Neurotensin Enhances Endogenous Extracellular Glutamate Levels in Primary Cultures of Rat Cortical Neurons: Involvement of Neurotensin Receptor in NMDA Induced Excitotoxicity

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Primary cultures of cortical neurons were employed to investigate the modulatory effects of neurotensin on glutamate excitotoxicity and the possible neuroprotective actions of the neurotensin receptor antagonist SR48692. NT(1–13) and its biologically active fragment NT(8–13) at 10 nM (30 min) increased endogenous glutamate levels. The inactive fragment NT(1–7) (10–100 nM; 30 min) was ineffective. SR48692, applied 20 min before NT and maintained in contact with cells during NT exposure as well as a low calcium medium (from the onset of the experiment) prevented the NT(1–13)-induced increase in extracellular glutamate levels. The addition of NMDA (0.01–10 μM; 10 min) to the medium concentration-dependently increased extracellular glutamate levels. When 0.1 nM NT(1–13) was added in combination with 0.01 μM NMDA, in concentrations by themselves ineffective, a significant increase in glutamate levels was observed. SR48692 at 100 nM counteracted the increase in glutamate levels induced by 0.1 nM NT(1–13) plus 0.01 μM NMDA. The inhibitor of the protein kinase C (PKC) calphostin C (0.1 μM; 10 min before NT) prevented the increase in glutamate levels induced by the combined treatments. The morphological analysis indicated that 10 nM NT(1–13) enhanced the glutamate (10 min)-induced apoptosis. The peptide was added 30 min prior to glutamate and maintained in contact with cells during the glutamate exposure. The presence of 100 nM SR48692 (20 min before NT) antagonized this effect of NT(1–13). These findings support the view of a pathophysiological role for NT in the cerebral cortex. Thus, under pathological conditions NT by enhancing glutamate outflow and by amplifying the NMDA-mediated glutamate signaling may be involved in increasing the degeneration of cortical neurons.

Keywords: cortical cell cultures, glutamate levels, neurotensin peptides, NMDA, SR48692.

Introduction

Evidence has been accumulated that glutamate, the major excitatory neurotransmitter in the central nervous system of vertebrates, is an important mediator of neuronal injury. The extracellular accumulation of glutamate and the excessive activation of glutamate receptors, particularly *N*-methyl-D-aspartate (NMDA) receptors (Choi *et al.*, 1988; Choi, 1992; Ikonomidou and Turski, 1995; Doble, 1999; Sattler and Tymianski, 2001), has been postulated to contribute to the neuronal cell death associated with chronic neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's diseases (Arias *et al.*, 1998; Sonsalla *et al.*, 1998; Schiefer *et al.*, 2002) and pathologic events such as hypoxia and ischemia (Johnston *et al.*, 2001). The mechanisms by which the excitotoxicity is exerted are, however, not completely understood, although there is ample evidence that cell death occurs by necrosis (Choi *et al.*, 1987; Sohn *et al.*, 1998) or by apoptosis (Bonfoco *et al.*, 1995; Finiels *et al.*, 1995). Recently it has been demonstrated in cultured cortical neurons that glutamate excitotoxicity, via NMDA receptors, induces apoptosis or necrosis depending on the intensity of the insult. Indeed, mild glutamate insult leads to an apoptotic cell death, while an intense glutamate insult induces predominantly a necrotic process (Cheung *et al.*, 1998). Therefore, endogenous compounds able to modulate the glutamatergic transmission may interfere with glutamate induced cell death.

It is well known that the tridecapeptide neurotensin (NT) plays an important role as a neuromodulator or neurotransmitter in neuronal signaling (see Kitabgi and Nemeroff, 1992). In this context, it has been shown that this peptide significantly enhances glutamatergic signaling both in *in vitro* (Ferraro *et al.*, 2000) and *in vivo* (Ferraro *et al.*, 1995, 1998, 2001) studies. These findings suggest a reinforcing action of NT on several functions exerted by glutamate in the central nervous system, in particular on the glutamate-mediated excitotoxicity. An involvement of NT in modulating glutamate excitotoxicity has recently been demonstrated in primary cultures of mesencephalic dopamine neurons (Antonelli *et al.*, 2002). In view of these findings it may be postulated that NT by enhancing glutamate signaling in several brain regions may be involved in the aetiology or progression of neurodegenerative disorders. Nevertheless, the effects of NT on glutamate transmission in the cerebral cortex, an important cerebral area damaged by pathological events like ischemia, are still undefined. On the basis of data obtained in cerebral cortical slices (Ferraro *et al.*, 2000) and in primary cultures of mesencephalic dopamine neurons (Antonelli *et al.*, 2002), the aim of the present study was to investigate the effect of NT and its receptor antagonist SR48692 on endogenous glutamate efflux in primary cultures of rat cerebral cortex. Since excessive activation of NMDA receptors has been postulated to be an important factor in the induction of glutamate-mediated neuronal damage and on the basis of our previous unpublished data suggesting a role for NMDA receptors in some NT effects, the possible relationship between NT and NMDA receptors was analysed. Furthermore, to verify the involvement of NT in the glutamate-induced apoptotic process, the effects of NT and SR48692 on the chromatin condensation were studied in primary cultures of rat cortical neurons exposed to glutamate.

