerve section follows very closely the time course of the appearance and disappearance of scavenging glial cells in the bulb (Graziadei and Monti-Graziadei, 1978), and presumably the same is true in the forebrain following bulbectomy. Similar observations have been noted by G. Jancsó (1978). Therefore, these responses are most likely due to a stress or injury response related to glial proliferation and dendritic remodeling.

The high levels of ODC activity we have observed in olfactory epithelium and bulb compared to other brain regions, coupled with the differential life span of putrescine and the aminopropyl portions of polyamines in brain as reported by Antrup and Seiler (1980), suggest that a study of the activity of S-adenosylmethionine decarboxylase and of polyamine turnover in olfactory tissues before and after lesion would contribute to our understanding of the role of ODC.

In conclusion, we have demonstrated unusually high levels of ODC activity in the olfactory epithelium and bulb, considered the possible role of ODC in this tissue and shown the response of this enzyme to a variety of lesions of the olfactory pathway. In the olfactory epithelium, the response of ODC is consistent with an absence of dramatic

mitotic stimulation following central lesions, while in the bulb, a role associated with terminal remodeling and/or gliosis seems probable.

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CHAPTER 3

MEMBRANE POTENTIAL OF OLFACTORY BULB SYNAPTOSOMAL FRACTIONS: CHARACTERIZATION WITH THE LIPOPHILIC CATION TETRAPHENYL-PHOSPHONIUM

ABSTRACT

The membrane potential of olfactory bulb synaptosomal fractions was monitored with the lipophilic cation tetraphenylphosphonium (TPP^+)[2], which has been reported to distribute across membranes according to the Nernst equation. The properties of the synaptosomal membrane potential as monitored with TPP^+ were similar to those reported for neural tissues using other measurement techniques. There is an electrical potential ($\Delta\Psi$) of -64 mV and -77 mV in the P_1 and P_2 synaptosomal fractions, respectively. This potential is due primarily to the K^+ diffusion gradient across the synaptosomal membrane.

The influence of ouabain on TPP^+ accumulation indicates that the Na^+,K^+-ATPase electrogenicity contributes about -20 mV to the resting synaptosomal membrane potential. Veratridine induced a decline in TPP^+ accumulation, which was blocked by tetrodotoxin or by the omission of Na^+ from the medium.

A significant mitochondrial contribution to TPP^+ accumulation was observed, which varied as a complex function of TPP^+ concentration in the medium in a manner indicating that TPP^+ interfered with maintenance of mitochondrial potential. This mitochondrial contribution could be eliminated by performing the experiments anaerobically in the presence of oligomycin. The results are discussed with relation to the future possible use of TPP^+ for $\Delta\Psi$ measurements in synaptosomal preparations.

INTRODUCTION

Nerve endings (synaptosomes), prepared by homogenization of brain tissue under conditions of moderate shear (for review; see Whittaker, 1969), are an important *in vitro* system for the study of processes associated with binding, uptake, metabolism and release of neurotransmitters (Cotman et al., 1976; for review, see Baldessarini and Karobath, 1973 and Levi and Raiteri, 1976). Such preparations have been shown to retain many of the metabolic (Bradford, 1969 and 1975), osmotic (White and Keen, 1970; Keen and White, 1971) and morphological properties of the neural tissue of origin and to contain one or more mitochondria (Whittaker, 1969). In addition, synaptosomes possess extremely active ion transport systems (Li and White, 1977), which maintain selective distribution of Na^+, K^+ and Cl^- across the synaptosomal membrane (Campbell, 1976).

An important question that has to be addressed is: Do synaptosomes maintain a $\Delta\Psi$ which regulates and is regulated by other components in a normal manner so as to provide a model system to study neuronal function? However, measurement of changes in synaptosomal membrane potential ($\Delta\Psi$) associated with the above processes cannot be performed by direct electrophysiological techniques. Evaluation of the synaptosomal plasma membrane $\Delta\Psi$ by monitoring changes in cyanine dye fluorescence (Blaustein and Goldring, 1975; Ng and Howard, 1978; Sen and Cooper, 1978) or calculations based on the Na^+, K^+ and Cl^- distribution across the synaptosomal membrane (Campbell, 1976) or the diffusion potential of Rb^+ (Scott and Nicholls, 1979 and 1980) each suffer from different problems (Sims et al., 1974; Simons, 1976; Kinnally and Tedeschi, 1977; Krasne, 1977; Montecucco et al., 1979; Hansson et al., 1980).

The radioactive permeant lipophilic cations such as methytriphenylphosphonium ($TPMP^+$) and tetraphenylphosphonium (TPP^+) have been used to measure membrane potential in mitochondria (Grinius et al., 1970) and bacteria and bacterial vesicles (H. R. Kaback, personal communication) and recently also in intrasynaptosomal mitochondria (Scott and Nicholls, 1979 and 1980), neuroblastoma glioma hybrid cells (Lichtstein et al., 1979) and guinea pig synaptosomes (Ramos et al., 1979; Creveling et al., 1980).

In the present paper, we have critically evaluated the utility of $[^3H]TPP^+$ for studying the membrane potential properties of mammalian synaptosomal preparations. In particular, we have studied preparations from rat olfactory bulbs. This is a brain area rich in dendrodendritic synapses (Shepherd, 1977; Kornguth et al., 1979; Quinn and Cagan, 1980), and about which we have recently learned a great deal regarding the occurrence and distribution of neurotransmitters and neurotransmitter-related biochemistry (Nadi et al., 1980a and 1980b; for reviews, see Margolis, 1974 and 1980).

MATERIALS AND METHODS

[^{3}H]TPP$^+$ (2.5 Ci/mmol) was generously supplied by Dr. H.R. Kaback (Roche Institute of Molecular Biology, Nutley, New Jersey). TPP$^+$ bromide was purchased from K & K Laboratories (Plainview, New York). Veratridine was from Sigma Chemical Co. (St. Louis, Missouri). Tetrodotoxin, ouabain, oligomycin and carbonyl-cyanide-m-chlorophenyl hydrazone (CCCP) were purchased from Calbiochem (La Jolla, California). The radioactive compounds: [^{3}H]H_2O (0.1 Ci/g), [methoxy-^{14}C]inulin (15.0 mCi/g), [1-^{14}C]β-alanine (55 mCi/mmol), [3,5-^{3}H] (Ring) tyrosine (53.2 Ci/mmol), [1-^{14}C]glutamate (23 mCi/mmol), [^{3}H-methyl]diazepam (39.1 Ci/mmol), [^{3}H-(N)-methylene]muscimol (12.1 Ci/mmol) and [1-phenyl-4-^{3}H]spiroperidol (26.4 Ci/mmol) were all from New England Nuclear (Boston, Massachusetts). The following were obtained from Amersham Corporation (Arlington Heights, Illinois): [1,7,8(N)-^{3}H]dihydromorphine (81 Ci/mmol) and [3-^{3}H]DL-quinuclidinyl benzilate (13 and 16 Ci/mmol). [^{3}H]Carnosine was synthesized in this laboratory from [3-^{3}H(N)]β-alanine (36 Ci/mmol) obtained from New England Nuclear and unlabeled L-histidine using the method of Hirsch et al. (1978), described previously. All other chemicals used were of analytical grade.

Olfactory bulbs were obtained from inbred WKY rats raised in a germ-free colony at Hoffmann-La Roche Inc. (Nutley, New Jersey). Two- to three-month old female rats were sacrified by exsanguination following CO_2 asphyxiation, and the olfactory bulbs were rapidly removed and placed on ice.

Synaptosomal Preparation

Olfactory bulbs were homogenized (according to Hajos et al., 1974; as adapted by Keller, 1975) in 10 volumes of 0.32 M sucrose, 1 mM $MgSO_4$ and

5 mM HEPES-Tris (pH 7.4) by 50 strokes with a Dounce homogenizer (Kontes) (H_0) followed by sequential filtration through 1000, 210 and 70 mM mesh nylon bolting cloth (H_1), with subsequent centrifugation at 1000 x g for 1 min to yield a pellet (P_0) and a supernatant (S_0). S_0 was recentrifuged at 1000 x g for 10 min to give pellet P_1 and supernatant S_1. S_1 was then centrifuged at 14,500 x g for 20 min to give pellet P_2 and final supernatant S_2. The pellets were gently resuspended in 1 ml of homogenization medium for each gram of starting material.

Synaptosomal Characterization

Synaptosomal fractions were prepared for electron microscopy according to a modification of the method of Cotman and Flansburg (1970) by fixation with 1% osmium tetroxide in Beem capsules, dehydration with increasing concentrations of acetone and embedding in Epon 812. Embedded pellets (1 mm thick) were sectioned in a plane perpendicular to the axis of centrifugation in order to be able to evaluate all the components of the fraction.

To monitor the distribution of newly synthesized carnosine in the synaptosomal fractions, animals were subjected to intranasal lavage with 25 μCi [1-^{14}C]β-alanine on the evening before death. The synaptosomal [^{14}C]carnosine content was determined as described previously by Margolis and Grillo (1977). Carnosine was determined chemically as the fluorescent 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) derivative essentially as previously described (Wideman et al., 1978) but using an isocratic rather than stepwise elution procedure. Glutamate decarboxylase activity was monitored as the release of $^{14}CO_2$ from [1-^{14}C]glutamate according to Baxter (1972) as modified by Margolis et al. (1974). Tyrosine hydroxylase

activity was evaluated by monitoring the formation of $[^3H]H_2O$, resulting from hydroxylation of 3,5[^{3}H]tyrosine as discussed by Nadi et al. (1980b).

Binding sites for the ligands spiroperidol (SPI), dihydromorphine (DHM), quinuclidinyl benzilate (QNB), diazepam (DZ), muscimol and carnosine were assayed as previously described by Nadi et al. (1980a).

DNA content was determined according to Burton (1956), and proteins were estimated by the dye binding method of Sedmak and Grossberg (1977) with BSA as standard. Insoluble proteins were dissolved in a small volume of formic acid and diluted for assay so that the final concentration of formic acid in the reaction mixture was one percent.

$[^3H]TPP^+$ Accumulation

Synaptosomal fractions P_1 and P_2 were individually resuspended in homogenization medium and 10 µl aliquots containing 50-120 µg protein were incubated at 37°C for 5 min in 200 µl of either low K^+ medium: 121.5 mM NaCl + 5 mM KCl, or high K^+ medium: 121.5 mM KCl + 5 mM NaCl or choline medium, 121.5 mM choline chloride + 5 mM KCl. In addition, all the incubation media contained 0.25 µCi of 5 µM $[^3H]TPP^+$, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1 mM sodium phosphate, 5.5 mM glucose and 45 mM HEPES buffered with tris base to pH 7.4. All incubations were in air unless otherwise indicated. Alterations in incubation time, additives and composition of the medium will be specified as appropriate. Incubation was stopped by the addition of 2.5 ml of cold 0.8 M NaCl and immediate rapid vacuum filtration through Millipore filters (EHWP cellotate), followed by an additional wash with 2.5 ml of 0.8 M NaCl. The total filtration time for each sample was less than 20 seconds. The filters were then dried at 50°C for 30 min and the radioactivity was determined in a liquid scintillation

spectrometer in the presence of 10 ml of Hydrofluor (National Diagnostics, Parsippany, New Jersey).

Synaptosomal Volume

The internal synaptosomal volume was evaluated using [methoxy-^{14}C]inulin as an indicator of the external volume and [^{3}H]H_2O as an indicator of the total volume, basically according to the procedure described by Schuldiner and Kaback (1975). The synaptosomal volume is defined as the calculated difference between the total volume and the external volume.

An aliquot of synaptosomal suspension (30 µl, 500 µl protein) was added to 470 µl of the same buffer composition as in the various TPP^+ accumulation studies, but which contained in addition 0.5 µCi [^{3}H]H_2O and 0.05 µCi [^{14}C]inulin. After rapid mixing, the samples were immediately centrifuged for 30 sec at about 15,000 x g in an Eppendorf Microfuge. An aliquot of supernatant was taken from each tube for the determination of the contents of [^{3}H] and [^{14}C] to evaluate the total input. After removal of the balance of the supernatant by aspiration, the pellet was dissolved in 0.5 ml of 1% SDS at room temperature overnight and transferred quantitatively to scintillation vials with 1 ml H_2O and counted in 10 ml Hydrofluor with the appropriate standards. Internal synaptosomal volumes of 6.6 ± 0.53 S.D. µl/mg protein for P_1 and 5.5 ± 0.51 S.D. µl/mg protein for P_2 were obtained. No significant variation in volumes were observed under isotonic conditions with changes in K^+ concentrations and drug additions.

Calculation of [^{3}H]TPP^+ Gradient and $\Delta\Psi$

The transmembrane potential ($\Delta\Psi$) of the synaptosomes was calculated based on the distribution of [^{3}H]TPP^+ according to an adaptation of the

Nernst equation:

$$\Delta\Psi = \frac{-RT}{ZF} \ln [TPP^+]_{in}/[TPP^+]_{out} \quad (1)$$

which at 37°C simplifies to:

$$\Delta\Psi = 61.5 \log [TPP^+]_{in}/[TPP^+]_{out} \quad (2)$$

where: $$[TPP^+]_{in} = [TPP^+]_{in}^{\text{low } K^+} - [TPP^+]_{in}^{\text{high } K^+} \quad (3)$$

This correction [equation (3)] is based on the assumption that the accumulation of $[^3H]TPP^+$ in the presence of depolarizing concentrations of extracellular potassium (122 mM) is not due to the synaptosomal membrane potential as discussed by Lichtstein et al. (1979). The ratio:

$$[TPP^+]_{in}/[TPP^+]_{out} = TPP^+ \text{ gradient.} \quad (4)$$

Equations (4) and (2) were the basic equations used in this study to calculate the $[^3H]TPP^+$ gradient and membrane potential, respectively. Certain modifications of these equations have been derived in the text in order to adapt them to the specific problems of $\Delta\Psi$ quantitation in olfactory bulb synaptosomes.

