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THE EFFECT OF CAPSAICIN SENSORY PRIMARY AFFERENTS
ON THE ERUPTION RATE OF RATS' MANDIBULAR INCISORS

By

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A thesis

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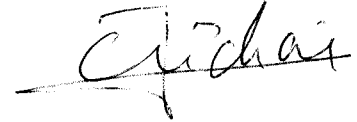
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ABSTRACT OF THESIS

Thesis Title: The effect of capsaicin sensory primary afferents on the eruption rate of rat's mandibular incisors

By: Marianne EL HAGE

Major: Human Morphology

Background and Aims: Tooth eruption is defined as the movement of a tooth from its site of development within the alveolar process to its functional position in the oral cavity. Several studies aimed to investigate the neural effect on the eruption mechanism. Most of reported results were descriptive based on clinical observations and were not supported by experimental evidence. The present study aims to investigate (1) the possible involvement of the sensory nervous system in the eruption rate of mandibular incisors in rats, and 2) the possible involvement of substance P in this process.

Materials and methods: Rats (adult female Sprague-Dawley) were divided into 5 groups; group 1, (n=7) had no chemical or surgical intervention (control group); group 2, (n=7) was subjected to the exposure and sectioning of the inferior alveolar nerve (**IAN**); group 3, (n=6) was subjected to selective ablation of the sensory afferent fibers to the incisor by topical application of capsaicin on the left IAN; group 4, (n=5), was the sham capsaicin group, where the capsaicin solvent was applied to the left IAN; group 5, (n=7) consisted of rats subjected to systemic ablation of their capsaicin sensitive primary afferents (**CSPA**); group 6, (n=8) was subjected to daily treatment with substance P antagonist, Spantide II.

The method adopted to measure tooth eruption was based on two fixed reference points; the first reference was localized, with a groove, at the junction of the buccal and distal surface of the tooth and the second was a tattoo mark on the gingiva at the tooth base. A digital caliper (accuracy 0.01mm) was used for measurements, which were performed every 48hr over a period of 144 hours. Each measurement was repeated three times on each rat. Rats were sacrificed under deep anesthesia and the brainstem of rats of three groups (Control, Spantide II, and systemic capsaicin ablation) were collected and processed for the determination of substance P by Immunohistochemistry (IHC). The result obtained for each time point was presented as the mean and standard error of the mean (SEM) of measurements made on all rats in the same experimental group. Statistics were made using GraphPad Instat 3, and the significance of variations were calculated.

Results: A general trend of reduction in the rate of eruption was observed in all groups. Significant reduction was observed in group 2 during the second (48-96hrs) and the third (96-144hrs) time segments, ($0.44 \pm 0.13\text{mm}$) and ($0.47 \pm 0.11\text{mm}$), respectively compared to control ($0.79 \pm 0.15\text{mm}$) and ($1.02 \pm 0.14\text{mm}$). Same pattern of attenuation was noticed in group 3 ($0.41 \pm 0.13\text{mm}$, $p < 0.01$) as compared to the control ($1.08 \pm 0.09\text{mm}$), as well as in group 6 ($0.73 \pm 0.08\text{mm}$, $p < 0.05$) compared to control ($1.08 \pm 0.09\text{mm}$) at time point 48-96 hrs group 5 showed a significant attenuation of the eruption rate of the mandibular incisors at the initial time segment ($0.64 \pm 0.04\text{mm}$, $p < 0.001$) as compared to the control group ($1.18 \pm 0.15\text{mm}$). IHC results showed a marked attenuation of substance P immunoreactivity in group 6 and a complete absence of immunoreactivity in G6 as compared to control.

Conclusion: Capsaicin sensitive primary afferents play a major role in the control of the eruption process; however, their absence does not produce a permanent impairment.

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LIST OF ABBREVIATIONS

APES:	Amino-propyl-tri-ethoxy-silane
BSA:	Bovine serum albumin
cAMP:	Cyclic AMP
CSF-1:	Colony stimulating factor 1
CGRP:	Calcitonin gene-related peptide
CSPA:	Capsaicin sensitive primary afferents
DAB:	Diaminobenzidine
DF:	Dental follicle
GAL:	Galanin
IAN:	Inferior alveolar nerve
IHC:	Immuno-Histochemistry
OPG:	Osteoprotegerin
RANKL:	Nuclear factor KB ligand
SEM:	Standard error of the mean
SP:	Substance P
SOM:	Somatostatin
TBS:	Tris-buffered saline
TRAP+:	Tartrate-resistant acid phosphatase-positive
VIP:	Vasoactive intestinal peptide

I. Introduction

A. Definition of eruption

Tooth eruption is a complex and tightly regulated process that involves cells of the tooth organ and the surrounding alveolus. It is defined as the vertical migration of a tooth from the developing alveolar bone to its functional position in the oral cavity (Massler and Schour, 1941).

In the past, various hypotheses on the mechanisms of tooth eruption have been suggested. Four basic mechanisms have been described as the major events underlying tooth eruption; these include:

1. Root formation (root elongation).
2. Changes in the hydrostatic pressure within the peri apical tissues pushing the tooth occlusally.
3. Pulling of the tooth in an occlusal direction by the cells and fibers of the periodontal ligament.
4. Alveolar bone remodeling.

B. Hypotheses of Tooth Eruption

1. Root formation:

Since root formation occurs at the time of eruption, it has long been considered the force responsible for eruption (Massler and Schour, 1941). However, root elongation cannot be expected to move a tooth in three-dimensional space. Yet rootless teeth do erupt. Several clinical studies have reported the presence of unerupted teeth with normal root development (Witkop, 1975). As illustration, Marks and Cahill (1984), showed that the tooth itself had no part in the eruptive process. In this study tooth germs

were removed and replaced with a metallic replica. The follicular changes and the path of eruption were similar to those in normal conditions and the metallic replica erupted. Therefore rootless teeth do erupt. In other words, root formation per se is not a mandatory requirement for tooth eruption. It may, however, accelerate eruption speed.

2. Hydrostatic pressure:

Several studies demonstrated the presence of a differential hydrostatic pressure between the tissues surrounding the erupting tooth. Van Hassel and McMinn (1972) have shown that the tissue pressure apical to the erupting tooth was greater than the occlusal pressure, hypothetically generating an eruptive force. However, no correlation was found between the magnitude of the force and the rate of eruption.

3. Periodontal ligament:

Formation and renewal of the periodontal ligament is associated with the continuous eruption of permanently growing rodent incisors (Berkovitz and Thomas, 1969; Berkovitz, 1971). However, for teeth with a limited period of growth, the presence of a periodontal ligament does not ensure eruption; it starts in the supra-osseous phase after the tooth has become out of the bone. In some conditions like osteopetrotic mutations (Marks, 1989) the periodontal ligament is present, but teeth do not erupt; while in cases of dentinal dysplasia type I, characterized by rootless teeth and absence of periodontal ligament, eruption occurs (Witkop, 1975; Marks, 1989; Shields et al., 1973). Therefore, the periodontal ligament cannot be considered as a key player in the tooth eruption process.

4. Alveolar bone remodeling:

Formation of bone apical to the tooth bud has long been proposed as one mechanism causing tooth eruption. The alveolar process forms during tooth development, and is locally deficient in sites where teeth fail to develop (Brash, 1928; Landsberger, 1924). Clinical situations such as in osteopetrotic mutations and cleidocranial dysplasia questioned the validity of the alveolar bone remodeling hypothesis in eruption since teeth formed but did not erupt, or erupted later and in different places (Marks, 1989; Jensen et. al, 1990).