Materials and Methods

Materials

The culture dishes were purchased from Nunc A/S (Roskilde, Denmark). Fetal calf serum and Eagle's basal medium were obtained from Gibco (Grand Island, NY). Poly-L-lysine, trypsin, soybean trypsin inhibitor, DNase, cytosine arabinoside, gentamycine sulfate, glutamine, L-glutamic acid and Hoechst 33258 were obtained from

Sigma Chemical Co. NT(1–13), NT(8–13) and NT(1–7) from Peninsula Laboratories Europe Ltd (St Helens, Merseyside, UK) were dissolved in Krebs solution just before testing and used only once. SR48692 (2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy-phenyl)pyrazol-3-yl)carboxy-lamino]tri-cyclo)3.3.1.1.^{3,7})-decan-2-carboxylic acid; Sanofi-Synthélabo Recherche, Toulouse, France) was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was <0.001%; when required the DMSO vehicle was added alone or together with NT peptides and no changes in glutamate efflux were observed. Calphostin C was purchased from Calbiochem Novabiochem Intl. (La Jolla, CA). NMDA was obtained from Tocris Cookson (Bristol, UK)

Primary Cultures of Rat Cortical Neurons

According to a previously described method (Alho *et al.*, 1988), primary cultures of cortical neurons have been prepared from 1-day-old Sprague–Dawley rats. After re-suspension in the plating medium, the cells were counted and then plated on poly-L-lysine (5μg/ml)-coated dishes at a density of 2.5×10^6 cells/dish. In the dishes used for Hoechst 33258 nuclear staining, the cells were plated on poly-L-lysine coated glass coverslips at a concentration of 2.5×10^6 cells/coverslip. The plating medium consisted of Eagle's Basal Medium supplemented with 10% inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamycine. Cultures were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cytosine arabinoside (10 μM) was added within 24 h of plating to prevent glial cell proliferation (Alho *et al.*, 1988). The cultures were maintained for 8 days *in vitro* (DIV) before experiments.

Determination of Endogenous Extracellular Glutamate Levels

On the day of the experiment, the cells were rinsed twice by replacing the culture medium with Krebs–Ringer bicarbonate buffer (37°C). Thereafter, five consecutive fractions were collected renewing this solution (400 μl) every 30 min. The first two samples have been used to assess basal glutamate levels (Ferraro *et al.*, 2001). NT(1–13), the biologically active fragment NT(8–13) and the inactive one NT(1–7) were applied at the onset of the third fraction and maintained for 30 min. When required, the NT receptor antagonist SR48692 was added 20 min before NT(1–13) and the inhibitor of the protein kinase C (PKC), calphostin C, was added to the cultures 10 min prior to NT and maintained in contact with the cells during NT exposure. In the set of experiments where NMDA was tested, the compound was added to the cultures 10 min before the collection of the third fraction.

Endogenous Glutamate Assay

Endogenous glutamate levels have been quantified using a high performance liquid chromatografy (HPLC)/fluorimetric detection system, including a precolumn derivatization *o*-phthaldialdehyde reagent and a Chromsep 5 (C18) column. The mobile phase consisted of 0.1 M sodium acetate, 10 % methanol and 2.5% tetrahydrofuran, pH 6.5. The limit of detection for glutamate was 30 fmol/sample.

Neurotoxicity Experiments

Following the removal of the growth medium, the cultures were exposed for 10 min to glutamate 30 μM in Mg^{2+}-free Krebs–Ringer bicarbonate buffer at 37°C in a 5% CO_2/95% air atmosphere. NT and its receptor antagonist SR48692, dissolved in Mg^{2+}-free Krebs–Ringer bicarbonate buffer, were added to the cultures using the following experimental protocol. NT 10 nM was added 30 min prior to glutamate and maintained in contact with cells during the glutamate exposure. The NT antagonist SR48692 100 nM was added 20 min prior to NT and maintained in contact with cells during NT and glutamate exposure. NT and SR48692 alone were also tested. The cultures were then returned to the incubator in their growth medium and the Hoechst 33258 nuclear staining for the determination of chromatin condensation was performed 24 h later. In parallel, these parameters were performed in control cultures.

Nuclear Staining with Hoechst 33258

Twenty-four hours after the brief (10 min) glutamate exposure, cells were fixed in 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS) and then incubated for 20 min at room temperature with Hoechst 33258 (1 μg/ml in PBS). After rinsing with PBS, coverslips were mounted on slides with a solution containing 50% glycerol in 0.044 M citrate and 0.111 M phosphate buffer (pH 5.5) and visualized under a fluorescence microscope. The percentage of cells showing chromatin condensation (apoptotic nuclei) was quantified by counting ≥ 3000 cells under each experimental condition (five randomly selected fields per well, nine or ten wells per condition per experiment and five independent experiments).