RESULTS

Synaptosomal Fraction Characterization

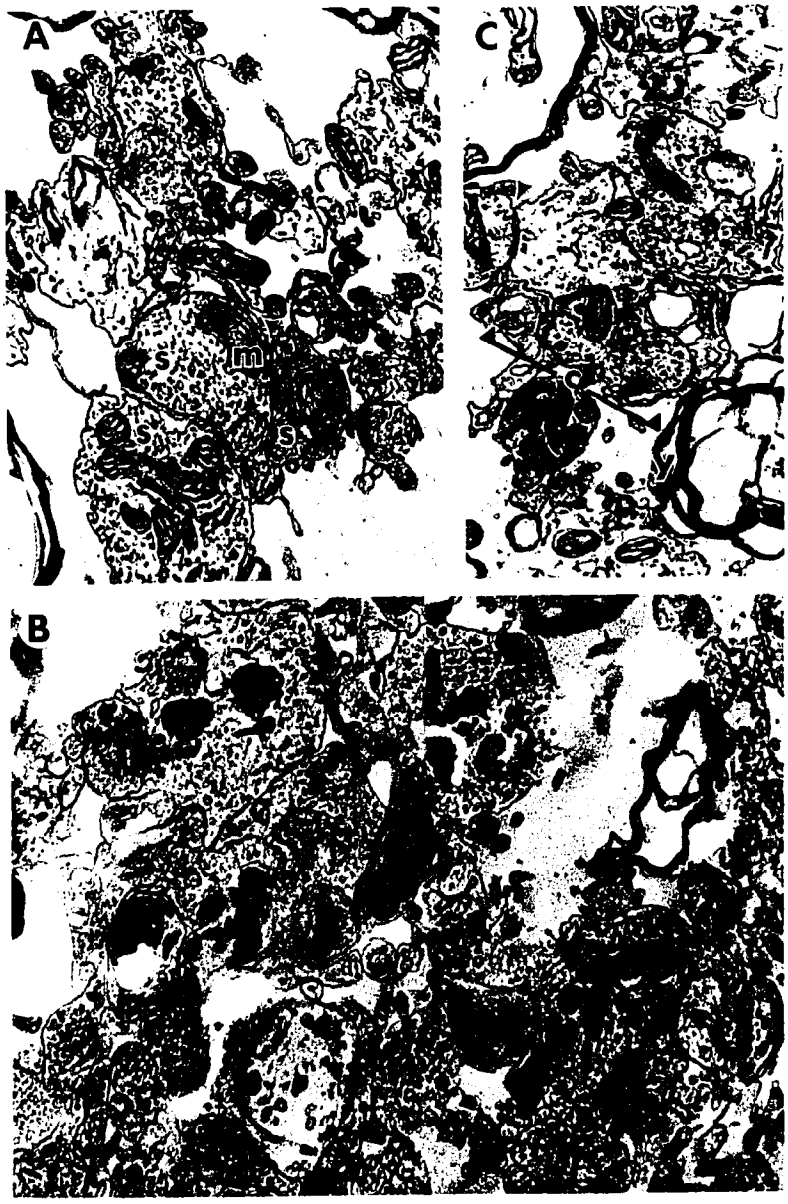
The olfactory bulb synaptosomal fractions have been characterized both morphologically and biochemically.

Electron microscopic observations (Fig. 1) indicated that fraction P_0 consists primarily of nuclei and unbroken cells. Fraction P_1 contains multisynaptosomal particles, occasional nuclei and some unidentified membranes. Fraction P_2 contains primarily individual synaptosomes as identified by their content of internal mitochondria, synaptic vesicles and postsynaptic densities, as well as some unidentified particles and occasional free mitochondria.

The distribution of DNA in the various homogenate subfractions (Table 1) supports the conclusion obtained from the EM study that most of the nuclei in the original homogenate (Ho) are removed into the P_0 fraction since only a small amount of the homogenate DNA is observed in the P_1 fraction, and no DNA could be detected in the P_2 or S_2 fractions. The distribution of the activity of the neurotransmitter-synthesizing enzymes, glutamate decarboxylase (GAD) and tyrosine hydroxylase (TH) [Table 1], shows that about 75% of GAD and 44% of TH activities are associated with the particulate fractions. Their distribution among P_0, P_1 and P_2 is uniform. Carnosine (β-alanyl-L-histidine) is specifically associated with olfactory neuron terminals in the bulb (Margolis et al., 1974; Margolis, 1974; Ferriero and Margolis, 1975; Hirsch and Margolis, 1979; Nadi et al., 1980a). The distribution pattern of this dipeptide among the particulate fractions is very similar whether measured chemically or as

Figure 1. *Electron micrographs of the olfactory bulb homogenate fractions.* Synaptosomal fractions prepared from rat olfactory bulbs by gentle homogenization in 0.32M buffered sucrose. After removal of most of the nuclei and unbroken cells into the P_0 pellet by centrifugation at 1000xg for 1 min, the P_1 fraction was obtained by subsequent centrifugation of S_0 for 10 min at 1000xg. The P_1 fraction (A, B, C) contains a few nuclei (not shown) and primarily synaptosomes (s) clustered together in synaptic complexes (c). Synaptic vesicle are observed as are synaptic contacts (arrow heads) with classical postsynaptic thickening of the membrane. Intrasynaptosomal mitochondria (m) and a few fragments of myelinated axons (y) are also observed.

Fraction P_2 (D, E) represents the classical crude synaptosomal pellet obtained by centrifugation of S_1 (the supernatant of P_1) for 20 min at 14,500g. The legend is the same as for P_1. However, P_2 contains conventional synaptosomes rather than multisynaptic particles, a few free mitochondria (m) are visible. No nuclei or myelinated fragments are observed. Marker bar = 1.0 micron.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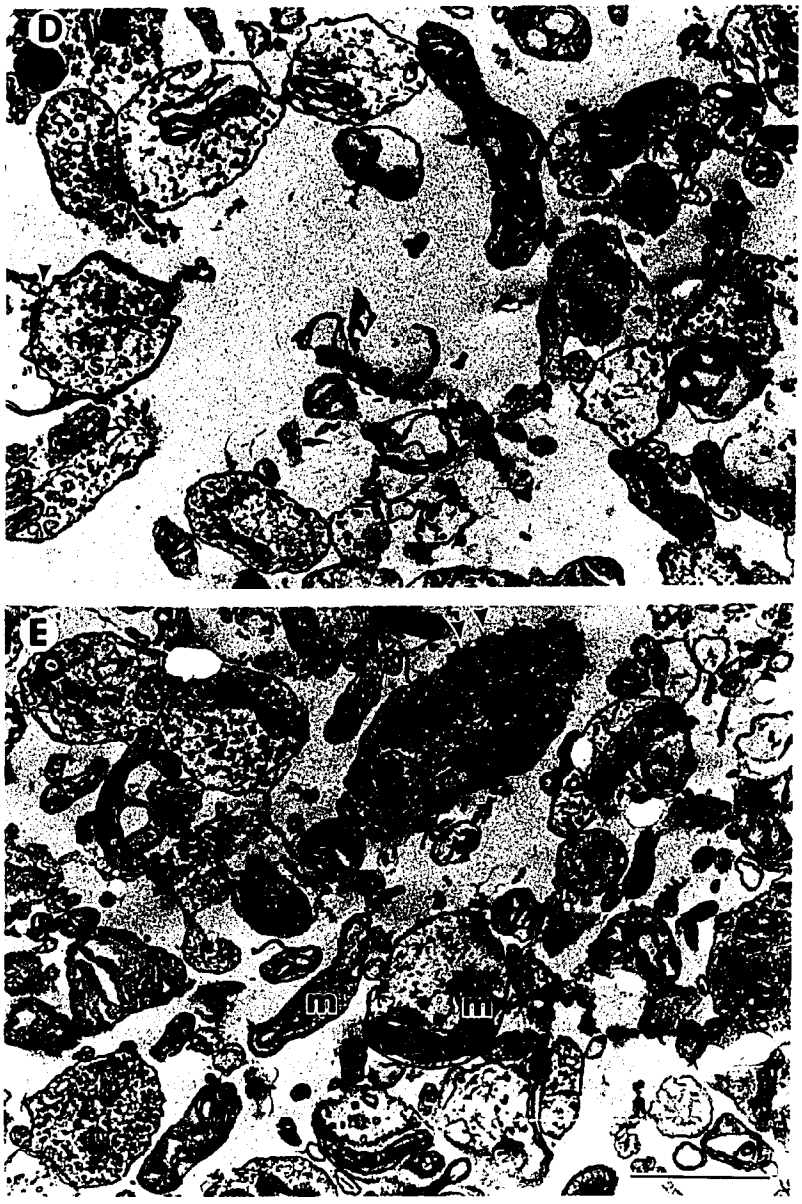
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TABLE 1. *Biochemical characterization of rat olfactory bulb homogenate fractions*

	Carnosine			Glutamic Acid Decarboxylase		Tyrosine Hydroxylase		DNA	
	Chemical analysis		*Radioactive analysis*						
	% Recovery[a]	nmol/mg Protein	% Recovery[b]	% Recovery[c]	nmol/min/ mg Protein	% Recovery[d]	pmol/min/ mg Protein	% Recovery[e]	µg DNA mg Protein
H_0		23.5			12.4		20.0		0.035
H_1		23.5			11.9		19.3		0.033
P_0	6.9	12.8	7.4	20.9	12.7	14.4	20.1	75.9	0.17
P_1	9.5	18.4	9.3	23.2	13.1	14.3	18.5	21.1	0.034
P_2	10.5	11.4	9.9	28.2	14.2	14.9	17.2	<4.4	<0.006
S_2	73.0	51.9	73.4	28.0	8.2	56.4	38.0	<3.0	<0.006

[a] Total carnosine in the tissue was 1.5 nmol/mg tissue.

[b] Represents the newly (16 h) synthesized carnosine at about 30,000 cpm/mg tissue.

[c] Initial activity for H_0 was 0.57 nmol/min/mg tissue.

[d] Initial activity for H_0 was 1.3 pmol/min/mg tissue.

[e] Represents percent of total DNA in tissue (3.15 µg DNA/mg tissue) that was recovered in each fraction.

All values are the averages of three separate preparations.

newly synthesized [^{14}C] carnosine (Table 1). Much more of the dipeptide carnosine is present in the final supernatant than is seen for either of the two enzyme activities studied.

The distribution of ligand binding sites (Table 2) among the olfactory bulb homogenate subfractions shows that the fractions can be differentiated according to their binding site compositions. Spiroperidol and carnosine binding are enriched in P_1, while QNB binding is highest in P_2. Muscimol, DHM and DZ binding distribute similarly among the P_0, P_1 and P_2 fractions. Thus, fractions P_1 and P_2 share many basic synaptosomal characteristics such as neurotransmitter binding sites, carnosine content, GAD and TH activity and some morphological properties, while they differ from one another in the morphological appearance of multi- versus monosynaptosomal particles and in the distribution of certain neurotransmitter binding sites. These may serve as an indicator of the area of origin of P_1 and P_2 particles in the olfactory bulb, which together with the resting membrane potential properties (to be described next), will facilitate further in vitro study of olfactory bulb function.

TPP$^+$ Accumulation in synaptosomes

When the olfactory bulb synaptosomal fractions P_1 and P_2 were incubated in low K^+ medium with 5 μM TPP$^+$, accumulation of this cation was rapid for the first 2 min (80% of max.), and achieved steady state levels by 5 min (see below) which were maintained for 1 hr. At this time the TPP$^+$ concentration gradients ($[TPP^+]_{in}/[TPP^+]_{out}$) were 25 for P_1 and 39 for P_2. Based on the report of Ramos et al. (1979) and on our studies (unpublished data) showing that at 5 μM TPP$^+$ accumulation is a linear function of

TABLE 2. *Distribution of ligand binding sites in olfactory bulb fractions*

Fraction	fmol/mg Protein[a]					
	[^{3}H]SPI* (Dopamine)	[^{3}H]DHM* (Levallorphan)	[^{3}H]QNB* (Atropine)	[^{3}H]DZ* (Diazepam)	[^{3}H]Muscimol (GABA*)	[^{3}H]Carnosine (Carnosine)
H_1	26	59	1153	757	901	216
P_0	106	24	581	469	459	97
P_1	171	40	1035	733	726	257
P_2	68	39	1377	668	706	147

*Abbreviations: Spiroperidol, SPI; dihydromorphine, DHM; quinuclidinyl benzilate, QNB; diazepam (DZ); γ-aminobutyric acid (GABA). The unlabelled competitor is indicated in parentheses below the labelled ligand. Binding was estimated as previously described (Nadi et al., 1980a).

[a] All values are the averages of two separate preparations.

synaptosomal protein concentration in the range from 50 μg to 150 μg protein per assay, a concentration of 5 μM TPP^+ was chosen for these experiments. When the protonophore CCCP was added at 5 min, about 90% of the TPP^+ associated with synaptosomes was lost in less than 1 min. Similar results were obtained when the detergent Triton X-100 was added to the incubation medium at a final concentration of 0.5% or when the synaptosomes were lysed with distilled water after the steady state level of TPP^+ accumulation was achieved. Furthermore, TPP^+ that is accumulated exhibits no alteration in the BuOH:H_2O distribution coefficient or mobility on TLC, indicating that TPP^+ is unaltered during these incubations. These results also indicate that the plateau levels of accumulation represent a reversible steady state accumulation of TPP^+ (rather than conversion of TPP^+ into a stable internal component of the synaptosomes).

Influence of pH on the Accumulation of TPP^+

The internal pH of nerve cells was reported to be 6.7-7.1 (Arieff et al., 1976; Sundt and Anderson, 1980), while the external pH value of body fluids is about 7.4. It is possible that a change in one of these values could cause a change in the polarity of the plasma membrane and subsequently in the accumulation of the cation TPP^+. TPP^+ uptake was independent of external pH in the range 7 to 7.6 (Table 3). Since TPP^+ uptake declined outside this range, it is clear that the optimum external pH for maintaining $\Delta\Psi$ in the synaptosomes is near that of normal extracellular pH.

Influence of Potassium Concentration on Membrane Potential and Mitochondrial Contribution

Intrasynaptosomal mitochondria might be expected to accumulate TPP+

TABLE 3. *pH Effect on TPP^+ Accumulation*

	Percent of TPP^+ Accumulation at pH 7.4*	
pH	P_1	P_2
6.7	80	74
7.0	100	92
7.2	95	94
7.4	100	100
7.6	101	101
7.8	88	87
8.0	88	90

* Synaptosomes incubated at 37°C for 5 min in low K^+ (4.5 mM) medium at various external pH and TPP^+ accumulations were compared to pH 7.4. Values are the averages of three separate preparations.

in a cascade fashion as a function of the cytoplasmic TPP^+ concentration, thus contributing to the total observed synaptosomal content of TPP^+. An indication of the mitochondrial contribution is the observation that $\Delta\Psi$ values decline in the presence of oligomycin/Argon. Thus, the mitochondrial proton electrochemical gradient complicates the determination of $\Delta\Psi$ with TPP^+. The effect of varying external potassium concentrations on the plasma membrane potential of rat olfactory bulb synaptosomal fractions was therefore evaluated in the absence and presence of oligomycin/Argon (Fig. 2).

