

Neurotensin Induces Tyrosine Hydroxylase Gene Activation through Nitric Oxide and Protein Kinase C Signaling Pathways

MUSTAPHA NAJIMI,¹ JEAN-JACQUES ROBERT, JACQUES MALLET, WILLIAM ROSTÈNE, and PATRICIA FORGEZ

Institut National de la Santé et de la Recherche Médicale U339, Hôpital Saint-Antoine, Paris, France (M.N., W.R., P.F.); and Centre National de la Recherche Scientifique Unité Mixte Recherche 9223, Hôpital de la Pitié-Salpêtrière, Paris, France (J.J.R., J.M.)

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ABSTRACT

The regulation of tyrosine hydroxylase (TH) represents an effective means to control the level of catecholamines, because TH is the major limiting enzyme of monoamine biosynthesis. The neuropeptide neurotensin (NT) is a neuromodulator of dopaminergic systems, and a direct interaction between NT and TH expression has been demonstrated *in vivo* and *in vitro*. In the present work, the molecular mechanisms and signaling pathways responsible for TH gene activation have been explored. In N1E-115 cells, NT agonist induced a TH protein level increase, correlating with a significant increase in TH mRNA abundance. This cellular response was the result of TH pro-

motor activation, via c-fos and Jun D binding at the AP-1 responsive element. Using selective protein kinase C and nitric oxide synthase inhibitors, we demonstrate, by quantitative reverse transcription-polymerase chain reaction, gel shift, and protein assays, that TH gene activation by NT agonist requires both protein kinase C stimulation and nitric oxide production. The two pathways exert distinct roles; whereas nitric oxide synthase inhibitors blocked c-fos expression, protein kinase C inhibitors blocked that of Jun D. The requirement for two distinct and concomitant pathways by NT demonstrates a very fine level of control of specificity on TH gene activation.

Tyrosine hydroxylase (TH) is the first and major rate-limiting enzyme of catecholamine biosynthesis in dopaminergic and noradrenergic neurons (Nagatsu et al., 1964). These neurons are involved in the regulation of several important brain functions including motor activity, stress, and emotional responses. A crucial role for this enzyme has been demonstrated in diseases caused by central dopaminergic neuronal damage, such as Parkinson disease, schizophrenia, or prolactinemia (Mallet, 1996; Haavik and Toska, 1998). TH regulation is generated when dopaminergic and noradrenergic systems are altered by a variety of factors induced by pharmacological, environmental, or physiological changes. This regulation affects TH activity through phosphorylation, which modulates both enzyme affinity for cofactor and maximal velocity (Kumer and Vrana, 1996). Additionally, TH protein levels can also be modulated by alterations in TH

gene transcription or TH mRNA stability (Kumer and Vrana, 1996). Heterologous TH regulation has also been described in particular through peptidergic neurotransmitters (Haycock, 1996). Despite the accumulation of evidence detailing the direct role of the second messenger cascades associated to G proteins in TH phosphorylation and TH gene stimulation, the elucidation of the overall intracellular mechanism remains fragmentary for each of the specific molecules leading to TH gene regulation.

Neurotensin (NT) is a 13-amino-acid peptide and is colocalized with TH in dopaminergic neurons of the hypothalamus, and mesencephalon (Hokfelt et al., 1984). Many studies have established diverse regulatory effects exerted by NT on dopaminergic systems (Berod and Rostene, 2002). The vast majority of the NT effects are mediated through a specific high-affinity NT receptor (NT-1 receptor), belonging to the G protein coupled receptor family (Tanaka et al., 1990). NT is very effective in enhancing [³H]dopamine release in primary mesencephalic neurons and rat striatal slices (Brouard et al., 1994). This role was confirmed *in vivo*, by microdialysis experiments, showing that chronically injected SR48692, an NT-1 receptor antagonist,

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¹ Present address: Laboratoire de Pharmacologie Experimentale, Université Catholique de Louvain, 54.10 Avenue Hippocrate 54, 1200 Bruxelles, Belgique.

ABBREVIATIONS: TH, tyrosine hydroxylase [tyrosine 3-monooxygenase: L-tyrosine, tetrahydropteridin:oxygen oxidoreductase (3-hydroxylating)]; NT, neurotensin; NT-1 receptor, high affinity neurotensin receptor; JMV 449, H-LysΨ(CH₂NH)Lys-Pro-Tyr-Ile-Leu-OH; AP, activator protein; PKC, protein kinase C; NO, nitric oxide; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; RT, reverse transcriptase; PCR, polymerase chain reaction; DTT, dithiothreitol; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; GF-109203X, bisindolylmaleimide I; L-NMMA, N-monomethyl-L-arginine, monoacetate salt; L-NAME, N-nitro-L-arginine methyl ester, Hydrochloride; D-NAME, N-nitro-D-arginine, methyl ester, hydrochloride; RLU, relative light units; H89, N-[2-((bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl]; H7, [1-5(isoquinolinesulfonyl)-2-methylpiperazine, 2HCl]; AG 1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline.

decreased dopamine release in the nucleus accumbens and in the dopamine rich regions of the rat central nervous system (Azzi et al., 1998). Interestingly, NT acts equally well on TH expression and TH activity, because NT increases protein and mRNA in neuroblastoma cell lines (Najimi et al., 1998), and TH activity via a protein kinase C pathway in cultured tuberoinfundibular dopaminergic neurons (Berry and Gudelsky, 1992). Together, these results suggest that NT plays a significant role in the establishment of the dopaminergic tone by affecting TH expression and TH activity.

