Dexamethasone-Induced Down-Regulation of Nerve Growth Factor Receptor p75\textsuperscript{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\textsuperscript{NTR} mRNA and protein levels, suggesting a transcriptional mechanism. This down regulation of p75\textsuperscript{NTR} was antagonized by the glucocorticoid type II receptor (GR-2), RU-38486 but not by the mineralocorticoid 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. Dexamethasone-activated down regulation of p75\textsuperscript{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\textsuperscript{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 prophylactically to pregnant women undergoing pre-term labor or directly to pre-term 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\textsuperscript{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\textsuperscript{NTR} receptors, a cellular process coincidently 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\textsuperscript{NTR} receptor process demonstrated in the present study, may represent a mechanism contributing to the corticosteroids-induced neuronal side effects.
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). 125I-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 anti-NGF antibodies anti-NGF 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-p75NTR 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% CO2 [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 37°C. 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 37°C 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.

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 65°C 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 80°C for 2 h and prehybridized according to manufacturer instructions. For hybridization, 0.5×10⁶ cpm/ml of 32P-human p75NTR (specific activity 1×10⁸ cpm/μg DNA) or 32P-cyclophilin (specific activity 1×10⁶ 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 (Qiogene/ 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 p75NTR 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 p75NTR 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: TCA-TGAA GATGAGCCTTGACATCCG-3' and antisense: CTT AGAA GCATTTGGCGGTGCAGATG-3', TrkA (232bp) sense: TGCC TGGCCTCTCTCTTCTA-3' and antisense:
GTGGTGAAACAGGCTCAC-3', p75NTR (663bp) sense: AGCCACCCAGACCCTGTGTG-3' and antisense: TTGCAGCTGTTCCACCTCT-3'. PCR products were analyzed by electrophoresis on agarose gel (2%) containing ethidium bromide for UV visualization. The quantitation of mRNA bands was estimated using QuantOne 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 125I-NGF binding was reduced by 50% after culturing in the presence of 0.5 μM Dex for 3 days (Fig. 1A). Reduction in 125I-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 125I-NGF binding indicating that the effect observed was specific for Dex and hydrocortisone (Fig. 1C).

The Dex-induced decrease in 125I-NGF binding was prevented completely by co-treatment of the cells with 1 μM of the glucocorticoid receptor antagonist RU-38486 but not the mineralocorticosteroid receptor type I (MR-1) antagonist RU-28318 (Table 1), indicating the effect was mediated by GR-2 receptors. Concentrations of Dex within the range of 10^-9 - 2x10^-8 M resulting in inhibition of 125I-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 catecholamine 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 to 10^-5 and * are statistically significant (p<0.05 by ANOVA).
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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 \( \mu \)M 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 \( \beta \)-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 \( \mu \)M 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 \( \mu \)M retinoic acid, 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(^{125}I)-NGF binding (fmol/mg protein)</th>
<th>Catecholamine content (ng/10^6 cell)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dopamine</td>
</tr>
<tr>
<td>Control</td>
<td>150 ± 13</td>
<td>211 ± 11</td>
</tr>
<tr>
<td>Ethanol</td>
<td>165 ± 12</td>
<td>205 ± 7</td>
</tr>
<tr>
<td>Dex</td>
<td>71 ± 8*</td>
<td>245 ± 4*</td>
</tr>
<tr>
<td>Dex/RU-28318</td>
<td>74 ± 17</td>
<td>239 ± 8</td>
</tr>
<tr>
<td>Dex/RU-38486</td>
<td>179 ± 9**</td>
<td>207 ± 9**</td>
</tr>
<tr>
<td>RU-28318</td>
<td>155 ± 8</td>
<td>189 ± 11</td>
</tr>
<tr>
<td>RU-38486</td>
<td>159 ± 11</td>
<td>202 ± 3</td>
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The values are mean ± SD of three experiments performed in triplicates. (*compared to control, ** compared to Dex, \( p<0.05 \) by ANOVA).