The membrane potentials observed in the presence of 5 mM external K^+ under oligomycin/Argon are -64 mV for P_1 and -77 mV for P_2 and are in good agreement with values reported in nervous tissue by various methods, while the $\Delta\Psi$ values observed in air are higher; -86 mV for P_1 and -98 mV for P_2, respectively. The decline in $\Delta\Psi$ in response to increasing external K^+ either in the presence or in the absence of oligomycin/Argon (Fig. 2) demonstrates the dependence of the membrane potential on the external potassium concentration, with limiting slopes somewhat smaller than the expected value of 61.5, assuming:

$$\Delta\Psi = -61.5 \log [K^+]_{in}/[K^+]_{out}.$$

The experimental data deviate from the Nernst equation above at concentrations of external K^+ below 10 mM, indicating the presence of an additional small contribution to the synaptosomal $\Delta\Psi$, possibly from Na^+ conductance.

In order to evaluate more carefully the contribution of the mitochondrial potential to the accumulation of $[^3H]TPP^+$ into the synaptosomal fractions, the calculated $\Delta\Psi$ values obtained in the absence of

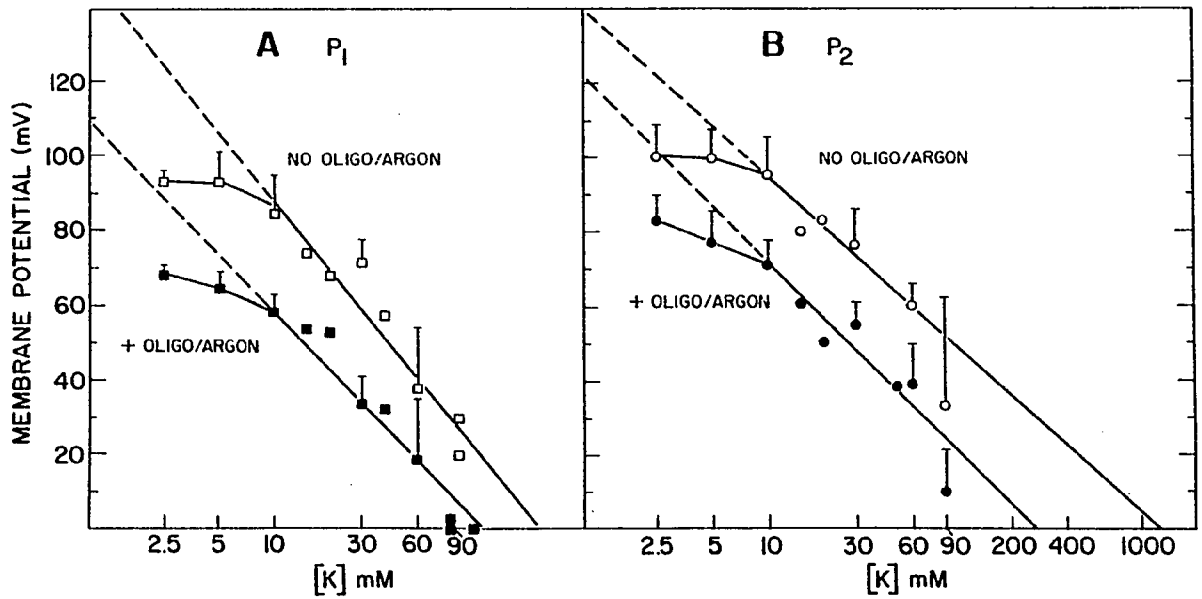
Figure 2. *Effect of potassium concentration of membrane potential.* TPP^+ accumulation was measured in the presence of 0 mM to 150 mM K^+ concentrations. The reaction was initiated by adding [^{3}H] TPP^+ (5 μM) to synaptosomes that were preequilibrated with K^+ buffer in air or in oligomycin/Argon for 5 min at 37°C. The reaction wasstopped after 5 min by rapid vacuum filtration. Data are presented as the averages of three determinations ± S.D. or of single determinations. The limiting slope was calculated using membrane potential values obtained for 10 mM K^+ or higher. Panel A = Fraction P_1 and Panel B = Fraction P_2. P_1 (□) and P_2 (o) = in air (no oligo/Ar) and P_1 (■) and P_2 (●) = with oligo/Ar. Calculation of the logarithmic regression of the Nernst equation:

$\Delta\Psi = a - b \log [K^+]_{out}$, where $\Delta\Psi = b \log [K^+]_{in} - b \log [K^+]_{out}$ resulted in the following regression parameters:

		a	b	R^{2*}
P_1	-oligo/Ar	149	60	0.78
	+oligo/Ar	109	50	0.74
P_2	-oligo/Ar	141	45	0.83
	+oligo/Ar	123	50	0.80

*R^2 is the correlation coefficient.

EFFECT OF POTASSIUM CONCENTRATION ON MEMBRANE POTENTIAL



oligomycin/Argon were compared with the $\Delta\Psi$ values obtained in the presence of oligomycin/Argon by linear regression analysis (Fig. 3) according to the equation:

$$\Delta\Psi(\text{-oligomycin/Argon}) = a + b\Delta\Psi(\text{+oligomycin/Argon}).$$

A linear correlation was obtained between the two sets of $\Delta\Psi$ values (R^2 = 0.91 for P_1 and 0.98 for P_2), with a slope of approximately 1, having the same increment in $\Delta\Psi_{(\text{-oligomycin/Argon})}$ as in the respective $\Delta\Psi(\text{+oligomycin/Argon})$. This indicates that measurement of [^{3}H] TPP^+ accumulation in the absence of oligomycin/Argon, although not quantitatively measuring the synaptosomal potential due to the presence of a mitochondrial contribution (which in this case represents about -25 mV), can nevertheless reflect changes in synaptosomal plasma membrane potential so long as mitochondrial potential changes are not involved. Consistent with this conclusion is the observation of a constant value for the mitochondrial contribution to total apparent synaptosomal $\Delta\Psi$ (Factor a), expressed as the differences between the $\Delta\Psi$ pairs over a wide range of plasma membrane potentials and external potassium concentrations.

Time Course of the Effect of Na^+,K^+-ATPase Blocker Ouabain on TPP^+ Accumulation

The cardiac glycoside drug ouabain is a specific inhibitor of Na^+,K^+-ATPase (Baker, 1965; Schatzmann, 1965; Smith and Harber, 1973; Dahl and Hokin, 1974; Glynn and Karlish, 1975; Wallick et al., 1979), and was reported to have no effect on mitochondrial ATPase or mitochondrial $\Delta\Psi$ (Scott and Nicholls, 1980). Thus, its effect on TPP^+ accumulation was studied in order to evaluate Na^+,K^+-ATPase contribution to the resting synaptosomal $\Delta\Psi$.

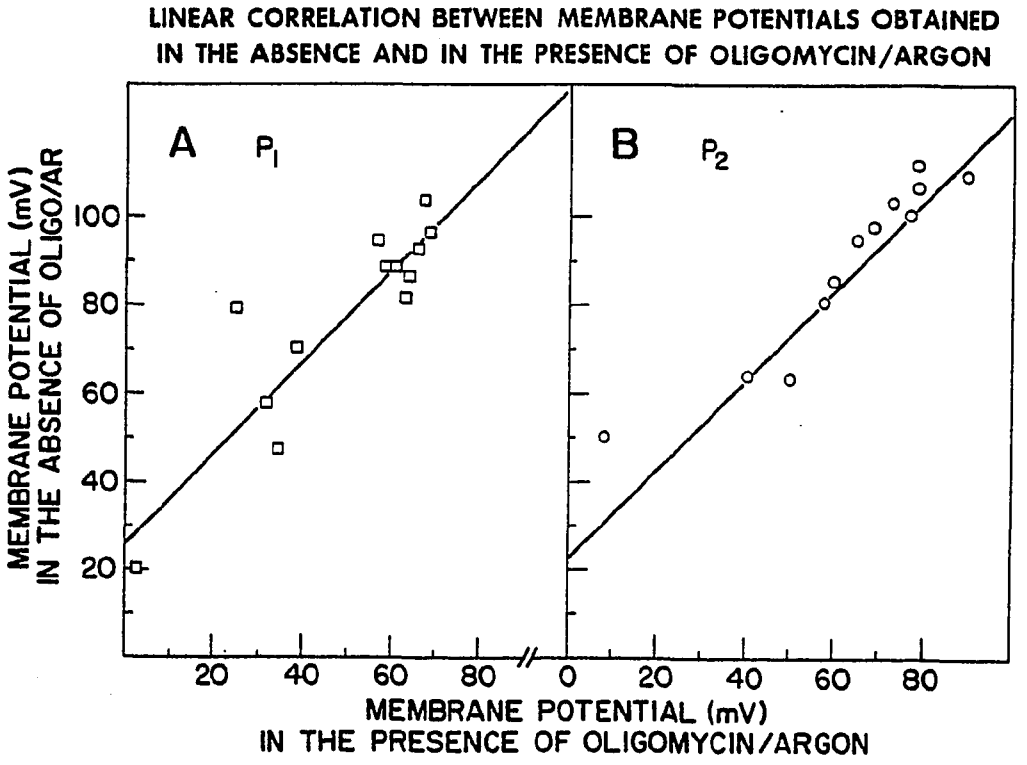


Figure 3. *Linear correlation between membrane potentials obtained in the absence and in the presence of oligomycin/Argon.* The experiment was performed as described in Figure 2. Membrane potentials obtained in air at each potassium concentration were plotted versus membrane potentials obtained under oligomycin/Argon for the same K^+ concentration. Panel A = Fraction P_1 and Panel B = Fraction P_2. The correlation line parameters obtained for the linear equation of $\Delta\Psi_{(-oligo/Arg)} = a + b\ \Delta\Psi_{(+oligo/Arg)}$ are: For P_1, a = 25,61 and b = 1.03 and the correlation coefficient $R^2 = 0.98$; and for P_2, a = 22.6 and b = 1.01 and the correlation coefficient $R^2 = 0.91$.

Two aspects of ouabain on [^{3}H]TPP^+ accumulation in P_1 and P_2 fractions were observed (Fig. 4). The first was a rapid effect, in which ouabain added after TPP^+ accumulation had achieved steady state, caused a very rapid release of 50% of the TPP^+. The second was a slowly developing effect, which became obvious after 20 min and almost completely eliminated TPP^+ accumulation by 1 hr after ouabain addition. When ouabain and TPP^+ are added to the synaptosomal fractions simultaneously, the extent of initial TPP^+ accumulation is reduced but not blocked.

The Effect of Increasing Concentrations of Veratridine on TPP^+ Accumulation

The neurotoxic alkaloid veratridine causes depolarization of nerve cells or synaptosomes (Blaustein and Goldring, 1975) by keeping the action potential sodium channel constantly open, permitting electrogenic Na^+ influx (Ulbricht, 1969; Catteral and Nirenberg, 1973; Li and White, 1977). Veratridine caused a dose-dependent reduction of TPP^+ accumulation into both P_1 and P_2 fractions suspended in low K^+ medium (Fig. 5). This resulted in a progressive fall in the [^{3}H]TPP^+ concentration gradient ($[TPP^+]_{in}/[TPP^+]_{out}$) to about 50% of control at a concentration of 50 μM veratridine and 25% of control at 100 μM. This decrease in TPP^+ accumulation in the presence of veratridine is consistent with veratridine acting as a depolarizing agent in our system. Based on these data, veratridine concentrations of 50 μM or 100 μM have been chosen for use in subsequent experiments, where competition or activity with other drugs was monitored.

Influence of $\Delta\Psi$-Modifying Drugs on TPP^+ Accumulation and $\Delta\Psi$

In order to further characterize the mechanisms involved in the determination of synaptosomal $\Delta\Psi$, we studied the effects of $\Delta\Psi$-modifying

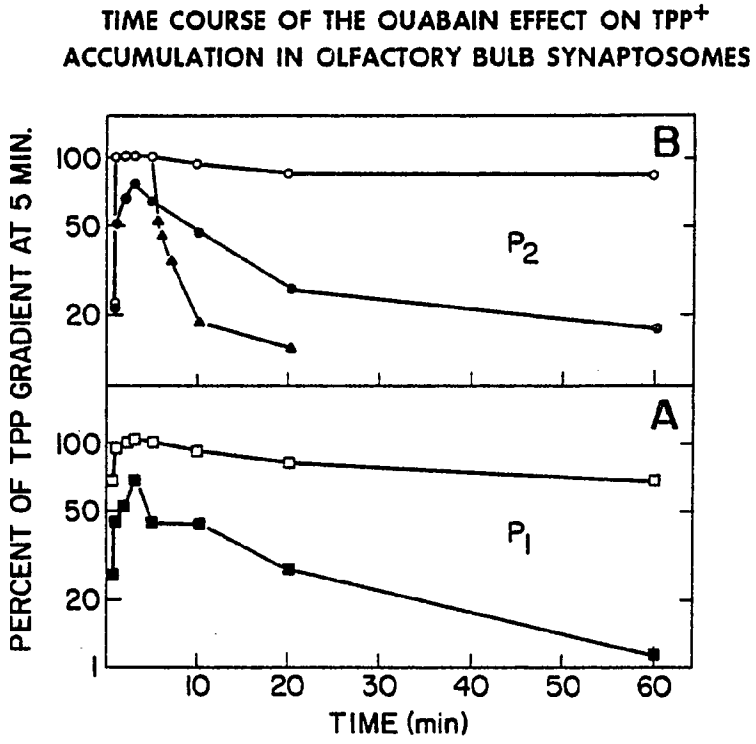


Figure 4. *Time course of the ouabain effect on TPP^+ accumulation.* Ouabain (10 mM) was added to the synaptosomes at time 0 (P_1-■, P_2-●) or at 5 min (P_2-▲) after the beginning of incubation with TPP^+. Incubation was stopped at different time points between 30 sec to to 60 min. Control (P_1-□, P_2-○). Panel A = Fraction P_1 and Panel B = Fraction P_2.

