

3-Nitropropionic acid modifies neurotrophin mRNA expression in the mouse striatum: 18S-rRNA is a reliable control gene for studies of the striatum

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Abstract: Objective The aim of the present study was to determine the changes in the mRNA levels of neurotrophins and their receptors in the striatal tissue of mice treated with 3-nitropropionic acid (3-NP). **Methods** At 1 and 48 h after the last drug administration, the mRNA expression of nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 as well as their receptors p75, TrkA, TrkB and TrkC, was evaluated using semi-quantitative (semi-Q) and real-time RT-PCR. β -actin mRNA and ribosomal 18S (18S rRNA) were tested as internal controls. **Results** 3-NP treatment did not affect mRNA expression of all neurotrophins and their respective receptors equally. Also, differences in neurotrophin and receptor mRNA expression were observed between semi-Q and real-time RT-PCR. Real-time RT-PCR was more accurate in evaluating the mRNA expression of the neurotrophins than semi-Q, and 18S rRNA was more reliable than β -actin as an internal control. **Conclusion** Neurotrophins and their receptors expression is differentially affected by neuronal damage produced by inhibition of mitochondrial respiration with 3-NP treatment in low, sub-chronic doses *in vivo*.

Keywords: neurotrophins; striatum; neurodegenerative disease; PCR; 18S; 3-nitropropionic acid

1 Introduction

Neurotrophins are growth factors that regulate the cellular and molecular mechanisms involved in the development and maintenance of the nervous system^[1]. In the mature nervous system, they play a major role in neuronal protection and the maintenance of cellular homeostasis; therefore, any change in their expression could be associated with neurodegeneration^[2].

There are difficulties in assessing the expression of neurotrophins in the nervous system; for example, in early reports, brain-derived neurotrophic factor (BDNF) mRNA was not detected in striatal tissue by *in situ* hybridization^[3], but soon after, nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3)^[4] and NT-4/5^[5] mRNA expression was described using the RNase protection assay, and in most of these experiments glyceraldehyde dehydrogenase (GAPDH) was selected as the control gene.

Another problem hindering the evaluation of neurotrophin expression is that the amount of mRNA or protein obtained can be misinterpreted. Furthermore, the type of reverse transcriptase-polymerase chain reaction (RT-PCR)

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technique used and the selection of the internal control gene are important for the assessment of neurotrophin mRNA. The gene used as an internal control should not vary under experimental conditions^[6]. Internal controls commonly used include β -actin, β -2-globulin, β -tubulin, GAPDH and 18S rRNA^[7].

The expression levels of neurotrophins and their receptors change in the presence of neuronal damage, e.g., after excitotoxic lesioning of the striatum^[4,8,9].

Mitochondrial dysfunction is known to underlie the cellular mechanisms in neurodegeneration^[10]; indeed, the application of mitochondrial toxins mimics the degeneration exhibited in Huntington disease (HD). For example, 3-nitropropionic acid (3-NP) induces striatal neuronal damage in humans and experimental animals^[11,12]. This mitochondrial toxin inhibits mitochondrial complex II in the respiratory chain, reduces ATP synthesis, increases Ca^{2+} influx, and activates proteases that cause cellular damage and the death of medium spiny neurons^[12–14]. In previous experiments we showed that systemic administration of low sub-chronic doses of 3-NP to C57/BL6 mice is enough to initiate damage of calbindin-positive neurons in the striatum^[15–17].

As neurotrophin levels are altered in animal models of HD, and 3-NP administration mimics the histopathology of HD, we aimed to evaluate changes in neurotrophin mRNA expression after sub-chronic (5-day) intraperitoneal 3-NP treatment at a low dose, to mimic early neuronal damage. mRNA expression was evaluated at 1 and 48 h after the final dose of 3-NP using semi-quantitative (semi-Q) RT-PCR and real-time RT-PCR. To select the internal control for gene expression, we avoided GAPDH because in previous experiments, we found changes in its expression in the striatum after 3-NP treatment^[15]. Instead, two genes, β -actin due to its stable expression in mouse models of neurodegeneration^[18] and 18S rRNA (a gene encoding a small sub-unit of the 18S ribosome) due to its stability in the mouse brain and other tissues^[7,19], were tested as internal controls.

2 Materials and methods

2.1 Animals Male C57/BL6 mice (30 days old) were pur-

chased from Harlan Laboratories Inc., Mexico, and housed in groups of five in Plexiglas boxes at room temperature (24–26 °C) under a 12:12 h light/dark cycle with free access to food and water. All experiments were performed following the National and International Guidelines and were approved by the Institutional Animal Care Committee. Two days after arrival, animals were randomly assigned to the 3-NP group ($n = 9$) to receive intraperitoneal injections (once per day, at ~11:30 a.m.) of 3-NP (15 mg/kg; Sigma-Aldrich, St. Louis, MO) or to the control group ($n = 9$) receiving vehicle (phosphate buffer, PB; 0.01mol/L; pH 7.4) for 5 days. The last drug administration was on day 5. 3-NP was dissolved in PB and pH adjusted with NaOH.

2.2 Tissue harvest The mice were sacrificed 1 or 48 h after the final treatment. The brain was removed under halothane anesthesia, and the striatal tissue was dissected out and kept at –70 °C for total RNA extraction.

2.3 RNA extraction and RT-PCR Total RNA was isolated from 0.5-g samples of frozen striatal tissue (3–6 brains from each experimental condition). RNA extraction was carried out using the TRIzol reagent method modified for cerebral tissue using the Total RNA Isolation Reagent (Invitrogen Life Technologies Inc., Carlsbad, CA) and precipitation with ethanol. The RNA concentration was calculated from the optical density at 260 nm, and the purity was determined by measuring the 260/280 nm absorbance ratio. cDNA was generated from 1.5 $\mu\text{g}/\mu\text{L}$ of total RNA with a mix of oligo (dT) 12–18 and random hexamer primers using a SuperScript I RT kit (Invitrogen Life Technologies Inc.). The reaction was performed on a Techne TC3000 thermocycler, at 37 °C for 60 min. cDNAs were kept at –20 °C before use.

2.4 Semi-Q RT-PCR Semi-Q RT-PCR for each gene was performed in a 20 μL reaction system containing 20 ng cDNA, a final concentration of 1 $\mu\text{mol/L}$ of each primer, and RED Taq DNA polymerase (Sigma-Aldrich). The primers and PCR conditions for NGF, BDNF, NT-3, NT-4/5, TrkA, TrkB, TrkC, 18S rRNA and β -actin are reported elsewhere^[20,21] and listed in Table 1. PCR products were subjected to electrophoresis on a 2% agarose gel, and stained with ethidium bromide (Sigma-Aldrich). The