In the present study, we investigate the molecular mechanisms responsible for the modulation of TH gene expression by NT in the murine neuroblastoma cell line N1E-115. This adrenergic cell line contains large amounts of TH, expresses high-affinity NT binding sites, and the intracellular signaling pathways activated by NT are well described (Gilbert et al., 1988; Bozou et al., 1989; Slusher et al., 1994). We demonstrate that TH gene expression is induced by the NT agonist JMV 449 (Doulut et al., 1992). This effect is mediated by the heterodimer complex formed by c-fos and Jun D on the AP-1 responsive element located in the TH proximal promoter. Furthermore, TH transcriptional activation by NT agonist occurs only if the two complementary signaling pathways, PKC and NO synthesis, are activated.

Materials and Methods

Cell Culture. N1E-115 mouse neuroblastoma cells, provided by Sanofi Recherche (Montpellier, France), were grown in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1; Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS), and 2 mM glutamine. At confluence, cells were routinely dispersed in trypsin-EDTA and subcultured at a 1:10 dilution. After 24-h subculture, cell differentiation was induced for 48 to 72 h in the same media containing 1.5% FCS and 1% DMSO. Differentiated cells were treated with 1 μ M JMV 449, a potent and stable pseudopeptide NT agonist (Doulut et al., 1992). All cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂.

Western Blotting. 2 \times 10⁵ cells were seeded in six-well culture dishes. After differentiation cells were washed twice with 10 mM phosphate-buffered saline, pH 7.4, and harvested by scraping in 200 μ l of 1% deoxycholic acid, pH 11.3. The extracts were boiled for 5 min and centrifuged. Aliquots of the supernatant were stored at -20°C until used. Cell extracts (6 μ g for TH and 15 μ g for c-fos, c-jun, and Jun D) were analyzed by 10% SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted to nitrocellulose membranes with a minitransblot electrophoretic transfer cell (Bio-Rad, Hercules, CA). After electroblotting, the nitrocellulose membranes were stained with 0.2% of ponceau S (Sigma-Aldrich Chimie, St. Quentin Fallavier, France) in 0.3% trichloroacetic acid. The quality of the electrophoresis and the reliability of sample loading were determined by scanning densitometry quantification of a specific protein band, using the software program RAG (Biocom, Les Ulis, France). If loaded samples were equivalent in all wells, the nitrocellulose membranes were treated with 5% dry milk in Tris-buffered saline (50 mM Tris, pH 8.1, 150 mM NaCl) buffer containing 0.05% Tween 20 for 1 h with gentle shaking at room temperature. TH and c-jun immunoreactive proteins were detected using monoclonal antibodies (1:1000 and 1:500, respectively) (Roche Applied Science, Meylan, France; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity of c-fos and Jun D proteins was detected using specific rabbit polyclonal antibodies (1:500) (Santa Cruz Biotechnology). The antigen-antibody complex was visualized with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Sigma-Aldrich Chimie) (1:500) and goat anti-rabbit immunoglobulins (Sigma-Aldrich Chimie) (1:3000)

antibodies and revealed with the enhanced chemiluminescence system (Amersham Biosciences, Saclay, France). Relative amounts of proteins were quantified by scanning densitometry using the software program RAG (Biocom).

RNA Extraction and TH mRNA Quantitative RT-PCR. Total RNA was extracted from cells by the acidic phenol/chloroform guanidine thiocyanate method as modified by Najimi et al. (1998). An additional ethanol precipitation was performed in buffer containing 150 mM NaCl, 15 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The RNA pellet was resuspended in 50 μ l of sterile diethyl pyrocarbonate-treated water. Aliquots were prepared and stored at -80°C. Total RNA recovery was measured by spectrophotometric absorbance at 260 nm.

Internal Control (cRNA) Preparation. TH cDNA was kindly supplied by Dr. N Faucon-Biguet (Paris, France). The plasmid pTH was constructed by inserting the coding region of rat TH cDNA (+172 to +1770) into the *Pst*I site of the vector pT7/T3 α 18 (Invitrogen). An oligonucleotide containing a poly(dA)₄₅ was inserted between the *Sal*I and *Bam*HI sites. The pTH Δ 70 plasmid was obtained by deleting 70 nucleotides between the *Eco*RI and *Sac*I sites. The internal control used in this study, cRNA Δ 70, was prepared by *in vitro* transcription of the linearized plasmid pTH Δ 70 at the *Sal*I site with T7 RNA polymerase (Invitrogen) and then purified on oligo(dT) columns (Sigma-Aldrich Chimie). After elution, the cRNA Δ 70 was ethanol precipitated and diluted to 1 \times 10⁷ molecules/ μ l in diethyl pyrocarbonate-treated water containing 0.5 U/ml of RNasin (Promega, Charbonnières, France), and stored at -80°C.