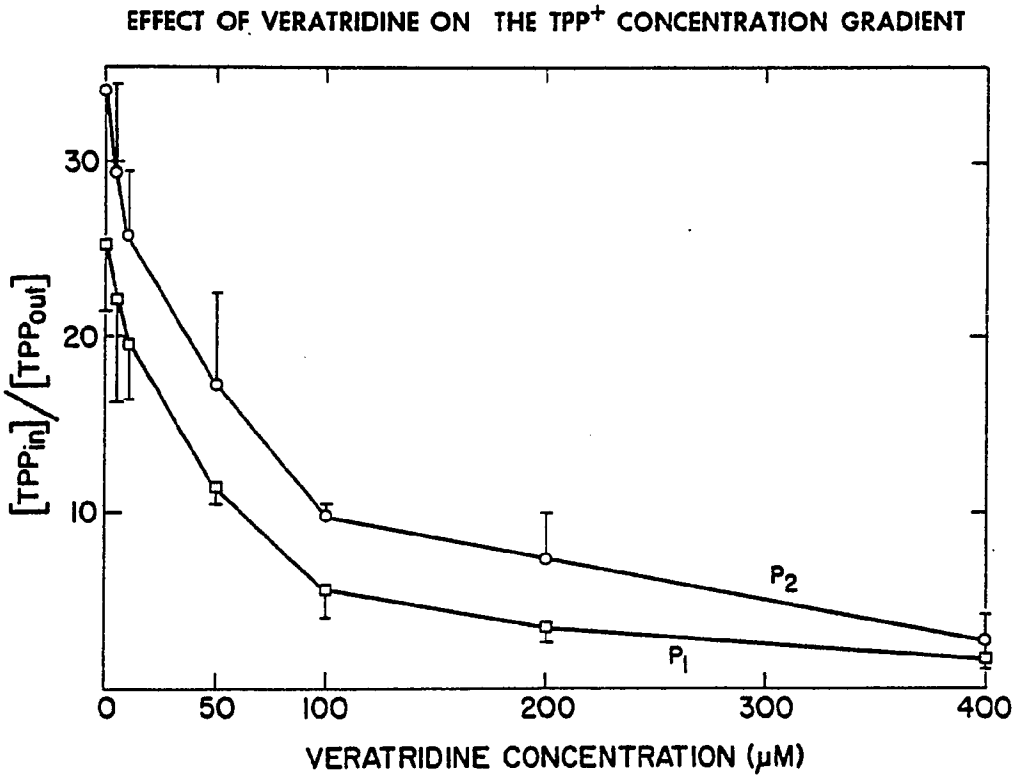


Figure 5. *Effect of veratridine on the TPP^+ concentration gradient.* Synaptosomes were incubated for 5 min in the absence or in the presence of increasing concentrations of veratridine. Fraction P_1 (□) and Fraction P_2 (○). The values represent mean ±S.D. for three preparations.

drugs on TPP^+ accumulation in high and low potassium media in the presence or absence of oligomycin/Argon. The specificity of this approach is derived from our previous experiments (Fig. 2). Incubation of synaptosomes in the presence of oligomycin/Argon reduces the TPP^+ accumulation in both high and low K^+ media (Fig. 6). This indeed indicates that there is TPP^+ accumulation in mitochondria, which is dependent upon the state of the mitochondria. These mitochondria continue to accumulate TPP^+, even in the presence of high K^+ in the medium. As indicated before, the cascade accumulation of TPP^+ into mitochondria leads to a higher apparent $\Delta\Psi$ in air than in oligomycin/Argon.

Vertridine is without effect on TPP^+ accumulation in the presence of high K^+ and oligomycin/Argon (i.e. under conditions where neither synaptosomal nor mitochondrial $\Delta\Psi$ exist). However, in the presence of low K^+ medium, veratridine causes the same proportional loss in TPP^+ in the presence of oligomycin/Argon as it causes in air (Fig. 6). No effect of veratridine is observed when Na^+ is replaced with $choline^+$. Tetrodotoxin (TTX) is known to be a specific blocker of action potential sodium channels (Evans, 1972; Narahashi et al., 1964), and by itself has no effect on TPP^+ accumulation in either high or low K^+ medium. However, TTX inhibits the effects of veratridine that are seen at low K^+ both in the presence and in the absence of oligomycin/Argon (Fig. 6). Thus, TTX has no effect on resting $\Delta\Psi$, but it can prevent the depolarizing effect of veratridine in synaptosomal fractions (Table 4).

Ouabain had no effect on TPP^+ accumulation at high K^+, but it reduced TPP^+ accumulation in low K^+ in the presence or in the absence of oligomycin/Argon (Figs. 4 and 6), indicating that ouabain reduces only the

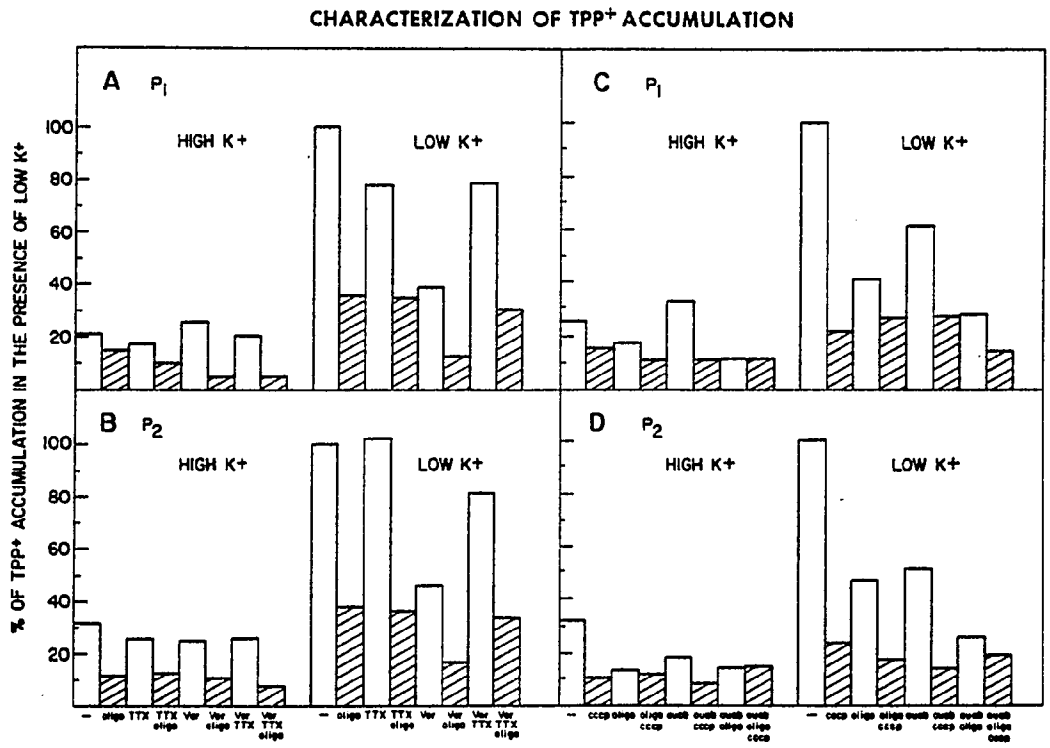


Figure 6. *Characterization of TPP^+ accumulation.* Synaptosomes were allowed to equilibrate with buffer, drugs and air or Argon for 5 min before TPP^+ addition. TPP^+ accumulation into Fractions P_1 or P_2 was measured in high K^+ or low K^+ medium. Relative accumulation under each condition was calculated using accumulation of TPP^+ under low K^+ and air with no drugs as 100% value. In Panel A (Fraction P_1) and Panel B (Fraction P_2), effects of tetrodotoxin (15 µM), veratridine (50 µM) and oligomycin (10 µg/ml) were compared. Empty bars = in air and no oligomycin. Filled bars = under oligomycin/ Argon. In Panel C (Fraction P_1) and Panel D (Fraction P_2), effects of oligomycin (10 µg/ml), ouabain (10 mM) and CCCP (10 µM) were compared. Empty bars = no CCCP. Filled bars = in the presence of CCCP.

TABLE 4. *Synaptosomal Membrane Potential Changes as a Result of Drugs*

A. *Effects of veratridine (Ver) and tetrodotoxin (TTX)*

Tissue Fraction	Oligomycin/Arg (10 µg/ml)	Control	Ver	TTX	Ver and TTX
		Membrane Potential (mV)*			
P_1	+	61.2	29.1	66.8	59.1
	-	95.9	43.2	91.5	84.3
P_2	+	69.0	31.9	67.1	69.7
	-	95.5	64.1	95.7	90.3

B. *Effects of ouabain (Oua) and carbonyl-cyanide-m-chlorophenyl hydrazone (CCCP)*

Tissue Fraction	Oligomycin/Arg (10 µg/ml)	Control	Oua	CCCP	CCCP and Oua
		Membrane Potential (mV)*			
P_1	+	56.5	47.2	45.1	18.8
	-	95.1	71.5	17.3	29.9
P_2	+	76.3	56.2	36.2	31.6
	-	89.6	79.2	46.1	33.7

* Membrane potentials were calculated from the difference of TPP^+ uptake in low and high potassium under each drug and are the average from two separate preparations (each in duplicate).

Experimental details described in legend to Figure 6.

synaptosomal $\Delta\Psi$, with no direct effect on the mitochondrial $\Delta\Psi$. The effect of ouabain at saturating levels and veratridine at unsaturating levels were additive. The protonophore CCCP, which is expected to collapse the synaptosomal plasma membrane $\Delta\Psi$ and the mitochondrial $\Delta\Psi$, reduced the accumulation of TPP^+ to very low levels in both high and low K^+ media. No further reduction of TPP^+ accumulation could be detected in the presence of CCCP when oligomycin/Argon or ouabain were added alone or in combination (Fig. 6). The cause of the residual TPP^+ accumulation in the presence of CCCP (Table 4) is unknown.

Influence of TPP^+ Concentrations on TPP^+ Accumulation

Synaptosomal TPP^+ accumulation was observed to behave as an inverse function of external TPP^+ concentration in the range of 0.5 to 100 µM, so that increasing external TPP^+ resulted in reduction of the apparent $\Delta\Psi$ (Fig. 7). A possible explanation for this phenomenon may be derived from the following consideration: If one assumes a plasma membrane potential of -66 mV (based on our oligomycin/Argon results) and a mitochondria $\Delta\Psi$ of -120 mV (according to Scott and Nicholls, 1980), then at 5 µM external TPP^+ the synaptic cytoplasm would contain about 60 µM TPP^+, while the concentration of TPP^+ in the intrasynaptosomal mitochondria would be about 6 mM. Furthermore, as the external TPP^+ concentration rises, the TPP^+ concentration in the intrasynaptosomal mitochondria would also be expected to rise and finally attain levels such that TPP^+ no longer behaves as an inert component in the system. This would lead to collapse of the electrical gradient existing across the mitochondrial membrane.

Thus, as the medium concentrations of TPP^+ increase, the fraction of intrasynaptosomal TPP^+, which is extramitochondrial, increases such that at external TPP^+ concentrations in excess of 50 µM virtually all the TPP^+

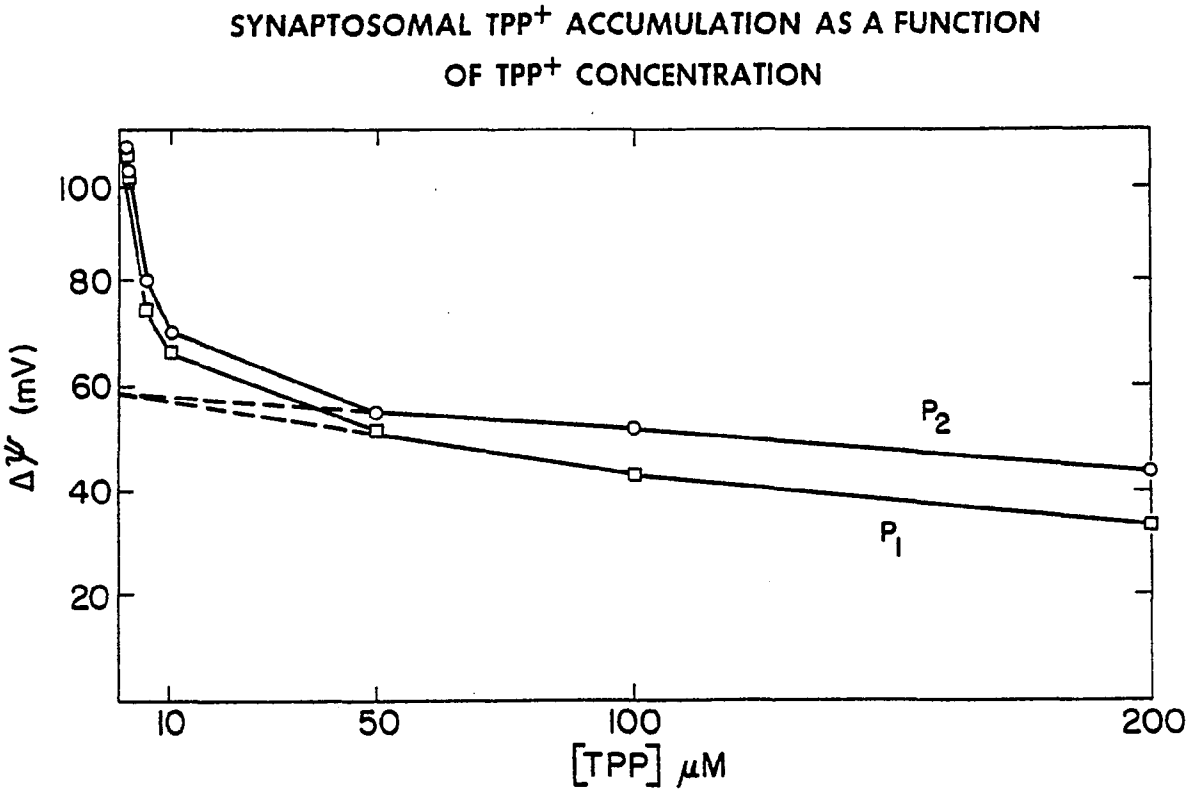


Figure 7. *Synaptosomal TTP^+ accumulation as a function of TPP^+ concentration.* TPP^+ accumulation proceeded for 5 min at 37°C in the presence of external TPP^+ concentrations ranging from 0.5 μM to 200 μM. ΔΨ was calculated for Fraction P_1 (□) and Fraction P_2 (o). Dotted lines is extrapolation of the plateau slope to zero TPP^+ concentration. Data are means of three preparations.

