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Creatine and neurotrophin-4/5 promote survival of nitric oxide synthase-expressing interneurons in striatal cultures

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Abstract

Nitric oxide (NO) mediates a variety of physiological functions in the central nervous system and acts as an important developmental regulator. Striatal interneurons expressing neuronal nitric oxide synthase (nNOS) have been described to be relatively spared from the progressive cell loss in Huntington's disease (HD). We have recently shown that creatine, which supports the phosphagen energy system, induces the differentiation of GABAergic cells in cultured striatal tissue. Moreover, neurotrophin-4/5 (NT-4/5) has been found to promote the survival and differentiation of cultured striatal neurons. In the present study, we assessed the effects of creatine and NT-4/5 on nNOS-immunoreactive (-ir) neurons of E14 rat ganglionic eminences grown for 1 week in culture. Chronic administration of creatine [5 mM], NT-4/5 [10 ng/ml], or a combination of both factors significantly increased numbers of nNOS-ir neurons. NT-4/5 exposure also robustly increased levels of nNOS protein. Interestingly, only NT-4/5 and combined treatment significantly increased general viability but no effects were seen for creatine supplementation alone. In addition, NT-4/5 and combined treatment resulted in a significant larger soma size and number of primary neurites of nNOS-ir neurons while creatine administration alone exerted no effects. Double-immunolabeling studies revealed that all nNOS-ir cells co-localized with GABA. In summary, our findings suggest that creatine and NT-4/5 affect differentiation and/or survival of striatal nNOS-ir GABAergic interneurons. These findings provide novel insights into the biology of developing striatal neurons and highlight the potential of both creatine and NT-4/5 as therapeutics for HD.

Keywords: Creatine; Neurotrophin-4/5; Ganglionic eminence; Tissue culture; Nitric oxide synthase; Differentiation

Interneurons, including the subpopulation of nNOS-expressing neurons in the striatum have been found to be relatively spared by the progressive cell loss observed in Huntington's disease (HD) [5]. In line with this observation, it was reported that HD-transgenic mice missing both copies of the nNOS gene show accelerated disease progression relative to wildtype mice and mice carrying one copy of the nNOS gene [11]. Moreover, cerebral neurons expressing nNOS are reported to be resistant to many neurodegenerative diseases and to certain forms of excitotoxicity [5,29]. Additionally, in peripheral nerve degeneration, NO activity seems to be essential for recovery and cell survival [18]. In the developing brain, nNOS was described to mediate a protecting effect against radiation [13]. Important to note, however, is that excess of NO also leads to neuronal cell death after stroke and other neurotoxic conditions [4,27] and is known to produce oxidative damage in HD [33].

NOS which co-localizes with somatostatin and neuropeptide Y in a subpopulation of striatal neurons is first detected in the rat brain at embryonic day (E) 15 in the hypothalamus and pons, and at E18 detection in the striatum was reported [12]. NO seems to be active during early stages of neuronal differentiation by initiating a switch to cytostasis [34]. Moreover, NO is believed to control proliferation and differentiation of mouse neuronal progenitor cells [10]. Therefore, it has been suggested that NO produced by nitrergic neurons in adult mouse subventricular zone and olfactory bulb, exerts a negative control on the size of the undifferentiated precursor pool and promotes neuronal differentiation [30].

Neurotrophin-4/5 (NT-4/5) [6] is a member of the neurotrophin family, which includes in mammals also nerve growth factor (NGF) [23], brain-derived neurotrophic factor (BDNF) [22] and neurotrophin-3 (NT-3) [25]. Binding of neurotrophins

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to the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases initiates many intracellular signaling cascades that involve stimulation of phosphatidylinositol hydrolysis and rapid phosphorylation of phospholipase-C gamma 1 [42] and promote cell survival and differentiation through Ras-MAP kinase and phosphotidylinositol 3-kinase activities [14,31]. All neurotrophins also bind to the non-tyrosine kinase low-affinity NGF receptor (p75NTR) [17,21]. NT-4/5, which activates TrkB, has been reported to promote the survival and differentiation of rat striatal GABAergic neurons, to elevate calretinin (CR) protein levels and number of CR-ir neurons [40]. NT-4/5 also stimulated GABAergic differentiation of cultured cerebral cortical neurons [41]. In former studies, NT-4/5 was found to prevent the death of striatal projection neurons in a rodent model of HD [32]. Furthermore, BDNF, NT-3 and NT-4/5 were shown to significantly increase survival of NADPH-diaphorase-positive neurons in spinal cord cultures [15].

