Dexamethasone-Induced Down-Regulation of Nerve Growth Factor Receptor $p75^{NTR}$ is Mediated by Glucocorticoid Type II Receptor in PC12 Cell Model

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Abstract: PC12 clones are established neuronal models to investigate mechanisms involved in the cross-talk between the neurotrophins and drugs. Chronic treatment of PC12 cells with the glucocorticoid agonist drug, dexamethasone (Dex), elicited a 50% decrease in the selective binding of ^{125}I-NGF along with a reduction in the NGF receptor $p75^{NTR}$ mRNA and protein levels, suggesting a transcriptional mechanism. This down regulation of $p75^{NTR}$ was antagonized by the glucocorticoid type II receptor (GR-2), RU-38486 but not by the minerallocorticoid receptor, RU-28318, antagonists. This process was associated with increased autophosphorylation of the NGF receptor, TrkA. Chronic treatment of PC12 with Dex abolished the NGF-induced proliferation of the cells after 20 hours and inhibited by 45% the neurite elongation after 96 hours. RU-38486 blocked Dex-induced shift of PC12 cells from dopaminergic to noradrenergic phenotype. Dex-induced down regulation of $p75^{NTR}$ receptor is mediated by GR-2 and is correlated with disruption of NGF induced proliferation and differentiation. This study may prove relevant with respect to the understanding of neuronal side-effects of corticosteroids.

Keywords: PC12, NGF, dexamethasone, $p75^{NTR}$, TrkA, corticosteroid type-2 receptor, down regulation.

INTRODUCTION

Naturally occurring glucocorticoids such as cortisol (hydrocortisone) as well as the synthetic congener dexamethasone, are known to have important effects on the nervous system including regulation of neurotransmitter synthesis [1] as well as modulation of neuronal survival, proliferation and differentiation [2, 3]. It is postulated that increased neuronal cell death in the brain associated with aging and certain neurodegenerative disorders may occur as a consequence of elevated blood levels of glucocorticoids [4]. Also, there are clinical observations that prenatal exposure to dexamethasone given prophylacticaly to pregnant women undergoing pre-term labor or directly to pre-termed infants in order to reduce the incidence of respiratory distress [5] may have detrimental influence on the development of the central nervous system [6]. The mechanisms responsible for these neurotoxic effects are unknown.

NGF, a prototype of the neurotrophin family, regulates proliferation and differentiation of specific neuronal tissues during both physiological and pathological processes [7]. Neurotrophins in general and NGF in particular represent the first line of defense in the central nervous system to provide neuroprotection and ensure survival against neurotoxic and neurodegenerative insults [7]. These NGF effects are mediated by two types of receptors: the non enzymatic $p75^{NTR}$ receptor and the tyrosine kinase receptor TrkA [8].

It is hypothesized that through chronic activation of their cognate receptors, corticosteroids may have an impact on neurotrophin receptors that may contributes to corticosteroids neurological side-effects which may impinge into neurodegeneration and neurotoxicity.

In the present study, the effect of glucocorticoids on NGF receptor expression and function in PC12 cells, a widely used *in vitro* neuronal model, was investigated [8]. Through activation of distinct molecular signaling pathways, these neurosecretory cells upon exposure to NGF or dexamethasone are induced to differentiate into either dopaminergic neuron or chromaffin noradrenergic-like phenotypes, respectively [9]. These properties of PC12 cells allows for investigation of possible cross-talk between steroids and neurotrophins-activated signaling pathways, which affect the differentiation outcome of the cell.

Present study demonstrates for the first time that the corticosteroid receptor type-2 (GR-2) activation is responsible for the transcriptional mediated-down regulation of $p75^{NTR}$ receptors, a cellular process coincidentally correlated with enhanced TrkA receptor autophosphorylation and disruption of NGF-induced proliferation and differentiation. Since chronic exposure to high glucocorticoid levels had been associated with attention, memory deficits and cerebral cortex development [10, 11], the dexamethasone induced-down regulation of $p75^{NTR}$ receptor process demonstrated in the present study, may represent a mechanism contributing to the corticosteroids-induced neuronal side effects.

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MATERIALS AND METHODS

Materials

Retinoic acid, dexamethasone and other steroids were purchased from Sigma Chemical Co, (St. Louis, MO). 2.5S mouse NGF was obtained from Alomone Labs., (Jerusalem, Israel). Horseradish peroxidase (HRP)-goat anti-rabbit and anti-mouse antibodies were acquired from Jackson Immune Research Laboratories, Inc., (West Grove, PA). ^{125}I-NGF (specific activity 1000-2000 Ci/mmol) was purchased from Amersham Biosciences (Little Chalfont, UK). RU-28318 and RU-38486 were obtained from Roussel-Uclaf (Romainville, France). The cyclophilin probe was kindly provided by Dr. G. Guroff (NIH, Bethesda, USA). The monoclonal antibodies anti-phosphotyrosine (4G10) and anti-p75^{NTR} were purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and Promega Co. (Madison, WI), respectively. The polyclonal anti-TrkA (C14) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell Cultures

wtPC12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7% fetal calf serum, 7% horse serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (Beit Haemek, Afula, Israel). The cultures were maintained in an incubator at 37°C in an atmosphere of 6% CO_2 [12]. PC12nnr5 cells, a PC12 variant lacking TrkA receptors, were grown on collagen-coated tissue culture dishes in RPMI 1640 medium [12]. PC12-6.24-I cells, a clone of PC12 over-expressing TrkA receptor, were grown as PC12 cells in the presence of 200 μg/ml G-418 [13]. The endothelial cell clone b.End3 was propagated in DMEM supplemented with 10% fetal calf serum as previously described [14]. In experiments conducted to investigate a possible steroid effect, the cells were grown on tissue culture plates uncoated or coated with rat tail type I collagen (0.1 mg/ml) in DMEM supplemented with 2% fetal calf serum (Hyclone, Logan, UT) that had been depleted of endogenous steroids using activated charcoal. Cultures were incubated in DMEM supplemented with 2% charcoal-stripped fetal calf serum for at least 1 day before initiation of steroid treatment and subsequently throughout the full course of the experiment.

