Aus dem
Zentrum für Zahn-, Mund-, und Kieferheilkunde
der
Heinrich-Heine-Universität Düsseldorf

Westdeutsche Kieferklinik
Poliklinik für Zahnerhaltung und Präventive Zahnheilkunde
Direktor: Prof. W. H.-M. Raab

Apoptosis in the Dental Pulp:
Influences of Pulpal Microcirculation and Pulpal Nociceptive Innervation

Als Inauguraldissertation gedruckt
mit Genehmigung der
Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

gez: Prof. Dr. W. H.-M. Raab
Dekan
Referent: Prof. Dr. W. H.-M. Raab
Korreferent: Prof. Dr. A. Herforth

In memory of my mother…

TABLE OF CONTENTS

1. Introduction

1.1   General Introduction

In trying to fully understand which physiological mechanisms of the dental pulp influence odontoblast function, one must look at several aspects of the complex cellular interplay within the pulpal environment. Our original line of interest in investigating the physiology of the odontoblast was to understand the importance of neuronal content and function of the odontoblast. This question, however, is often closely associated with the vascular circulation in pulpal tissue, as the neuronal and vascular components of dental pulp are two anatomical and physiological systems which support the primary pulpal purpose; to provide a sensory and nutritional system to the tooth. As one looks at the presence or absence of the neuronal component, one must ask how the neurotransmitters produced by sensory nerve fibers may influence its surrounding tissues---specifically its vascular tissue. This interplay of cell and tissue types within the pulp produces the complex pulpal physiology which we are trying to understand. Therefore, it is crucial to investigate study models which isolate these physiological components of different tissue types within the pulpal structure in order to gain the desired information pertaining to odontoblast function.

The neonatally desensitized capsaicin-treated animal provides an interesting study model to evaluate neuronal dependence in the dental pulp. Capsaicin is a molecule found in hot peppers that has been consumed in Mexico since the prehistoric times (Lembeck 1983). The first crystallization of capsaicin from paprika and its chemical analysis were performed in the late 19th century (Russell and Burchiel 1984; Lembeck 1987). With further examination of this molecule, the unique characteristic of specific desensitization of sensory neurons was discovered when capsaicin was applied to mammalian physiological systems. In previous studies we

have looked at the development of dentin in animals which have been neonatally and systemically desensitized with capsaicin. In these animals we found profound dentinal defects (Fig. 1-4)(Krage et al.1999a; Raab et al. 1996).
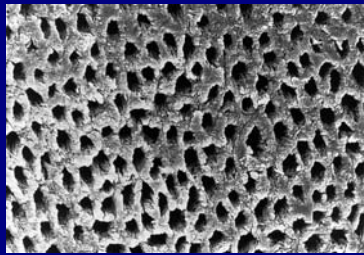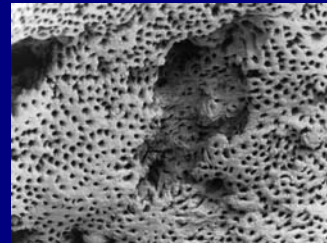
Fig. 1
Dentin in Control Rat at 150 days
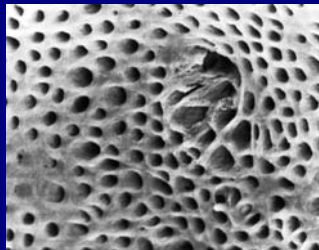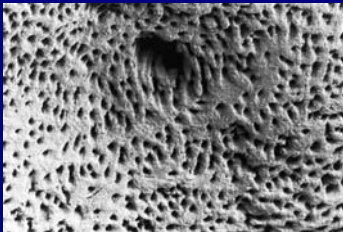
Fig. 2, Fig. 3, Fig 4
Dentin in Capsaicin-Treated Rat at 150 days of Age

While we may interpret these dentinal defects to be the result of an inactivation of nociceptive neuronal content, we must also consider that the lack of the neuropeptides known as calcitonin gene related peptide (CGRP) and substance-P (SP) will produce a relative vasoconstriction compared to physiological conditions of the surrounding tissues. This is due to the fact that CGRP and SP are normal vasodilators to assist in the healing response to a pathogenic or painful stimulus and when eliminated from a system, this response does not take place, leading to a reduction in microcirculation to its peripheral tissues (Byers et al. 1987; Raab 1992, Heyeraas et al. 1993; Berggreen and Heyeraas 1996; Gazelius et al. 1987).

It is not clear if developmental dental defects seen in rats desensitized with capsaicin are solely the result of functional changes in the interaction between the odontoblasts and sensory pulpal fibers or if they are due to the reduction in microcirculation of the pulpal tissue following either the absence of CGRP and SP, or compression of apical vasculature due to increased biting force as a result of reduced sensory feedback. One possibility to answer this question is to look at the pulpal content and tooth development of the nitric oxide synthase–3 (NOS-3) knockout mouse. The NOS-3 isoform of nitric oxide synthase is necessary for the endothelium to regulate microcirculation (Bevan et al. 1995). This isoform has been genetically inactivated in the knockout mouse, producing a reduction in peripheral systemic microcirculation. With examination of the dentinal development of the NOS-3 knockout mouse, we were surprised to discover that the dentin was quite different from that of its wild type (WT). Our investigations lead us to conclude that there is a 2-fold increase of the diameter of the dentin tubuli in the NOS-3 knockout compared to its WT, as well as a "honey comb" pattern of the tubuli that was not exhibited in the WT (Fig. 5-8)(Krage et al. 2001).
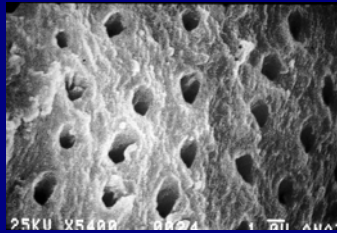
Fig. 5
Dentin in Wild Type Mouse at 150 days
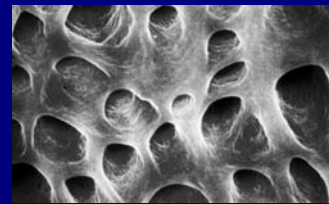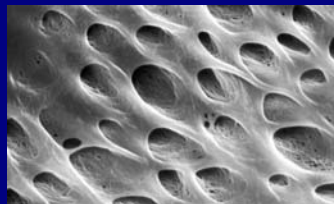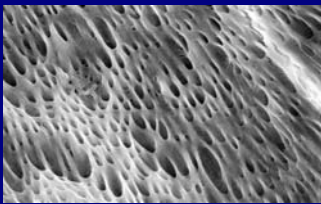
Fig. 6, Fig. 7, Fig. 8
Dentin in NOS-3 Knockout Mouse at Wild Type Mouse at 150 days

These differences in the dentinal development of the NOS-3 knockout mouse, however, did not compare to the profound dentinal irregularities found in the neonatally nociceptive desensitized animals. Therefore, we concluded from these studies that the loss of neuronal content in pulpal tissues has a separate influence on odontoblast functions compared to the reduction in microcirculation alone (Krage et al. 2002a).

With these preliminary results concerning dentinal development in the capsaicin-treated rat and the NOS-3 knockout mouse we have gained much knowledge in relation to influences pulpal microcirculation and pulpal nociceptive innervation have on odontoblast function. With this knowledge, however, come many more questions. It is necessary to further investigate how these two factors influence a change in cellular function of the odontoblast.

As we know that there is no direct synapse between the odontoblasts and nerve fibers (Norlin et al. 1999), we must look for the exact cellular mechanism that might be influenced by the microcirculation and innervation of the pulpal tissue.

For example, in the literature it is unclear how trophic factors, such as epithelial growth factor (EGF)--associated with nerves and odontoblast, alike--influence cell differentiation and odontoblast function. It has been reported by Cobo and colleagues that a very weak immunostaining for EGF is observed in the dental germ cells during the bud, cap, and bell teeth stages, as well as in few ectomesenchymal cells (Cobo et al. 1992). In developed, but not erupted teeth, a moderate immunoreactivity for EGF and EGFr (epithelial growth factor receptor) is present in the odontoblasts, in the ameloblasts and in the internal epithelial cells, but it is stronger in the dentin. Interestingly, the presence of EGF/EGFr is also observed in the intercalated ducts of salivary glands, primarily the submaxillary gland, in the maxillary bone cells, and in

the cells of the peripheral and central nervous system. These observations suggested that EGF has little or no effect during the early periods of tooth differentiation, whereas it is probably involved in the production of dentin. Moreover, EGF seems to participate in the maturation and differentiation of other embryonic tissues, such as tissues of the nervous system and bone (Cobo et al. 1992). Therefore, this trophic factor and others may be a possible link between odontoblasts and their nociceptive environment. This possible link, however, is poorly understood and, at this point, only speculative.

In hopes of better understanding the cellular mechanisms of the dental pulp we have returned to our two study models, the capsaicin-treated rat and the NOS-3 knockout mouse, where we may further investigate what is going on within the cellular mechanism of the odontoblast itself. As it has been reported that capsaicin produces an increase in apoptosis (programmed cell death) in certain tissues (Surh 2002; Smith and Liu 2002; Jung et al. 2001; Lee et al. 2000), one may question if the rate of apoptosis is also increased in pulpal tissue. With these questions we may also further question if it is the reduction in microcirculation that is causing any change in rate in apoptosis.

1.2   Special Introduction

1.2.1.   Capsaicin

1.2.1.1.   General History

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the pungent ingredient in chili peppers obtained from the plant genus *Capsicum* (Fig. 9).
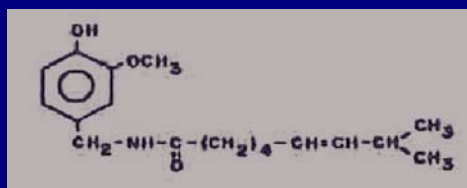
Fig. 9
Chemical Configuration of Capsaicin Molecule

Chili peppers have historically been used as food additives and preservatives, as well as ingredients in certain social rituals and herbal medicines for symptoms ranging from itch and pain to constipation. After noting such physiological effects in Hungarian school children in the late 1940s, the Hungarian pharmacologist, Nicholas Jancso, and his laboratory began the first extensive studies of the pharmalogical effects of capsaicin (Buck and Burks 1986). These studies were continued by Janos Szolcsanyi, Gabor Jansco, and Aurelia Jancso-Gabor after Nicholas Jancso's death in 1966. This study group established that the physiological application of capsaicin produces an initial intense excitation of certain sensory neurons that is followed by a prolonged period of insensitivity to neuronal stimuli (Buck and Burks 1986). This early work with capsaicin may be found in the review articles from Jancso, Virus and Szolcsanyi (Jancso 1968; Virus and Gebhart 1979; Szolcsanyi 1982).

1.2.1.2. Physiological Mechanism of Systemic Capsaicin Application

Capsaicin elicits responses specifically to nociceptive C fibers and A-δ fibers (Holzer 1991; Szolcsanyi et al. 1993). When topically applied to skin and mucosa, capsaicin produces a local desensitization that recovers after a few days (Szolcsanyi et al. 1990; Maggi 1991). This shows to be irreversible when applied systemically at the neonatal phase of life (Szolcsanyi et al. 1990; Maggi 1991). Therefore, when capsaicin is locally applied to the skin or mucosa in an adult, it has a transient excitatory and desensitizing effect. When applied neonatally and systemically, capsaicin can become a neurotoxin, in which its desensitizing action is permanent. Maggi further established that this irreversible desensitization is produced by an excessive calcium influx in the cell body which, in turn, blocks the conduction of action potentials of the neuron (Maggi 1991). Furthermore, others have established that the neonatal systemic application with capsaicin induces an estimated functional

loss of 85% of afferent C-fibers and approximately 30% of the A-δ fibers in the skin (Buck and Burks 1986).

Several studies have shown that the stimulation of sensory nerve fibers in the pulp causes vasodilation, resulting from a release of neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) (Kim 1990; Olgart et al.1991; Heyeraas and Kvinnsland 1992; Heyeraas et al. 1993; Kvinnsland et al. 1991). With the loss of the nociceptive function after capsaicin application, this physiological vasodilation normally caused by the presence of neuropeptides can not take place due to the lack of sensory nerve stimulation and lack of neuropeptide release. Ultimately, this lack of nociceptive function in the pulp causes a physiological reduction in the microcirculation of pulpal tissue (Raab 1990; Lawson 1989). While it is assumed that there is a reduction in microcirculation of the dental pulp in the neonatally and systemically capsaicin-treated rat through a reduction in the neuropeptides CGRP and SP, it is not clear if this is the main factor for the physiological consequence of a change in function of the odontoblast in this experimental group, or if, perhaps, the physiologically significant factor for this functional effect is due to a detrimental change in the physiological signaling between neuronal components of pulpal tissue and the odontoblasts in the capsaicin-treated rat.

1.2.2.   Nitric Oxide Synthase

1.2.2.1   Physiological Mechanisms

It has been established that the endothelium produces nitric oxide (NO) which in turn produces a relaxation of the underlying vascular smooth muscle (Huang et al. 1995; Mombouli and Vanhoutte 1999; Shesely et al. 1996). Furthermore, the endothelial cells achieve this NO production by expressing NO synthase 3 (NOS-3)

which is the third isoform of NOS that generates NO using L-arginine as a substrate. NO synthase is enhanced after Ca^{2+} / calmodulin binding and is an NADPH-dependent oxygenase that requires tetrahydrobiopterin, FAD and FMN as cofactors (Moncada et al.1991; Forstermann et al. 1994). In endothelial cells the NOS enzyme is localized preferentially in caveolin following post-translational acylation (Michal 1997, Feron et al. 1998).

1.2.2.2 NOS-3 Knockout Mouse

One possibility to understand the significance of microcirculation on odontoblast function is to compare the physiological function and structure of dental tissue in the NOS-3 knockout mouse, also known as the eNOS knockout mouse, with its wild type control group. The NOS-3 isoform of nitric oxide synthase plays a key role in endothelial-dependent vasodilation of arteries and arterioles (Fleming and Busse 1999). The NOS-3 isoform of nitric oxide synthase has been genetically inactivated in the knockout mouse, producing a reduction of peripheral systemic microcirculation. The NOS-3 knockout mouse, therefore, is the perfect study model to determine if microcirculation is the key factor in physiological and developmental changes seen in capsaicin-treated animals.

1.2.3. Apoptosis

1.2.3.1. Cellular Morphology of Cell Death

The term "apoptosis" was first applied in the late 1960s and early 1970s by Kerr, Wyllie and Currie in their analysis of programmed cell death. In this early work they were the first to recognize that apoptosis and necrosis were two distinctly different cellular processes (Kerr et al., 1972). Necrosis involves a swelling of the cytoplasm which leads to a degradation of the cell membrane, thus leading to a

disintegration of the cellular structure and a "spilling" of the cellular contents. In the swelling stage of necrosis the chromatin is not altered (Fig. 10). As the cell disintegrates, the debris is cleaned up by macrophages.