Statistics

The effects of the treatments on endogenous extracellular glutamate levels during the third fraction were reported and expressed as percentage changes of basal values, as calculated by the means of the two fractions collected prior to treatment. The statistical analysis was carried out by one- or two-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons.

Results

Neurotensinergic Modulation of Extracellular Glutamate Levels

Effects of the Parent Peptide NT(1–13) and NT(8–13) and NT(1–7) Fragments on Extracellular Glutamate Levels

Extracellular glutamate levels in control cortical cell cultures were 0.107 ± 0.012 μM (as resulted from the mean of total control cells: n = 31) and remained essentially stable over the duration of the experiment (five collected fractions; 150 min). The addition of NT(1–13) (0.1–100 nM; 30 min) to the medium during the third fraction was associated with a bell-shaped concentration-dependent increase in extracellular glutamate levels (Fig. 1*A*). In fact, the peptide significantly increased glutamate levels to a similar extent at 1 and 10 nM concentrations, while at the lower (0.1 nM) and the higher (100 nM) concentrations NT(1–13) was ineffective (Fig. 1*A*).

As shown in Figure 1*B*, the biologically active fragment NT(8–13) induced at 10 nM (30 min) an increase of endogenous glutamate levels, which was similar to that obtained with the parent peptide (10 nM). On the contrary, the biologically inactive fragment NT(1–7) (10 and 100 nM; 30 min) was ineffective.

Effects of SR48692 and a Low Calcium Medium Alone on the Glutamate Response to NT(1–13)

The effect of NT(1–13) (10 nM) in the presence of the selective NT receptor antagonist SR48692 was also tested. As shown in Figure 2*A*, SR48692 (100 nM; 20 min before NT and maintained in contact with cells during NT exposure), which was ineffective by itself, completely prevented the NT(1–13)-induced increase in extracellular glutamate levels.

When NT(1–13) (10 nM; 30 min) was added to cortical cell cultures maintained from the onset of the experiment in a low calcium medium (0.2 mM Ca^{2+}), the increase in glutamate levels induced by NT(1–13) at 10 nM was prevented (Fig. 2*B*).

NT–NMDA Receptor Interactions

Based on the hypothesis that NT via its receptor activation may exert an enhancement of NMDA-receptor signaling in response to glutamate, the effects of NT and its antagonist have also been evaluated on the endogenous glutamate release in cortical cell cultures during the activation of NMDA receptors by NMDA.

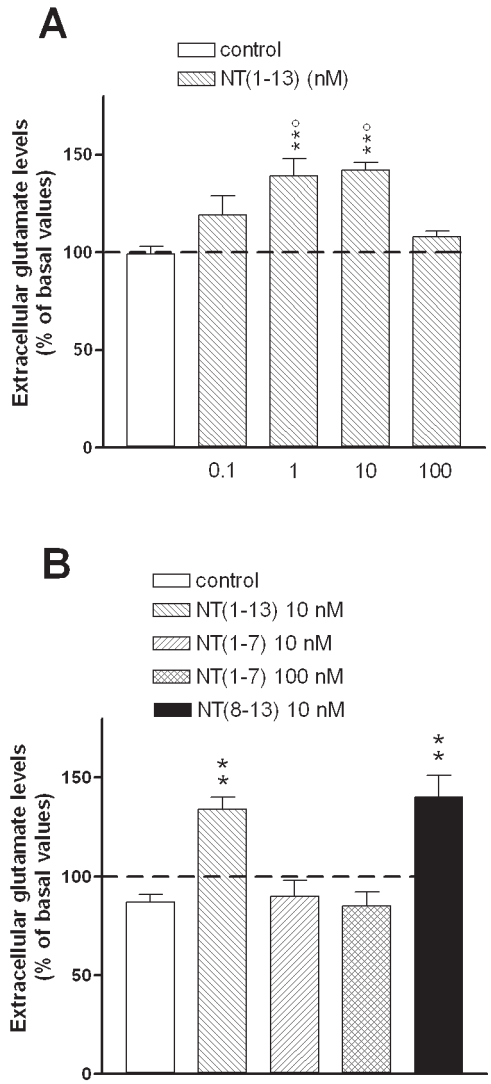

Figure 1. Effects of (*A*) NT(1–13) and its related peptides (*B*) NT(1–7) and NT(8–13) on extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values calculated from the mean of the first two fractions. NT (1–13) and its fragments were applied at the onset of the third fraction and maintained for 30 min. (*A*) Basal glutamate levels were: 0.098 ± 0.012 μM. Each column represents the mean ± SEM of 14 experiments for each group. $^{**}P < 0.01$ significantly different from control; $^{\circ}P < 0.05$ significantly different from 0.1 and 100 nM NT(1–13), according to the one-way ANOVA followed by the Newman–Keuls test for multiple comparisons. (*B*) Basal glutamate levels were: 0.103 ± 0.015 μM. Each column represents the mean ± SEM of 12 experiments for each group. $^{**}P < 0.01$ significantly different from the other groups, according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

