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The Somatotopic Organization of the Rat Trigeminal Ganglion

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THE SOMATOTOPIC ORGANIZATION OF THE RAT TRIGEMINAL GANGLION

by

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DEDICATION

To my wife Deb, whose love, patience, and encouragement has sustained me throughout all of my endeavors.

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VITA

The author, Scott Brian Shellhammer, was born in Ashtabula, Ohio on the seventeenth day of July, 1950.

He obtained his elementary education at Saybrook elementary school, Saybrook, Ohio and his secondary education at Harbor High School, in Ashtabula, Ohio where he graduated in June of 1968.

In September of 1968 he entered Kent State University in Kent, Ohio and obtained two years of pre-pharmacy education. In September 1970 he transferred to Ohio State University, Columbus, Ohio for one year of pharmacy school.

In September of 1971 he entered the Ohio State University College of Dentistry where he graduated in December of 1974 with the degree of Doctor of Dental Surgery. Honors included membership to Omicron Kappa Upsilon and the United States Air Force Senior Dental Student Scholarship.

In January of 1975 he was stationed at Keesler Air Force Base, Biloxi, Mississippi and remained there until his discharge in June of 1978. He spent the last two years of his tour providing care to the hospital personnel assigned there. Upon discharge he received the Air Force Commendation Medal.

In September of 1978 he entered Loyola University College of Dentistry and began a dual course of study leading to the didactic

degree of Master of Science in Oral Biology and a Certificate of Specialty Training in Endodontics.

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CHAPTER I

INTRODUCTION

Pain is a subject of extreme importance to all practitioners in the health-care field. Dentists have to deal with all of its aspects on a daily basis. In this respect dentists need to become "pain experts" in the sense that the more information they gain concerning this entity, the better service they can render their patients.

The subject of pain can be approached in many different ways. The objective of this study is to extend basic knowledge in the area of the use of axonal transport for studies of neuronal connectivity.

Since the pulp cavity is of particular interest to the dentist, it has been selected as the model system for this study. Because horseradish peroxidase can be taken up at the periphery of an axon and be transported in a retrograde manner to its nerve cell body, this protein tracer technique permits one to somatotopically locate the neuronal cell bodies within the trigeminal ganglion as they relate to individual teeth in the rat.

Two incubation techniques for horseradish peroxidase will be used and compared, i.e., the DAB technique of Graham and Karnovsky (1) and the Hanker and Yates (2) method.

CHAPTER II

REVIEW OF THE LITERATURE

Review of Trigeminal Ganglion Literature

Study of the trigeminal ganglion has been of interest to anatomists in general and to neuroanatomists and dentists in particular. Somatotopic and functional representations of the trigeminal ganglion have been described for various species.

In 1924 Allen (3) mapped out the somatotopic organization of the cat trigeminal ganglion by studying chromatolytic cell groups following transection of the various divisions of the trigeminal nerve. Kruger and Michel (4) studied the receptive fields of 788 neurons in the cat in order to reconstruct the relation of the spatial projection of the integument of the face onto different portions of the sensory trigeminal nuclear complex. Eisenman, Landgren, and Novin (5) reported on mechanoreceptor modalities by recording responses to physiologic mechanical stimuli from the main sensory and spinal nuclei of the trigeminal in the brain stem of the cat. The response of trigeminal ganglion neurons to physiologic stimuli has been studied by a single unit recording method in the cat by Kerr and Lysak (6). Darian-Smith, Mutton, and Proctor (7) evaluated the functional organization of tactile cutaneous afferents within the semilunar ganglion and trigeminal spinal tract of the cat. Beaudreau and Jerge (8) reported on a physiological study on the question of the somatotopic organization of the cat trigeminal

ganglion in 1968. In 1975 Arvidsson (9) evaluated the location of cat trigeminal ganglion cells innervating the dental pulp of upper and lower canines as revealed by retrograde axonal transport of horseradish peroxidase. All of these studies indicate that the cell bodies of the mandibular nerve are located posterolaterally, those of the ophthalmic nerve anteromedially, and those of the maxillary nerve in an area between these two locations.

Strassburg (10) studied the morphologic reaction of the trigeminal ganglion of the rabbit following experimental surgery on the maxillodental region. He found that mandibular fractures should not normally result in permanent damage to the trigeminal ganglion cells.

Carmel and Stein (11) evaluated cell changes in the trigeminal ganglion of the monkey following proximal and distal nerve section. Kerr and Lysak (6) studied the response of trigeminal ganglion neurons to physiological mechanical stimuli as revealed by a single-unit recording method in this same animal. They noted a mild degree of contralateral innervation at the midline with this technique. In 1977 Cox, Chiego, Avery, and Bradley (12) reported on horseradish peroxidase transport from primate dental pulps. Their results will be detailed at a later time.

The trigeminal ganglion of the rat has also been rather extensively studied from various aspects. In 1963 Dixon (13) did an electron microscopic study of the features of normal neurons and their satellite cells in the trigeminal ganglion as part of a wider investigation into the nervous pathways associated with nerve terminations and plexuses in the

oral mucosa. During the same year he published an article (14) describing the principal features of the ultrastructure of the normal trigeminal ganglion of the rat, with special reference to its content of myelinated and unmyelinated nerve fibers. In 1966, he studied nerve cell reactions to lesions of branches of the trigeminal nerve (15). Burnette and Dixon (16) investigated stereotaxically-induced lesions in the root of the trigeminal nerve of the albino rat. They attempted to devise a technique for the production of precise lesions in this area and to determine subsequent histological changes in the trigeminal ganglion.

Zucker and Welker (17), 1969 used electrophysiological methods to localize the cell bodies of neurons associated with vibrissae in rats. They noted that the more dorsal vibrissae projected to medial ganglion areas while the ventral vibrissae projected to lateral areas.

Tewari and Bourne (18) investigated the distribution of simple esterase, as well as specific and non-specific cholinesterase in the trigeminal ganglion cells of the rat. In 1969 Matsuura, Mori, and Kawakatsu (19) did a histochemical and electron-microscopic study in the same animal. They found three cell types, i.e., clear cells, dark cells, and transitional cells. The diameter of the cells varied from 30-60 μ. They feel that there is a transformation of the nerve cell types in various biological states from dark cell to clear cell.

In 1972 Mazza and Dixon (20) published a detailed description of chromatolytic cell group locations in the trigeminal ganglion of young adult Wistar rats following surgical division of the inferior alveolar,

mental, infraorbital, external nasal, and superior labial nerves. The animals were sacrificed 10 days post-operatively, the heads fixed in Bouin's fluid, and histologic sections prepared. They then made tracings of the trigeminal ganglia from photomicrographic enlargements of alternate sections in each slide series selected for study and plotted the location of the chromatolytic cells. It was noted that the external nasal and superior labial cells were located respectively at the medial and lateral sides of the ophthalmic-maxillary part of the ganglion with some overlap ventrally. Inferior alveolar nerve cell bodies were closely arranged in a posterolateral protuberance at the side of the ganglion, with the mental nerve cells concentrated in the dorsal part of this group.

It should be noted that the trigeminal ganglion of the rodent is different from other species in that the ophthalmic and maxillary nerve cells and fibers are combined to form a single division rather than two separate divisions.

Furstman, Saporta, and Kruger (21) used the technique of retrograde axonal transport of horseradish peroxidase to study the trigeminal ganglion in rats. They injected horseradish peroxidase into the pulp cavity of one or more teeth in 12 rats on one side of the mouth in various combinations ranging from a single tooth to all the upper and lower teeth that could successfully be opened with a fine dental drill. A 5 µl volume of 0.9N NaCl containing 1 mg Sigma type VI horseradish peroxidase was injected into each tooth and the rats were sacrificed after 1 or 2 days by cardiac perfusion with a solution of 1% glutaraldehyde-1% paraformaldehyde buffered with sodium cacodylate (pH 7.5). Both trigeminal

ganglia were dissected free and immersed in the same perfusate for 24 hours. The tissue was then immersed for an equal period of time in 5% sucrose buffered with 0.1M sodium cacodylate (pH7.5). Serial frozen sections were cut at 60 μ m and incubated in 3, 3'-diaminobenzidine tetrahydrochloride and H_2O_2 for HRP. The contralateral ganglia served as a control.

Two experimental and two control ganglia were processed as whole tissue blocks to reveal the presence of HRP. The tissue was treated in the same manner as the frozen section material with the exception that it was incubated en bloc for 3 hours at room temperature. The tissue was then washed through 3 changes of distilled water, dehydrated, and embedded in paraffin for serial sectioning, and Nissl stained.

The peroxidase reaction product was present in a limited number of ganglion cells in the ipsilateral trigeminal ganglion. No labeled (HRP-containing) cells were found in the contralateral trigeminal ganglion. The distribution of labeled neurons for a single incisor injection was limited to a compact zone containing a maximum of five labeled neurons on the periphery of the ganglion. Neurons labeled by multiple tooth injections, however, were not distributed in separate discrete zones and determination of topographical relationships were difficult to reconstruct.

The HRP reaction product for the two incubation procedures was slightly different. Free-floating frozen section incubation revealed large dark granules of HRP. Incubation en bloc produced a fine granular reaction product which made recognition of HRP- containing cells more

difficult than recognition of the large granule reaction product present in cells following incubation of sections. However, the reaction product in both procedures is essentially similar and ganglion cells containing HRP are easily recognized. This study demonstrated the feasibility of tracing first order afferents via the retrograde axonal transport of horseradish peroxidase.

Aldskogius and Arvidsson (22) published an article in 1978 in which they studied the trigeminal ganglia of normal rats which had been subjected to unilateral transection of the infraorbital nerve. Both light and electron microscopy were used in this study. Counts of ganglion cells in ganglia on the operated and unoperated sides were made following long postoperative survival times. The ultrastructural changes in ganglia of the operated sides were studied from 3 to 70 days postoperatively. Their quantitative observations revealed that a considerable loss of ganglion cells takes place on the operated side. They noted no signs of ganglion cell degeneration or death in normal trigeminal ganglia or trigeminal ganglia from the unoperated side.

Development of Neuroanatomical Research Techniques

Studies of neuronal architecture and connectivity have had a varied history. Camillo Golgi introduced the metallic impregnation technique in the late 19th century (23). In the early 1950's Nauta and Gygax (24) introduced the method for the selective impregnation of degenerating fibers, now commonly referred to as the "Nauta method". Fink and Heimer (25), 1967 demonstrated variants of this technique to

neuroanatomy.