RT-PCR. The quantitative RT-PCR was carried out under the conditions described by Souazé et al. (Souazé et al., 1996). The primer RT-TH (5'-GTCCAGGATGAGCTG-3') located at position 1447 to 1461 was used for reverse transcription. One microgram of total N1E-115 RNA and various amounts of cRNA Δ 70 were reverse transcribed into cDNA in a 30- μ l reaction mixture containing 50 pmol of RT-TH primer, 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂, 10 mM DTT, 1 mM dNTP, 1 U/ml of Moloney murine leukemia virus reverse transcriptase (Invitrogen), and 1 U/ml of RNasin, for 1 h at 37°C. PCR was performed by using primers sense S-TH (5'-CATGCCTCCTCACCTATGCA-3') and antisense AS-TH (5'-CGGTTCTGTTCGAGTCCT-3') at positions 931 to 949 and 1321 to 1339, respectively. The size of the PCR product was 409 base pairs for the endogenous molecule. The PCR amplification was performed on 1:5 (v/v) of the RT reaction in a mixture containing 16 mM Tris-HCl, pH 8.3, 40 mM KCl, 1.5 mM MgCl₂, 0.2 mM concentrations of each dNTP, 25 pmol of each primer (S-TH and AS-TH), 1 U of *Taq* DNA polymerase (Applied Biosystems, Les Ulis, France) and 10⁶ cpm of the AS-TH in a final volume of 50 μ l. The amplification profile was divided in 24 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. The PCR cycles were preceded by denaturation for 5 min and immediately followed by a final extension at 72°C for 10 min. The RNA quantification was performed in two steps. In the first step, various dilutions of cRNA Δ 70 and 1 μ g of total N1E-115 RNA were reverse transcribed and a PCR was performed on each RT reaction. This experiment gives an estimation of the number of TH mRNA molecules contained in the sample. A more precise quantification was performed in the second step (Souazé et al., 1996). Total N1E-115 RNA was mixed with an exact number of cRNA Δ 70 molecules, which was previously estimated from the titration assay. This mixture was reverse-transcribed and six tubes of a three-fold dilution of this reaction were amplified by PCR under the same conditions as described above. Using these two experimental steps in conjunction with the conditions described by Souazé et al., we were able to estimate differences between samples less than 2-fold with an accuracy of 90% (Souazé et al., 1996).

PCR Product Analysis. Each PCR product (20 μ l) was loaded on 5% polyacrylamide gel in buffer containing 90 mM Tris-borate and 2 mM EDTA and electrophoresed at 150 V for 3 h. After migration, the bands were stained in ethidium bromide. The bands were cut out

from the gel and counted in a β -scintillation counter (Model LS 6000sc; Beckman Coulter, Fullerton, CA). The amount of radioactivity (cpm) recovered from the excised gel bands was plotted against the number of known cRNA Δ 70 control molecules or the quantity of total RNA. Linear regressions of both curves were calculated and the absolute number of target molecules (number of TH mRNA molecules) was estimated by extrapolating the value of 1 μ g of total RNA to the internal control. Results are expressed as number of target molecules per microgram of total RNA. The absence of contaminating DNA in the RNA preparation was tested by performing a PCR under standard conditions.

Transient Cell Transfections and Luciferase Assay. Cells were plated to yield 10^5 cells/well in 24-well plates. Reporter plasmids pTH-5300-Luc, pTH-250-Luc, and pTH-200-Luc, which included 5300, 250, and 200 base pairs, respectively, of the upstream sequence of the rat TH promoter fused to luciferase cDNA, were transfected into N1E-115 cells. The transfection was performed by the calcium phosphate coprecipitation method for 16 h at 37°C (Sambrook et al., 1989). Positive (pCMV-LUC) and negative (pTH-0) controls (a promoter-less plasmid) were also transfected. After transfection, the medium was removed, and fresh medium was added for 24-h prior exposure to 1 μ M JMV 449 for 5 h. Transfected cells were harvested with lysis buffer (25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, and 1% Triton X-100). After 30 min of incubation on ice, cells were centrifuged at 12,000g for 5 min, 10 μ l of the supernatant was added to 365 μ l of measure buffer (25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT, and 5 mM ATP) and 200 μ l of 1 mM luciferin (Sigma-Aldrich Chimie) were added to the tube. The peak luminescence was measured after 30 s. All experiments were repeated 3 to 5 times (triplicates per experiments).

Gel Shift Assay. N1E-115 nuclear protein extracts were isolated as reported previously (Therrien and Drouin, 1993). A synthetic 22-mer oligonucleotide containing the TH rat native AP-1 (−210 to −189 bp) (5′-GAGGGTGATTGAGGAGGCAGGTG-3′) was end-labeled with T₄ polynucleotide kinase and [γ -³²P]ATP (3000 Ci/mmol; Amersham Bioscience, Saclay, France). The reactions were performed in 20 μ l containing 25 mM HEPES, pH 7.2, 150 mM KCl, 5 mM DTT, 10% glycerol, 1 μ g of poly(dI-dC), 10 μ g of fresh nuclear extract, and 2×10^5 cpm of [³²P]AP-1 probe for 20 min at RT. For competition experiments, 100-fold excess of unlabeled competitors were preincubated with the nuclear extracts 5 min before the probe was added to the sample. The sequences of the primers were: mutated AP-1 (5′-GAGGGTGAGCCAGGAGGCAGGTG-3′), Sp-1 (5′-ATCGATCGGGGCGGGGCGAGC-3′), ERE (5′-GATCCCGCGACGATCGCCAGGACCTGCGCGCTGCATATGG3′), and Tcf (5′-CCCTTTGATCTTACC-3′). Antibody competition assays were performed by incubating 10 μ g of N1E-115 cell nuclear protein extracts with 2 μ l of specific rabbit antibodies against c-fos, c-jun, Jun D, or Tcf-4 (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C. The antibody-protein mixture was then incubated with radiolabeled AP-1 probe and processed for the gel shift assay as described above. Control and treated samples were operated in the same experiment. DNA-protein complexes were resolved on 5% non-denaturing polyacrylamide gels at 150 V in 0.25 \times Tris-borate/EDTA buffer (see *PCR Product Analysis*) for 90 min at 22°C. The gels were dried and autoradiographed with intensifying screens.

Statistics. Statistical analyses were performed using the Student's *t* test. Data are expressed as the mean \pm S.E.M.

