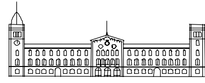

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Departament de Biologia Cel·lular i Anatomia Patològica

ESTUDIO DE LOS MECANISMOS IMPLICADOS EN LA NEURODEGENERACIÓN ESTRIATAL EN MODELOS MURINOS DE LA ENFERMEDAD DE HUNTINGTON

Tesis presentada por Jesús Fernando Torres Peraza

para optar al título de Doctor por la Universidad de Barcelona

RESULTADOS

TRABAJO 2

Mice heterozygous for neurotrophin-3 display enhanced vulnerability to excitotoxicity in the striatum through increased expression of N-methil-D-aspartate receptors

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MICE HETEROZYGOUS FOR NEUROTROPHIN-3 DISPLAY ENHANCED VULNERABILITY TO EXCITOTOXICITY IN THE STRIATUM THROUGH INCREASED EXPRESSION OF *N*-METHYL-D-ASPARTATE RECEPTORS

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Abstract—The striatum is one of the brain areas most vulnerable to excitotoxicity, a lesion that can be prevented by neurotrophins. In the present study, intrastriatal injection of the *N*-methyl-D-aspartate receptor (NMDAR) agonist quinolinate (QUIN) was perfomed in mice heterozygous for neurotrophin-3 (NT3 +/−) or brain-derived neurotrophic factor (BDNF +/−) to analyze the role of endogenous neurotrophins on the regulation of striatal neurons susceptibility to excitotoxic injury. QUIN injection induced a decrease in dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa (DARPP-32) protein levels that was higher in NT-3 +/− than in BDNF+/− or wild type animals. This enhanced susceptibility was specific for enkephalin- and tachykinin-positive projection neurons, and also for parvalbumin-positive interneurons. However the excitotoxic damage in large interneurons was not modified in NT-3 +/− mice compared with wild type animals. This effect can be related to the regulation of NMDARs by endogenous NT-3. Thus, our results show that there is an age-dependent regulation of NMDAR subunits NR1 and NR2A, but not NR2B, in NT-3 +/− mice. The deficit of endogenous NT-3 induced a decrease in NR1 and NR2A subunits at postnatal day (P) 0 and P3 mice respectively, whereas an upregulation was observed in 12 week old NT-3 +/− mice. This differential effect was also observed after administration of exogenous NT-3. In primary striatal cultures, NT-3 treatment induced an enhancement in NR2A, but not NR2B, protein levels. However, intrastriatal grafting of NT-3 secreting-cells in adult wild type mice produced a down-regulation of NR2A subunit. In conclusion, NT-3 regulates the expression of NMDAR subunits modifying striatal neuronal properties that confers the differential vulnerability to excitotoxicity in projection neurons and interneurons in the striatum. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: brain-derived neurotrophic factor, quinolinate, DARPP-32, enkephalin, tachykinin, parvalbumin.

An accurate neuronal circuit design is induced by the interaction of various trophic factors. These molecules show both overlapping and complementary effects on the regulation of growth, differentiation, and survival of neurons during development as well as in adulthood. In the striatum, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are the most relevant neurotrophins. Their receptors, trkB and trkC, are expressed by striatal GABAergic projection neurons, which represent about 90% of the total neuronal population (Merlio et al., 1992). However, the effects of these two neurotrophins differ depending on the neuronal population, developmental stage or pathological situation (Alberch et al., 2004). Although early *in vitro* studies showed that BDNF, but not NT-3, promotes the differentiation of striatal GABAergic neurons (Mizuno et al., 1994), other studies have reported that NT-3 also increases the survival and differentiation of these neurons (Ventimiglia et al., 1995). Moreover, BDNF (Ivkovic et al., 1997; Ivkovic and Ehrlich, 1999; Gavaldà et al., 2004) and NT-3 (Nakao et al., 1996) strongly regulate the development of markers for striatal projection neurons, such as calbindin and the dopamine and cyclic AMP-regulated phosphoprotein of 32 kDa (DARPP-32) proteins.

In vivo studies using mice carrying a mutation in the gene encoding BDNF or NT-3 have provided new evidences about the function of these neurotrophins. Mice with null mutation in the BDNF gene express low levels of calbindin (Jones et al., 1994), DARPP-32 (Ivkovic et al., 1997) and parvalbumin (Grobe et al., 2005) in postnatal striatum. The generation of a forebrain-restricted BDNF mutant mouse allowed study of the effects of BDNF absence, with aged animals showing a reduction in the striatal volume and the number of striatal projection neurons (Baquet et al., 2004). However, in the adult BDNF heterozygote mice striatal volume and cell number are not affected (Canals et al., 2004). Furthermore, these animals show increased striatal dopamine levels that are associated with altered behavioral responses involving the nigrostriatal dopaminergic system (Dluzen et al., 2001; Pineda et al., 2005). About NT-3, very little is known how its gene deletion affects the CNS. Recent studies showed that the volume and the number of projection neurons in the striatum are slightly lower in the adult NT-3 +/− mice (Canals et al., 2004).

Neurotrophins also induce neuroprotective effects in some pathological situations. After excitotoxic injury in the striatum, the expression of BDNF, NT-3 and their receptors is differentially regulated. Intrastriatal kainate injection increases the expression of BDNF, but not NT-3, whereas

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Abbreviations: BDNF, brain-derived neurotrophic factor; DARPP-32, dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa; GAD, glutamic acid decarboxylase 67; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; NT-3, neurotrophin-3; P, postnatal day; PBS, phosphate-buffered saline; PPE, preproenkephalin; PPTA, preprotachykinin A; QUIN, quinolinate.

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AMPA treatment enhances BDNF and decreases NT-3 (Canals et al., 1998). Furthermore, trkB expression is up-regulated by the stimulation of *N*-methyl-D-aspartate (NMDA) or non-NMDA glutamate receptors, but trkC is not modified (Canals et al., 1999). These differential effects are also observed when exogenous neurotrophins are administered. BDNF or NT-3 protects striatal projection neurons against quinolinate- (QUIN) (Pérez-Navarro et al., 1999, 2000) or kainate-induced toxicity (Gratacos et al., 2001), but their effects are different. Intrastriatal grafting of BDNF-secreting cells has a stronger survival effect on striatal neurons against excitotoxicity than those releasing NT-3 (Pérez-Navarro et al., 2000). However, NT-3 is more potent inducing the expression of tachykinin, and also enkephalin, in the lesioned striatum with QUIN (Pérez-Navarro et al., 1999). Similar differential effects between these two neurotrophins have been observed in other models. After neonatal hypoxia, BDNF and NT-3 are neuroprotective for striatal neurons with BDNF as the most potent (Galvin and Oorschot, 2003).

