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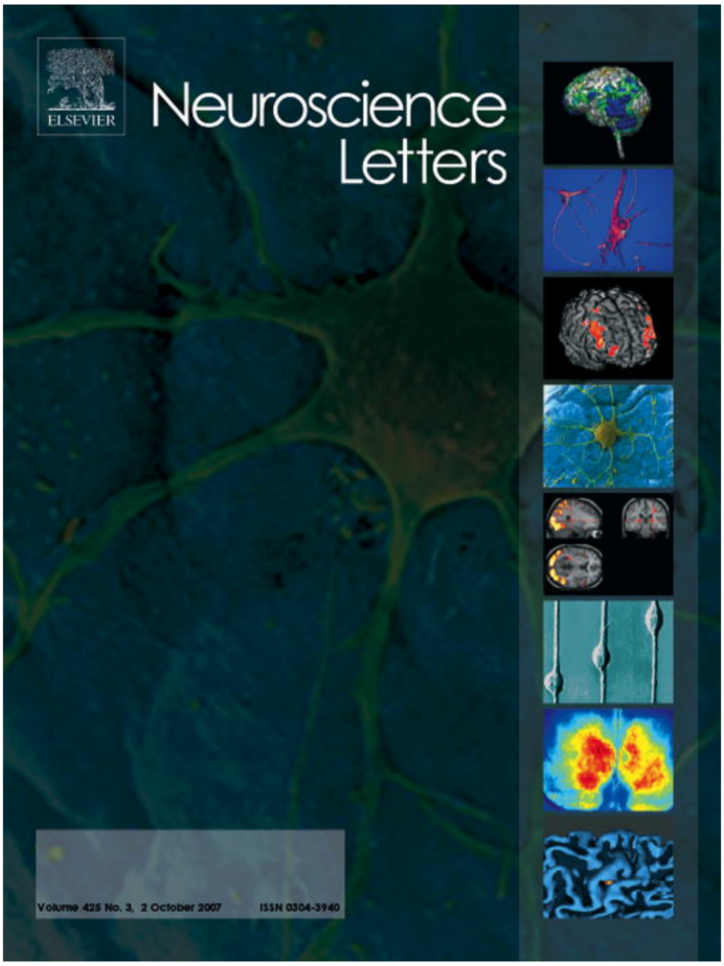

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IGF-1 exacerbates the neurotoxicity of the mitochondrial inhibitor 3NP in rats

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Abstract

Insulin-like Growth Factor 1 (IGF-1) has broad-range neuroprotective effects and is a therapeutic candidate for Huntington's disease (HD). IGF-1 protects striatal neurons from the toxicity of mutated huntingtin *in vitro* and improves neuronal survival *in vivo* in a phenotypic model of HD involving excitotoxic cell death. Because HD is a multifactorial disease, it is important to evaluate the neuroprotective role of IGF-1 in other pathological situations involved in HD progression. We have evaluated the neuroprotective effects of IGF-1 *in vivo*, using the 3-nitropropionic acid (3NP) rat model which replicates the mitochondrial dysfunction observed in HD. Continuous intracerebroventricular infusion of recombinant IGF-1 at a low dose (0.025 μg/h for 5 days) did not alleviate motor impairment and weight loss induced by 3NP treatment. In addition, histological evaluation and quantification of DNA fragmentation evidenced no improvement in neuronal survival. Of interest, we found that a higher concentration of IGF-1 (0.25 μg/h) resulted in an exacerbation of 3NP toxicity on striatal neurons. These results suggest that intracerebral delivery of IGF-1 may not provide a fully effective therapeutic strategy for HD or other disorders involving mitochondrial impairment.