Results

The NT Agonist JMV 449 Increases the Level of TH Protein and mRNA. N1E-115 neuroblastoma cells are widely studied for a number of adrenergic neuronal properties, including catecholamine synthesis, electrically excitable membranes, and physiological neuron characteristics (Braun-

tigam et al., 1982). This cell line was also proposed as model to study TH expression and TH activity regulation (Richelson, 1973). It was established previously that N1E-115 cells differentiated with 1.5% serum and 1% DMSO for 48 h express functional NT-1 receptors (Cusack et al., 1991). In our experiments, differentiated N1E-115 cells were triggered by NT agonist, JMV 449, a pseudopeptide analog of NT (8–13) corresponding to the active portion of the peptide (Doulut et al., 1992). Western blot analysis using TH monoclonal antibody showed a single band, corresponding to the expected TH molecular mass of 60 kDa, which increased and reached a plateau at 5 h with 1 μ M JMV 449 treatment (Fig. 1, top). In a control experiment, nondifferentiated cells were exposed to 1 μ M NT agonist for 5 h. As expected, the TH protein level was not altered because NT-1 receptor was not expressed at the cell surface (Fig. 1, bottom). Using scanning densitometry, TH increase was quantified after 5 h of 1 μ M JMV 449 treatment. Results from eight independent experiments showed a $191 \pm 40\%$ ($p < 0.001$ relative to control) increase of TH immuno-reactivity intensity. The dose response experiment showed an increase in TH protein level at 1 pM and 1 nM, compared with control (Fig. 1, top), with maximum effect reached at 10 nM. Note that the K_d for [¹²⁵I]-NT in N1E-115 cells is 66.3 ± 26.2 pM (Najimi et al., 1998); therefore, at 10 nM JMV 449, the membrane binding capacity is saturated. We have previously demonstrated that N1E-115 cells are rapidly desensitized after JMV 449 treatment; consequently, the increase in TH protein levels was transient and returns to the control level after prolonged NT agonist treatment (Najimi et al., 1998). In N1E-115, the rise in TH protein level was correlated with a $93 \pm 7\%$ ($p < 0.001$ relative to control) increase in TH mRNA levels in cells treated with 1 μ M JMV 449 for 1 h.

Increase of TH Level by NT Agonist is the Result of TH Gene Activation. To elucidate the regulatory mechanism of NT agonist stimulation on TH gene expression, we measured the activity of different lengths of TH promoter fused to the reporter gene luciferase. N1E-115 cells were transfected with plasmids containing TH promoter, then incubated for 5 h with 1 μ M JMV 449. As shown in Fig 2A, luciferase activity increased two-fold with plasmids contain-

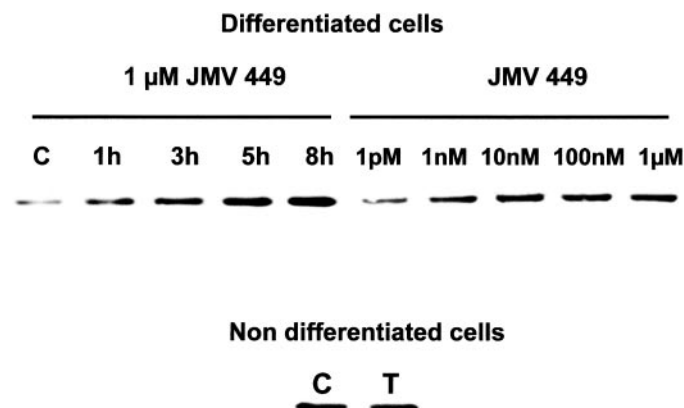


Fig. 1. Effect of JMV 449 on TH protein levels in N1E-115 cells. Top, time course and dose response on N1E-115 cells differentiated for 48 h with 1% DMSO and 1.5% FCS and treated with 1 μ M JMV 449 for 1, 3, 5, or 8 h or with 1 pM, 1 nM, 10 nM, 100 nM, and 1 μ M for 5 h. Bottom, TH western blot from nondifferentiated N1E-115 cells. C, control; T, treated with 1 μ M JMV 449 for 5 h.

ing 5300 and 250 base pairs of the TH promoter, respectively. No changes in luciferase activity were observed when cells were transfected with a plasmid containing only the 200 proximal base pairs of TH promoter. The basal expression level of the pTH-200-Luc was 10 fold higher than the negative control, indicating that the lack of regulation was not caused by the weakness of expression of the plasmid. The comparison between these results suggests that the major responsive genomic element(s) required for the activation of TH gene by NT agonist treatment are located between nucleotides -200 and -250 of the TH gene. In this region, two consensus elements have been reported, AP-1 and AP-2, in positions -199 to -205 and -214 to -220, respectively. We studied the role of each element by gel shift assays, using TH native AP-2 or AP-1 responsive elements. When the TH native AP-2 element was employed, a retained band was observed with the same intensity using nuclear extracts from control cells or cells treated with 1 μ M JMV 449 for 1 h,

