J Periodont Res 2005; 40; 28–35

JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2004.00765.x

Chronic treatment with the glutamate receptor antagonist MK-801 alters periodontal disease susceptibility

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Breivik T, Gundersen Y, Osmundsen H, Opstad PK, Fonnum F. Chronic treatment with the glutamate receptor antagonist MK-801 alters periodontal disease susceptibility. J Periodont Res 2005; 40: 28–35. © Blackwell Munksgaard 2004

Objective: Previous experiments in rats suggest that hypothalamic–pituitary–adrenal (HPA) axis over-responsiveness, which leads to increased secretion of immunoregulatory glucocorticoid hormones, increases periodontal disease susceptibility, whereas HPA axis under-responsiveness is associated with increased resistance to the disease. The present study was designed to investigate whether MK-801 (dizocilipine malate), an antagonist of the glutamate receptor *N*-methyl-D-aspartate (NMDA) in the brain, which has been found to play an important role in the regulation of the HPA axis, would influence the outcome of experimental ligature-induced periodontal disease in a rat model.

Methods: Experimental periodontal disease was induced in periodontal disease susceptible and HPA axis high-responding Fischer 344 rats 2 days before chronic treatment with MK-801(1 mg/kg intraperitoneally). The periodontal breakdown was assessed after the ligatures had been in place for 23 days. Following intraperitoneal Gram-negative bacterial lipopolysaccharide stimulation (*Escherichia coli*, 250 μg/kg), concentrations of glucocorticoid receptors (GRs) in the hippocampus, and levels of the cytokine tumour necrosis factor α (TNF-α), as well as the HPA axis-derived hormone corticosterone, were measured in serum.

Results: Compared to vehicle-treated controls, MK-801-treated rats had significantly increased periodontal tissue destruction ($p < 0.01$). MK-801-treated rats also showed significantly increased expression of GRs in the hippocampus ($p < 0.05$), elevated levels of corticosterone ($p < 0.001$) and reduced levels of TNF-α ($p < 0.01$) in serum 2 h after lipopolysaccharide stimulation.

Conclusion: These findings may implicate glutamate receptor-dependent mechanisms in periodontal disease, and support the concept of a bidirectional immune–brain–immune regulatory network with importance for periodontal health and disease.

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Key words: cytokines; glucocorticoids; lipopolysaccharide; periodontal disease

Accepted for publication July 21, 2004

Periodontal disease is the result of tissue destructive inflammatory responses in the gingival connective tissues to increased colonization of pathogenic dental plaque microorganisms, termed periodontopathogens (1–4). The disease is characterized by destruction of the dental attachment apparatus, including loss of periodontal attachment

fibres and resorption of the alveolar bone. In the most severe cases tooth loss may result. A change in the composition of dental plaque microflora towards species with potent ability to invade the host is clearly a triggering event. However, most of the cellular damage seems to be caused by tissue destructive agents such as reactive oxygen species and matrix metalloproteinases, released by cells belonging to the innate immune system as a response to the increased colonization of these pathogens (5–7).

Clinical observations from dental practise suggest that there are great variations in periodontal disease susceptibility. Smokers, diabetics, or individuals exposed to negative life events are especially vulnerable. Devastating cases are frequently observed in people suffering from pathological grief related to conjugal loss combined with poorly developed psychological coping strategies to negative life experiences (8–10). This relationship has recently been confirmed by epidemiological studies performed by our group as well as others (10–12). A positive association has also been established with genetics (13), increasing age (14, 15), and insulin deficiency (16). A fundamental question in periodontal disease is therefore how these risk factors can facilitate the growth of the invasive pathogens and thereby enhance the subsequent immune-induced tissue destruction. Previous investigations indicate that effects on the hypothalamic–pituitary–adrenal (HPA) axis may bias the immune response towards a too weak T helper 1 (Th1) response, and thus allow the periodontal pathogens to multiply (9, 16–23).

Activation of the HPA axis by danger signals, whether they are of psychological, physical, chemical or immunological origin, leads to an increased production and release of the corticotropin-releasing hormone and arginine vasopressin from the parvocellular neurones in the paraventicular nucleus (PVN) of hypothalamus. These hormones stimulate cells in the pituitary to secrete the adrenocorticotropic hormone (ACTH), which in turn enhances the secretion of glucocorticoids (predominantly cortisol in man and corticosterone in rodents) from the adrenal cortex. The powerful immunoregulatory properties of these hormones include suppression of Th1 responses and a subsequent down-regulation of cellular immunity (24, 25).