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Keywords: IGF-1; Neuroprotection; Huntington's disease; 3-Nitropropionic acid; Mitochondrial impairment; Striatum

Huntington's disease (HD) is an inherited neurodegenerative disease characterized by the preferential death of striatal neurons. HD patients exhibit progressive motor, psychiatric and cognitive abnormalities, and usually die within 10–20 years. The mutation responsible for HD was identified more than 10 years ago as an expanded polyglutamine tract in the N-terminal part of a protein named huntingtin (Htt) [27]. In spite of this well-defined molecular alteration, HD appears as a highly complex and multifactorial disease [17]. Many factors have been implicated in the degeneration of striatal neurons in HD including excitotoxicity, oxidative stress, protein misfolding as well as alterations in Ca^{2+} homeostasis, transcription, intracellular signaling, axonal transport and synaptic transmission [17]. Impairment in energy metabolism and mitochondrial defects also seems to play a central role in HD pathogenesis [7].

Metabolic alterations in HD are evidenced by the fact that patients are cachexic and exhibit striatal hypometabolism as well as increased lactate levels before marked atrophy of the striatum [6]. Recent studies in patients and genetic models of HD have evidenced several mitochondrial anomalies involved in disease progression [1,4,25]. These include preferential defects in mitochondrial complex II (succinate dehydrogenase, SDH) [4,25], which is consistent with the observation that intoxication of rodents and primates with 3-nitropropionic acid (3NP), an irreversible inhibitor of SDH, reproduces several histopathological and clinical features of HD [7].

There is currently no effective treatment for HD despite intense efforts to develop alternative therapeutic strategies. The 3NP models do not reproduce the genetic mutation responsible for HD. However, unlike transgenic models of HD, they involve a marked degeneration of the striatum that makes them valuable tools to evaluate neuroprotective strategies [7]. So far, these models have allowed for the assessment of several neuroprotective approaches such as inhibition of glutamate signaling [24], supplementation with energetic substrates [22],

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inhibition of proteases [5], and gene transfer of neurotrophic factors [23].

Along these lines, insulin-like growth factor 1 (IGF-1) is an interesting neuroprotective candidate for HD. IGF-1 reduces the toxicity of mutated Htt towards striatal neurons by inducing its phosphorylation [16]. In addition, we have previously shown that an intracerebroventricular (i.c.v.) infusion of recombinant IGF-1 has neuroprotective effects against excitoxicity using the quinolinate rat model of HD [12]. Here, we aimed to further evaluate neuroprotective effects of IGF-1 in the 3NP rat model of HD.

Three-month-old male Lewis rats (weighing ∼400 g, IFFA Credo, France) were used. For surgical procedure, animals were anesthetized with a mixture of ketamine (15 mg/kg) and xylazine (1.5 mg/kg). All experimental procedures were carried out in compliance with the recommendations of the EEC (86/609/EEC) and the guidelines of the French National Committee (87/848) for the care and use of laboratory animals. All chemicals were purchased from Sigma (Saint-Louis, MO) unless otherwise specified.

On day 0, rats were simultaneously implanted in the left lateral ventricle with a canula connected to a minipump filled with either IGF-1 at 0.025 μg/μl ($n = 10$), IGF-1 at 0.25 μg/μl ($n = 10$) or vehicle ($n = 10$) and with a subcutaneous pump filled with 3NP. Additional controls included sham-operated animals ($n = 9$).

On days 4 and 5, rats were evaluated using a clinical index as described previously (normal = 0, very symptomatic = 8) [23]. On day 5, animals were killed by decapitation, and the brain was rapidly removed from the skull. The left hemisphere was frozen in isopentane, and used for histochemistry to assess the activities of cytochrome oxidase (COX) and succinate dehydrogenase (SDH). The striatum and somatosensory cortex were dissected out from the right hemisphere in five animals per group for the purpose of biochemical analysis (quantification of IGF-1 and free oligonucleosomes levels).