The aim of the present study was to analyze how decreased levels of endogenous NT-3 or BDNF can affec the susceptibility of striatal neurons to the excitotoxicity induced by the NMDA glutamate receptor agonist QUIN To evaluate the mechanisms that regulate differences in the vulnerability of the striatal neuronal populations, NMDA receptor (NMDAR) subunits were examined.

EXPERIMENTAL PROCEDURES

Animals

NT-3 +/− (Ernfors et al., 1994a) and BDNF +/− (Ernfors et al., 1994b) mice were kindly provided by Dr. Patrik Ernfors (Karolinska Institute, Stockholm, Sweden). All mice used had the same genetic background (BalbC). Mice were housed together in numerical birth order in groups of mixed genotypes with access to food and water *ad libitum* in a colony room kept at a constant temperature (19–22 °C) and humidity (40–50%) on a 12-h light/dark cycle. Experiments were performed in male littermates to avoid sex differences. All analyses were conducted in a blind-coded manner with respect to genotype. Data were recorded for analysis by microchip number. For genotyping, DNA was obtained from tail biopsy and processed for PCR, as described elsewhere (Canals et al., 2004). Animal-related procedures were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the local animal care committee of the Universitat de Barcelona and by the Generalitat (Autonomous Government) of Catalonia. All efforts were made to minimize the number of animals used and their suffering.

Excitotoxic lesions

Mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and 12 nmol of QUIN (Sigma Chemical Co., St. Louis, MO, USA), diluted in 0.5 μl of sterile phosphate-buffered saline (PBS), or the same volume of PBS in control animals was stereotaxically injected into the left striatum at the following coordinates: AP +0.6 and L −2.0 from bregma, and −2.7 from dura. The needle was kept in the injection place for three minutes and then slowly removed. All analyses were performed 7 days after QUIN injection.

Cell grafting

Fischer-344 rat fibroblasts transfected with NT-3 and mock-transfected Fischer-344 rat 3T3 fibroblasts were used as described previously (Pérez-Navarro et al., 1999). A microinjection cannula was implanted into the left striatum of 9-week old immunodeficient mice (Swiss *nu/nu*, Charles River Laboratories, Paris, France) and 1 μl containing 2.5×10^5 cells were injected (1 μl/min) at the following coordinates: AP +0.6 and L −2.0 from bregma and −2.7 from dura. Seventy-two hours later, striatum was removed and processed for Western blot analysis. Five animals were used for each experimental condition.

Cell culture

Certified time-pregnant C57BL6 dams (Charles River Laboratories) were deeply anesthetized on gestational day 18, and fetuses were rapidly removed from the uterus. Striatal cells were plated at a density of 2×10^6 cells onto 60-mm or 200,000 cells onto 24-well plates which were pre-coated with 0.1 mg/ml poly-D-lysine (Sigma Chemical Co.), for Western blot or immunocytochemistry analysis, respectively. Neurobasal™ medium (Gibco-BRL, Renfrewshire, Scotland, UK) supplemented with B-27 (Gibco-BRL) and GlutaMAX™ (Gibco-BRL) was used to grow the cells in serum-free conditions. Plated cell cultures were maintained in an incubator h 5% CO_2 at 37 °C. NT-3 (50 ng/ml; Peprotech EC Ltd., ndon, UK) was added to the cultures at 3 days *in vitro*. Immuchemical and biochemical analysis were performed 24 h after -3 addition, as described previously (García-Martínez et al., 2006) employing primary antibodies directed against NR1, NR2A and NR2B (all from Chemicon, Temecula, CA, USA).

Western blot analysis

Animals (n=4 for each genotype) were deeply anesthetized using a CO_2 chamber, striatum was dissected and rapidly frozen, and stored at −80 °C until processing. Total protein homogenates were obtained and loaded in SDS-PAGE as described elsewhere (Canals et al., 2004). Then, proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA), incubated for 1 h with 5% BSA and 5% fat-free dry milk in Tris-buffered saline containing 0.1% Tween-20, and incubated overnight at 4 °C with primary antibodies against DARPP-32 (1:5000), NR1 (1:500), NR2A (1:1000) or NR2B (1:1000) all from Chemicon. Next, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:3000; Promega, Madison, WI, USA). To standardize total protein content in each lane, membranes were incubated with actin (1:3000; ICN Biomedicals Inc., Irvine, CA, USA) or α-tubulin (1:100,000; Sigma Chemical Co.) primary antibodies, followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody (1:3000; Promega). The reaction was finally viewed with the ECL Western blotting analysis system (Amersham Biosciences Europe GmbH, Roosendaal, The Netherlands). Western blot replicates were scanned and quantified using the Phoretix 1D Gel Analysis (Phoretics International Ltd., Newcastle, UK).

Immunohistochemistry analysis

Mice (n=4 for each genotype) were deeply anesthetized with pentobarbital (60 mg/kg, i.p.) and intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and post-fixed for 2 h in the same solution, cryoprotected with 10–30% sucrose in PBS with 0.02% sodium azide and frozen at −20 °C. Serial coronal sections (30 μm) obtained with a cryostat were collected in PBS as free-floating sections and pre-incubated for 1 h with PBS containing 10% methanol and 0.35% H_2O_2. Sections were then washed three times in PBS and blocked for 1 h with 2–10% normal horse serum in PBS. Tissue was then

incubated with the corresponding primary antibody diluted in PBS containing 2% normal goat serum for 16 h at 4 °C, except for DARPP-32 that was incubated at room temperature. The antibodies used were: anti-NeuN 1:100 (Chemicon); anti-DARPP-32, 1:10,000 (Chemicon) and antiparvalbumin, 1:1250 (Sigma Chemical Co.). Sections were washed three times and incubated with a biotinylated secondary antibody (1:200, Pierce) for 2 h at room temperature in the same buffer as the primary antibody. The immunohistochemical reaction was developed using the ImmunoPure Ultrasensitive ABC Staining Kit (Pierce, Woburn, MA, USA). No signal was detected in controls in which primary antibodies were omitted.