The corticotropin-releasing hormone/arginine vasopressin production in the parvocellular PVN neurones in the hypothalamus, which drives the HPA axis, is regulated by a number of inhibitory and excitatory pathways from various brain centres (26, 27). These multiple inputs allow the HPA axis to react to psychological, physical, chemical and immunological challenges (28, 29). The HPA axis responsiveness is also regulated by a feedback mechanism where glucocorticoids rapidly enter the brain, particularly the hippocampus, and bind to two types of corticosteroid receptors, namely the high affinity mineralocorticoid receptors, and the 10-fold lower affinity glucocorticoid receptors (GRs) (30).

Within the brain, the neurotransmitter glutamate has been found to play a significant role in generating and limiting responses initiated by danger signals that are interpreted as threatening for the organism (31). HPA axis activation stimulates the production of glutamate in the hippocampus, and the alteration in glutamate neurotransmission may be responsible for the negative feedback effect of the HPA axis via the hippocampus during dangerous situations (32). Thus, glutamatergic input from the hippocamus to the hypothalamic PVN seems to be involved in the negative feedback regulation of the HPA axis, which may also include the signals coming from pathogen-stimulated immune cells (33).

One of the major glutamate receptors at excitatory synapses in the brain, the *N*-methyl-D-aspartate (NMDA) receptor subtype, is importantly involved in HPA axis regulation (31). By means of various NMDA receptor agonists and antagonists it is possible to manipulate the glutamatergic neurotransmission, and thereby alter a wide spectrum of functional responses to environmental stimuli. MK-801 (dizocilpine maleate) is a glutamate receptor antagonist that binds to a non-competitive site within the NMDA receptor channel, and high doses of MK-801 are found to stimulate the HPA axis (34).

The aim of this study was to investigate whether chronic treatment with MK-801 would influence the development and progression of periodontal disease in HPA axis over-responding and periodontal disease susceptible Fischer 344 rats. We also wanted to measure whether MK-801 treatment influences GR expression in the hippocampus, corticosterone output, and cytokine responses to a robust lipopolysaccharide challenge.

Material and methods

Animals

Twenty male Fischer 344 rats, weighing approximately 300 g at the time of periodontal disease induction, were obtained from Möllegaard Breeding Center (Ejby, Denmark), and used after 2 weeks of acclimatization. The rats were housed in groups of three to four, and had free access to standard rat pellets and tap water. The animals were maintained under a 12–24 h light/dark cycle (light on 07.00 h to 19.00 h) with temperature and humidity at 22°C and 40–60%, respectively. The experiments were registered and approved by the Norwegian Experimental Animal Board (NEAB).

Experimental periodontal disease model

The animals were anaesthetized with a subcutaneous injection of Hypnorm-Dormicum (fentanyl/fluanisone, midazolam), 0.2 ml/100 g body weight. A sterile silk ligature (Ethicon Permahand® Seide, Norderstedt, Germany) was tied around the neck of the maxillary right 2nd molar teeth. The ligatures were left in the same position during the entire experiment and served as a retention device for oral microorganisms. Two hours before decapitation all animals were injected intraperitoneally with lipopolysaccharide (250 μg/kg, *Escherichia coli* Serotype 0111:B4, Sigma, St Louis, MO,

USA) to induce a robust immune and HPA axis response.

MK-801 treatment

The rats were randomly assigned to two groups ($n = 10$ in each group). Two days after periodontal disease induction the animals belonging to group 1 were injected daily intraperitoneally with MK-801 [1 mg/(kg day)] dissolved in sterile water, ethanol, and 0.1 N HCl for 2 days consecutively, rested for 2 days, injected with the same dosage for another 5 days consecutively, rested for 9 days, and ultimately injected for another 4 days consecutively with a reduced dosage [0.4 mg/(kg day)]. Group 2 had the same amount of vehicle and served as controls.

Blood serum preparation and storage

After decapitation of the rats, the blood samples were collected (6–10 ml from each animal) in vacutainer tubes (10 ml without additives) and allowed to clot on ice for 1 h. Thereafter, the samples were centrifuged for 20 min at 2000 *g*. The serum samples were removed, aliquoted and stored at −20°C prior to analysis of cytokines and corticosterone.

Isolation of RNA from hippocampus and reverse transcription–polymerase chain reaction (RT-PCR) assay of mRNA for the glucocorticoid receptor

The hippocampus was isolated immediately after decapitation of the rats and transferred into 1 ml of RNA-later (Ambion Inc., Austin, TX, USA) for later processing. RNA was extracted using RNA-Whiz (Ambion Inc.), yielding RNA preparations with A_{260}/A_{280} of 1.7–1.8. These were DNAase treated (DNA-Free, Ambion Inc.) prior to use in RT–PCR analysis.

RT–PCR primers were designed from the published base-sequence of mRNA for the rat glucocorticoid receptor (Accession no. Y12264) using the Oligo program (version 6) (Molecular Biology Insights Inc., Cascade, CO, USA). For internal standard, the signal from primers designed from the mRNA sequence of rat ribosomal protein L27 (Accession no. NM 022514) was preferred to those from rat β-actin (Accession no. V01217 J00691) signal. Primers for L27 and β-actin were also designed using Oligo version 6.