Lyophilized recombinant human IGF-1 (R&D systems Inc., Minneapolis, MN) was dissolved to a final concentration of 0.25 and 0.025 μg/μl in a vehicle composed of 10 mM acetic acid with 0.1% bovine serum albumin in 0.1 M phosphate buffer saline (PBS). Continuous i.c.v. delivery of IGF-1 was made via an osmotic minipump (1 μl/h, 2001 model, Alzet, Palo Alto, CA) connected to a canula (stereotaxic coordinates: Antero-posterior, −0.7 mm; lateral, 1.5 mm left from bregma; ventral, 3.6 mm from dura with tooth bar set at −3.3 mm; "brain infusion kit", Alzet) which was secured to the skull bone with dental cement as previously described [5]. Previous experiments have shown that IGF-1 degradation in the minipump was minimal, at least during the first 2 days after implantation [12].

3NP (Fluka, Saint-Louis, MO) was prepared as previously described and systemically administered via subcutaneous osmotic pumps (2ML1 model, Alzet) delivering 56 mg/kg/day for 5 days [5]. Control rats (no 3NP treatment) were similarly anesthetized and subject to identical surgical procedures. One rat of the group receiving the highest dose of IGF-1 was found dead on day 5 and was excluded for the calculation of the behavioral score at day 4.

The lateral striatum and the somatosensory cortex were rapidly dissected out from acutely prepared coronal sections (2 mm thick) prepared with a steel rat-brain matrix. Tissue samples were homogenized using a 1 ml glass–Teflon-homogenizer (900 rpm, 20 strokes) in 300 μl of buffer (25 mM HEPES pH 7.6, 0.1% Triton X-100, 5 mM $MgCl_2$, 1.3 mM EDTA, 1 mM EGTA, with protease inhibitor cocktail "complete", Roche, Indianapolis, IN). Homogenates were pooled by experimental group and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was collected and stored at −80 °C until analysis.

Striatal and cortical supernatants were used for the quantification of IGF-1 concentrations using the Quantikine Human IGF-1 colorimetric Sandwich ELISA kit (R&D) as described previously [12]. Quantification was made in duplicate for each group and each cerebral region.

Striatal supernatants were used for the quantification of free oligonucleosomes using the Cell Death detection ELISA plus kit (Roche) [5]. The measure was made in triplicate and was normalized to the protein content of each sample as measured by the microBCA kit (Pierce, Rockford, IL).

The left frozen hemispheres were cut serially into 40 μm-thick sections using a cryostat and stored at –20 °C before histochemistry. For each animal, 10–12 serial sections encompassing the striatum were examined (interspace 400 μm). SDH and COX histochemistry were performed as previously described, to quantify the regional V_{max} of these enzymes [5,14]. Non-specific staining was evaluated by incubating adjacent sections without substrate (succinate and cytochrome *c*, respectively). To precisely evaluate the loss of striatal COX activity due to striatal neurodegeneration, striatal COX activity was normalized to COX activity in the cortex, because cortical cells are generally unaffected by 3NP treatment.

Sections labeled for COX histochemistry were also used to evaluate the volume of striatal lesions. Lesioned areas were measured by delineating the external border of the lesion seen as a pale staining on digitized images. From these areas, the volume of the striatal lesion was determined using the Cavalieri method as previously described [12].

The blood was collected from decapitated animals and the plasma was recovered by a 3 min centrifugation at 3000 rpm. Samples were stored at −80 °C until analysis. Plasma glucose concentrations were measured using the glucose oxidase method (Beckman Coulter, Fullerton, CA, USA).

Results are expressed as mean values ± S.E.M. Statistical analysis included one-way analysis of variance (ANOVA) followed by a *post-hoc* Scheffé's test. Motor scores were compared using non-parametric tests: Kruskall–Wallis followed by a *U* Mann–Whitney test. The level of significance was set at $p < 0.05$.