Fig. 10
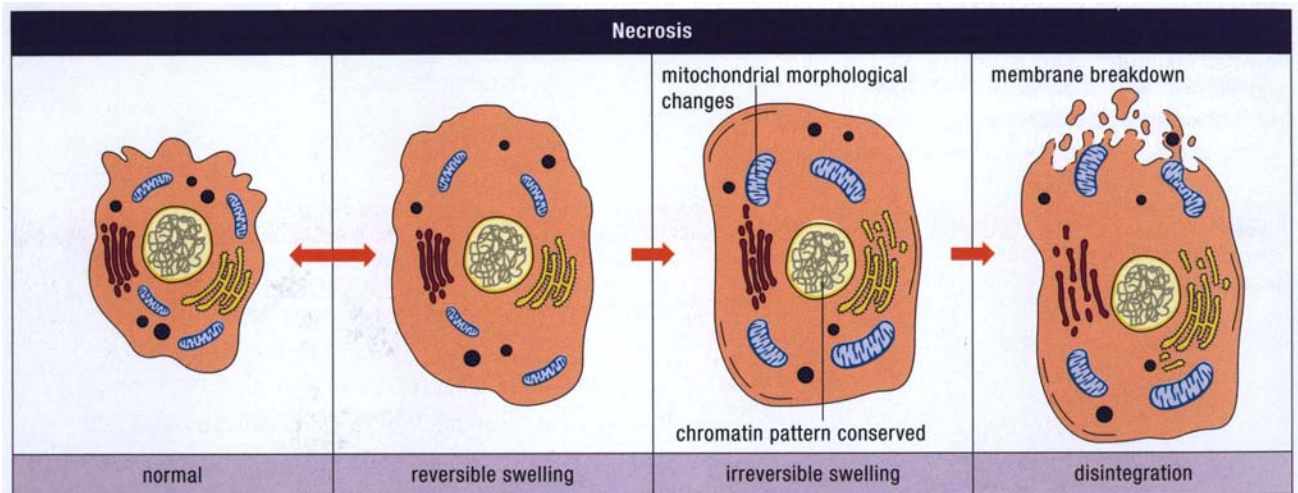
Illustration modified from Boehringer Mannheim (1998) Apoptosis and Cell Proliferation 2nd Edition.

Fig. 11
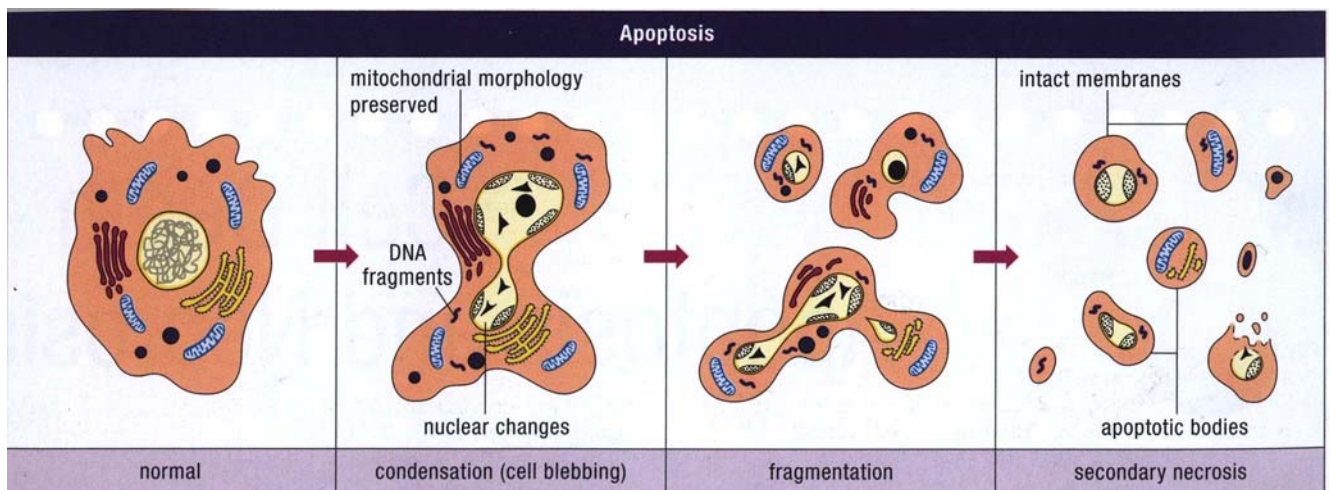
Illustration modified from Boehringer Mannheim (1998) Apoptosis and Cell Proliferation 2nd Edition.

Apoptosis, on the other hand, was first described by Kerr and his colleagues as "necrobiosis" or "coagulative necrosis". In this process there is an initial condensation of the nuclear chromatin which then leads to a convolution of the plasma membrane. Eventually the cell breaks up into subunits called apoptotic bodies and these units are cleaned up by macrophages or other surrounding cells (Fig. 11).

1.2.3.2 Genetics of Apoptosis

The recognition of apoptosis as a programmed cellular process led to several further investigations of the genetic component to apoptosis in the 1970s and 1980s. The initial genetic investigations were carried out on the roundworm, *Caenorhabditis elegans* (*C. elegans*) where every cell birth and cell death could be followed in this transparent organism (Sulston and Horvitz 1977; Kimble and Hirsch 1979; Sulston et al. 1983). This led to the breakthrough from Ellis and Horvitz in which they determined that the wild-type function of two genes, named ced-3 and ced-4, are required for the initiation of apoptosis in this worm (Metzstein et al. 1996). In the absence of these genes cells normally programmed to die instead survive and differentiate. Their work went on to establish that the gene named ced-9, negatively regulates ced-3 and ced-4, thereby protecting cells from undergoing apoptosis. Today we know that when a cell does not receive an apoptotic signal, ced-9 is coupled with ced-4, thereby preventing ced-4's interaction with ced-3. When the cell receives the apoptotic signal, ced-4 is released from its interaction with ced-9 and then is available to activate ced-3.

1.2.3.3. Molecular Biology of Cell Death

With the investigations of ced-3, ced-4, and ced-9 in *C. elegans* in the late 1980s, the mammalian homologs to ced-3 were quickly established with the new interest in the mammalian apoptotic pathway. Currently there are 14 mammalian homologs of ced-3. These homologs are called caspases, as they are enzymes which were originally called cysteine-dependent aspartate–specific proteases (Vanags et al. 1996). Seven of these caspases are involved in processing of pro-inflammatory cytokines and are not involved in apoptosis. Four of these caspases are involved in initiating apoptosis, while 3 are effector caspases which are responsible for cleaving various cellular proteins (Mirkes 2002)(Table 1):

Table 1

Group 1: cytokine processing:	Group 2: initiator caspases:	Group 3: effector caspases:
CASPASE-1 CASPASE-4 CASPASE-5 CASPASE-11 CASPASE-12 CASPASE-13 CASPASE-14	CASPASE-2 CASPASE-8 CASPASE-9 CASPASE-10	CASPASE-3 CASPASE-6 CASPASE-7

The apoptotic caspases, group 2 and group 3, exist in mammalians as pro-caspases which have not yet been activated. When an apoptotic stimulus occurs, the pro-enzyme is proteolytically cleaved into subunits that reconfigure to form the active form of the caspase.

After the cells receive the apoptotic signal, the initiator capsases (group 2) are activated via one of two major pathways of apoptosis (Chang and Yang, 2000). These 2 possibilities include the extrinsic pathway, or receptor mediated pathway,

and the intrinsic pathway, also known as the mitochondrial pathway. The extrinsic pathway is initiated by the activation of the pro-caspase 8 and 10, after a clustering of the Fos receptor in the cell membrane with the FADD, a cytosolic adaptor protein, which can then bind these pro-enzymes to activate them and initiate the cell death. The binding of these components together with the activated initator caspases 8 and 10 is called the death-inducing signaling complex (DISC). This process leads to activation of the effector enzyme, caspase 3, which is the central point of apoptosis where the intrinsic and the extrinsic pathways come together (Mirkes 2002)(Fig. 12).

In the intrinsic pathway, the cell death process is initiated by the release of cytochrome C from the mitochondria, which in turn activates Apaf-1 (Apoptosis protease-activating factor-1) which then couples with pro-caspase 9, thus activating it to an initiator caspase. This process leads to the activation of the effector caspases, where caspase-3 is again a key player in apoptosis (Mirkes, 2002)(Fig. 13). As the effector caspases take charge of the apoptotic process, cleavage of protein targets results in cellular changes specific to the apoptotic process. These changes include cell shrinkage and membrane disconfiguration, chromatin condensation, and ultimately DNA fragmentation. As the cell degrades, cellular subunits form known as apoptotic bodies and are cleared from the cell by phagocytes such as macrophages (Mirkes 2002).

Fig. 12

Apoptosis Extrinsic Pathway flow chart

Apoptosis Induction

↓

Receptor Activation
In Cell Membrane
(FADD/Fas)

also
(FADD/TRADD/TNFR1)
(FADD/TRADD/ DR3)
(FADD/TRADD/ APO-3)
(FADD/TRADD/DR4/5)

↓

Activation of Caspases
(Initiator Caspase 8/10)

↓

Phosphatidylserine Exposure

↓

Cell Morphological Changes

↓

DNA Fragmentation

Fig. 13

Apoptosis Intrinsic Pathway flow chart

Apoptosis Induction

↓

Cytorchrome C Production and
Excretion from Mitochondria

↓

Cytochrome C / Apaf 1 Complex
formation in Cytoplasm

↓

Decreased Mitochondrial Potential

↓

Activation of Caspases
(Initiator Caspase 9)

↓

Phosphatidylserine Exposure

↓

Cell Morphological Changes

↓

DNA Fragmentation

1.3　　　　Aim of Study

While the dental pulp is made up of a complex physiological environment where several different cell types interplay, the presence of apoptosis in this organ system may play a key role in this organ's physiological outcome. The rate of apoptosis, like odontoblast function, may be significantly influenced by the microcirculation and nociceptive innervation of the dental pulp. Furthermore, the apoptotic rate in the pulpal tissue may have a direct effect on the odontoblast function within the pulp. Therefore, the purpose of the following study was to determine any changes in rate of apoptosis in pulpal tissue of two study groups; the capsaicin-treated rat compared to rats without capsaicin application, and NOS-3 knockout compared to its WT, in order to better understand pulpal physiological influences in odontoblast function.

2.   Materials and Methods

2.1.   Animal Experiments

2.1.1   Neonatal Capsaicin Application

In order to have irreversible systemic desensitization of the C-fibers and the A-δ fibers, it is necessary to systemically apply capsaicin to the rats at the 1st to 3rd post-natal day, while it is at this time point that the development of the nervous system in the rat is not yet completed. If the capsaicin is applied after this developmental period, the animal would only experience a reversible desensitization of sensory nerve fibers after capsaicin application. Therefore, we injected 10 Wistar rats with capsaicin at a dose of 40mg/kg body weight on the first postnatal day. We experienced a 50% loss of the initial experimental group due to death immediately after capsaicin application, which is a common occurrence and is normally due to the extreme asthma that the rats encounter upon exposure to the systemic capsaicin application. Gaseous O_2 and an asthma spray (Aarane$^{®}$ N) were applied to each animal after capsaicin application in order to improve the survival rate. Finally, 5 capsaicin-treated rats served as our experimental rat group and 5 untreated rats served as a control group. After day 150, the systemic desensitization in the experimental group was tested by observation of the blink reflex after local application of mustard oil to the eye. All animals were then anaesthetized and intravitally perfused with a 4% paraformaldehyde solution containing 0.2% picric acid (Zamboni Solution). The jaws of all animals were dissected and decalcified in 10% EDTA for 5 to 10 days.

2.1.2   NOS-3 Knockout Mice

The NOS-3 knockout mouse population and WT population were provided by Dr. Gödecke from the Physiology Department at the Heinrich Heine University in Düsseldorf. This knockout mouse was produced at this institution via the cloning of the Murine eNOS gene and construction of a target vector. The detailed protocol concerning the production of this genetically altered mouse may be found in Dr. Gödecke's article "Coronary Hemodynamics in Endothelial NO Synthase Knockout Mice" (Gödecke et al 1998).

The jaws of 5 NOS-3 knockout mice and 5 WT mice were donated to our research lab to carry out our apoptosis investigations. Other tissues (i.e. heart) of each animal were used by Dr. Gödecke's working group for their cardiac physiology studies and only the oral tissues of these animals were supplied to us for our research purposes.

All animals from the mouse groups (5 NOS-3 knockout and 5 WT mice) were euthanized at day 150, as described above. The jaws of all animals were dissected and decalcified in EDTA for 5 to 10 days.

2.2.   Apoptotic Detection Procedures

2.2.1.   Method Selection

Several methods have been developed to study apoptosis in individual cells and specific tissue types. These methods are focused on two key events in the cell that is undergoing apoptosis:

i)   DNA fragmentation is a key event in apoptosis of the cell and assays that measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death.

ii)   Alterations in plasma membrane may also be a detection of cell death. For instance, during apoptosis, phosphatidylserine translocates from the cytoplasmic side of the membrane to the extracellular side and can be detected through special scientific method.

As we searched for the best test to analyze a change in rate of apoptosis in our different tissue types, we found that the measurement of DNA fragmentation was the most appropriate aspect of early apoptotic events. The methods used to assess DNA strand breaks are based on immunohistological labeling DNA, which is subsequently analyzed by flow cytometry, fluorescence microscopy, or light microscopy (Gavrieli and Sherman 1992). Furthermore, sections that have been labeled can be further processed for transmission electron microscopy analysis to confirm the accuracy of the immunohistolocalization of DNA fragmentation in the cell nucleus.

2.2.2   TUNEL Reaction

The TUNEL enzymatic labeling assay is the analysis we have chosen to identify and measure the fragmentation of DNA as an early cellular apoptotic process. In this early stage, the cleavage of DNA may yield double-stranded, low molecular weight (LMW) DNA fragments (mono- and oligonucleosomes) as well as single strand breaks, also referred to as "nicked DNA", in high molecular weight DNA (Garczyca et al. 1993). These DNA strand breaks can be detected by immunohistological labeling of the 3'-OH termini with modified nucleotides. A suitable nucleotide for this labeling of the "nicked" site in the DNA single strand change would be X-dUTP coupled with DNA polymerase. Using only these nucleotides, however, to label the single strand breaks in the DNA (know as the In Situ Nick Translation from Roche) has its disadvantage, in that it is not only detecting apoptotic DNA, but is also

detecting the random fragmentation of DNA by multiple endonucleases occurring in cellular necrosis (Fig. 14).