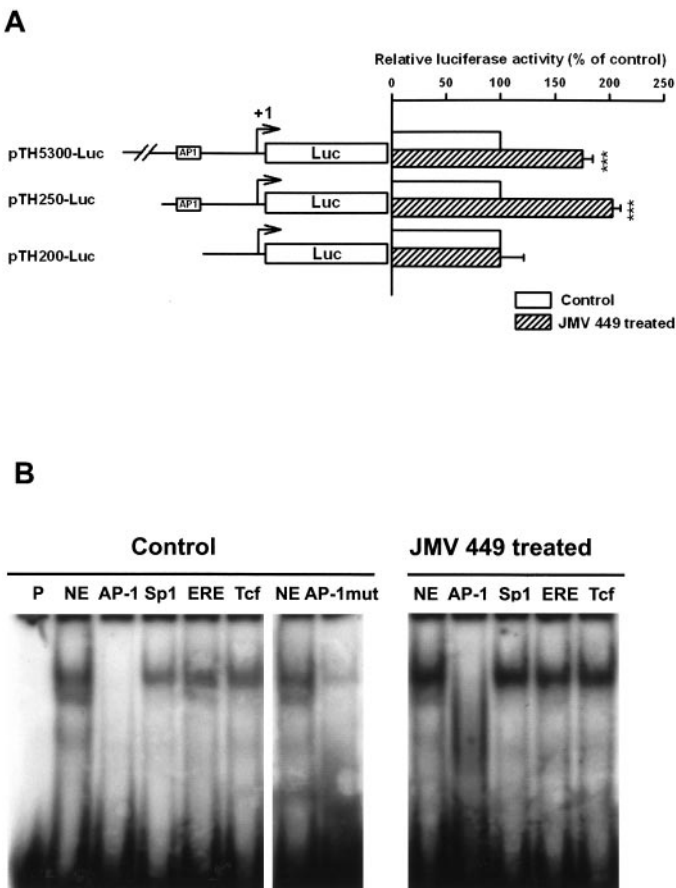


Fig. 2. JMV 449 increases TH gene transcription by binding of nuclear proteins to the native AP-1 site of the TH gene. **A**, luciferase assays were performed on lysates from differentiated N1E-115 cells transfected for 16 h with a nested deletion of the TH proximal promoter (□) and exposed to 1 μ M JMV 449 for 5 h (▨). The relative luciferase units (RLU) values of the control condition were arbitrarily set at 100%. The variation caused by the treatment was calculated as the percentage of control for each plasmid constructions. Values are the mean \pm S.E.M. of four separate experiments. ***, $P < 0.001$ relative to control. **B**, gel mobility shift experiments using nuclear extracts from control and 1 h JMV 449 treated N1E-115 cells. Nuclear extracts (NE) of N1E-115 cells cultured with or without 1 μ M JMV 449 revealed a binding activity to the 5' end labeled double-stranded TH native AP-1 (-210 to -189 bp), which can be completely displaced by a 100-fold excess of equivalent cold probe (AP-1) and partially with mutated AP-1 probe (AP-1mut), but not with 100-fold excess of irrelevant probes (Sp1), (ERE), and (Tcf). P, probe.

suggesting that this element was not involved in TH transcriptional regulation by NT agonist (data not shown). We confirmed the role of the AP-1 *cis*-acting element by analyzing, on gel retardation and competition assays, the interaction between nuclear extract proteins, and an oligonucleotide corresponding to the native sequence (-193 to -216 bp) of TH promoter. As demonstrated in Fig. 2B, the intensity of the shifted band increased when the probe was incubated with nuclear proteins extracted from cells treated with JMV 449 for 1 h. In control and treated cells, the complex could be displaced with an excess of unlabeled TH native AP-1 oligonucleotide, but not with Sp1, ERE, and Tcf consensus elements (Fig. 2B). We confirmed the specificity of the retained

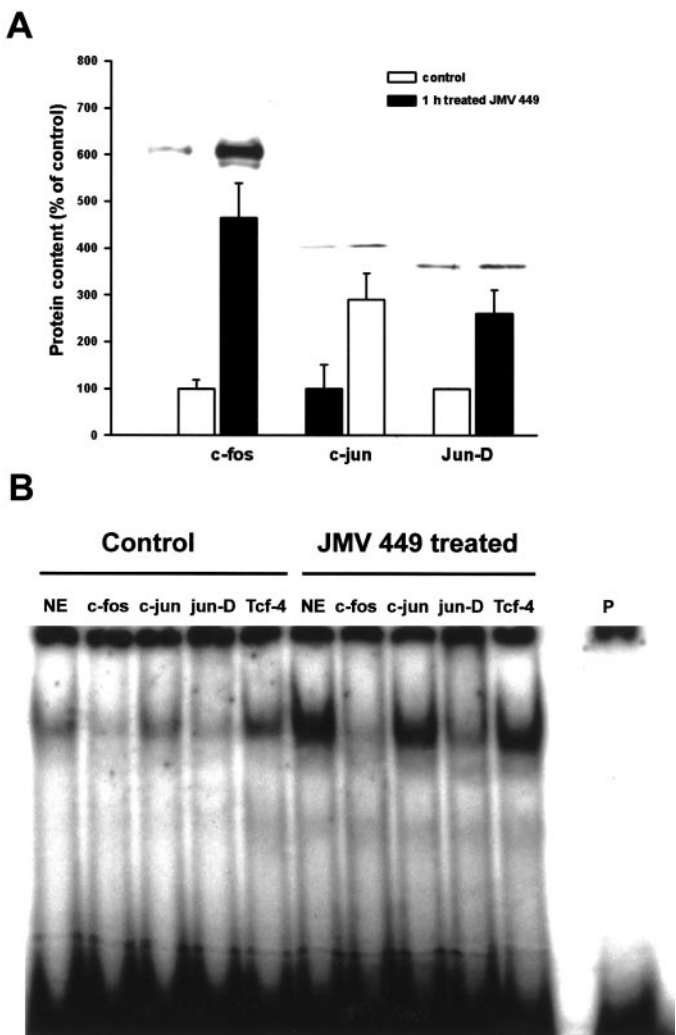


Fig. 3. Among the oncogenes c-fos, c-jun, and Jun D stimulated by JMV 449, only c-fos and Jun D bind to the native AP-1 element. **A**, differentiated N1E-115 cells were treated for 1 h with 1 μ M JMV 449. Cell extracts were analyzed by Western blot, with antibodies directed against c-fos, c-jun, or Jun D, as described under *Materials and Methods*. The relative amounts of proteins were quantified by scanning densitometry using the software program RAG (Biocom). Values are the mean \pm S.E.M. of three to four separate experiments. Shown above is an example of the corresponding blots. **B**, an example of the gel shift mobility experiments using nuclear extracts from control or N1E-115 cells treated with 1 μ M JMV 449 for 1 h. The binding of the protein nuclear complex (NE) can be prevented by preincubating the nuclear extract with antibodies directed against c-fos and Jun D but not with c-jun or Tcf-4 antibodies. This gel autoradiogram is representative of three independent experiments. P, probe.

band by using a mutated AP-1 oligonucleotide (T→G and T→C in positions -202 and -201, respectively). In nuclear extract from control cells, the retained band is partially displaced by 100-fold excess of the mutated AP-1 oligonucleotide (Fig. 2B). The increase in the intensity of the shifted band was evaluated by scanning densitometry using the software program RAG, and a significant increase of $203 \pm 14\%$ ($p < 0.01$ relative to control) was detected over four separate experiments. We conclude that the transcriptional activity of TH gene is the result of an increase in the binding activity of nuclear proteins at the AP-1 site.