In conclusion, none of the previously cited mechanisms can account alone for the complete process of tooth eruption.

C. Potentials of Animal Studies:

Several studies on the mechanisms of tooth eruption have been conducted on different species (rabbits, rats etc.). Some of these species are characterized by continuously growing teeth. Three distinct types of mammalian tooth eruption have been described (Steedle and Proffit, 1985):

1. Continuously growing teeth characterized by lack of distinction between the crown and the root structures. Attrition of the incisal border is continuously compensated for, at a rapid rate, by new root formation.
2. Continuously extruding teeth, where teeth will ultimately extrude from the alveolar supporting tissues, exposing an increasing amount of root surface. In animals having this type of eruption, attrition results in the eventual loss of teeth towards the end of their life.

3. Continuously erupting teeth, where the alveolar supporting structures follow the occlusally directed development of the tooth without any exposure of root surface.

When wear takes place, the occlusal level may remain constant, with the alveolar bone coming closer to the occlusal plane.

D. Requirements for Tooth Eruption

In order for a tooth to erupt, two requirements are needed, the dental follicle and the bone turnover.

1. Dental follicle for eruption

During the early 1980s several studies have been carried out to define which tissue was needed for eruption. Experiments in which the dental follicle (**DF**), a loose tissue sac that surrounds the unerupted tooth, was surgically removed resulted in a failure of tooth eruption (Landsberger, 1924). More dramatically, when the DF was left intact but the tooth replaced with a metallic replica, that replica did erupt (Marks, 1989). Other studies revealed regional differences in the DF; as illustration, when the coronal one-half of the follicle was removed but the basal (apical) one-half was left intact, alveolar bone resorption did not occur and the tooth did not erupt (Cahill and Marks, 1980). On the other hand, when the basal one-half of the DF was removed and the coronal one-half left intact, alveolar bone resorption occurred but the tooth did not erupt due to the absence of alveolar bone formation at the base of the crypt. Thus, it was concluded that the coronal region of the DF regulates the osteoclastogenesis and bone resorption needed for eruption whereas the basal one-half regulates the osteogenesis and bone formation needed for eruption.

2. Turnover of adjacent alveolar bone

Bone modeling and remodeling are important in forming the shape and maintaining the balance in the bony skeleton. Bone turnover and remodeling are mediated through osteogenesis and osteoclastogenesis. Development and differentiation of osteoblasts and osteoclasts are locally controlled by growth factors and cytokines produced in the bone marrow microenvironment, as well as by adhesion molecules that mediate cell-cell and cell-matrix interactions. Signals derived from the endocrine and autonomic nervous systems also exert potent effects on osteoclast and osteoblast development and differentiation (Cardinali et al., 2003).

Various molecules control bone turnover, and are in turn controlled by different mechanisms including various neurotransmitters and substances involved in their release and expression. These molecules vary in their role; some molecules play a key role in the osteoclastogenesis process (e.g. RANKL), while others function in modulating the actions of those major molecules (e.g. CSF-1, OPG, MCP-1 and others). A member of the TNF ligand family, a receptor activator of nuclear factor KB ligand (RANKL) present on the osteoclast precursors, along with its receptor (RANK) present on the surface of the osteoblast stromal cells, plays a major role in osteoclastogenesis. Intercellular signaling involves the binding of RANKL to RANK which results in recruitment of various members of TNF receptor-associated factors (TRAFs) within the osteoclast precursor. As RANKL bind to RANK, preosteoclasts are promoted, which result in osteoclast formation, fusion, activation and survival, and then resulting in bone resorption (Oshiro et. al, 2002). Figure 1 summarizes the major biological actions of the OPG/RANKL/RANK system.

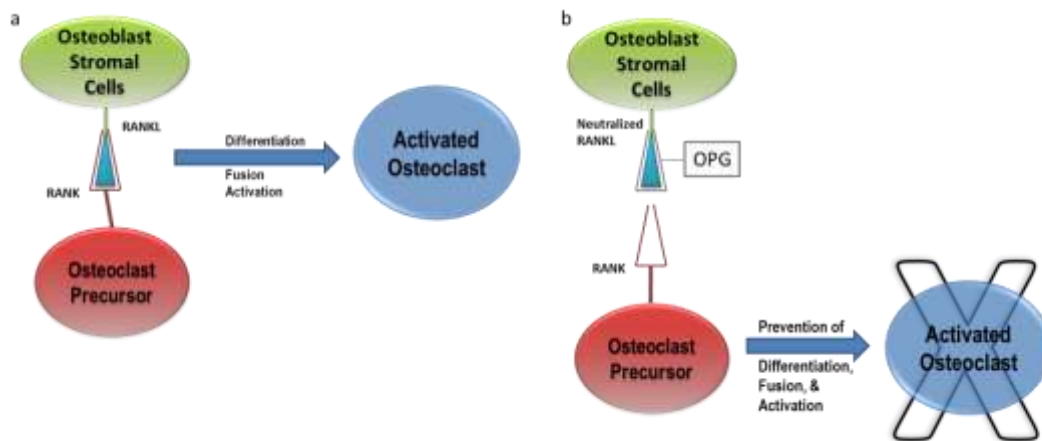


Fig.1: The major biological actions of the OPG/RANKL/RANK system:
a) activation of osteoclast precursors by binding of RANKL and RANK, b) neutralization of RANKL by OPG and prevention of RANKL-RANK interaction.

Colony stimulating factor 1 (CSF-1) up regulates RANK gene expression in osteoclast precursors (Cielinski et. al, 1994), and has chemotactic role in recruiting osteoclast precursors (Que et. al, 1997; Volejnikova et. al, 1997). The growth and differentiation of mononuclear pre-osteoclasts are also dependent upon CSF-1 (Sandy et. al, 1995).

Negative feedback signaling and mechanism for osteoclastogenesis is necessary to maintain balance. Osteoprotegerin (OPG) plays a major role in controlling the effect of RANKL; binding of OPG to RANKL prevents the binding between the latter and RANK on the cell membrane of osteoclast precursors, which will inhibit osteoclastogenesis (Kwon et. al, 1998).

The effect of the nervous system on tooth eruption is mediated through the control of osteogenesis and osteoclastogenesis that are required for tooth eruption. Osteoclasts are needed to resorb the alveolar bone as the tooth erupts. The formation of eruption pathways is considered a distinctive feature in bone. Even if the tooth is stopped from eruption, the pathway still forms (Cahill, 1969a; Wise et. al, 2008). Osteoclasts

responsible of creating this pathway arise from the dental follicle at specific time prior to eruption (Wise et. al, 1989). Bone resorption is essential in tooth eruption, and therefore, the interference with the resorptive process leads to the inhibition or the slowing down of eruption (Cielinski et al., 1994). Figure 2 summarizes the molecular regulation of osteoclastogenesis for eruption.