To eliminate this problem, we have chosen to use the procedure that not only labels these single strand breaks in the DNA, but also labels the Terminal deoxynucleotidyl transferase (TdT) at the blunt ends of double strand DNA breaks independent of a template. This end-labeling has been termed TUNEL (TdT-mediated X-dUTP nick end labeling)(Bortner and Rosenberg 1995)(Fig. 14).

In Situ Nick Translation (ISNT) Template Dependent
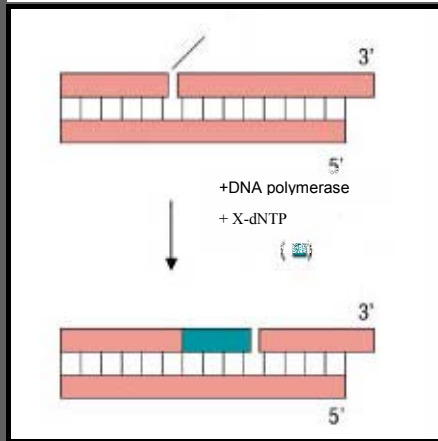
In Situ End Labeling (TUNEL) Template Independent
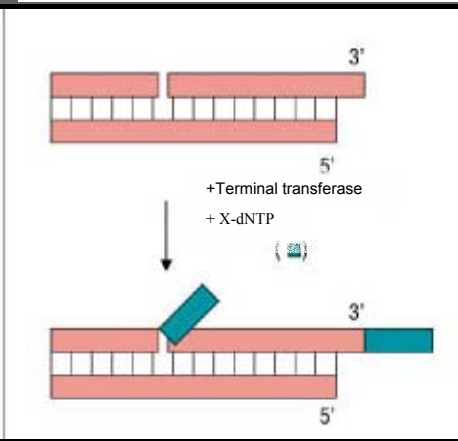
+DNA polymerase
+ X-dNTP

+Terminal transferase
+ X-dNTP

Figure 14:
Two enzymatic DNA labeling methods Nick Translation vs. End Labeling (Modified figure from *Apoptosis and Cell Proliferation, 2nd Edition* Boehringer Mannheim, Germany, 1998: p. 25.)

The TUNEL method is, therefore, more sensitive and faster than the ISNT method. In addition, in early stages cells undergoing apoptosis were preferentially labeled by the TUNEL reaction, whereas necrotic cells were identified by ISNT. Thus, experiments suggest the TUNEL reaction is more specific for the apoptosis and the combined use of the TUNEL and the nick translation techniques may be helpful to differentiate cellular apoptosis and necrosis (Gold et al 1994). Therefore, the *In Situ Cell Death Detection Kit, POD* (Roche, Germany Cat. No. 1 684 817) was used to detect early stages of apoptosis in all tissue types chosen for this study. With this kit we were able to achieve end-labeling of DNA with fluorescein-dUTP, followed by detection of incorporated fluorescein with an antibody and visualization of the antibody using a label-enhancement reaction.

2.2.3. Specimen Cryosectioning

In preparation for the TUNEL labeling, all specimens were removed from the Zamboni fixative solution within 24 hours after jaw preparation and washed over night in phosphate buffered saline (PBS). They were then saturated in 10% sucrose for 24 hours before being transferred to 30% sucrose where they were further saturated for at least 3 days before being pre-chilled with liquid nitrogen and kept at –80°C until cryosectioned. The cryosectioning was carried out on a *Leica CM3050* cryostat at a thickness of both 25 μm and 40 μm, giving us a range in the field of evaluation and observation of the TUNEL positive cell bodies. All sections were collected in a serial order into tissue culture wells containing a PBS wash solution until ready to carry out the enzymatic DNA labeling procedures.

2.2.4.   Enzymatic DNA Labeling

After cryosections had been washed in PBS for at least 30 minutes they were incubated in a blocking solution containing 3% H_2O_2 in methanol for 10 minutes at 15-25°C. This was followed by a second wash in PBS. Then the sections were further incubated in a permeabilisation solution made up of 0.1% Triton X-100 and 0.1% sodium citrate for a period of 2 minutes on ice (2-8°C). These steps were carried out to prepare the tissue sections for the enzymatic TUNEL reaction to take place.

The sections were then brought onto slides where the rest of the TUNEL reaction procedure took place. 50μl of TUNEL enzyme solution was added to 450μl Label Solution, which was prepared in the kit, and this substrate was a sufficient amount for 10 sections to sample. The slides were then rinsed twice in PBS. 50μl of the enzyme mixture was then applied to each section and incubated for 60 minutes at 37°C in a humidified chamber in the dark. After the incubation was complete, rinses in PBS were carried out 3 times again. This concluded the enzymatic labeling reaction.

The next step was to complete the signal conversion. This can be carried out with either the Converter-POD which is supplied in the kit, or with 3'3-diaminobenzidine, also know as DAB (Sigma, Germany), coupled with Nickel (Ni). We chose the Ni-DAB substrate as our label-enhancement, where we can attach Ni to the TUNEL positive reaction and obtain an obvious black staining. This was carried out by adding 50μl of the Ni-DAB substrate to each section for a period of 2-5 minutes, until the label-enhancement was visible, and then each reaction was stopped in a PBS wash. PBS washes were repeated twice.

Following the Ni-DAB reaction, the tissue sections were rinsed in PBS, mounted on gelatin-coated slides, air-dried and counterstained with a Richardson II stain at a dilution of 1:200 for 2 minutes. They were then dehydrated in graded alcohol, cleared in xylene, and coverslipped.

2.3 Microscopy

2.3.1 Light Microscopy (LM)

The TUNEL reaction of all sections of all study groups was thoroughly analyzed with a *Leitz Laborlux D* light microscope and digital imaging was taken at 100X, 250X and 400X magnifications.

2.3.2 Transmission Electron Microscopy (TEM)

Tissue sections chosen for further analysis under the transmission electron microscope were stored at 4°C in phosphate buffered saline (PBS). Sections were then rinsed in 0.1M sodium cacodylate buffer at pH 7.4 2X for 10 minutes. Sections were saturated in 1% osmium tetroxide (OsO_4) for 2 hours. This was followed again by a 10 minute rinse with 0.1M sodium cacodylate buffer. The sections must then be dehydrated, where they were briefly brought into 35% ethanol for 30 seconds, 50% ethanol for 10 minutes, 70% ethanol for 15 minutes, 95% ethanol 2X for 10 minutes, and finally in 100% ethanol 2X for 10 minutes. The sections were then ready for embedding in araldite (120ml Eponate-12, 72ml araldite, 270ml DDSA) on foil-covered slides. Sections were then further sectioned into ultra-thin sections of 100nm and mounted on grids. The uranyl acetate contrasting medium that is routinely used in TEM was not applied to our sections in order to insure that misinterpretation of the artifacts were not a risk in our data analysis. The ultra-thin sections were examined

using a Philips EM 208S electron microscope and images were digitally recorded.

Measurements of cellular structure were made with the Analysis® imaging system.

3. Results

3.1   TUNEL Cell Reaction

3.1.1   Control Rat Group vs. Capsaicin-Treated Rat Group

With the analysis of the TUNEL reaction in $25\mu m$ cryosections, it was determined that the control rat group showed a moderate TUNEL positive cell reaction in the pulpal tissue, made up mostly of a fibroblast cell population. In contrast, the capsaicin-treated rat group demonstrated an extremely positive TUNEL cell reaction. This staining was not only profound in the pulpal tissue (i.e. fibroblast cell population), but was also extreme in the odontoblast layer of the molars. The odontoblast layer of the control rat group did not show a profound positive TUNEL positive reaction (Fig. 15 – 18).
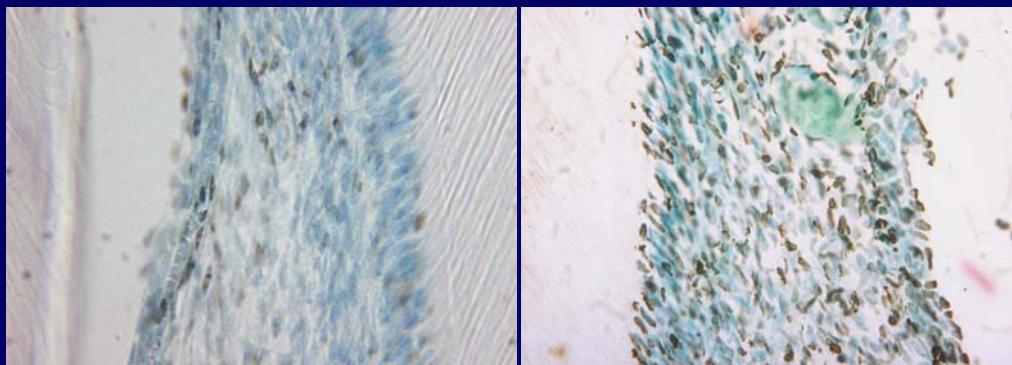
Fig. 15-16

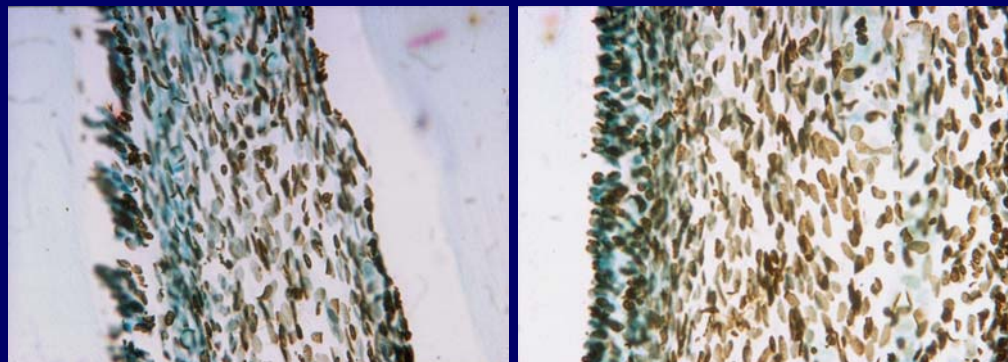TUNEL Reaction in Control Rat in 25μm cryosection

Fig. 17-18:

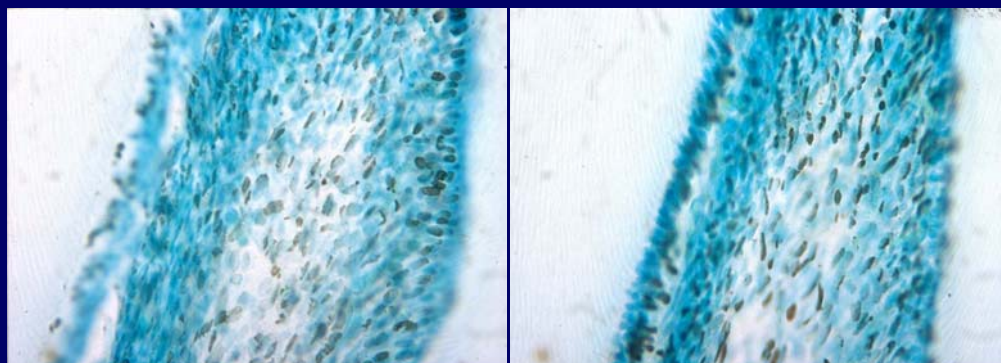TUNEL Reaction in Capsaicin-treated Rat in 25μm cryosection

Fig. 19-20:

TUNEL Reaction in Control Rat in 40μm cryosection
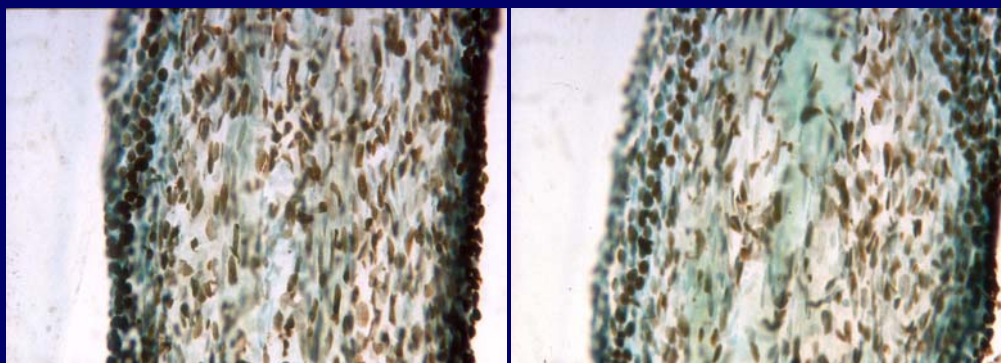
Fig. 21-22:

TUNEL Reaction in Capsaicin-treated Rat in 40μm cryosection

To analyze the contrast between our control group (rats not desensitized with capsaicin) and experimental group (capsaicin-treated rats), cryosections of 40 μm were also analyzed (Fig. 19-22).

3.1.2 WT Mouse Group vs. NOS-3 Knockout Mouse Group

In the WT mice group, the 25 μm cryosections demonstrated a moderate rate of TUNEL-positive cells in the pulpal tissue and these cells were made up mostly of a fibroblast cell population (Fig. 23-26). In contrast, the NOS-3 knockout mice group showed a greater intensity of TUNEL-positive cells in the pulpal tissue compared to that observed in the WT mice group, but the TUNEL-positive cell reaction in the NOS-3 knockout mouse was not as severe as that which was initially observed in the capsaicin-treated rat. Neither in the WT mice group, nor the NOS-3 knockout mice group, were there a pronounced TUNEL-positive cell rate of the odontoblast layer of the molar pulps, as was initially observed in the capsaicin-treated rat.