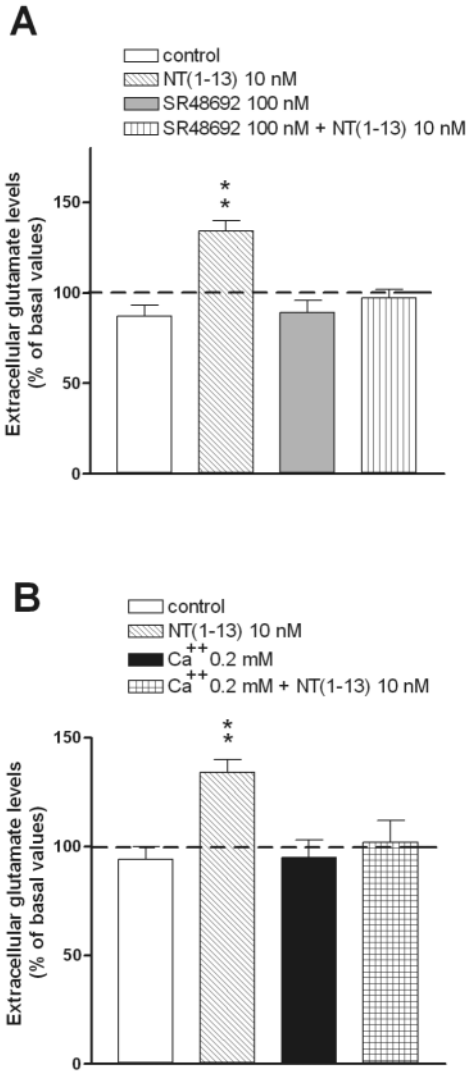

Figure 2. (*A*) Effects of NT(1–13) and SR48692 alone and in combination on extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.123 ± 0.010 μM), calculated from the mean of the first two fractions. NT (1–13) was applied at the onset of the third fraction and maintained for 30 min, while SR48692 was added 20 min before the agonist. Each column represents the mean ± SEM of six or seven experiments for each group. $^{**}P < 0.01$ significantly different from the other groups, according to the two-way ANOVA [$F(1,21) = 17.11$] followed by the Newman–Keuls test for multiple comparisons. (*B*) Effects of a low-calcium medium (Ca^{2+} 0.2 mM) on NT(1–13)-induced increases of extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.094 ± 0.012 μM), calculated from the mean of the first two fractions. NT (1–13) was applied at the onset of the third fraction and maintained for 30 min. The culture medium was replaced with Krebs–Ringer solution containing 0.2 mM Ca^{2+} from the onset of the experiment and it was maintained until the end of the experiment. Each column represents the mean ± SEM of six experiments for each group. $^{**}P < 0.01$ significantly different from the other groups, according to the two-way ANOVA [$F(1,20) = 10.21$] followed by the Newman–Keuls test for multiple comparisons.

Effects of NMDA on Extracellular Glutamate Levels

Extracellular glutamate levels in control cortical cell cultures were 0.111 ± 0.015 μM (n = 24) and remained essentially stable over the duration of the experiment (five collected fractions; 150 min). The addition of NMDA (0.01–10 μM; 10 min) to the medium during the third fraction was associated with a concentration-dependent increase in extracellular glutamate levels (Fig. 3). At the higher 30 μM concentration, NMDA induced a marked increase in glutamate levels (461 ± 21% of basal values; data not shown).

Effects of NT(1–13) on the NMDA Induced Rise of Extracellular Glutamate Levels in the Absence and in the Presence of SR48692

As previously reported (see above) 0.1 nM NT(1–13), when applied alone, did not modify extracellular glutamate levels in

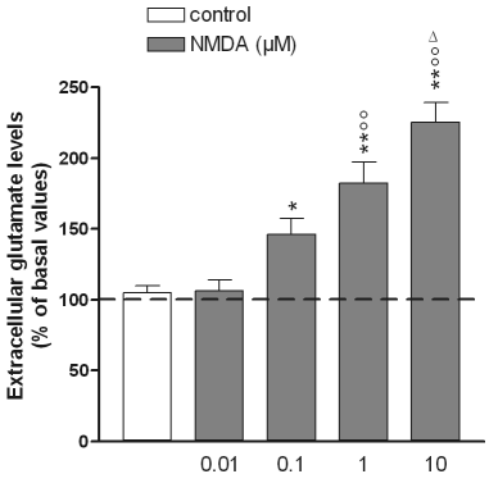

Figure 3. Effects of NMDA on extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.097 ± 0.014 μM), calculated from the mean of the first two fractions. NMDA was added to the Krebs–Ringer solution 10 min before the end of the third fraction. Each column represents the mean ± SEM of 14 experiments for each group. $^{*}P < 0.05$; $^{**}P < 0.01$ significantly different from control and 0.01 μM NMDA; $^{\circ\circ}P < 0.01$ significantly different from 0.1 μM NMDA; $\Delta P < 0.05$ significantly different from 1μM NMDA, according to the one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

cortical cell cultures. Interestingly, when NT(1–13) (0.1 nM; 30 min) was applied in combination with NMDA (0.01 μM; 10 min), by itself ineffective, a significant increase in glutamate levels was observed. Furthermore, NT(1–13) (0.1 nM; 30 min) significantly potentiated the effect of NMDA (0.1 μM; 10 min) on glutamate levels (Fig. 4*A*).