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 \( \mu \)g cell protein from samples of untreated cells (control), cells treated for 5 days with 0.5 \( \mu \)M Dex in the absence (Dex) or presence of 1 \( \mu \)M RU-38486 (Dex+RU) or cells treated for five days with 5 \( \mu \)M 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\textsuperscript{NTR} transcript by 40%. In PC12nnr5 cells that lack TrkA receptors, the level of p75\textsuperscript{NTR} transcript is low but detectable, as expected [22]. Dex treatment reduced the level of p75\textsuperscript{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\textsuperscript{NTR} transcript in these cells. The selective inhibition of p75\textsuperscript{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\textsuperscript{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\textsuperscript{NTR} transcription in these PC12 cell clones.

Fig. (3). The inhibitory effect of dexamethasone (Dex) on the expression of p75\textsuperscript{NTR} mRNA in wtPC12 cells (A) and PC12nnr5 cells (B). The cells were treated for 5 days with either 0.5 \( \mu \text{M} \) Dex in the absence (DEX) or presence of 1 \( \mu \text{M} \) RU-38486 (DEX+RU-38486) or 5 \( \mu \text{M} \) retinoic acid (RA) or left untreated (Control). Northern blot analysis was carried out using poly(A\textsuperscript{+}) RNA (20 \( \mu \text{g/lane} \)). The blot was hybridized with \( ^{32}\text{P} \)-labeled human p75\textsuperscript{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\textsuperscript{NTR} transcript by about 50% but not the level of TrkA receptor, as compared by the ratio to \( \beta \)-actin transcripts levels. Interestingly, Dex treatment resulted also with 85% inhibition of the mRNA for MAP-2 as compared by the ratio to \( \beta \)-actin transcripts levels. MAP-2 is coding for an important cytoskeletal 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 of p75\textsuperscript{NTR} mRNA in wtPC12 cells (A) and b.End3 endothelial cells (B). The cells were treated for 4 days with 1 \( \mu \text{M} \) Dex. mRNA was extracted and RT-PCR carried out using 50 \( \mu \text{g mRNA} \). Similar amounts of PCR products were loaded on the agarose gel for electrophoresis. For normalization the bands intensities were compared to the levels of \( \beta \)-actin. The mean value of transcript level ratio to \( \beta \)-actin is indicated on top of the bands.

It maybe questioned whether Dex induced decrease of p75\textsuperscript{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 \( \mu \text{M} \) Dex and measured the level of p75\textsuperscript{NTR} transcripts. Fig. (4B) clearly indicates that in endothelial cells Dex induced 50% reduction in p75\textsuperscript{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\textsuperscript{NTR} is implicated in the regulation of TrkA receptor tyrosine phosphorylation activity [22]. To determine if a Dex-induced reduction in p75\textsuperscript{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 \( \mu \text{M} \) Dex and/or 1 \( \mu \text{M} \) 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 occurs 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) was measured (Fig. 6A, 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 p75NTR receptor, a process correlated with increased TrkA receptor autophosphorylation and disruption of NGF-induced proliferation and differentiation. Dex-induced down regulation of p75NTR 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 3H-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 p75NTR are most probably mediate mainly by GR-2 since progesterone didn’t affect

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**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 tran- script (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.

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 hyp- campal 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 Alz-heimer’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 re- sponsible in part for the observed neuropathologies.

LIST OF ABBREVIATIONS

- NGF = Nerve Growth Factor
- RA = retinoic acid
- Dex = dexamethasone
- GR-2 = glucocorticoid receptor type-2
- \( p75^{NTR} \) = neurotrophin r eceptor (NTR) subtype
- TrkA = tropomyosin-related tyrosine kinase re- ceptor
- RU-28318 = mineralocorticosteroid type 1 receptor (MR-1) antagonist, (spironolactone) 3,3-ofoxo-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 nmr5 = cells lacking TrkA
- PC12-6.24.-I = cells over-expressing TrkA
- Ef = elongation factor
- PRC = percentage of responsive cells

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