Histology

To identify large interneurons, brain slices were serially mounted in slides and processed for Nissl staining as previously described (Canals et al., 2004). Large neurons were identified following parameters described previously by Oorschot (1996).

In situ hybridization studies

Animals (n=5 for each group) were killed by decapitation, brains were removed, frozen using CO_2 pellets, and stored at −80 °C. Cryostat coronal sections (14 μm) through the whole striatum were serially collected on silane-coated slides, fixed with 4% paraformaldehyde, dehydrated in graded ethanol, treated with chloroform, and air dried. Consecutive sections were processed for *in situ* hybridization with oligonucleotide probes for rat glutamic acid decarboxylase (GAD) 67, preproenkephalin (PPE), or preprotachykinin A (PPTA) as described elsewhere (Pérez-Navarro et al., 1999). The slides were exposed to Phospho Imager for 7–20 days, dipped in LM-1 emulsion (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 2 months at 4 °C, developed in D-19 (Eastman Kodak, Rochester, NY, USA), fixed, and lightly counterstained with Cresyl Violet.

Cell counting

Neuronal densities of ipsilateral and contralateral striatum were stereologically measured using an optical dissector/Cavalieri combination as described elsewhere (Oorschot, 1996). Unbiased stereological counts of striatal cells were obtained from the entire striatum using the Computer Assisted Stereology Toolbox (CAST) software (Olympus, Ballerup, Denmark A/S). The dissector counting method was employed to analyze every eight sections stained by immunohistochemistry or Nissl, and every 20 coronal sections processed by *in situ* hybridization. The counting frames were randomly sampled and the coefficient of variation was between 0.03–0.05. All cell counts were performed blind with respect to genotype.

Statistical analysis

Analysis between groups was made using ANOVA followed by the least significant difference (L.S.D.) post hoc test or Student's t-test for unpaired samples.

RESULTS

Reduced levels of endogenous NT-3 increases vulnerability of striatal cells to excitotoxic injury

To determine whether decreases in endogenous NT-3 or BDNF modify the susceptibility of striatal neurons to excitotoxicity, we analyzed levels of DARPP-32 protein in the striatum of NT-3 +/− and BDNF +/− mice after QUIN injection. Western blot analysis showed a decrease of DARPP-32 protein levels after intrastriatal QUIN injection that was significantly higher in NT-3 (49.8±4) than in wild type (27.5±6) mice (Fig. 1A, B). In contrast, the striatum of BDNF +/− mice showed a similar reduction in DARPP-32 protein levels to wild type mice (20.6±5%; Fig. 1A, C).

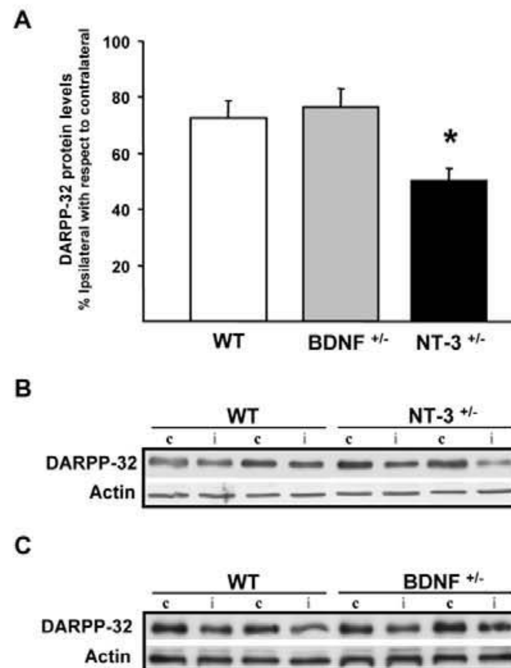
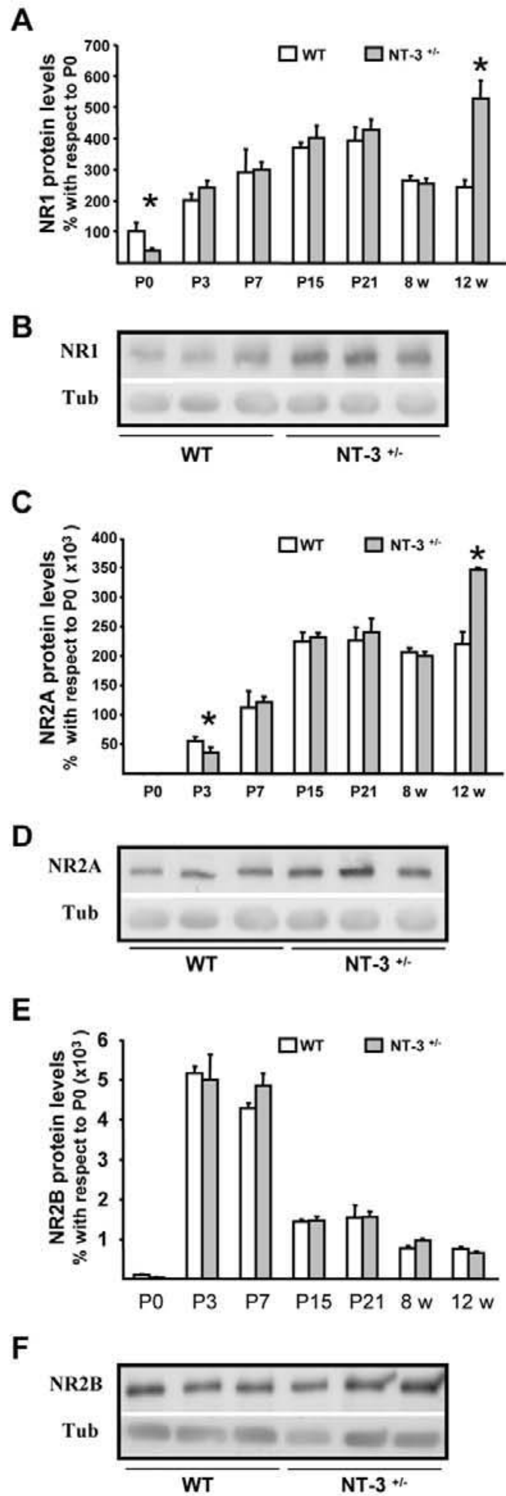