TABLE 5. *TPP^+ Toxicity to Mitochondria (P_2 Fraction)*

Time after Addition of TPP^+	TPP^+ (μM)	$-\Delta\Psi$ Total		$-\Delta\Psi$ [a] Mitochondria	$[TPP^+]_{in}$ Mitochondria (mM)	
		+Oligo/Arg	-Oligo/Arg		Calculated[a]	Expected[b]
5 min	1	60.3	110.0	106.7	0.52	1.2
	5	60.2	92.6	84.4	1.13	6.0
	50	46.9	63.4	57.2	2.46	60.0
10 min	1	56.6	114.3	108.5	0.66	1.2
	5	54.9	96.1	96.3	1.44	6.0
	50	54.5	57.7	6.4	0.49	60.0

[a] ΔΨ Mitochondira was calculated from the difference between TPP^+ accumulation in the absence and in the presence of oligomycin/argon, assuming the mitochondrial volume to be 10% of synaptosomal volume. This gave $[TPP^+]_{in}$ mitochondria. The synaptosomal $[TPP^+]_{in}$ in the presence of oligomycin was taken as the mitochondrial $[TPP^+]_{out}$. ΔΨ was calculated as:

$$-61.5 \log \frac{[TPP^+]_{in} \text{ mitochondria}}{[TPP^+]_{out} \text{ mitochondria.}}$$

[b] The expected concentration of TPP^+ inside the mitochondria was calculated from the TPP^+ concentration inside the synaptosomes, assuming a mitochondrial potential of 120 mV or TPP^+ gradient of 100.

accumulation would be extramitochondrial. To test this hypothesis, the accumulation of TPP^+ into the synaptosomal fractions was monitored as a function of medium TPP^+ concentration in the presence and in the absence of oligomycin/Argon. However, in the presence of oligomycin/Argon TPP^+ accumulation was independent of the external TPP^+ concentration (Table 5). The mitochondrial $\Delta\Psi$ has been calculated based on (i) the difference in TPP^+ accumulation in the presence versus the absence of oligomycin/Argon (Table 5) and (ii) the assumption that the intrasynaptosomal mitochondria represent about 10% of the total internal volume (according to Scott and Nicholls, 1980). At 1 μM TPP^+ the observed mitochondrial $\Delta\Psi$ of -110 mV is in excellent agreement with values reported in the literature (Mitchell and Moyle, 1969). The mitochondrial content of TPP^+ does not increase proportionally as the external TPP^+ concentration rises, indicating that the mitochondrial $\Delta\Psi$ declines progressively with increasing TPP^+ concentrations (Table 5), presumably due to dissipation of the mitochondria $\Delta\Psi$ by the high internal TPP^+ concentration[3].

DISCUSSION

The data in this paper demonstrate that the plasma membrane potential of olfactory bulb synaptosomal preparations can be quantitated from measurements of the steady state accumulation of the lipophilic cation tetraphenylphosphonium. However, due to a significant and variable contribution of mitochondrial TPP^+ accumulation to total TPP^+ accumulation in the synaptosomal preparation an accurate calculation of plasma membrane $\Delta\Psi$ can be performed only under certain conditions. A contribution of the mitochondrial potential to total TPP^+ accumulation was expected (Mitchell and Moyle, 1969; Nicholls, 1974; Rottenberg, 1979) as mitochondria exhibit a negative internal potential. The mitochondrial contribution can be derived by comparing TPP^+ accumulation under air versus its accumulation in the presence of oligomycin/Argon. If one inserts both the mitochondrial and the plasma membrane potential contribution to TPP^+ accumulation into the Nernst equation (as was described by Scott and Nicholls, 1979) [s = synaptosomal, e = external, $\Delta\Psi_p$ = $\Delta\Psi$ of plasma membrane, $\Delta\Psi_m$ = $\Delta\Psi$ of mitochondria, V_c = cytoplasmic volume and V_m = mitochondrial volume], one obtains:

$$\frac{[TPP^+s]}{[TPP^+e]} = \frac{V_c \cdot 10^{\Delta\Psi_p/61.5} + V_m \cdot 10^{(\Delta\Psi_m + \Delta\Psi_p)/61.5}}{V_c + V_m}$$

Resolving the equation for the measurable parameters of $\Delta\Psi_{(-\text{oligomycin/Argon})}$ and $\Delta\Psi_{(+\text{oligomycin/Argon})}$, this reduces to:

$$\frac{[TPP^+s]}{[TPP^+e]} = \frac{V_c + V_m \cdot 10^{\Delta\Psi_m/61.5}}{V_c + V_m} \cdot 10^{\Delta\Psi_p/61.5}$$

$$\text{or:} \qquad 61.5 \log \frac{[TPP^+_s]}{[TPP^+_e]} = 61.5 \log \frac{V_c + V_m \cdot 10^{\Delta\Psi_m/61.5}}{V_c + V_m} + \Delta\Psi_p$$

which simplified to:

$$\Delta\Psi_{(-\text{oligomycin/Argon})} = a + \Delta\Psi_{(+\text{oligomycin/Argon})},$$

where parameter a replaces the mitochondrial contribution factor. Parameter a is constant when the mitochondrial potential and the volume are constant. Under this condition, this equation predicts a linear relationship between $\Delta\Psi_{(-\text{oligomycin/Argon})}$ and $\Delta\Psi_{(+\text{oligomycin/Argon})}$. This relationship was actually obtained with a slope of 1 as predicted by the equation, and gave values of -25 mV for P_1 and -22 mV for P_2 (Fig. 3). The mitochondrial accumulation of TPP^+ decreased as a function of increasing concentration of TPP^+ in the range from 0.1 μM to 200 μM TPP^+, while no such change was observed in plasma membrane $\Delta\Psi$ dependent accumulation of TPP^+. This demonstrates that TPP^+ itself interferes with the mitochondrial membrane potential under aerobic conditions.

Therefore, changes in the plasma membrane potential of olfactory bulb synaptosomes can be evaluated by TPP^+ accumulation quantitatively in the presence of oligomycin/Argon, where the mitochondrial contribution is eliminated, or qualitatively in the absence of oligomycin/Argon, if the drug in question does not affect the mitochondrial potential. Our data suggest that a second approach to overcome the mitochondrial contribution to TPP^+ accumulation is to use high enough TPP^+ concentrations to block mitochondrial function and subsequent back extrapolation to estimate plasma membrane $\Delta\Psi$ (Fig. 6). This is technically much simpler than the oligomycin/Argon approach, and in some situations may be equivalent.

Utilizing this approach, our results for rat olfactory bulb synaptosomes agree with the qualitative evaluation of the lipophilic cation gradients in guinea pig synaptosomes presented by Ramos et al. (1979) for TPP^+ and by Creveling et al. (1980) for $TPMP^+$. In addition, by characterizing and eliminating the mitochondrial contribution, we have achieved an accurate quantitative biochemical measurement of synaptosomal membrane $\Delta\Psi$, which previously could be estimated only qualitatively with other biochemical techniques.

The properties of the olfactory bulb synaptosomal plasma membrane potential were found to be similar to those of nerve cells according to the following criteria: (A) There is a negative electrical potential ($\Delta\Psi$) of -64 mV in P_1 and -77 mV in P_2 synaptosomes. (B) These $\Delta\Psi$ are primarily due to a K^+ diffusion gradient across the synaptosomal membrane, as indicated by increased depolarization as a result of increasing external concentrations of K^+, and no large change when Na^+ is replaced by $choline^+$. A correlation of $\Delta\Psi$ with the logarithm of external K^+ concentration resulted in a limiting slope of 50 instead of 61.5 as predicted by the Nernst equation. This difference is similar to that noted previously by Hodgkin and Keynes (1955) for the squid giant axon. The deviation from the linear relationship that was observed for this synaptosomal preparation at external concentrations of K^+ below 10 mM was similar to that observed by electrophysiological measurements in other neural cells (Hodgkin and Keynes, 1955; Gorman and Marmor, 1970; Eyzaguirre and Fidone, 1975) and has been attributed to a small sodium permeability. (C) A minor role for the contribution of active sodium transport to $\Delta\Psi$ was expressed in our studies by the depolarization which occurred in the presence of ouabain, as well as by the slight hyperpolarization observed when choline replaced Na^+ in the

incubation medium. (D) A role for Na^+,K^+-ATPase in maintaining the synaptic plasma membrane potential based on the inhibition of the Na^+,K^+-ATPase by ouabain. Ouabain causes a rapid depolarization with a $t_{1/2}$ of 30 sec without any detectable loss of internal K^+, indicating an electrogenic role for the Na^+,K^+-ATPase in synaptosomes which contributes about -10 to -20 mV to the membrane potential. This finding agrees with Gorman and Marmor (1970) and Thomas (1972), whose studies also suggested an electrogenic role for the Na^+, K^+-ATPase. The slow depolarizing effect of ouabain is most likely due to the inability of ouabain-inhibited synaptosomes to reaccumulate K^+that has leaked out with the resultant lower $[K^+]_{in}/[K^+]_{out}$ ratio. The 80% loss of internal K^+ as observed by atomic absorption spectrometry 1 hr after ouabain addition to synaptosomes (unpublished observations) supports this hypothesis. (E) The existence of a veratridine-sensitive sodium channel through which veratridine caused a decrease in TPP^+ accumulation into synaptosomes. This effect of veratridine was blocked by tetrodotoxin and was not observed when sodium was replaced by choline in the extrasynaptosomal medium. These observations are consistent with the existence of an action potential sodium channel of the the type known in whole nerve cells (Narahashi et al., 1964; Ulbricht, 1969; Evans, 1972; Caterall and Nirenberg, 1973).

In summary, measurements of the distribution of the lipophilic cation tetraphenylphosphonium have been used to characterize the plasma membrane potential of synaptosomal fractions from rat olfactory bulbs. When the mitochondrial contribution to TPP^+ accumulation was eliminated, synaptosomal plasma membrane $\Delta\Psi$ properties were found to be similar to neuronal $\Delta\Psi$ obtained by electrophysiological measurements. As the TPP^+ measurements

must be carried out at steady state in order to be used to quantitate $\Delta\Psi$, this probe is not suitable for the study of the effects of neurotransmitters which result in rapid (msec) and reversible changes in the membrane potential. The influence of various drugs with long-term effects on the plasma membrane potential and the role of certain ions in maintaining the plasma membrane potential can be studied conveniently with this lipophilic cation, as exemplified by our studies. Thus, TPP^+ accumulation into synaptosomes can be used to monitor long-term changes in neuronal membrane $\Delta\Psi$ generated by slowly-acting hormones or neuromodulators with time courses of seconds to hours. It would be of interest to evaluate the use of TPP^+ in the millisecond range (using a stop flow system under non-steady state conditions) as a $\Delta\Psi$ probe for the detection of short-term events. Lastly, as the olfactory bulb was found to contain several types of peptide neurotransmitters and hormones, the application of TPP^+ as a $\Delta\Psi$ probe should be very useful in studying the role of these peptides in the olfactory pathway.

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CHAPTER 4

CALCIUM DEPENDENT DEPOLARIZATION STIMULATED CARNOSINE RELEASE SUPPORTS CARNOSINE ROLE AS A NEUROEFFECTOR

ABSTRACT

The dipeptide carnosine (β-alanyl-L-histidine) has been proposed as a neurotransmitter in the mammalian olfactory pathway. Therefore, the efflux of in vivo synthesized [^{14}C] carnosine from mouse olfactory bulb synaptosomes was investigated. The synaptosomal preparation used in these studies maintained a potassium dependent transmembrane potential of -70mV. Carnosine was found to be released from the olfactory bulb synaptosomes by two mechanisms. The first is a slow spontaneous process which is independent of depolarization. The rate of this release was doubled in the presence of 1 mM external carnosine. Release by the second mechanism was markedly stimulated in the presence of calcium by depolarization with either 60 mM K^+ or 300 μM veratridine. Omission of calcium abolished the stimulatory effect of both of these agents. Furthermore, blockage of the veratridine induced depolarization by TTX also inhibited carnosine release. These results are consistent with the hypothesis that carnosine acts as a neurotransmitter in the mouse olfactory pathway.

INTRODUCTION

Over the last decade, substantial biochemical evidence has been accumulated indicating that the dipeptide carnosine (β-alanyl-L-histidine) may play a crucial role as a neurotransmitter in the rodent olfactory pathway (for review see Margolis 1980). Many of the biochemical criteria essential for the determination of its role as a neurotransmitter in this system have been met. Carnosine has been found to be compartmentalized solely in the olfactory receptor neurons (Margolis and Grillo, 1978; Margolis, et al., 1974; Neidle and Kandera, 1974; Ferriero and Margolis, 1975; Harding, et al., 1977, 1978) where it is synthesized by a specific synthetase (Horinishi, et al., 1978; Harding and Margolis, 1976; Harding, et al., 1977; Ng and Marshall, 1978). Carnosine is transported by axoplasmic flow (Margolis and Grillo, 1977) into layers of the olfactory bulb containing the olfactory nerve axons and terminals (Nadi, et al., 1980; Burd, et al., 1980). In addition, the olfactory pathway contains a very active and specific degradative enzyme, carnosinase (Margolis, et al., 1979; Harding and Margolis, 1976) which is extraneuronally located (Harding, et al., 1977; Farbman, Grillo and Margolis, unpublished observations). Carnosinase may, in turn, act as an inactivating system in a fashion analogous to the enzymes responsible for the degradation of other neurotransmitters, e.g., acetylcholine and the enkephalins (Sparf, 1973; MacIntosh and Collier, 1976; Hambrook, et al., 1976; Jacquet, et al., 1976). Finally, membrane fractions of olfactory bulbs exhibit reversible, saturable, and

stereospecific binding of carnosine (Hirsch and Margolis, 1979; Hirsch, et al., 1978) strongly suggesting that specific receptors for carnosine exist in this system.

One remaining criterion necessary for establishing carnosine as a neurotransmitter is the demonstration that it plays a physiological role in olfaction. Preliminary studies have attempted to investigate this question by evaluating the electrophysiological responses of olfactory bulb neurons to carnosine (Gonzales-Estrada and Freeman, 1980; Tonosaki and Shibuya, 1979; Nicoll, et al., 1980; MacLeod and Straughan, 1979). These studies have not, however, reached a common conclusion.

In this paper we have approached the question of a physiological role for carnosine as a neuroeffector in the mouse olfactory pathway by establishing that the release of in vivo labeled carnosine from synaptosomal preparations is stimulated by depolarization, a significant fraction of which is calcium dependent, providing further support for its classification as a neurotransmitter.