Fig. 23-24:

TUNEL Reaction in Wild Type Mice in 25µm cryosection

Fig. 25-26:

TUNEL Reaction in NOS-3 Knockout Mice in 25µm cryosection
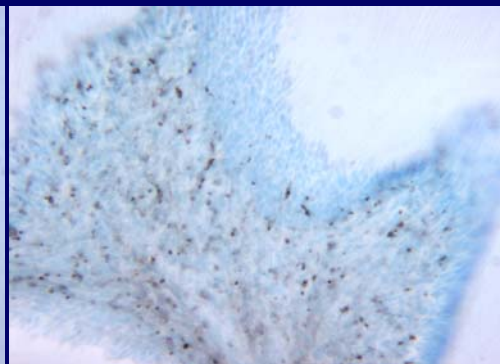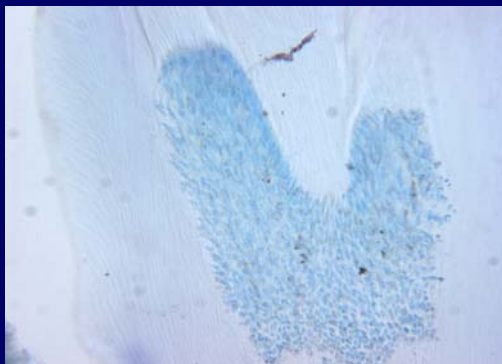
Fig. 27-28:

TUNEL Reaction in Wild Type Mice in 40µm cryosection
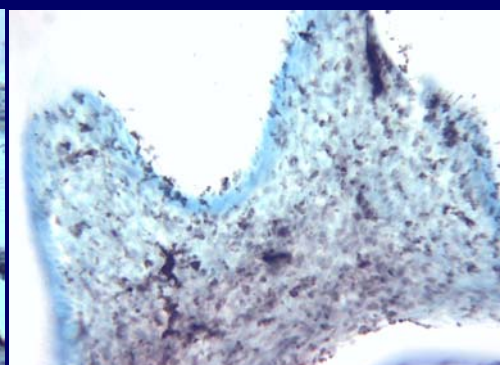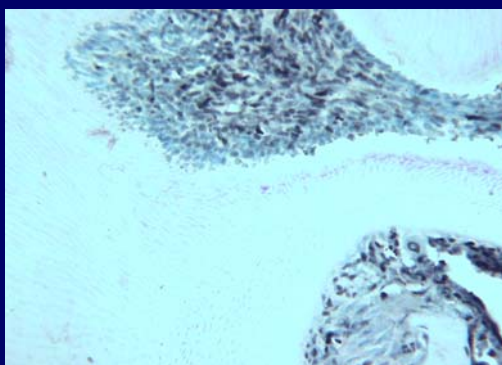
Fig. 29-30:
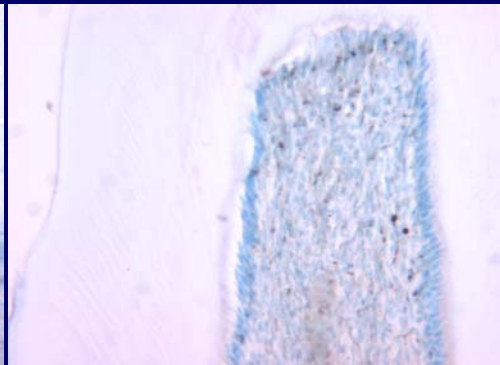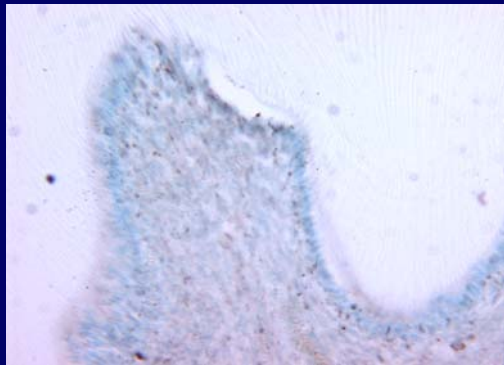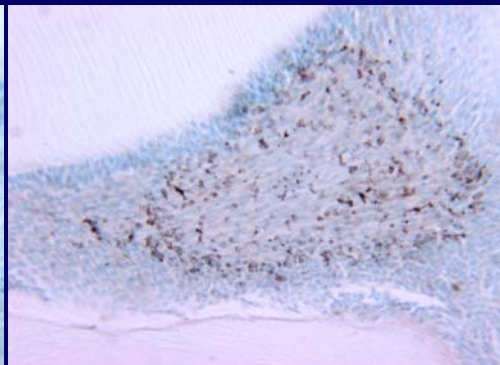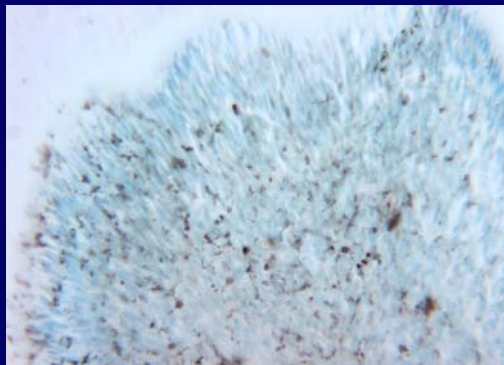
TUNEL Reaction in NOS-3 Knockout Mice in 40µm cryosection

To analyze the contrast between our control group (WT mice) and experimental group (NOS-3 knockout mice), cryosections of 40 μm were also analyzed (Fig. 27-30).

3.2   Transmission Electron Microscopy

3.2.1   Capsaicin-Treated Rat Group vs. NOS-3 Knockout Mice

Due to the extreme positive labeling in the odontoblasts of the capsaicin-treated rat group, we selected sections from each animal of this group for further analysis under the transmission electron microscope to confirm that our TUNEL reaction was accurate. TEM was not carried out to observe the odontoblasts of the control rats, as TUNEL staining was absent in these cells. Furthermore, our TEM analysis was extended to the pulpal tissue of the NOS-3 knockout mouse. This procedure enabled us to insure in both experimental groups that the TUNEL-positive cell reaction was not a false-positive one, by confirming that the TUNEL marking was indeed taking place in the nucleus where it should be labeling only the nicked DNA of the cell. The TEM analysis also enabled us to determine if the cellular morphology (i.e. apoptotic bodies, condensation of chromatin, and plasma membrane disconfiguration) that one would expect at the fragmented DNA stage of cellular apoptosis was coinciding with the TUNEL-positive cell reaction.

In the capsaicin-treated rat, the Ni-DAB labeling of the TUNEL-positive cell was in fact located in the nuclei of the odontoblasts. These cells, however, did not show cellular signs of apoptosis. As described in the introduction, at this stage of apoptosis in which single strand breakage occurs, parallel observations of cellular changes such as "blebbing" of the cell membrane, condensation of the chromatin, deformation of the nucleus, and development of apoptotic bodies are expected

(Mirkes 2002). On the contrary, in the odontoblasts of the capsaicin-treated rat we observed round, regular shaped nuclei with no chromatin condensation and no apoptotic body formation (Fig. 31-33). Therefore, the location of our TUNEL labeling in these odontoblasts may indicate that there is damage to DNA in these cells, but this DNA damage has not produced the apoptotic signaling to the cell in order for the cell to undergo further apoptotic processes that ultimately lead to apoptotic body formation and cell degeneration. It must be questioned, then, if this DNA damage may be producing other cellular signaling in the physiological functioning of these cells and this point is further addressed in the discussion of these results.
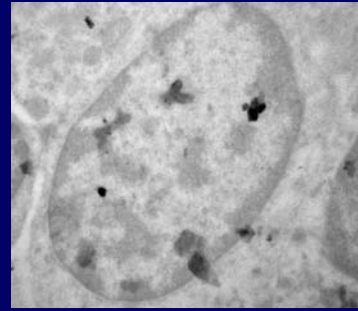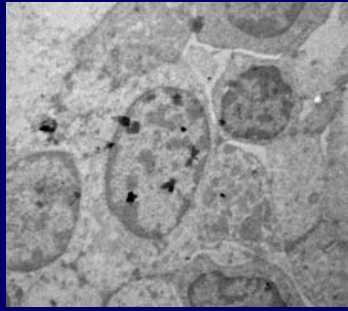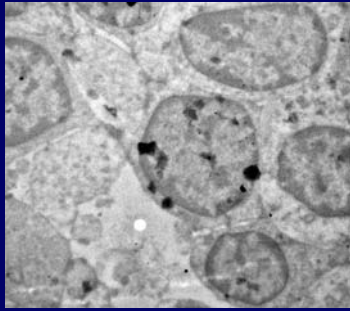
Fig. 31-33:

Nickel/DAB labeling of nicked DNA (TUNEL reaction) in nucleus of capsaicin-treated rat at 150 days. Cell morphology appears normal.

150 days
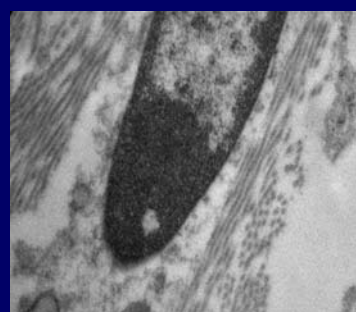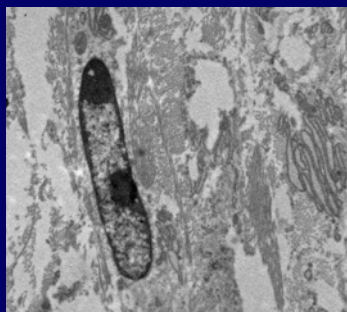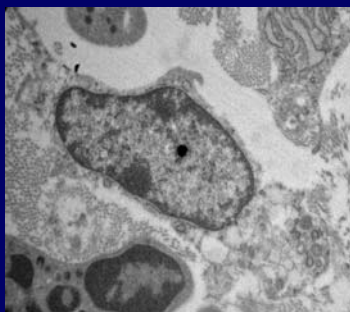
Fig. 34-36:

Nickel/DAB labeling of nicked DNA (TUNEL reaction) in fibroblast nucleus of NOS-3 knockout mice at 150 days. Chromatin condensation and deformation of the nucleus were observed.

In the NOS-3 knockout mouse, labeling was not noted in the odontoblast layer. In the TUNEL-positive fibroblast of this experimental group, the Ni-DAB labeling of the TUNEL reaction was also found within the nucleus. In these NOS-3 knockout mice, however, the expected cellular signs of the apoptosis at the fragmented DNA stage were detected with TEM (Fig. 34-36). Therefore, we have established in this TEM analysis of the NOS-3 knockout mouse that the TUNEL-positive labeling in the fibroblasts of the NOS-3 knockout mouse is a true labeling of an apoptotic process in these cells.

Furthermore, as a control, other cell types which are expected to have very high rates of apoptosis due to increased cell cycle rates (i.e. gingival and junctional epithelium) (Thomson et al. 2001) were examined in the NOS-3 knockout mouse in order to compare the cellular morphological changes in these TUNEL-positive nuclei. In these cell types of the NOS-3 knockout mouse, the expected cellular changes of apoptosis were also observed in the TUNEL-positive cells (Fig. 37-40).
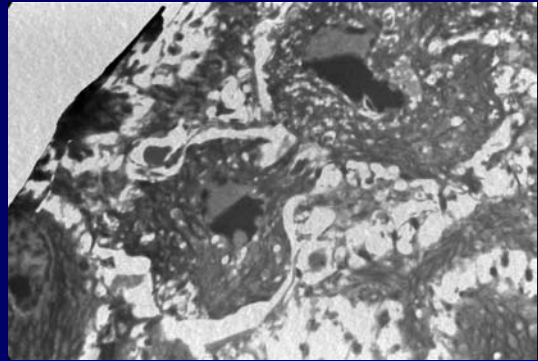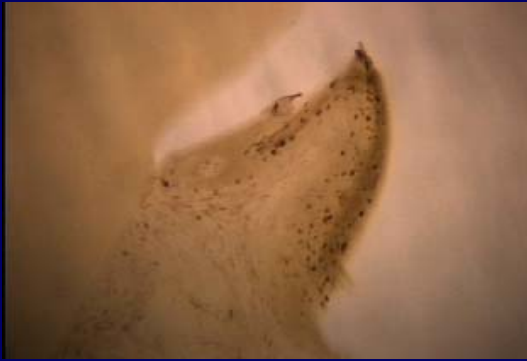
Fig. 37-38:

Nickel/DAB labeling of nicked DNA (TUNEL reaction) in gingival tissue of NOS-3 knockout mice at 150 days. Chromatin condensation in nucleus and formation of apoptotic bodies
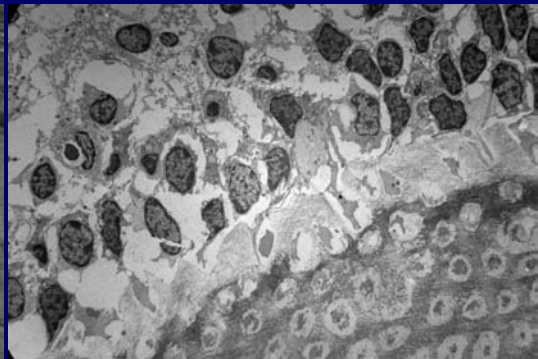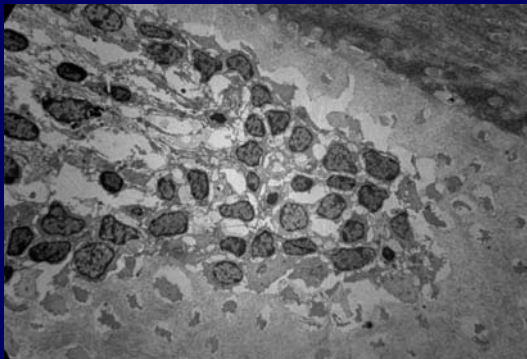
150 days

Fig. 39-40:

Nickel/DAB labeling of nicked DNA (TUNEL reaction) in junctional epithelium of NOS-3 knockout mice at 150 days. Chromatin condensation in nucleus and formation of apoptotic bodies

150 days

4. Discussion

4.1 Physiological and Pathological Aspects of Apoptosis

It has been stated that apoptosis plays a significant role in embryological development and tissue maintenance. The idea that programmed cell death is important in embryological development was first introduced to science by Gluckmann and his colleagues in 1951, where they demonstrated that cell death is an integral part of the normal development of virtually every tissue and organ (Gluckmann 1951). For example, it has been established that programmed cell death may be observed at very early stages in the development of the central nervous system, i.e., closure of the neural tube. Cell death is a common occurrence in the CNS development, with approximately 50% of neurons generated being eliminated by cell death (Oppenheim 1991). The most well recognized example, however, of programmed cell death in early development is during limb development and digit formation. Cells in the interdigital region are removed as part of the process leading to formation of digits. In these examples, apoptosis plays a role in the fusion of two epithelial sheets (neural tube closure), removal of excess cells (CNS development), and sculpting of tissues (digit formation) (Mirkes 2002). With the recent advances in recombinant DNA technology, it is clear today that normal development requires the process of apoptosis in a physiological system. This has been established in studies where various cell death genes have been deleted in mice by homologous recombination (Zheng et al. 1999). Transgenic mice in which the effector caspase-3 is knocked out, for example, exhibit profoundly abnormal brain development characterized by ectopic cell masses located between the cerebral cortex, the hippocampus and the striatum (Kuida et al. 1996). Furthermore, it has been recognized that the phenotype of the caspase-3 knockout is caused primarily

by a decrease of apoptosis. Although this mutant clearly showed that normal brain development required caspase-3-mediated cell death, other organs that exhibit apoptosis, such as heart, lung, liver, kidney, spleen and testis, exhibit no discernible histological abnormalities. These results suggest that other effector caspases, e.g., capsase-6 and/or caspase-7, perhaps perform a redundant function in unaffected organs but not in the brain (Mirkes 2002).