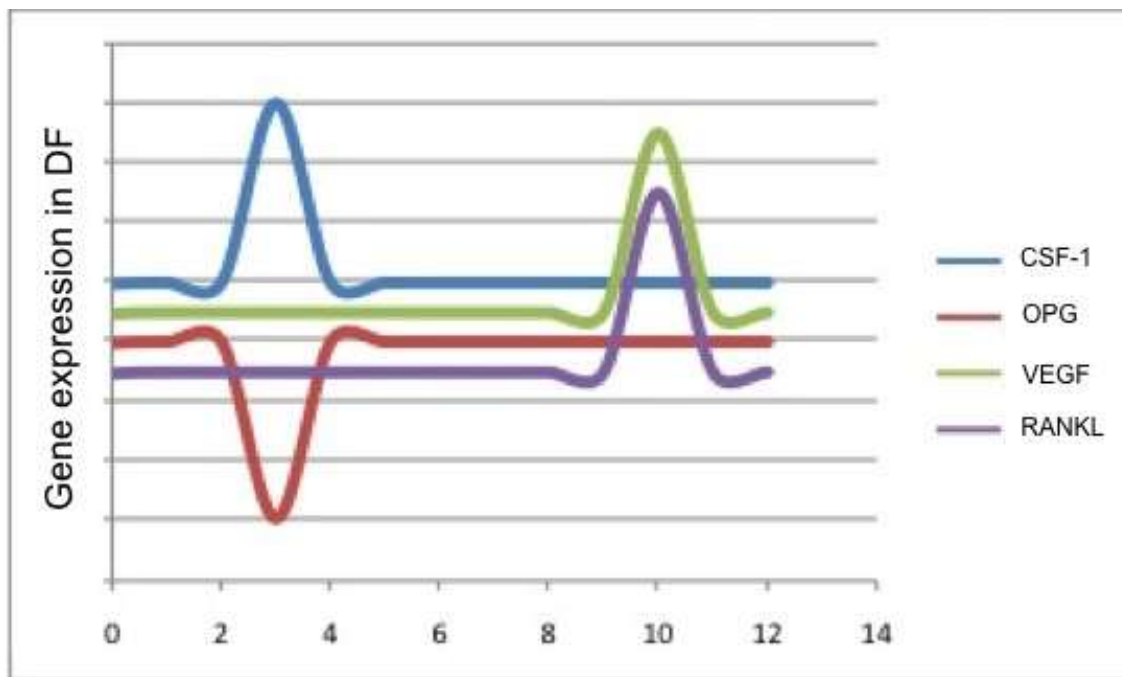


Fig.2: Expression of genes in the dental follicle of the rats' incisor at various days post-natally.

At day 3, the major burst of osteoclastogenesis, osteoprotegerin is downregulated such that a favorable RANK/osteoprotegerin ratio is established to promote osteoclastogenesis. At this time, CSF-1 is maximally expressed, both to down-regulate osteoprotegerin expression and to promote osteoclast precursor recruitment and osteoclastogenesis. At day 10, the minor burst of osteoclastogenesis, VEGF is maximally expressed to stimulate RANK expression on osteoclast precursors, as well as to interact with RANKL and CSF-1 to promote osteoclastogenesis. At this time, RANKL is up-regulated such that a favorable RANKL/osteoprotegerin ratio could exist to stimulate this minor burst of osteoclastogenesis.

E. Neuronal Regulation of Bone Turnover

Bone regulates its own metabolism according to systemic and local variations such as hormones and growth factors. Injuries to the central and peripheral nervous systems have harmful effects on the bone metabolism in various clinical (Chantraine et al., 1986) and experimental (Hill et al., 1993) situations. Both sensory and sympathetic nervous system are directly involved in bone metabolism through the dense network of nerve fibres supplying the periosteum, the bone marrow and the osteochondral junction of the growth plate (Bjurholm et al., 1988; Hill et al., 1991; Hukkanen et al., 1992).

1. Effect of Substance P (SP)-containing nerve endings on bone turnover

Regardless of the abundant bony innervation, the physiological effect of the sensory innervation in bone is still not clearly elucidated. Some nerve-derived factors, called neuropeptides, have been identified for their role in regulating bone metabolism. Substance P (SP) belongs to this category of neuropeptides. It is abundantly distributed in the sensory nerve fibers (Hokfelt et al., 2000) along with other neuropeptides such as Calcitonine Gene Related Peptide (CGRP). The SP-containing nerve fibers innervate the medullary tissues of bone as well as the periostium and may be found in the bone marrow in association with blood vessels (Imai et al., 1997). Substance P is thought to be involved in a cascade of immune responses and may be a chronic mediator of bone metabolism (Lundy et al., 2004; Abd El-Aleem et al., 2004). Resembling CGRP, SP promotes cAMP production by osteoclasts (Bjurholm et al., 1992) and stimulates bone formation (Shih et al., 1997). However, CGRP and SP seem to regulate the activity of osteoclasts. The osteoclasts usually express neurokin-1 receptors, through which SP

induces transduction signals (Gotoh et al., 1998). SP promotes the differentiation of osteoclasts in periodontal tissue due to the fact that the action of SP was observed in the gingival cervical fluid in periodontitis patients (Lundy et al., 2000). In human periodontal ligament cells, continuous SP secretion by sensory neurons in painful chronic periodontitis or tooth movement increases RANKL expression and decreases OPG expression, thus promoting osteoclastogenesis (Lundy et al., 2000).

2. Effect of sensory denervation on bone turnover

Several attempts of selective denervation have been adopted to define and clarify the role of neuropeptides, specifically SP, in bone physiology. A number of methods have been used; including the surgical destruction of the related anatomical structures such as the inferior alveolar nerve and chemical denervation using selective neurotoxins. For example, neurotoxins such as capsaicin led to the systemic depletion of sensory fibers (Imai et al., 1994; Wimalawansa, 1993). The effects of denervation on bone metabolism differ depending on the chemical substance used. The effect of sensory denervation on bone metabolism is manifested as a downregulation of osteoclastic activity thus reducing resorption (Imai et al., 1994).

F. Effect of Drug- Induced Sensory Denervation on Osteoclastic Resorption

1. Capsaicin

a. Definition and medical use

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the major pungent ingredient of hot peppers of the plant genus *Capsicum*, has been known for many years to alter certain sensory functions in laboratory animals. The first study on the pharmacological effects

of capsaicin reported that it produced a burning sensation with hyperemia when dropped on the skin (Monsereenusorn et al., 1982). Currently, the effects of capsaicin are thought to be dependent on the method of application (systemic or topical), dosage, age of the animals at the time when capsaicin was administered and differences in animal species. Topical application produced a localized inflammatory reaction apparently accompanied by painful irritation after which there was an insensitivity of the area to chemical irritants. Systemic administration of capsaicin produced a similar insensitivity that lasted from several weeks to months in the treated animals. This neurotoxin effect has apparently, resulted from a selective effect of the compound on primary afferent neurons mediating certain kinds of peripheral stimuli.

b. Mechanism of action

In the rat, capsaicin markedly reduced the sensitivity to nociceptive chemicals without affecting responsiveness to touch or mechanical or thermal pain (Jancso, 1968). This action on sensory neurons in the adult rat was reported to be accompanied by alterations in mitochondrial structure and depletion of cytoplasmic vesicles in peripheral processes of sensory neurons and in type B dorsal root ganglion cells. The morphological changes were long-lasting, but no evidence of actual neuronal degeneration was showed (Joo et al., 1969; Szolcsanyi et al., 1975). Systemic treatment of neonatal rats with capsaicin, however, produced a permanent insensitivity to chemical irritation that was accompanied by degeneration of small sensory neurons (Jancso et al., 1977).

Regarding the effect of capsaicin on bone metabolism, studies have reported a decrease of 21% of bone resorption in adulthood, when rats were treated with capsaicin

at birth (Hill et al., 1991). On the other hand, Adam et al. (1999), marked a relatively higher rate of reduction in resorption (by 40 %), after capsaicin treatment administered to adult rats. This difference was explained by the fact that the destruction of the sensory innervation at birth was compensated for with time (Hill et al., 1991) or that the sensory nervous system had a minor contribution to bone resorption.