Radioreceptor Assays

NGF binding studies were carried out as previously described [12]. PC12 cell cultures were washed and equilibrated with fresh serum-free DMEM containing 0.1% bovine serum albumin for 1 h at 37ºC. Total binding was measured by adding saturating concentrations of radioactive NGF while nonspecific binding was evaluated by adding 100-fold excess unlabeled ligand. The incubation was carried out at 37ºC for 45 min. At the end of incubation, the cells were washed, solubilized with 1 N NaOH and cell associated radioactivity was counted. Data points represent specific binding expressed as fmol/mg protein and are the mean ± SD of six replicate samples.

Immunoprecipitation and Immunoblotting

The cells were cultured for 3-5 days in 10 cm tissue culture dishes in DMEM containing 2% charcoal-stripped fetal calf serum. Fresh media and tested compound were added every 2 days. Following treatment with 10 ng/ml NGF for 5 min, the cultures were extracted on ice for 30 min in the RIPA lysis buffer and protein content was determined by Bradford assay [12]. Samples of 200 μg protein were subjected to immunoprecipitation using anti-TrkA antibody for 12 h at 4ºC followed by an additional 2 h incubation with protein A-Sepharose. Precipitates were washed, boiled for 5 min in SDS sample buffer and subjected to electrophoresis by loading onto 10% SDS-polyacrylamide gels. After electrophoresis, proteins were electrophoretically transferred to immobilon membranes. Immunoblots were probed overnight at 4°C with the anti-phosphotyrosine antibody and target proteins detected using a horseradish peroxidase-coupled secondary antibody and ECL. The level of TrkA protein was determined by stripping the blots and reprobing with anti-TrkA rabbit polyclonal antibody. p75^{NTR} was directly analyzed by western blotting using 20 μg protein of lysate samples. Photographic films were developed and densitometric analysis performed using a computerized image analysis program [15].

mRNA Preparation, Northern Blotting and RT-PCR

For Northern blotting PC12 cell mRNA was prepared [16] using the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). The amount of mRNA was estimated by optical absorbance measured at 260 nm and 20 μg samples were heated at 65ºC for 5 min in loading buffer. Thereafter samples were analyzed by electrophoresis on 7% agarose gel containing 2.2 M formaldehyde, 20 mM MOPS, pH 6.8, 5 mM sodium acetate and 1 mM EDTA. The samples were transferred overnight to Hybond-N membranes (Amersham Biosciences, UK) using a solution of 0.15 M NaCl containing 0.015 M sodium citrate. The membranes were heated at 80ºC for 2 h and prehybridized according to manufacturer instructions. For hybridization, 0.5x10^{6}cpm/ml of ^{32}P-human p75^{NTR} (specific activity 1x10^8 cpm/μg DNA) or ^{32}P-cyclophilin (specific activity 1x10^6 cpm/μg DNA) cDNA were added. The probes were labeled using Prime-It random primer commercial kit (Stratagene, Cedar Creek, TX) and purified using a GeneClean kit (Qbiogene/ MP biomedical products, Morgan Irvine, CA). The content of mRNA was estimated by measuring within the linear range of intensity the ratio between the peak densitometric area of p75^{NTR} normalized to the peak area of cyclophilin transcript [16].

RT-PCR assay was performed as described earlier [15]. Briefly, total RNA was isolated using the SV total RNA isolation system (Promega, Madison, WI, USA). A quantity of 1 μg of total RNA was reverse transcribed using the Reverse Transcription System (Promega), according to the manufacturer's instructions. Then PCR was applied containing 5 μg cDNA, 50 pmoles of upstream sense and downstream sense primers, using GoTaq® Green Master Mix (Promega). The cDNA for β-actin was amplified by 35 cycles; for TrkA and p75^{NTR} a 40 cycle amplification was used. For generation of various cDNA fragments, a Mastercycler gradient (Eppendorf, Germany) was used programmed as follows: denaturation at 94°C for 1 min, annealing at 65°C for 1 min and elongation at 72°C for 2 min. To identify the following primers were used: β-actin (285bp) sense: TCATGAAGTGTGACGTTGACATCCGT-3' and antisense: CTT AGAAGCATTTGCGGTGCACGATG-3', TrkA (232bp) sense: TGCCTGCCTCTTCCTTTCTA-3' and antisense:

GTGGTGAACACAGGCATCAC-3', $p75^{NTR}$ (663bp) sense: AGCCAACCAGACCGTGTGTG-3' and antisense: TTGCAGCTGTTCCACCTCTT-3', PCR products were analyzed by electrophoresis on agarose gel (2%) containing ethidium bromide for UV visualization. The quantitation of mRNA bands was extimated by using QuantiOne software (BioRad, USA).

Measurement of Catecholamine Content

PC12 clones were grown to confluency in 12-well plates and incubated in DMEM containing 2% charcoal-stripped fetal calf serum in the presence or absence of Dex (1 M) for 3 days. The cells were washed, extracted with 0.1 M perchloric acid containing 0.1 mM EDTA and the amount of dopamine and norepinephrine was estimated by reverse phase HPLC coupled with electrochemical detection [17].

Measurement of Cell Proliferation

The effect of NGF on PC12 proliferation was performed in cultures grown at low density in 96 wells in 2% charcoal stripped fetal serum. After 72 hours of culturing the cells MTT was added for 1 h. Following incubation with MTT (Sigma®) the cells were lysed with DMSO and the absorbance was measured at 570 nM using Tecan® spectrofluorometer. The percent of proliferation was calculated compared to untreated cultures [18].

Measurement of Morphological Differentiation (Neurite Outgrowth)

The NGF-induced neuronal differentiation of PC12 cells cultured in the presence or absence of Dex was estimated as previously described [13]. The percentage of PC12 cells elaborating neurite outgrowths and their length was evaluated by digital acquisition of data and computer analysis. Two parameters were calculated: (1) Percentage of responsive cells (PRC), defined as the percentage of cells with neurites, out of all cells measured, and (2) Elongation of neurites (Ef), defined as the ratio between the neurite length and the cell diameter. A ratio of less than 2.0 indicated a lack of NGF-induced differentiation, and was not considered in the results.

Statistics

The results are presented as the mean ± S.D of 3-9 experiments (n=3-6). Determination of statistically significant differences between experimental groups was performed using analysis of variance program (ANOVA), and differences in results between groups compared were considered significant when *p values < 0.05 or less were obtained.