AP-1 Site, c-fos, and Jun D Are cis- and trans-Acting Elements Involved in the Activation of TH Gene by NT Agonist. The transcription factors c-fos, c-jun, and Jun D are known to be *trans*-acting factors of the AP-1 responsive element (Sassone-Corsi et al., 1988; Hirai et al., 1989). Therefore, these three factors are potential candidates to act as effectors in the modulation of the TH promoter by NT agonist. We first verified that JMV 449 regulates the protein expression level of c-fos, Jun D, and c-jun. As revealed by Western blot analysis, JMV 449 increased c-fos, c-jun, and Jun D protein levels compared with control conditions (Fig. 3A). The stimulation occurred with treatments varying from 15 min to 120 min (data not shown) and the highest levels were detected after 1-h treatment (Fig. 3A). We identified the factors binding to the native AP-1 element by using c-fos, c-jun, and Jun D antibodies. As shown in figure 3B, the complex of nuclear extracts from control or treated cells and labeled probe was strongly diminished by preincubating the nuclear extracts with 2 μ l of c-fos and Jun D antibodies. In contrast, the antibodies directed against c-jun or Tcf-4 did not change the intensity of the shifted band. From these

results, we conclude that NT activates c-fos and Jun D, which act as *trans*-acting factors on the native TH AP-1 element.

TH Gene Activation by NT Agonist Requires Two Complementary Signaling Pathways: PKC Activation and NO Synthesis. It was previously reported that in N1E-115 cells, NT-1 receptor mediates phospholipase C activation and, subsequently, inositol phosphate hydrolysis (Amar et al., 1987) and NO and cGMP accumulation (Gilbert et al., 1988). To determine the second messenger pathways responsible for the TH gene transcription alteration, we analyzed the effect of various selective pharmacological inhibitors of intracellular signaling cascades. Because it is well known that the TH gene is sensitive to PKC activation (Vyas et al., 1990), we first studied the effect of PKC inhibitors on luciferase activity of the pTH-250-Luc induced by JMV 449. As shown in Fig. 4A, incubation of N1E-115 cells with 10 μ M H7 and 1 μ M staurosporine, two nonselective protein kinase inhibitors, or with 10 μ M D-sphingosine, 1 μ M Gö 6976, and 1 μ M GF 109203X, selective PKC inhibitors, completely block the activation of pTH-250-Luc by NT agonist. Inhibitors of NO synthase were also tested, because it was previously shown that NT-induced cGMP accumulation requires the production of NO (Slusher et al., 1994). Similarly, an inhibitor of the three isoforms of NO synthase, L-NMMA (10 μ M), as well as the specific inhibitor of endothelial nitric oxide synthase, L-NAME (100 nM) completely abolished the NT-induced TH promoter activity. In contrast, the inactive enantiomer, D-NAME (10 μ M) did not affect the TH promoter activity, attesting to the specificity of L-NAME. As control experiments, we tested a PKA inhibitor, H89, and a specific epidermal growth factor receptor inhibitor, tyrphostin AG 1478. Alone, these two inhibitors reduce the basal expression