As research in these areas increased, new methods were developed. One important technique, which allowed for the direct visualization of physiologically identified neurons, was the method of intracellular staining with certain fluorescent dyes, such as Procion yellow.

In 1850 Waller (26) discovered that if an axon is interrupted, the portion distal to the lesion degenerates. Inherent in this study is the concept that the soma of a neuron constitutes the "trophic center" for the maintenance of its various processes. In 1948 Weiss and Hiscoe (27) showed that if a peripheral nerve was constricted at some point along its length, there was a progressive distention of the nerve proximal to the constricted region and a marked narrowing just distal to it. When the constriction was subsequently removed, a "bolus" of material, could be followed down the nerve at a rate of about 1-2 mm/day. Weiss introduced the term axonal flow to this phenomenon. Taylor and Weiss (28) 1965 completed one of the first definitive experiments using axonal transport for tracing connections in the nervous system by injecting a tritium-labeled amino acid ([^{3}H] leucine) into the vitreous body of mice and subsequently following the movement of the labeled proteins autoradiographically.

Work was done indicating that injection of ^{3}H-labeled amino acids into a discrete population of neurons, such as a dorsal root ganglion, could result in the selective transport of labeled protein into the central nervous system along the processes of the cells, and that this could be followed as clearly, and with the same degree of

precision, as the degeneration that follows the interruption of a dorsal root. Advantages of the autoradiographic method over degeneration techniques include; (1) since it does not involve the placement of destructive (and generally unselective) lesions, it can be used in conjunction with physiological studies that require the preservation of the tissue; (2) as the label is preferentially incorporated by the neuronal somata, the involvement of fibers passing through the area under study should not present a complicating factor; (3) the discrete quality of silver grains enables precise estimates to be made of the grain densities in different regions of the autoradiographs, and to this degree the presence or absence of a projection can be objectively assessed. Important developments during the use of this method included demonstration that radioactively labeled proteins transported down axons could be readily demonstrated in the electron microscope and the application of this method to the study of the more rapidly transported axoplasmic proteins. There are numerous disadvantages to the autoradiographic technique. At the light microscopical level the method tells one little about the actual morphology of the fibers and terminals being studied, since all one actually observes is the distribution of silver grains in the emulsion over-lying the section due to the radioactivity in the most superficial 2-3μ m of the section. A second disadvantage is the fact that it is generally difficult, if not impossible, to label an entire population of neurons in a large structure, or if the origin of a pathway is very spread out. It is also not very well suited for studying short axonal connections since

the isotope spreads by diffusion over a distance of 100 μ m or more. Another problem inherent in the autoradiographic method is the difficulty of tracing lightly labelled-pathways over long distances. A further difficulty is that it is not known how heavily labelled a cell has to be before a significant amount of radioactivity can be detected in its axon and axon terminals. Lastly, there is an extremely long exposure time required for electron microscope autoradiography (3-6 months), and this can be an extremely frustrating wait, especially if something goes wrong with the technique.

Development of HRP Techniques Based on Axonal Transport

A short time after Weiss and Hiscoe's (27) 1948 discovery that a large amount of material is continuously being produced in the perikarya of neurons for transport down their axons, the question was raised as to what happens to the transported materials. It seems plausible that some might be utilized and subsequently broken down either in the axon or in its terminals, and that some might be selectively transported from the terminals of the axons to the cells with which they are in contact. The third possibility suggested was that some fraction of the materials might be returned to the cell soma by a process of retrograde axonal flow. At that time there was no direct evidence for this third possibility. Some indirect evidence put forth included the point that the spread of certain neurotopic viruses could more readily be explained on the grounds that at some point they gained access to peripheral nerves and then moved to the neuronal perikarya where they grew and multiplied (Good Pasture, (29) 1925).

The first direct evidence for retrograde movement in axonal processes came from the observation of neurites in tissue culture. It was observed that mitochondria and various vesicular organelles appeared subject to rapid bidirectional movements, often with a net displacement towards the cell body (Hughes (31), 1953 Pomerat (30), 1967). In 1971 Kristensson and Olson (32) reported the discovery that certain large macromolecules, such as Evans blue-labelled albumin were readily taken up by the terminals of an axon and were rapidly transported back to the neuronal soma where they appeared to accumulate.

LaVail and LaVail (33) 1972 were among the first to take advantage of the retrograde axonal transport of an exogenous marker as a potential neuroanatomical method. Using the enzyme horseradish peroxidase (HRP), they were able to show that the enzyme was preferentially transported in the retrograde direction in the visual system of young chicks. This study was of particular interest since it indicated that this approach could be used to study the origin of neuronal pathways in the central nervous system. This initial report has been subsequently followed by two studies describing in more detail the electron microscopic appearance of the transported marker, and the optimal conditions for using it in neuroanatomical studies (LaVail et al., (34) 1973; LaVail and LaVail (35) 1974).

Studies have also shown that HRP has great promise in the marking of peripheral axons. Kristensson and Olsson (36) in 1971 reported a study on the subcellular localization of horseradish peroxidase in hypoglossal neurons following injection of HRP into the tongue muscles of

mice. They reported that the protein presumably entered the nerve trunks at the myoneural junctions, was taken up by the axons, and then in a retrograde fashion was transported to the perikaryon. They noted that the accumulation of exogenous peroxidase was most probably the result of pinocytotic uptake and retrograde axonal transport of protein.

In 1975 Furstman et al., (21) reported that HRP could be demonstrated in the trigeminal ganglion of the rat after deposition in the dental pulp. This investigation appears to be the first in which the tracing method based upon uptake and retrograde transport of HRP has been applied to dental structures. They used a small number of animals (12) and did not chart the location of the HRP-labelled nerve cells in the ganglion. They did note that the peroxidase reaction product was present in a limited number of ganglion cells in the ipsilateral trigeminal ganglion, but that no labelled cells were present in the contralateral trigeminal ganglion.

In that same year Arvidsson (9) reported on an investigation concerning the location of cat trigeminal ganglion cells innervating dental pulps of upper and lower canines studied by retrograde transport of horseradish peroxidase. Nerve cell bodies innervating the lower canine were found exclusively in the posterolateral part of the ganglion, while those innervating the upper canine were found exclusively in the middle part of the ganglion. He noted that all labelled neurons in this study were found ipsilateral to the HRP injections, but he did not rule out the possibility of contralateral innervation of the dental pulp since only one section out of three was examined on the non-operated

side.

Anderson and Rosing (37) 1977 used horseradish peroxidase to determine the location of cat trigeminal ganglion cells innervating maxillary canine teeth. They routinely found HRP-labelled cells in both ipsilateral and contralateral ganglia. In an abstract by Cox et al., (12) in 1977, these researchers reported on an investigation of retrograde axonal transport of HRP from primate dental pulps. Localization of HRP in this study was found by light and electron microscopy in both ipsilateral and contralateral trigeminal ganglia. They also noted that a group of four to ten cells in the ipsilateral pons were found to be HRP positive at the level of entry of the root of the trigeminal nerve. They feel that in primate dentitions there may be a sensory overlap via the mental, or other nerves. The pontine localization indicates primary afferent innervation from cell bodies that are located within the central nervous system, since no evidence of trans-synaptic migration of HRP is known.

In a recent article by Fuller, Wilson, and Winfrey (38) 1979, they indicated that they were unable to show peripheral transmedian innervation of feline mandibular canine teeth as determined by horseradish peroxidase. It can be noted from this article as well as the previous ones cited, that more research in the area of transmedian innervation of tooth pulps is necessary for a thorough understanding of the subject.

Horseradish Peroxidase Histochemistry

A major step is the use of HRP as a neuroanatomical tracer involves

the demonstration of the marker by histochemical means. Graham and Karnovsky (1) 1965 introduced 3,3'-Diaminobenzidine (DAB) as a histochemical substrate for peroxidase activity. It allows for localization of activity when observed by either light or electron microscopy. It is especially good for ultrastructural cytochemistry due to the great electron opacity of its reaction product. It should be noted that DAB, is a borderline carcinogen. Some of the benzidine derivatives which can be used as a substrate and which have been tested for carcinogenic potency include DAB, dimethoxybenzidine (o-dianisidine), and dimethylbenzidine (o-tolidine). Of those three, DAB may be the least hazardous (Griswold, Casey, Weisburger and Weisburger (39) 1968; Hadidian, Fredrickson, Weisburger, Weisburger, Glass and Mantel, (40) 1968). Despite the lack of proof that DAB is carcinogenic, discretion demands that it be treated as such (Federal Register (41), 1974). DAB is denatured by sodium hypochlorite which is a principal ingredient in laundry bleach. Before disposal, all DAB solutions should be denatured by adding 5.25% sodium hypochlorite. All utensils and areas exposed to DAB should be cleaned with the same solution.

Recent studies (Hanker, Anderson, and Bloom (42), 1972; Hanker and Rabin (43), 1975) have suggested that oxidative coupling reactions of aromatic amines in the presence of phenols might provide a suitable substitute for DAB. These reactions yield deeply-colored synthetic melanin-like compounds which are osmiophilic and sufficiently unsoluble to be suitable end products for histochemistry. To utilize such

a reaction for a substitute for DAB in the demonstration of exogenous HRP, the reaction must be sufficiently rapid to deposit the end product at the cell or tissue sites of the plant hydroperoxidase alone. This was realized by Hanker, Yates, Metz, and Rustioni (2) 1977 when they found that the peroxidation of P-phenylenediamine (PPD) was greatly accelerated by the presence of pyrocatechol (PC). The copolymer formed as a result of the oxidative coupling reaction was osmiophilic and bluer than oxidized DAB. It was insoluble and conformed well to biological ultrastructure. Incubation times for this medium are generally much shorter than those required for adequate staining with DAB. It was also noted that erythrocyte staining, which is intense with DAB medium due to hemoglobin, peroxidase, or catalase, was much less prominent with the PPD-PC medium. The reagent as described has so far been shown to be non-carcinogenic.

Hardy and Heimer (44) 1977 introduced a procedure in which tetramethyl benzidine (TMB) was used as a substrate for the detection of intra-axonal transport of HRP. TMB is supposedly safer in regards to carcinogenicity than the previously mentioned benzidine derivatives. The two main problems with this medium is that it is prone to crystal formation and it does not seem to give optimal histological definition. Use of this medium would require trial procedures in order to obtain maximum sensitivity without disturbing crystal formation.