MATERIALS AND METHODS

Radioactive Labeling of Synaptosomal Fractions

[^{14}C]-carnosine was synthesized in vivo from [^{14}C]-β-alanine that was administered by intranasal irrigation (20μCi/20 μl/mouse) as previously described (Margolis and Grillo, 1977). The [^{14}C]-carnosine, thus formed, was transported to the nerve terminals of the olfactory receptor neurons in the olfactory bulb (Margolis and Grillo, 1977; Burd, et al., 1980).

[^{3}H]-GABA loading was carried out in vitro by incubating 200 μl (2 mg Protein) aliquots of resuspended synaptosomal fractions (see below) with 5 μM [^{3}H]-GABA (1.78 μCi) in 1 ml final volume of HEPES buffered 5 mM K^+ superfusion medium (see below) containing amino-oxyacetic acid (2 μg/ml) at 37°C for 5 min. Incubation was terminated by the addition of 2.5 ml cold buffer followed by rapid filtration and an additional wash with 2.5 ml cold buffer. GABA uptake was found to proceed in a temperature dependent manner (the residual uptake at 0°C was 4%), and was inhibited by 100 μM GABA (10% residual uptake), 100 μM diaminobutyric acid (50% residual uptake) and only partially inhibited by 100 μM β-alanine (75% residual uptake). [^{3}H]-GABA loaded synaptosomes to be used in release studies, were incubated with [^{3}H]-GABA for 15 min. Incubation was terminated by dilution in 5 ml cold HEPES buffer containing100 μM GABA followed by centrifugation at 14,500g for 10 min. The supernatant was discarded and the pellet was resuspended in 800 μl of 5 mM K^+ superfusion medium, The re-suspended pellet served as synaptosomal source for GABA release

studies. The input per release sample contained about 36,000 cpm/100 µg Protein/50 µl. [^{3}H]-Leucine uptake was performed as above for [^{3}H]-GABA at 10 µM leucine using 10 µCi/ml/2 mg synaptosomal protein. Leucine uptake was found to be temperature dependent (30% residual uptake at 0°C). The synaptosomal input per release study was about 27,000 cpm [^{3}H]-leucine/100 µg protein/50 µl suspension.

Time Course of [^{14}C]-Carnosine Labeling

CD-1 female mice were irrigated with [^{14}C]β-alanine (1 µCi/100 µl/mouse) and killed by CO_2 asphyxiation followed by exsanguination at the indicated times. The olfactory bulbs were dissolved in 1 ml of formic acid and after the addition of 2 ml of water and 10 ml of hydrofluor, radioactivity was measured in a liquid scintillation spectrometer. The radioactivity in the olfactory bulbs was evaluated for carnosine content by chromatographic separation on Dowex 50 columns according to Margolis and Grillo (1977) and by high pressure liquid chromatography (HPLC) as the fluorescent 2-methoxy-2,4-diphenyl-3(2H)-furanone(MDPF)derivative according to Wideman, et al., (1978). At least 95% of the incorporated counts were associated with carnosine.

Preparation of Synaptosomes

Synaptosomes were prepared from mouse olfactory bulbs (according to Hajos, et al., 1974 as adapted by Rochel, Chapter 3, This work) by gentle homogenization in a Dounce homogenizer (Kontes Co.) in 0.32 M sucrose, 5 mM HEPES, (pH 7.4), 1 mM $MgSO_4$. The homogenate was

filtered sequentially through 1000, 210, and 70 μM nylon bolting cloth. The pellet removed by centrifugation for 1 min at 1000 x g_{max} was discarded and the supernatant centrifuged at 14,500 x g_{max} for 20 min and the pellet used as the source of the synaptosomes in these studies. About 13% of the total radioactivity present in the bulb was recovered in this fraction. The pellet was routinely resuspended in the homogenization buffer at a concentration equvalent to 1 mg of original tissue per μl and used within 1.5 hours.

Release of [^{14}C]-Carnosine from Synaptosomes

[^{14}C]-carnosine release was studied in a superfusion system modified from Muler, et al., (1975). Superfusion medium contained 50 mM HEPES buffered to pH 7.4 with NaOH, 5.5 mM D-glucose, 0.8 mM $MgSO_4$ and 1 mM sodium phospate.

In the 5 mM K^+ medium, the NaCl content was 135 mM; in other experiments, the content of NaCl was adjusted in order to maintain constant osmolality. When present, calcium was 2 mM $CaCl_2$. The resuspended synaptosomal pellet fractions (50 μl containing 28-40 x 10^3 cpm and 0.8 to 1 mg protein) were layered on top of a 0.8 x 1.0 cm G-15 Sephadex column formed in a 3 ml disposable syringe barrel after preequilibration with superfusion buffer at 37°C. These columns were superfused at 37°C with continuously oxygenated medium at 0.75 ml/min using a peristaltic pump (Buchler Instruments). Superfusate fractions were collected at 3 min intervals and the radioactivity was monitored in the presence of 10 ml Hydrofluor (National Diagnostics Corp.) in a liquid scintillation spectrometer. The

superfusion protocol followed was: 1) superfusion with 5 mM K^+ medium for 15 min to allow spontaneous release of carnosine to achieve a low, stable rate, 2) a 6 min superfusion with test compounds followed by superfusion with 5 mM K^+ medium for 6 additional min, 3) a wash of 0.1 M HCl for 3 min in order to release the remaining carnosine from the synaptosomes. When the synaptosomes were superfused with distilled water, 92% of the radioactivity eluted in the first wash and a total of 99.5% eluted in the first and second washes. This indicates that the [^{14}C]-carnosine is not irreversibly bound to some internal structure or to the G-15 column, and that it can be released when the synaptosomes are lysed with distilled H_2O. A further indication that carnosine does not bind to the G-15 column results from the observation that in the absence of synaptosomes, more than 99% of the radioactive carnosine is eluted from the G-15 column in the first fraction. The release of [^{14}C]-carnosine is expressed as the ratio of the radioactivity released in a specific fraction to the total radioactivity present in the synaptosomes (as a percentage) at the beginning of that 3 min release period.

Materials

[1-^{14}C]-β-alanine (55.2 mCi/mmol), [2,3-^{3}H(N)]-GABA (35.6 Ci/mmol) and [4,5-^{3}H(N)]-leucine (51.6 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.) Veratridine, EGTA, choline chloride, HEPES, capsaicin, and bestatin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Tetrodotoxin was from Calbiochem-Behring (San Diego, Cal.) G-15 sephadex was obtained from Pharmacia Fine

Chemicals (Piscataway, N. J.). All other reagents were of analytical grade. Female exbreeder CD-1 mice were obtained from Charles River Farms and were maintained on water and food pellets ad libitum, with a 12-hour night/day cycle.

RESULTS

Time Course of [^{14}C]-Carnosine Appearance in the Olfactory Bulb

Mice were intranasally irrigated with [^{14}C]β-alanine and sacrificed at the indicated times following treatment, and the radioactivity in the olfactory bulb monitored (Fig. 1). The accumulation of [^{14}C]-carnosine in the olfactory bulb reached a peak at about 9 hours and decreased slowly thereafter consistent with the reported half life of ~20 hours (Margolis and Grillo, 1977). Animals used for carnosine release studies were always sacrificed within the 12-16 hour period following irrigation with [^{14}C]β-alanine. Approximately 0.5% of the radioisotope administered appeared as carnosine in the bulb, 15 hr after irrigation.

Stimulation of Carnosine Release by 60 mM K^+

Olfactory bulb synaptosomes were superfused for 15 min with non-depolarizing medium containing 5 mM K^+ in the absence of calcium. The stimulation of carnosine release was initiated by replacing this medium with depolarizing media containing 60 mM K^+ in the presence or absence of 2 mM Ca^{2+} (Fig. 2). The spontaneous release of carnosine from the olfactory bulb synaptosomes (during superfusion with 5 mM K^+ medium) reached a low, steady state 9 min after the onset of the superfusion and remained stable for at least 40 min. The rate of spontaneous carnosine release was about 1%/3 min of the total radioactivity in the synaptosomal fraction and represented about 10 times background.

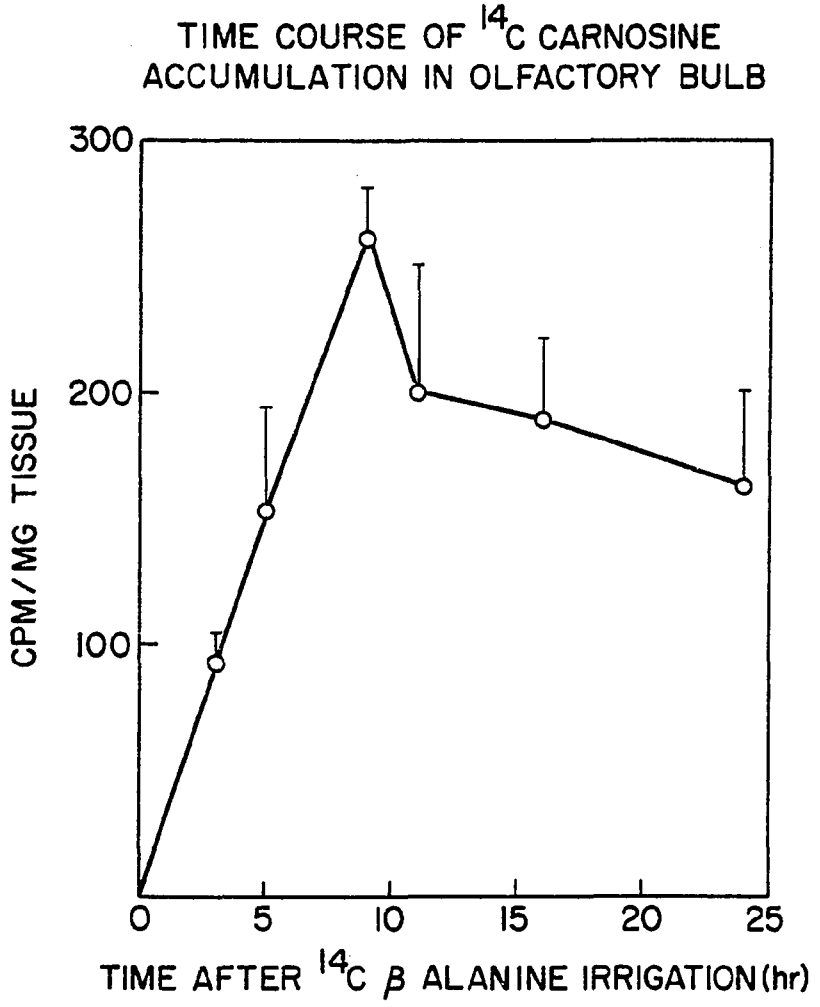

Figure 1. *Time course of [^{14}C]-carnosine accumulation in the mouse olfactory bulb.* [^{14}C]β-alanine was administered by intranasal irrigation (1μCi/100μl). Mice were killed at the indicated times after irrigation, and the radioactivity in the olfactory bulb was determined. The results are the average of four determinations ±S.D.

A marked increase in carnosine release (3-fold) occurred following exposure of the synaptosomes to depolarizing concentrations of potassium (60 mM) in the superfusion medium. An even greater increase was observed (5-fold) when the 60 mM K^+ medium also contained 2 mM $CaCl_2$. (Fig. 2).

When a second depolarizing stimulus of 60 mM K^+ was applied after the synaptosomes were allowed to recover their $\Delta\Psi$ in 5 mM K^+ for 6 min, a second pulse of carnosine release was induced (Fig. 3). Thus, a single depolarizing treatment does not totally deplete the synaptosomes of all the releasable carnosine. Successive depolarizing stimuli can be used to generate a set of sequential carnosine releases from the synaptosomal fractions.

Comparison of Efflux Parameters for Carnosine, GABA and Leucine

The parameters of carnosine efflux from olfactory bulb synaptosomes were compared to those of a transmitter amino acid (GABA) and a nontransmitter amino acid (leucine) (Table 1). Only 45% of carnosine or 33% of GABA were lost during the equilibration period of the superfusion and, within 9 min, the efflux had reached a very low, steady state of 1.3% for carnosine and 2.7% for GABA per 3 minutes. In contrast, the synaptosomes were very leaky for the nontransmitter amino acid leucine with a 90% loss of leucine from synaptosomes occurring during the initial equilibration, followed by a continuing, very high rate of efflux (>8%). In addition, when release was stimulated by depolarization with 60 mM K^+, the efflux of both GABA and carnosine increased 2.7 fold, while leucine efflux was only 1.5 times

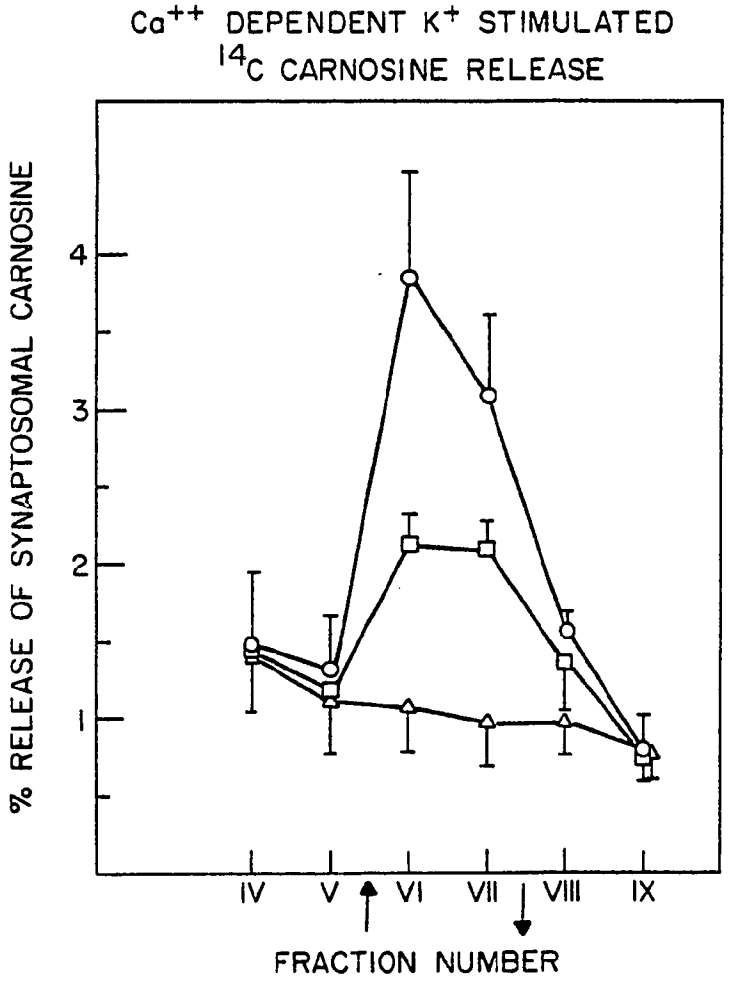

Figure 2. [^{14}C]-*Carnosine release as a function of potassium and calcium.* Synaptosomes were superfused 15 min with 5 mM K^+ medium in the absence of Ca^{2+}. At 15 min (↑) the superfusion medium was replaced by either 60 mM K^+ (□) or by 60 mM K^+ + 2 mM Ca^{2+} (○) or by 5 mM K^+ (Δ), after 6 min in the superfusion media were changed back to 5 mM K^+ (↓). [^{14}C]-carnosine released in each fraction is expressed as the percent it represents of the total [^{14}C]-carnosine in the synaptosomes at the beginning of the three min release period. The results are average of 8 determinations ±S.D.