Creatine is a substrate for cytosolic and mitochondrial creatine kinases (CK), and thereby equilibrating ATP levels in the cell by increasing the phosphocreatine (PCr) pool [44] and enhancing the function of a cellular energy shuttle, coupling sites of ATP production and ATP consumption [7,39]. In addition, there is evidence for a direct anti-apoptotic effect of elevated cellular creatine concentrations by inhibition of the mitochondrial permeability transition pore opening [36]. Since neurons have only a very low capacity to synthesize creatine [8], they rely on creatine supply from glia cells or from the blood stream [38]. Administration of exogenous creatine has previously been shown to result in higher cerebral PCr levels [26], which are reduced in HD patients [3]. In line with this observation using a transgenic mouse model of HD it has been demonstrated that dietary creatine supplementation significantly improved survival, slowed development of brain atrophy and delayed atrophy of striatal interneurons [1,35]. Recently, we demonstrated that creatine promoted the GABAergic phenotype of cultured striatal neuronal precursors [2].

In the present study, we therefore aimed at investigating the effects of creatine and NT-4/5 treatment on nNOS-ir neurons in striatal cultures. For that purpose, E14 fetuses (E0 = day of vaginal plug) were removed by cesarean section from pregnant Wistar rats (Janvier, France), the whole ganglionic eminences dissected bilaterally and cell suspensions prepared as previously described [2,40]. Medium was changed 24 h after plating and, subsequently, every other day. Cells were grown for 7 days in vitro (DIV7) and randomly assigned to either the control or to the treatment groups. Creatine monohydrate (Creapure^{TM}; kindly provided by SKW Trostberg; [5 mM]), NT-4/5 (kindly provided by Regeneron [10 ng/ml]), or the combination thereof, were added to the conditioning medium at DIV0 and at each medium change. The concentration of 5 mM was chosen due to the maximal effects found on survival of GABAergic neurons in striatal cultures [2]. All animal procedures were approved by the Animal Research Ethics Committee of the Canton Berne, Switzerland.

At DIV7, cultures (12 cultures per group for each analysis, three independent experiments) were either utilized for examination of viable cell numbers as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [2,40] or fixed for immunohistochemical analyses. For the latter, cultures were exposed to primary antibodies directed against nNOS (1:1000, BD Transduction Laboratories) in 0.1 M PBS/0.1% Triton X-100/2.5% horse serum overnight at 4 °C [2]. Visualization of bound antibodies was done by using the ABC method including a metal enhanced 3′,3-diaminobenzidine substrate kit (Pierce, No. 34065, IL, USA). Cell counts were carried out in six randomly chosen areas (sized 0.9 mm^2) per culture under bright light illumination and cell density was calculated per mm^2 [2]. Morphological parameters, i.e., soma size and number of primary neurites emerging from the cell body per single neuron were assessed using a microscope connected to a digital image processing system (Nikon). For analysis of morphological parameters 72 neurons (from 12 cultures; three independent experiments) of each treatment group were investigated. Immunoblot analyses were carried out as previously described [28]. Briefly, SDS–PAGE was performed using 8% SDS–polyacrylamide separating gel and then transferred to PVDF membranes (Bio-Rad). Blots were blocked and then incubated with the primary antibodies (rabbit polyclonal anti-nNOS, 1:250; Sigma, or mouse monoclonal anti-α-tubulin, 1:20,000; Boehringer) overnight at 4 °C and subsequently incubated with appropriate affinity purified peroxidase-coupled secondary antibodies (Jackson Immunoresearch Laboratory). Specific bands were visualized by amplified chemiluminescence and quantified using a digital imaging system (ImageStation; Kodak, USA). α-Tubulin signal was used as loading control. For statistical analysis, a commercially available software package was used (Statistica 5.0; StatSoft). The experimental groups were compared by nonparametric Kruskal–Wallis ANOVA, followed by post hoc Dunn's multiple comparisons test or the Mann–Whitney test. Differences were considered statistically significant at $p < 0.05$. Values are presented as mean ± S.E.M.