Other studies, however, have shown that the number of osteoclast precursors relative to the total osteoclast population was greatly reduced in the capsaicin-treated group (Adam et al. 1990). This indicates that sensory innervation is involved in the recruitment and/or differentiation of osteoclast precursors.

Moreover, monocytic cells, whose lineage belongs to the osteoclasts, express receptors for substance P (Ho et al., 1997); it is thus likely that the systemic reduction in substance P contributed to the disturbances in osteoclast precursor recruitment and differentiation.

2. Spantide

a. Definition and Medical use

Spantide was introduced as a tachykinin antagonist in 1984 and has served as a starting point in the design of new antagonists and has proved to be more effective with no neurological side effects. It has been used to study the role of action of tachykinins in peripheral and central nervous system (Folkers et al., 1984; Fujino et al., 1987; Togashi et al., 1987).

b. Mechanism of action

Spantide, is one of the most effective tachykinin antagonists in the spinal cord (Rosell et al., 1983; Folkers et al., 1984), and has often been used to examine possible neurotransmitter roles of tachykinins in the peripheral and central nervous systems (Folkers et al., 1984; Fujino et al., 1987; Bartho et al., 1989). Several studies suggested that spantide is an antagonist with preference for NK1-tachykinin receptors with some affinity to NK2 receptor, (Chahl, 1985; Buck, 1988; Maggi et al., 1991; Bartho et al., 1989) without causing any neural damage or signs of motor impairment or neurotoxicity.

Intrathecal administration of Spantide usually causes weak facilitation of the flexor reflex, especially at lower doses (10–100 ng) and at higher doses (1–10 µg) it sometimes depressed the reflex (Wiesenfeld-Hallin et al., 1990). Pre-treatment with spantide (1, 3 or 10 µg) effectively antagonized the facilitatory effect of SP on the flexor reflex for about 30 min (Wiesenfeld-Hallin et al., 1990). The facilitation of the reflex induced by intrathecal administration of other neuropeptides present in primary afferents, such as somatostatin (SOM), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and galanin (GAL), was not influenced by spantide (Wiesenfeld-Hallin et al., 1990).

G. Aim of the study

The aim of the study is to investigate the possible involvement of the sensory nervous system in the eruption rate of mandibular incisors in rats.

In an attempt to determine the effect of capsaicin sensitive primary afferents (CSPA) on their targeted bone and alveolae. Previous studies have suggested an increase in bone resorption after treatment with capsaicin (Hill et al., 1991), others after performing neurotomies found out different an increase in resorption and thus an increase in eruption rate (Sandhu et al., 1987). Our study aimed at investigating the involvement of the sensory nervous system in the eruption rate of mandibular incisors by targeting the capsaicin sensitive primary afferents (CSPA) through either systemic ablation of sensory fibers using capsaicin, total denervation of the inferior alveolar nerve, topical application of capsaicin on the inferior alveolar nerve or treatment with Spantide II, tackykinin NK1 antagonist.

This study also aimed at investigating the effect of substance P on the rate of tooth eruption. As the resorption process is mediated by the osteoclasts, control of osteoclasts differentiation might affect the eruptive process. We hypothesized that the effect of the CSPA on osteoclasts can be mediated through the activation of RANKL system by the release of SP from the sensory nerve endings.

II. Material and Methods

A. Animals

All experiments were performed on adult Sprague–Dawley female rats (250–300 g) kept in groups in plastic cages under standard colony conditions (12 h dark/light cycle, temperature 22 ± 2 °C) and free access to food and water. All procedures were carried out with strict adherence to the guidelines for pain experimentation in animals (Zimmermann, 1983) and were approved by the Institutional Animal Care Committee. Experiments were conducted during day hours, between 8 AM and 6 PM.

B. Experimental groups

All rats were chosen for the experiment with the mandibular incisor completely developed and intact. Rats were divided into 5 groups; Group 1 (n=7) was composed of rats who did not undergo any chemical or surgical intervention and it served as a control group. Group 2 (n=6) consisted of rats that had the sensory fibers of the left inferior alveolar nerve (IAN) selectively blocked by topical application of capsaicin on the exposed left inferior alveolar nerve. Group 3 (n=5) was the sham capsaicin rat group, where the capsaicin solvent was applied to the left IAN. Group 4 (n=7) consisted of rats subjected to systemic ablation of their capsaicin sensitive primary afferents and then treated with Guanethidine; Group 5 (n=8) consisted of rats treated with Spantide II.

In all the rats, except for the groups that received systemic administration of the drug, the right (R) side served as the control side and the left (L) side as the experimental side.

C. Experimental procedures

The experiments were performed under deep general anesthesia achieved by injection of atropine (Atropine sulfate, Laboratoire Aguetant) (dilution 1:10 in saline, 0.05mg/kg) and chlorpromazine (Largactil®, 8mg/kg) intra-peritoneally (i.p.), followed 10 min later by intraperitoneal (*i.p.*) injection of ketamine (Ketalar®, 50mg/kg).

1. Exposure of the inferior alveolar nerve (IAN)

The technique adopted for the exposure of the IAN was similar to that described by Dreyer and Rerief (1969) with some modifications to satisfy some experimental demands.

Three imaginary lines were identified on the left side of the rat's face (Fig.3); (1) line A-B; (2) E-F runs parallel to A-B line; (3) C-D line runs in the middle between the A-B and E-F, and is where the incision will be performed; (4) G-H line bisects the C-D line and represents the first point of access to the masseters muscle fibers.

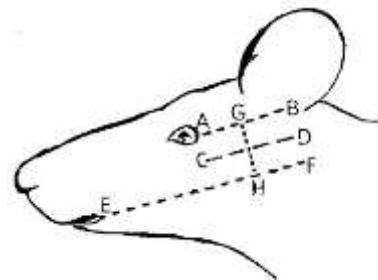
Fig.3: Schematic representation of the surgical landmarks

AB: outer canthus of the eye to the base of the ear;

EF: elongation of the labial commissure

CD: incision line (equals to AB)

GH: bisector of AB, vertical releasing incision



Skin incision was performed and the masseteric fascia is exposed. Once the masseter muscle was uncovered, the tip of the scissors was inserted until bone contact was obtained. Then the muscular fibers were separated from each other without cutting them. The displaced muscle was maintained in position using metallic hooks attached to

weights, and the ascending ramus of the mandible was then exposed. The bony prominence was identified and the pathway of the IAN was identified by transparency on a bony ridge extending from the prominence to the condyle. With a round carbide bur (Meisinger – germany) of 1mm in diameter mounted on a straight hand piece, the outer part of the cortex just distal to the bony prominence was removed exposing 2-3 mm of the inferior alveolar nerve. At this stage, the IAN was exposed to topical application of capsaicin or sham topical capsaicin.



Fig.4: Exposure of the inferior alveolar nerve on the left side

2. Axotomy

After exposing the left inferior alveolar nerve, the nerve was removed from its bony canal using glass probes; the nerve bundle was cut leaving the two ends of the nerve separated at a distance of about 2 mm. Care was taken to keep the vascular bundle intact.

3. Selective blocking of sensitive fibers with capsaicin topical application

This procedure was based on the technique described by Wall et al. (1970). Briefly, A small piece of cotton soaked with a solution of 10% of capsaicin (8-methyl-N-vanillyl-non-anamide; cat. no. M1022, Sigma, St. Louis, MO, USA) dissolved in 10% of Tween

80 and 90% of olive oil, was applied on the exposed IAN for 30 min. Then, the nerve was rinsed thoroughly with sterile saline for 10 min before suturing the wound.