The carnosine release induced by 60 mM K^+ and 60 mM K^+ + calcium was 3 times and 5 times the release at 5 mM K^+, respectively.

TABLE 1. *Comparison of efflux parameters for carnosine, GABA and leucine.*[a]

Ligand[b]	% ligand lost[c] during equilibration (first 9 min)	% efflux during[d] steady state (per 3 min)	% released by 60 mM K^+ / % released by 5mM K^+ [e]	
			$-Ca^{2+}$	$+Ca^{2+}$
^{3}H-GABA	33.54±7.9 (35)	2.66±0.48 (8)	1.66±0.32 (5)	2.71±0.56 (5)
^{3}H-Leucine	90.5±1.7 (12)	8.5±0.63 (12)	1.23±0.10 (4)	1.51±0.13 (4)
^{14}C-Carnosine	45.0±2.3 (14)	1.31±0.14 (11)	1.95±0.40 (4)	2.70±0.62 (21)

(a) Synaptosomal fractions containing the appropriate compound were superfused with 5 mM K^+ buffer at 37°C, effluent was collected in 3 min fractions. Stimulation of release with 60 mM K^+ started 15 min and terminated at 21 min. All the results are expressed as mean ±S.D. (n).

(b) Synaptosomes were labelled by *in vitro* uptake of compound for [^{3}H]GABA or [^{3}H]leucine or by *in vivo* labelling with[^{14}C]-β-alanine as precursor as described in the text for [^{14}C]carnosine.

(c) Expressed as % of total compound in the synaptosomes in the beginning of the superfusion.

(d) Expressed at % of content in synaptosomes at the beginning of the 3 min release period.

(e) Ratio between % released as expressed in (d).

control (Table 1). This demonstrates that the carnosine efflux parameters show greater similarity to those of GABA than they do to those of leucine.

Effects of EGTA on Carnosine Release

The role of Ca^{2+} in the release of carnosine was further evaluated by either omitting Ca^{2+} from medium, or chelating it with 3 mM EGTA during depolarizing stimulation (Fig. 3). EGTA blocked the calcium dependent component of carnosine release but did not significantly ($P \leq 0.5$) alter carnosine release induced by 60 mM K^+ in the absence of Ca^{2+}. This effect of EGTA further implies that calcium ions play a major role in carnosine release induced by depolarization.

Relationship Between Synaptosomal Potential and Carnosine Release

The relationship between carnosine release and the plasma membrane potential was investigated by determining the extent to which carnosine efflux was stimulated by varying concentrations of K^+ ions in the presence of calcium (Fig. 4). Potassium-stimulated carnosine release increased in direct proportion to the depolarization of the synaptosomal plasma membrane imposed by increasing concentrations of external potassium.

Veratridine Effect on Carnosine Release

The neuroactive alkaloid veratridine was used as a specific depolarizing agent which mimics the physiological depolarization via the action potential sodium gate (Fig. 5). Under conditions

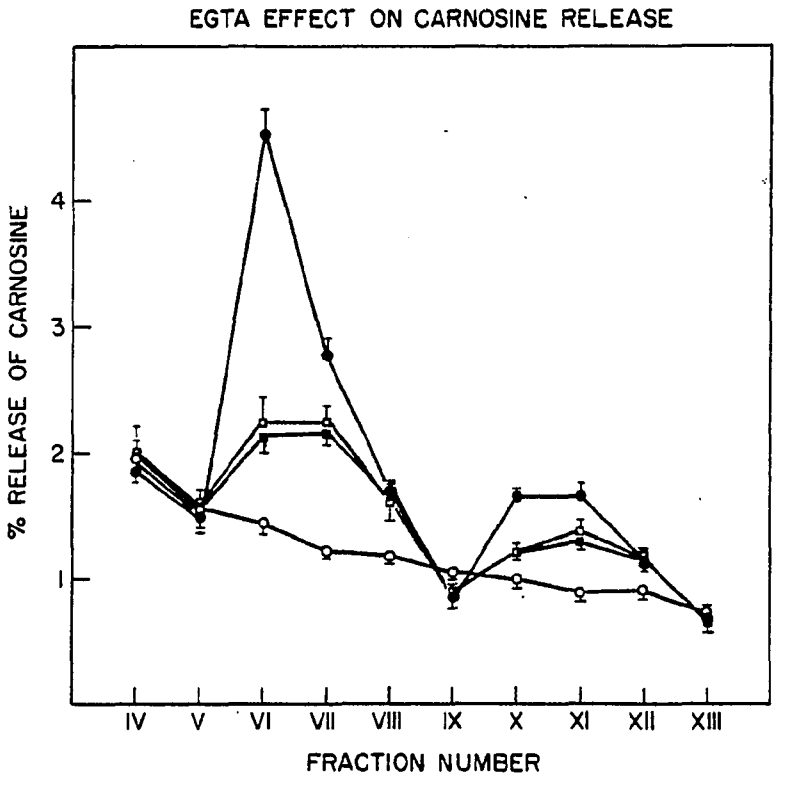

Figure 3. *EGTA effect on carnosine release.* Synaptosomes were superfused as described in the legend of Figure 2. The treatment was initiated at 15 min with media containing either 60 mM K^+ EGTA (□), or 60 mM K^+ + Ca^{2+} ± EGTA (-EGTA, o; +EGTA, ■) or 5 mM K^+ control (o). A second treatment was initiated at 27 min. (↑) indicates the beginning of the treatments; (↓), their end. The results are the average of 4 determinations ±S.D.

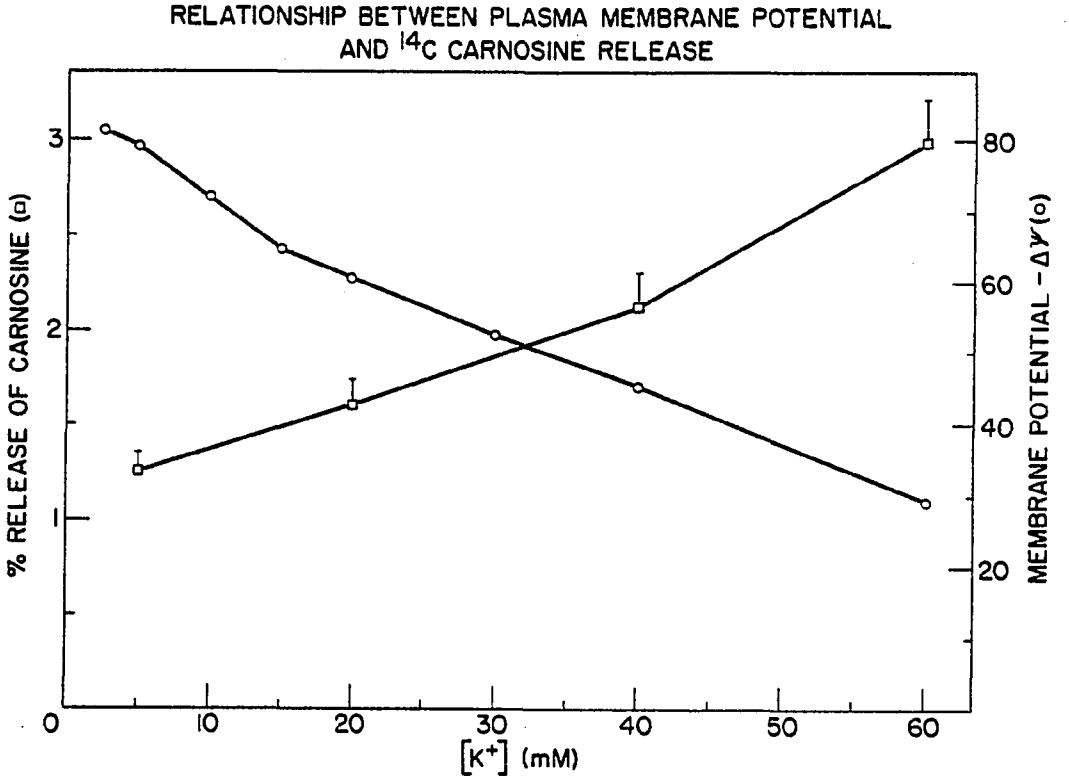

Figure 4. *Relationship between membrane potential and carnosine release.* Synaptosomes were superfused as described in Methods section. Caronsine release was stimulated with various concentrations of K^+. The percent of carnosine released (□) during the stimulation period (fraction VI + VII) and the membrane potential (○) obtained are plotted as functions of the external potassium concentrations. Membrane potentials of the synaptosomes were evaulated in previous studies (Rochel, et. al., 1980) with the lipophilic cation tetraphenyl phosphonium. The results are the average of 4 determinations ±S.D.

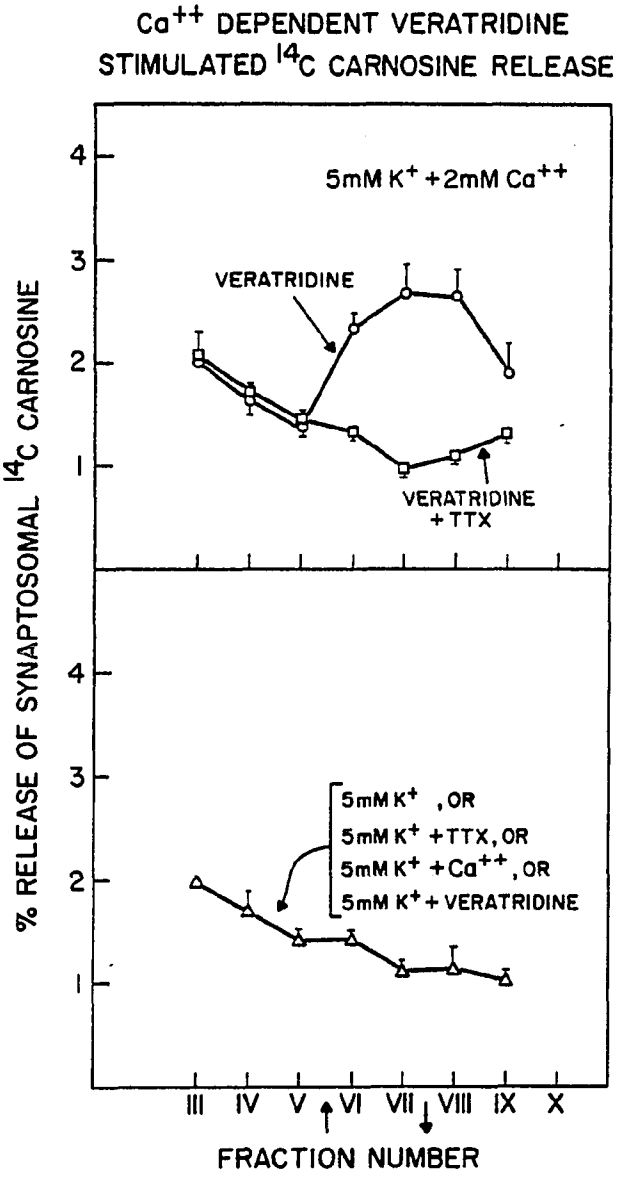

Figure 5. *Stimulation of carnosine release by veratridine.* The legend as in Figure 2. Stimulation of carnosine release was initiated by media containing 5 mM K^+ with or without: 300 μM veratridine, Ca^{2+}, TTX (15 μM) and their combinations. Beginning of treatment (↑); its end (↓). The results are means of four determinations ±S.D.

similar to those used here, veratridine was shown to depolarize olfactory bulb synaptosomes (Rochel, et al., 1980, 1981) and to be blcoked by tetrodotoxin. Veratridine stimulated the release of [^{14}C]-carnosine three-fold in the presence of calcium but had no effect in the absence of calcium. The stimulatory effect of veratridine was entirely abolished in the presence of the sodium gate blocker tetrodotoxin (15 μM). Tetrodotoxin itself did not affect the rate of spontaneous carnosine release. This indicates that the effect of veratridine on carnosine release is mediated via the depolarization of the synaptosomal plasma membrane.

Homoexchange of Carnosine

Addition of carnosine (1 mM) to the superfusion medium resulted in a two-fold increase in the rate of the spontaneous carnosine release which occurred in the presence of 5 mM K^+ and 2 mM Ca^{2+} (from 1% to 2% total carnosine/3 min). When carnosine was added under depolarizing conditions, a similar increase (of 1%) was observed in addition to the increase brought about by the 60 mM K^+. Replacement of sodium by choline in the superfusion medium did not significantly alter the increase of carnosine release induced by external carnosine.