SR48692 at 100 nM, applied 20 min before NT and maintained in contact with cells during NT-NMDA exposure, completely prevented the increase in glutamate levels induced by the treatment with 0.1 nM NT(1–13) plus 0.01 μM NMDA (Fig. 4*B*).

Effects of Calphostin C on the Rise of Extracellular Glutamate Levels after Combined Treatment with NT(1–13) and NMDA

In view of previous data (Skeberdis *et al.*, 2001) demonstrating the existence of a mGluR1-mediated potentiation of NMDA receptors involving the activation of PKC and in order to clarify the interaction between NT and NMDA receptors, the role of the PKC activity in the interactions between NT and NMDA receptors was also analysed. To this end, the inhibitor of PKC, calphostin C (CalC; 0.1 μM) was added to the cultures 10 min prior to NT and maintained in contact with cells during NT and NMDA exposure. As shown in Figure 5, the increase in glutamate levels induced by the combined treatment with 0.1 nM NT(1–13) and 0.01 μM NMDA was fully prevented by CalC, by itself ineffective.

NT and Glutamate-induced Apoptosis

Nuclear Staining of Glutamate-treated Cortical Cell Cultures

The specific DNA stain, Hoechst 33258, was used to assess changes in the chromatin structure following exposure to glutamate. As reported in Figure 6*A*, nuclei in control cortical cells observed by fluorescence microscopy were large and exhibited diffuse staining of the chromatin. In contrast, nuclei

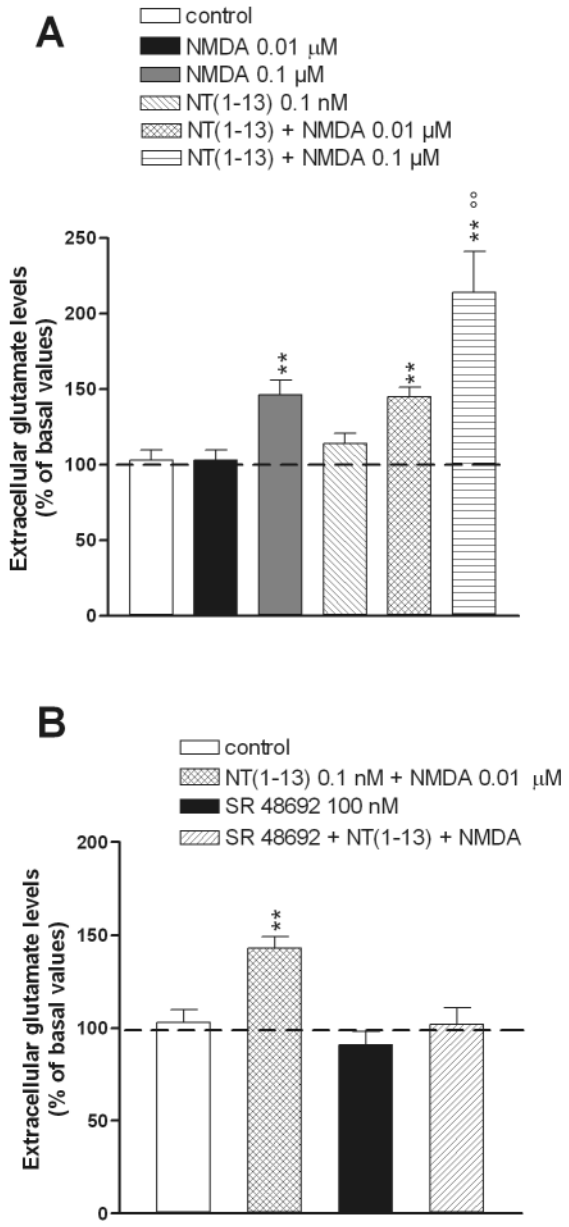

Figure 4. (*A*) Effects of treatments with 0.1 nM NT(1–13) and 0.01 and 0.1μM NMDA, alone and in combination, on extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.099 ± 0.003 μM), calculated from the mean of the first two fractions. NT (1–13) was applied at the onset of the third fraction and maintained for 30 min, while NMDA was added to the Krebs–Ringer solution 10 min before the end of the third fraction. Each column represents the mean ± SEM of 13 or 14 experiments for each group. $^{**}P < 0.01$ significantly different from control, 0.01 μM NMDA as well as 0.1 nM NT(1–13); $^{\circ\circ}P < 0.01$ significantly different from 0.1 μM NMDA and NT(1–13) + 0.01 μM NMDA, according to the two-way ANOVA [$F(2,74) = 6.78$] followed by the Newman–Keuls test for multiple comparisons. (*B*) Effects of 100 nM SR48692 on the 0.1 nM NT(1–13) plus 0.01 μM NMDA-induced increase in extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.093± 0.011 μM), calculated from the mean of the first two fractions. NT (1–13) was applied at the onset of the third fraction and maintained for 30 min, while SR48692 was added 20 min before the agonist. NMDA was added to the Krebs–Ringer solution 10 min before the end of the third fraction. Each column represents the mean ± SEM of seven or eight experiments for each group. $^{**}P < 0.01$ significantly different from the other groups, according to the two-way ANOVA [$F(1,24) = 12.46$] followed by the Newman–Keuls test for multiple comparisons.