Table 1. Primers and PCR conditions

Neurotrophin	Sequence	No. of cycles	T _m (°C)
NGF	Forward 5'-TAGCGTAATGTCCATGTTGT	35	58
	Reverse 5'-CCCACACACTGACACTGTCA		
BDNF	Forward 5'-GAAGAGCTGCTGGATGAGGAC	40	60
	Reverse 5'-TTCAGTTGGCCTTTTGATACC		
NT-3	Forward 5'-CTCATTATCAAGTTGATCCA	35	55
	Reverse 5'-CCTCCGTGGTGAATGTTCTATT		
NT-4/5	Forward 5'-CCCTGCGTCAGTACTTCTTCGAGAC	40	65
	Reverse 5'-CTGGACGTCAGGCACGGCCTGTTC		
β-actin	Forward 5'-TGGTGGGTATGGGTCAGAAGGACTC	30	60
	Reverse 5'-CATGGCTGGGGTGTGAAGGTCTCA		
18S	Forward 5'-GGGAGCCTGAGAAACGGC	20	60
	Reverse 5'-GGGTCGGGAGTGGGTAATTT		
TrkA	Forward 5'-GTGCTCAATGAGACCAGC-TTC	40	60
	Reverse 5'-CTTCAGTGCCTTGACAGCCAC		
TrkB-	Forward 5'-CATGGATCCTGACCCACTCCCCACCTTG	50	68
	Reverse 5'-CATAAGCTTCGACTCCAGGCCGCCCCATG		
TrkB+	Forward 5'-GGGGATCCGGTATCACCAACAGCCAGCTCAAGCCG	50	68
	Reverse 5'-CCAAGCTTCTCGGTGGGCGGGTTACCCTCTGCCATC		
TrkC	Forward 5'-TGGACTGGATAGTCACTGG	45	60
	Reverse 5'-TGGGTCACAGTGATAGGAG		
p75	Forward 5'-GAGCCACCAGAGCGTGTG	35	60
	Reverse 5'-GGGGATGTGGCAGTGGAC		

The nucleotide sequences used to assay gene expression were determined according to those previously reported^[20,21]. All primers were synthesized by Sigma-Aldrich Inc., St. Louis, MO. TrkB-, truncated isoform; TrkB+, full-length isoform.

area and density of the PCR product bands were measured by chemoluminescence using a Fujifilm FLA-5000 scanner and digitized with the Image Reader FLA-5000 V2.1 program. The control mRNA level served as an internal standard to ensure equal loading of RNA, and neurotrophin mRNA levels were normalized to those of the internal control. The resulting measurements were expressed as arbitrary densitometric units and statistically analyzed.

2.5 Real-time PCR Real-time quantitative RT-PCR was performed to quantify changes in neurotrophin and neurotrophin receptor expression levels due to 3-NP treatment. Real-time quantitative PCR for 18S RNA, neurotrophins and their respective receptors was performed with 20 ng

cDNA, 1 μmol/L of each primer, and SYBR Green Jump-Start Taq ReadyMix. The primers were the same as those used for semi-Q RT-PCR (Table 1). Reactions of each sample were performed in three replicates, on a Rotor Gene RG 3000 thermocycler system (Corbett Research, Australia), and the fluorescence was analyzed using the Rotor-Gene Analysis Software 6.1 for SYBR Green. Relative gene expression was calculated from the cycle threshold (C_t) values, within the log-linear phase, as $2^{-\Delta\Delta C_t[23]}$, and normalized to 18S rRNA expression.

2.6 Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay TUNEL assay was carried out to evaluate apoptotic damage in the cortex

above the dorsal striatum. The assay was performed at 48 h after the last dose following the methodology in our previous report^[15] using an apoptosis detection kit (TA300, R&D Systems, Minneapolis, MN). To count TUNEL-labeled cells, images were obtained using an image analysis system (Metamorph 4.0, Universal Imaging, West Chester, PA) connected to a microscope equipped with a video camera coupled to a computer monitor to capture and digitize the cortical and striatal images. The analysis consisted of obtaining squared probes of 1 mm² at 40× magnification to count cells with densely labeled, small particles in the cytoplasm (apoptotic bodies), and different types of chromatin condensation around the margin of the nucleus forming either crescent caps or rings, under a light microscope. The number of damaged cells was averaged from 10 randomly selected fields of 3 sections from each mouse brain.

2.7 Data analysis Data were analyzed with Sigma Stat 3.1 software (Softeck, SPSS Inc., San José, CA) and plotted using Microcal Origin 7 (Microcal Origin Lab Corp., Northampton, MA) and Adobe Illustrator 10 and CS5 (Adobe Systems Inc., San José, CA). Statistical analysis was conducted with a parametric *t*-test or a non-parametric Mann-Whitney test if the data did not display a normal distribution. *P* < 0.05 was considered statistically significant.

3 Results

3.1 Selection of the internal control β -actin mRNA and 18S rRNA were tested as internal controls (Fig. 1). First, β -actin mRNA expression in striatal tissue did not change between 1 and 48 h after administration of the final dose in control conditions ($t_6 = -1.259$; *P* = 0.255); however, there was a significant difference in β -actin mRNA expression in tissue from 3-NP-treated mice between 1 and 48 h ($t_9 = -2.845$, *P* = 0.019). This variation suggested that β -actin was not a good internal control in our experimental conditions. In addition, when all data (1 and 48 h) were pooled, there was no difference in β -actin mRNA expression in the striatal tissue between control and 3-NP-treated mice ($t_{17} = 0.1081$, *P* = 0.49). The expression of 18S rRNA in the striatum of mice was not significantly different between

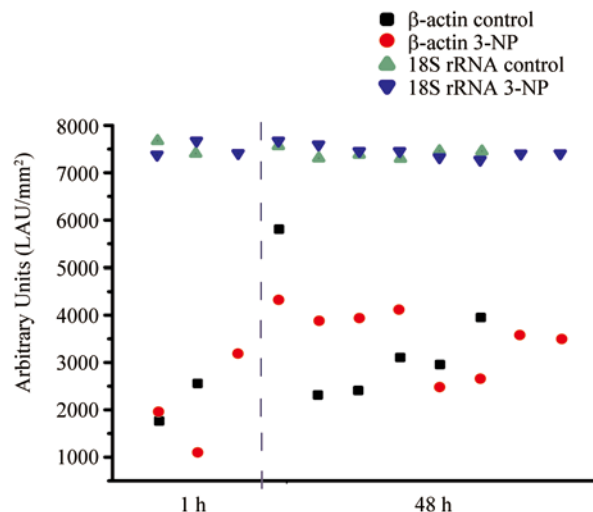


Fig. 1. Analysis of reference gene expression by densitometry (LAU/mm²). The graph displays the absolute expression of ribosomal 18S rRNA and β -actin mRNA in striatal tissue from control and 3-NP-treated mice at 1 (control, *n* = 2; 3-NP, *n* = 3) and 48 h (control, *n* = 6; 3-NP, *n* = 8) after the last dose. Data were analyzed by measuring the area and density of the band widths obtained by electrophoresis and are expressed in arbitrary units. Each point is the absolute mRNA or rRNA expression of β -actin or 18S from one mouse striatum. Note that the absolute expression of 18S rRNA remained stable throughout the experimental conditions.

the evaluated times, 1 and 48 h (Fig. 1), either after vehicle ($t_6 = 1.252$, *P* = 0.257) or after 3-NP ($t_9 = 0.525$, *P* = 0.612). Comparison between control and 3-NP-treated tissues when data (1 and 48 h) were pooled did not present statistical difference ($t_{17} = 0.001$, *P* = 0.999, *t*-test). This result suggested that 18S rRNA was a good internal control for our study.

The relative expression levels of neurotrophins and their receptors were normalized to each of the internal controls (β -actin and 18S). Significant differences were obtained upon comparison of the relative expression in the control group normalized to β -actin and that in the control group normalized to 18S rRNA at 1 h (Fig. 2A, C) and 48 h after the final dose (Fig. 2B, D; Table 2).

