

## ORIGINAL ARTICLES

## Effects of nerve and fibroblast growth factors on the production of nitric oxide in experimental model of Huntington's disease

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*The role of nitric oxide (NO) in neurological diseases represents one of the most studied, yet controversial subjects in physiology. The aim was to examine the effects of intrastriatal injection neurotrophins (nerve growth factors-NGF, fibroblast growth factors-FGF) in order to investigate the possible involvement of NO in quinolinic acid (QA) induced striatal toxicity in the rat model of Huntington's disease (HD). QA was administered unilaterally into the striatum of adult Wistar rats in a single dose of 150 nM. The other two groups of animals were pretreated immediately before QA application with NGF and FGF, respectively. Control group was treated with 0.9% saline solution in the same manner. Animals were decapitated 7 days after the treatment. Nitrite levels were significantly decreased both in the ipsi- and contralateral striatum and forebrain cortex of NGF- and FGF-treated animals compared with QA treatment. These results indicated a temporal and spatial propagation of oxidative stress and spread protective effects of NGF and FGF on the forebrain cortex, the distant structure, but tightly connected with striatum, the place of direct neurotoxic damage. Neurotrophins could be the potential neuroprotective agents in HD.*

**Key words:** Huntington disease; disease models, animal; quinolinic acid; nitric oxide; nerve growth factor; fibroblast growth factor; oxidative stress.

### Introduction

Huntington's disease (HD) is dominantly inherited, untreatable neurological disorder featuring a progressive loss of striatal output neurons that results in dyskinesia, cognitive decline and ultimately, death (1). HD gene mutation is an expanded and unstable trinucleotide repeat in a new gene, which encodes a 349 kd protein. Huntingtin is a widely expressed protein (e.g., in the testicles, heart, liver, skeletal muscle, and lung), and its expression is high in the brain, particularly in the hippocampus, cerebellar granular cell layer, and Purkinje cells. Mutant huntingtin (and possibly the polyglutamine tract itself) may induce the defective mitochondrial function (2). The question then arises as to the relevance of the mitochondrial defect to cell death in the striatum. The striatum is known to be an area highly dependent on oxidative phosphorylation for energy, and the

increased huntingtin binding in the striatum (e.g., via huntingtin binding proteins such as huntingtin-associated protein 1 [HAP-1]) may cause the impaired oxidative phosphorylation, and in that way predispose neurons to the cell death by its excitotoxicity. However, the cascade of events in HD pathogenesis is likely to be much more complicated and involve factors specific to the biochemistry and pharmacology of the striatum (3, 4).

Besides the striatum, the forebrain cortex is also included in the selectively vulnerable brain structures. Massive afferents from all areas of the cortex represent the most important source of excitatory amino acids, whereas the intrinsic circuits provide the striatum with acetylcholine, GABA, nitric oxide and adenosine. All these neurotransmitter systems interact with each other and with voltage-dependent conductances to regulate the efficacy of the synaptic transmission within this nucleus. The integrative ac-

tion exerted by the striatal projection neurons on this converging information dictated the final output of the striatum to the other basal ganglia structures (5).

Quinolinic acid (QA) is a selective agonist at receptors for the glutamate analogue N-methyl-D-aspartate (NMDA) and has become a widely used tool for the study of neuronal damage resulting from the activation of these receptors (6). Since it is an endogenous metabolite of tryptophan, it has also become a focus of interest for understanding of the pathological processes underlying neuronal damage in HD (7). The mechanism by which QA produces neuronal damage remains uncertain. The neurotoxicity of QA is considerably greater than can be accounted for by the activation of NMDA receptors. There are qualitative, as well as pharmacological differences between the neurotoxic effects of NMDA and QA, which suggest the involvement of mechanisms other than NMDA receptor activation (8).