Various articles and authors have reported on some of the technical considerations on the use of horseradish peroxidase as a neuronal

marker. Different types of commercially prepared HRP are available, with almost all authors using Sigma Type VI (Sigma Chemical Company, St. Louis, Mo.). The fixative is also of extreme importance. To minimize the inactivation of HRP between the death of the animal and the incubation of sections, fixation should be done in glutaraldehyde alone, (Kim and Strick (45) 1976, Malmgren and Olsson (46) 1977), because glutaraldehyde stabilizes HRP, whereas at room temperature formaldehyde reduces HRP activity.

Many histologists infiltrate the brain in a sucrose solution to reduce ice crystallization when the tissue is frozen for sectioning (Adams (47), 1977). This treatment should be as brief as possible to prevent inactivation of the enzyme. The procedure affords cryo-protection and helps to remove residual fixative from the tissue, the need for which has been stressed by LaVail (48) 1975.

Techniques for increased visualization of the reaction end-products have been proposed by various authors. Adams (47) 1977 feels that localization of faint granules of reaction product in both stained and unstained sections is facilitated by the use of phase contrast microscopy, which, at low magnification, shows the granules as intense black dots. Malmgren and Olsson (49) 1978 state that one can markedly increase the contrast in DAB containing procedures by using appropriate filters for light-field microscopy. They feel that inexpensive gelatin filters such as the Kodak Wratten No. 46 considerably improve the visibility of labelled neurons in a DAB procedure. Visualization may also

be increased by lightly counterstaining the neurons themselves. Another method of changing the brown DAB reaction product to one that is dark blue or black is to process the tissue in cobalt chloride ($CoCl_2$) prior to incubation in DAB. The darker reaction product gives the added advantage of extending the sensitivity of the HRP method and it obviates the need for dark-field illumination or phase microscopy.

The rate at which the protein tracers are taken up and transported by axons to become histochemically visible in the nerve cell bodies appears to be relatively rapid. The rate of retrograde axonal movement of HRP in the chick visual system has been estimated to be at least 72 mm per day in retinal ganglion cells (LaVail and LaVail (33), 1972) and at least 84 mm per day in isthmo-optic nucleus neurons (LaVail and LaVail (35), 1974). In the mammalian nervous system, Kristensson (23) 1975 has estimated a rate of approximately 48-72 mm/day. This is in good agreement with an estimated rate of 2.4 mm/hr for the neural spread of poliomyelits virus in monkey sciatic nerve (Bodian and Howe (50), 1941). Hansson (51) 1973 found a faster rate of about 120 mm/day in retinal ganglion cells of rats and Edstrom and Hanson (52) 1973 found HRP transported at 60 mm/day by frog sciatic nerves maintained at 18°C in vitro. In his study on cats Arvidsson (9) 1975 noted that the shortest postoperative survival time that gave labelling of cells in the trigeminal ganglion was 24 hours. With an approximate distance of 70 mm between a lower canine and the trigeminal ganglion this would indicate a rate of transport of at least 70 mm/day. In any

case, these approximate rates serve to indicate that, in general, survival times of 1-3 days should be sufficient to mark cell bodies of origin of fibers in the central nervous system.

Bunt et al., (53) 1974 and LaVail and LaVail (35) 1974 noted that post-injection times longer than 3-4 days may frustrate the interpretation of results of some experiments because disappearance of HRP from retinal ganglion cells appears to be fairly complete 3-4 days following tectal injection. Kristensson and Olsson (54) 1973 noticed the disappearance of HRP from hypoglossal neurons by 6 days following injection into the tongue of suckling mice. Therefore, the shortest survival time which gives maximum accumulation of tracer should be determined for each experimental system.

CHAPTER III

MATERIALS AND METHODS

Thirty Sprague-Dawley albino rats were used in this study. They were equally divided according to sex and each weighed between 220-250 grams. Throughout the experiment they were housed two to a cage at the Loyola University College of Dentistry. The animals were under continuous supervision and maintained on a diet of standard laboratory meal and water ad libitum.

SEDATION AND ANESTHESIA

Each animal was anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal) 50 mg/ml. A dose of 4 mg per 100 grams of body weight was adequate for light surgical anesthesia.

Six animals, three male and three female, were utilized per session with a total of four sessions. A session is defined as all the procedures accomplished during a specific time frame. The experimental techniques performed each session were the same, the only exception being that two of the sessions utilized the DAB processing technique as proposed by Graham and Karnovsky (1), while the other two sessions utilized the processing technique proposed by Hanker and Yates (2). Each session was accomplished in three successive days.

On day one, three male and three female animals were selected.

They were coded according to the tooth being treated in the following manner.

maxillary right central	-	a single front foot red
maxillary right 1st molar	-	a single back foot red
maxillary right 2nd molar	-	both back feet red
mandibular right central	-	a single front foot black
mandibular right 1st molar	-	a single back foot black
mandibular right 2nd molar	-	both back feet black

Throughout the four sessions the animals were divided according to sex so that two males and two females were utilized per each tooth treated. The experimentally treated teeth were always on the right side.

The animals were weighed and anesthetized according to the method noted previously. A fresh solution of horseradish peroxidase (HRP) (Sigma type VI, Sigma Chemical Co., St. Louis, Mo.) was prepared. Five mg of HRP was combined with 10 microliters of distilled water to make a 50% solution of HRP.

The tooth to be treated was prepared as aseptically as possible with a 1/2 round bur mounted in a dental handpiece until the pulp chamber (figures 1 & 2) was reached. The maxillary and mandibular central incisors were entered from the buccal aspect while the molars were all entered from the occlusal aspect. Entry into the pulp chamber was indicated by the presence of slight hemorrhage. A sterile paper point (Johnson and Johnson, East Windsor, New Jersey) was then inserted into the chamber to slightly reduce the blood volume to allow for the

placement of the HRP solution. Following removal of the paper point 1 µl (one microliter) of a 50% solution of HRP was carefully injected into the pulp chamber with a microsyringe (Hamilton, Reno, Nevada) (figures 3 & 4). The access opening was then sealed off by the use of a dental temporary cement (Cavit, Premier Dental) (figure 5). This sequence was repeated on each of the five remaining teeth in the group.

Following the experimental procedure the animals were placed back in their cages to recuperate. They were only allowed water post-operatively so that they would not dislodge the temporary filling placed in their tooth.

On the morning of the second day of each session a fresh fixative solution of 1% glutaraldehyde in 0.1M sodium cacodylate buffered to pH 7.4 was prepared. The animals were sacrificed one at a time by an intraperitoneal overdose of sodium pentobarbital 24 hours following the placement of the HRP solution in the tooth.

Following decerebration of the animal, the mandible was removed, the neurocranium opened, and the trigeminalganglia exposed. This procedure took less than sixty-seconds. The specimens were then immersed in the buffered 1% glutaraldehyde solution for 1 1/2 - 2 hours at room temperature.

Following the initial fixation the ipsilateral and contralateral trigeminal ganglia were dissected free using 3x binocular loops. During this procedure the specimens were kept continually wet with fixative solution. The trigeminal ganglion was located in a bony depression in the base of the skull, roofed by the dura mater. The cavernous sinus

and the basi-sphenoid bone lay on the medial aspect of the ganglion, while laterally a spur of the petrous temporal bone separated the ganglion from the internal carotid artery and the mandibular division of the trigeminal nerve.

Following isolation the trigeminal ganglia were immersed overnight at room temperature in buffered 1% glutaraldehyde. During the entire fixation and dissection procedures the specimens were handled with extreme care so as not to injure the delicate tissues.

On the 3rd day all specimens, both ipsi and contralateral ganglia, were removed from fixative solution and mounted for the preparation of frozen sections. The proper orientation of the sections of the isolated ganglia was determined by reference to the anatomical relationships of the ganglia in the in situ preparations. The frozen specimens were sectioned at a thickness of 60 microns.

The sections were collected in 0.1M sodium cacodylate buffer, 7.4. The sections were collected in sequential order and were placed in numbered compartments in a special holder which could then be transferred to the various solutions required by the incubation technique of choice.

It is at this point that the sessions altered slightly. Two sessions used the incubation procedure proposed by Graham and Karnovsky (1) while the other two sessions used the procedure proposed by Hanker and Yates (2). Sessions 1 and 3 utilized the Hanker/Yates (2) technique while sessions 2 and 4 utilized the method proposed by Graham and Karnovsky (1).

The Hanker-Yates technique included the collection of frozen

sections in buffered sodium cacodylate; these sections were then incubated in Hanker-Yates solution for 15 minutes. The Hanker/Yates solution was prepared by mixing Hanker/Yates reagent, 75 mg. (Polysciences, Inc. Warrington, Pa.) with 50 ml of 0.1 M tris- HCl at pH 7.4 and 0.5 ml of 1% hydrogen peroxide. This was adjusted so that an adequate final volume was present for the tissue sections.

The sections were then passed through two, five minute rinses in buffered 0.1 M sodium cacodylate. The sections were mounted on gelled slides and allowed to air dry. The sections were then dehydrated in the following manner.

5 minutes	95% alcohol
5 minutes	100% alcohol
5 minutes	100% alcohol
5 minutes	Xylene
15 minutes	Xylene

The sections were then coverslipped. Great care was exercised to orient the sections properly and also to keep them in sequential order.

As noted previously, sessions 2 and 4 utilized the incubation procedure proposed by Graham and Karnovsky (1). The basic procedure was modified slightly by the addition of cobalt chloride for enhancement as noted by Adams (47) 1977. The sections were again collected in buffered 0.1 M sodium cacodylate, pH 7.4. The sections were then transferred for five minutes to 0.1 M tris-HCl buffer at pH 7.5. From here the sections were soaked in 0.5% cobalt chloride for five minutes. Following the cobalt chloride enhancement the sections were rinsed twice at five minute intervals in 0.1 M tris- HCl buffer at pH 7.5.

The sections were then incubated in a solution of 100 mg DAB (Polyscience, Inc., Warrington, Pa.), 200 ml of buffered 0.2 M sodium cacodylate, 2 ml of 3% H_2O_2 , and 4 ml of distilled water for 15 minutes. Because of its possible carcinogenicity, the DAB solution was prepared and used under a hood. Following the incubation procedure, the sections were again rinsed for two five minute intervals in buffered 0.1 M sodium cacodylate, pH 7.4. The sections were collected and mounted in sequence on gel slides, dehydrated to 100% as noted previously, xylened, and coverslipped.

