# Effects of Matrix Metalloproteinase Inhibition on Long-Term Plasticity at Perforant Pathway/ Dentate Gyrus Synapses: in vivo Study

## Matriks Metalloproteinaz İnhibitörlerinin Perforan Yol - Dentat Girus Sinapslarındaki Uzun Süreli Potansiyasyonlara Etkisi: in vivo Çalışma

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**Abstract**
**Purpose:** The aim of the study was to explore whether matrix metalloproteinases (MMPs) is a requisite for efficient hippocampal-dependent learning in the perforant pathway/dentate gyrus synapses. With this aim, we infused FN-439 which is a MMPs inhibitor into the dentate gyrus.
**Material and Methods:** The experiments were carried out on 18 male Wistar rats. Rats were anesthetized with urethane, and the head was fixed in stereotaxic head-holder. A bipolar stimulating electrode was placed on the medial perforant path. One of the barrels of double-barreled glass micropipette was filled with aCSF or FN-439 and applied to dentate gyrus before the EPSP recordings. Other barrel was filled with 3 M NaCl and field excitatory postsynaptic potentials (fEPSPs) were recorded from this barrel. The high frequency stimulation (HFS) protocol was applied.
**Results:** Infusion of FN-439 resulted in a clear inhibition of the fEPSP slope and population spike amplitude in the early and maintenance phase. The HFS protocol caused significantly less potentiation of dentate synapses subjected to FN-439 when compared to infusion of aCSF.
**Conclusion:** Acute application of FN-439 significantly inhibited long-term plasticity in the perforant patway/dentate gyrus neurons of anaesthetized rats. Thus, we showed that MMPs are necessary for long term potentials which are important model for learning.

Key words: **Dentate Gyrus; Long-Term Potentiation; Matrix Metalloproteinases.**

**Özet**
**Amaç:** Çalışmanın amacı matriks metalloproteinlerinazların (MMPs) perforan yol/dentat girusda hipokampal bağımlı öğrenmede gerekli olup olmadığını araştırmaktır. Bu amaçla, bir MMPs inhibitörü olan FN-439 dentat girusa uygulandı.
**Gereç ve Yöntemler:** Deneyler 18 erişkin erkek sıçanda gerçekleştirildi. Her kayıt fazı öncesinde, sıçanlar uretan ile uyutuldu. Uyutulan sıçan, başından stereotaksik alete sabitlendi. Bipolar uyarıcı elektrot medyal perforan yola yerleştirildi. İki kanallı cam mikropipetin kanallarından biri yapay BOS veya FN-439 ile dolduruldu ve eksitator postsinaptik alan potansiyellerin (EPSP) kaydından önce dentat girusa verildi. Diğer kanal 3M NaCl ile dolduruldu ve EPSP'ler bu kanaldan kaydedildi. Uzun süreli potansiyellerin uyarılması için yüksek frekanslı uyarı (YFU) protokolü uygulandı.
**Bulgular:** FN-439'un uygulanması erken ve sürdürme fazında EPSP ve populasyon spike'larının inhibisyonu ile sonuçlandı. Uzun süreli potansiyeller, yapay BOS'a göre anlamlı olarak daha az potansiyelize oldular.
**Sonuç:** FN-439 un akut uygulanması, anestezili sıçanların perforan yol/dentat giruslarında uzun süreli potansiyelleri anlamlı olarak inhibe etti. Böylece MMP'lerin öğrenmede önemli bir model olan uzun süreli potansiyasyonda gerekli olduğu gösterilmiştir.

Anahtar kelimeler: **Dentat Girus; Matriks metalloproteinazlar; Uzun-Süre Potansiyasyon.**

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## Introduction

Matrix metalloproteinases (MMPs) modulate the structure and function of extracellular matrix (ECM) with resulting effects on synaptic plasticity and learning (1, 2). MMPs are known to play an important role in synaptic remodeling, which results from hippocampal differentiation (3). They are critical for hippocampal-dependent learning (4).

Long-term potentiation (LTP), a long-lasting increase in the efficacy of synaptic transmission induced by trains of high-frequency stimulation, has long been believed to represent a mechanism involved in information storage during learning and memory (5, 6). Owing to the close association between the impairment of hippocampal long-term potentiation (LTP) and the deficits of behavioral learning and memory, the hippocampal LTP has been widely used as a neuronal model of activity-dependent synaptic plasticity (7-11).