Fig. 1. DARPP-32 protein levels in NT-3 +/− mice striatum are more affected by NMDAR-mediated lesion. Changes in striatal DARPP-32 protein levels were analyzed by Western blot after QUIN-induced lesion in wild type, BDNF +/− and NT-3 +/− mice striatum. (A) Intrastriatal QUIN injection induced a decrease in DARPP-32 levels in all genotypes analyzed. However it was higher in the NT-3 +/− striatum. Results are expressed as mean±S.E.M of percentages of injected with respect to contralateral (non-lesioned) striatum in four independent experiments. (B) Representative blots showing changes in DARPP-32 levels in wild type vs. NT-3 +/− mice (C) Representative blots showing changes in DARPP-32 levels in wild type vs. BDNF +/− mice. C, contralateral striatum; I, ipsilateral striatum. * P<0.05 compared with wild type, n=4 animals.

Age-dependent regulation of NMDAR expression by endogenous NT-3, but not BDNF

To determine whether endogenous NT-3 modulates striatal expression of NMDAR subunits we analyzed protein levels of NR1, NR2A and NR2B subunits during postnatal development and adulthood in wild type and NT-3 +/− mice. Interestingly, we found that NT-3 differentially modulates levels of NMDAR subunits in an age-dependent manner. NR1 subunit increased its expression during development. However, there were significant differences in NR1 expression in wild type and NT-3 +/− striata. The deficit of NT-3 produced a reduction of NR1 levels at postnatal day (P) 0 (38±6% relative to wild type, P<0.05; Fig. 2A, B), whereas enhanced levels were observed at 12



weeks of age (217±25% relative to wild type, $P<0.05$). Nevertheless, no differences were detected from 3 days to 8 weeks of age between both genotypes. Similar temporal developmental profile was observed in NR2A expression. Since the levels of NR2A are very low at birth, a transient reduction of this subunit was detected at P3 in the striatum of NT-3 +/− mice (62±6% with respect to wild type, $P<0.05$; Fig. 2C, D). In older ages, no differences in NR2A levels were observed until 12 weeks of age, when an increase of 157±3.8% respect to wild type ($P<0.05$; Fig. 2C, D) was detected. The over-expression of NR1 and NR2A subunits correlates with higher NMDAR-mediated excitotoxicity found in NT-3 +/− mice at 12 weeks of age. It is noteworthy that reduction of endogenous NT-3 levels selectively affected NR1 and NR2A subunits, since protein levels of NR2B were not different between NT-3 +/− and wild type mice at any age analyzed (Fig. 2E, F).

To confirm the specificity of NT-3 in regulation of striatal NMDAR expression we measured NMDAR protein levels in BDNF +/− mice. Interestingly, we found that reduced levels of endogenous BDNF did not modify expression of NMDAR subunits in adult mice (NR1: 96.7±6%, NR2A: 91.8±24% and NR2B: 96±8% with respect to wild type, Fig. 3A–F). Furthermore, we analyzed NR1, NR2A and NR2B at 4 and 8 weeks of age and there were no differences between wild type and BDNF +/− striata (data not shown).

Exogenous NT-3 modulates the expression of NMDAR subunits

According to the differential regulation observed above in early postnatal and adulthood, we next studied whether exogenous NT-3 is able to regulate NMDAR subunits using two different approaches: a) administration of NT-3 in primary striatal cell cultures, and b) grafting of NT-3 secreting cells in adult striatum of wild type mice.

First, we added NT-3 (50 ng/ml) to striatal cell culture at 3 days *in vitro*, and 24 h later NMDAR protein levels were analyzed by Western blot. NT-3 treatment enhanced NR1 and NR2A levels respect to control cultures, although the increase was higher in NR2A levels (181±25 relative to control; Fig. 4A, B). Immunostaining for NR2A subunit

Fig. 2. Endogenous NT-3 selectively modulates NMDAR expression in an age-dependent manner. NMDAR protein levels were measured by Western blot during postnatal development and adulthood in striatum from wild type and NT-3 +/− mice. (A) Time course of NR1 expression show that NT-3 +/− mice express lower protein levels of NR1 at P0 while, at 12 weeks of age these mice show increased expression of NR1. (B) Representative blots showing changes in NR1 levels in wild type vs. NT-3 +/− mice at 12 weeks of age. (C) Time course of NR2A expression shows that NT-3 +/− mice express lower levels of NR2A protein at P3 in striatum while, at 12 weeks of age these mice show increased expression of NR2A. (D) Representative blots showing changes in NR2A levels in wild type vs. NT-3 +/− mice at 12 weeks of age. (E) Time course of NR2B expression shows no differences between wild type and NT-3 +/− mice at any age analyzed. (F) Representative blots of NR2B in wild type and NT-3 +/− mice at 12 weeks of age. Results in graphs are expressed as mean±S.E.M of percentages with respect to wild type at P0. * $P<0.05$ compared with age-matched wild type, $n=4$–5 animals.