Two animals which did not receive HRP were also sacrificed. The ganglia were fixed in the same manner noted above. One set of ganglia was prepared by the Graham and Karnovsky (1) incubation technique with cobalt chloride enhancement. Frozen sections were then prepared and mounted. The second set of ganglia was embedded in paraffin, sectioned at seven microns, and stained with hematoxylin and eosin to observe normal histological architecture.

Light microscopic sections were evaluated and the somatotopic organization of the trigeminal ganglia observed and plotted. The sections were first observed under 40x power to locate areas of HRP reaction product. These areas were then observed under 100x and 250x to be sure that HRP granules were actually present within neuronal cell bodies.

To enhance the visualization of HRP granules a continuous running filter monochromator (Carl Zeiss) was utilized. The main feature of the continuous running filter monochromator is a special type of interference filter. This filter is a longitudinally banded filter which

transmits light in spectral selectivity from violet (approximately 400 mμ) to infrared (approximately 70 mμ).

In each session, six teeth on the right side were treated. Both ipsilateral and contralateral ganglia were observed for the presence of HRP granules. Composite pictures showing labeled neurons were assembled for both experimental and control ganglia. These composite pictures not only showed the position of the neuronal cell bodies innervating the different teeth, they also gave a quantitative evaluation as to the number of neuronal cell bodies tagged by a single intra-pulpal injection of HRP.

During the study 3 animals died from what appeared to be respiratory arrest. They all had seemed to tolerate the procedure well, but quit breathing approximately 15-20 minutes post-operatively. Each of the animals was on the lower limit of the weight range utilized during the experiment. Each of the animals was replaced by a new animal of the same sex and the experimental procedure repeated. The results of the study were not altered by this complication.

CHAPTER IV

RESULTS

The results of this study confirm those of Furstman (21), who revealed that in the rat there is an uptake and retrograde axonal transport of HRP to trigeminal sensory neurons after deposition of this protein into the dental pulpal tissues.

The normal gross architecture of the rodent trigeminal ganglion was first observed. The trigeminal ganglion in many other animals is composed of three separate divisions, ophthalmic, maxillary, and mandibular; the rodent ganglion is composed of only two divisions, a combined ophthalmic maxillary division and a mandibular division. The rodent ganglion is basically a flat structure.

The normal histologic morphology of the rodent trigeminal ganglion was observed. For this the trigeminal ganglia which had been embedded in paraffin, sectioned at seven microns, and stained with hematoxylin and eosin were utilized. Normal trigeminal ganglionic neurons in the rat, when stained with H & E, typically present large, centrally-placed nuclei and a coarsely granular cytoplasm (fig. 6). The neurons tended to be grouped in long linear arrays in the ophthalmic-maxillary division while the mandibular division neurons appeared to form contiguous groups. (fig. 7 & 8).

Although each section of each ganglion, both ipsilateral and

contralateral, was examined separately, it was found that for final reporting purposes it would be desirable to make composite drawings. A composite drawing was completed for each of the six teeth treated, i.e., maxillary central, maxillary first molar, maxillary second molar, mandibular central, mandibular first molar, and mandibular second molar. The results from each of the four experimental sessions were combined. HRP-positive neurons looked like those in figures 9 & 10.

MANDIBULAR CENTRAL INCISOR (fig 11)

The HRP-positive neurons for the mandibular central incisor were located in a fairly linear arrangement beginning from the postero-lateral protuberance and heading to the bifurcation of the ophthalmic-maxillary and mandibular divisions. In the experimental ganglion fourteen HRP labelled cells were noted fairly well dispersed throughout the area described above. In the control ganglia four HRP labelled cells were observed closer to the postero-lateral protuberance.

MANDIBULAR FIRST MOLAR (fig 12)

The HRP positive neurons for the mandibular first molar were found in three areas. They were noted in the postero-lateral protuberance area, in the bifurcation area of ophthalmic-maxillary and mandibular divisions, and antero-laterally in the mandibular division itself. There were sixteen HRP labelled cells in the experimental ganglia and eight HRP labelled cells in the control ganglia. The HRP labelled cells in the control ganglia were again closer to the postero-lateral protuberance of the ganglia

while the experimental HRP labelled cells were fairly well dispersed in the area described.

MANDIBULAR SECOND MOLAR (fig 13)

The mandibular second molar HRP positive neurons were fairly equally distributed around the bifurcation area of the ophthalmic-maxillary and mandibular divisions. There were sixteen HRP-positive cells in the experimental ganglia and five HRP-positive cells in the control ganglia. Almost all of the experimental HRP-positive cells were located right around the bifurcation area or antero-laterally in the ophthalmic-maxillary division. The HRP-positive cells in the control ganglia were more widely dispersed.

MANDIBULAR CENTRAL, FIRST MOLAR, SECOND MOLAR (fig 14)

As the experimental procedure advanced from anterior (central) to posterior (second molar) in the mandibular teeth, the HRP labelled cells appeared to move antero-medially as a group.

MAXILLARY CENTRAL INCISOR (fig 15)

The HRP-positive labelled cells for the maxillary central incisor were distributed in a fairly linear arrangement from anterior to posterior in the middle of the ophthalmic-maxillary division. A few cells were found at the lateral border of this division above the bifurcation area. Sixteen HRP labelled cells were observed in the experimental ganglia and fourteen cells in the control ganglia. The labelled cells from the control ganglia were found in closer proximity to one another than those labelled cells from the experimental ganglia.

MAXILLARY FIRST MOLAR (fig 16)

The HRP labelled cells in the maxillary first molar were arranged in a more diagonal linear arrangement from the central anterior portion of the ophthalmic-maxillary division postero-laterally to below the bifurcation area. There were sixteen HRP-positive cells in the experimental ganglia and seven such cells in the control ganglia. The labelled cells in the control ganglia were in a more linear arrangement than those found in the experimental ganglia.

MAXILLARY SECOND MOLAR (fig 17)

The maxillary second molar injection labelled the greatest number of cells seen in the experiment. The HRP labelled cells were found in a very diagonal linear arrangement from the central anterior portion of the ophthalmic-maxillary division postero-laterally to well-below the bifurcation area. Twenty-seven labelled cells were observed in the experimental ganglia and six were noted in the control ganglia.

MAXILLARY CENTRAL, FIRST MOLAR, SECOND MOLAR (fig 18)

For the most part, the labelled cells from all three of these teeth remained linearly arranged in the central portion of the ophthalmic-maxillary division of the trigeminal ganglion. Slight deviations were observed in the maxillary central incisor, where four labelled cells were noted in an area on the lateral side of the ophthalmic-maxillary division. In the maxillary first molar four labelled cells were seen surrounding the bifurcation area.

COMPARISON OF HRP INCUBATION PROCEDURES

The second portion of the study compared two HRP processing techniques, the Hanker-Yates (2) method and the Graham and Karnovsky (1) method with the addition of cobalt chloride for enhancement.

Positive results were obtained irrespective of the incubation technique utilized. What was noted was that the technique proposed by Hanker and Yates (2) yielded a much fainter reaction product than that produced by the Graham and Karnovsky (1) method. The Hanker/Yates (2) technique was much less time consuming and required fewer transfers of the specimens. Probably the most important factor favorable to the Hanker/Yates (2) technique is that it utilizes a chromogen that is non-carcinogenic in nature.

The major problem with the Graham and Karnovsky (1) method is that it does utilize a borderline carcinogen, namely 3,3'-diaminobenzidine or DAB. With this in mind, greater care must be exercised when utilizing this procedure. The DAB solution must be prepared and used under a hood and all equipment that it comes in contact with must be denatured by sodium hypochlorite. The incubation procedure also takes a longer time to accomplish with this technique. The major point in favor of this technique was the superior reaction product which resulted. A much darker reaction product resulted which greatly aided visualiztion within the cells.

Following utilization of both incubation procedures in this study, it was observed that the superior reaction product resulting from the Graham and Karnovsky (1) incubation procedure is well worth the extra

time and care required in its use.

CHAPTER V

DISCUSSION

The retrograde axonal transport of horseradish peroxidase has become an important neuroanatomical research tool. This study indicates that it is possible to somatotopically map out the organization of the trigeminal ganglion by that method. Further research is necessary, not only to confirm the results found here, but to expand current knowledge in the area.

As noted previously there are relatively few articles pertaining to the somatotopic organization of the rat trigeminal ganglion, although extensive literature is available concerning this area in other animal species. Although Furstman (21) studied the trigeminal ganglion following deposition of horseradish peroxidase into dental pulps, he did not chart the location of the HRP-labelled nerve cells in the ganglion. His only notation as to somatotopic organization was that the area of distribution of labelled neurons for single incisor injections was limited to a compact zone containing a maximum of five labelled neurons on the periphery of the ganglion. He did not report on the distribution of multiple tooth injections. He reported finding no HRP-labelled cells in the contralateral (control) trigeminal ganglion.

Zucker and Welker (17) recorded the responses of ophthalmic-maxillary division neurons to stimulation of vibrissae in the rat.

Using single neuron recordings they found that the more dorsal vibrissae (those closer to the midline) projected to the medial ganglion area, and that the ventral vibrissae projected to the lateral area.

The most extensive study of the rat trigeminal ganglion organization was undertaken by Mazza and Dixon (20). They made surgical divisions of the inferior alveolar, mental, infraorbital, external nasal, or superior labial nerves, and studied the resulting chromatolytic cell groups in the ganglion.

The results of this study closely resemble those noted by Mazza and Dixon (20). In horizontal sections of the ganglion they noted "A dense mass of nerve cell bodies on the lateral aspect of the base of the mandibular division extended antero-medially and contrasted sharply with the more distally placed columns of cells associated with the ophthalmic-maxillary division." Hutchens, White, and Dixon 1966 (15) in a preliminary study of chromatolytic cell changes in the trigeminal ganglion following various types of nerve lesions found that cell bodies of the superior labial and external nasal neurons seemed to constitute parallel columns of cells in the ophthalmic-maxillary part of the ganglion, while inferior alveolar and mental nerve cells appeared to form contiguous groups at the commencement of the mandibular division. In their present study Mazza and Dixon (20) found that external nasal and superior labial chromatolytic cells were located respectively at the medial and lateral sides of the ophthalmic-maxillary part of the ganglion, with some overlap noted ventrally. The inferior alveolar

chromatolytic nerve cell bodies were closely arranged in a postero-lateral protuberance at the side of the ganglion, with the mental nerve cells concentrated in the dorsal part of this group.