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent endopeptidases that includes over 25 distinct enzymes. The primary role of MMPs is to dynamically degrade and alter ECM structure and function (1). Some researches predicted that MMPs function is critical for hippocampal-dependent learning (12-16). Others employed MMPs inhibitors to determine whether the participation of MMPs was required for LTP and learning (3, 17-21). Some experiments in vivo and in vitro have shown that some MMPs inhibitors significantly inhibit LTP induction in the hippocampus of rats. However, the mechanisms underlying the suppressive action of MMPs or its fragments on LTP are controversial (22).

MMPs activity is inhibited with FN-439, a commercially available peptidyl hydroxamic acid, developed for its action on human MMPs (21). This compound is water soluble and retains its inhibitory activity in the presence of neutral proteinases, making it a good choice for the modulation of MMPs activity in vivo (17, 19). FN-439 resulted in impairments in paired-pulse facilitation, theta-burst facilitation, and long-term depression in acute hippocampal slices (14).

As far as it is known, there is no study on the effect of MMPs inhibition on plasticity at the perforant pathway with FN-439 in vivo. Thus, the primary goal of this study was to test the hypothesis that FN-439 could exhibit similar suppressive effects on the hippocampal LTP in vivo, as they did in brain slices in vitro (14,23).

## Materials and Methods

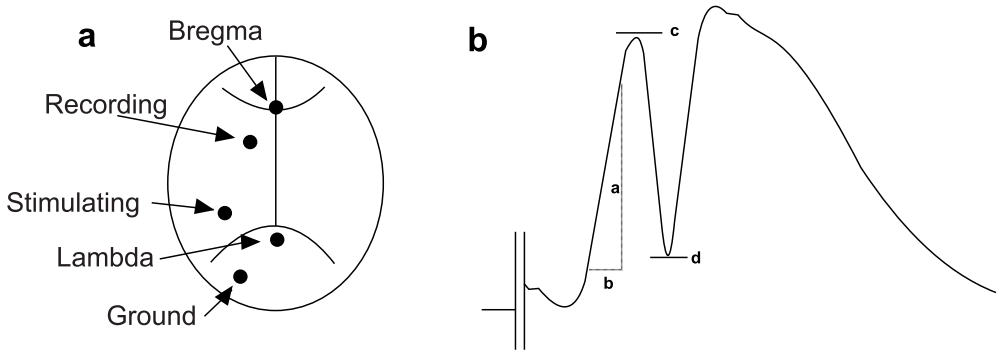
Experimental Animals and Groups. The acute experiments were carried out on 18 adult male Wistar rats, at the age of 3-4 months, weighing 290-320 g, fed with tap water and purina rodent chow. The experiments were carried out after receiving approval by the Committee on Ethics in Animal Experimentation of Erciyes University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

On the day of the experiment, the rats were randomly divided into three groups: artificial cerebrospinal fluid infused (aCSF-infused, no high frequency stimulation (HFS), n=6), aCSF+HFS (aCSF-infused, high frequency stimulation, n=6), and FN-439+HFS (FN-439 infused, high frequency stimulation, n=6) groups.

**Drug application.** FN-439 is a synthetic peptide hydroxamate with the capacity to chelate Zn+2, thereby blocking the active site of metalloproteinases (19, 21). The drug exhibits specificity for interstitial collagenase, MMP-9, and MMP-3. Reeves and co-workers used the FN-439 in vivo to block both MMP-3 and -9 gene expressions. Using a commercial fluorescent substrate assay for MMP-3 activity, they determined percentage inhibition of purified MMP-3 over a range of FN-439 doses (75, 150, and 750 M), inclusive of the published IC50 concentration. Their results showed 39.7, 51.6, and 69.3% inhibition, respectively, confirming 150 M as the FN-439 IC50 value for MMP-3 and identifying 750 M as a reasonable dosage for significant MMP-3 inhibition. Their subsequent in vitro zymography experiments showed that exposure of EC lesion hippocampal preparations to 150 M FN-439 blocked up to 98.8% of the MMP-9 gelatinolytic activity relative to matched untreated samples. Based on these findings, 720 M dose was chosen for FN-439 to target both MMP-3 and -9 in vivo (3). 7.2 mM stock solution of FN-439 (180 lmol/L; Sigma Chemical, St Louis, MO, USA) was dissolved in sterile aCSF. Total injected volume to experimental group was 0.1 cc.