of glutamate-treated cortical cells (Fig. 6*B*,*D*) showed a variety of abnormal morphologies, including highly condensed and fragmented chromatin, which is a typical feature of chromatin condensation (i.e. apoptosis). The number of apoptotic cells increased after glutamate exposure (10 min) relative to control cells and NT at 10 nM, added 30 min prior to glutamate and maintained in contact with cells during the glutamate exposure, enhanced the glutamate-induced increase in the number of the apoptotic cells (Figs 6*C* and 7). Finally, the selective NT receptor antagonist SR48692 (100 nM; 20 min before NT and maintained in contact with cells during NT and glutamate exposure) prevented the enhancing effect of NT on the glutamate induced increase (Fig. 7).

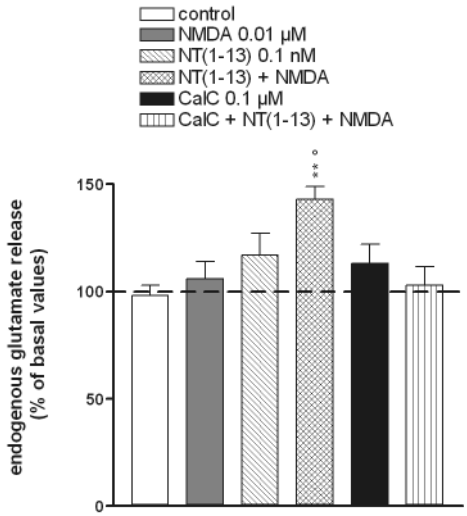

Figure 5. Effects of the PKC inhibitor CalC 0.1 μM on the rise induced by combined treatment with 0.1 nM NT(1–13) and 0.01 μM NMDA on extracellular glutamate levels in primary cultures of rat cerebral cortex neurons. Glutamate levels measured in the third (30 min) fraction are reported and expressed as percentage of the basal values (0.091 ± 0.010 μM), calculated from the mean of the first two fractions. NT(1–13) was applied at the onset of the third fraction and maintained for 30 min, while CalC was added 10 min before NT(1–13). NMDA was added to the Krebs–Ringer solution 10 min before the end of the third fraction. Each column represents the mean ± SEM of six or seven experiments for each group. $^{**}P < 0.01$ significantly different from control, 0.01 μM NMDA and CalC + NT(1–13) + NMDA; $^{\circ}P < 0.05$ significantly different from 0.1 nM NT(1–13), 0.1 μM CalC, according to the two-way ANOVA [$F(2,33) = 7.25$] followed by the Newman–Keuls test for multiple comparisons.

Discussion

The present experiments indicate for the first time that NT may be involved in increasing the degeneration of cortical neurons by interfering with glutamate signals. In fact, NT increases the basal endogenous glutamate release from rat cortical cell cultures. In fact, the parent peptide NT(1–13) and the biologically active fragment NT(8–13) induce an increase in extracellular glutamate levels, displaying a bell-shaped concentration–response curve. These data are consistent with previous findings (Faggin *et al.*, 1990; Ferraro *et al.*, 2000) which demonstrated, by using the same range of concentrations, that the NT-induced increases in K^+-evoked glutamate release and in electrically stimulated [^{3}H]dopamine release show bell-shaped concentration–response curves. The reduced responsiveness of glutamatergic neurones to the highest concentrations of NT(1–13) as observed in the present study could be due to a rapid desensitization of the NT receptors during the treatment period. This phenomenon seems to occur also when the effects of the peptide were assessed on other neuronal systems in various brain regions (Faggin and