One such mechanism may involve free radicals. Activation of glutamate receptors is known to induce an influx of calcium ions into neurones, which starts a destructive sequence of events within the cell, possibly leading to the generation of reactive oxygen species (9). Recent work has shown that QA may lead to the generation of free radicals, such as the release of nitric oxide (NO) in the brain (10). The role of NO in these circumstances was controversial because the physiological chemistry of NO was complex and encompassed numerous potential reactions. It is convenient to separate NO chemistry into the direct and indirect effects (11). Direct effects are those reactions in which NO interacts directly with a biological molecule, whereas the indirect effects are those reactions mediated by NO-derived intermediates such as reactive nitrogen oxide species derived from the reaction of NO with O<sub>2</sub> or superoxide (O<sub>2</sub><sup>-</sup>). Direct effects such as interaction of NO with metal-containing proteins or with organic free radicals may occur at the lower concentrations or fluxes of NO, whereas higher NO fluxes can result in the indirect effects such as nitrosation, nitration and oxidation.

The delivery of neurotrophins (NTF) or their genes to the CNS has shown a promise as a rational therapy for the treatment of brain injury and neurodegenerative disorders (12). A remarkably large number of proteins that mediate development and responses to the injury were found to protect neurons from excitotoxicity and to stimulate CNS repair. These include nerve growth factor (NGF), fibroblast growth factor (FGF), brain derived neurotrophic factor (BDNF), neurotrophin (NT-3), neurotrophin 4/5 (NT-4/5) and ciliary neurotrophic factor (CNTF) (13). In this paper it was assumed that NGF and FGF could have the protective effect on the propagation of oxidative stress caused by QA in the rat model of Huntington's disease mediated by NO.

## Methods

The investigation was performed on adult Wistar rats of both sexes, with body mass about 250 g. Animals were

classified into four groups and were put in macrolen cages (Erath, FRG). Animals had free access to food and water. Average microclimate conditions were the following: room temperature 23±2° C, air humidity 55±10%; air was conditioned by 10–50 exchanges per hour, and light regime was in 12 hours cycles from 7 to 19 hours.

QA was administered unilaterally into the striatum in the single dose of 250.7 µg (150 nM) using stereotaxic instrument for small animals and coordinate for the striatum (8.4; 2.4; 5.0 mm). The second and the third group were treated with QA and NGF (NGF in the dose of 7 ng), and QA and FGF (FGF in the dose of 4 ng), respectively. Neurotrophic factors were applied immediately after the neurotoxin. Control group was treated with 0.9% saline solution in the same manner. For all treated animals injected intracerebral volumes were 10 µl.

Before the treatment animals were anesthetized by pentobarbital sodium i.p. in a dose of 40.5 mg/kg b.m.

Animals were sacrificed by decapitation 7 days after the treatment.

Prepared crude mitochondrial fraction of the striatum and forebrain cortex was used for the appropriate biochemical analysis (14).

NO is an important intra- and intercellular mediator but its half-life *in vivo* is only a few seconds. Most of the NO is oxidized to nitrite/nitrate, and the concentrations of these anions were used as the quantitative indices of NO production. The simplest and most widely used technique is spectrophotometric measurement of nitrite using the Griess reagent, which consisted of naphthylethylenediamine dihydrochloride in water and sulwocanilamide in phosphoric acid. Griess reagent formed a purple azo dye with nitrite, which could be measured with a spectrophotometer (15).

Mean values and SD were calculated for each parameter of interest. Differences between groups were examined using Student's independent *t*-test. The results were considered to be significant at  $p < 0.05$ .

## Results

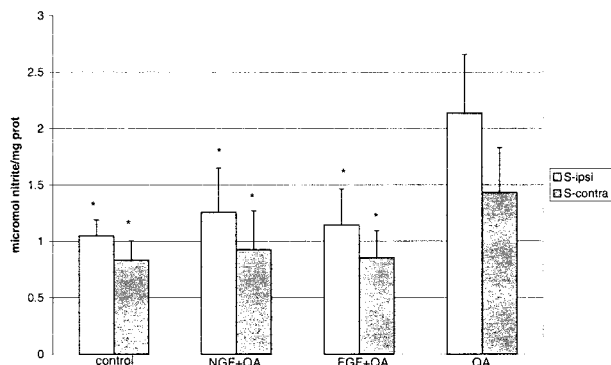
There was no significant difference in nitrite concentrations between ipsi- and contralateral side striatum and cortex of control animals (Fig. 1, 2).

Nitrite level was evidently increased in the ipsi- and contralateral striatum and forebrain cortex of QA treated animals compared to controls ( $p < 0.0001$ ). The difference of nitrite concentration in the striatum between ipsilateral and contralateral side of QA treated animals was highly significant ( $p < 0.0001$ ) but in the forebrain cortex there was no difference.