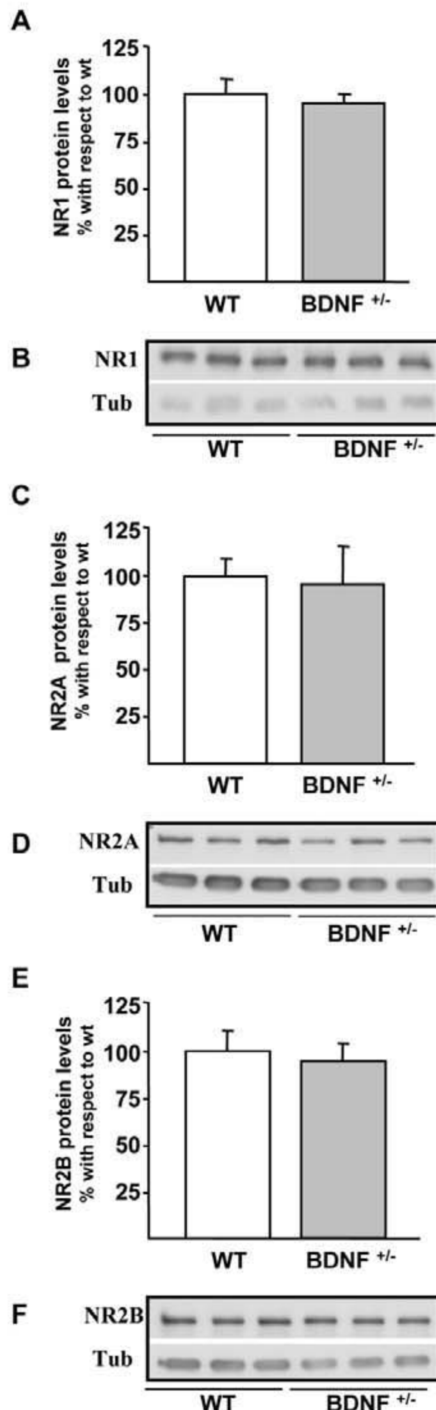


Fig. 3. Normal expression of NMDAR subunits in striatum from BDNF +/− mice. Striatal expression of NMDAR subunits from BDNF +/− mice was analyzed by Western blot. Bars show that BDNF +/− mice have equal protein levels of NR1 (A), NR2A (C) and NR2B (E) in striatum than wild type mice at 12 weeks of age. Results are expressed as mean±S.E.M. of percentages with respect to wild type. B–D and F show representative blots of NR1, NR2A and NR2B respectively, from wild type and BDNF +/− animals, $n=4$–5 animals.

also showed a stronger signal in cells treated with NT-3 (Fig. 4C, D). However, NR2B levels remained unchanged after NT-3 treatment (Fig. 4A, B).

Next, we studied the effect of exogenous NT-3 in the adult striatum. The administration of exogenous NT-3 by grafted cells induced a specific decrease of NR2A protein levels (36+/−17% relative to mock cells grafted striatum, $P<0.05$; Fig. 5). However, protein levels of NR1 and NR2B subunits remained unaltered in striatum grafted with NT-3 secreting cells (Fig. 5).

Striatal projection neurons and parvalbumin-positive interneurons from NT-3 +/− mice are more vulnerable to excitotoxicity

Our results show that the deficit of NT-3 did not modify the total number of striatal neurons in non-lesioned animals. Stereological analysis demonstrated that the density of total neurons (NeuN-positive cells), striatal projection neurons (DARPP-32-positive neurons), striato-pallidal neurons (PPE-positive neurons), striato-nigral neurons (PPTA-positive neurons) and interneurons (both parvalbumin-positive and large neurons) was not different in wild type and NT-3 +/− mice (see contralateral striata in Fig. 6C–F).

To evaluate differences in the lesion size and neuronal density after QUIN injection in the striatum of NT-3 +/− and wild type mice, NeuN immunostaining was performed. The striatum of NT-3 +/− mice showed an approximately twofold greater increase in lesion size than wild type mice did (wt: 2.5+/−0.2 mm^3 and NT-3 +/−: 4.6±0.6 mm^3, $P<0.05$; Fig. 6A, B). In accordance with this, the density of total striatal neurons was lowered more in NT-3 +/− (50.1±7%) than in wild type mice (34.6±5%) by intrastriatal QUIN injection (Fig. 6C). We also analyzed susceptibility of striatal projection neurons by immunohistochemistry for DARPP-32. The density of DARPP-32-positive neurons after QUIN injection was more decreased in NT-3 +/− (41.3±5%) mice than in wild type mice (27.5±3%) (Fig. 6D).

We also examined changes in the susceptibility to excitotoxicity of different subpopulations of GABAergic (GAD-positive) striatal projection neurons by *in situ* hybridization. As expected, intrastriatal QUIN injection induced a greater decrease in GAD-positive neurons density in NT-3 +/− than in wild type mice (wild type: 20.1±3%, NT-3 +/−: 43.8±0.6%, $P<0.05$, Fig. 7A). In addition, both subpopulations of striatal GABAergic projection neurons, striatopallidal (PPE-positive, Fig. 7B) and striatonigral (PPTA-positive, Fig. 7C) neurons, from NT-3 +/− mice were more susceptible to QUIN-induced excitotoxicity than those from wild type mice (wild type, PPE: 16.4±5%; PPTA: 15.5±4%; NT-3 +/−, PPE: 35.1±6%; PPTA: 37.9±7%, $P<0.05$).