The same treatment regimen was adopted for the sham group by applying the solvent, excluding capsaicin from the solution prepared.

At the end of the surgical intervention, the skin was sutured with a non-resorbable 3-0 suture material (MERSILK, ETHICON, Auneau-France). Antibiotic ointment (bacitracin zinc and neomycin sulphate 250 IU 5000 IU) was applied topically to the wound after closure.

4. Capsaicin sensitive primary afferents block

The procedure for chemical systemic ablation of CSPA followed the technique described by Saadé et al., 2002 with minor modifications. Capsaicin (8-methyl-N-vanillyl-non-anamide; cat. no. M1022, Sigma, St. Louis, MO, USA) was dissolved in a 10% Tween-80 and 90% olive oil solution, then injected subcutaneously at the concentration of 25mg/kg into the left leg, using a sterile insulin syringe (U-40micro five-IV, Becton Dickenson, Franklin Lakes, NJ, USA). The second injection was carried out after 8 hours at a concentration of 50mg/kg and the third dose at same concentration (50mg/kg) was administered 32hours after the first injection. The rats were allowed to heal for 2 weeks. To assess if the ablation was successful, the eye-wiping test was performed. It consisted of on applying a drop of capsaicin solution (10mg/kg) in one eye and evaluating the behavioral response of the rat (Hammond & Ruda, 1991).

5. Substance P antagonist, Spantide II

The SP antagonist Spantide II (Peninsula Lab cat. no. 7461-180299, Belmont, CA, USA) was subcutaneously injected in the left leg at a concentration of 0.2g/kg in (0.1ml), using the same procedure described for capsaicin (Massaad et al., 2002).

D. Measurement of tooth eruption

The method adopted was based on two fixed reference points; the first reference was localized on the tooth itself, at the junction of the buccal and distal surface of the tooth (clear differentiation in enamel's color). This technique would allow having a stable and reproducible guide on all animals. The groove was produced using a metallic disc (HI-FLEX, EDENTA AG, Hauptstrasse 7, CH-9434 AU/SG Switzerland) mounted on a straight hand piece. The second reference point was a tattoo (Drawing Ink, Rotring, Sanford GmbH D-22510 Hamburg, Germany) placed on the distal margin of the fixed attached gingiva.

The distance between the tattoo on the gingiva and the groove on the tooth was labeled (a), while the distance between the tattoo and the incisal edge of the tooth was (b) (Fig.5). A digital caliper (Electronic Digital Caliper, accuracy 0.01mm) was used for measurements. The measurements were obtained every 48hr over a period of 144 hours. Each measurement was repeated three times on each rat and the average was used as final value for the indicated time interval.

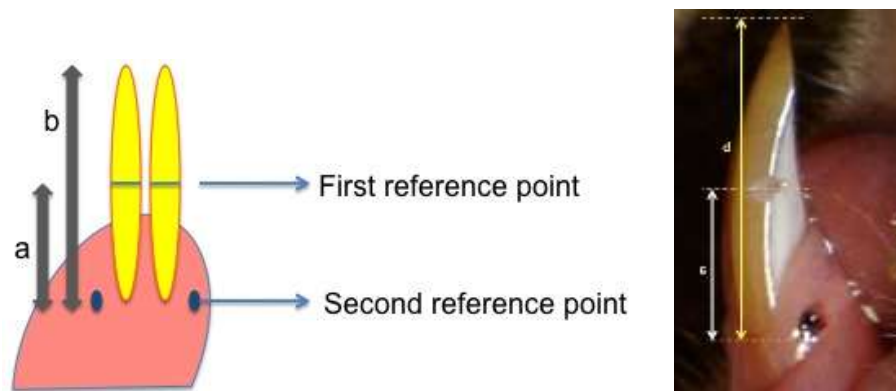


Fig.5: Representation of the landmarks placed on the incisor and attached gingiva: a) Distance between the groove produced on the tooth (first reference point) and the tattoo on the attached gingiva, b) Distance between the tattoo on the attached gingiva and the incisal edge.

E. Animal sacrifice

At the end of each experiment, each animal received a lethal injection of ketamine (Ketalar®, 100mg/kg) (i.p.) followed by a cervical dislocation. Mandibles and brainstems were sampled and kept under cold temperature (deep freeze, -80° C).

F. Immuno-Histochemistry (IHC): staining for substance P

The protocol of IHC was adopted in this study to target the existence of substance P in control rats or in rats after CSPA ablation. The primary antibody used was rabbit polyclonal to Substance P with the concentration of (1:10) (Anti-Substance P antibody (ab51900), Rabbit polyclonal to Substance P, abcam®, 330 Cambridge Science Park, Cambridge, CB4 0FL, UK).

The brainstems of rats from groups treated with spantide, systemic capsaicin treatment, and control were collected after sacrifice and tissues were fixed in a formaldehyde solution for 48 hours. After fixation, the tissue block were embedded in paraffin, then

cut in a microtome to 5 microns thickness. Cuts were performed at the level of the trigeminal nucleus, where substance P is targeted. Tissue sections were best mounted on positively charged or APES (amino-propyl-tri-ethoxy-silane) coated slides. Before proceeding with the staining protocol, the slides were deparaffinized (20 min in oven) and rehydrated.

All incubations were carried out in a humidified chamber to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. A shallow, plastic box with a sealed lid and wet tissue paper in the bottom were used as an adequate chamber for incubation.

Sections were washed twice for 5 minutes each in TBS plus 0.025% Triton X-100, and then the blocking agent was applied for 2 hours at room temperature (10% normal serum with 1% BSA in TBS). Slides were kept to drain for few seconds, and the primary antibody diluted to 1/10 in TBS with 1% BSA. Incubation was carried out overnight at 4°C. At day 2, the slides were washed with gentle agitation and were incubated in 0.3% H₂O₂ in TBS for 15 min. Then, the enzyme-conjugated secondary antibody diluted to 1/500 in TBS with 1% BSA was applied to the slides and kept incubated for 1 hour at room temperature. DAB (3,3'-Diaminobenzidine) was then applied, and the slides were put under running water for 10 min. Hematoxilline staining was carried out, the slides were dehydrated and stabilized with a mounting medium. At this point, the slides were ready to be analyzed under the microscope.

G. Qualitative assessment of substance P

Slides were observed and analyzed under the light microscope to detect qualitatively the presence of substance P. The positive immunoreactivity was designated by a brownish band (DAB stain) in the dorsal horn of the brainstem.

H. Statistical analysis

The rate of eruption is represented by the differences between (a) and (b) (shown in Fig. 5). Four time points were registered for each experimental group: at 0h, 48 hours, 96 hours and 144 hours. At each time point, measurements were recorded made three times then averaged for each rat. The result obtained for each time point was presented as the mean and standard error of the mean (SEM) of measurements made on all rats in the same experimental group.

Statistics were made using GraphPad InStat 3 (GraphPad Software Inc. San Diego, CA, USA) and the significance of variations were calculated. The data were represented in graphs using the GraphPad Prism 3 (GraphPad Software Inc. San Diego, CA, USA).

III. Results

A. Temporal evolution of the eruption rate of intact rats' mandibular incisor

The general trend of eruption rate of intact rats' mandibular incisors shows a biphasic variation throughout the observational period (Fig. 6). At the initial time segment (0-48 h), the eruption rate of intact incisors in (control group) showed a rapid phase of acceleration (mean \pm SEM: 1.18 ± 0.15 mm) compared to the second and third time segment where the eruption rate showed a progressive decline with time. The average amount of eruption was 1.08 ± 0.09 and 0.79 ± 0.15 mm, during the second and the third time segments (48-96 and 96-144h), respectively.