It was much later established that apoptosis is also induced by many teratogens in tissues that develop abnormally and give rise to structural malformations (Scott 1977; Knudsen 1997). For example, over 1,200 chemical and physical agents are known to disrupt development leading to structural and/or functional malformations in experimental animals (Shepard 2001). It has also been established that teratogens often induce cell death in areas of normal apoptosis, suggesting a mechanistic link between apoptosis and teratogen-induced cell death (Menkes et al. 1970; Milaire and Rooze 1983; Sulik et al. 1988). To understand why some cells die and others do not after exposure to a teratogen, it is first necessary to understand the molecular mechanisms of teratogen-induced cell death. When it became clear in the 1990s that toxicant-induced cell death was apoptotic in nature, an initiation by researchers to study these processes was onset and is intensively been investigated still today (Mirkes 2002).

4.2   Apoptosis and Reduction in Nitric Oxide

It has been established in the literature that reduction in nitric oxide and consequent tissue-ischemia in specific tissue types is associated with an increased apoptotic rate. This pathological apoptotic process, furthermore, may be influenced by biological factors and is still today not completely understood. For example, researchers have established that acute renal failure may occur due to

44

ischemia/reperfusion injury and remains a major case of morbidity and mortality among patients in the intensive care unit (Weisberg et al. 1997). It has been observed that this pathology is due to apoptosis and necrosis, alike. Furthermore, this process can be prevented to a certain degree with the supplementary application of melatonin (Kunduzova et al. 2003).

It has also been widely established that besides necrosis, apoptosis is the other major mode of cardiomyocyte loss in ischemic cardiovascular disease. This was well demonstrated with the application of L-NAME, a biochemical agent used to physiologically block NOS-3 in specific tissue types. Liu and colleagues established that L-NAME enhances microcirculatory congestion and cardiomyocyte apoptosis during myocardial ischemia-perfusion in rats (Liu et al. 2002). Another recent study demonstrated that nitric oxide mediates the anti-apoptotic effects of insulin in myocardial ischemia-reperfusion (Gao et al. 2002).

This interplay between ischemia, NOS-3 and apoptosis is, however, quite complex and still poorly understood. While a lack of NO through inhibition of NOS can result in increased apoptotic processes, an increased NO level has also been found to increase apoptotic rates. This was well demonstrated by Qui and colleagues, where they looked at metastatic cancer cells that seed the lung via blood vessels. Because endothelial cells generate nitric oxide (NO) in response to shear stress, this working group postulated that the arrest of cancer cells in the pulmonary microcirculation causes the release of NO in the lung. They concluded in their study involving the arrest of B16 melanoma cells that tumor cell arrest in the pulmonary microcirculation induces eNOS-dependent NO release by the endothelium adjacent to the arrested tumor cells and that NO is one factor that causes tumor cell apoptosis, clearance from the lung, and inhibition of metastasis (Qiu et al. 2003).

4.2.1 Detection of Pulpal Apoptosis in the NOS-3 Knockout Mouse

The TUNEL reaction was apparent in the dental pulp of both the WT and NOS-3 knockout study groups, but was more pronounced in the NOS-3 knockout mouse compared to its WT. We do expect to see minimal apoptotic cells in the dental pulp given a normal physiological situation (i.e. WT), as programmed cell death is a normal physiological process in order to eliminate mutated or weakened cells from the organ system. Furthermore, it has been established that apoptosis contributes to normal dental pulp formation and maintenance (Nishikawa and Sasaki 1999). In both the control group (WT) and the study group (NOS-3 knockout), the majority of the TUNEL positive cells were of the pulpal fibroblast population, and little if any cells were labeled in the odontoblast and sub-odontoblast cell population. The differences in TUNEL positive cell rates between the WT and the knockout group, however, illustrate that the genetic elimination of NOS-3 in our experimental group does in fact influence a change in the balance of healthy cells to those with a nicked DNA, as this is specifically what the TUNEL reaction labels. With this information, it was important to question if the pronounced TUNEL staining in the NOS-3 knockout mice was in fact a true marker of the apoptotic process, where a fragmentation of DNA leads to cellular morphological changes. In order to determine the accuracy of the TUNEL reaction in the knockout mouse group, we examined these immuno-labeled sections under the transmission electron microscope.

While we know that the TUNEL reaction specifically labels the 3'-OH termini of the single strand breaks in DNA, all immuno-labeling that is detected outside the nucleus of the TUNEL positive cell would be considered a "false-positive" immuno-labeling. When examining the pronounced TUNEL labeling in the NOS-3 knockout mice, the Ni-DAB labeling-enhancement was detected only in the nucleus of the TUNEL positive cells. Furthermore, the majority of cells labeled within the dental pulp were fibroblasts and not odontoblasts. The expected histological alterations of apoptosis at the DNA fragmentation stage were noted in all TUNEL positive cells examined with the TEM (Fig. 34-36). The histological changes noted were condensation of chromatin, formation of apoptotic bodies, and disconfiguration of the cellular plasma membrane. Neither TUNEL staining, nor apoptotic cell morphology, was detected in the odontoblasts of the NOS-3 knockout mice.

Other oral tissue types, such as gingival and junctional epithelium, contain cell populations that undergo rapid cell cycles in order to protect the organism from exhausted or mutated cell systems (Thomson et al. 2001). With this rapid cell cycle, a higher apoptotic rate is expected in these tissue types. Therefore, the examination of these tissues is a good control of the TUNEL reaction and a good reference for the morphological changes of their rapid apoptotic process. With the TEM analysis of the TUNEL positive cell bodies within the gingival tissue and junctional epithelium of the NOS-3 knockout mice, we were able to further demonstrate the expected cellular alterations that are coupled with the DNA fragmentation stage of apoptosis (Fig. 34-36). From our TEM analysis of the TUNEL positive cells in the NOS-3 knockout

mouse we may conclude that the TUNEL reaction in these cells is labeling cells that are undergoing a true apoptotic process.

4.3   Apoptosis after Neonatal Application of Capsaicin

As it has often been demonstrated that there is a physiological link between insufficient microcirculation and rate of apoptosis in various tissue types, the same holds true for inactivation and/or denervation of tissue and change in apoptotic rate. In the literature it has been repeatedly demonstrated that the lack of nociceptive innervation is associated with increased cellular apoptosis. For example, it is well known in the literature that neuronal death due to apoptosis after neonatal application of capsaicin is a common occurrence. Sugimoto and colleagues nicely demonstrated that DNA fragmentation can be detected via a TUNEL labeling of neurons in the trigeminal nerve within 24 hours after neonatal application of capsaicin and this early sign of apoptosis was further confirmed with apoptotic morphological changes in the neurons under the transmission electron microscope (Sugimoto et al. 1999). It has also been established, however, that the neonatal application of capsaicin produced apoptotic processes in other cell types. For example, it has been shown that systemical application of capsaicin produces programmed cell death in mitogen activated human T cells (Macho et al. 1999). In this work it is discussed that capsaicin is a vanilloid quinone analog that inhibits the plasma membrane electron transport system and induces apoptosis in transformed cells. Furthermore Lee and colleagues demonstrated that there is a pronounced apoptosis in human glioblastoma cells after capsaicin application (Lee et al. 2000). Jung and colleagues took this one step further and demonstrated the chemoprotective potential of capsaicin in hepatocarcinoma cells by showing the downregulation of Bcl-2 (an anti-

apoptotic biochemical factor) and the activation of caspase-3 (Jung et al. 2001). Again Smith and Liu demonstrated an impairment in wound healing after capsaicin application and discussed the effects of a change in cell proliferation and increased apoptosis (Smith and Liu 2002). While all of these examples demonstrate an association between capsaicin application and increased apoptosis, the exact biochemical mechanism responsible for these phenomena is still very poorly understood.

4.3.1   Detection of Pulpal Apoptosis in the Capsaicin-Treated Rat

The TUNEL reaction was, again, apparent in both the control rat group and the capsaicin-treated rat group, as was detected in the WT and NOS-3 knockout mice groups. The TUNEL reaction in the neonatally desensitized capsaicin-treated rat group, however, demonstrated a severe TUNEL positive cell reaction in both the pulpal fibroblasts and the odontoblast layer (Fig. 15-22). Furthermore, nearly 100% of the odontoblasts in the capsaicin-treated group demonstrated a positive TUNEL reaction at 150 days of age. With these results one must question if the TUNEL reaction is correctly marking the early stages of apoptosis, while these rats were exposed to the desensitization with capsaicin at the 1st post natal day and neither odontoblasts, nor odontoblast-like cells, exhibit a high proliferative cellular rate, as do other cell types (i.e. fibroblasts) (Casasco et al. 1997). Therefore, our results would conclude that either the complete layer of odontoblasts has spontaneously undergone early stages of cell death at 150 days of life, which would be unlikely in the entire experimental group, or that the TUNEL reaction is labeling a cell process that is not representative of apoptosis.   These results, therefore, are quite questionable and further analysis of the TUNEL reaction in the odontoblast layer of

the capsaicin-treated rat was necessary to fully understand the puzzling labeling of the TUNEL reaction in this cell type.

With the described TUNEL results in the capsaicin-treated group, one would expect that it is likely that the TUNEL reaction in this experimental group is representative of a false-positive reaction in the odontoblast layer, in which the TUNEL may be labeling cell structures outside the nucleus. Upon examination of these odontoblasts with TEM, however, we were surprised to observe that the TUNEL labeling is, in fact, confined within the nucleus. More astonishing upon examination with TEM, however, was the cellular structure of the TUNEL-positive odontoblasts in the capsaicin-treated group. Cellular signs of apoptosis, such as chromatin condensation, cell membrane disconfiguration, or apoptotic body formation, were not demonstrated in these cells (Fig. 31-33). It has been questioned in the literature if odontoblasts do in fact show such signs of apoptosis, while we know that these cells may exist as primary odontoblasts for the entire adult life of the organism. It has been demonstrated in organ culture of human teeth, however, that such signs of apoptosis may also be exhibited in the odontoblast layer (Fig. 45-47) (Krage et al. 2002e).
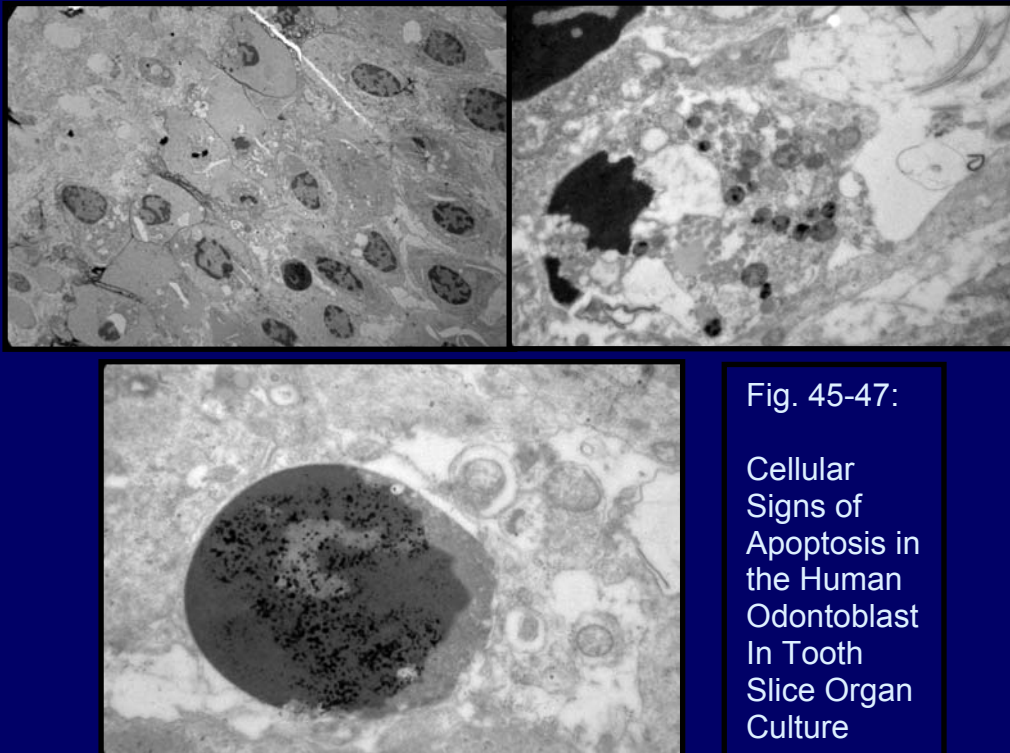
Fig. 45-47:

Cellular Signs of Apoptosis in the Human Odontoblast In Tooth Slice Organ Culture

Thus, our TUNEL reaction may be interpreted in one of two ways:

i.   The TUNEL labeling is indeed a false-positive.

ii. The TUNEL labeling is labeling the 3'-OH termini of the single strand breaks in the DNA of the odontoblasts, but these cells are not undergoing apoptosis after partial DNA damage has occurred.

While the TUNEL labeling is confined to the nuclei, it is unlikely that the labeling is a false-positive one. Further analysis of the odontoblasts in the capsaicin-treated rat group, however, is necessary to determine the specific DNA damage that might be occurring in these cells and whether an apoptotic process is truly beginning in these cells.

4.4   Comparison of Two Study Models Influencing Physiological Factors in the Dental Pulp

In our analysis of the two chosen animal models for this study we hoped to separate the significance between two physiological systems within the pulpal environment, namely the pulpal microcirculation and the pulpal nociceptive innervation. While we have determined, through TUNEL reaction analysis and confirmation of labeling with TEM, that the NOS-3 knockout mouse demonstrates a higher rate of apoptosis in its pulpal fibroblast, we suspect that the reduction in microcirculation results in an increase in apoptosis in pulpal fibroblasts. In the capsaicin-treated rat, on the other hand, we know from previous studies that the neonatally capsaicin-treated rat presents us with a model that exhibits desensitization of the pulpal nociceptive system and, consequently, with a reduction in the release of neuropeptides (i.e. CGRP, SP) which may influence the microcirculation in the pulpal environment (Krage et al. 2002c). In this model, our TUNEL reaction was detected in

both pulpal fibroblasts and odontoblasts. Therefore, we may suspect that the elimination of the nociceptive system in this model is the additional element which is resulting in the TUNEL reaction in the odontoblast layer. Due to the fact that the capsaicin-treated rat does not present a physiological system with the elimination of the nociceptive system alone, it is difficult to interpret the extent of nociceptive influences on physiological microcirculation.