Since this study evaluated the somatotopic organization of the trigeminal ganglion by observing the neuronal cell bodies from six separate teeth, 3 maxillary and 3 mandibular, the results may be more specific than those obtained by chromatolytic studies. This technique, utilized on an animal model such as the monkey, may result in a more accurate representation of the somatotopic organization of the trigeminal ganglion in humans.

A very interesting finding in the study was the constant presence of HRP-positive neurons in the control, unoperated side, ganglia. Contralateral presence of HRP was observed in each tooth treated and following either incubation technique. HRP-positive neurons were always present in the contralateral ganglia although usually in a greatly reduced number. This observation has received considerable attention in the literature and has led to quite a bit of controversy.

In Furstman's (21) study of the retrograde axonal transport of HRP from rodent pulps, he noted that no HRP-labelled cells were found in the contalateral trigeminal ganglion. In this article he does not state if he examined the contralateral ganglion as carefully as he did the experimental ganglion. He also utilized a less concentrated solution of HRP for injecting than in our study. These may be important factors to consider. Aldskogius and Arvidsson (22) did a study in which the trigeminal ganglia of normal rats and of adult rats subjected to

unilateral transection of the infraorbital nerve were studied by light and electron microscopy. The unoperated side did not show any signs of nerve cell or nerve fiber degeneration. Fuller, Wilson, and Winfrey (38) injected HRP into the mandibular canine teeth of nine cats. There were no HRP-positive cells in the contralateral ganglia. A possible problem in their study was that they left the HRP solution in the open cavity for 30-45 minutes before sealing the tooth. This could result in possible inactivation of the HRP, especially since they only used a 15% solution to begin with. Matthews and Lisney (55) recorded compound action potentials from the pulps of canine teeth in cats during stimulation of the inferior dental nerve, trigeminal ganglion or brain stem on one side. Recordings were also made from the inferior dental nerve while stimulating the canine pulps. Evidence was lacking for pulpal fibers crossing the midline.

There have been numerous articles in favor of transmedian innervation. Using electrophysiological studies Kerr and Lysak (6), Darian-Smith et al., (7), and Anderson and Pearl (56) have all reported transmedian innervation in the trigeminal system of the cat.

In Mazza and Dixon's (20) 1972 study of the chromatolytic cell groups in the trigeminal ganglion of the rat following unilateral nerve division of various branches, they indicated that some chromatolytic cells were found in the trigeminal ganglia on the unoperated side, but that this was probably a normal occurence and not related to the experimental procedure performed. They noted "Small neurons that demonstrated

some of the characteristic features of chromatolytic cells were observed in both the mandibular and superior labial areas. This type of cell, reminiscent of autonomic ganglion cells, was seen ubiquitously in both experimental and control ganglia and was not recorded as chromatolytic on the tracings." More recent studies using HRP indicate that these neuronal findings may actually be present due to transmedian innervation of dental pulps.

In 1977 Anderson and Rosing (37) published a study on the location of feline trigeminal ganglion cells innervating maxillary canine teeth using HRP. Following exposure of 8 maxillary canine tooth pulps to HRP solution by two different methods, they observed HRP granules in both ipsilateral and contralateral trigeminal ganglia. They noted that since there are approximately 44,000 cells in a feline trigeminal ganglion, their study indicated that about 0.37% of the cells in the trigeminal ganglion send peripheral processes to an ipsilateral canine tooth and about 0.22% of the cells supply a contralateral canine tooth.

Cox et al., (12) published an abstract in 1977 concerning a study of HRP transport from primate dental pulps. Five monkeys were used in the study. Localization of HRP was observed with both light and electron microscopy in the ipsilateral and contralateral trigeminal ganglia. He also noted that a group of 4 to 10 cells in the ipsilateral pons were also found to be HRP positive at the level of entry of the root of the trigeminal nerve in all the monkeys. This pontine localization seems to indicate a primary afferent innervation from cell bodies that are

located within the central neurons system, since no evidence of trans-synaptic migration of HRP has been proven.

The localization of HRP in the contralateral trigeminal ganglion as noted above as well as in the present study seems to indicate that there is a sensory overlap via various nerves supplying the dentition. From a clinical standpoint in the practice of dentistry these findings would seem to give some reason for those cases in which clinical signs of profound anesthesia are present, yet the patient still feels pain.

The studies indicate that HRP has great potential as a neuroanatomical research tool. They also indicate that we have only scratched the surface in our understanding of the organization and function of the trigeminal ganglion. Further research is necessary in this area.

As a final note concerning incubation procedures, Mesulam and Rosene (57) did an intensive study of the nine most popular methods for HRP neurohistochemistry and suggested that HRP sensitivity is determined by multiple factors which include the method of fixation, post-fixation storage, the choice of chromogen, the incubation parameters, the type of HRP enzyme that is administered, and the postreaction treatment. It is very important that preliminary tests be run to determine these factors for the animal model as well as the neural tissues to be studied before a full investigation is begun.

CHAPTER VI

SUMMARY AND CONCLUSIONS

In an effort to map out the somatotopic organization of the trigeminal ganglion of the rat, a protein tracer (horseradish peroxidase) was injected into the dental pulps of twenty-four albino Sprague-Dawley rats. The protein tracer was allowed sufficient time to travel in a retrograde manner to the neuronal cell bodies of origin in the trigeminal ganglia. Following sacrifice of the animals, frozen sections were prepared of the experimental (injected side) and control (unoperated side) trigeminal ganglia. The frozen sections were then run through two separate incubation procedures for the visualization of HRP granules. Following the incubation procedure, the sections were observed under light microscopy and the somatotopic organization of the trigeminal ganglion of the rat described as accurately as possible.

Under the conditions of this experiment, the following conclusions could be drawn:

a.) The feasibility of tracing first order afferents via the retrograde axonal transport of HRP was reconfirmed.

b.) The use of the dental pulp as an injection site for HRP was excellent for determining neuronal cell bodies of origin for the various teeth.

c.) The neuronal cell bodies of origin of the fibers innervating the various mandibular teeth are distributed throughout the entire mandibular division of the trigeminal ganglion.

d.) The neuronal cell bodies of origin of the fibers innervating the various maxillary teeth are distributed in a linear array throughout the length of the ophthalmic-maxillary division of the trigeminal ganglion.

e.) The presence of HRP-positive neurons in both the experimental (injected side) and control (unoperated side) trigeminal ganglia was a consistent occurrence, indicating a possible transmedian innervation in the rodent dentition.

f.) The use of 3,3'-diaminobenzidine along with the addition of cobalt chloride for enhancement allowed for excellent visualization of the HRP granules.

g.) The Hanker/Yates technique for the incubation of HRP gave positive results, but not of the intensity obtained with DAB.

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FIGURES

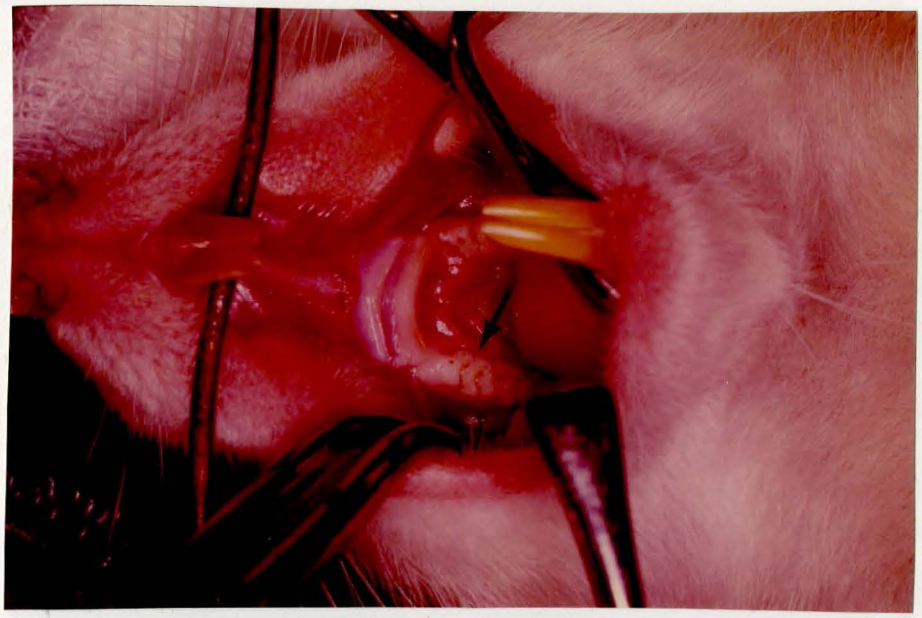

Figure I
Maxillary Right First Molar → Pre-operatively

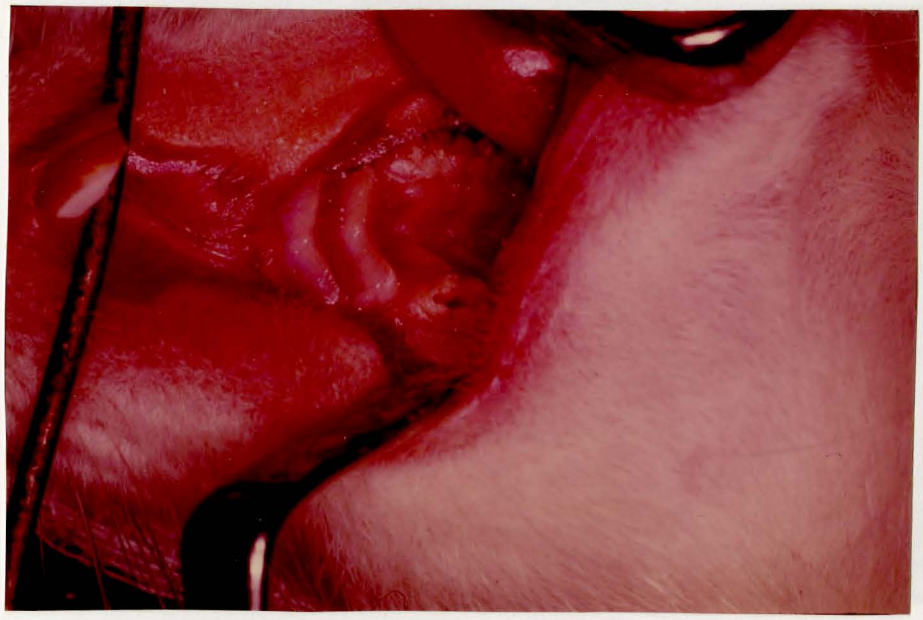

Figure II

Maxillary Right First Molar → Pulpal Access
Note occlusal access used and the presence of hemorrhage indicating that the pulp has been reached