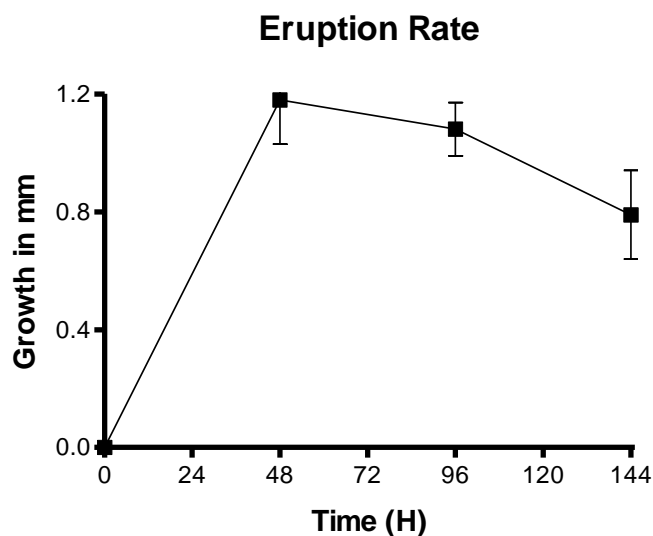


Fig. 6: Temporal evolution of eruption rate of intact rats' mandibular incisors. Each data point represents the mean \pm SEM of measurements made on 7 rats for the indicated time interval. All rats had the measurements taken under deep general anesthesia. Note the initial phase of acceleration followed by a progressive decline.

B. Effect of sectioning the left inferior alveolar nerve (axotomy) on the eruption rate of the ipsilateral rats' mandibular incisors

The mean amount of eruption in the incisors with cut inferior alveolar nerve, was 0.90 ± 0.19 mm during the first time segment and did not differ from that measured in the sham group (0.79 ± 0.15 mm, $n = 5$). The mean amount of eruption was significantly reduced during the second (48-96h) and the third (96-144h) time segments, to 0.44 ± 0.13 and 0.47 ± 0.11 mm, respectively. There were no significant differences in the total amount of eruption as well as in the eruption rates measured in the intact side (right side) and the sham group in each time segment and at the total amount of eruption ($p > 0.05$, Fig. 7).

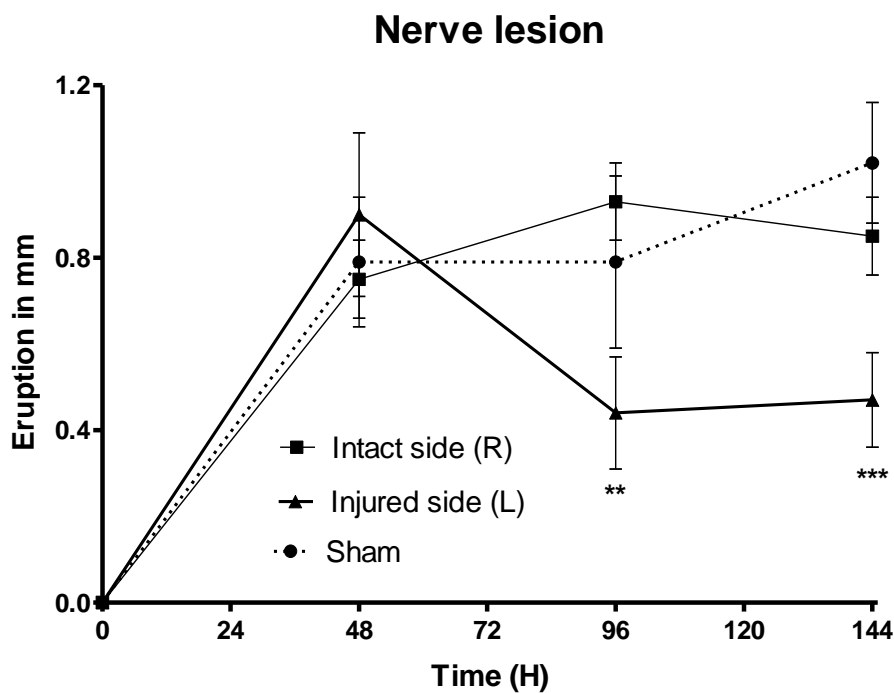


Fig. 7: Lesion of the left IAN significantly reduced the rate of eruption of the denervated incisors

Each data point represents the mean \pm SEM of amount of eruption measured at a specific time segment for the rats sustaining a lesion of the left inferior alveolar nerve ($n=7$), compared to sham group ($n=5$). All rats had the measurements taken under deep general anesthesia. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

C. Effect of systemic ablation of capsaicin sensitive primary afferents (CSPA) on the eruption rate of rats' mandibular incisors

Systemic ablation of CSPA fibers in a group of 7 rats produced a significant attenuation of the eruption rate of the mandibular incisors over the initial time segment (0-48 h., Fig. 8). The mean amount of eruption in the treated animals, at the initial time segment, was 0.64 ± 0.04 mm, as compared to the control group ($n=7$; 1.18 ± 0.15 mm, $P < 0.001$). The mean amounts of incisors eruption at the second (48-96 h, mean amount of eruption (mm) \pm SEM: 1.02 ± 0.09) and third time segments (96-144 h, mean amount of eruption (mm) \pm SEM: 0.92 ± 0.09) of the treated animals were not statistically significant compared to the control group ($p > 0.05$).

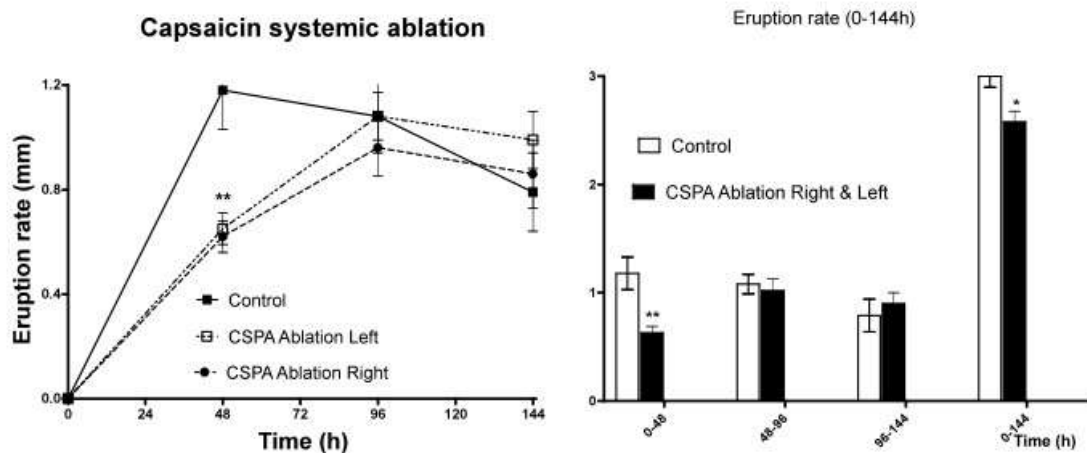


Fig. 8: Systemic ablation of CSPA reduced the eruption rate of rats' incisors

The eruption rate of the rats' mandibular incisors treated with capsaicin injection showed a significant decrease ($p < 0.001$) in the initial time segment (0-48h), and returned close to controls during the remaining time intervals

Each data point represents the mean \pm SEM of measurements made in each group at the indicated time interval ($n = 7$ for each group). Panel A, illustrates the time course of eruption of each incisor in treated rats; panel B illustrates the average of changes in both incisors versus time and the total change over 144 h. All rats had the measurements taken under deep general anesthesia. (* $P < 0.05$, ** $P < 0.01$).