RESULTS

Dexamethasone-Induced Decrease of NGF Binding is Mediated by GR-2

The level of PC12 cell ^{125}I-NGF binding was reduced by 50% after culturing in the presence of 0.5 μM Dex for 3 days (Fig. **1A**). Reduction in ^{125}I-NGF binding affected by Dex treatment was concentration dependent (Fig. **1B**) with the maximal effect observed at 1 μM Dex. Treatment of PC12 cells for up to 3 days with 1 μM of different steroid receptor agonists [19] did not significantly reduce ^{125}I-NGF binding indicating that the effect observed was specific for Dex and hydrocortisone (Fig. **1C**).

The Dex-induced decrease in ^{125}I-NGF binding was prevented completely by co-treatment of the cells with 1μM of the glucocorticoid receptor antagonist RU-38486 but not the mineralocorticosteroid receptor type I (MR-1) antagonist RU-28318 (Table **1**), indicating the effect is mediated by GR-2 receptors. Concentrations of Dex within the range of 10^{-9} - $2x10^{-8}$M resulting in inhibition of ^{125}I-NGF binding (Fig. **1A**) are similar to concentrations reported for saturation of the corticosteroid receptor [20].

To investigate the effect of RU-38486 on PC12 differentiation towards the chromaffinergic phenotype, the cells were treated for 3-5 days with Dex and the cathecholamine content was evaluated. It has been found that the content of dopamine and norepinephrine was increased by 16% and 448%, respectively (Table **1**) thereby confirming Dex-elicited chromaffin differentiation of the cells compared to control (or ethanol) cells [9]. The Dex-induced increase in catecholamine content was blocked by co-treatment of the cells with GR-2 antagonist RU-38486 but not RU-28318 while treatment with the steroid receptor antagonist alone did

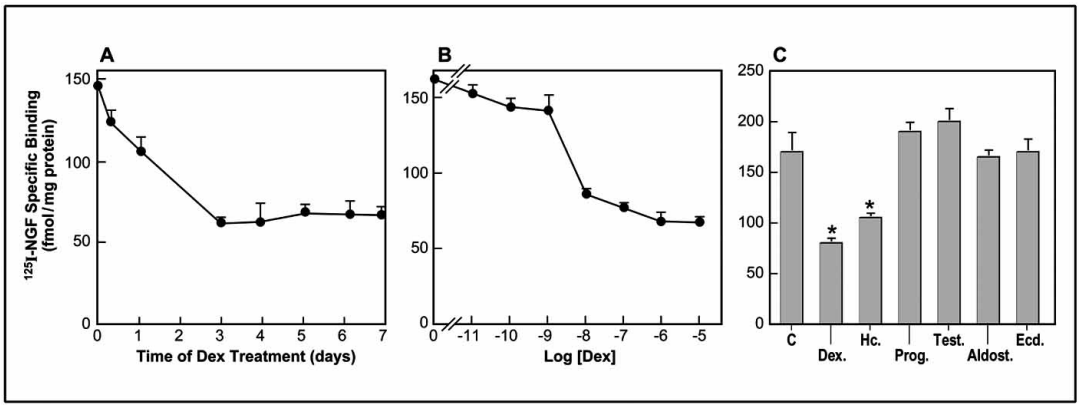

Fig. (1). Dexamethasone (Dex)-induced down –regulation of NGF receptors as measured by binding assays .wtPC12 cells were treated for different periods of time with 0.5μM Dex (**A**) or treated for 3 days with different Dex concentrations (**B**) or 1 μM of different steroids (**C**). Hc, hydrocortisone; Prog, progesterone; Test, testosterone; Aldost, aldosterone; Ecd, ecdysone; Binding assay was done as described in Materials and Methods. Values represent mean ± SD of four experiments each one performed in sixplicates; in A, values at 3-7 days and in B, values from 10^{-8}-10^{-5} and * are statistically significant ($p<0.05$ by ANOVA).

Table 1. The Effect of MR-1 and GR-2 Receptor Antagonists on the Dexamethasone- Induced Decrease of ^{125}I-NGF Binding and Increase in Catecholamine Content in PC12 Cells[a]

Treatment	^{125}I-NGF binding (fmol/mg protein)	Catecholamine content (ng/10^{6}cell)	
		Dopamine	Norepinephrine
Control	150 ± 13	211 ± 11	31 ± 3
Ethanol	165 ± 12	205 ± 7	35 ± 7
Dex	71 ± 8*	245 ± 4*	139 ± 9*
Dex/RU-28318	74 ± 17	239 ± 8	145 ± 13
Dex/RU-38486	179 ± 9**	207 ± 9**	41 ± 7**
RU-28318	155 ± 8	189 ± 11	38 ± 6
RU-38486	159 ± 11	202 ± 3	24 ± 6

[a]wtPC12 cells were treated for 3 days with either 500 nM of dexamethasone in the presence or absence of RU antagonists (5 μM) or 0.01% ethanol (carrier used for drug solubilization) or left untreated (control). The binding assays performed in six replicates and the catecholamine determinations performed in triplicates were done as described in Materials and Methods. The values are mean ± SD of three independent experiments. (*compared to control, ** compared to Dex, p<0.05 by ANOVA).

not affect cathecolamine content compared to control (Table **1**).

Dexamethasone-Induced Decreases in p75^{NTR} Protein and mRNA Level is Mediated by GR-2

Analysis of p75^{NTR} protein by Western blotting showed that incubation of wtPC12 cells for 3 days with 0.5 μM Dex resulted in a 20% decrease in the level of p75^{NTR} protein when compared to control cells (Fig. **2A**). Although the results varied between experiments, the decreased p75^{NTR} protein level was usually between 20-55% of control (n=9 experiments, p<0.05). Similar findings were observed when the ratio of p75^{NTR} protein to β-actin expression was calculated (data not shown). This effect of Dex on p75^{NTR} protein expression was blocked by RU-38486 (Fig. **2A**). Treatment with 5 μM retinoic acid, known to stimulate p75^{NTR} expression [21], was performed as a positive control yielding a 25 ± 8% increase in p75^{NTR} protein level (Fig. **2A**). To determine whether the effect of Dex on p75^{NTR} protein level is related to TrkA receptor expression [22], Dex-induced decrease of p75^{NTR} protein was evaluated in several PC12 cell variants that either lacked or over-expressed TrkA receptors without altering p75^{NTR} protein expression [12]. As indicated in (Fig. **2B** and **2C**), Dex treatment reduced by 50% the expression of p75^{NTR} protein in these PC12 clones, a process completely abolished by RU-38486, but not RU-28318 (data not shown). Similar findings were obtained by radioreceptor assay (data not shown). Therefore, it seems likely that Dex-induced decrease of p75^{NTR} protein in these three PC12 cell clones is independent of the level of expression of TrkA and is mediated by activation of GR-2.