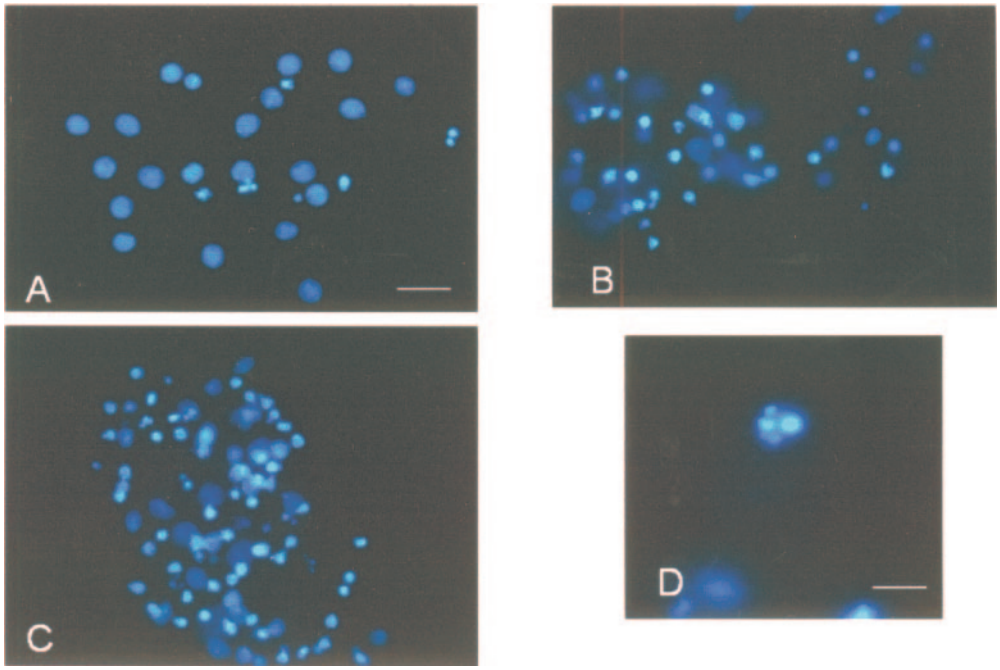

Figure 6. Fluorescence photomicrographs of primary cultures of rat cerebral cortex neurons labelled with the nuclear fluorescent marker Hoechst 33258. The cells were exposed to 30 μM Glu for 10 min. NT (10 nM) was added 30 min prior to Glu and maintained in contact with the cells during the Glu exposure. Twenty-four hours after Glu exposure, the cells were labelled with the nuclear fluorescent marker Hoechst 33258 for the determination of chromatin condensation. Control (*A*); 30 μM Glu (*B*); 10 nM NT + 30 μM Glu (*C*); typical sign of neuronal apoptosis, such as condensation of nuclear chromatin (*D*). Scale bars:, 12 μm (*A–C*); 5 μm (*D*).

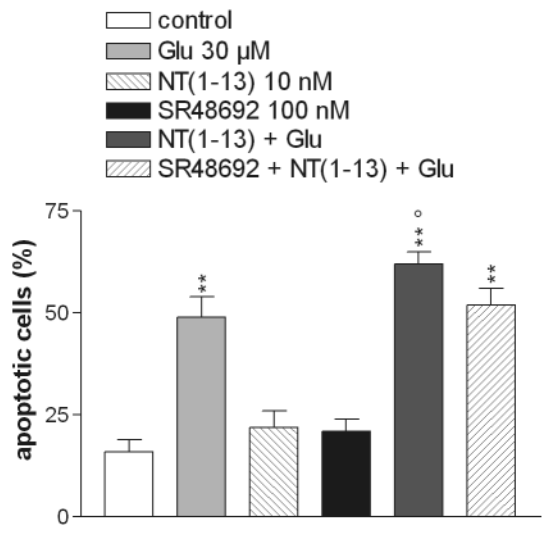

Figure 7. Effects of 10 nM NT(1–13) with or without 100 nM SR48692 on the 30 µM glutamate-induced apoptotic cell death in primary cultures of rat cerebral cortex neurons. The cells were exposed to 30 µM Glu for 10 min. NT (10 nM) was added 30 min prior to Glu and maintained in contact with the cells during the Glu exposure, while 100 nM SR48692 was added 20 min prior to NT and maintained in contact with the cells during the NT and glutamate exposure. Twenty-four hours after Glu exposure, the cells were labelled with nuclear fluorescent marker Hoechst 33258 for the determination of chromatin condensation. Each column represents the mean ± SEM of nine or ten experiments for each group. $^{**}P < 0.01$ significantly different from control, NT (1–13) and SR48692; $^{\circ}P < 0.05$ significantly different from Glu and SR48692 + NT(1–13) + Glu, according to the two-way ANOVA [$F(2,33) = 17.11$] followed by the Newman–Keuls test for multiple comparisons.

Cubeddu, 1990; Deutch and Zahm, 1992; Heaulme *et al.*, 1997). Since the selective non-peptide NT receptor antagonist SR48692 counteracts facilitatory effects of NT(1–13), it seems reasonable that NTR1 receptors, directly and/or indirectly, may control the functional activity of cortical glutamate neurons. The involvement of NT receptors appears to be further supported by the evidence that the NH_2-terminal NT(1–7) fragment, which has been shown to lack affinity for NT receptors in binding studies and to possess negligible activity on dopamine and glutamate release in functional investigations (St Pierre *et al.*, 1981; Faggin and Cubeddu, 1990; Ferraro *et al.*, 2000), failed to affect spontaneous glutamate release under the present experimental conditions. The observation that the replacement of the normal Krebs–Ringer bicarbonate buffer with a low-calcium medium (0.2 mM) reduced the NT (1–13)-induced increase in extracellular glutamate levels, suggests the involvement of a calcium-dependent mechanism in this effect. These findings strengthen the evidence for a functional role of NT receptors in modulating the neuronal activity of glutamatergic nerve cells in the cerebral cortex.