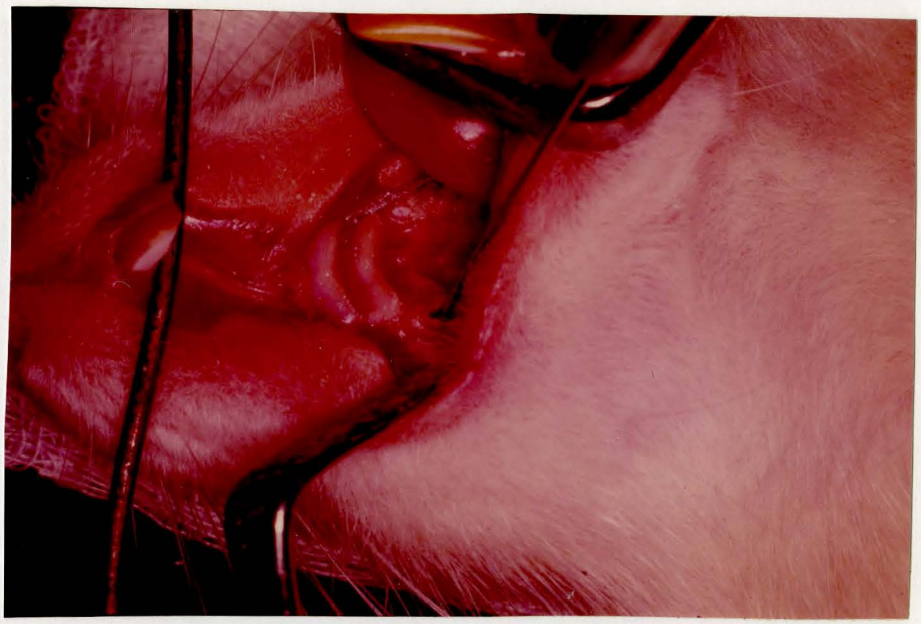

Figure III
Maxillary Right First Molar → Microsyringe in place

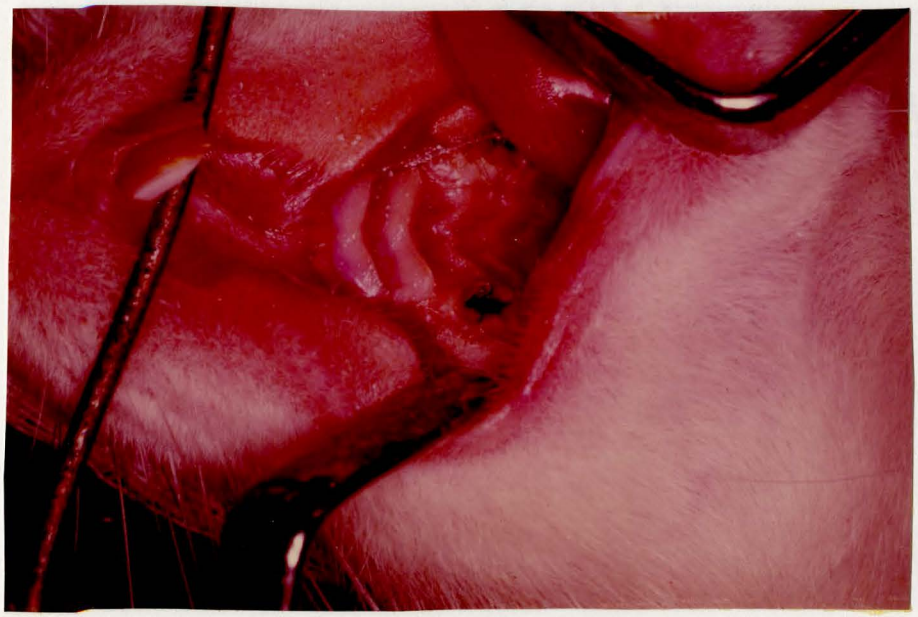

Figure IV
Maxillary Right First Molar → HRP in pulp chamber

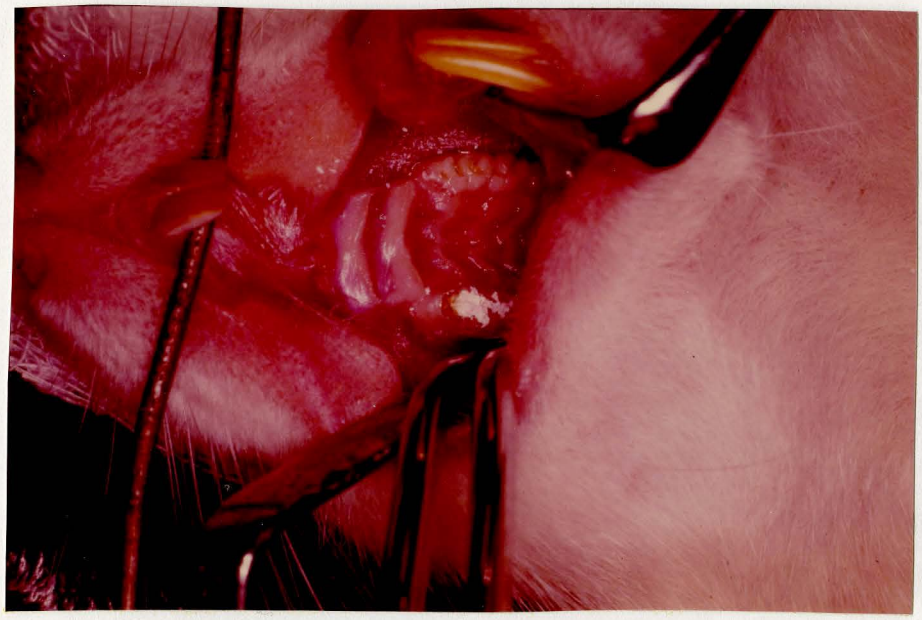

Figure V
Maxillary Right First Molar → Temporary filling in place

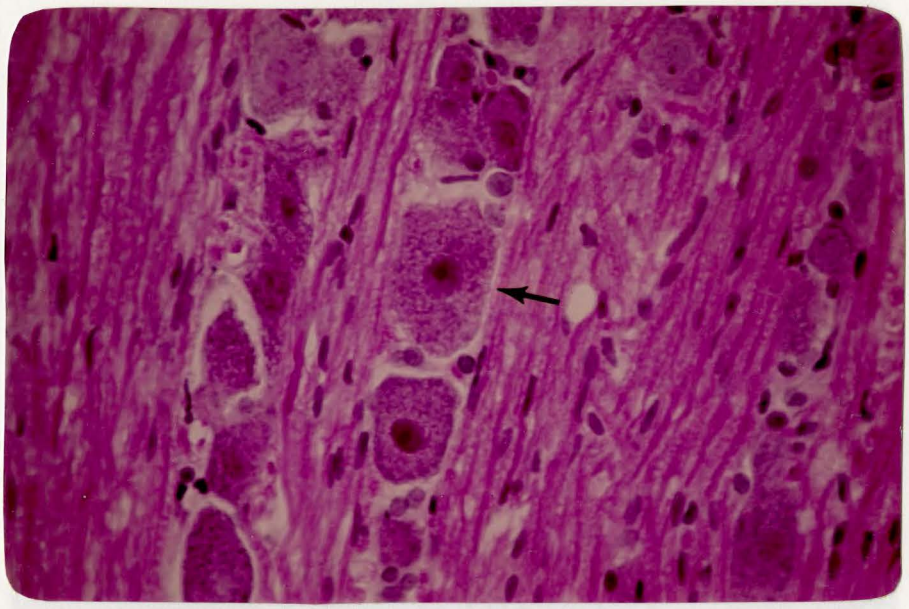

Figure VI
Rat Trigeminal Ganglionic Neurons
Hematoxylin and Eosin Stain (126x)
Note large centrally placed nuclei and
coarsely granular cytoplasm

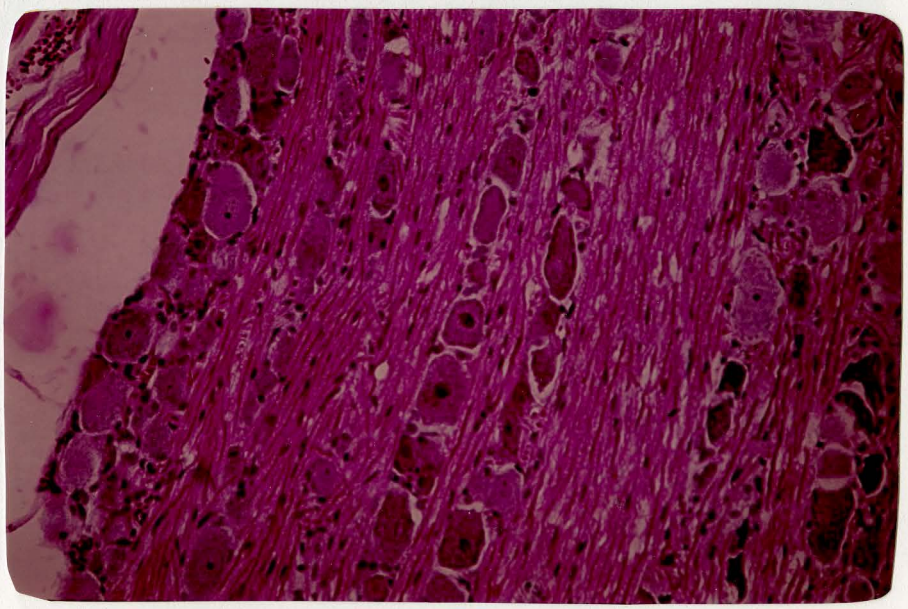

Figure VII
Linear Arrangement of Trigeminal ganglionic neurons found in the ophthalmic-Maxillary division. Hematoxylin and eosin stain (56x)