Northern blot analysis was conducted to determine whether the level of p75^{NTR} mRNA was altered by Dex treatment. In wtPC12 cells, Dex reduced the level of p75^{NTR} transcript by 45%, an effect reversed by approximately 80% in the presence of RU-38486 (Fig. **3A**). 5 μM retinoic acid,

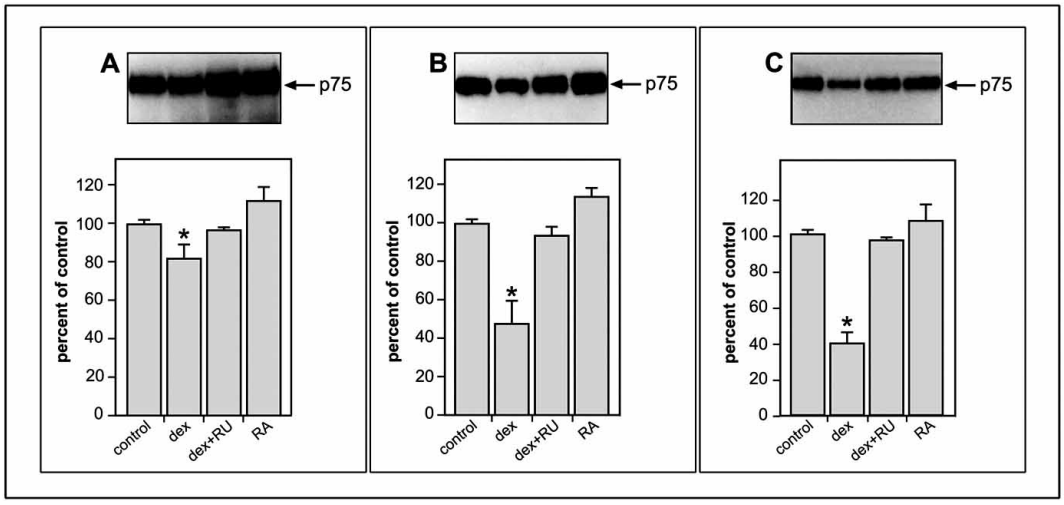

Fig. (2). Dexamethasone (Dex)-induced, down regulation of NGF- receptors in wtPC12 cells (**A**), PC12-6.24-I cells (**B**), PC12nnr5 cells (**C**) as measured by Western blotting. The immune reaction with anti p75^{NTR} antibody was performed with 20 μg cell protein from samples of untreated cells (control), cells treated for 5 days with 0.5 μM Dex in the absence (Dex) or presence of 1 μM RU-38486 (Dex+RU) or cells treated for five days with 5 μM retinoic acid (RA). p75 arrow indicate the position of p75^{NTR}. The intensity of the bands is presented as mean ± SD of three experiments performed in duplicates (*p< 0.05 by ANOVA).

used as positive control, increased the level of $p75^{NTR}$ transcript by 40%. In PC12nnr5 cells that lack TrkA receptors, the level of $p75^{NTR}$ transcript is low but detectable, as expected [22]. Dex treatment reduced the level of $p75^{NTR}$ transcript by 92%, an effect antagonized by 80% upon treatment with RU-38486 (Fig. **3B**). Retinoic acid caused a 20% increase in the level of $p75^{NTR}$ transcript in these cells. The selective inhibition of $p75^{NTR}$ transcript level caused by Dex treatment and precise mRNA normalization is evident from the lack of any effect of Dex on cyclophilin transcript level (Fig. **3A**, **B**-lower part). Although interexperimental variation was observed, the consistent decrease of $p75^{NTR}$ transcript level by 40-90% as compared to control cultures (n=6, $p<0.05$), clearly suggest a TrkA–independent, GR-2 mediated effect of Dex on $p75^{NTR}$ transcription in these PC12 cell clones.

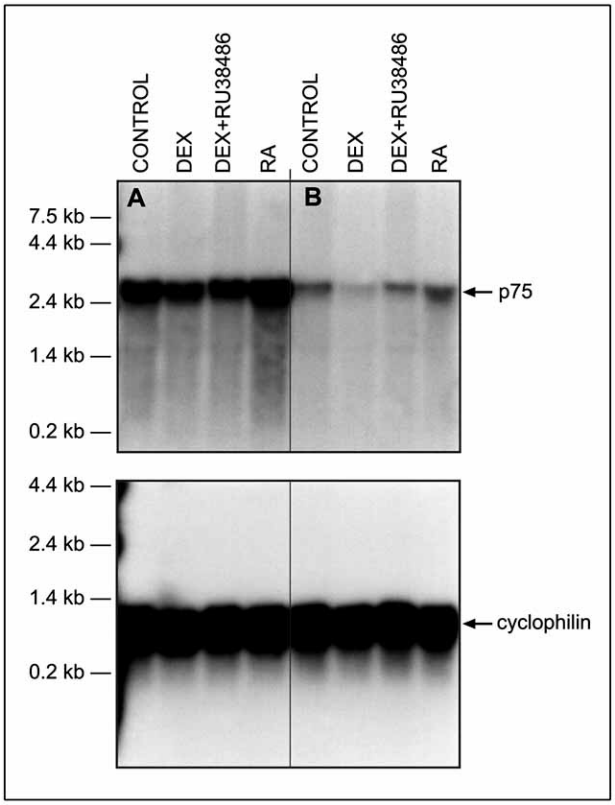

Fig. (3). The inhibitory effect of dexamethasone (Dex) on the expression of $p75^{NTR}$ mRNA in wtPC12 cells (**A**) and PC12nnr5 cells (**B**). The cells were treated for 5 days with either 0.5 μM Dex in the absence (DEX) or presence of 1 μM RU-38486 (DEX+RU-38486) or 5 μM retinoic acid (RA) or left untreated (Control). Northern blot analysis was carried out using poly(A^+) RNA (20 μg/lane). The blot was hybridized with ^{32}P-labeled human $p75^{NTR}$ cDNA (Top); the same blot was stripped and hybridized with a probe for cyclophilin mRNA (Bottom). Migration of markers of different kilobase size is shown on the left. p75 and cyclophilin arrows indicate transcripts position. This experiment was performed four times with essentially identical results.