RT–PCR was carried out using the Qiagen One-Step RT–PCR kit (Qiagen GmbH, Hilden, Germany). RT–PCR products were analysed by agarose-electrophoresis using Gelstar fluorescence dye (BMA Inc., Cambrex Corp., New Jersey, NJ, USA) for detection. Fluorescence signals were quantified by using the ProExpress imager (Perkin Elmer Life and Sciences Inc., Boston, MA, USA). The RT–PCR analysis was carried out so as to ensure linearity between amount of RT–PCR product and integrated fluorescence signal.

Jaw preparation and storage

The maxillae were excised and fixed in 4% formaldehyde.

Analysis of corticosterone and tumour necrosis factor α (TNF-α)

The separated plasma corticosterone samples were thawed and measured with ^{125}I radioimmunoassay coat-A-count kit from Diagnostic Products Corporation (Los Angeles, CA, USA, catalogue number TKRC1). The detection limit was 5.7 ng/ml. The levels of TNF-α in the serum samples were measured by means of enzyme-linked immunosorbent assay (ELISA) kits (catalogue numbers RTA00, R & D Systems Inc., Minneapolis, MN, USA). The minimum detectable concentration of TNF-α was less than 12.5 pg/ml.

Radiographic examination of periodontal bone loss

The maxillary jaws were placed and stabilized with dental wax on a Trophy digital X-ray sensor, orientated with the axis of the teeth parallel to the sensor surface. The distance between the cemento-enamel junction and the alveolar bone on mesial and distal surfaces of the second molars was displayed digitally. The examiner was unaware of whether the specimens came from experimental or control animals/teeth. The reliability of the method was tested earlier (17) and shown to have a standard error of the mean (SEM) difference between two readings of 0.16 mm.

Histological examination of periodontal tissue loss

After the radiographic examinations, the specimens were decalcified in 10% EDTA for approximately 2 weeks, until complete decalcification could be confirmed radiographically. The specimens were then dehydrated in graded alcohol, carefully orientated and embedded in paraffin with the axis of the teeth parallel to the cutting direction. The blocks were cut in serial sections of 5-μm thickness in a mesio-distal direction. The most central section from each tooth, i.e. the one that comprised the centre of the dental pulp, was selected for analysis, stained with haematoxylin and eosin, and mounted.

The sections were placed in a Nikon microscope (Nikon GmbH, Dusseldorf, Germany) with a camera and a TV monitor (Sony Entertainment TV, Oslo, Norway). Sections from experimental sites were magnified 120 × in order to measure the cemento-enamel junction to fibre attachment and cemento-enamel junction to alveolar bone distance on drawings made from the monitor screen. The reliability of the method was tested earlier (17) and revealed that the SEM difference between the two readings were 0.003 mm and 0.005 mm for cemento-enamel junction to fibre attachment and cemento-enamel junction to alveolar bone, respectively.

Statistical methods

The effect of saline and MK-801 treatment on serum corticosterone and tissue destruction was assessed with two-factorial repeated measures ANOVA test. The animal is used as the analytic unit, the data are expressed as

means ± standard deviation (SD), and α level set at $p < 0.05$.

Results

Effect of MK-801 on periodontal disease destruction

The mean attachment fibre loss at the experimental sites in the vehicle-treated control animals was 0.38 ± 0.10 mm, compared with 0.50 ± 0.10 mm in the MK-801-treated rats ($p < 0.01$) (Fig. 1). The corresponding bone losses as measured histometrically were 0.63 ± 0.08 mm vs. 0.67 ± 0.07 mm (not significant between groups). Radiographically the difference in bone loss in the vehicle-treated controls was 0.50 ± 0.11 mm. The same measurements were 0.62 ± 0.06 mm in MK-801-treated animals ($p < 0.01$). Also, the spontaneous attachment fibre loss, as measured on the control teeth without ligature, was significantly less severe in the controls (0.02 ± 0.01) than in the MK-801-treated animals (0.05 ± 0.02) ($p < 0.01$).

Effects of MK-801 on corticosterone serum levels

In the vehicle-treated rats the average plasma corticosterone levels at death was 1327 ± 111 ng/ml, compared with 1798 ± 220 ng/ml in their MK-801-treated counterparts (Fig. 2, $p < 0.001$ between groups).

Effect of MK-801 on TNF-α serum levels

Levels of TNF-α in serum collected 2 h after i.p. injection of lipopolysaccharide (250 μg/kg) on the day of death, 4 weeks after application of the ligatures, were significantly reduced in the MK-801-treated rats (MK-801-treated, 481.0 ± 274.6 pg/ml; controls, 911.9 ± 334.0 pg/ml, $p < 0.01$, Fig. 3).