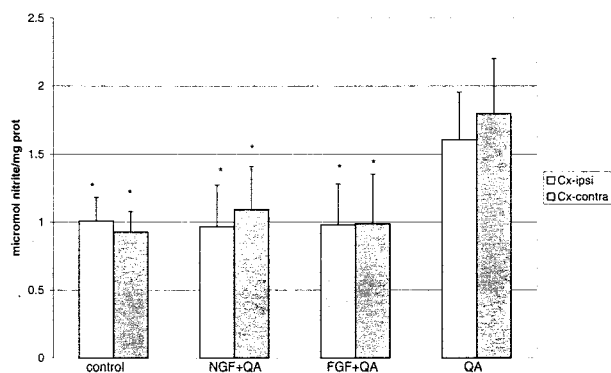
In both groups of animals treated with neurotrophins there was no significant change in the nitrite level compared to the controls (Fig. 1, 2).

Nitrite levels were significantly decreased in the ipsi- and contralateral striatum and forebrain cortex of neurotrophins treated animals. The differences of nitrite con-

centration of NGF-treated and FGF-treated animals compared to QA-treated animals were significant (ipsilateral  $p < 0.002$  vs. NGF and FGF) and contralateral ( $p < 0.02$  vs. NGF,  $p < 0.0003$  FGF).



**Fig. 1** – Nitrite levels in the ipsi- and contralateral striatum of QA- and NTF-treated Wistar rats. (Si, Sc=striatum ipsi-, contralateral). Values are given as  $\mu\text{mol nitrite/mg proteine Mean} \pm \text{SD}$  \*- Significance to corresponding values of QA-treated group.  $p < 0.05$ ).



**Fig. 2** – Nitrite levels in the ipsi- and contralateral forebrain cortex of QA- and NTF-treated Wistar rats. (Cxi, Cxc=forebrain cortex ipsi-, contralateral). Values are given as  $\mu\text{mol nitrite/mg proteine Mean} \pm \text{SD}$  \*- Significance to corresponding values of QA-treated group. ( $p < 0.05$ ).

## Discussion

Nitric oxide (NO) is widely described as a neuronal messenger showing both neuroprotective and neurotoxic effects. It is also clear that NO is involved in many physiological processes such as learning and memory, long-term potentiation and apoptosis, among other functions. Under pathological conditions, NO may promote oxidative stress and cell damage (16). NMDA receptor activation increases NO synthesis probably by increasing intracellular calcium which binds to calmodulin, allowing it to activate nitric oxide synthase - NOS. Striatal application of QA is associated to neural damage through NMDA receptor activation and increased NOS activity. NMDA receptor-mediated cell

death provided firm evidence that NO modulated the NMDA channel in a manner consistent with both physiological and pathophysiological role (17).

Under the pathological conditions, NO acts as a calcium dependent neuromodulator and retrograde messenger following stimulation of the NMDA receptor complex. Our results indicated that the nitrite levels were increased in the ipsi- and contralateral striatum of QA-treated animals (Fig. 1). It was speculated that NO interacted with superoxide anions resulting ultimately in the production of neurotoxic hydroxyl radicals and nitrogen dioxide (18). The neurodestructive effect of NO was shown to occur due to various chemical stages such as nitrosonium ion, peroxy nitrite, etc.

NO was implicated as a mediator of cell injury in HD. Thus, NO as a free radical, might be involved in QA-induced neurotoxicity and oxidative stress. Nitrite levels were increased in the ipsi- and contralateral forebrain cortex of QA-treated animals (Fig. 2). These results indicated a temporal and spatial propagation of oxidative stress in the forebrain cortex, the structure distant, but tightly connected with striatum, the place of direct neurotoxic damage. Affection of the contralateral forebrain cortex, as well as contralateral striatum might be due to the cascade reactions of reactive oxygen species and activated mechanism of excitotoxicity, as a sequel of defect in energy metabolism (19). An inability to maintain cellular ATP levels might lead to partial neuronal depolarization, relief of the voltage dependent  $\text{Mg}^{2+}$  block of NMDA receptors, and persistent activation of ambient glutamate levels (20). Glutamate-induced calcium accumulation, as a result of slow excitotoxicity, correlated with subsequent neuronal degeneration.