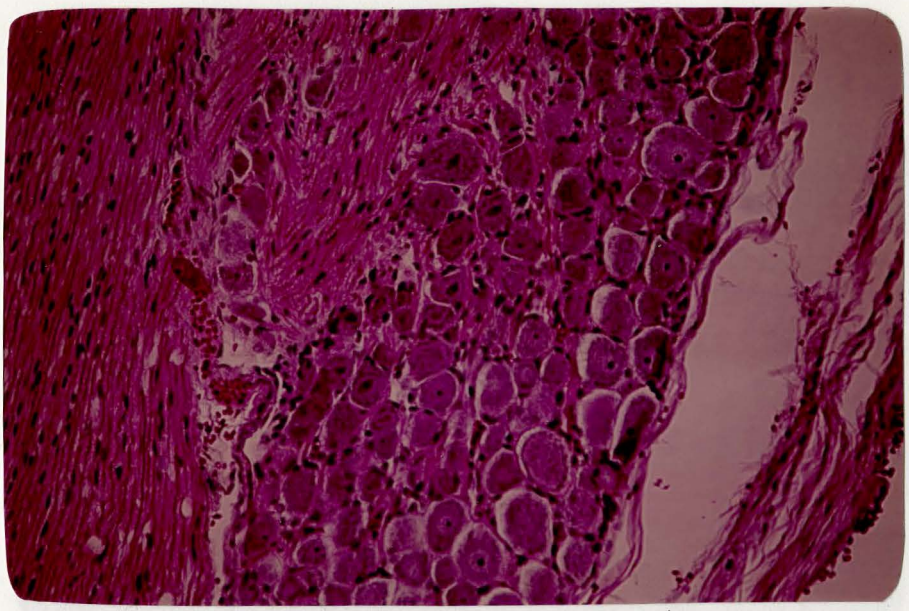

Figure VIII
Aggregation of trigeminal ganglionic neurons found in the lateral protuberance area of the mandibular division. Hematoxylin and eosin stain (56x)

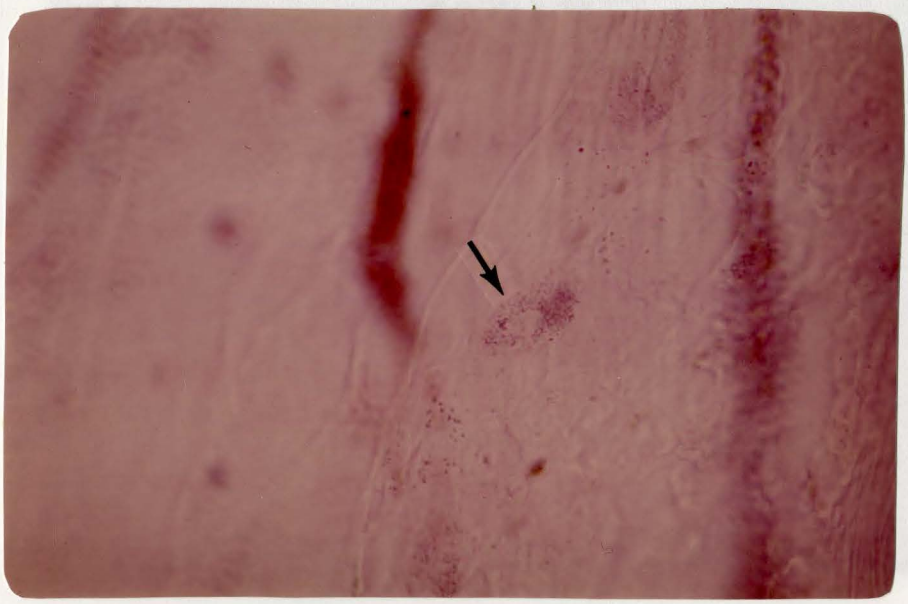

Figure IX
Isolated neuron with positive HRP granules present in cytoplasm. Graham and Karnovsky incubation procedure (126x).

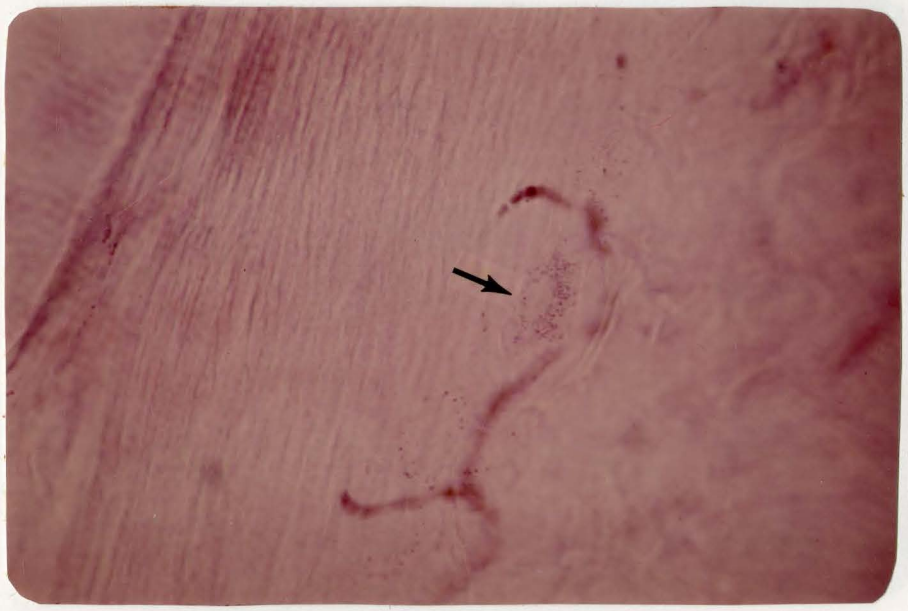

Figure X
Isolated neuron with positive HRP granules present in cytoplasm. Graham and Karnovsky incubation procedure (126x).

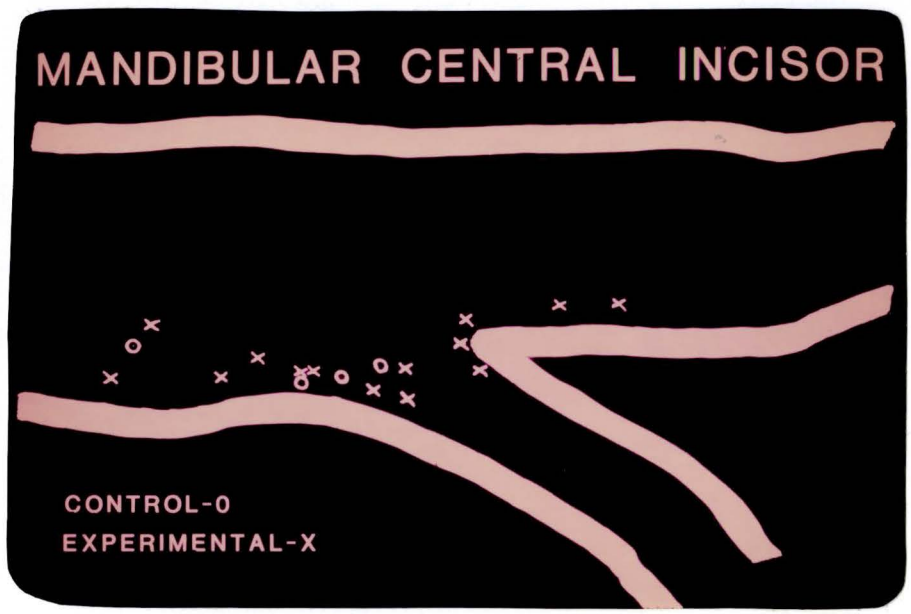

Figure XI
Mandibular central incisor → composite HRP diagram.

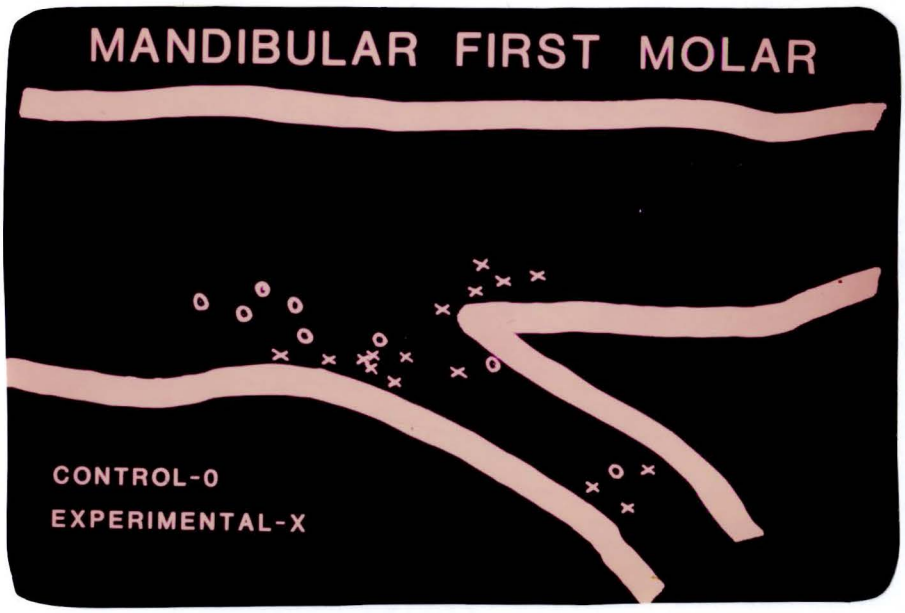

Figure XII
Mandibular first molar → composite HRP diagram.

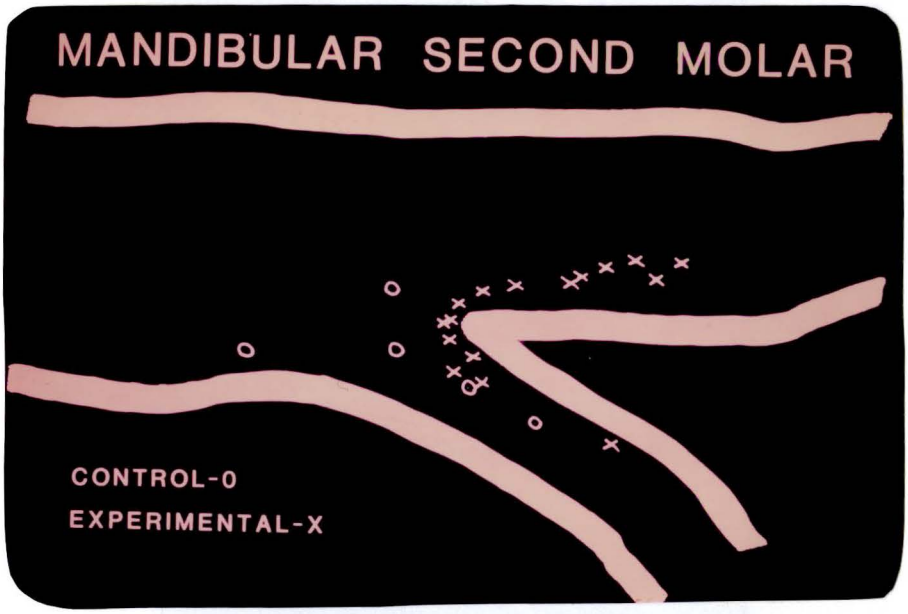

Figure XIII
Mandibular second molar → composite HRP diagram.