Effect of MK-801 on hippocampal expression of mRNA for the GR

The integrated fluorescence signals of the PCR-products obtained with the primers for glucocorticoid receptor were expressed as a fraction of the signals obtained using the primers from ribosomal protein L27 (housekeeping gene, used as internal control). The mean ratio obtained for the glucocorticoid receptor was 6.7 ± 0.5 in vehicle-treated controls and 29.0 ± 7.8 in the MK-801-treated

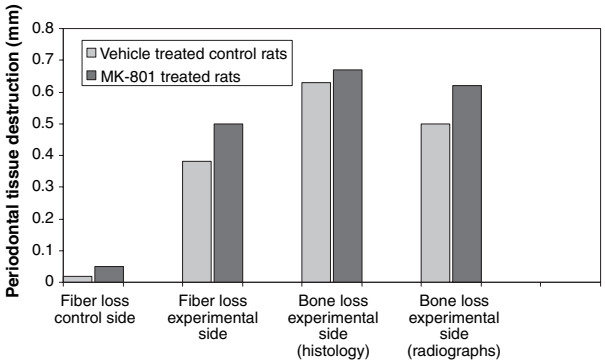

Fig. 1. The mean distance from the cemento-enamel junction to the most coronal fibre attachment as well as to the alveolar bone crest (B) in mm in vehicle-treated control rats and MK-801-treated rats. $p < 0.01$, fibre loss control side; $p < 0.01$, fibre loss experimental side; $p > 0.05$, bone loss experimental side, histology; $p < 0.01$, bone loss experimental side, radiographs.

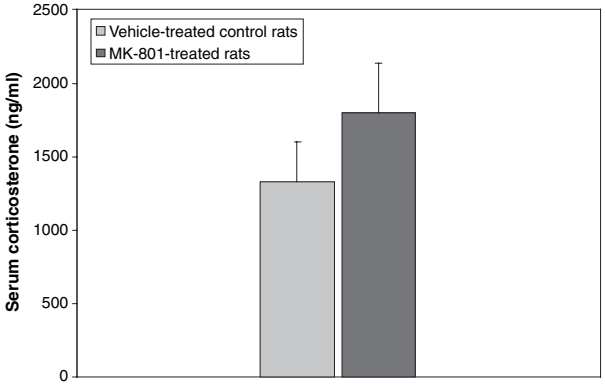

Fig. 2. Levels of corticosterone in serum 2 h after intraperitoneal injection of lipopolysaccharide (250 μg/kg). $p < 0.001$.

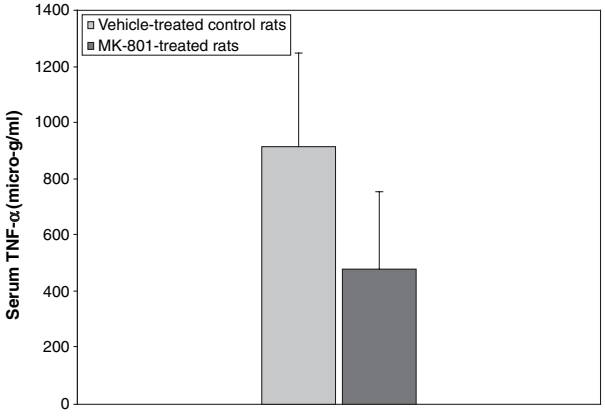

Fig. 3. Levels of tumour-necrosis factor α (TNF-α) in serum 2 h after intraperitoneal injection of lipopolysaccharide (250 μg/kg). $p < 0.001$.

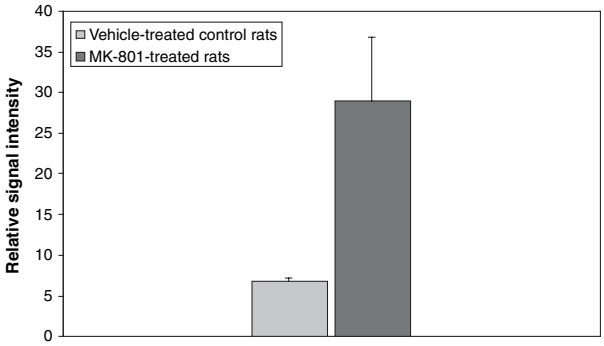

Fig. 4. Relative levels of mRNA for the glucocorticoid receptor in hippocampus expressed relative to the level of ribosomal protein L27 (house-keeping gene used as internal control).

rats (Fig. 4, $p < 0.05$ between groups).