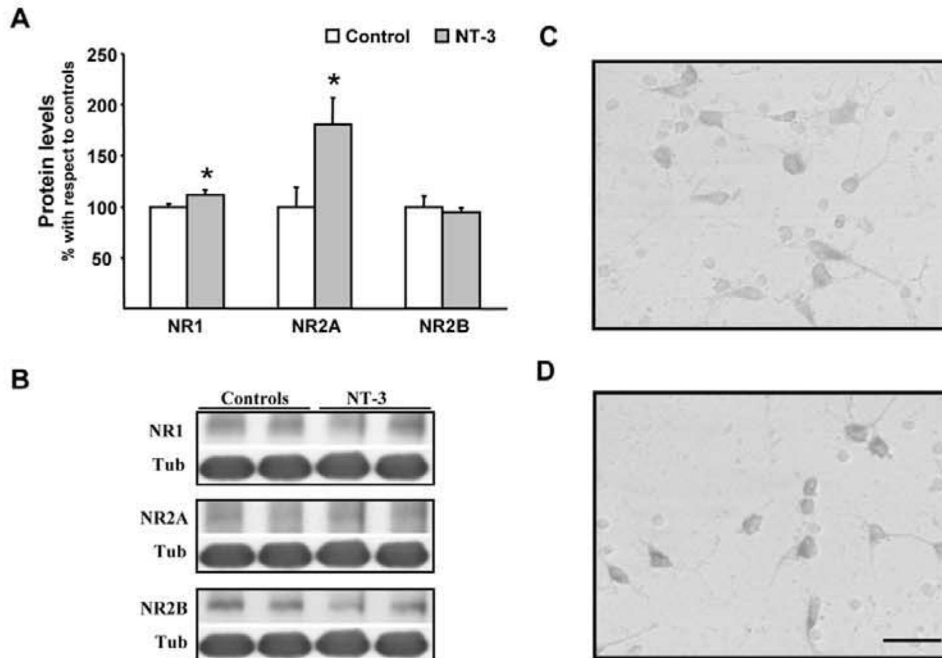


Fig. 4. NT-3 treatment selectively increases NMDAR subunit expression *in vitro*. Striatal neurons were treated at 3 days *in vitro* with NT-3 during 24 h and protein levels of NMDAR subunits were determined by Western blot. (A) Bars show that NT-3 treatment induces an increase of NR1 and NR2A protein levels however, the effect of NT-3 is higher in NR2A than NR1 expression. In contrast, NT-3 does not induce changes in NR2B protein levels. Results are expressed as mean±S.E.M of percentages with respect to non-treated controls. * $P<0.05$ compared with controls, $n=4-5$ in two independent experiments. (B) Representative blots of NR1, NR2A and NR2B from controls and NT-3-treated cultures. The effect of NT-3 was also analyzed by immunocytochemistry for NR2A, showing strong signal in NT-3-treated cells (D) compared with control cells (C). Scale bar=30 μm.

Next, we studied whether the excitotoxic susceptibility of two striatal interneuron subpopulations, parvalbumin-positive (GABAergic) and large (cholinergic) interneurons, was modulated by NT-3. Interestingly, the density of parvalbumin-positive neurons after QUIN-induced lesion was lowered in NT-3 +/− mice than in wild type mice (wild type: 64.7±6%, NT-3 +/− : 47.6±7%, $P<0.05$; Fig. 6E), whereas, the density of large interneurons was equally reduced after excitotoxic lesion (wild type: 72.4±9%, NT-3 +/− : 71.3±4%, Fig. 6F).

DISCUSSION

The present study shows that the deficit of endogenous NT-3, but not BDNF, enhances the vulnerability of the striatum to excitotoxic stimulus. Striatal projection neurons and parvalbumin-positive interneurons from NT-3 +/− mice were more affected by QUIN injection, whereas the sensitivity to excitotoxic damage of large interneurons was not modified. Changes in NMDAR expression may be a possible mechanism that produces the differential susceptibility to excitotoxicity in NT-3 deficient mice. We observed an age-dependent NT-3 effect on NMDAR expression, since NR1 and NR2A were decreased postnatally, while they were upregulated in 12 week old NT-3 +/− mice.

There is strong evidence that BDNF and NT-3 have trophic effects on striatal neurons (Ventimiglia et al., 1995; Mizuno et al., 1994; Gavaldà et al., 2004). However, the distinct spatio-temporal developmental profiles of trkB and trkC suggest that their respective ligands BDNF and NT-3 may have specialized functions in striatal neuronal development (Jung and Bennett, 1996). In line with this view, previous studies described that BDNF and NT-3 oppose one another in regulating the dendritic growth of pyramidal neurons in the cortex (McAllister et al., 1997). These two neurotrophins also have different patterns of expression in the cortex and striatum after intrastriatal injection of different glutamate receptor agonists (Canals et al., 1998, 1999; Checa et al., 2000, 2001). Our present results show that BDNF and NT-3 have different effects in modulating the sensitivity of striatal neurons to excitotoxicity. Intrastriatal QUIN injection produces greater lesion in DARPP-32- and NeuN-positive neurons of NT-3 heterozygote mice than was observed in wild type and BDNF +/− mice. The effect of NT-3 modulating excitotoxicity is specific for striatal enkephalinergic and tachykininergic projection neurons, and also for parvalbumin-positive interneurons. However, the deficit of NT-3 did not modify the sensitivity to excitotoxicity in large cholinergic interneurons. This specificity could be related with the presence of TrkC receptors in each neuronal population. TrkC is predominantly located in projection neurons (Merlio et al., 1992), whereas the

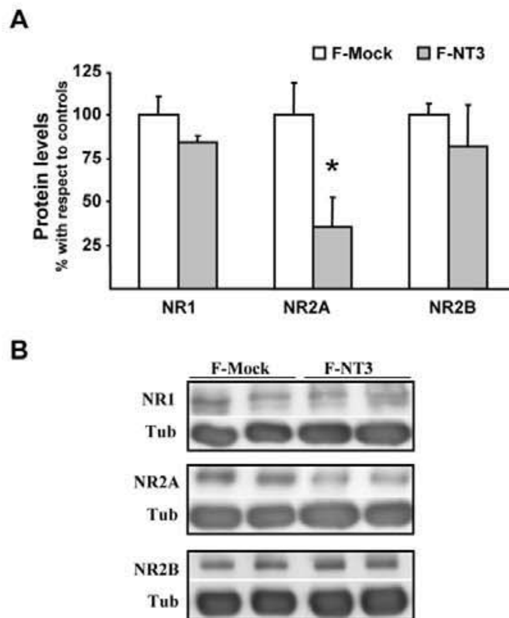


Fig. 5. Exogenous NT-3 selectively modulates NMDAR subunit expression *in vivo*. NT-3-overexpressing (F-NT3) and mock (F-Mock) fibroblasts were grafted into the striatum of adult immunodeficient mice and NMDAR protein levels were measured by Western blot 3 days after transplantation. (A) Bars show that striatal cells respond to exogenous NT-3 selectively decreasing NR2A protein levels. NR1 and NR2B protein levels are not affected by administration of exogenous NT-3. Results are expressed as mean±S.E.M of percentages with respect to controls (F-Mock grafted striatum). * $P<0.05$ compared with controls, $n=5$ animals. (B) Representative blots of NR1, NR2A and NR2B from animals grafted with F-Mock and F-NT3 cells.

neurotrophin receptor most expressed in striatal cholinergic interneurons is trkA (Holtzman et al., 1995).