The expression of neurotrophins, Trks, and p75 receptors normalized to β -actin was higher than that normalized to 18S in striatal tissue from 3-NP treated mice 1 h after the final administration (Fig. 2E, G), and their expression exhibited discrete differences at 48 h after the final dose when

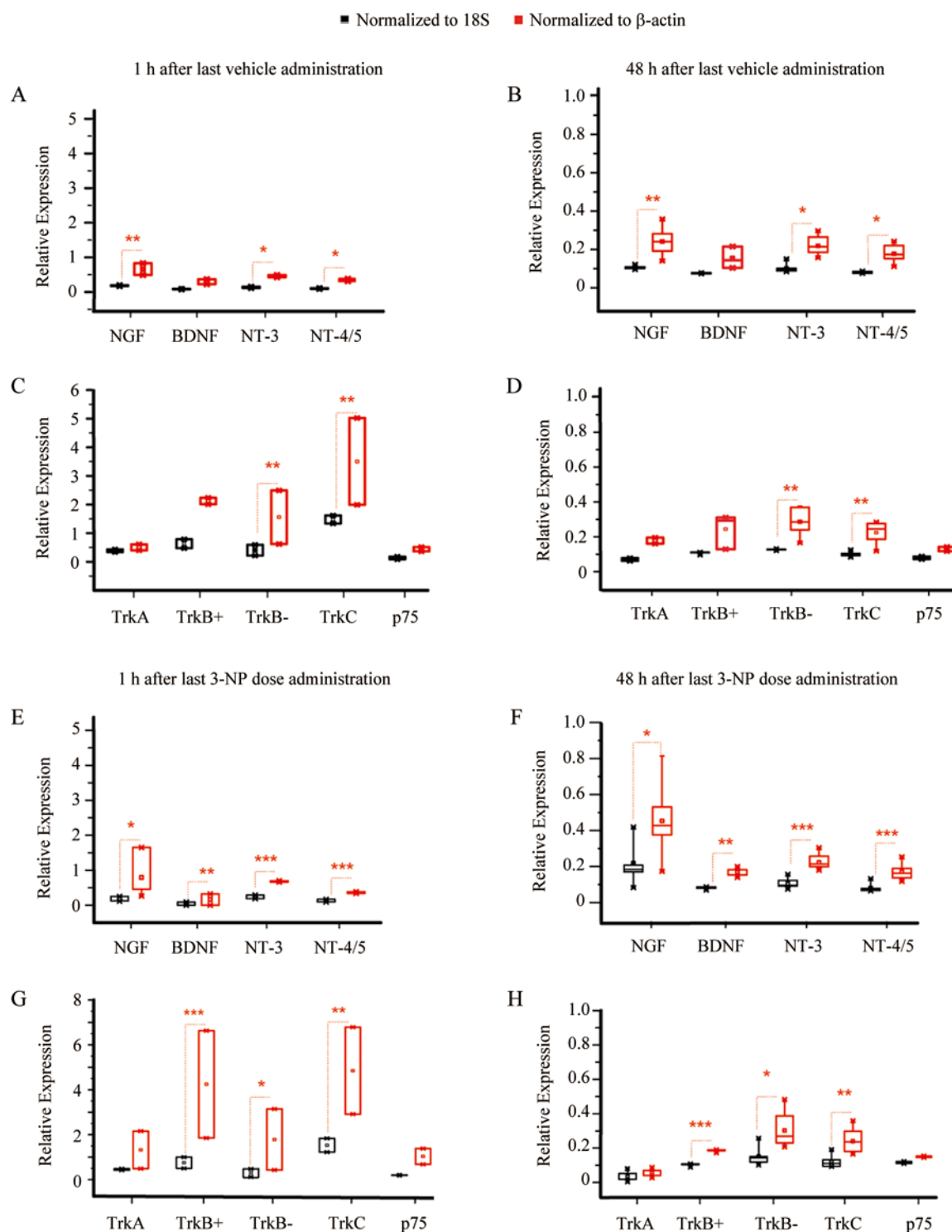


Fig. 2. Comparison of the relative expression of neurotrophins and neurotrophin receptors normalized to β -actin mRNA *versus* ribosomal 18S rRNA in control conditions and after 3-NP administration. The relative mRNA expression of the neurotrophins (A, B) and neurotrophin receptors (C, D) normalized to 18S rRNA were compared with those normalized to β -actin mRNA, at 1 (A, C) and 48 h (B, D) after the last vehicle administration. Similar comparisons were made in the 3-NP treatment group (E–H). Note that the relative mRNA expression of the neurotrophins and their receptors was overestimated when normalized to β -actin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. TrkB+, full-length isoform; TrkB-, truncated isoform.

Table 2. Comparisons of the mRNA expression in control and 3-NP groups normalized to β -actin or to 18S rRNA at 48 h after final treatment dose

Groups	Genes	<i>t</i> -test value	<i>P</i> value
β -actin control vs 18S rRNA control	NGF	$t_{10} = 0.134$	0.001
	BDNF	$t_4 = 0.076$	0.118
	NT-3	$t_{10} = 0.118$	0.001
	NT-4/5	$t_{10} = 0.097$	0.001
	TrkB+	$t_4 = 0.151$	0.089
	TrkB-	$t_{10} = 0.160$	0.001
	TrkC	$t_{10} = 0.124$	0.001
β -actin 3-NP vs 18S rRNA 3-NP	NGF	$t_{14} = 0.234$	0.011
	BDNF	$t_6 = 0.088$	0.001
	NT-3	$t_{12} = 0.118$	0.000 1
	NT-4/5	$t_{14} = 0.091$	0.000 4
	TrkB+	$t_4 = 0.082$	0.000 3
	TrkB-	$t_{10} = 0.151$	0.013
	TrkC	$t_{10} = 0.120$	0.006

TrkB+, full-length isoform; TrkB-, truncated isoform.

normalized to β -actin or 18S rRNA (Fig. 2F, H; Table 2).

3.2 Neurotrophin gene expression in the striatum after 3-NP/vehicle treatment: semi-Q RT-PCR The semi-Q RT-PCR evaluations for all neurotrophins are shown in Fig. 3. There were no differences between the 3-NP and control groups in mRNA expression of NGF (Fig. 3C), BDNF (Fig. 3E), NT-3 (Fig. 3G), and NT-4/5 (Fig. 3I) normalized to both control genes, as evaluated 1 h after the last dose.

At 48 h after the final 3-NP dose, only NGF mRNA expression normalized to β -actin ($t_{12} = 2.569$, $P = 0.025$) and 18S rRNA ($t_{12} = 2.656$, $P = 0.021$) showed a significant increase (Fig. 3D) compared to that in control group. The mRNA expression of BDNF ($t_5 = 0.684$, $P = 0.524$; Fig. 3F), NT-3 ($t_{11} = 0.429$, $P = 0.6$; Fig. 3H) and NT-4/5 ($t_{12} = 0.04$, $P = 0.963$; Fig. 3J) normalized to that of β -actin did not change. Similar results were obtained with normalization to 18S rRNA for BDNF ($t_5 = 2.229$, $P = 0.075$; Fig. 3F), NT-3 ($t_{11} = -0.066$, $P = 0.948$; Fig. 3H) and NT-4/5 ($t_{12} = 0.852$, $P = 0.411$; Fig. 3J).