Discussion

The study demonstrates that repeated administrations of the non-competitive NMDA receptor antagonist MK-801, which blocks the major glutamate receptors at the excitatory synapses in the central nervous system, significantly increase tissue breakdown at the control side without ligature (naturally accruing periodontal disease), and in the experimental side with ligature-induced periodontal disease. Other investigators have suggested that MK-801 may stimulate the HPA axis by increasing the biosynthesis of corticotropin-releasing hormone in parvocellular nerve cells of the hypothalamic paraventricular nuclei (34). We measured significantly enhanced serum levels of corticosterone after a robust lipopolysaccharide challenge in the MK-801-treated rats, thus confirming increased HPA responsiveness.

Chronic MK-801 treatment was also found to alter the GR expression in the hippocampus, as well as TNF-α cytokine response to lipopolysaccharide stimulation. The increased hippocampal GR expression may represent a compensatory up-regulation of these receptors and reduce the MK-801-induced corticosterone secretion. Together with increased occupancy of GRs due to higher levels of corticosterone, this up-regulation has been found to mediate feedback action aimed at restoring disturbances in homeostasis, whether environmentally or experimentally induced (30). The reduced TNF-α serum levels after lipopolysaccharide stimulation may, in part, be a result of the increased corticosterone levels induced by MK-801.

It is well established that HPA axis activation importantly modulates immunologic responses. Increased levels of glucocorticoides down-regulate the release of pro-inflammatory cytokines, such as TNF-α, from immune cells activated by bacterial products or other inflammatory challenges, and the overall effect seems to protect the host from 'overshooting' Th1-mediated immune responses (25).

The dose of MK-801 employed during the first week of the present experiment was the same as used in a study showing that MK-801 stimulates the HPA axis (35). However, high doses of MK-801 also induce a number of other effects, including increased locomotor activity, ataxia and learning impairment (36). After 5 days of MK-801 treatment, our rats showed dramatic motor hyperactivity (37). The administration of MK-801 was therefore stopped for 9 days until normal behaviour was re-established. After that the MK-801 dosage was reduced from 1 mg/(kg day) to 0.4 mg/(kg day) for the remaining period prior to death. Using this protocol we still obtained a significant increase in serum corticosterone levels, and there was a clear effect of MK-801 treatment on the degree of periodontal tissue loss. These findings support our previous studies indicating that the glutamate and the HPA responsiveness are involved in the development and progression of periodontal disease (21). They also corroborate that rats responding to stressful stimuli with a high HPA axis reactivity and subsequent high blood levels of glucocorticoid hormones, develop more severe periodontal breakdown than do low responding rats (17–23, 38). Both genetically determined and experimentally induced HPA over-responsiveness were found to increase susceptibility to disease, whereas under-responsiveness diminished both development and progression. The results are in line with recent reports showing that genetically determined, age-related and environmental stress-induced variations in the magnitude of the HPA response are important factors in determining the severity of pathogen-induced inflammatory diseases (39, 40).

Glutamate has been shown to be a primary excitatory amino acid and neurotransmitter in the brain. Together with its NMDA receptors it may be part of an important neurochemical system regulating hypothalamic excitatory synaptic activity, including the secretion of corticotropin-releasing hormone from the parvocellular nerve cells in the PVN of hypothalamus (41, 42). As local administration of glutamate into the PVN stimulates the HPA axis via the release of corticotropin-releasing hormone (42), non-competitive NMDA receptor antagonist drugs, such as MK-801, would be expected to inhibit (rather than stimulate) the activity of the HPA axis. However, other authors have found that MK-801 administration markedly increases the expression of corticotropin-releasing hormone in the PVN of hypothalamus, and stimulates the secretion of corticosterone (34). These results are supported by the present study, as illustrated by significant elevated blood corticosterone level in response to lipopolysaccharide challenge.

The effects of MK-801 treatment on periodontal disease outcome may, in part, be explained by stimulation of the HPA axis and the subsequent hypersecretion of glucocorticoids. When binding to their intracellular receptors within immune and tissue cells involved in the inflammatory process, glucocorticoids can alter the production of cytokines (43, 44), adhesion

molecules (45), as well as cellular suicide or apoptosis (46, 47). An excessive glucocorticoid response will change the Th1/Th2 balance towards a Th2 cytokine-secreting profile (48), and thus be partly responsible for increased progression of periodontal disease. This suggestion is supported by studies showing that macrophage activation, which is an indicator of a Th1 response, is reduced in people with periodontal disease (49), and local Th2-type cytokine expression is enhanced in inflamed tissues from patients with periodontal disease (50). Moreover, a vaccine (*Mycobacterium vaccae*) that is able to prevent Th2 responses and stimulate Th1 responses reduces periodontal disease development and progression in rats (51–53). This effect of glucocorticoids on the Th1/Th2 balance has been found to be partly a direct effect (54), and partly an indirect effect by suppressing interleukin-12 (IL-12) and enhancing IL-10 production by antigen-presenting cells (43, 44).