These results mean that NT-3 is regulating some kind of intrinsic neuroprotective mechanism in some populations of striatal neurons, though the mechanism by which NT-3 is protective is not fully understood. However, the selective regulation of NMDARs can modulate the vulnerability of neuronal populations to excitotoxicity. NR1, NR2A and NR2B are the most widely distributed subunits in the mature forebrain (Wang et al., 2004). The levels of these subunits determine the magnitude of the NMDA response by altering neuronal calcium homeostasis: over-activation induces neuronal death. In fact, the enrichment of NR1, NR2A and NR2B protein levels in cortical cultures with respect to striatal cultures confers enhanced vulnerability to NMDA toxicity (Kovacs et al., 2001). In the present paper, we show that a deficit of NT-3 induces a selective regulation of NMDAR subunits NR1 and NR2A, but not NR2B, in an age-dependent manner. NT-3 +/− mice only showed changes in NR1 and NR2A subunits in early postnatal development and in the adult, but in an opposite way. NR1 and NR2A were decreased at P0 and P3 respectively, whereas these two subunits were increased in 12 weeks old NT-3 +/− mice relative to age matched wild type mice. Previous studies already reported that the vulnerability of striatal neurons to excitotoxic damage can change with age. Thus, the abundance of glutamate receptors on striatal neurons (Magnusson and Cotman, 1993; Villares and Stavale, 2001) and their responsiveness to NMDA (Cepeda et al., 1996) decline with age. NMDAR selective excitotoxins cause smaller striatal lesions in adult than in juvenile rats (McDonald et al., 1988; Figueredo-Cardenas et al., 1997). These effects are selective for each neuronal population, since it has been described that enkephalinergic neurons become less affected by QUIN than substance P-containing striatal neurons as rats age (Sun et al., 2003). Our results suggest that NT-3 participates in the regulation of the different sensitivity to excitotoxicity modulating the levels of NR1 and NR2A. This was confirmed analyzing the effect of exogenous NT-3 by two different approaches. NT-3 treatment increases the expression of NR1 and NR2A, but not NR2B, in striatal cell cultures. However, when NT-3 was administered in adult animals, the levels of NR2A subunit decreased. It is noteworthy that in all experimental conditions analyzed only NR1 and NR2A were modified. These results and other studies support the possibility that NR2A is more involved in glutamate neurotoxicity than NR2B. In fact, the vulnerability of certain hippocampal neurons to glutamate follows the postnatal expression of NR2A subunits (Marks et al., 1996). Moreover, mice with null mutation of NR2A have an attenuation of the volume lesion induced by transient focal ischemia and the same mice with NR2B heterozygosis do not result in further reduction in injury volume (Morikawa et al., 1998). Moreover, it has been described that all human striatal neurons have NMDARs, but different populations have different subunit compositions that may affect function as well as selective vulnerability (Kuppenbender et al., 2000). Here, we show that the high basal levels of NR1 and NR2A subunits in the adult confer on NT-3 +/− mice enhanced vulnerability to excitotoxicity in the striatum. However, the regulation of the expression of NMDAR composition by neurotrophins varies depending on the neuronal populations. Previous studies show that cultured hippocampal and cortical neurons pre-treated with BDNF have higher levels of NR1 and NR2A, while NR2B levels decrease, which is associated with increased calcium response to NMDA (Glazner and Mattson, 2000; Small et al., 1998). However, BDNF induces an acute decrease in both NR2A and NR2C protein levels in cerebellar granule neurons without affecting NR2B or NR1 levels (Brandoli et al., 1998). In the present study, we observed that changes in endogenous BDNF levels do not modify the protein levels of NMDAR subunits that do not alter vulnerability to excitotoxicity in the striatum, although this neurotrophin is a potent protective agent for striatal neurons (Pérez-Navarro et al., 1999, 2000). Therefore, these results suggest that BDNF activates other mechanisms independently of alterations in NMDARs to protect striatal neurons. These mechanisms may involve antioxidant systems (Mattson et al., 1995) or antiapoptotic proteins (Pérez-Navarro et al., 2005). In addition, endogenous NT-3 regulates the expression of NR1 and NR2A