First, we determined the cell types in our control cultures. We found that the overall total neural cell number was 434.2 ± 20.4 cells/mm^2, including 147.7 ± 6.7 GABAergic cells/mm^2 and 9.6 ± 2.0 nNOS-ir cells/mm^2 corresponding to 32.8 ± 1.9% and 2.2 ± 0.1% of total neural cells, respectively ($n = 8$, two independent experiments, mean ± S.E.M.). This result is in accordance with that reported by Kumar [20]. Chronic creatine exposure did not result in changes of overall viable cell numbers as assessed using the MTT assay, whereas NT-4/5 and a combination treatment significantly increased general viability (Fig. 1A). We found that chronic treatment of striatal cultures by creatine resulted in significantly higher densities of nNOS-ir neurons as compared to untreated controls. Similarly, NT-4/5 exposure showed a significant increase. Interestingly, the combination of creatine and NT-4/5 did not further increase nNOS-ir cell densities (Figs. 1B and 2). Based on our previous report showing that NT-4/5 stimulated the morphological differentiation of CR-expressing neurons in striatal cultures [42], we wondered whether similar effects would be seen for creatine and NT-4/5 on nNOS-ir neurons. We observed that number of primary neurites/nNOS-ir neuron was significantly increased in the NT-4/5 and combination exposed cultures but not in the creatine group (Fig. 3A). Soma size of nNOS-ir neurons was found to

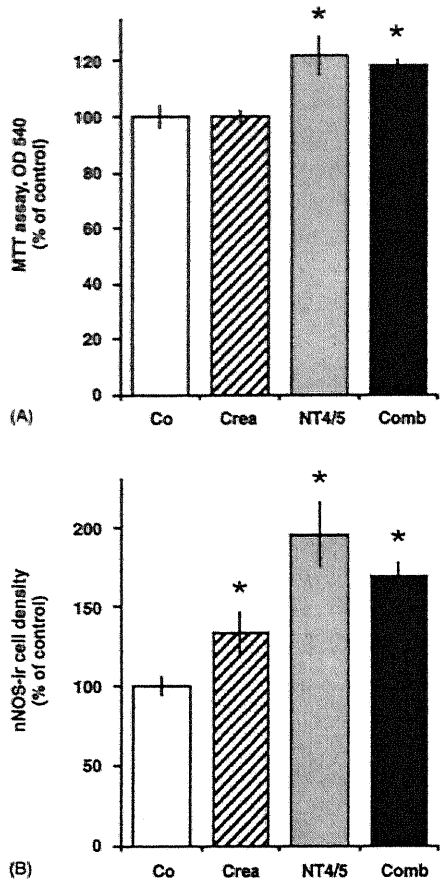

Fig. 1. Effects of creatine (Crea, hatched bar), NT-4/5 (gray bar) or a combination thereof (Comb, filled bar) on measures of the MTT assay (A) and on cell density of nNOS-immunoreactive (-ir) neurons (B) in striatal cultures grown for 7 days in vitro. Untreated cultures served as controls (Co, open bars). Data are given as mean ± S.E.M. and are expressed as percentage of the corresponding controls. *Indicates significance at $p < 0.05$ compared to control.

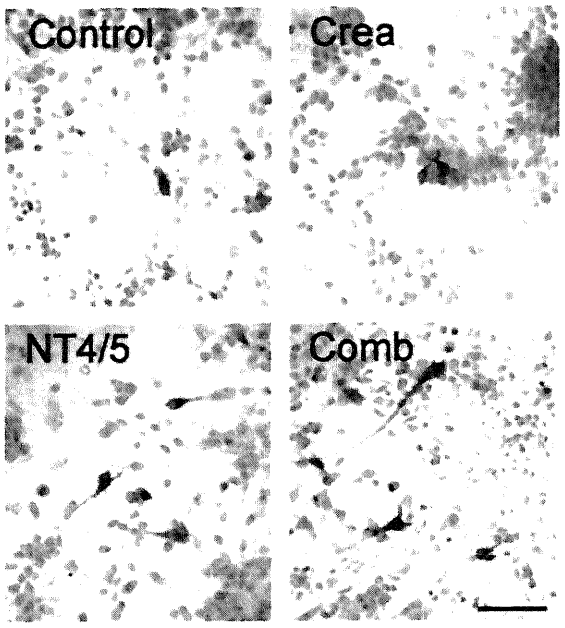

Fig. 2. Representative digitalized photomicrographs of striatal nNOS-ir neurons cultured for 7 days in absence (control) or presence of creatine, NT-4/5 or creatine and NT-4/5 (Comb). Scale bar: 50 μm.