To get a more complete picture the northern results were confirmed by semi-quantitative RT-PCR experiments presented in (Fig. **4**). Dex reduced the level of $p75^{NTR}$ transcript by about 50% but not the level of TrkA receptor, as compared by the ratio to β-actin transcripts levels. Interestingly, Dex treatment resulted also with 85% inhibition of the mRNA for MAP-2 as compared by the ratio to β-actin transcripts levels. MAP-2 is coding for an important cytoskeleton protein in PC12 cells required for neurite outgrowth [23]. Therefore these results also indicate effects of Dex on the cytoskeletal protein expression.

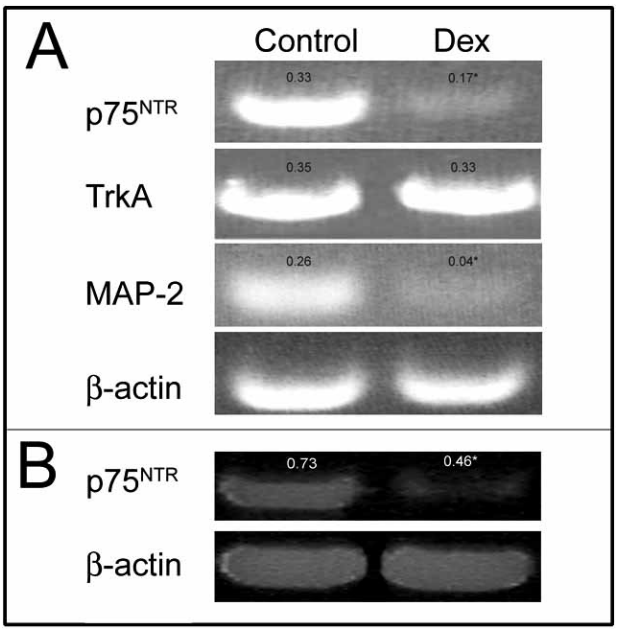

Fig. (4). The inhibitory effect of Dex on the expression pf $p75^{NTR}$ mRNA in wtPC12 cells (**A**) and b.End3 endothelial cells (**B**). The cells were treated for 4 days with 1 μM Dex. mRNA was extracted and RT-PCR carried out using 50 μg mRNA. Similar amounts of PCR products were loaded on the agarose gel for electrophoresis. For normalization the bands intensities were compared to the levels of β-actin. The mean value of transcript level ratio to β-actin is indicated on top of the bands.

It maybe questioned whether Dex induced decrease of $p75^{NTR}$ transcripts are exclusively detected in PC12 or maybe also found in non-neuronal NGF-responsive cells. To answer this question we treated for 4 days endothelial cells which express both NGF receptors [24] with 1 μM Dex and measured the level of $p75^{NTR}$ transcripts. Fig. (**4B**) clearly indicates that in endothelial cells Dex induced 50% reduction in $p75^{NTR}$ mRNA as similarly measured in wtPC12 cells (Fig. **4A**) indicating a general effect of the corticosteroid.

Dexamethasone-Induced Increase in TrkA Autophosphorylation is Mediated by GR-2

$p75^{NTR}$ is implicated in the regulation of TrkA receptor tyrosine phosphorylation activity [22]. To determine if a Dex-induced reduction in $p75^{NTR}$ receptor protein is related to TrkA receptor kinase activity, PC12-6.24-I cells, overexpressing TrkA receptor were used and not wtPC12 in order to facilitate TrkA phosphorylation activity measurements. PC12-6.24-I cells were cultured for 5 days in the absence or presence of 0.25 μM of DEX and/or 1 μM RU-38486 were treated with 10 ng/ml NGF for 5 min prior to cell harvesting in order to stimulate TrkA receptor phosphorylation activity. Thereafter, group samples were immunoprecipitated with anti-TrkA antibody followed by western blotting detection using phosphotyrosine antibody to probe for TrkA autophosphorylation of tyrosine residues (Fig. **5**). In untreated cells the level of TrkA phosphorylation activity was low, increas-

ing by approximately four- fold upon NGF stimulation (Fig. **5**). In the cultures treated for 5 days with Dex, strong basal tyrosine phosphorylation activity of TrkA was detected which could not be increased further upon acute NGF stimulation (Fig. **5**). The Dex-induced phosphorylation effect was inhibited by 80% using RU-38486 (Fig. **5**), but not RU-28318 (data not shown). In cells treated for 5 days with 1 μM RU-38486 alone, but not RU-28318 (data not shown), basal tyrosine phosphorylation activity of TrkA was increased by four-fold compared to the control PC12 cells and upon acute NGF stimulation a strong hyperphosphorylation effect was observed (Fig. **5**). There are at least two plausible explanations for these findings: (i.) in the absence of corticosteroid, RU-38486, functions as an agonist therefore possessing mixed agonist-antagonist functionality [25]; (ii.) basal activity of TrkA receptor is also under the control of GR-2, therefore, upon RU-38486 antagonist addition, disinhibition occurrs resulting in TrkA hyperphosphorylation. Irrespective of the mechanism involved, these data support the concept that cross-talk occurs between GR-2 and TrkA receptors. Unlike results observed for treatment with RU-38486 only, addition of NGF to cultures treated with both RU-38486 and Dex failed to stimulate additional tyrosine phosphorylation of TrkA beyond the level determined for non-NGF treated cells indicating Dex treatment elicits maximum TrkA receptor kinase activity. These findings clearly suggest that cross-talk between GR-2 and TrkA receptors occurs.