Neurotrophins were proposed as the candidates for the treatment of neurodegenerative disease. NGF and FGF differentially protect striatal and cortical projection neurons and interneurons against the injection of the NMDA receptor agonist, QA. Nitrite levels were evidently decreased in both ipsi- and contralateral striatum and forebrain cortex of NTF-treated animals (Fig. 1, 2). Therefore, it was proposed that neurotrophins might have an important role in the protection against neuronal damage following brain insults. Changes in trk expression levels could be responsible for the differences in the vulnerability of striatal neuronal populations observed after QA application.

The main protective influence of NGF included its direct and indirect participation in NO metabolism and its effects to cellular homeostasis. NGF could have the protective effect on the propagation of oxidative stress caused by QA (21).

A report that FGF can protect against glutamate neurotoxicity, and that the FGF receptor (FGFR3), with its gene located in the HD region on chromosome 4, appeared in striatal neurons, made it tempting to speculate on a possibly important role for FGF-FGFR3 interactions in HD pathology (22).

The present results indicated that striatal lesions induced changes in the functional activity of basal ganglia nuclei and that the NTF partly reversed the alterations in the functional state of the basal ganglia circuitry (23). It could be proposed that the lesion-induced morphological changes in the striatum and atrophic changes due to transsynaptic degeneration, would be also less extensive in the NGF- and FGF-treated animals.

NO is clearly a unique biological effector molecule but has very complex physiological chemistry. Under the conditions of normal release, NO is a neuronal messenger molecule. With excessive release such as in the rat model of Huntington's disease, NO may function as a cytotoxic molecule mediating QA-induced cell death as well as being involved in neurotoxicity and oxidative stress.

### Conclusion

The results that were obtained at the 7<sup>th</sup> day after the intrastriatal unilateral application of NTF (NGF, FGF),

given before QA, were contrary to those induced by QA only, such as:

- decrease of nitrite level in ipsi- and contralateral striatum,
- decrease of nitrite level in ipsi- and contralateral forebrain cortex.

Considering that NGF and FGF mediate their activity across glutamergic synapse/nitric oxide, and beside others have also antioxidative effects, the obtained results indicate on their protective role towards functional defects caused by QA. In our study the *in vivo* cytoprotective effects of NTF against striatal excitotoxic lesions suggest that these molecules could be used as potential neuroprotective agents in HD.

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### Апстракт

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#### УТИЦАЈ НЕРВНОГ И ФИБРОБЛАСТНОГ ФАКТОРА РАСТА НА ПРОИЗВОДЊУ АЗОТ ОКСИДА У ЕКСПЕРИМЕНТНОМ МОДЕЛУ ХАНТИНГТОНОВЕ БОЛЕСТИ

Улога азот оксида (NO) у неуродегенеративним обољењима је и поред интензивног изучавања још увек контроверзна. Циљ рада био је да се испитају ефекти интрастријатне примене неуротрофина на моделу Хунтингтонове болести (ХБ) код пацова коју изазива хинолинска киселина (ХК) у смислу сагледавања улоге NO. ХК је апликована интрастријатно, унилатерално у дози од 150 nM. Друге две групе животиња су непосредно пре неуротоксина добиле нервни фактор раста (НФР), односно фибробластни фактор раста (ФФР). Контролна група третирана је 0,9% физиолошким раствором на исти начин. Животиње су жртвоване декапитацијом 7 дана након третмана. Концентрација нитрита је снижена у ипси- и конралатералном стријатуму и кортексу животиња третираних неуротрофинима. Ови резултати би могли да упуте на временску и просторну пропацију оксидативног стреса и проширеног протективног ефекта НФР и ФФР на кору предњег мозга, структуру која је одвојена, али богато повезана са стријатумом, местом директног неуротоксичног оштећења. Неуротрофини могу бити потенцијални неуропротективни агенси у ХБ.

**Кључне речи:** Хантингтонова болест; болест, модели на животињама; хинолинска киселина; азот, оксид; фактор раста, нервни; фактор раста, фибробластни; стрес, оксидативни.