On day 5 of 3NP treatment, IGF-1 concentration was quantified in striatal and cortical homogenates. 3NP treatment associated with an i.c.v. infusion of the vehicle did not alter IGF-1 basal concentrations ($p > 0.6$ between Control and vehicle groups). Chronic infusion of IGF-1 at a rate of 0.025 and 0.25 μg/h in the lateral ventricle increased IGF-1 striatal concentration by 30% and 144%, respectively (Table 1). A significant increase in IGF-1 concentration was also detected in the

Table 1
I.C.V. infusion of IGF-1 increases striatal and cortical IGF-1 concentrations

	Control	Vehicle	IGF-1 0.025 µg/h	IGF-1 0.25 µg/h
[IGF-1] pg/mg total protein				
Striatum	49.6 ± 0.4	50.2 ± 0.2	64.7 ± 1.4[a]	122.8 ± 0.5[b]
Cortex	35.8 ± 1.1	37.8 ± 0.9	42.7 ± 0.9[c]	59.2 ± 0.9[a]

ELISA measurement of IGF-1 in striatal and cortical extracts after 5 days of an i.c.v. infusion of IGF-1 at 0.025 or 0.25 µg/h or vehicle. Brain IGF-1 levels were significantly increased by infusion of recombinant IGF-1 at both concentrations. Measurements were made in duplicate and expressed as mean values ± S.E.M.

[a] $p < 0.005$ vs. control and vehicle groups.

[b] $p < 0.0001$ vs. control, vehicle and IGF-1 at 0.025 µg/h groups.

[c] $p < 0.05$ vs. control and IGF-1 at 0.25 µg/h groups, ANOVA and Scheffé's test.

somatosensory cortex (+20% and 64% for a delivery dose of 0.025 and 0.25 µg/h, respectively, Table 1).

To check that the i.c.v. infusion of IGF-1 did not directly interfere with 3NP and its inhibition of the mitochondrial enzyme SDH, SDH activity was measured on brain slices by histochemistry at the end of 3NP treatment. Optical densities were measured in the unlesioned somatosensory cerebral cortex, to have an index of SDH inhibition independent of SDH depletion due to neuronal death [5]. Treatment with 3NP led to a significant inhibition of SDH activity in the cerebral cortex ($p < 0.0001$ versus control). Levels of SDH inhibition were similar in all 3NP-treated groups ($p = 0.9$ and 0.1 between vehicle and IGF-1 at 0.025 and 0.25 µg/h, respectively), demonstrating that IGF-1 did not directly interfere with 3NP. We then evaluated IGF-1 neuroprotective effects against metabolic impairment using clinical, histological and biochemical indices.

Chronic treatment with 3NP leads to a sustained metabolic inhibition associated with a progressive weight loss. At day 5 of 3NP treatment, animals in all 3NP-treated groups had weight loss, but the decrease in body weight was significantly exacerbated in the group receiving the highest dose of IGF-1 (Fig. 1A). Sham-operated animals had stable body weight over the entire 5-day period (data not shown).

Chronic 3NP treatment also induced progressive motor impairment (dystonia, dyskinesia and hypokinesia) which was quantified by a behavioral scale. Progressive motor disabilities were first detected in all groups after 4 days of 3NP infusion. IGF-1 at the lowest dose tested (0.025 µg/h) had no detectable effect compared to vehicle (Fig. 1B). In the group receiving 0.25 µg/h IGF-1, motor disabilities were significantly exacerbated at day 5 ($p < 0.001$ versus vehicle and 0.025 µg/h IGF-1).

To evaluate the effects of IGF-1 on the survival of striatal neurons, we first evaluated the activity of the mitochondrial enzyme COX, a sensitive index of neuronal integrity, using histochemistry [5,14]. Rats treated with 3NP had a visible lesion in the striatum, characterized by low COX activity (Fig. 2A). Cortical COX activity was not affected by either 3NP or IGF-1 ($p = 0.39$, data not shown). Conversely, striatal COX activity was significantly decreased in all 3NP treated groups (Fig. 2B). However, animals receiving 0.25 µg/h IGF-1 displayed exacerbated loss of striatal COX activity (Fig. 2B). The highest dose of IGF-1 also resulted in a significant increase in the size of striatal lesion while IGF-1 at low doses did not have any significant effect compared with vehicle (Fig. 2C).