NMDA receptors have been found even on osteoblasts and osteoclasts (55). Both cell types are highly involved in periodontal bone production and breakdown, and a direct and/or indirect effect of MK-801 on osteoblast and osteoclast function may contribute to the observed protective effects. However, a recent study indicates that glutamate does not seem to play a major role in controlling bone formation and resorption (56).

Interestingly, periodontal disease risk factors such as smoking (57), insulin deficiency (17), and depressive mood states (11, 58, 59), induce sustained HPA reactivity (60–62). Moreover, the HPA reactivity is genetically determined (30) and normally increases with age (63). In both instances, an association with increased periodontal disease exists (13–15). It is therefore plausible that these risk factors may, in part, increase disease susceptibility via a common inappropriate HPA regulatory mechanism that constantly modulates immune responses to dental plaque bacteria.

The findings in this experiment support the now well-established concept that the immune system and the CNS are bi-directional linked by a complex network of bi-directional signals via the nervous and the endocrine systems. Immune responses to bacterial (or other antigenic challenges) convey signals to the brain about contact with an antigen, and the brain regulates the immune response via its peripheral arms, including the HPA axis (64, 65).

In conclusion, we have described a potent enhancing effect of the NMDA receptor antagonist MK-801 on periodontal breakdown and thus have identified a novel mechanism that may be involved in periodontal disease development and progression. This finding may suggest that the central nervous and immune systems are functionally linked by glutamatergic regulation of the HPA axis, and that this mechanism can be a major contributor to the immunodeficiency associated with the increased growth of pathogens in dental plaque in patients exposed to conditions associated with HPA axis hyperactivity. NMDA receptor involvement in the pathogenesis of periodontal disease has, to our knowledge, not been documented by other investigators. A better understanding of the mechanisms by which CNS regulates the neuroendocrine system, including HPA axis activation by Gram-negative bacteria, will likely improve treatment of periodontal disease.

References

1. Lindhe J. *Textbook of Clinical Periodontology*. Copenhagen: Munksgaard, 1995.
2. Socransky SS, Haffajee AD. The bacterial aetiology of destructive periodontal disease. *J Periodontol* 1992;**63:**322–331.
3. Sela MN. Role of *Treponema denticola* in periodontal disease. *Crit Rev Oral Biol Med* 2001;**12:**399–413.
4. Kamma JJ, Slots J. Herpes virus–bacterial interaction in aggressive periodontitis. *J Clin Periodontol* 2003;**30:**420–426.
5. Birkedal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. *J Periodont Res* 1993;**28:**500–510.
6. Ding Y, Haapasalo M, Kerosuo E, Lounatmaa K, Kotiranta A, Sorsa T. Release and activation of human neutrophil matrix metallo- and serine proteinases during phagocytosis of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Treponema denticola*. *J Clin Periodontol* 1997;**24:**237–248.
7. Lohinai Z, Benedek P, Feher E *et al*. Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. *Br J Pharmacol* 1998;**123:**353–360.
8. Breivik T, Thrane PS, Murison R, Gjermo P. Emotional stress effects on immunity, gingivitis and periodontitis. *Eur J Oral Sci* 1996;**104:**327–334.
9. Breivik T, Thrane PS. Psychoneuroimmune interactions in periodontal disease. In: Ader R, Felten DL, Cohen N, eds. *Psychoneuroimmunlogy*, 3rd edition, Chapter 63. San Diego, CA: Academic Press, 2001: 627–644.
10. Hugoson A, Ljungquist B, Breivik T. The relationship of some negative events and psychological factors to periodontal disease in an adult Swedish population 50–80 years of age. *J Clin Periodontol* 2002;**29:** 247–253.
11. Genco RJ, Ho AW, Kopman J, Grossi SG, Dunford RG, Tedesco LA. Models to evaluate the role of stress in periodontal disease. *Ann Periodontol* 1998;**3:**288–302.
12. Wimmer G, Janda M, Wieselmann-Penkner K, Jakse N, Polansky R, Pertl C. Coping with stress: Its influence on periodontal disease. *J Periodontol* 2002;**73:** 1343–1351.
13. Michalowicz B. Genetic and heritable risk factors in periodontal disease. *J Periodontol* 1994;**65:**479–488.
14. Burt BA. Periodontitis and ageing: reviewing recent evidence. *J Am Dent Assoc* 1994;**125:**273–279.
15. Hugoson A, Jordan T. Frequency distribution of individuals aged 20–70 years according to severity of periodontal disease. *Community Dent Oral Epidemiol* 1982;**10:**187–192.
16. Thorsteinson H, Hugoson A. Periodontal disease experience in adult long-duration insulin-dependent diabetics. *J Clin Periodontol* 1993;**20:**352–358.
17. Breivik T, Opstad PK, Gjermo P, Thrane PS. Effects of hypothalamic-pituitary-adrenal axis reactivity on periodontal tissue destruction in rats. *Eur J Oral Sci* 2000;**108:**115–122.
18. Breivik T, Sluyter F, Hof M, Cools A. Differential susceptibility to periodontitis in genetically selected Wistar rat lines that differ in their behavioral and endocrinological response to stressors. *Behav Genet* 2000;**30:**123–130.
19. Breivik T, Thrane PS, Gjermo P, Opstad PK. Glucocorticoid receptor antagonist RU-486 treatment reduces periodontitis in Fischer 344 rats. *J Periodont Res* 2000;**35:** 285–290.
20. Breivik T, Thrane PS, Gjermo P, Opstad PK, Pabst R, von Horsten S.