3.3 Neurotrophin receptor gene expression in the stri-

tum after 3-NP/vehicle treatment: Semi-Q RT-PCR

The expression of neurotrophin receptor mRNA, normalized to either β -actin or 18S rRNA, was not statistically different between 3-NP and control groups at the evaluated times (Fig. 4).

3.4 Neurotrophin and receptor mRNA expression in the striatum after 3-NP treatment: Quantitative real-time RT-PCR

3.4.1 p75 receptor The relative expression of p75 mRNA, a low-affinity receptor for all neurotrophins, increased significantly at both 1 (175.17%, $t_3 = -3.973$, $P = 0.029$, *t*-test, Fig. 5A) and 48 h (521.42%, $t_6 = -2.931$, $P = 0.026$, *t*-test) after the last drug exposure (Fig. 5B).

3.4.2 NGF and TrkA receptor NGF mRNA levels were not altered at 1 ($t_3 = -1.599$, $P = 0.208$, *t*-test; Fig. 6A) or 48 h ($t_9 = 34$, $P = 0.537$, Mann-Whitney test; Fig. 6B) after the last dose of 3-NP. TrkA receptor mRNA level was not altered at 1 h ($t_3 = 1.94$, $P = 0.146$, *t*-test; Fig. 6C), but increased by 108% at 48 h after the last drug dose ($t_7 = 30$, $P = 0.016$; Mann-Whitney test; Fig. 6D).

3.4.3 BDNF, NT-4/5 and TrkB receptor BDNF mRNA expression was not significantly altered 1 h after the last dose of 3-NP ($t_3 = -1.472$, $P = 0.237$, *t*-test; Fig. 7A), but was decreased (37.6%) at 48 h ($t_8 = 3.041$, $P = 0.016$, *t*-test, Fig. 7B), compared with control. The neurotrophin NT-4/5, which has the same Trk receptor as BDNF, did not show changes in mRNA expression at 1 or 48 h after the last 3-NP dose ($t_3 = 2.894$, $P = 0.063$; $t_9 = -1.144$, $P = 0.282$, respectively, *t*-test; Fig. 7C, D).

Moreover, mRNA expression of TrkB was evaluated in full-length (TrkB+) and truncated (TrkB-) isoforms. TrkB+ showed no differences between the groups at either time point (1 h, $t_3 = 1.348$, $P = 0.270$, Fig. 7E; 48 h, $t_8 = -1.571$, $P = 0.155$; *t*-test; Fig. 7F). TrkB- showed a slight, but insignificant increase in mRNA expression at both 1 and 48 h after the last dose ($t_3 = -1.743$, $P = 0.180$, Fig. 7G; $t_9 = -1.729$, $P = 0.118$, Fig. 7H; respectively).

3.4.4 NT-3 and the TrkC receptor The NT-3 mRNA expression level exhibited an insignificant change 1 h after the last 3-NP dose ($t_3 = 2.617$, $P = 0.079$, *t*-test; Fig. 8A) but increased (46.72%) in the 3-NP group at 48 h after the

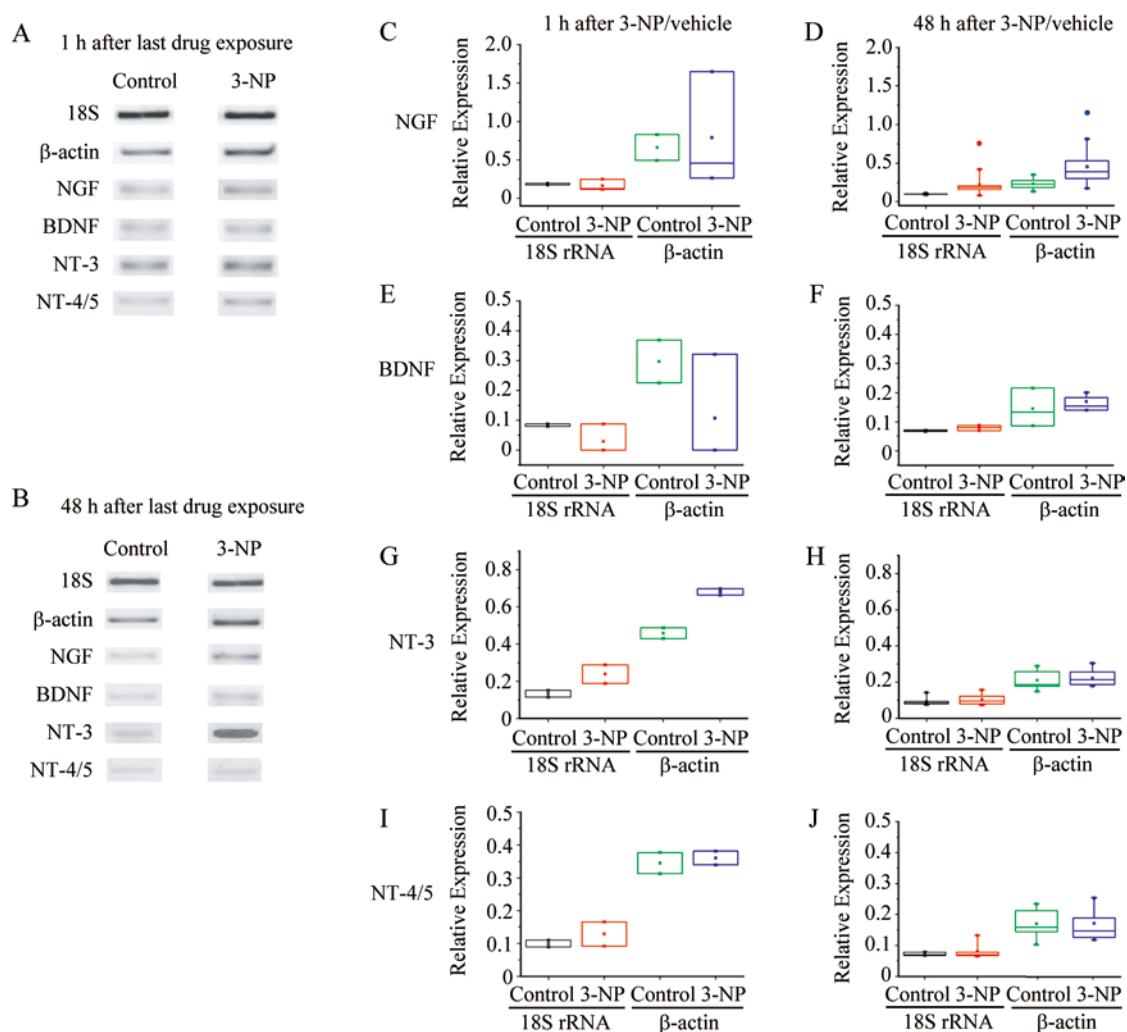


Fig. 3. Semi-quantitative RT-PCR of neurotrophins in striatal tissue from control and 3-NP-treated mice at 1 and 48 h. The total RNA was reverse-transcribed and amplified with PCR using primers that specifically target the neurotrophins, 18S rRNA and β -actin mRNA. The internal controls were 18S rRNA and β -actin mRNA. Reaction products are shown in the electrophoresis panel as 2% agarose gels stained with Sybr Green at 1 (A) and 48 (B) h after the last drug exposure. Box plots show the relative mRNA expression of each neurotrophin in control and 3-NP conditions, normalized to 18S rRNA or β -actin mRNA. Data obtained after 1 h are displayed in the left panels, and after 48 h in the right panels for NGF (C, D), BDNF (E, F), NT-3 (G, H), and NT-4/5 (I, J). Normalization to 18S rRNA or β -actin is indicated in each graph.

last dose, compared with the control group ($t_8 = -2.365$, $P = 0.046$, t -test; Fig. 8B). The NT-3 receptor TrkC showed a similar pattern of mRNA expression, i.e., an insignificant decrease ($t_3 = 0.204$, $P = 0.852$, t -test; Fig. 8C) at 1 h but an increase (168%) at 48 h after the last 3-NP dose ($t_8 = -4.204$, $P = 0.003$, t -test; Fig. 8D).