Additionally, we measured levels of free oligonucleosomes in striatal extracts at the end of 3NP treatment as an index of ongoing cell death. Treatment with 3NP induced significant increases in free oligonucleosomes compared with control animals (Fig. 2D). Levels of free oligonucleosomes were further increased in 3NP-treated rats receiving IGF-1 at both doses (Fig. 2D).

Because IGF-1 is involved in the regulation of peripheral energy metabolism [15], we measured blood glucose levels at the end of 3NP treatment. IGF-1 at 0.25 µg/h significantly increased blood glucose levels compared to controls (Fig. 2E).

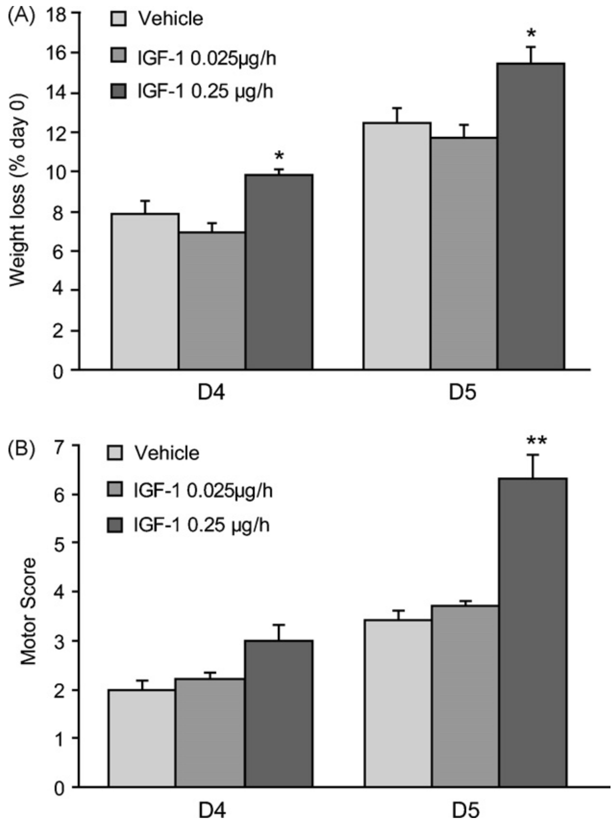

Fig. 1. IGF-1 exacerbates 3NP effects on weight loss and motor symptoms. (A) Rat infused with IGF-1 at 0.25 µg/h lost more weight during 3NP treatment. *$p < 0.05$ vs. vehicle and IGF-1 at 0.025 µg/h, ANOVA followed by Scheffé's test. (B) The severity of motor symptoms was evaluated at day 4 (D4) and day 5 (D5) of 3NP treatment using a motor score. Rats receiving IGF-1 at 0.25 µg/h exhibit more significant motor deficits. IGF-1 at 0.025 µg/h has no beneficial effects. **$p < 0.001$ vs. vehicle and IGF-1 at 0.025 µg/h, Kruskall–Wallis followed by *U* Mann–Whitney test.