D. Effect of local ablation of capsaicin sensitive primary afferents on the eruption rate of the mandibular incisors

Topical application of capsaicin on the inferior alveolar nerve produced a trend of attenuation in the rate of eruption over the first and the second time segments in both mandibular incisors (Fig. 9A). At the first time segment (0-48 hrs) the amount of eruption in both mandibular incisors was reduced (left incisor: 0.87 ± 0.11 mm; right incisor: 0.91 ± 0.10 mm), but did not reach significance as compared to control group (n=7; 1.18 ± 0.15 mm). During the second time segment (48-96 hrs), the amount of eruption in the left mandibular incisor was significantly reduced (0.41 ± 0.13 mm, $p < 0.01$) as well as for the right mandibular incisor (0.59 ± 0.15 mm, $p < 0.05$) as compared to the control (1.08 ± 0.09 mm) (Fig. 9 A). At the third time segment (96-144 hrs), the amount of eruption of the right mandibular incisor was close to the control (0.84 ± 0.19 mm), and the left incisor's rate of eruption was less than the right and the control but not significant (0.6 ± 0.10 mm).

The total amount of eruption in the treated left mandibular incisor (Fig. 9B) shows a significant reduction 1.88 ± 0.11 mm compared to the contralateral tooth (2.33 ± 0.09 mm, $p < 0.01$) and to the sham group (n=5; 2.02 ± 0.23 mm, $p < 0.01$).

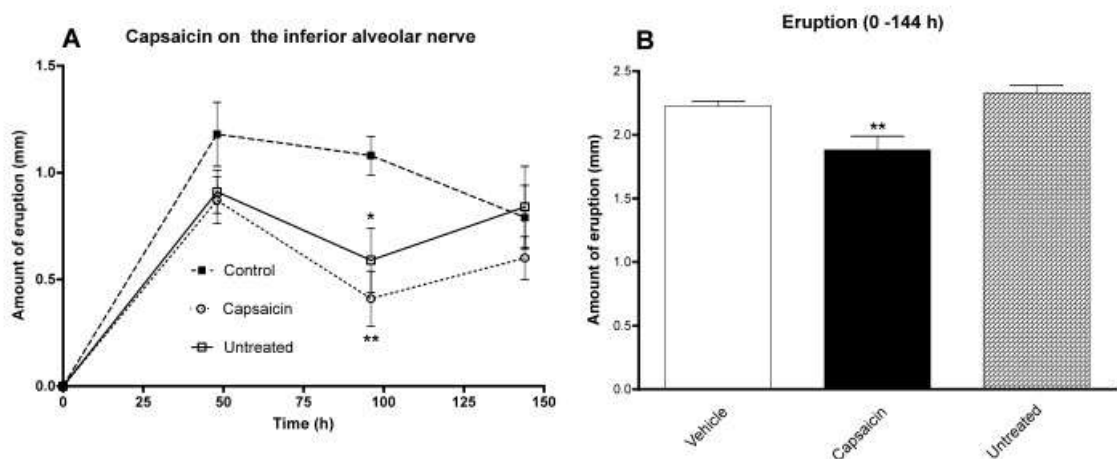


Fig. 9: Topical capsaicin application on the left IAN reduced the rate of eruption on both ipsilateral and contralateral incisors.

Capsaicin application on the left inferior alveolar nerve (A) produced a significant reduction in the eruption rate of mandibular incisors at (48-96h; $p < 0.01$) The total amount of eruption (B) was significantly lower in the experimental group compared to the sham group ($p < 0.01$).

E. Effect of treatment with Spantide II on the eruption rate of rat's mandibular incisors

Treatment of rats with the substance P antagonist (Spantide II, 0.2 g/kg), elicited a general trend of attenuation in the eruption rate over the initial two time segments (Fig. 10). At the initial time segment (0-48 h), the mean amount of eruption of the treated animals was not significantly reduced (0.99 ± 0.05 versus 1.18 ± 0.15 mm in control; $p > 0.05$). The mean amounts of incisor eruption at the second time segment (48-96 h.) was 0.73 ± 0.08 mm in treated and 1.08 ± 0.09 mm in control rats ($p < 0.05$). At the third time segment (96-144 h) the amount of eruption in the treated group was higher (1.11 ± 0.19 mm) than the control animals (0.79 ± 0.16 mm), but the difference was not significant ($p > 0.05$).

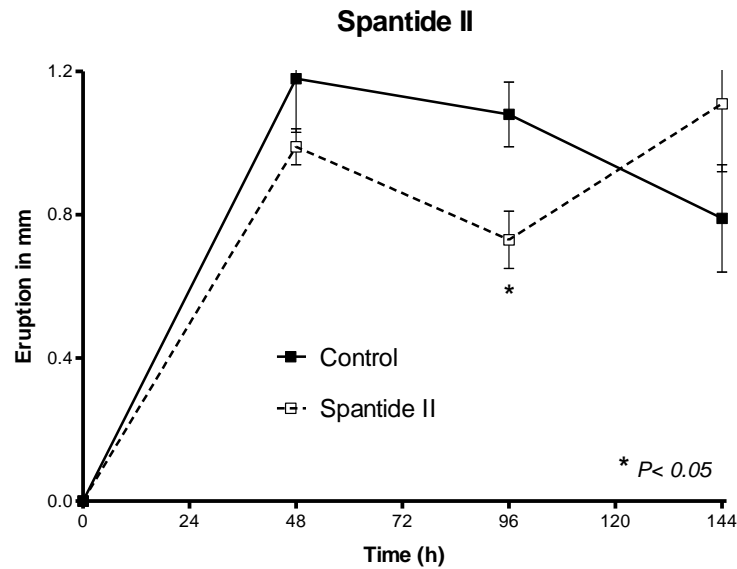


Fig. 10: Treatment with substance P antagonist (Spantide II) reduced the eruption rate of rats'

Each data point represents the mean \pm SEM of amount of eruption measured on both mandibular incisors (left and right) at the indicated time interval in a group of treated rats (n=8) as compared to control (n=7) taken under deep general anesthesia.

F. Substance P immunoreactivity

Immunohistochemical staining on sections obtained at the level of the trigeminal nucleus in the control group showed positive reactivity presented by a brownish (DAB) band in the dorsal horn of the brainstem (arrows, Fig. 11-A). Sections obtained from animals treated with substance P antagonist (Spantide II) showed less expression of immunoreactivity (light brown band compared to the control, Fig. 11-B). On the other hand, no immunoreactivity was observed on sections obtained from animals treated with systemic administration of capsaicin (Fig. 11-C).