All the results of quantitative real-time PCR evaluation of neurotrophin and receptor mRNA expression in the striatum after 3-NP treatment are summarized in Table 3.

As a lack of neurotrophic factors like BDNF in the cortico-striatal pathway induces cell damage, TUNEL assay was performed to document this. 3-NP increased the number of apoptotic cells in the striatum and, to a lesser extent, in the cortex at 48 h after the last dose (Fig. 9).

4 Discussion

The results of the present study showed that systemic administration of 3-NP induced cell damage, and differen-

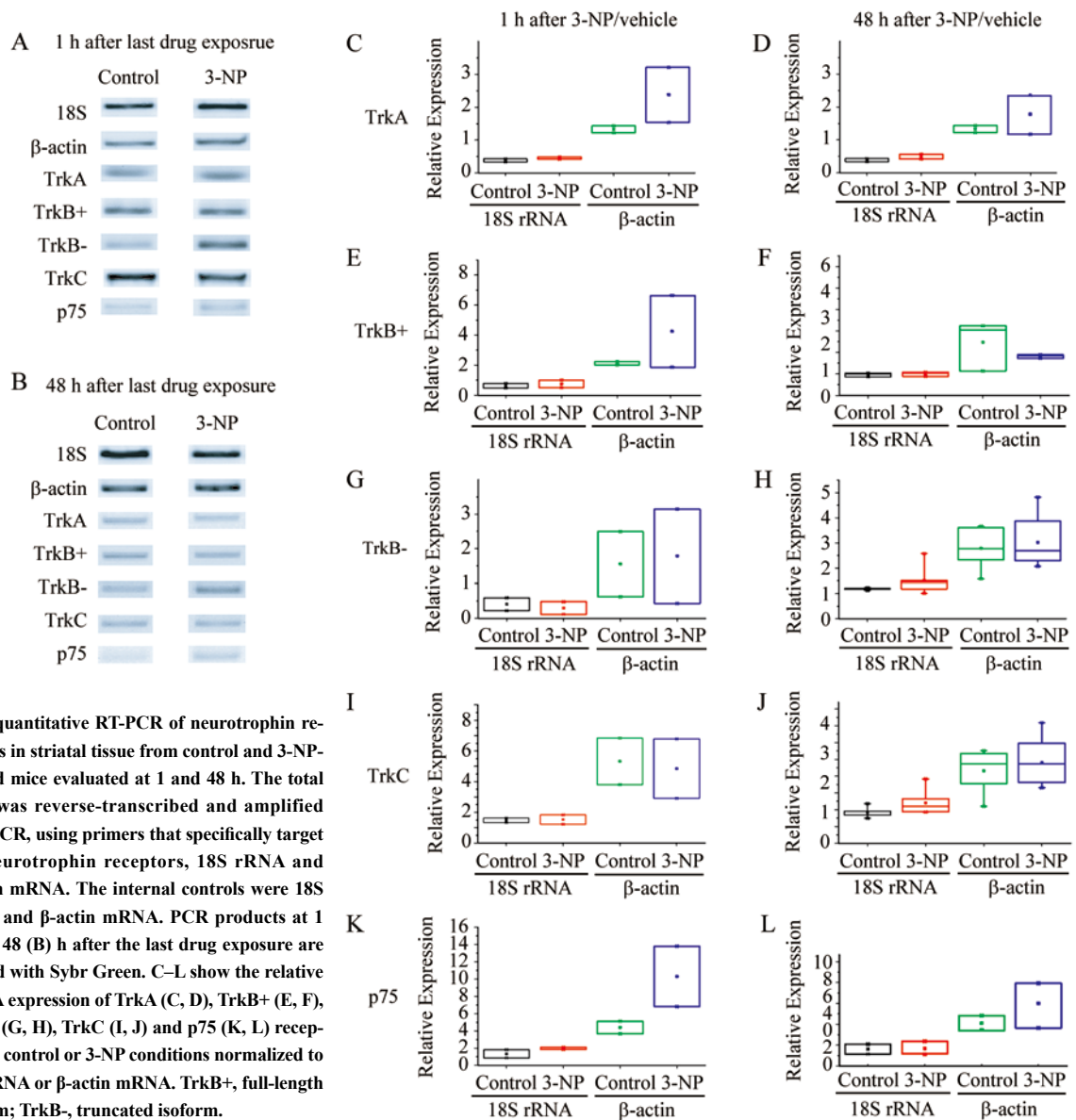


Fig. 4. Semi-quantitative RT-PCR of neurotrophin receptors in striatal tissue from control and 3-NP-treated mice evaluated at 1 and 48 h. The total RNA was reverse-transcribed and amplified with PCR, using primers that specifically target the neurotrophin receptors, 18S rRNA and β -actin mRNA. The internal controls were 18S rRNA and β -actin mRNA. PCR products at 1 (A) or 48 (B) h after the last drug exposure are stained with Sybr Green. C–L show the relative mRNA expression of TrkA (C, D), TrkB+ (E, F), TrkB- (G, H), TrkC (I, J) and p75 (K, L) receptors in control or 3-NP conditions normalized to 18S rRNA or β -actin mRNA. TrkB+, full-length isoform; TrkB-, truncated isoform.

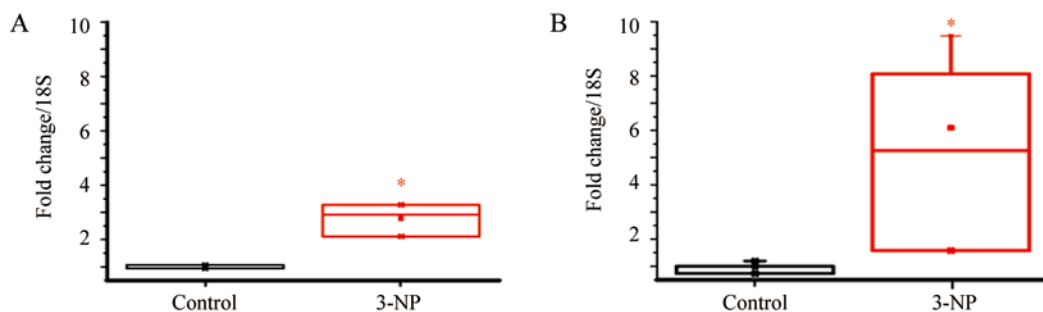


Fig. 5. Fold change of p75 mRNA expression determined by quantitative RT-PCR. A: p75 mRNA expression 1 h after the last 3-NP dose. Fold change in expression is with respect to the 18S internal control ($t_3 = -3.973$, $*P = 0.029$). B: p75 mRNA expression 48 h after the last drug dose ($t_6 = -2.931$, $*P = 0.026$).