**Electrodes and Stereotaxic Manipulation.** Rats were anesthetized with urethane (1.5-g/kg i.p.) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). A bipolar tungsten electrode (stainless steel, Teflon coated, 127-m in diameter, insulated except at its tips) was used to stimulate the medial perforant path (from bregma, in mm: AP, 8.0; ML, 4.4; DV, 2 2.5 below dura) of the left hemisphere (24). The stimulation electrode was connected

to the output of an isolator (SS-201J Isolator, Nihon Kohden, Tokyo, Japan) connected with a stimulator (SEN 3201 Electronic Stimulator, Nihon Kohden, Tokyo, Japan). A double-barreled glass micropipette (Borosilicate, o.d., 1.5-mm; 10-cm length, catalog no.: BT150-10, Sutter Instruments) was inserted into the granule cell layer of the ipsilateral dentate gyrus (in mm: AP, 3.5; ML, 2.15; DV, 2.53 below dura). The tips of both barrels were at the same level. One of the barrels was filled with 3 M NaCl (tip resistance, 2-10 M Ω), and field excitatory postsynaptic potentials (fEPSPs) were recorded from this barrel. The other barrel was filled with aCSF (total injected volume 0.1 cc) or FN-439. The infusion barrel was connected to a Hamilton syringe (25µL) driven by a syringe pump (5µL/min; 10µL total volume; Stoelting Co, Wood Dale, Illinois, USA). An Ag AgCl disc electrode was positioned under the neck skin and served as the reference electrode. The active and reference electrodes were connected to an amplifier (VCC600 single channel epithelial voltage/current clamp system, Physiological Instruments, Harvard Apparatus, Holliston, MA, USA) using a head-stage. The entire system was grounded using a Faraday cage. The depth of recording was adjusted to obtain a large positive fEPSP, and a superimposed negative-going population spike (PS) was evoked (see Fig. 1 for a typical response of the granule cell layer of dentate gyrus) with a 0.1 mm step. After the typical response was recorded, the final depth-stimulating electrodes were adjusted to maximize the PS amplitude in response to the perforant path stimulation.




| (B) | AP (mm) | ML (mm) |
|---|---|---|
| Stimulating (LPP) | -8.0 | 4.4 |
| Recording (DG) | -3.5 | 2.15 |

**Figure 1.** a. Coordinates of recording and stimulating electrodes. b: Measurement of fEPSP and PS from a typical dentate gyrus response. The slope of fEPSP = a/b. The magnitude of PS = c-d. LPP: lateral perforant patway. DG: dentate gyrus.

The Scope program (ADInstruments, Colorado Springs, CO, USA) was used to control stimulation and recording. Monophasic 10 V and 0.175-ms pulses were generated by the A/D board (Powerlab/8SP, ADInstruments, Colorado Springs, CO, USA) of a computer and triggered to a stimulator connected with an isolator. Biological signals were amplified (1000) on a pre-amplifier at a bandwidth of 0.1-10 kHz. Waveforms were digitized on-line at a rate of 40 kHz for 20 ms, displayed on a computer monitor and stored using Scope for offline analysis.

**Experimental Protocol.** Each experiment started with recording an inputoutput curve consisting of 15 stimuli ranging from 0 to 525 µA, every 10 sec. For each time point, three evoked responses were averaged. The relation between stimulus intensity and the fEPSP slope or PS amplitude was described by a sigmoidal curve; from this curve the half maximal stimulus intensity was determined. Stimulus intensity that produced 50% of maximum response (i.e., test pulse) was used in subsequent experiments. The measured test pulse for different animals was between 100 and 400 µA.