4.4.1   Reduction of Microcirculation in the NOS-3 Knockout Mouse

With the observation of an increased apoptotic rate in the NOS-3 knockout mouse compared to its WT, we must ask 2 questions:

i. What physiological significance does the genetic alteration of systemic NOS-3 in the knockout mouse have on the pulpal tissue and what physiological pathway may link the reduction of NOS-3 in the tissue to an increased rate of apoptosis?

ii. Does this increased rate of apoptosis in the NOS-3 knockout mouse effect the odontoblast function within the pulp, and if so, is this a direct or indirect effect?

To answer the first question, one must consider the importance of NO on cell respiration. We know that NO is a key signaling molecule in cell respiration where, at physiological concentrations, it inhibits the mitochondrial enzyme cytochrome c oxidase (complex IV) in competition with oxygen (Brown and Cooper 1994; Cleeter et al. 1994). Furthermore, it has been suggested that the interplay between oxygen and NO allows cytochrome c oxidase to act as an oxygen sensor in cells (Clementi et al. 1999). Keeping this in mind, we must question which physiological compensation mechanisms have been developed by the NOS-3 knockout mouse in order to maintain a physiological cell respiration rate. It has been observed in one of our early

studies concerning the pulpal tissue of the NOS-3 knockout mouse that the odontoblast cell body and process contain 3-fold more mitochondria than that of odontoblasts in the WT (Fig. 41-44) (Krage et al. 2002d).
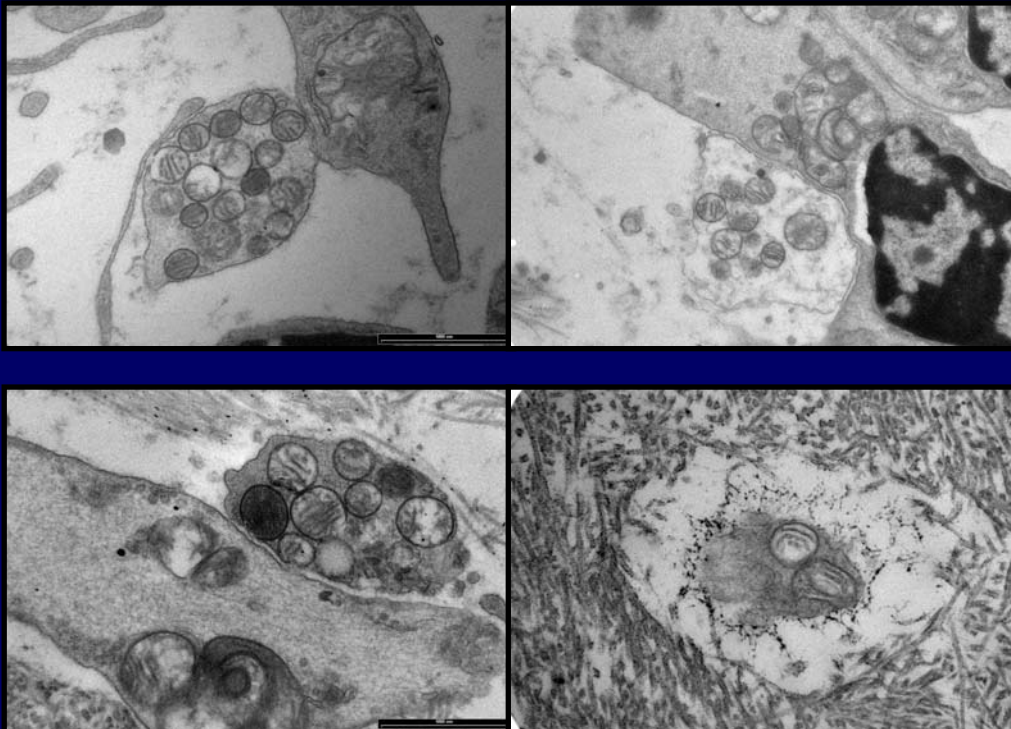
Fig. 41-44:

High Mitochondrial Density in the Odontoblast of NOS-3 Knockout Mice

150 days

While other cell types were not investigated in this previous study concerning mitochondrial density, it does suggest that the NO and cellular mitochondrial function are a significant physiological component in the NOS-3 knockout mouse. Furthermore, the mitochondrion is a key organelle in the control of cell death, via the intrinsic pathway, as was described in the introduction. Here, NO inhibits complex IV in the respiratory chain and is reported to possess both pro-apoptoic and anti-apoptoic actions (Beltràn et al. 2000). Therefore, the concentration of NO in a physiological system is highly determinant of which role NO plays in the apoptotic process. While the NOS-3 knockout mouse is deficient in the endothelial-derived NO and NO is normally a major regulator of vascular tone, one must keep in mind that NO is released by a large variety of cell types and has even been reported to be released from the mitochondrion itself (Lopez-Figueroa et al. 2000). Furthermore, it has recently been reported that mitochondrial membranes generate NO after treatment with different apoptotic stimuli and that this correlates with mitochondrial respiratory impairment as an early phenomenon of apoptosis (Bustamante et al. 2000). Whether or not the apoptosis found in the pulpal tissue of the NOS-3 knockout mouse is one of the intrinsic or extrinsic pathway can not be determined by apoptotic detection through a TUNEL reaction, as this only labels DNA fragmentation which is a final stage of apoptosis. In order to better understand the exact process and pathway of apoptosis in the NOS-3 knockout mouse, a primary antibody which may target an initiator caspase, or other pre-apoptotic stage---prior to the activation of caspase-3---would be necessary to analyze the exact activity and pathway in this early apoptotic process. Therefore, in order to understand the exact correlation of NO and apoptosis in the pulpal tissue of the NOS-3 knockout mouse, further investigations concerning earlier stages of the apoptotic process are necessary.

While we do know, however, that there is an increased apoptotic rate of the fibroblasts in the pulpal tissue and not in the odontoblast layer of the NOS-3 knockout mouse, we must again ask what direct or indirect effect the increased rate of apoptosis may have on the odontoblast function of the NOS-3 knockout mouse. As previously mentioned in the introduction, the adult NOS-3 knockout mouse does demonstrate an alteration in its dentin structure compared to its WT, suggesting an alteration in the odontoblast function of this study model. While we have observed in this study that there is no direct effect on the apoptotic rate of the odontoblast layer itself, there may still be an indirect influence of the increased apoptotic rate of the supporting pulpal fibroblasts on the odontoblast layer. The question of the significance of the cellular interplay of the pulpal tissue is a much more complex one and is still not very well understood in the literature. The previous investigations concerning an alteration in dentin structure of the NOS-3 knockout mouse concluded that the genetic elimination of NOS-3 from a physiological system does in fact effect odontoblast function, but whether the increased apoptotic rate of the pulpal fibroblast is a key player in this alteration of odontoblast function is still yet to be determined.

4.4.2 Reduction of Nociceptive Innervation in the Capsaicin-Treated Rat

It has been established in previous studies that the neonatal application of capsaicin has profound effects on odontoblast function (Fig. 2-4)(Raab et al. 1996, Krage et al. 2002). In these previous studies, the appearance of the crater-like defects in the dentin of nociceptive-inactivated adult rats have lead investigators to suggest that groups of odontoblasts may be missing or presenting temporary malfunction in the odontoblast layer of these animals, thus producing these obvious

voids in dentin production. When one looks at the odontoblast layer in these animals histologically, however, one finds a homogeneously thick odontoblast layer with healthy appearing cell structure. Therefore, these craters in the coronal dentin are not produced by an absence of odontoblasts and it may then be considered that the existing odontoblasts have been altered in function due to the application of neonatal capsaicin. The question is why the function may be altered in these cells.

To answer this question, it is important to consider what physiological significance nociceptive innervation has on the pulpal environment. It has been reported in previous studies that the nociceptive innervation is a crucial element in the regulation of cutaneous cell function and growth (Holzer 1988; Holzer and Maggi 1998; Sann and Pierau 1998). Furthermore, wound tissue contains a rich nerve supply (the cicatricial plexus) (Aldoskogius et al. 1987; Kishimoto et al. 1981; Liu and Luo 1999; Marfurt et al. 1993; Reynolds and Fitzgerald 1995), and rendering innervation deficient through pathological or experimental means reportedly impairs healing of some types of cutaneous wounds (Basson and Burney 1982; Engin et al. 1996; Kjartansson et al. 1987b; Peskar et al. 1995; Westerman et al. 1993). There is accumulating evidence that sensory neuropeptides may mediate the actions of sensory nerves in wound repair, as healing is improved by their exogenous application (Engin 1998; Kjartansson and Dalsgaard 1987a) and impaired by their depletion (Khalil and Helme 1996; Kjartansson et al. 1987b; Peskar et al. 1995). These studies provide a basis for suggesting that peptides released from sensory nerves are important in wound healing. Furthermore, it has been demonstrated that capsaicin---when applied locally---protects the gastric mucosa against experimental injury, while capsaicin desensitisation reduces the rate of gastric ulcer healing (Kang et al. 1996). Not only does this work further demonstrate that the local (transient) and systemic (permanent) effects of capsaicin are physiologically quite different, but this

also demonstrates that nociceptive desensitization with systemic capsaicin application produces detrimental effects on wound healing in a variety of organ systems. Therefore, it would be feasible to consider that the supply of neuropeptides from the nociceptive system in the pulpal tissue is also acting as a regulating factor on the odontoblast layer. This would be one explanation for the production of reparative dentin in the wound healing process of pulpal tissue. With this in mind, the critical question is what exactly is happening to the odontoblast, both directly and indirectly, in the absence of these vital neuropeptides? From our TUNEL results, we have established that---due to the absence of cellular signs of apoptosis in the TUNEL-positive odontoblasts of the capsaicin-treated group---it is very unlikely that these odontoblasts are truly undergoing an apoptotic process. Therefore, it is most likely that the TUNEL is truly labeling the nicked DNA in the nuclei of these odontoblasts, but these cells appear to be surviving with this alteration of their DNA. Furthermore, the likelihood that these cells have altered their function as a result of damage to their DNA is great. Further investigation of the exact location in the DNA sequence which is damaged is necessary, however, to fully understand its significance on the odontoblast function.

In the literature it has been demonstrated in a recent study that there is a profound off-set in the interplay between apoptosis and cell proliferation in the wound healing process of the skin of capsaicin-treated animals (Smith and Liu 2002). Smith and his colleagues have demonstrated that the granulation tissue mitosis rate is drastically increased in the capsaicin-treated rat. An increased mitotic rate would lead to enlarged wound volume only if it is not offset by an increased rate of cell death. Apoptosis is important in the later stages of would healing in the skin for converting highly cellular granulation tissue into relatively acellular mature scar tissue. In Smith's study, the numbers of TUNEL-stained cells were identical in innervated and

denervated preparations, despite the greater mitotic rate after capsaicin treatment. This implies that mechanisms regulating granulation cell death are downregulated below that which is necessary to insure appropriate reductions in cellularity during the transition to a mature scar. Therefore, the failure of apoptosis to keep pace with augmented rates of cell division contributes to greater wound volume, and ultimately a larger scar, in denervated wounds of the skin. Keeping in mind that teeth are derived from the same embryological origin as skin, it is interesting to ponder how this information may apply to the odontoblast. While we did not see final stages of apoptosis in the odontoblast of the capsaicin-treated rat, we also know that the odontoblast does not undergo normal cell proliferation and cell circulation as do fibroblasts of the skin or pulp. Therefore, a failed signaling system between neurons and odontoblasts could be presenting again an offset in which apoptosis is not allowed to be carried out to its final stages.

4.5   Outcomes and Conclusions

4.5.1   Conclusions of the Study

After thorough LM and TEM analysis of TUNEL reaction in our two study models---the capsaicin-treated rat and the NOS-3 knockout mouse---we may conclude the following;

     i.   The NOS-3 knockout mouse has a higher pulpal apoptotic rate compared to its WT, and this finding is most likely due to a reduction in microcirculation in this animal model.

     ii.   The apoptotic rate is increased in the capsaicin-treated rat compared to its control rat, but an apoptotic process in the odontoblasts of the capsaicin-treated rat with TUNEL labeling can not be determined from this study and further investigation is necessary to correlate apoptosis in odontoblasts with pulpal nociceptive innervation.

4.5.2 Future Aspects

While this study has uncovered elements of the pulpal environment where microcirculation and nociceptive innervation come into play, it has also created new questions concerning pulpal physiology and odontoblast function. We must ask ourselves these questions:

i. Is it the loss of neuropeptides that is the significant factor in the capsaicin-treated rat which is altering its odontoblast function ?

ii. Are the loss of the nociceptive innervation and the reduction of microcirculation coupling together to effect odontoblast function in the capsaicin-treated rat, or is this a separate function of the elimination of the nociceptive system?

iii. Is the loss of the nociceptive innervation responsible for the single-strand breakage of DNA in the nuclei of the odontoblast in the capsaicin-treated rat?

iv. Is it possible for odontoblasts with damaged DNA to survive, and if so, how does this effect their function?

These and many more questions concerning pulpal physiology are necessary to answer before one can fully understand the physiology of the odontoblast. In order to answer these questions, systems must be developed in which we can separate each individual physiological system and analyze its sole significance in odontoblast function. In recent years several in vitro (Sloan et al. 1998; Magloire et al. 1985) systems have been refined which will enable us to better answer these questions. It is important, however, not to loose sight of the significance that exists in the interplay of these physiological systems.

5.   References

Aldoskogius H, Hermanson A, Jonsson CE. (1987)
Reinnervation of experimental superficial wounds in rats.
Plast Reconstr Surg. 79(4): 595-599.

Basson MD, Burney RE. (1982)
Defective wound healing in patients with paraplegia and quadriplegia.
Surg Gynecol Obstet. 155(1): 9-12.

Beltràn B, Orsi A, Clementi E, Moncada S. (2000)
Oxidative stress and S-nitrosylation of proteins in cells.
Br J Pharmacol. 129(5): 953-960.

Berggreen E., Heyeraas K. (1996)
Effect of capsaicin treatment or inferior alveolar nerve resection on dentin formation and calcitonin gene-related peptide- and substance P-immunoreactive nerve fibers in rat molar pulp.
Archs oral Biol. 41: 1121-1131.

Bevan JA, Kaley G, Rubanyi GM. (1995)
Flow-dependent regulation of Vascular Function.
New York: Oxford University Press.

Boehringer Mannheim. (1998)
Apoptosis and cell proliferation 2nd Edition. Germany. Boehringer Mannheim GmbH, Biochemica: 2-31.

Bortner DM, Rosenberg MP. (1995)
Overexpression of cyclin A in the mammary glands of transgenic mice results in the induction of nuclear abnormalities and increased apoptosis.
Cell Growth Differ: 6(12): 1579-1589.