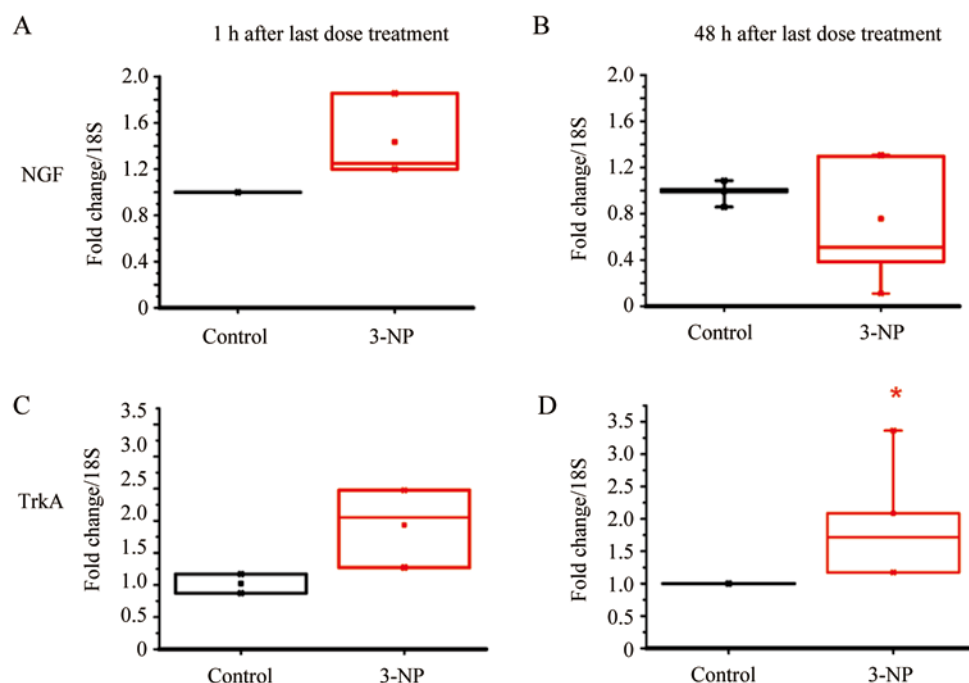


Fig. 6. Fold change of NGF and TrkA receptor mRNA expression detected by quantitative RT-PCR. A and B: NGF mRNA expression 1 h (A) and 48 h (B) after the last dose. C and D: TrkA mRNA expression 1 h (C) and 48 h (D) after the last dose. Note that the TrkA expression evaluated at 48 h after the last 3-NP dose is the only value that is significantly different ($t_7 = 30$, $*P = 0.016$).

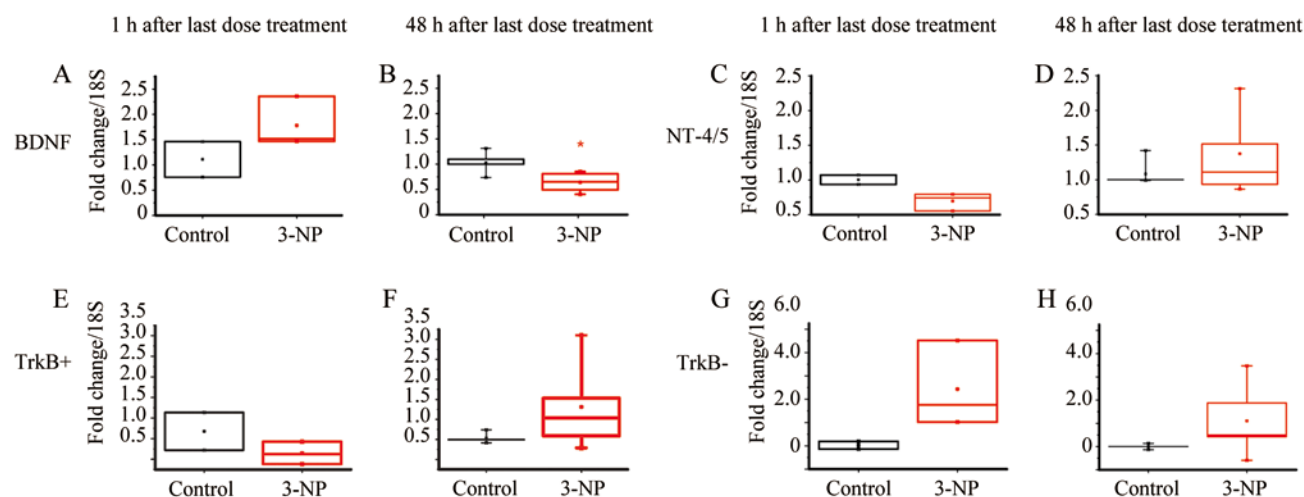


Fig. 7. Fold change of BDNF, NT-4/5 and TrkB receptor mRNA expression detected by quantitative RT-PCR. A and B: BDNF mRNA expression 1 h (A) and 48 h (B) after the last dose of 3-NP. C and D: NT-4/5 mRNA expression 1 h (C) and 48 h (D) after the last dose. E and F: Full-length TrkB (TrkB+) mRNA expression 1 h (E) and 48 h (F) after the last dose. G and H: Truncated TrkB (TrkB-) mRNA expression 1 h (G) and 48 h (H) after the last dose. Note that BDNF expression at 48 h after the last drug administration was the only value that reached statistical significance ($t_8 = 3.041$, $*P = 0.016$).

tially affected the relative mRNA expression of neurotrophins and their receptors. The study also identified varia-

tions in the mRNA expression depending on evaluation time (1 or 48 h after the last dose); and that mRNA expres-