be slightly larger after creatine treatment; however, this increase was not significant, while soma size was significantly increased in the NT-4/5 and combination-treated cultures (Fig. 3B). In similarity to the outcome seen for analysis of cell numbers and morphological complexity, nNOS-protein levels were found to be significantly elevated after NT-4/5 exposure as compared to controls (Fig. 4). Creatine exposure only resulted in moderate upregulation of nNOS-protein levels and accordingly combination treatment did not further alter the outcome seen for NT-4/5 exposure alone. Finally, and in order to investigate whether the nNOS positive neurons do also contain GABA, double-immunofluorescence studies were performed. We observed that nNOS-ir neurons co-localized with GABA (data not shown), which demonstrates that in our cultures nNOS-ir neurons represent a subpopulation of striatal GABAergic cells of 6.5 ± 0.3% ($n = 8$, two independent experiments, mean ± S.E.M.).

In accordance with our previous studies [2,40], we found that general viability as assessed by the MTT assay was significantly higher after NT-4/5 treatment and unchanged after creatine exposure alone. The combination treatment failed to further enhance the readout of the MTT assay, which suggests that creatine and the phosphagen energy system, respectively, do not directly affect the NT-4/5 augmenting effects on general viability of striatal neurons. Furthermore, the finding indicates that NT-4/5 supported different neuronal subpopulations in the cultures, since nNOS-ir neurons represent only a small portion of striatal cells [20]. Creatine, NT-4/5 and combination treatment, however, resulted in significant higher nNOS-ir cell densities. Our results for NT-4/5 treatment in the present study are in agreement with the report showing that BDNF provided significant neurotrophic support to nNOS-ir striatal interneurons in adult rats [19]. Interestingly, nNOS-ir cell densities were not, as it could be expected, higher in the combination group as compared to single treatment of NT-4/5. This let us to speculate that either both factors used similar intracellular signaling pathways or that NT-4/5 used partially similar mechanisms for inducing its effects as it was the case for creatine. Based on the report that NO inactivates creatine kinase by promoting *S*-nitrosylation [43]; however, it may also be possible that the robust increase in nNOS-ir cell numbers and as a result high levels of NO induced by NT-4/5, inhibited an effect of creatine. While creatine treatment exerted no significant effects on the morphology of nNOS-ir neurons, NT-4/5- and combination-treated groups showed significantly higher number of primary neurites/neuron and an increased soma size. This indicates that NT-4/5 interacts with the morphological differentiation of nNOS-ir neurons. Furthermore, NT-4/5 treatment increased expression of nNOS protein in our cultures. The idea of a differentiation inducing effect on nNOS-ir neurons by NT-4/5 is supported by former cell culture studies showing that NT-4/5 promoted survival, morphological and biochemical differentiation of striatal and

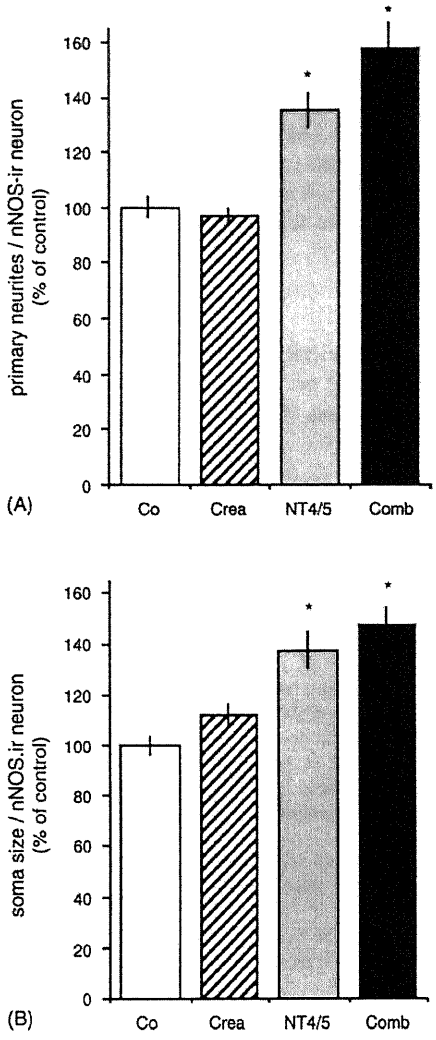

Fig. 3. Effects of creatine (Crea, hatched bar), NT-4/5 (gray bar), or a combination thereof (Comb, filled bar) on number of primary neuritis per nNOS-immunoreactive (-ir) neurons (A), and on soma size (B). Untreated cultures served as controls (Co, open bars). Data are given as mean ± S.E.M. and are expressed as percentage of the corresponding controls. *Indicates significance at $p < 0.05$ compared to control.