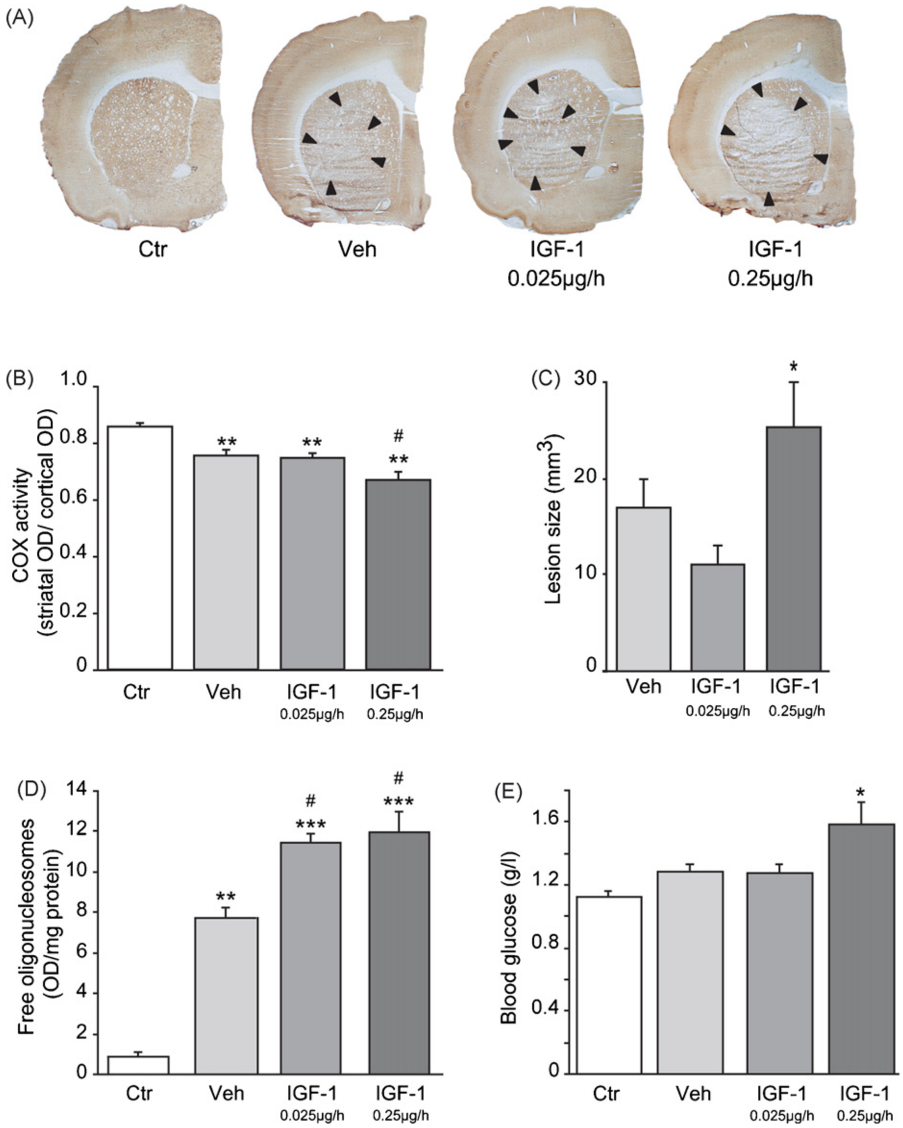

Fig. 2. IGF-1 increases 3NP-mediated neuronal death in the striatum and alters glycemia. (A) Images of representative brain sections stained for COX histochemistry of control rats (Ctr) and rats chronically treated with 3NP and receiving vehicle (Veh), IGF-1 at 0.025 and 0.25 μg/h. The lesion, delimited by black arrows, appears larger with the highest dose of IGF-1. (B) COX activity was quantified by densitometry and expressed as a ratio between striatal and cortical COX activities. 3NP treatment induces a significant decrease in COX activity due to striatal lesion. $^{**}p < 0.01$ vs. control group (Ctr) $\#p < 0.05$ vs. vehicle group (Veh), ANOVA and Scheffé's test. (C) Lesion sizes were quantified at the end of 3NP treatment on serial sections stained for COX histochemistry. IGF-1 at 0.25 μg/h significantly increases the size of the lesion. At lower doses, IGF-1 tended to reduce the lesion induced by 3NP compared to vehicle (Veh), without reaching significance. $^{*}p < 0.05$, ANOVA and Scheffé's test. (D) The striatal content in free oligonucleosomes was assessed at the end of 3NP treatment. 3NP significantly increases the level of free oligonucleosomes in all groups. IGF-1 significantly exacerbates this effect at both concentrations. $^{**}p < 0.001$, $^{***}p < 0.0001$ vs. control group (Ctr). $\#p < 0.05$ vs. vehicle group (Veh). ANOVA and Scheffé's test. (E) Blood glucose levels were measured at the end of 3NP treatment. The highest dose of IGF-1 resulted in hyperglycemia. $^{*}p < 0.01$ vs. control group. ANOVA and Scheffé's test.