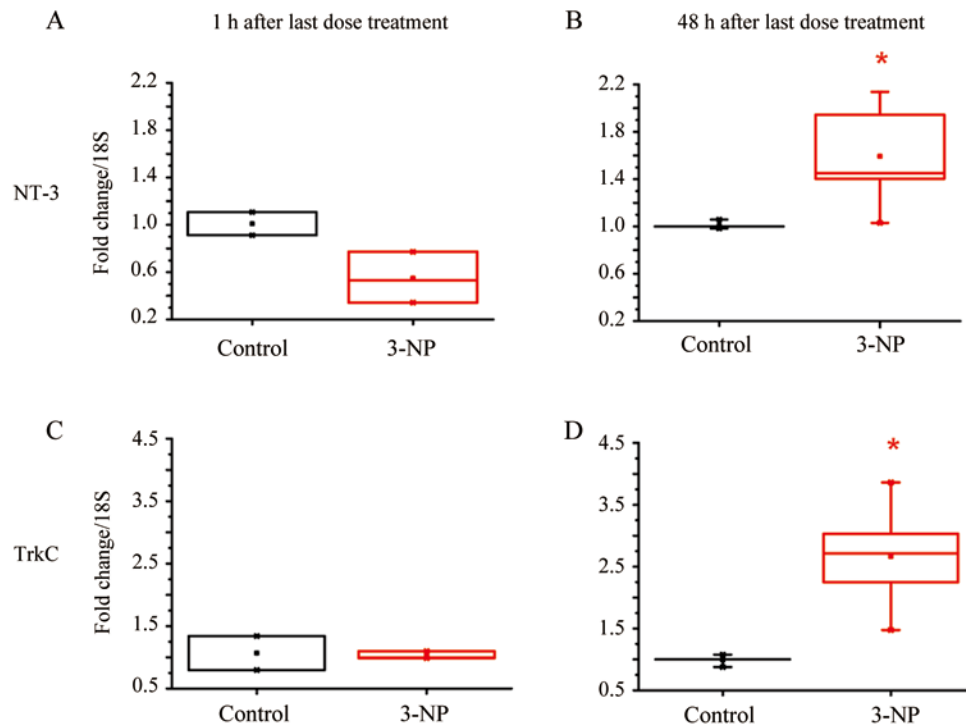


Fig. 8. Fold change of NT-3 and TrkC receptor mRNA expression detected by quantitative RT-PCR. A and B: NT-3 mRNA expression 1 h (A) and 48 h (B) after the last dose. C and D: TrkC mRNA expression 1 h (C) and 48 h (D) after the last dose. Note that both NT-3 and TrkC were markedly different at 48 h after the last dose between control and 3-NP groups (NT-3, $t_8 = -2.365$, $*P = 0.046$; TrkC, $t_8 = -4.204$, $*P = 0.003$).

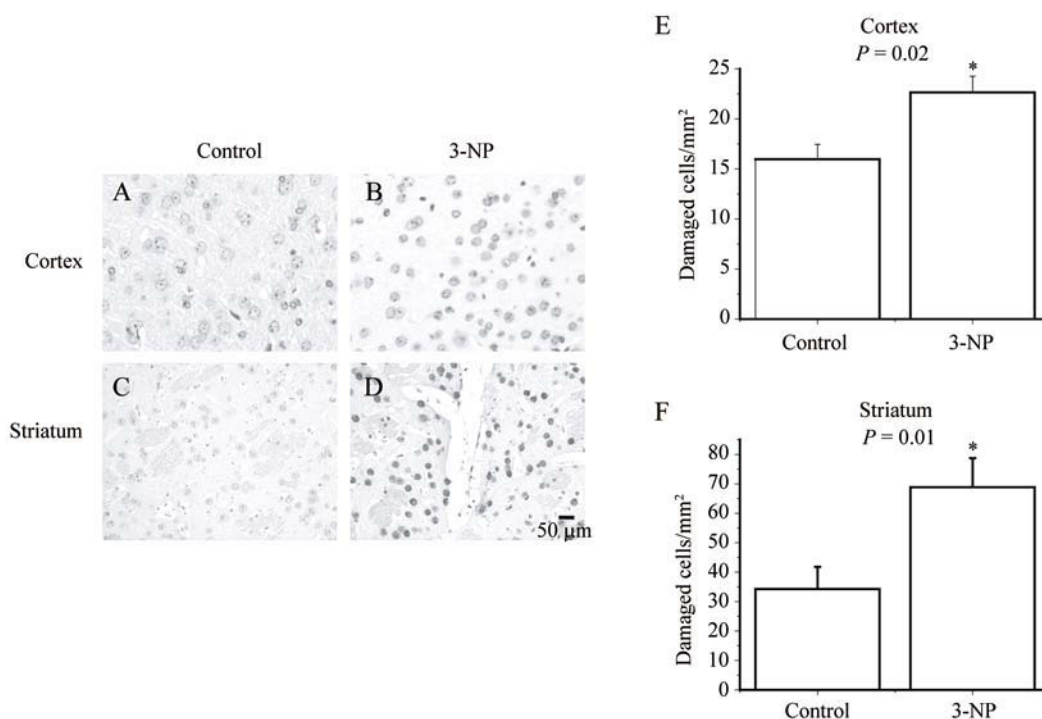


Fig. 9. TUNEL assays in the cortex and striatum of 3-NP-treated and control mice. Left panel illustrates photomicrographs of control (A, C) and 3-NP treated sections (B, D) from cortex (A, B) and striatum (C, D) processed with the TUNEL assay. E and F show the numbers of damaged cells in the experimental conditions in both areas. Scale bar, 50 μm .

Table 3. Summary of mRNA expression obtained through quantitative RT-PCR

Changes in expression level	Neurotrophin				Receptor				
	NGF	BDNF	NT-3	NT-4/5	TrkA	TrkB+	TrkA	TrkC	P75
1 h after the last drug exposure	↑	↑	↓	↓	↑	↓	↑	↓	↑↑*
48 h after the last drug exposure	↓	↓**	↑*	↑*	↑**	↑	↑	↑**	↑↑*

mRNA expression “↑”, increase; “↓”, decrease. * $P \leq 0.05$, ** $P \leq 0.01$. TrkB+, full-length isoform; TrkB-, truncated isoform.

sion differed depending on whether β -actin or 18S rRNA was used as the internal control. Furthermore, mRNA expression was different when assessed through Semi-Q-RT-PCR or quantitative real-time RT-PCR. Because differences were found to be related to PCR techniques and control genes, these are discussed first and changes in neurotrophins and receptors expression are noted later.

4.1 Semi-Q PCR versus quantitative RT-PCR The PCR technique is ideal for detecting changes in gene expression through measurement of the mRNA abundance corresponding to specific genes in different physiological states^[22]. However, accurate results depend on the technique used. The results obtained in the present study differed depending on which technique was used and which gene was selected as an internal control.

The Semi-Q RT-PCR method was less sensitive in estimating low levels of mRNA, and it overestimated gene expression levels generated as increased expression of neurotrophin mRNAs after 3-NP treatment. Conversely, quantitative RT-PCR showed significant changes in the relative expression levels of BDNF, NT-3, TrkA, TrkC and p75 mRNA, confirming that this method is more sensitive in estimating subtle changes in the expression of specific genes^[23,24], such as those of the neurotrophins.

The difference between semi-Q RT-PCR and quantitative RT-PCR is well recognized^[25]; however, as much of the information related to neurotrophin mRNA expression in the striatum was obtained with semi-Q RT-PCR or *in situ* hybridization, we used this as a reference for comparison with previous reports in the same region^[3-5]. We are aware that the primers used in the present study were designed for semi-Q RT-PCR^[20,21]. However, in our experi-

mental conditions they worked with the same efficiency for quantitative RT-PCR, showing efficient amplification and one single product.

4.2 Selection of reference genes To address errors in the estimation of cDNA amplification, the relative quantification is normalized to the expression of an internal control template in a control sample; this manipulation reduces experimental error^[26]. An ideal reference gene would have consistent, non-regulated and independent expression. Commonly-used internal controls include those involved in vital functions of the cell such as metabolism (GAPDH) or cell structure (actin or tubulin); however, not all internal controls are abundant or expressed homogeneously throughout the organism^[18,27]. We previously demonstrated that GAPDH levels vary with the experimental protocol^[15], therefore we evaluated β -actin and ribosomal 18S in the present study.

Although β -actin is a common internal control in RT-PCR studies of the nervous system, there is much evidence pointing out its inconvenience^[18]. In the present study, we found that β -actin mRNA expression was unstable and heterogeneous throughout all experimental conditions. In addition, β -actin expression has been shown to vary among different regions in the brain^[18] and therefore is not always a good control^[28]. With regard to our results, it is known that the transport of β -actin mRNA is controlled by neurotrophins; therefore, if 3-NP affects neurotrophin expression, it also affects β -actin transport^[29], which may change its final expression.