After the stimulation intensity for the test pulse was determined, the experiment was started. During 40 min the test stimuli were applied every 30 sec, and evoked

responses were recorded from dentate gyrus. During the last 20 min of this period, FN-439 or aCSF were infused. The mean value of fEPSP or PS during the first 20 min period was evaluated as 100 percent. Each EPSP and each PS was expressed percentage of baseline value. The following step started with application of the high frequency stimulation (HFS: 100-Hz, 1-sec, four train, intertrain interval: 5 min) and following delivery of tetanic stimuli, application of the test stimuli was continued up to the 180$^{th}$ min every 30 sec.

**Data Analysis and Statistics.** For statistical analysis, the entire experimental period was divided into nine 20 min sub-periods. The mean value of fEPSP or PS during first 20 min period was evaluated as 100%. Each EPSP and each PS was expressed as the percentage of the baseline value. A mean value of responses at 40 time points was defined as the baseline. Subsequent data were expressed as the percentage change from the baseline.

The slope of fEPSP was calculated as the amplitude change at 20-80% of the voltage difference between the start of the waveform and the fEPSP amplitude at the onset of PS. The PS amplitude was measured from the first positive peak to a negative peak (Figure 1). Slope and amplitude data for each epoch were compared among the groups using one way ANOVA followed by Scheffe's test. All values were taken as the mean±S.E.M. Probabilities less than 0.05 were considered to be significant.

**Results**

No significant differences were noted among the groups in the mean absolute value of PS amplitude or the fEPSP slope during the first sub-period while infusing aCSF and perforant pathway stimulated every 30 sec. During period 2 (20$^{th}$ and 40$^{th}$ minutes), perforant pathway was stimulated every 30 second and infused FN-439 or aCSF to the rats. There were no significant differences among the groups in the mean absolute value of the fEPSP slope during the second sub-period (P>0.05; 48±38.4% in aCSF group; 47±33.3% in FN-439 group). There were also no significant differences for PS amplitudes of groups. Then, we applied the HFS protocol to FN-439 and aCSF groups between 40$^{th}$ and 60$^{th}$ minutes (period 3).

Immediately after tetanization, the slopes of fEPSP were significantly increased in the aCSF infused group (192±56.1%) when compared with the FN-439 infused group (62±32.9%). There was a significant difference between these groups at this time point (60th min, F=683, P<0.001). Significant differences between groups continued to the end of the experimental period. At the 80$^{th}$ min, the fEPSP slopes of FN-439 infused group were decreased, while the fEPSP slopes of aCSF infused group were increased. There were highly stable fEPSP slopes between the 100-180 min. At the 180$^{th}$ min, fEPSP slopes of aCSF infused group (150±45.1%) were statistically higher than FN-439 infused group (81±40.3%, F=291, P<0.001) (Figure 2).

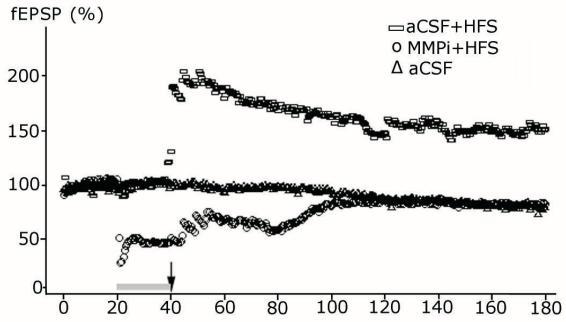



**Figure 2.** Effect of MMP inhibitor on field excitator postsinaptic potential (fEPSP). x abscis: time. y abscise: slope of fEPSP. arrow: starting of HFS. Grey bar: infusion period. aCSF: artificial cerebrospinal fluid; MMPi: matrix metalloproteinase inhibitor; HFS: High frequency stimulation.

The amplitude of PS was also significantly increased in the aCSF infused group (217±41.2%) when compared with the FN-439 infused group (76±49.9%) after the tetanization. There was a significant difference between these groups at this time point (60$^{th}$ min, F=1011, P<0.001). Significant differences between groups continued to the end of the experimental period. After the HFS protocol, the amplitude of PS of FN-439 infused group were decreased, while the the amplitude of PS of aCSF infused group were increased during the experiment period. At the 180$^{th}$ min, the amplitude of PS of aCSF infused group (167±48.7%) was statistically different from FN-439 infused group (115±76.9%, F=216, P<0.001) (Figure 3).