Several arguments support the therapeutic use of IGF-1 in HD. IGF-1 is a neurotrophic factor with broad-range neuroprotective effects [11], and specifically protects cultured striatal neurons exposed to mutated Htt [16]. In addition, IGF-1, which belongs to the insulin family, is involved in the regulation of peripheral and central energy metabolism [15], both of which have been identified as abnormal in HD patients [28]. Specifically, patients and transgenic HD mice commonly suffer from hyperglycemia that may be attributable to decreased insulin production or sensitivity [13,18]. In addition to protecting neurons, IGF-1 may thus restore normal insulin signaling, reverse hyperglycemia and improve the metabolic status of HD patients.

We found that IGF-1 at low doses was ineffective at preventing cell death and, at high doses, was toxic for striatal neurons subjected to impairment of energy metabolism. The absence of neuroprotective effects of 0.025 μg/h IGF-1 was unlikely due to an insufficient striatal concentration of IGF-1, because we found increased levels of free oligonucleosomes at this dose. This observation also suggests that while low doses of IGF-1 do not increase lesion volume, they exacerbate the severity of neurodegenerative processes.

Increasing IGF-1 striatal concentrations to twice the control level (5-day infusion of IGF-1 at 0.25 μg/h) exacerbated 3NP-induced weight loss, motor disabilities, and cell death in the lateral striatum. Importantly, the level of 3NP-mediated inhibition of SDH was similar in all groups, ruling out a direct interaction of IGF-1 with 3NP. It rather seems that chronic i.c.v. infusion of IGF-1 selectively increases the vulnerability of the striatum to 3NP. We have previously shown that the same delivery system of IGF-1 was neuroprotective against an intrastriatal injection of the NMDA receptor agonist quinolinate, which triggers excitotoxic processes and Ca^{2+} overload [12]. Excitotoxicity and Ca^{2+} deregulation are also involved in the 3NP-mediated neuronal death. However, they are thought to result from an altered ability of mitochondria subjected to metabolic impairment to buffer Ca^{2+} getting into neurons through NMDA receptors [19]. Therefore, it is likely that 3NP and quinolinate contribute to neuronal death via different mechanisms, which may explain why IGF-1 is protective against quinolinate and not 3NP, as reported for other neuroprotective agents such as minocycline [2].

IGF-1 plays a key role in several physiological functions such as growth and metabolic regulation [29]. Concentration of IGF-1 in the brain is relatively low, while it can be released at high levels from the liver after stimulation by the growth hormone produced by the pituitary gland [26]. As IGF-1 exerts a negative feedback on the hypothalamo-pituitary axis [26], a chronic i.c.v. delivery of IGF-1 may alter the equilibrium between central and peripheral IGF-1 concentrations, leading to general metabolic disturbances, as supported here by the development of hyperglycemia. IGF-1 protoxic effects in presence of metabolic impairment may therefore result from peripheral and/or central effects. Further studies should evaluate the precise mechanisms involved.

Other delivery modes of IGF-1 to the nervous system such as intranasal infusion [21], diffusion from subcutaneous microspheres [8] or viral gene transfer [20] have proven efficient and may represent useful alternative strategies. IGF-1 levels are also increased by physical exercise, which may lead to a more physiological upregulation of IGF-1 cerebral concentrations [9].

IGF-1 has been proposed to be a therapeutic candidate for amyotrophic lateral sclerosis [20] and Alzheimer's disease [10], two neurodegenerative diseases characterized by metabolic disturbances [3]. It is therefore important to evaluate IGF-1 neuroprotective potential in animal models which reproduce metabolic defects found in patients and to test alternative modes of delivery of IGF-1 to the brain. Our results suggest that a central delivery of IGF-1 might not be beneficial in pathological conditions associated with impairment in energy metabolism.

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