Adoption of the housekeeping gene 18S rRNA as reference is strongly supported by other studies^[7,27,28,30]. After 18S rRNA was shown to be more reliable as a control gene

than β -actin in semi-Q RT-PCR, the subsequent real-time RT-PCR experiments only used 18S rRNA as an internal control. Since the 18S rRNA sequence in eukaryotes does not have a PolyA tail, we used a mix of oligo (dT) 12–18 and random hexamers for the reverse-transcription reaction^[30] to guarantee its hybridization.

4.3 Early versus long-term changes in the expression of neurotrophins and their receptors after 3-NP administration Although the mRNA expression levels of neurotrophins and their Trk receptors in striatal tissue changed 1 h after the last 3-NP dose, these variations were not significant when assessed using semi-Q RT-PCR or quantitative RT-PCR. These results are in agreement with previous reports, where significant changes in neurotrophin expression were observed only 6 h after an excitotoxic lesion^[4] or one week after a mechanical injury^[31]. The lack of early changes (less than 6 h after lesion) in neurotrophin mRNA expression has also been reported in other neuronal populations^[32]. In the present study, greatest changes were detected at 48 h after the last dose, suggesting that damage is progressive and that low sub-chronic doses of 3-NP mimics the progression of neurodegeneration very well.

4.4 Neurotrophin mRNA expression measured by semi-Q RT-PCR In the present study, semi-Q RT-PCR only detected increases in the relative expression of NGF mRNA at 48 h after the last 3-NP treatment with both reference genes, confirming that this method is less sensitive when the cellular target is expressed at low levels^[23].

With regard to NGF, it is possible that its increased mRNA expression is associated with striatal neurodegeneration and/or the provision of trophic support^[33,34], although overestimation of NGF mRNA levels can be attributed to the PCR method.

4.5 NGF and TrkA mRNA expression measured by quantitative RT-PCR Contrary to the result obtained with semi-Q RT-PCR, the mRNA levels of NGF did not change when measured using quantitative RT-PCR at 48 h after the last 3-NP dose. Thus, the difference between the two PCR methods might be explained by the fact that the chemoluminescence bands captured from the gels of semi-Q RT-PCR represent the amplification of the final product;

this does not always correspond to the optimal efficiency of the reaction and therefore is less precise^[25].

The relative expression of TrkA mRNA increased significantly in the 3-NP group at 48 h after the last dose. The differential expression pattern between TrkA receptors and their endogenous ligand indicates that when neuronal damage occurs, NGF-sensitive cells may require additional available receptors; an increase in the number of receptors potentially triggers signaling to facilitate the survival of NGF-sensitive neurons^[35]. In the striatum, the NGF-responsive cells are cholinergic interneurons^[4,8], and our results confirmed that 3-NP does not affect all striatal cell populations equally.

4.6 BDNF, NT-4/5 and TrkB expression measured by quantitative RT-PCR BDNF mRNA expression was reduced significantly 48 h after the last 3-NP dose. Therefore, 3-NP treatment in systemic, low (15 mg/kg) and sub-chronic doses (5 days of treatment) generates cellular damage which limits access of the BDNF supply to the striatum as reported previously with toxic high doses of this neurotoxin and in other experimental models of neurodegeneration^[4,36]. Our BDNF results are also consistent with numerous studies carried out in animal models of HD under different experimental conditions^[37–39] and in postmortem studies of HD patients^[40], suggesting that attenuation of BDNF leads to the selective death of striatal neurons and contributes to the development of HD pathology^[36]. We provided evidence of how 3-NP treatment may affect BDNF levels in the striatum; TUNEL assays showed that not only striatal, but also cortical cells showed 3-NP-induced apoptotic damage. As BDNF is synthesized in the cortex, any damage in the cortico-striatal pathway reduces cortico-striatal trophic support, leading to striatal degeneration.

In contrast to the effects on BDNF, 3-NP did not change NT-4/5 mRNA expression at the evaluated times. Similar to BDNF, NT-4/5 binds to TrkB receptors, and the neuronal protection provided by both neurotrophins depends on TrkB receptor expression^[41] and activation of the full-length TrkB isoform, which has tyrosine kinase activity^[42]. In this regard, we did not find a significant change in

the expression of the full-length and truncated isoforms of TrkB receptors in the striatum of the 3-NP-treated groups. Since NT-4/5 was not affected by the early striatal damage induced by low sub-chronic doses of 3-NP, NT-4/5 should be tested as a rescue factor in animal models of striatal degeneration because this neurotrophin also activates TrkB signaling and perhaps favors neuronal protection in the early stages of neuronal degeneration, maintaining the function of TrkB receptors. Certainly, some studies have proposed that NT-4/5 can replace BDNF deficiency to promote the survival^[43] of specific striatal neuronal populations^[44]. We have demonstrated that NT-4/5 becomes detectable in striatal cells after BDNF during postnatal development^[45], and since there is no evidence that it comes from the cortex, it is possible that NT-4/5 is produced by cells that are not as sensitive to 3-NP as those that respond to BDNF.

4.7 NT-3 and TrkC receptor mRNA expression measured by quantitative RT-PCR The mRNA levels of NT-3 and its receptor TrkC did not change 1 h after the last 3-NP dose; however, the NT-3 mRNA expression in 3-NP-treated mice significantly increased 48 h after the last drug administration. This increase suggests that NT-3 plays an important trophic role in striatal function. NT-3 may be released into the striatum from corticostriatal afferents^[45] and protect striatal cells^[46] by activating the TrkC receptor and MAPK/ERK and PI-3K/Ark signaling pathways^[41]. NT-3 is able to bind to the other Trk receptors, so if NT-3 is over-expressed, it could bind to TrkA and TrkB receptors and trigger alternative signaling pathways to promote cell survival or regeneration^[41,46]. The level of TrkC receptor mRNA also increased at 48 h, which supports the hypothesis that TrkC and NT-3 play a neuroprotective role in the striatum. However, the TrkC receptor increase could be associated with apoptosis induction, as proposed elsewhere^[46,47]. We have evidence that 3-NP administered as in the present study triggers apoptosis; hence a TrkC increase due to apoptosis cannot be ruled out.

4.8 p75 mRNA expression level as measured by quantitative RT-PCR The relative mRNA expression of p75 increased at both times as determined by real-time RT-

PCR. The explanation for the increase may be that the p75 receptor interacts biochemically and functionally with Trk receptors^[48] and, depending on the cellular context, its expression varies. For example, p75 expression is reduced during development^[49], but in the mature nervous system its increase is associated with caspase activation^[9,50] and cellular damage^[51]. As such, the p75 expression increases in our experimental conditions may be related to the striatal apoptotic injury documented in Fig. 9; more experiments are needed to test this idea.

5 Conclusion

The present study shows that the expression of neurotrophins and their receptors is differentially affected by neuronal damage induced by inhibition of mitochondrial respiration with 3-NP in low sub-chronic doses *in vivo*. Our data also show that 18S rRNA can be used as a reference gene for studies of neurotrophin expression in the striatum when the underlying damage is related to mitochondrial dysfunction. Furthermore, β -actin and GAPDH should be avoided in 3-NP studies because their use may affect the accuracy of gene expression results.

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