Hypothalamic-pituitary-adrenal axis activation by experimental periodontal disease in rats. *J Periodont Res* 2001;**36:**295–300.
21. Breivik T, Thrane PS, Gjermo P, Fonnum F. Postnatal glutamate-induced central nervous system lesions alter periodontal disease susceptibility in adult Wistar rats. *J Clin Periodontol* 2001;**28:**904–909.
22. Breivik T, Thrane Gjermo P, Cools A, Myhrer T. Effects of hippocampal lesioning on experimental periodontal disease in Wistar rats. *J Periodont Res* 2002;**37:**360–365.
23. Breivik T, Stephan M, Brabant GE, Straub RH, Pabst R, von Horsten S. Postnatal lipopolysaccharide-induced illness predisposes to periodontal disease in adulthood. *Brain Behav Immun* 2002;**16:**421–438.
24. Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med* 1995;**332:**1351–1361.
25. Elenkov IJ, Chrousos GP. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends Endocrinol Metab* 1999;**10:**359–368.
26. Beaulieu S, Pelletier G, Vaudry H, Barden N. Influence of the central nucleus of the amygdala on the content of corticotropin-relasing factor in the median eminence. *Neuroendocrinology* 1989;**49:**255–261.
27. Lightman SL, Windle RJ, Ma XM *et al*. Hypothalamic-pituitary-adrenal function. *Arch Physiol Biochem* 2002;**110:**90–93.
28. Borovikova LV, Ivanokova S, Zhang M, Yang H, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000;**405:**458–462.
29. Turnbull AV, Rivier CL. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: Actions and mechanisms of action. *Physiol Rev* 1999;**79:**1–71.
30. De Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M. Brain corticosteroid receptor balance in health and disease. *Endocrinol Rev* 1998;**19:**269–301.
31. Ziegler DR, Herman JP. Local integration of glutamate signalling in the hypothalamic paraventricular region: regulation of glucocorticoid stress responses. *Endocrinology* 2000;**141:**4801–4804.
32. Abraham I, Juhasz G, Kekesi KA, Kovacs KJ. Corticosterone peak is responsible for the stress-induced elevation of glutamate in the hippocampus. *Stress* 1997;**2:**71–181.
33. Espey MG, Basil AS. Glutamate augments retrovirus-induced immunodeficiency through chronic stimulation of the hypothalamic-pituitary-adrenal axis. *J Immunol* 1999;**162:**4998–5002.
34. Lee S, Rivier C, Torres G. Induction of c-*fos* and CRF mRNA by MK-801 in the parvocellular paraventricular nucleus of the rat hypothalamus. *Mol Brain Res* 1994;**24:**192–1998.
35. Pechnick RN, George R, Poland RE. Characterisation of the effects of acute and repeated administration of MK-801 on the release of adrenocorticotropin, corticosterone and prolactin in the rat. *Eur J Pharmacol* 1989;**164:**257–263.
36. Hesselink MB, Smolders H, De Boer AG, Breimer DD, Danysz W. Modifications of the behavioural profile of non-competitive NMDA receptor antagonists, memantine, amantadine and (+) MK-801 after chronic administration. *Behav Pharmacol* 1999;**10:**85–98.
37. Harkin A, Morris K, Kelly JP, O'Donnell JM, Leonard BE. Modulation of MK-801-induced behaviour by noradrenergic agents in mice. *Psychopharmacology* 2001;**154:**177–188.
38. Sluyter F, Breivik T, Cools A. Manipulation in the maternal environment reverse genetically predisposed periodontitis in rats. *Clin Diagn Lab Immunol* 2002;**4:**931–932.
39. Kavelaars A, Heijnen CJ, Ellenbroek B, van Loveren H, Cools A. Apomorphine-susceptible and apomorphine-unsusceptible Wistar rats differ in their susceptibility to inflammatory and infectious diseases: a study on rats with group-specific differences in structure and reactivity of hypothalamic-pituitary-adrenal axis. *J Neurosci* 1997;**17:**2580–2584.
40. Howards AD, Zwilling BS. Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines. *Clin Exp Immunol* 1999;**115:**428–434.
41. Van den Pol AN, Trombley PQ. Glutamate neurons in hypothalamus regulate excitatory transmission. *J Neurosci* 1993;**13:**2829–2836.