Brown GC, Cooper CE. (1994)
Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase.
FEBS Lett. 356(2-3): 295-298.

Buck SH, Burks TF. (1986)
The neuropharmacology of capsaicin: Review of some recent observations.
Pharmacological Reviews 38(3): 179-226.

Bustamante J, Bersier G, Romero M, Badin RA, Boveris A. (2000)
Nitric oxide production and mitochondrial dysfunction during rat thymocyte apoptosis.
Arch Biochem Biophys 376(2): 239-247.

Byers MR. (1992)
Effects of inflammation on dental sensory nerves and vice versa.
Proc Finn Dent Soc.88: 499-506.

Byers MR, Mecifi KB, Kimberly CL. (1987)
Numerous nerves with calcitonin gene-related peptide-like immunoreactivity
innervate junctional epithelium of rats.
Brain Res. 419: 311-314.

Casasco A, Casasco M, Calligaro A, Ferrieri G, Brambilla E, Strohmenger L,
Alberici R, Mazzini G. (1997)
Cell proliferation in developing human dental pulp. A combined flow cytometric
and immunohistochemical study.
Eur J Oral Sci. 105(6): 609-613.

Chang HY, Yang X. (2000)
Proteases for cell suicide: functions and regulation of caspases.
Microbiol Mol Biol Rev. 64: 821-846.

Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH. (1994)
Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the
mitochondrial respiratory chain, by nitric oxide. Implications for
neurodegenerative diseases.
FEBS Lett. 345(1): 50-54.

Clementi E, Brown GC, Foxwell N, Moncada S. (1999)
On the mechanism by which vascular endothelial cells regulate their oxygen
consumption.
Proc Natl Acad Sci U S A. 96(4): 1559-1562.

Cobo J, Hernandez LC, del Valle ME, Vijande M, Vega JA. (1992)
Immunohistochemical localization of epidermal growth factor and its receptor
during odontogenesis in the rat.
Eur J Orthod.14(5): 333-338.

Engin C, Demirkan F, Ayhan S, Atabay K, Baran NK. (1996)
Delayed effect of denervation on wound contraction in rat skin.
Plast Reconstr Surg. 98(6): 1063-1067.

Engin C. (1998)
Effects of calcitonin gene-related peptide on wound contraction in denervated
and normal rat skin: a preliminary report.
Plast Reconstr Surg. 101(7): 1887-1890.

Feron O, Dessy C, Opel DJ, Arstall MA, Kelly RA, Michel T. (1998)
Modulation of the endothelial nitric-oxide synthase-caveolin interaction in
cardiac myocytes. Implications for the autonomic regulation of heart rate.
J Biol Chem. 273(46): 30249-30254.

Fleming I, Busse R. (1999)
NO: the primary EDRF.
J Mol Cell Cardiol. 31: 5-14.

Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H. (1994)
Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions.
Hypertention 23 : 1121-1131.

Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL. (2002)
Nitric Oxide mediates the antiapoptotic effect of insulin in myocardial ischemic-reperfusion: the roles of P13-kinase, Akt, and endothelial nitric oxide synthase phosphorylation.
Circulation Mar 26:105(12): 1497-1502.

Garczyca W, Gong J, Darzynkiewicz Z. (1993)
Detection of DNA strand breaks in individual apoptotic cells by in situ terminal deoxynucleotidyl transferase and nick translation assay.
Cancer Res. 52: 1945-1951.

Gavrieli Y, Sherman SA. (1992)
Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation.
J. Cell Biol. 119: 493-501.

Gazelius B, Edwall B, Olgart L. (1987)
Vasodilatory effects and coexistence of calcitonin gene-related peptide (CGRP) and substance P in sensory nerves of the rat dental pulp.
Acta-Physio-Scand. 130(1): 33-40.

Gluckmann A. (1951)
Cell deaths in normal vertebrate ontogeny.
Biol Rev. 26: 59-86.

Gödecke A, Decking KM, Ding Z, Hirchenhain J, Bidmon HJ, Gödecke S, Schader J. (1998)
Coronary Hemodynamics in Endothelial NO Synthase Knockout Mice.
Circ Res. 82: 186-194.

Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassmann H. (1994)
Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques.
Lab. Invest. 71(2): 219-225.

Heyeraas KJ, Kvinnsland I. (1992)
Tissue pressure and blood flow in pulpal inflammation.
Proc Finn Dent Soc. 88(1): 393-401.

Heyeraas K, Kim S, Raab WH-M, Byers MR, Liu M. (1993)
Effect of electrical tooth stimulation on blood flow, interstitial fluid pressure and substance P and CGRP- immunoreactive nerve fibers in the low compliant cat dental pulp.
Microvascular Research 47: 329-343.

Holzer P. (1988)
Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides.
Neuroscience. 3: 739-768.

Holzer P. (1991)
Capsaicin: cellular targets, mechanism of action, and selectivity for thin sensory neurones.
Pharmcol Rev. 43: 143-201.

Holzer P, Maggi CA. (1998)
Dissociation of dorsal root ganglion neurons into afferent and efferent-like neurons.
Neuroscience 86(2): 389-398.

Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. (1995)
Hypertension in mice lacking the gene for endothelial nitric oxide synthase.
Nature 377: 239-242.

Jancso N. (1968)
Desensitization with capsaicin and related acylamides as a tool for studying the function of pain receptors.
Pharmacology of Pain-Proceedings of Third International Congress in Pharmacology, ed. by R.K.S. Lim, Pergamon Press, Oxford: 33-55.

Jung MY, Kang HJ, Moon A. (2001)
Capsaicin-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves Bcl2 downregulation and caspase-3 activation.
Cancer Lett. 165(2): 139-145.

Kang JY, Teng CH, Chen FC. (1996)
Effect of capsaicin and cimetidine on the healing of acetic acid induced gastric ulceration in the rat.
Gut 38(6): 832-836.

Kerr JFR, Wyllie AH, Currie AR. (1972)
Apoptosis: a basic phenomenon with wide-ranging implications in tissue kinetics.
Br J Cancer 26: 239-257.

Khalil Z, Helme R. (1996)
Sensory peptides as neuromodulators of wound healing in aged rats.
J Gerontol A Biol Sci Med Sci. 51(5): B354-B361.

Kimble J, Hirsch D. (1979)
The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*.
Dev Biol. 70: 396-417.

Kim S. (1990)
Neurovascular interaction in the dental pulp in health and inflammation.
J. Endodontics 16: 48-53.

Kishimoto H, Simon JR, Aprison MH. (1981)
Determination of the equilibrium dissociation constants and number of glycine binding sites in several areas of the rat central nervous system, using a sodium-independent system.
J Neurochem. 37(4): 1015-1024.

Kjartansson J, Dalsgaard CJ. (1987a)
Calcitonin gene-related peptide increases survival of a musculocutaneous critical flap in the rat.
Eur J Pharmacol. 142(3): 355-358.

Kjartansson J, Dalsgaard CJ, Jonsson CE. (1987b)
Decreased survival of experimental critical flaps in rats after sensory denervation with capsaicin.
Plast Reconstr Surg. 79(2): 218-221.

Knudsen TB. (1997)
Cell death. In: Kavlock RJ, Daston CG, editors.
Drug toxicity in embryonic development I. Vol. 124/I.
New York: Springer-Verlag: 211-244.

Krage T, Stiefel A, Raab WH-M. (1999a)
Changes in dentinal and predentinal development after desensitization with capsaicin.
J Dent Res. 78 (IADR Abstracts): #277.

Krage T, Telli C, Raab WH-M. (1999b)
Changes in the formation of reparative dentin after desensitization with capsaicin.
J Dent Res. 78 (IADR Abstracts): #278.

Krage T, Zanger K, Raab WH-M. (2001)
SEM Study of Dentinal Development in the NOS-3 Knockout Mouse.
J Dent Res. 80 (IADR Abstracts): #1424.

Krage T, Lambrichts I, Zanger K, Raab WH-M. (2002a)
Capsaicin-treated animals vs. NO-knockout mice: a SEM and TEM comparison.
Proceedings to the International Conference on Dentin/pulp Complex 2001, Quintessence Publishing, Editor: Ishikawa et al., Tokyo: 187-189.

Krage T, Vandenabeele F, Lambrichts I, Raab WH-M. (2002b)
A TEM analysis of pulpal sensory content in capsaicin-treated rats.
Proceedings to the International Conference on Dentin/pulp Complex 2001, Quintessence Publishing, Editor: Ishikawa et al., Tokyo: 185-186.

Krage T, Lambrichts I, Raab WH-M. (2002c)
Capsaicin-treated animals vs. the NO-knockout mouse: an immunohistological comparison.
Proceedings to the International Conference on Dentin/pulp Complex 2001, Quintessence Publishing, Editor: Ishikawa et al., Tokyo: 119-122.

Krage T, Vandenabeele F, Lambrichts I, Raab WH-M. (2002d)
Odontoblast microstructure and possible compensation in the NOS-3 knockout mouse.
J Dent Res. 81 (Spec Iss A): #0272.

Krage T, Lambrichts I, Vandenabeele F, Goedecke A, Raab WH-M. (2002e)
Apoptosis in pulpal tissue of the NOS-3 knockout mouse.
J Dent Res. CED/PAN Meeting, Cardiff, Whales: #448

Küln R, Rajewsky K, Müller W. (1991)
Generation and analysis of interleukin 4 deficient mice.
 Science 254: 707-710.

Kuida K, Zheng TS, Na S, Kuan C-Y, Yang D, Karasuyama H, Rakic P, Flavell RA. (1996)
Decreased apoptosis in the brain and premature lethality in CPP-32 deficient mice.
Nature 384: 368-372.

Kunduzova OR, Escourrou G, Seguelas MH, Delagrange P, De La Farge F, Cambon C, Parini A. (2003)
Prevention of apoptotic and necrotic cell death, caspase-3 activation, and renal dysfunction by melatonin after ischemia/perfusion.
FASEB journal online:10.1096/fj.02-0504fje.

Kvinnsland I, Heyeraas K J, Byers MR. (1991)
Regeneration of calcitonin gene-related peptide (CGRP) immunoreactive nerves in replanted rat molars and their supporting tissues.
 Arch Oral Biol. 36: 815-825.

Lawson SN. (1989)
The morphological consequences of neonatal treatment with capsaicin on primary afferent neurons in adult rats.
Acta Physiol Hung. 69(3-4): 315-321.

Lee YS, Nam DH, Kim JA. (2000)
Induction of apoptosis by capsaicin in A172 human glioblastoma cells.
Cancer Lett. 161(1):121-130.

Lembeck F. (1983)
Sir Thomas Lewis's nocifensor system, histamine and substance-P-containing primary afferent nerves.
TINS: 106-108.

Lembeck F. (1987)
Columbus, capsaicum and capsaicin: past, present and future.
Acta Physiol. Hung. 69: 265-273.

Liu WG, Luo YX. (1999)
The early protective effects of basic fibroblast growth factor on acute spinal cord injury in rats.
Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 13(5): 291-294.

Liu P, Xu B, Forman LJ, Carsia R, Hock CE. (2002)
L-NAME enhances microcirculatory congestion and cardiomyocyte apoptosis during myocardial ischemia-reperfusion in rats.
Shock Mar. 17(3): 185-192.

Lopez-Figueroa MO, Caamano C, Morano MI, Ronn LC, Akil H, Watson SJ. (2000)
Direct evidence of nitric oxide presence within mitochondria.
Biochem Biophys Res Commun. 272(1): 129-133.

Macho A, Calzado MA, Munoz-Blanco J, Gomez-Diaz C, Gajate C, Mollinedo F, Navas P, Munoz E. (1999)
Selective induction of apoptosis by capsaicin in transformed cells: the role of reactive oxygen species and calcium.
Cell Death Differ Feb. 6(2): 155-165.

Maggi CA. (1991)
Capsaicin and primary afferent neurons: from basic science to human therapy?
J Auton Nerv Syst. 33(1): 1-14.

Magloire, H, Hartmann DJ, Couble ML, Joffre A, Grimaud JA, Herbage D, Ville G. (1985)
Cytodifferentiation of human dental pulp cells in explant culture. In: Tooth Morphogenesis and Differentiation. (Eds. A.B. Belcourt, J.V. Ruch)
Colloque. INSERM. Paris. 125: 119 -126.

Mansour SL, Thomas KR, Capecchi MR. (1986)
Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes.
Nature 336: 743-748.

Marfurt CF, Ellis LC, Jones MA. (1993)
Sensory and sympathetic nerve sprouting in the rat cornea following neonatal administration of capsaicin.
Somatosens Mot Res. 10(4): 377-398.

Marsden PA, Heng HHQ, Scherer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC, Schappart KT. (1993)
Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene.
J. Biol Chem. 268:17478-17488.

Menkes B, Sandoe S, Ilies A. (1970)
Cell death in teratogenesis. In: Wollam DH, editor. Advances in teratology.
New York: Academic Press: 169-215.

Metzstein MM, Hengartner MO, Tsung N, Ellis RE, Horvitz HR. (1996)
Transcriptional regulator of programmed cell death encoded by Caenorhabditis elegans gene ces-2.
Nature 382(6591): 545-547.

Michal M. (1997)
Benign epithelial structures on the surface of appendices.
Pathology 29(3): 267-269.

Milaire J, Rooze M. (1983)
Hereditary and induced modifications of the normal necrotic patterns in the developing limb buds of the rat and mouse; facts and hypotheses.
Arch of Biol. (Bruxelles) 94: 459-490.

Mirkes PE. (2002)
2001 Warkany Lecture: To die or not to die, the role of apoptosis in normal and abnormal mammalian development.
Teratology 65: 228-239.

Mombouli J, Vanhoutte P. (1999)
Endothelial Dysfunction: From Physiology to Therapy.
J Mol Cell Cardiol. 31: 61-74.

Moncada S, Palmer RMJ, Higgs EA. (1991)
Nitric oxide: physiology, pathophysiology, and pharmacology.
Pharmacol Rev. 43: 109-142.

Nilsson J, von Euler A, Dalsgaard CJ. (1985)
Stimulation of connective tissue and cell growth by substance P and substance K.
Nature 315: 61-63.

Nishikawa S, Sasaki F. (1999)
Apoptosis of dental pulp cells and their elimination by macrophages and MHC class II-expressing dendritic cells.
J Histochem Cytochem. 47: 303-311.

Norlin T, Hilliges M, Brodin L. (1999)
Immunohistological demonstration of exocytosis regulating proteins within rat molar dentinal tubules.
Arch. Oral Biol. 44(3): 223-231.

Olgart L, Edwall L, Gazelius B. (1991)
Involvement of afferent nerves in pulpal blood-flow reactions in response to clinical and experimental procedures in the cat.
Arch Oral Biol. 36: 575-581.