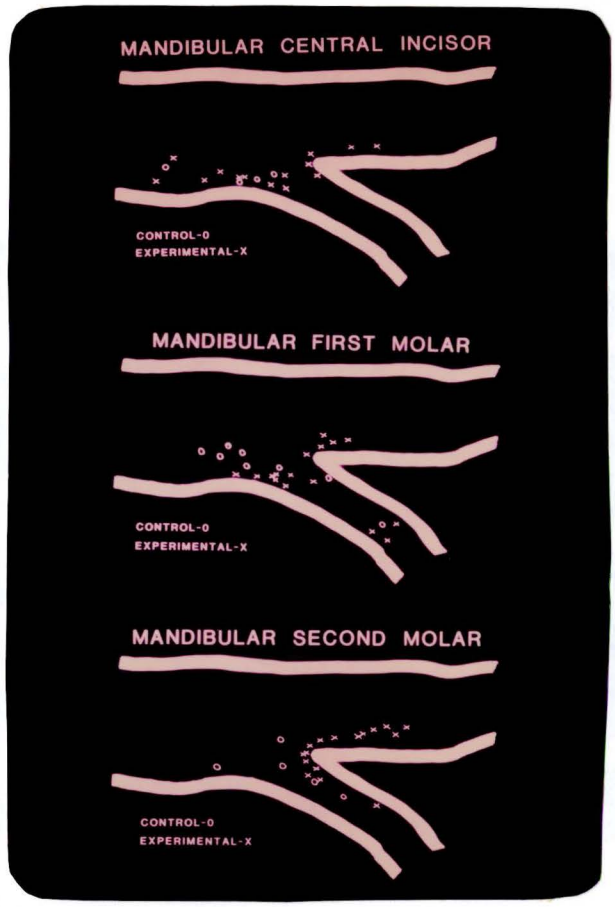

Figure XIV
Composite diagrams of HRP positive neurons found in each of the mandibular teeth examined in the study.

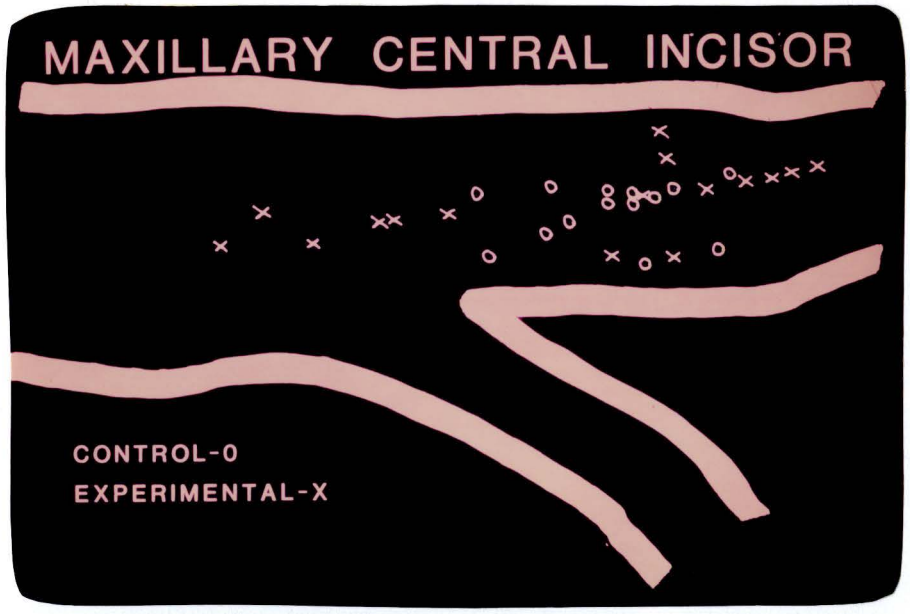

Figure XV
Maxillary central incisor → composite HRP diagram.

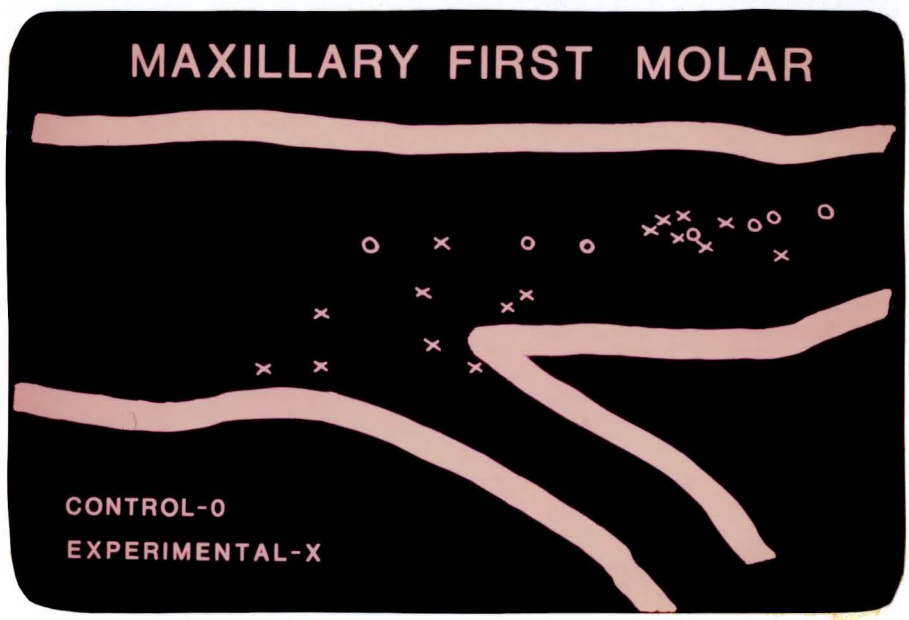

Figure XVI
Maxillary first molar → composite HRP diagram.

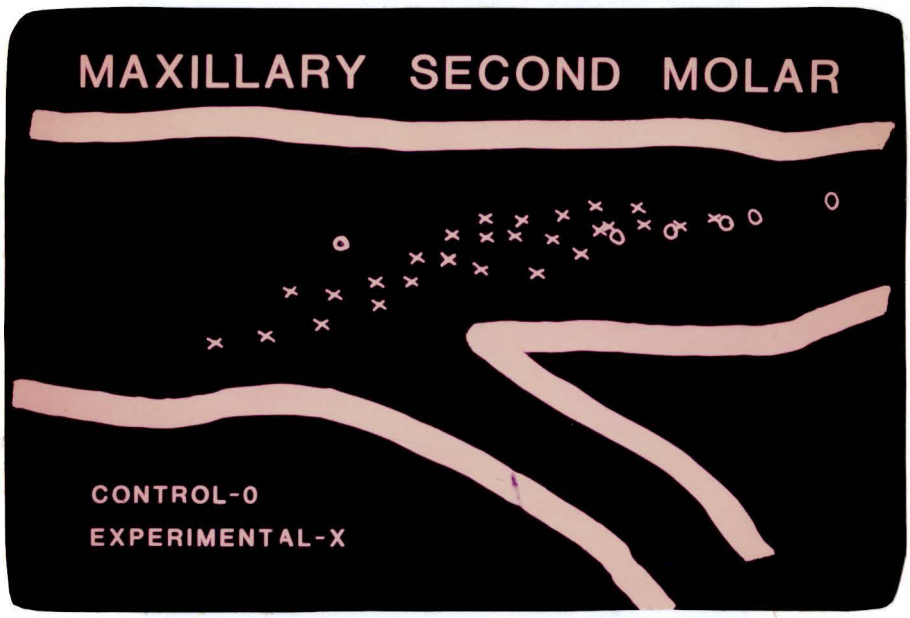

Figure XVII
Maxillary second molar → composite HRP diagram.

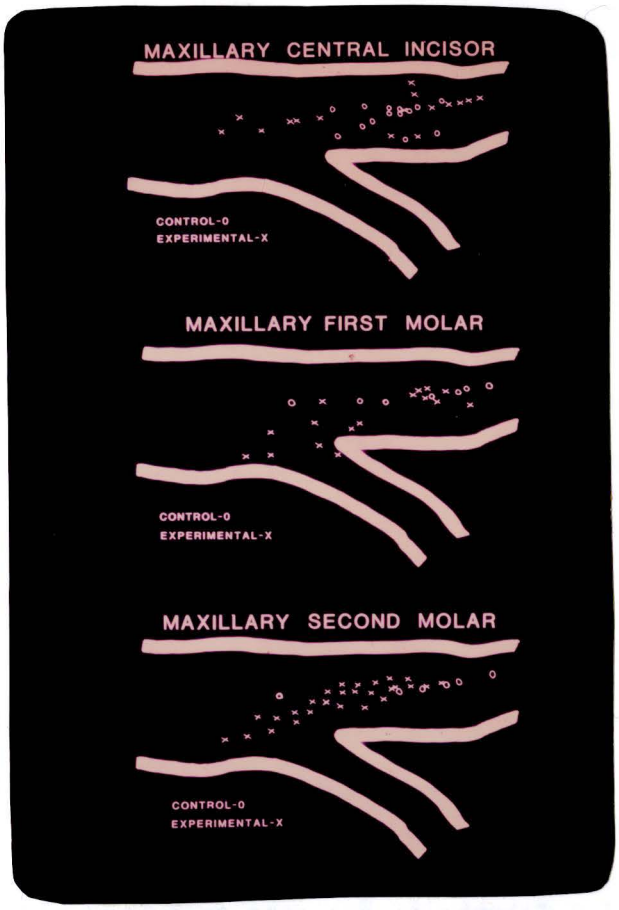

Figure XVIII
Composite diagrams of HRP positive neurons found in each of the maxillary teeth examined in the study.

APPROVAL SHEET

The thesis submitted by Scott B. Shellhammer, D.D.S., has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

April 7, 1970
Date

Joseph M. Gowgiel
Director's Signature