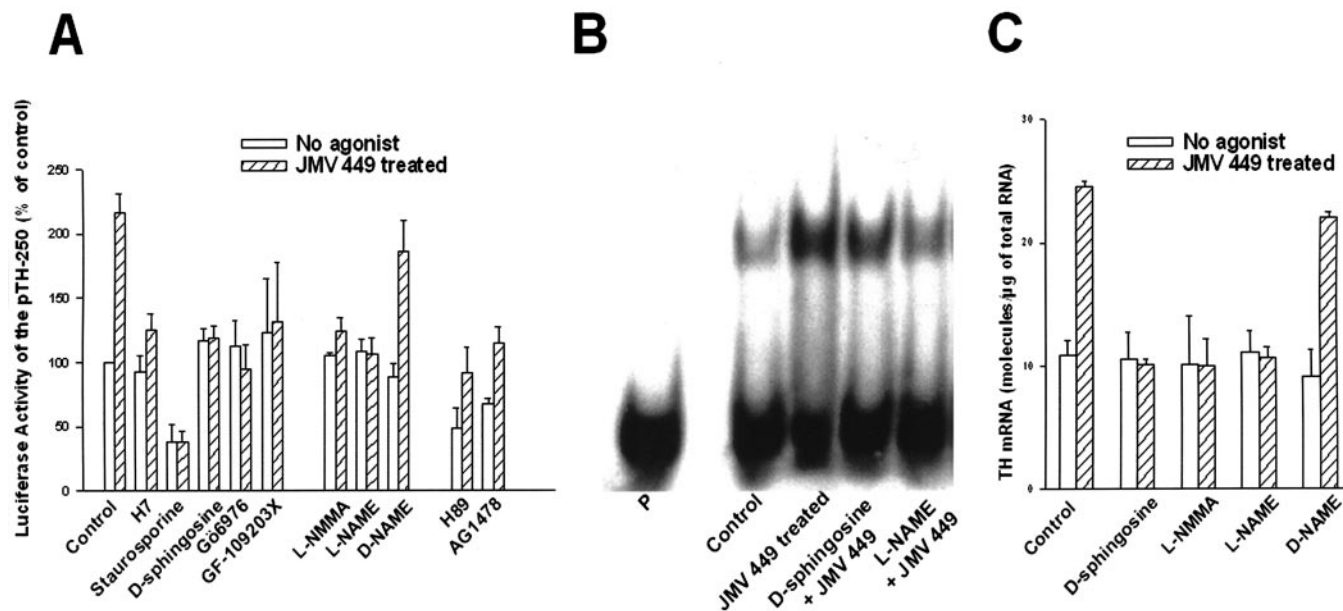


Fig. 4. TH gene stimulation by JMV 449 is mediated by NO production and PKC activation. A, differentiated N1E-115 cells were transfected for 16 h with the plasmid pTH-250-Luc and incubated in the absence (□) or in the presence of 1 μ M JMV 449 (▨) with or without 10 μ M H7, 1 μ M staurosporine, 10 μ M D-sphingosine, 1 μ M Gö 6976, 1 μ M GF 109203X, 10 μ M L-NMMA, 100 nM L-NAME, 100 nM D-NAME, 10 μ M H89, or 0.25 μ M AG1478 for 5 h. The RLU value of the control condition was arbitrarily set at 100%. The variation of the number of RLU, corresponding to treatment with inhibitors, with or without JMV 449, was calculated as percentage of control. Data are the mean \pm S.E.M. of at least three separate experiments performed in triplicate. B, typical autoradiogram of a gel shift experiment using nuclear extracts from differentiated N1E-115 cells incubated with 10 μ M D-sphingosine or 100 nM L-NAME in the presence of 1 μ M JMV 449 for 1 h. P, probe, C, TH mRNA ($\times 10^6$ molecules/ μ g of total RNA) was measured by quantitative RT-PCR on cells treated for 1 h with 10 μ M D-sphingosine, 100 nM L-NAME, 100 nM D-NAME, or 10 μ M L-NMMA in the presence (▨) or in the absence (□) of 1 μ M JMV 449. Data shown represent means \pm S.E.M. of at least three independent experiments.

of pTH-250-luc but do not block the increase in TH promoter activity induced by 1 μ M JMV 449. We confirm the effect of PKC and NO pathways on native AP-1 element stimulation, because the enhanced binding of the nuclear factors observed after 1 h of 1 μ M JMV 449 is abolished in the presence of either PKC or NO synthase inhibitors (Fig. 4B). The same result was found on the TH endogenous gene, because the increase in TH mRNA induced by JMV 449 was inhibited in the presence of either D-sphingosine or L-NAME (Fig. 4 C). We conclude that activation of TH gene by NT agonist is mediated by NO production and PKC activation resulting in the induction of *c-fos* and Jun D, which bind to the AP-1 element of the TH gene.

PKC and NO Signaling Pathways Play Independent Roles on the Activation of the *trans*-Acting Element *c-fos* or Jun D. To clarify the role of PKC activation and NO production in TH gene activation, we tested the effect of the two inhibitors D-sphingosine and L-NAME on NT agonist induced *c-fos* and Jun D expression. Interestingly, the two inhibitors act differently on the expression of these transcription factors. As shown in figure 5, A and B, the activation of *c-fos* expression is inhibited only by the NO synthase inhibitor, L-NAME, whereas Jun D expression activation is abolished only by the PKC inhibitor, D-sphingosine. In contrast, H89, a specific PKA inhibitor, has no effect on either *c-fos* or

Jun D increased expression by NT agonist (Fig 5A). These results demonstrate that the two signaling pathways act independently on *c-fos* and Jun D protein expression to induce TH gene activation.

Discussion

The TH proximal promoter contains a number of regulatory sequences known to be activated by effectors stimulated through the G protein-coupled receptors. Among these sequences, the AP-1 region of TH gene is known to be induced by different ligands, such as angiotensin II and carbachol (Stachowiak et al., 1990; Chae et al., 1996). The AP-1 sequence is recognized by a group of inducible transcription factors organized in two families: fos-related antigen and jun proteins (Pennypacker et al., 1994). These transcription factors are usually expressed at low basal levels but they can be dramatically augmented by a large variety of stimuli. The specificity of the gene activation via the AP-1 responsive element is offered by the multiplicity of homo and heterodimers formed between the AP-1 factors. In addition, a secondary level of specificity is conferred by the diversity of signaling pathways, which activate the AP-1 factors. In support of this concept, we report that NT agonist activates the TH gene through different and concomitant pathways. Indeed, NT induced TH gene activation is the result of AP-1 factor binding via specific Jun D and *c-fos* complexes; *c-fos* activation is strictly dependent on the NO production and Jun D being conditioned on PKC activation.

Up to now, most studies describing the role of the AP-1 factor complex in gene activation focused on a single partner within the complex. Particular attention concentrated on *c-fos* and its activation by PKC pathway. However, other reports have described that NO activates *c-fos* in a cGMP-independent or -dependent manner (Idriss et al., 1999; Wu et al., 2000). Vyas et al. (1990) have shown that PKC stimulation is involved in TH gene activation on the AP-1 element. Our data are in agreement with this result because a specific PKC inhibitor prevents both TH gene activation and the binding of AP-1 protein complex on the AP-1 responsive element (Fig. 4). However, when the AP-1 protein complex was analyzed in more detail, we found that the two partners of this complex, *c-fos* and Jun D, were not regulated by the same signaling pathway. We observed that TH gene activation is sensitive to PKC inhibitor because Jun D is activated by PKC (Figs. 3 and 5). Compared with other genes of this family, few results are available concerning the *Jun D* activation processes. *Jun D* transcription was first described to be insensitive to phorbol ester treatment (Hirai et al., 1989). Nevertheless, in Ca^{2+} -stimulated keratinocytes and lead-treated PC12 cells, it was shown that the Jun D gene was induced after PKC activation (Rutberg et al., 1996; Chakraborti et al., 1999). It is therefore relevant that the activation of the other partner, *c-fos*, is strictly dependent on NO production (Fig. 5).