Dexamethasone Disrupts NGF-Induced Differentiation

The effect of Dex treatment on NGF-induced neuronal differentiation (Fig. **6A-D**) and proliferation (Fig. **6E**) was evaluated using a PC12 morphologic bioassay and MTT assay, respectively (Fig.6).In control untreated wtPC12 cells, NGF stimulated neurite outgrowth (Fig. **6A-C**) as expected [13]. In cells treated for 4 days with 1 μM Dex no neurite outgrowth was observed (Fig. **6A-C**) as also expected [26]. Upon concomitant treatment of the cells for 4 days with 50 ng/ml NGF and 1 μM Dex a significant 45% inhibition of neurite length (E_f) was measured (Fig. **6 A, C**) with no significant effect on the percentage of cells responding with neurite outgrowth (PRC) (Fig. **6A**, **B**) as described in earlier report [26]. While NGF moderately increased cell diameter due to the hypertrophy of the cells [27] Dex treatment did not affect significantly cell diameter (Fig. **6D**), which is used in the calculation of the Ef parameter; therefore we can conclude that Dex treatment significantly affects NGF-induced elongation of neurites, i.e., disruption of NGF-induced differentiation (Fig. **6A-C**). In the first 48 h upon NGF treatment PC12 cells double in number before cessation of growth and initiation of neurite outgrowth [28]. Fig. (**6E**) depicts experiments to verify possible effects of Dex treatment on NGF-induced doubling of the cells. It is evident that Dex treatment completely blocked the NGF-induced doubling of the cells indicating an interference with the NGF-induced effects on PC12 cell cycle.

DISCUSSION

The present study indicates that chronic treatment with Dex of PC12 neuronal model induced down-regulation of $p75^{NTR}$ receptor, a process correlated with increased TrkA receptor autophosphorylation and disruption of NGF-induced proliferation and differentiation. Dex-induced down regulation of $p75^{NTR}$ receptors has been documented *in vitro* [26, 29, 30] and *in vivo* [30, 31]. However, this is the first study to document the involvement of GR-2 in mediating the effects of Dex by using RU-38486, a pharmacologic antagonist with greater selectivity for glucocorticoid *vs* other steroid receptors [32]. The ability of RU-38486, but not of RU-28318 a MR-1 antagonist, to abolish Dex effects observed in PC12 cells is consistent with previous results from our laboratory characterizing the ^{3}H-Dex binding sites in the wtPC12 cells as being of the GR-2 receptor subtype [20]. Although, RU-38486 (Mifepristone) is used clinically as antiprogestine for medical abortion [33] and as antiglucocorticoid for treatment of hypercortisolism [34] in PC12 model its effects on Dex-induced down regulation of $p75^{NTR}$ are most probably mediate mainly by GR-2 since progesterone didn't affect

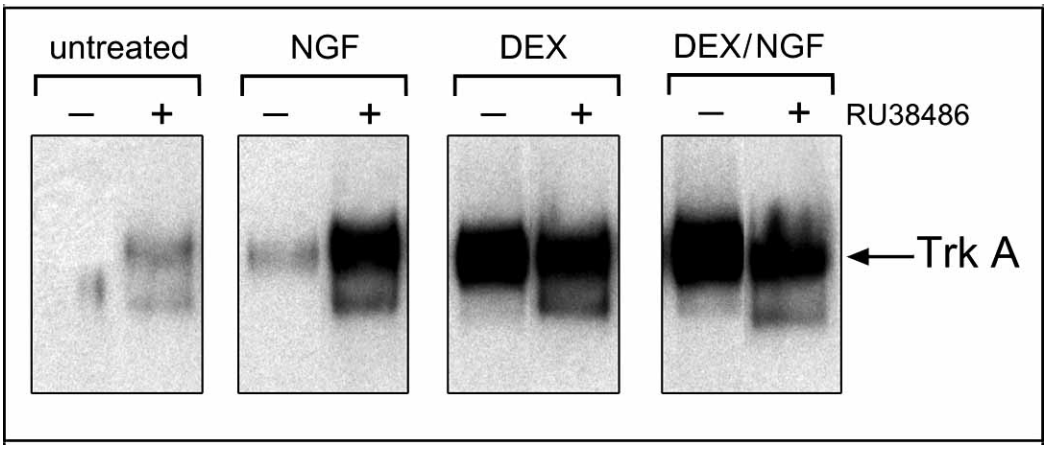

Fig. (5). The stimulatory effect of dexamethasone (Dex) on TrkA tyrosine phosphorylation activity in PC12-6.24-I cells. The cells were treated for 5 days with 0.25 μM Dex (DEX) in the absence (-) or presence (+) of 1 μM RU-38486 or left untreated (Untreated). At the conclusion of the incubation period, selected, untreated (NGF) or dex-treated (DEX/NGF) cultures were incubated for 5 min with NGF (10 ng/ml) prior harvesting cells. The cells were lysed, the TrkA immunoprecipitates prepared and separated on 7.5 % SDS-polyacrylamide gels and TrkA tyrosine phosphorylation (arrow) was analyzed by Western blotting as described under Materials and Methods. Thereafter the blots were reprobed with pan-antibodies for TrkA indicating 95% similarity in the levels of immunoprecipitated receptors. These experiments were done three times with essentially identical results.

the level of NGF binding (Fig. **1**). Dex-induced down regulation of $p75^{NTR}$ appears to be mediated by a transcriptional mechanism and is independent of the level of TrkA transcript (Fig. **4A**) and protein (Fig. **5**). GR antagonists, such as RU-38486, have been shown to be effective at blocking gene-transcription mediated by glucocorticoids [32]. This selective inhibitory effect of RU-38486 is confirmed by the impact of the antagonist on Dex-induced down regulation of $p75^{NTR}$ in PC12 cells. Interestingly, with chronic Dex treatment of PC12 cells, a causal relationship between the Dex-induced TrkA phosphorylation activity and down regulation of $p75^{NTR}$ receptors was uncovered.