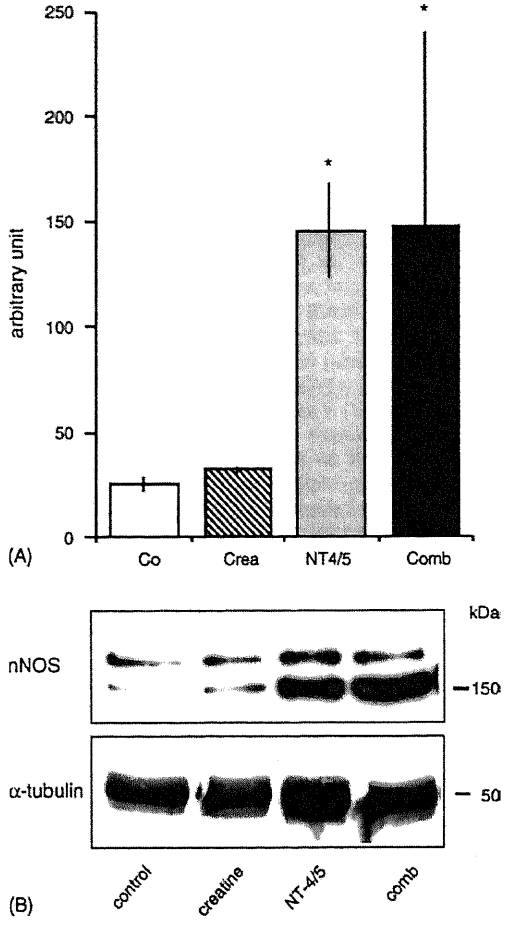

Fig. 4. Effects of creatine (Crea, hatched bar), NT-4/5 (gray bar), or a combination thereof (Comb, filled bar) on protein levels of nNOS in striatal cultures grown for 7 days in vitro. Untreated cultures served as controls (Co, open bar). Densitometric analyses demonstrated significantly higher intensities in the NT-4/5 and combination groups (A). Membranes were reprobed with α-tubulin as loading control (B). Data are given as mean ± S.E.M. and are expressed as percentage of control. *Indicates significance at $p < 0.05$ compared to control.

cortical GABAergic neurons [40,41]. Altogether, NT-4/5 seems to act as a survival and/or differentiation factor for several subpopulations of striatal neurons. In agreement with our findings a phenotype-inducing role of NT-4/5 for NADPH-diaphorase neurons in spinal cord cultures was described [15,16]. In contrast to NT-4/5, we did not find an effect of creatine treatment on general viability, while survival of nNOS-expressing neurons was still increased. We assume that the low abundance of nNOS-ir neurons in the cultures (see above) has masked an effect of creatine exposure on general viability. This result favors the view of an induction of nNOS-ir neuron differentiation and maturation after creatine treatment rather than an increase in the number of neurons due to cell proliferation. A similar outcome was reported for creatine effects on cultured striatal GABAergic neurons [2]. The missing effects on morphological differentiation in combination with little effects on nNOS protein levels, however, do not strongly support such an assumption. Hence, it seems as creatine acted rather as a neuroprotective than a survival-promoting factor on nNOS-expressing neurons. Support to this notion is given by our previous study demonstrating that creatine exposure protected GABAergic cells in striatal cultures against 3-nitropropionic acid (3-NP)-induced toxicity and metabolic insult [2]. In line with this hypothesis are two recent reports where significant protection against 3-NP by high doses of creatine has been found in the adult rat [26,37]. Notably, creatine has been suggested to mediate neuroprotection through inhibition of apoptotic pathways triggered by mitochondrial permeability transition [36]. Whether the effects of creatine supplementation were caused directly by improving cellular energy metabolism or by affecting release or synthesis of growth factors as observed in skeletal muscle cells [24] remains to be elucidated. In line with this notion using hippocampal cell cultures, creatine supplementation has been

shown to increase the phosphocreatine to ATP ratio, therefore, providing effective energy shuttle [9].

In sum, our observations that creatine and NT-4/5 induced the differentiation and/or survival of striatal nNOS-expressing interneurons, suggest that creatine and NT-4/5 play an important role in the developmental regulation of striatal GABAergic interneurons and it can be speculated that these factors also protect interneurons in vivo from adverse events.

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