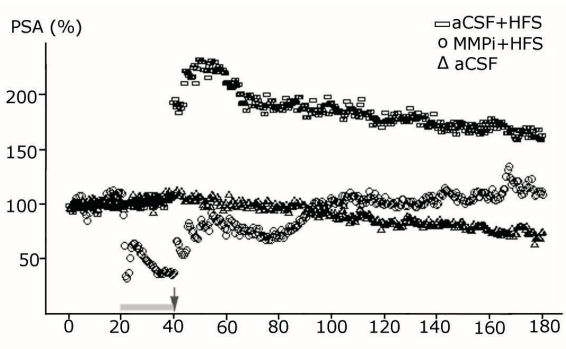



**Figure 3.** Effect of MMP inhibitor on population spike amplitude (PSA). x abscis: time. y abscise: slope of PSA. arrow: starting of HFS. Grey bar: infusion period. Grey bar: infusion period. aCSF: artificial cerebrospinal fluid; MMPi: matrix metalloproteinase inhibitor; HFS: High frequency stimulation.

The mean absolute value of PS amplitude or the fEPSP slope in aCSF group (no HFS) started to decrease from period 4, and declined values were obtained from the MMPs inhibitor-infused group.

**Discussion**

Although it is becoming increasingly evident that MMPs participate in processes necessary for synaptic plasticity, little is understood regarding the mechanisms by which they contribute. Here is a reliable protocol established for in vivo rat recordings of synaptic plasticity. This is the first in vivo study in which FN-439 was applied to healthy dentate gyrus to investigate LTP. The present investigation indicates that FN-439 inhibits long-term plasticity in perforant pathway/dentate gyrus neurons of anaesthetized rats. The HFS protocol caused significantly less potentiation of dentate synapses subjected to FN-439 when compared to those subjected to aCSF.

In recent years, it was observed that hippocampal MMP -3 and -9 increased transiently during water maze acquisition as assessed by Western Blotting and mRNA analysis (13, 14, 25). Furthermore, inhibition of mmPs activity with MMP-3 and -9 antisense oligonucleotides and/or MMPs inhibitor FN-439 altered long-term potentiation and prevented acquisition in the Morris water maze. Reeves et al. applied FN-439 as intraventricular infusion at 30 min postlesion, followed by electrophysiological and electron microscopic analysis at 7 d survival, and they found deafferentation-induced collateral sprouting in the dentate gyrus (3).

In our experiment, infusion of FN-439 resulted in a clear inhibition of fEPSP and PS in the early and end phases. It has been presumed that LTP consisted of several phases, including the induction phase, early and late maintenance phases, and different phases were mediated by different mechanisms (26). Early maintenance of LTP was thought to be mediated mainly by phosphorylation of some protein kinases, such as protein kinase C and cGMP-dependent protein kinase etc (27, 28). According to some hypotheses, MMPs promote stability of early-phase LTP necessitated identifying the particular cell adhesion molecules (CAMs) influenced by plasticity-associated MMPs activity. Of the CAMs demonstrably important for hippocampal plasticity, the three families that receive the most attention are neural cell adhesion molecules (NCAMs), cadherins, and integrins (29-31). Although MMPs have been shown to influence the function of each of these families of CAMs (32-34) some researchers focused on integrins (35). Specifically, neither FN-439 nor RGD compounds are required to be present during tetanization to affect LTP stability, and loss of blockage efficacy progresses for each of these inhibitors if LTP is allowed to develop for approximately 15 min prior to administration (with a complete loss of efficacy occurring after 30 min). This is in contrast to a NCAM blockage, given that administration immediately following LTP induction is inconsequential to the magnitude and stability of LTP. The inconsistencies between MMPs inhibition and the inhibition of cadherins and NCAMs make it unlikely that the effects of MMPs inhibition on LTP stability during early-phase maintenance are related to impairment of NCAM or cadherin function (14). Further studies needed for investigation of molecular mechanism related with NCM or cadherin function of MMPs.

As a conclusion, in this experiment, acute application of FN-439 to anesthetized rats significantly inhibited the formation of LTP. So, MMPs are necessary for long term potentials which are important model for learning.

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