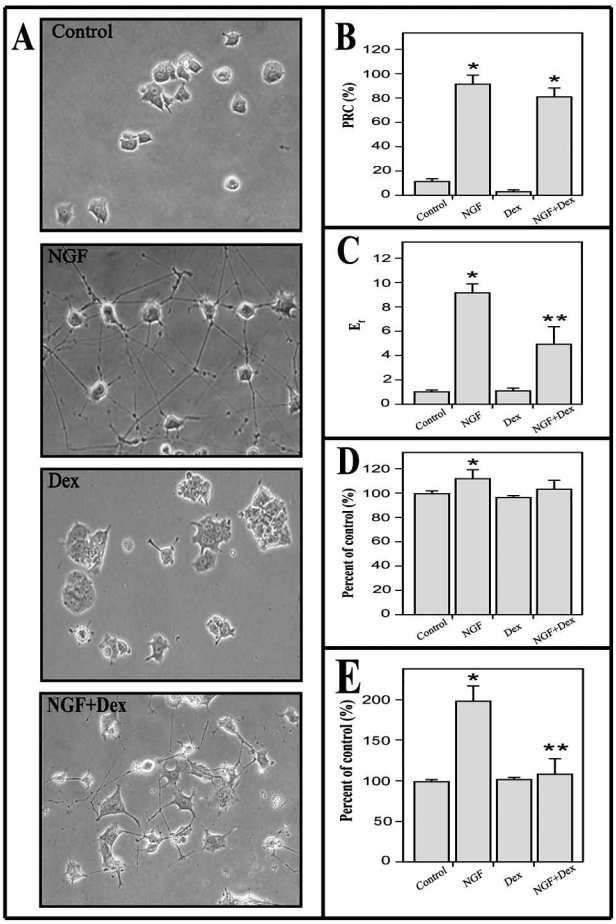

Fig. (6). The inhibitory effect of Dex on NGF-induced differentiation and proliferation of wtPC12 cells. **A** – photographs of X200 magnification of cells untreated (control), or treated with 50 ng/ml NGF (NGF) in the presence (NGF+Dex) or absence (Dex) of 1 μM Dex for 4 days. The effect of Dex on cel diameter (**D**) and differentiation measured by PRC (**B**) and E_f (**C**) parameters. The effect of Dex on cell proliferation measured by MTT assay (**E**). The values are presented as mean ± SD of three experiments performed in six-plicates; * - compared to control ($p<0.01$); ** - compared to NGF ($p< 0.05$).

Two major molecular mechanisms may account for this interactive relationship: (i.) Dex-induced augmentation of TrkA activity through regulatory phosphorylation pathways; (ii.) Dex-induced down regulation of $p75^{NTR}$ receptors is directly responsible for the increased TrkA phosphorylation. This possibility we favored upon considering that $p75^{NTR}$ directly interacts with [35] and regulates TrkA phosphorylation activity [36] and is consistent with the dogma that $p75^{NTR}$ acts like a regulator that modulates the affinity and selectivity of NGF for the TrkA receptor [37], resulting in signaling that leads to either cell death or survival [38]. Dex induced $p75^{NTR}$ down regulation is a marker for existence of a cross-talk between signaling cascades regulated by Dex and NGF receptors, in line with reports for other steroids [39, 40].

A correlation was found between Dex-induced down regulation of $p75^{NTR}$ and disruption of NGF-induced proliferation and differentiation of the cells. Dex completely blocked the NGF-induced effect on cell proliferation while partially affected the neurite extension but not the percentage of cells responding to NGF; thus reinforcing recent similar neurotoxic effects in Dex-treated PC12 cells [41], or hyppocampal neurons [42, 43]. Since Dex treatment induced additional cellular effects such as downregulation of MAP-2, crucial for NGF-induced neurite outgrowth [23], it also appear plausible that Dex inhibits the expression of proteins necessary for TrkA-mediated neurite outgrowth which would uncouple the TrkA activity from neurite outgrowth.

These findings may be relevant for investigations in psychiatric and neurodegenerative disorders in which high levels of circulating glucocorticoids have been described and in which a beneficial therapeutic effect of RU-38486 has been reported in patients suffering from depression [44] or Alzheimer's disease [45]. It is tempting to propose that in these neurological disorders glucocorticoids induce similar cross talk processes that impact NGF receptors which may be responsible in part for the observed neuropathologies.

LIST OF ABBREVIATIONS

NGF	=	Nerve Growth Factor
RA	=	retinoic acid
Dex	=	dexamethasone
RA	=	retinoic acid
GR-2	=	glucocorticoid receptor type-2
$p75^{NTR}$	=	neurotrophin receptor (NTR) subtype
TrkA	=	tropomyosin-related tyrosine kinase receptor
RU-28318	=	mineralocorticosteroid type 1 receptor (MR-1) antagonist, (spironolactone) 3,3-oxo-7 propyl-17-hydroxy-androstan-4-en-17yl-propionic acid lactone
RU-38484	=	GR-2 antagonist (mifepristone), 17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-propynylestra-4,9-diene-3-on
wtPC12	=	wild type pheochromocytoma cells
PC12 nnr5	=	cells lacking TrkA
PC12-6.24.-I	=	cells over-expressing TrkA
Ef	=	elongation factor
PRC	=	percentage of responsive cells