Effects of Various Neurotransmitters on Carnosine Release

In the olfactory bulbs, dopamine (Halasz, et al., 1977, 1978, 1979) and GABA (Ribak, et al., 1977) have been suggested to be the neurotransmitters of the periglomerular cells, whilst aspartate and glutamate are thought to be transmitters of the mitral cells

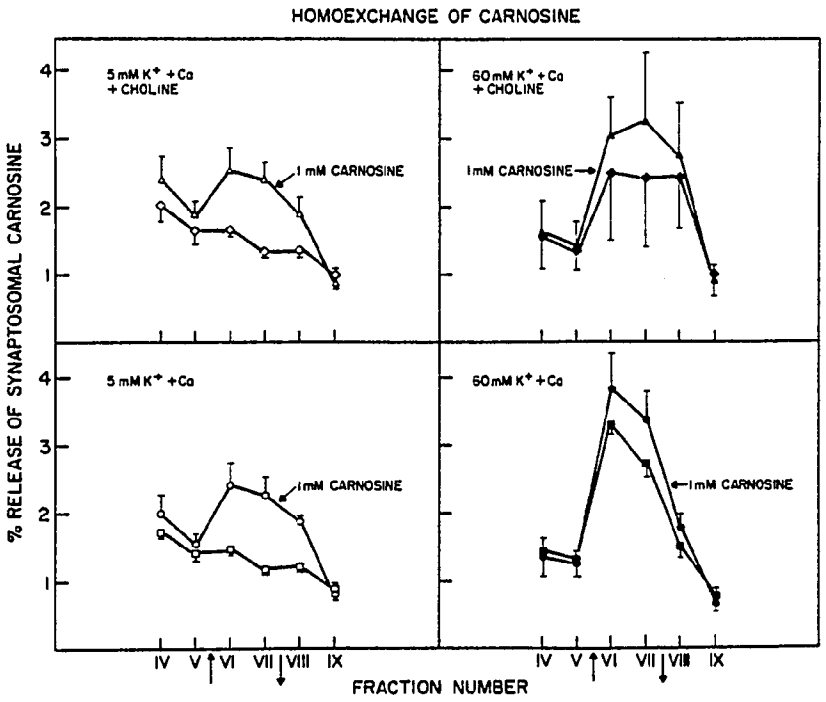

Figure 6. *Homoexchange of carnosine.* The legend as in Figure 2. During treatment, the superfusion media were supplemented with 1 mM carnosine and contained either 5 mM K^+ + Ca^{2+} or 60 mM K^+ + Ca^{2+} in the presence or absence of sodium. (Na^+ was replaced by choline). Beginning of stimulus (↑); end of stimulus (↓). The results are average of four determinations ±S.D.

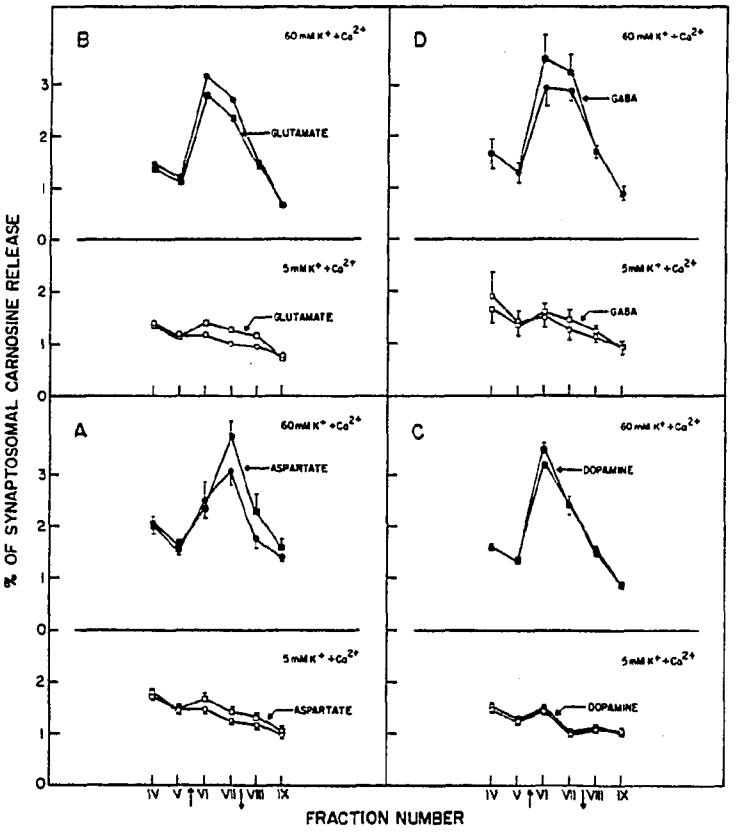

Figure 7. *Modulation of carnosine release by various neurotransmitters.* The legend as in Figure 2. Stimulation of carnosine release was initiated (↑) by the addition of aspartate (100 μM, A), or glutamate (100 μM, B), or dopamine (100 μM, C), or GABA (100 μM, D) in 5 mM K^+ medium or in 60 mM K^+ medium. (↓) indicates end of stimulus. Results are mean of four determinations. 5 mM K^+ (o), 5 mM K^+ + drug (□), 60 mM K^+ (●), 60 mM K^+ + drug (■).

(Collins, 1979a, 1979b, 1981) with GABA being implicated also in the granule cells (Ribak, et al., 1977). Since the first two groups of cells form anatomical contacts with the olfactory receptor neurons and because transsynaptic regulation of tyrosine hydroxylase activity and of dopamine levels is mediated by the receptor neurons as indicated by denervation studies (Nadi, et al., 1981; Kawano, et al., 1981; Margolis, et al., 1981), it was of interest to investigate whether these neuroactive agents influence carnosine release (Fig. 7). Dopamine (100 μM) did not alter the rate of carnosine release from olfactory bulb synaptosomes in the presence of either 5 mM K^+ + 2 mM Ca^{2+} or 60 mM K^+ + Ca^{2+} medium. Addition of glutamate (100 μM or 1 mM) produced a slight increase of carnosine release in the presence of 5 mM K^+ + 2 mM Ca^{2+} or 20 mM K^+ 2 mM Ca^{2+}. However, glutamate slightly decreased the stimulatory effect of 60 mM K^+ + 2 mM Ca^{2+}. Aspartate (500 μM) slightly increased the carnosine release both in the presence of 5 mM K^+ + 2 mM Ca^{2+} and 60 mM K^+ + 2 mM Ca^{2+}. Superfusion with GABA (100 μM) also caused slight stimulation of carnosine release in both depolarizing (60 mM K^+) and non-depolarizing (5 mM K^+) media. Capsaicin (8-N-methyl-N-vanillyl-nonenamide) is a drug known to induce substance P release from primary afferents of chemosensory neurons in the spinal cord (Alarie and Keller, 1973; Akagi, et al., 1980; Jancso, 1966). In analogy to substance P, carnosine is also a putative neurotransmitter peptide located in primary afferent chemosensory neurons. However, capsaicin did not alter carnosine release in the presence of non-depolarizing potassium concentrations (Table 2).

TABLE 2. *Effect of bestation and capsaicin on carnosine release.*

PRE-TREATMENT	TREATMENT			[^{14}C]CARNOSINE RELEASE
Medium	Medium	Capsaicin 1 µM	Bestatin 1 µg/ml	Treatment / Control
EXPERIMENT I:				
5 mM K^+ + Ca^{2+}	5 mM K^+ + Ca^{2+}	-	-	1.0
"	"	+	-	1.0
"	60 mM K^+ + Ca^{2+}	-	-	2.12
EXPERIMENT II:				
5 mM K^+ + Ca^{2+}	5 mM K^+ + Ca^{2+}	-	-	1.03
"	"	-	+	1.04
"	60 mM K^+ + Ca^{2+}	-	-	2.43
"	"	-	+	2.41

The legend as in Figure 2. When present, Bestatin was included in the medium continuously from the onset of the superfusion, treatment was initiated at 15 min by replacing the 5 mM K^+ medium with 60 mM K^+ + Ca^{2+} medium containing bestatin in the treatment superfusion. Medium was not changed in the controls. Capsaicin was applied on the onset of treatment in 5 mM K^+ superfusion media. The results are average of 4 determinations.

Effect of a Carnosinase Inhibitor on Observed Release of [^{14}C]-Carnosine

Bestatin, ([(2S,3R)-3-amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine), inhibits the Mn^{2+} stimulated membrane bound carnosinase acitivity, of brain both in vivo and in vitro (Margolis, Kawano and Grillo, unpublished observations). Administration of bestatin in the superfusion media (1 µg/ml) did not affect the apparent carnosine efflux either during spontaneous release (5 mM K^+ + 2 mM Ca^{2+}), or during depolarization stimulated release (60 mM K^+ + 2 mM Ca^{2+}) (Table 2). These observations indicate that all of the [^{14}C]-carnosine released from the snyaptosomes is detected in this system and that there is no significant loss of radioactivity as a result of hydrolysis of carnosine by a dipeptidase with subsequent reuptake of radioactive β-alanine.

DISCUSSION

The role of the putative neurotransmitter carnosine in the olfactory pathway was investigated with respect to its release from olfactory bulb synaptosomes in response to depolarizing stimuli and to neurotransmitter application.

A calcium dependent depolarization stimulated release of carnosine by olfactory bulb synaptosomes was demonstrated and characterized. The process of depolarization stimulated Ca^{2+} dependent release of ligands by nerve endings has been previously described for many neuroactive agents including acetylcholine (Katz and Miledi, 1967), catecholamines (Pellegrino de Iraldi, 1980, Smith and Winkler, 1972; Stjarne, 1970; Folko and Haggendal, 1970; Geffen, et al., 1970; Gronbald, et al., 1980); enkephalins (Henerson, et al., 1979; Bayon, et al., 1978) and GABA (Cotman and Hyacock, 1976). This process has been correlated with specific vesicle exocytosis resulting from Ca^{2+} influx in response to depolarization of the presynaptic membrane either by electrical stimulus or by specific membrane potential drugs (Katz and Miledi, 1965, 1967; Miledi, 1973; Heuser and Reese, 1973, 1976; Gray, 1975; Jeuser, 1977). The data presented in this paper have demonstrated a very close relationship between carnosine release and plasma membrane potential ($\Delta\Psi$) imposed by varying the concentration of external potassium. Depolarization of the plasma membrane by the active sodium gate drug veratridine (Ulbricht, 1969) induced carnosine release. However, veratridine was unable to induce carnosine release when the depolarization of

the plasma membrane was blocked by the sodium gate inhibitor tetrodotoxin (Narahashi, et al., 1964; Evans, 1972; Li and White, 1977). Calcium enhanced the carnosine release produced by depolarization with 60 mM K^+, and its presence was absolutely required for producing the veratridine dependent efflux..

These data suggest that a mechanism similar to the Ca^{2+} dependent exocytosis described above is functional in carnosine release from the olfactory nerve endings in the olfactory bulb.

The synaptosomal preparation used in this study represents a heterogeneous population. However, as a result of the unique process of carnosine labeling, (i.e., [^{14}C]- β-alanine uptake by olfactory neurons, and transport of the in vivo synthesized [^{14}C]-carnosine to the olfactory nerve endings), [^{14}C]-carnsine is restricted to a specific subpopulation of terminals. Thus, carnosine release can occur only from olfactory neuron synaptosomes. In contrast, both [^{3}H]-GABA and [^{3}H]-leucine were loaded in vitro after the synaptosomes were isolated. Since no uptake system for carnosine has been detected (unpublished observations) it could not be loaded in vitro. Furthermore, GABA is presumably taken up by nerve endings of periglomerular and granule cells different from those containing carnosine (Halasz, et al., 1977), whereas leucine is taken up by a less restricted subpopulation of synaptosomes.

Keeping these differences in mind, the parameters of carnosine efflux were compared to those of GABA and leucine. Carnosine was found to be compartmentalized in the synaptosomal preparation in a manner that immobilized it to the same extent as GABA in this study

and as reported for other neuroactive agents such as catecholamines which are present in specific vesicles within the nerve endings (Cotman and Haycock, 1976). In contrast to the low rate of basal efflux seen for carnosine and GABA, the synaptosomal fraction was very leaky for the nontransmitter amino acid leucine and was similar to that reported for lysine efflux (Cotman and Haycock, 1976). In addition, 60 mM K^+ depolarization stimulated release of GABA and carnosine to a similar extent while leucine release was almost unresponsive. These observations imply that carnosine is located in synaptosomes in a restricted manner as seen for various other neurotransmitters.

The effect of exogenous carnosine also supports the hypothesis that the Ca^{2+} dependent release is by specific vesicle exocytosis. External carnosine at 1 mM caused an increase of [^{14}C]-carnosine release under both non-depolarizing conditions (5 mM K^+) and depolarizing conditions (60 mM K^+,Ca^{2+}). The increment was similar in both cases and, in the latter, was in addition to the depolarization induced release. This implies that the effect of external carnosine is achieved by a mechanism different from the one responsible for the increased efflux produced by depolarization with 60 mM potassium.

The apparent increase in [^{14}C]-carnosine release in the presence of 1 mM carnosine cannot be accounted for by competitive inhibition of carnosinase by the non-radioactive carnosine, since there was no increase in the presence of the carnosinase inhibitor bestatin. Furthermore, inhibition of carnosinase activity should result in the same proportional increase of [^{14}C]-carnosine release in the presence

of 60 mM K^+ as in 5 mM K^+. However, neither of these effects were detected in the olfactory bulb synaptosomes thus, strongly arguing against carnosinase involvement as responsible for the effect of 1 mM carnosine.

These results, in addition to the observations that the exogenous carnosine induced [^{14}C]-carnosine release is Na^+ independent, indicate that the carnosine effect is most likelydue to homoexchange with cytoplasmic carnosine.

Two models for the effects of depolarization by 60 mM K^+ or veratridine can be considered. The first involves depolarization induced carnosine release from a cytoplasmic pool. Such a process must be affected by external carnosine through an exchange mechanism. In the second mechanism, the depolarization effect proceeds through specific vesicular exocytosis. This process should be Ca^{2+} dependent and unaffected by the presence of external carnosine. Our results are most consistent with the second model, that induction of carnosine release by depolarization occurs through specific vesicle exocytosis. This conclusion is in disagreement with the claim put forward by Harding and O'Fallon (1979) that carnosine is not located in vesicles derived from synaptosomal fractions of olfactory bulbs. However, they did not demonstrate the presence of any known transmitter compound in their vesicle fractions.

To evaluate the functional relationship between adjacent neuronal structures and the carnosine containing afferent neurons, the effects of several neurotransmitters present in these postsynaptic structures were studied.

Since the stimulation by either GABA, glutamate or aspartate was extremely small and since dopamine had no influence at all on carnosine release (Fig. 7), it appears that none of these agents is a major modulator of carnosine release by the olfactory neuron terminals. These results are consistent with the anatomical evaluation of olfactory bulb organization (Shepherd, 1972; Pinching and Powell, 1971a, b; White, 1971, 1973) which did not detect any presynaptic contacts on the olfactory nerve axons and nerve endings.

In conclusion, the release mechanism provides a physiological clue to the role of carnosine as a neurotransmitter in the olfactory pathway. Biochemical and anatomical evidence of carnosine localization, synthesis and transport in the olfactory neuron and its extraneuronal degradation as well as the presence of carnosine binding sites in the glomerular region, have been previously reported. (For review, see Margolis, 1980). Together with the calcium dependent carnosine release mechanism described in the present paper, these all provide a strong argument in support of carnosine as a neurotransmitter in the olfactory pathway.

However, to unequivocally establish the physiological role of carnosine in the olfactory neurons, it still remains essential to demonstrate that this presynaptically released dipeptide has a physiological effect on the post-synaptic neurons.

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