The precise mechanism by which NO pathway regulates *c-fos* remains unclear. It is known that *c-fos* expression is potentiated by Elk-1 after phosphorylation of extracellular signal-regulated kinases 1 and 2 (Vanhoutte et al., 1999). In parallel to this study, we have also observed an inhibition of NT agonist induced Elk-1 expression by NO synthase inhibitor (data not shown). However, this observation is too premature to suggest that Elk-1 is the target of NO to activate

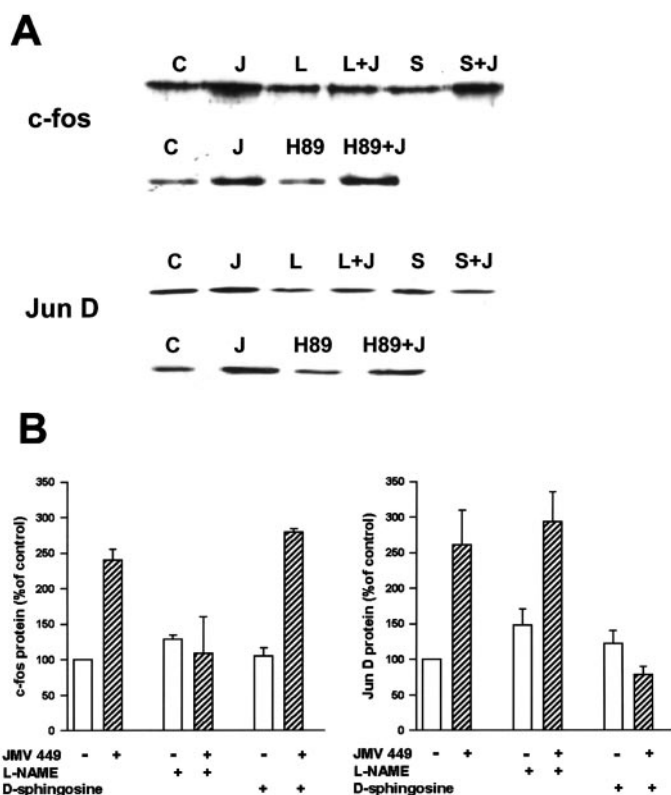


Fig. 5. Effect of D-sphingosine and L-NAME on the JMV 449-induced increase in *c-fos* and Jun D protein levels. A, representative *c-fos* and Jun D Western blots of differentiated N1E-115 cells, control (C) or treated with 1 μ M JMV449 (J) for 1 h with: first line, 100 nM L-NAME (L), 10 μ M D-sphingosine (S), L-NAME and JMV 449 (L+J), or D-Sphingosine (S) and JMV 449 (S+J); second line, 10 μ M H89 or H89 and JMV 449 (H89+J). This latter experiment was performed twice. B, relative expression of *c-fos* or Jun D (left and right, respectively) was estimated from Western blots by scanning the film by densitometry using the software program RAG (Biocom). Data are expressed as percentage of control cells. Values are the mean \pm S.E.M. of three separate experiments.

c-fos. The concept of the involvement of several independent signaling pathways in the activation of *fos* and *jun* has been evoked (Ding et al., 1998; Qiao et al., 2000). For instance, in the case of muscarinic receptors, ligand activation induced c-fos stimulation via PKC pathway and Jun D by a signaling Ca^{2+} /calmodulin dependent kinase II (Ding et al., 1998). This study provides further evidence of this type of regulation.

The co-localization of NT with catecholaminergic and particularly dopaminergic neurons provides a neuro-anatomical basis for a direct stimulatory effect of NT on TH expression (Szigethy and Beaudet, 1989). *Ex vivo* studies reported NT action on dopamine release in dopaminergic neurons (Brouard et al., 1994). Furthermore, our laboratory has previously demonstrated that in the nucleus accumbens and the mesencephalon, a chronic treatment with a NT-1 receptor antagonist increased TH expression with a loss of dopamine levels (Azzi et al., 1998). Under the same experimental conditions, NT-1 receptor antagonist treatment was also associated with a major increase of NT-1 receptor expression (Azzi et al., 1994). More likely, these experiments revealed the role of endogenous NT than the effect of the blockade of NT transmission. Taken together, these observations and those from data showing that NT can induce c-fos expression in vivo (Lambert et al., 1996), suggest that the mechanism described in the present work represents a close approach to explain TH regulation by NT in vivo. One additional role implicated in the NT induction of TH gene expression would be the consequence on the enzyme activity. In fact, it has been demonstrated that in TIDA neurons, NT increases this parameter (Berry and Gudelsky, 1992). Therefore, it may be of a particular interest to investigate in more detail the role of NT in modulating catecholamine levels.

The findings presented in this study are the first description of the molecular mechanisms implicated in the activation of TH by a NT agonist. The data revealed a molecular fine-tuning of TH expression by NT, which would be related to the fact that NT is a modulator of dopaminergic neurotransmission.

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Address correspondence to: Dr. P. Forgez, INSERM U339, Hôpital Saint-Antoine, 184 rue du Faubourg St-Antoine 75012 Paris, France. E-mail: forgez@adr.st-antoine.inserm.fr