The potential modulation by NT of the glutamatergic receptor signaling, namely the responsiveness of the NMDA receptors, was also evaluated. The possible existence of a reciprocal interaction between NT and NMDA receptor mediated signals could play a relevant physio-pathological role in cortical neuronal function, the NMDA receptors being especially important for the toxic actions of glutamate. The application of NMDA to cortical cell cultures induced a concentration-dependent increase in endogenous glutamate extracellular levels. Interestingly, this effect of NMDA was augmented in the presence of an ineffective concentration of NT(1–13). These results suggest that NT enhances the NMDA-receptor signaling, probably mediated by the NTR1 subtype as shown by the SR48692-induced counteraction of this action. Since (i) phospholipase C–PKC–IP3 pathway is the major signal transduction pathway known to be activated by NTR1 (Vincent *et al.*, 1999) and (ii) Skeberdis *et al.* (2001) recently demonstrated the existence of an mGluR1-mediated potentiation of NMDA receptors involving the activation of PKC, the role of the PKC activity in the interactions between NT and NMDA receptors was also analysed. The effect of the PKC inhibitor calphostin C was therefore tested on the NTR1-mediated potentiation of NMDA-induced increases of extracellular glutamate levels. The observation that calphostin C prevented this effect suggests that the NT-mediated potentiation of NMDA receptor signaling may be mediated by phosphorylation(s) of the NMDA receptors. In fact, the enhancement of NMDA receptor mediated excitatory postsynaptic potentials in striatal slices after metamphetamine sensitization may be related to activation of protein kinase C together with protein kinase A (Moriguchi *et al.*, 2002). It should also be underlined that there is a dependency of extracellular calcium since a low-calcium medium reduced the NT-induced rise of glutamate release. Thus, it could be suggested that the activation of phospholipase C by 0.1 nM NT leads to a rise of intracellular calcium (Gully *et al.*, 1993) which is not sufficient to optimally release glutamate unless sufficient influx of calcium takes place through activated NMDA channels (see Matsuyama *et al.*, 2002). NT can also induce a rise of intracellular calcium levels in astrocytes from the ventral tegmental area, but this NT receptor is only partially sensitive to the blocking activity of SR48692 (Trudeau, 2000).

The above-reported NT-induced increase of endogenous extracellular glutamate levels together with the NT-mediated amplification of NMDA receptor signal in cortical cell cultures could suggest a possible involvement of NT in glutamate-induced excitotoxicity. In fact, the substantial elevation in extracellular glutamate and, consequently, the excessive stimulation of glutamate receptors, especially NMDA receptors, are implicated in the neuronal cell death during degenerative processes. An involvement of apoptosis in glutamate-induced excitotoxicity has been proposed by various authors, particularly Cheung *et al.* (1998), demonstrating that mild concentrations (20–50 µM) of glutamate caused a mixture of injury patterns with apoptosis predominating considerably over necrosis in cultured cortical neurons. In view of these findings, we challenged our cultures with 30 µM glutamate and were able to induce a prevalent apoptotic neuronal death as shown by the presence of nuclear chromatin condensation. Under these conditions the treatment of the cultures with 10 nM NT increased the number of apoptotic nuclei, indicating the involvement of NT in the glutamate-induced apoptosis. Although Hoechst staining does not distinguish between neurons and astrocytes, it is worth noting that in our preparation (i.e. cerebral cortex neurons in cultures) cytosine arabinoside was added within 24 h of plating to prevent glial cell proliferation. Following this procedure (Alho *et al.*, 1988), 92–94 % of total cells from 5 to 14 DIV exhibited NF-immunoreactivity (specific marker for neurons) and only 6–8% exhibited GFAP immunoreactivity (specific marker for astrocytes). Thus, in our opinion, the contribution of non-neuronal cells in the present apoptosis results is negligible. Finally, the finding that SR48692 counteracts the effect of the NT suggests that

NTR1 receptors play a key role in the potentiation of glutamate toxicity. In this context it is worth noting that previous studies demonstrated that NT potentiates the glutamate-induced neurotoxicity also in mesencephalic dopaminergic neurons (Antonelli *et al.*, 2002), an effect fully prevented by SR48692. The evidence that the peptide shows the same profile of action in different brain areas strengthen the view of a relevant pathophysiological role for NT in glutamate-induced neurodegeneration.

In conclusion, it is hypothesized that under pathological conditions NT may be involved in increasing the degeneration of cortical neurons. This effect could be due either to a NT-induced enhancement of glutamate outflow, probably via a rise of intracellular calcium or to an amplification of the NMDA-mediated glutamate signaling. The latter event probably occurs through a post-junctional phosphorylation mechanism, by involving interalia PKC, favouring the depolarization and the entry of calcium. The finding that the NTR1 receptor type may be involved in the amplification of glutamate-induced injury, through indirect NTR1/NMDA receptor interactions via PKC mechanisms and interactions at the intracellular calcium level, encourages experiments aiming at testing the possible use of selective NT receptor antagonists, such as SR48692, in the treatment of acute and chronic neurodegenerative disorders.

Notes

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