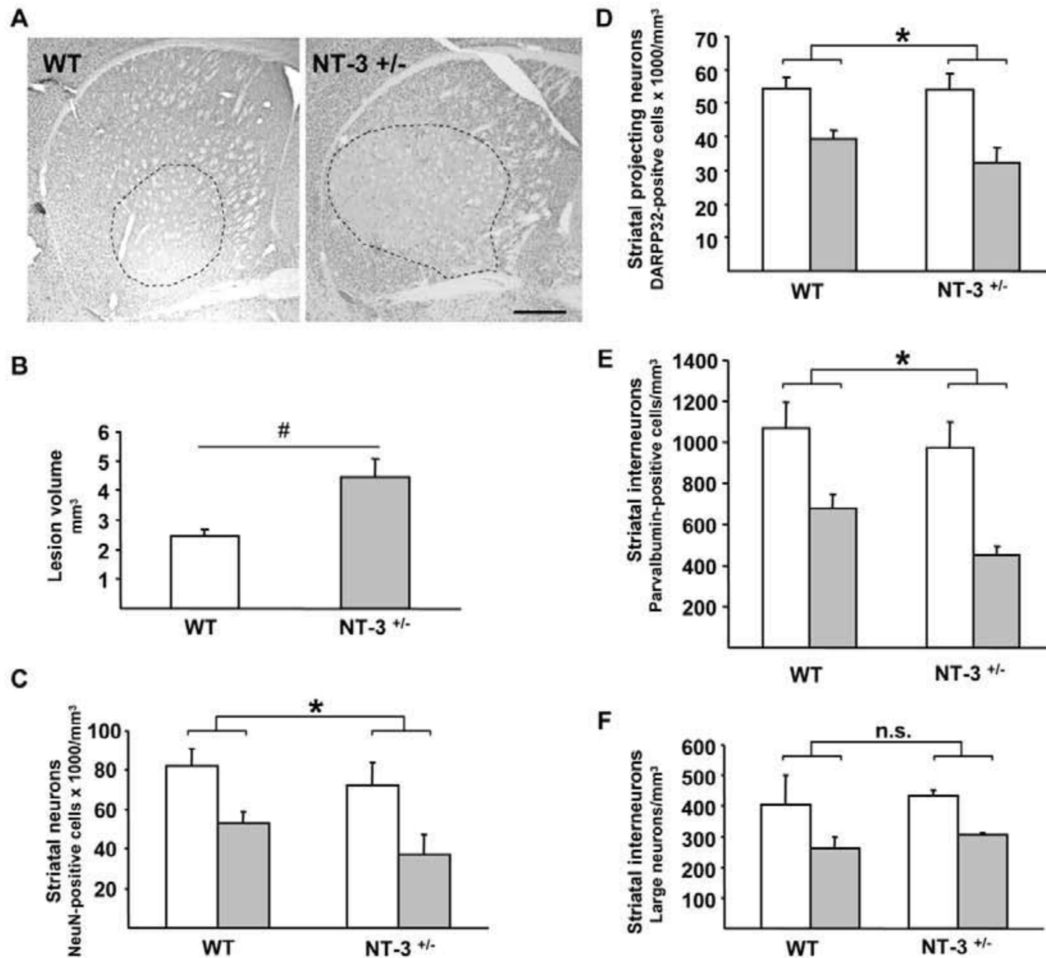


Fig. 6. Viability of striatal projecting neurons and parvalbumin-positive interneurons after QUIN injection is more affected in NT/3 +/− than in wild type mice. QUIN-induced lesion was examined by immunohistochemistry. (A) Representative NeuN-immunostained coronal sections show that the area of striatal lesion (dotted areas) in NT-3 +/− mice is larger than in wild type mice. (B) Bars show that volume of QUIN-induced lesion is larger in NT-3 +/− than in wild type mice; # $P<0.05$ compared with wild type mice. (C) Stereological analysis demonstrates that NT-3 +/− mice show a largest reduction in the density of NeuN-positive cells after intrastriatal QUIN injection. (D) The reduction in the density of striatal projection neurons (DARPP-32-positive neurons) was also higher in NT-3 +/− than in wild type mice. However, interneurons were differentially affected by reduced levels of NT-3. Thus, NT-3 +/− mice show a largest reduction in the density of parvalbumin-positive interneurons than wild type mice after QUIN injection (E), while large interneurons were equally affected in NT-3 +/− and in wild type mice (F). Values are given as mean±S.E.M. of number of neurons/mm^3 in the injected (filled bars) and the contralateral (open bars) striatum. * $P<0.05$ comparing percentage of decrease in cell density of injected with respect to contralateral striatum between wild type and NT-3 +/− mice, $n=4$ animals. Scale bar=500 μm in A.

subunits in the striatum, with different neuroprotective mechanisms than BDNF's. It was recently described that NT-3 treatment does not alter mRNA levels for NR2A and NR2B subunits in neurons derived from murine embryonic stem cells (Lee et al., 2005). Therefore, the regulation of NMDARs could be a cell-specific neuroprotective mechanism activated by trophic factors to modulate susceptibility to excitotoxicity.

Other mechanisms may also participate in the protective effects of NT-3. Some experimental evidence indicates that the protective action of NT-3 against various insults is mediated via stabilization of neuronal calcium homeostasis (Cheng and Mattson, 1994). NT-3 prevents the rise of intraneuronal free calcium levels and promotes neuronal survival in hippocampal cultures exposed to glutamate (Cheng and Mattson, 1994). This stabilization of calcium homeostasis could be mediated through upregulation of calcium-binding proteins, such as calbindin and parvalbumin. Actually, NT-3 increases the number of calbindin-positive neurons in striatal cultures (Nakao et al., 1995; Ventimiglia et al., 1995). Furthermore, NT-3 is a potent neuroprotective agent for calbindin- and parvalbumin-pos-

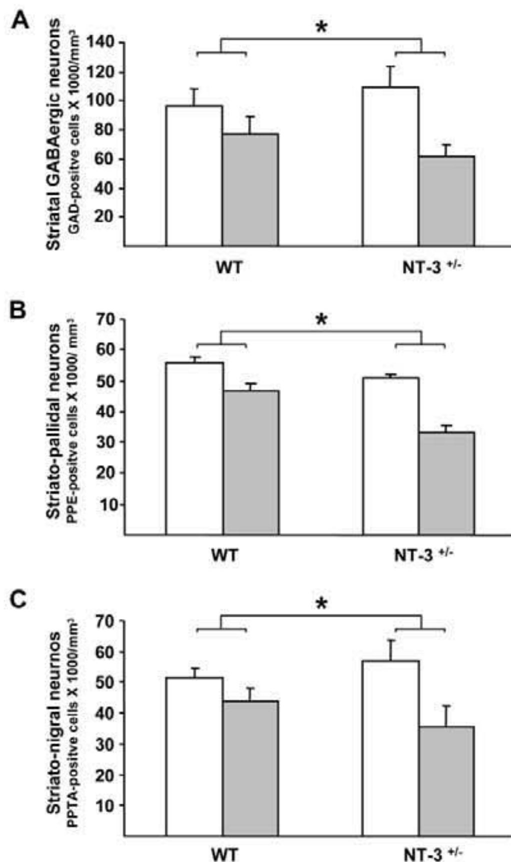


Fig. 7. Low levels of endogenous NT-3 increase the susceptibility of both striatal projection neuron populations to excitotoxic insult. The expression of striatal neuronal markers was analyzed by *in situ* hybridization after NMDAR-mediated toxicity. Intrastriatal QUIN injection induced a higher reduction of GABAergic (GAD-positive cells, A), striatopallidal (PPE-positive cells, B) and striatonigral (PPTA-positive cells, C) neurons in NT-3 +/− than in wild type mice. Values are given as mean±S.E.M. of neurons/mm³ in the injected (filled bars) and the contralateral (open bars) striatum. * $P<0.05$ comparing percentage of decrease in cell density of injected with respect to contralateral striatum between wt and NT-3 +/− mice, $n=4$ animals.

itive cells in the striatum after QUIN or kainate intrastriatal injection (Gratacos et al., 2001). In line with these data, our results also show that NT-3 deficit induces greater vulnerability in striatal projection neurons and parvalbumin-positive interneurons after QUIN treatment.

CONCLUSION

In conclusion, NT-3 is important to establish the definitive maturation of glutamate receptors and to preserve the functional integrity of the corticostriatal pathway. In addition, the selective regulation of NR1 and NR2A subunits participate in the neuroprotective effect of NT-3, but not BDNF, on striatal neurons against excitotoxicity.

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