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REFERENCES

[1] McEwen, B.S.; deKloet, E.R.; Rostene, W. *Physiol. Rev.*, **1986**, *66*, 1121.
[2] Tanapat, P.; Gould, E. *Biol. Psychiat.*, **1999**, *46*, 1472.
[3] Glick, R.D.; Medary, I.; Aronson, D.C.; Scotto, K.W.; Swendeman, S.L.; La Quaglia, M.P. *J. Pediatr. Surg.*, **2000**, *35*, 465.
[4] Hoschl, C.; Hajek, T. *Eur. Arch. Psychiat. Clin. Neurosci.*, **2001**, *251*, 81.
[5] NIH Consensus Development Panel. *J. Am. Med. Assoc.*, **1995**, *273*, 413.
[6] Kreider, M.L.; Aldridge, J.E.; Cousins, M.M.; Oliver, C.A.; Seidler, F.J.; Slotkin, T.A. *Neuropsychopharmacol.*, **2005**, *30*, 1841.
[7] Sofroniew, M.V.; Howe, C.L.; Mobley, W.C. *Ann. Rev. Neurosci.*, **2001**, *24*, 1217.
[8] Vaudry, D.; Stork, P.J.; Lazarovici, P.; Eiden, L.E. *Science*, **2002**, *296*, 1648.
[9] Fujita, K.; Lazarovici, P.; Guroff, G. *Environ. Health Perspect.*, **1989**, *80*, 127.
[10] Hibberd, C.; Yau, J.L.; Seckl, J.R. *J. Anat.*, **2000**, *197*, 553.
[11] Wolkowitz, O.M.; Reus, V.I.; Weingartner, H. *Am. J. Psychiat.*, **1990**, *147*, 1297.
[12] Lazarovici, P.; Oshima, M.; Shavit, D.; Shibutani, M.; Jiang, H.; Monshipouri, M.; Fink, D.; Movsesyan, V.; Guroff, G. *J. Biol. Chem.*, **1997**, *272*, 11026.
[13] Katzir, I.; Shani, J.; Regev, K.; Shabashov, D.; Lazarovici, P. *J. Mol. Neurosci.*, **2002**, *18*, 251.
[14] Omidi, Y.; Campbell, L.; Barar, J.; Connell, D.; Akhtar, S.; Gumbleton, M. *Brain Res.*, **2003**, *990*, 95.
[15] Jiang, H.; Movsesyan, V.; Fink, D.W. Jr.; Fasler, M.; Whalin, M.; Katagiri, Y.; Monshipouri, M.; Dickens, G.; Lelkes, P.I.; Guroff, G.; Lazarovici, P. *J. Cell Biochem.*, **1997**, *66*, 229.
[16] Oshima, M.; Sithanandam, G.; Rapp, U.R.; Guroff, G. *J. Biol. Chem.*, **1991**, *266*, 23753.
[17] Slingerland, R.J.; Van Kuilenburg, A.B.; Bodlaender, J.M.; Overmars, H.; Voute, P.A.; VanGennip, A.H. *J. Chromatogr. B Biomed. Sci. Appl.*, **1998**, *716*, 65.
[18] Yang, T.T.; Tsao, C.W.; Li, J.S.; Wu, H.T.; Hsu, C.T.; Cheng, J.T. *Neurosci. Lett.*, **2007**, *426*, 45.
[19] von Langen, J.; Fritzemeier, K.J.; Diekmann, S.; Hilisch, A. *Chembiochem.*, **2005**, *6*, 1110.
[20] Abu-Raya, S.; Bloch-Shilderman, E.; Shohami, E.; Trembovler, V.; Shai, Y.; Weidenfeld, J.; Yedgar, S.; Gutman, Y.; Lazarovici, P. *J. Pharmacol. Exp. Ther.*, **1998**, *287*, 889.
[21] Scheibe, R.J.; Wagner, J.A. *J. Biol. Chem.*, **1992**, *267*, 17611.
[22] Rankin, S.L.; Guy, C.S.; Mearow, K.M. *Neurosci. Lett.*, **2005**, *383*, 305.
[23] Greene, L.A.; Liem, R.K.; Shelanski, M.L. *J. Cell Biol.*, **1983**, *96*, 76.
[24] Lecht, S.; Puxeddu, I.; Levi-Schaffer, F.; Reich, R.; Davidson, B.; Schaefer, E.; Mercinkiewicz, C.; Lelkes, P.I.; Lazarovici, P. In: Maragoudakis, M.E.; Papadimitriou, E. Eds. Angiogenesis: Basic Science and Clinical Applications. ISBN: 978-81-7895-302-1. Kerala, Transworld Research Network. **2007**; *in press*.
[25] Meyer, M.E.; Pornon, A.; Ji, J.W.; Bocquel, M.T.; Chambon, P.; Gronemever, H. *EMBO J.*, **1990**, *9*, 3923.
[26] Tocco, M.D.; Contreras, M.L.; Koizumi, S.; Dickens, G.; Guroff, G. *J. Neurosci. Res.*, **1988**, *20*, 411.
[27] Huff, K.; End, D.; Guroff, G. *J. Cell Biol.*, **1981**, *88*, 189.
[28] Rudkin, B.B.; Lazarovici, P.; Levi, B.Z.; Abe, Y.; Fujita, K.; Guroff, G. *EMBO J.*, **1989**, *8*, 3319.
[29] Atouf, F.; Tazi, A.; Polak, M.; Czernichow, P.; Scharfmann, R. *J. Neuroendocrinol.*, **1995**, *7*, 957.
[30] Yakovlev, A.G.; De Bernardi, M.A.; Fabrazzo, M.; Brooker, G.; Costa, E.; Mocchetti, I. *Neurosci. Lett.*, **1990**, *116*, 216.
[31] Brandoli, C.; Shi, B.; Pflug, B.; Andrews, P.; Wrathall, J.R.; Mocchetti, I. *Mol. Brain Res.*, **2001**, *87*, 61.
[32] Agarwai, M.K. *Pharmacol. Ther.*, **1996**, *70*, 183.
[33] Spitz, I.M.; Bardin, C.W. *N. Engl. J. Med.*, **1993**, *329*, 404.
[34] Nieman, L.K.; Chrousos, G.P.; Kellner, C.; Spitz, I.M.; Nisula, B.C.; Cutler, G.B.; Merriam, G.R.; Bardin, C.W.; Loriaux, D.L. *J. Clin. Endocrinol. Metab.*, **1985**, *61*, 536.
[35] Esposito, D.; Patel, P.; Stephens, R.M.; Perez, P.; Chao, M.V.; Kaplan, D.R.; Hempstead, B.L. *J. Biol. Chem.*, **2001**, *276*, 32687.
[36] Kahle, P.; Barker, P.A.; Shooter, E.M.; Hertel, C. *J. Neurosci. Res.*, **1994**, *38*, 599.
[37] Heampstead, B.L. *Curr. Opin .Neurobiol.*, **2002**, *12*, 260.
[38] Mamidipudi, V.; Wooten, M.W. *J. Neurosci. Res.*, **2002**, *68*, 373.
[39] Hasan, W.; Smith, H.J.; Tiang, A.Y.; Smith, P.G. *J. Neurobiol.*, **2005**, *65*, 192.
[40] Tirassa, P.; Thiblin, I.; Agren, G.; Vigneti, E.; Aloe, L.; Stenfors, C. *J. Neurosci. Res.*, **1997**, *47*, 198.
[41] Jameson, R.R.; Seidler, F.J.; Qiao, D.; Slotkin, T.A. *Neuropsychopharmacol.*, **2006**, *31*, 1647.
[42] Kim, J.B.; Ju, J.Y.; Kim, J.H.; Kim, T.Y.; Yang, B.H.; Lee, Y.S.; Son, H. *Brain Res.*, **2004**, *1027*, 1.
[43] Cereseto, M.; Reinés, A.; Ferrero, A.; Sifonios, L.; Rubio, M.; Wikinski, S. *Eur. J. Neurosci.*, **2006**, *24*, 3354.
[44] Belanoff, J.K.; Flores, B.H.; Kalezhan, M.; Sund, B.; Schatzberg, A.F. *J. Clin. Psychopharmacol.*, **2001**, *21*, 516.
[45] Kim, K.T.; Park, D.H.; Joh, T.H. *Neurology*, **2002**, *58*, 1436.

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