Oppenheim RW. (1991)
Cell death during development of the nervous system.
Annu Rev Neurosci. 14: 453-501.

Peskar BM, Lambrecht N, Stroff T, Respondek M, Muller KM. (1995)
Functional ablation of sensory neurons impairs healing of acute gastric mucosal damage in rats.
Dig Dis Sci. 40(11): 2460-2464.

Qiu H, Orr FW, Jensen D, Wang HH, McIntosh AR, Hasinoff BB, Nance DM, Pylypas S, Qi K, Song C, Muschel R, Al-Mehdi AB. (2003)
Arrest of B16 melanoma cells in the mouse pulmonary microcirculation induces endothelial nitric oxide synthases-dependent nitric oxide release that is cytotoxic to the tumor cells.
Am J Path. 162: 403-412.

Raab WH-M. (1990)
Sensorische und entzündliche Reaktionsmechanismen der Zahnpulpa.
Dtch Zahnärztl Z. 45(1): 20-22.

Raab WH-M. (1992)
Temperature related changes in pulpal microcirculation.
Proc Finn Dent Soc. 88(1): 469-479.

Raab WH-M, Stiefel A, Müller-Raab K. (1996)
Changes in the formation of dental hard tissues after desensitization with capsaicin.
Proceedings to the International Conference on Dentin/pulp Complex 1995, Quintessence Publishing, Editor: Shimono et al., Tokyo: 90-92.

Reynolds ML, Fitzgerald M. (1995)
Long-term sensory hyperinnervation following neonatal skin wounds.
J Comp Neurol. 358(4): 487-498.

Russell LC, Burchiel KJ. (1984)
Neurophysiological effects of capsaicin.
Brain Res. Rev. 8: 165-176.

Sann H, Pierau FK. (1998)
Efferent functions of C-fiber nociceptors.
Z Rheumatol. 57(2): 8-13.

Scott WJ. (1977)
Cell death and reduced proliferative rate
In: Wilson JG, Fraser FC, editors. Handbook of teratology. Vol. 2.
New York: Plenum Press: 81-98.

Shepard TH. (2001)
Catalog of teratogenic agents.
Tenth edition. Baltimore: The Johns Hopskins University Press.

Shesely,EG., Maeda. N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman
PA, Sessa WC, Smithies O. (1996)
Elevated blood pressures in mice lacking endothelial nitric oxide synthase.
Proc Natl Acad Sci USA. 93: 13176-13181.

Sloan, AJ, Shelton, RM, Hahn, AC, Moxham, BJ, Smith, AJ. (1998)
An in vitro approach for the study of dentinogenesis by organ culture of the
dentin/pulp complex for rat incisor teeth.
Archs Oral Biol. 43: 421-430.

Smith PG, Liu M. (2002)
Impaired cutaneous wound healing after sensory denervation in developing
rats: effects on cell proliferation and apoptosis.
Cell Tissue Res. 307(3): 281-291.

Sugimoto T, Takeyama A, Xiao C, Takano-Yamamoto T, Ichikawa H. (1999)
Electron microscopic demonstration of nick-end labeled DNA fragments during
capsaicin-induced apoptosis of trigeminal primary neurons in neonatal rats.
Brain Res Feb. 6:818(1): 147-152.

Sulik KK, Cook CS, Webster WS. (1988)
Teratogens and craniofacial malformations: relationships to cell death.
Development 103(Suppl): 213-232.

Sulston JE, Horvitz HR. (1977)
Post-embryotic cell lineage of the nematode *Caenorhabditis elegans*.
Dev Biol. 56: 110-156.

Sulston JE, Schierenberg E, White JG, Thomson JN. (1983)
The embryonic cell lineage of the nematode *Caenorhabditis elegans*.
Dev Biol. 100: 64-119.

Surh YJ. (2002)
More than spice: capsaicin in hot chili peppers makes tumor cells commit suicide.
J Natl Cancer Inst. 94(17): 1263-1265.

Szolcsanyi J. (1982)
Pungent agents producing pyrexia. In Handbook of Experimental Pharmacology, Pyretics and Antipyretics, ed. by A.S. Milton, Springer-Verlag, Berlin. 60: 437-478.

Szolcsanyi J, Nagy J, Petho G. (1993)
Effect of CP-96,345 a non-peptide substance P antagonist, capsaicin, resiniferatoxin and ruthenium red on nociception.
Regul Pept. 46(1-2): 437-439.

Szolcsanyi J, Szallasi A, Szallasi Z, Joo F, Blumberg PM. (1990)
Resiniferatoxin: an ultrapotent selective modulator of capsaicin-sensitive primary afferent neurons.
J Pharmacol Exp Ther. 255(2): 923-928.

Thomson PJ, Potten CS, Appleton DR. (2001)
In vitro labeling studies and the measurement of epithelial cell proliferation activity in the human oral cavity.
Arch of Oral Biol. 46(12): 1157-1164.

Vanags DM, Porn-Ares MI, Coppola S, Burgess DH, Orrenius S. (1996)
Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis.
J Biol Chem. 271: 31075-31085.

Virus RM, Gebhart GF. (1979)
Minireview-pharmacologic actions of capsaicin. Apparent involvement of substance P and serotonin.
Life Science 25: 1273-1284.

Weisberg LS, Allgren RL, Genter FC, Kurnik BR. (1997)
Cause of acute tubular necrosis affects ist prognosis. The Auriculin Anaritide Acute Renal Failure Study Group.
Arch Intern Med. 157: 1833-1888.

Westerman RA, Carr RW, Delaney CA, Morris MJ, Roberts RG. (1993)
The role of skin nociceptive afferent nerves in blister healing.
Clin Exp Neurol. 30: 39-60.

Wyllie A, Donahue V, Fischer B, Hill D, Keesey J, Simone M. (1998)
Apoptosis and cell proliferation, 2nd Edition. Boehringer Mannheim, Biochemica, Germany: 1-31.

Zhang JL, Petal JM, Li YD, Block ER. (1996)
Reductase domain cysteines 1048 and 1114 are critical for catalytic activity of human epithelial cell nitric oxide synthase as probed by site-directed mutagenesis.
Biophys Res Commun. 226: 293-300.

Zheng TS, Hunot S, Kuida K, Flavell RA. (1999)
Caspase knockouts: matters of life and death.
Cell Death Differ. 6: 1043-1053.

6. Acknowledgements

Curriculum Vitae

Persönliche Daten

Name: Tracy Krage (DMD-USA)
Geburtstag: 12.10.1968
Geburtsort: San Francisco, Kalifornien, USA
Nationalität: Amerikanerin

Schule/Universität

1974-1980:   Grundschule „Grimmer Grammer School"
                  Fremont, Kalifornien, USA

1980-1982:   Mittelstufenschule „Walters Junior Highschool"
                  Fremont, Kalifornien, USA

1982-1986:   Hochstufenschule „J.F. Kennedy Highschool"
                  Fremont, Kalifornien, USA

1986-1991:   University of California at Davis, USA
                  Abschluss: Bachelor of Science in Physiology (BS)

1991-1995:   Tufts University, School of Dental Medicine, Boston, USA
                  Abschluss: Doctor of Medical Dentistry (DMD)

Examen/Approbation/Promotion

Juli 1993:   "US National Dental Board Examination" Teil I
Dezember 1994:   "US National Dental Board Examination" Teil II
August 1995:   "Combined Regional Examination" (NERB & CRDTS)
August 1995:   Approbation als Zahnärztin in den USA

März 1996-Sept. 1997:   Berufserlaubnis als Zahnärztin, Niedersachsen, Deutschland
Sept. 1997-Mai 2001:   Berufserlaubnis als Zahnärztin, Nordrhein-Westfalen,
                         Deutschland

Mai. 2001:   Approbation als Zahnärztin in Deutschland

Klinische Erfahrung

März 1996-August 1997:   Zahnärztin, Praxis Dres. Stockleben, Hannover, Deutschland

Sept. 1997 bis heute: Wissenschaftliche Mitarbeiterin
                          Heinrich-Heine-Universität Düsseldorf
                          Poliklinik für Konservierende Zahnerhaltung
                          und Präventive Zahnheilkunde

Wissenschaftliche Erfahrung

1987-1991:	Labortechnische Assistentin, Renal Devision Abteilung für Humanphysiologie, Prof. Dr. L. Rabinowitz UC Davis, School of Medicine, California, USA
1989-1991:	Labortechnische Assistentin Abteilung für Bakteriologie, Prof. Dr. R. Walker UC Davis, School of Vet. Medicine, California, USA
6/1992-9/1992:	Biomedizinisches Austauschprogramm, Prof. Dr. H. Stolte Abteilung für Radiologie, Medizinische Hochschule, Hannover
9/1997 bis heute:	Forschungsprojekttätigkeit „der neuronale und der Einfluss der Mikrozirkulation auf die Odontoblastenfunktion" Abteilung für Konservierende Zahnheilkunde Heinrich-Heine-Universität Düsseldorf
9/1998:	Immunhistologische Weiterbildung Abteilung für Physiologie, Prof. Dr. Heyeraas Universität in Bergen, Belgien
2/2001:	Zell-und Organkulturen Fortbildung Abteilung für orale Biologie, Prof. AJ Smith Universität in Birmingham, England
7/2002-8/2002:	Sommerkurs in „Clinical Dental Research" Department of Dental Public Health, Prof. Timothy DeRouen Universität in Washington, Seattle, USA
6/2002-9/2002:	Immunhistologische Weiterbildung Abteilung für Anästhesiologie, Prof. Margaret Byers Universität in Washington, Seattle, USA

Auszeichnungen

7/2001:	Quintessence Award internationale Konferenz des Dentin/Pulpa Komplex 2001 Kazusa, Japan
9/2002:	Senior Robert Frank Award (ausgewählt als Kandidatin für den Hatton Award beim IADR in Schweden 6/2003) „International Assiciation of Dental Research" Central European Division Cardiff, Wales

---

Ort und Datum

Apoptose in der Zahnpulpa: Einflüsse der pulpalen Mikrozirkulation und der pulpalen nozizeptiven Innervation.

Tracy Krage, DMD (USA)
Poliklinik für Zahnerhaltung und Präventive Zahnheilkunde, Heinrich-Heine-Universität Düsseldorf.

Es ist bis heute ungeklärt, welche physiologischen Auswirkungen die nozizeptive Innervation und die pulpale Mikrozirkulation auf die Funktion der Odontoblasten hat. Deshalb sind Tierstudienmodelle, in denen selektiv spezifische physiologische Faktoren ausgeschaltet werden, sehr hilfreich bei der Untersuchung der Abhängigkeiten der Odontoblasten-Physiologie. Die neonatale Behandlung mit Capsaicin erzeugt bei Ratten ein Tiermodell, das eine selektive Inaktivierung des nozizeptiven Systems zeigt. Darüberhinaus ist die Stickstoffoxid-Synthase-3-Knockout-Maus, die auch als eNOS-Knockout-Maus bezeichnet wird, ein wertvolles physiologisches System, um die Auswirkungen von Veränderungen der pulpalen Mikrozirkulation auf die Odontoblastenfunktion zu studieren. Zurückliegende Studien haben gezeigt, dass es Veränderungen in der Odontoblastenfunktion jedes dieser Tiermodelle gibt. Eine physiologische Erklärung für diese Veränderungen fehlt noch immer. Apoptose, der programmierte Zelltod, könnte ein wichtiger physiologischer Faktor in der zellulären Interaktion innerhalb der Zahnpulpa sein. Ziel der vorliegenden Studie war es, das Ausmaß der zellulären Apoptose im Pulpagewebe dieser beiden Tiermodelle zu studieren, um die Bedeutung der nozizeptiven Funktion und der pulpalen Mikrozirkulation in der zellulären Physiologie der Zahnpulpa besser zu verstehen. Dazu wurde die TdT-abhängige X-dUTP-nick-end-labeling(TUNEL)-Reaktion in pulpalem Gewebe durchgeführt, um DNA-Einzelstrangbrüche zu erkennen. Fünf Capsaicin-behandelte Ratten, denen vom ersten bis dritten Lebenstag eine Dosis von 40~mg/kg Körpergewicht an Capsaicin systemisch verabreicht wurde, und fünf unbehandelte Ratten, die als Kontrollgruppe dienten, stellten die Capsaicin-Studiengruppe dar. Fünf eNOS-Knockout-Mäuse und fünf Wildtyp(WT)-Mäuse dienten als eNOS-Studiengruppe. Alle Tiere wurden im Alter von 150 Tagen geopfert und ihre Kiefer präpariert. Dazu wurde der als TUNEL bezeichnete enzymatische Labeling-Test, der auch als „In Situ Nick Translation Kit" (Fa. Roche, Deutschland), bekannt ist, angewendet. Als Kontrolle unserer Ergebnisse wurden an ausgewählten Geweben weitere zelluläre Untersuchungen mit Hilfe des Transmissionselektronenmikroskop (TEM) durchgeführt. Vergleicht man die Capsaicin-behandelten Ratten mit ihrer Kontrollgruppe, so zeigen unsere Ergebnisse eine erhöhte TUNEL-positive Zellreaktionsrate in den pulpalen Fibroblasten und in der Odontoblasten-Zellschicht. Obwohl in der NOS-3-Knockout-Maus, im Vergleich zu ihrer WT Kontrollgruppe, eine erhöhte TUNEL-positive Zellreaktionsrate beobachtet wurde, fanden wir bei der eNOS-Knockout-Maus im Vergleich zu ihrer WT Kontrollgruppe keine Erhöhung in der TUNEL-Zellreaktionsrate in der Odontoblastenschicht. Darüberhinaus zeigte die TEM-Analyse Anzeichen von Apoptose auf zellulärer Ebene in den TUNEL-positiven Fibroblasten beider Tiermodelle. In den TUNEL-positiven Odontoblasten der Capsaicin-behandelten Gruppe wurden bei der TEM-Analyse keine zellulären Anzeichen von Apoptose gefunden. Diese Ergebnisse deuten darauf hin, daß das TUNEL Enzymlabeling von Odontoblasten in der Capsaicin-behandelten Gruppe einen DNA Einzelstrangbruch im Kern nachweist, und diese nachgewiesene DNA-Beschädigung führt nicht zum erwarteten apoptotischen Prozess. Wir schließen aus unseren Ergebnissen, dass das nozizeptive System und die Mikrozirkulation der Zahnpulpa eine wichtige physiologische Bedeutung auf die apoptotische und zelluläre Interaktion der pulpalen Umgebung haben. Darüberhinaus könnten diese zellulären Einflüsse funktionelle Veränderungen in den Odontoblasten der Capsaicin-behandelten Ratte und der eNOS-Knockout-Maus herbeiführen.

Unterschrift des Referenten Prof.Dr.W.H.-M. Raab