42. Feldman S, Weidenfeld J. Hypothalamic mechanisms mediating glutamate effects on the hypothalamo-pituitary-adrenocortical axis. *J Neural Transm* 1997;**104:**633–642.
43. Vieira PL, Kalinski P, Wierenga EA, Kapsenberg ML, de Jong EC. Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential. *J Immunol* 1998;**161:**5245–5251.
44. Visser J, van Boxel-Dezaire A, Methorst D, Brunt T, de Kloet ER, Nagelkerken L. Differential regulation of interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. *Blood* 1998;**91:**4255–4264.
45. Eguchi K, Kawakami A, Nakashima M *et al*. Interferon-alpha and dexamethasone inhibit adhesion of T cells to endothelial cells and synovial cells. *Clin Exp Immunol* 1992;**88:**448–454.
46. Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J Immunol* 1995;**154:**4719–4725.
47. Schmith M, Pauels H-G, Lugering N, Lugering A, Domschke W, Kucharzik T. Glucocorticoids induce apoptosis in human monocytes. Potential role of IL-1β. *J Immunol* 1999;**163:**3484–3490.
48. Rook GA. Glucocorticoids and immune function. *Baillieres Best Pract Res Clin Endocrinol Metab* 1999;**13:**567–581.
49. Chapple CG, Srivastava M, Hunter N. Failure of macrophage activation in destructive periodontal disease. *J Pathol* 1998;**186:**281–286.
50. Tokoro Y, Matsuki Y, Yamamoto T, Suzuki T, Hara K. Relevance of local Th2-type cytokine mRNA expression in immunocompetent infiltrates in inflamed gingival tissue to periodontal diseases. *Clin Exp Immunol* 1997;**107:**166–174.
51. Breivik T, Rook GA. Prevaccination with SRL172 (heat-killed *Mycobacterium vaccae*) inhibits experimental periodontal disease in Wistar rats. *Clin Exp Immunol* 2000;**120:**463–467.
52. Breivik T, Rook GA. Treatment with SRL172 (heat-killed *Mycobacterium vaccae*) inhibits progression of established experimental periodontal disease in Wistar rats. *J Periodont Res* 2002;**37:**210–214.
53. Breivik T, Rook GA. Oral treatment with SR299 (killed Mycobacterium vaccae) inhibits experimental periodontal disease in Wistar rats. *J Clin Periodontol* 2003;**30:**931–936.
54. Daynes RA, Meikle AW, Araneo TA. Locally active steroid hormones may facilitate compartmentalization of immunity by regulating the types of lymphokines produced by helper T cells. *Res Immunol* 1991;**142:**40–45.
55. Skerry TM, Taylor AF. Glutamate signalling in bone. *Curr Pharm Des* 2001;**7:**737–750.
56. Gary C, Marie H, Arora M *et al*. Glutamate does not play a major role in controlling bone growth. *J Bone Miner Res* 2001;**16:**742–749.
57. Martinez-Canut P, Lorca A, Magan R. Smoking and periodontal disease severity. *J Clin Periodontol* 1996;**22:**743–749.
58. Montairo da Silva AM, Oakly DA, Newman HN, Nohl FS, Lloyd HM. Psychosocial factors and adult onset rapidly progressive periodontitis. *J Clin Periodontol* 1996;**23:**789–794.
59. Moss ME, Beck JD, Kaplan BH *et al*. Exploratory case-control analysis of psychosocial factors and adult periodontitis. *J Periodontol* 1996;**67:**1060–1069.
60. Charlton BG, Ferrier IN. Hypothalamo-pituitary-adrenal axis abnormalities in depression: a review and a model. *Psychol Med* 1989;**19:**331–336.
61. Matta SG, Foster CA, Sharp BM. Nicotine stimulates the expression of cFos

protein in the parvocellular paraventricular nucleus and brainstem catecholaminergic regions. *Endocrinology* 1993;**132:** 2149–2156.

62. Schwartz MW, Strack AM, Dallman MF. Evidence that elevated plasma corticosterone levels are caused of reduced hypothalamic corticotropin-releasing hormone gene expression in diabetes. *Regul Pept* 1997;**72:**105–112.
63. Friedman M, Green MF, Sharland DE. Assessment of hypothalamic-pituitary-adrenal function in the geriatric age group. *J Gerontol* 1969;**24:**292–297.
64. Ader R, Cohen N, Felten D. Psychoneuroimmunology: Interactions between the nervous system and the immune system. *Lancet* 1995;**345:**99–103.
65. Blalock JE. The syntax of immune-neuroendocrine communication. *Immunol Today* 1994;**15:**504–511.