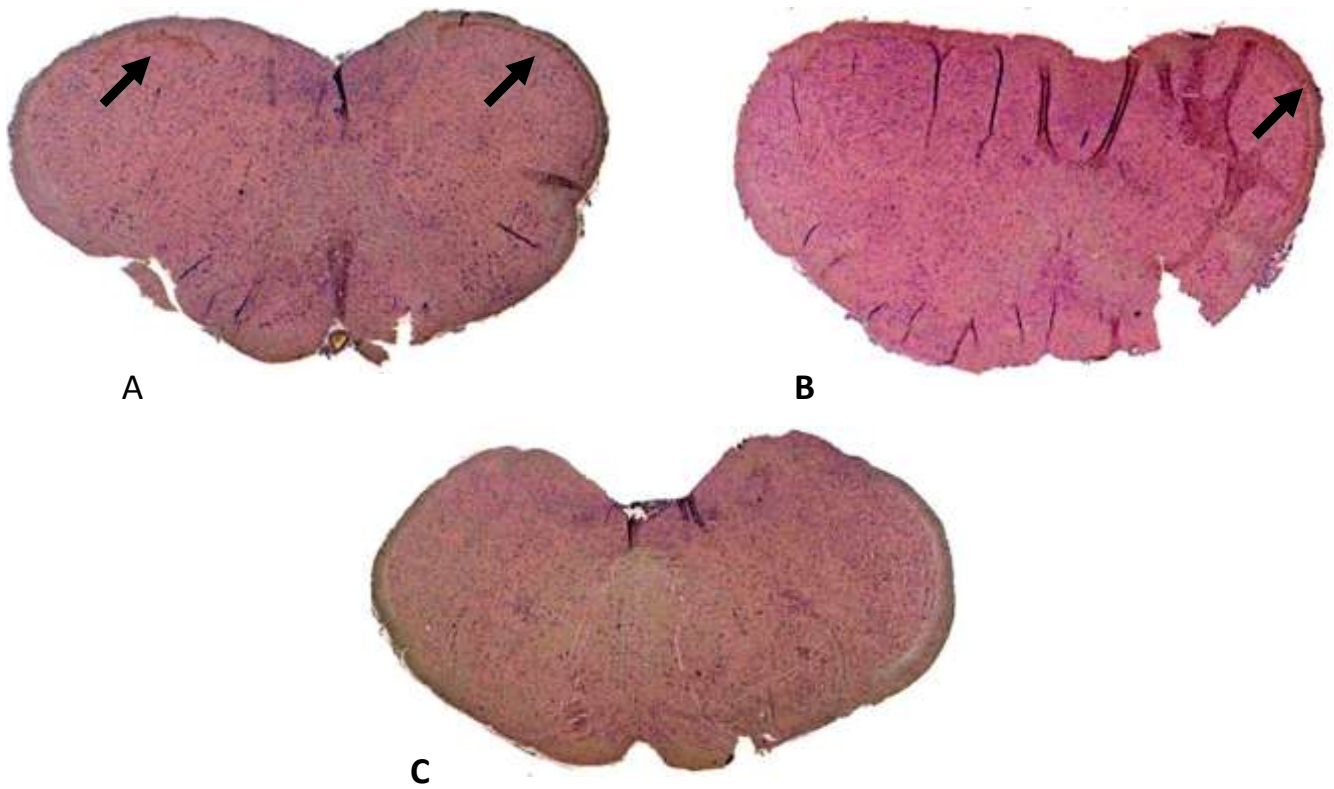


Fig. 11: Substance P immunoreactivity at the level of the trigeminal nucleus. Light microscopy of (A); control group, note the presence of immunoreactivity indicated by the arrows. (B) Spantide II group, (C); systemic capsaicin group. (B) and (C) show absence of substance P at the level of the trigeminal nucleus in the dorsal horn of the brainstem.

IV. Discussion

The initial aim of the study was to investigate the role of innervations, and in particular the role of the capsaicin sensitive primary afferents (CSPA) on the eruption rate of teeth. Although the number of these fibers is not as important as in other organs, yet they constitute the major component of sensory afferents and seem that to play an important role in regulating the eruption process of the teeth.

The most restrictive anatomic feature of the connective tissue of the tooth pulp is that it is encased in rigid mineralized tissue (dentin). This provides the pulp a low-compliance environment in which nutrients and wastes are almost entirely supplied and withdrawn via vessels traversing the narrow apical foramen. Previous study has reported a dramatic and sustained decrease of pulpal blood flow following application of inflammatory mediators (Kim et. al, 1992). However, more recent studies indicate that the pulp has physiologic feedback mechanisms that act to oppose increases in tissue pressure (e.g. increased lymph flow and absorption of interstitial fluid into capillaries in noninflamed areas) (Heyeraas et. al, 1999).

Substance P and CGRP have been reported to exert trophic effects on the growth of pulp fibroblasts in vitro (Bongenhielm et. al, 1995). Moreover, sectioning of the inferior alveolar nerve or capsaicin treatment, both of which caused a decrease in the number of nerves containing substance P and CGRP and resulted in reduced secondary dentin deposition in rat molars (Jacobsen et. al, 1996).

A characteristic feature of rodent's teeth is the presence of continuously growing incisors. Tooth substance lost at the incisal edge during tooth wear (physiologic

attrition) is replaced by new tissue production at the odontogenic base, maintaining a more or less constant tooth length in the adult. In our study, the temporal evolution of the eruption rate of intact rats' mandibular incisors was characterized by an initial phase of acceleration followed by a progressive deceleration with time (Fig.6).

Sectioning of the inferior alveolar nerve (IAN) was performed to investigate the possible involvement of tooth innervations in the eruption process (Fig. 7). Ladizesky et. al, 2001 reported first an increase in the eruption rate of the maxillary incisor after superior cervical ganglionectomy. Jacobsen et. al, 1996 described a decrease in SP and CGRP levels and reduction in dentin formation and eruption rate after denervation. Our results are in agreement with the second report and prompted us to investigate further the role of nerve fibers secreting substance P and CGRP

Capsaicin has been used in the previous study to investigate the effect of sensory innervation on bone metabolism and tooth eruption. Capsaicin application on the IAN (or ablation of CSPA fibers in the IAN) has been shown to cause a consistent reduction in the numbers of CGRP- and substance P immunoreactive fibers in the jaws. (Jacobsen et al., 1996). Other studies have shown a reduction in bone resorption in rats treated with capsaicin (Adam et. al, 2000), and a reduction in the overall bone mass (Wang et. al, 2009). This inhibitory effect on bone resorption, that is required for tooth eruption, suggests a possible reduction in the eruption rate.

In the present study, topical application of capsaicin on the IAN, resulted in slight reduction of the eruption rate of both mandibular incisors that was more pronounced during the second time segment (Fig. 9). This delay in the peak of attenuation on the left side can be attributed to the progressive degeneration of the synaptic terminals and depletion of neurotransmitters due to CSPA ablation. On the other hand, the right

mandibular incisor showed an attenuation in the eruption rate due to the effect of surgery. As the surgical site was healing, the rate of eruption on the right side went back to normal.

On the other hand, systemic administration of capsaicin caused more marked attenuation in the eruption rate starting at the initial time segment (Fig. 8). This difference in timing between local and systemic CSPA ablation can be attributed to differences in the timing of measurement of teeth eruption and the procedures used for CSPA ablation. The systemic ablation of CSPA fibers was confirmed by the important reduction of SP immunoreactivity in the trigeminal nucleus of the treated as compared to intact rats. Therefore one may speculate that the reduced eruption induced by CSPA ablation might be related to the role of SP in osteoclast differentiation, which constitutes a key factor in tooth eruption (Sohn, 2005). This interpretation is further supported by the observed reduction of eruption following treatment with the SP antagonist Spantide II (Fig. 11).

In conclusion, the nervous system plays a key role in the control of the eruption process of rats' mandibular incisors. CSPA fibers constitute a major component of tooth innervations and appear to have an important contribution in the eruption process. Evidence for this contribution was provided by the reduction of the eruption rate following CSPA systemic or local ablation and by the treatment with the SP antagonist spantide II.

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