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# Delayed translocation of NGFI–B/RXR in glutamate stimulated neurons allows late protection by 9-*cis* retinoic acid

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ABSTRACT

Nuclear receptor and apoptosis inducer NGFI-B translocates out of the nucleus as a heterodimer with RXR in response to different apoptosis stimuli, and therefore represents a potential pharmacological target. We found that the cytosolic levels of NGFI-B and RXRα were increased in cultures of cerebellar granule neurons 2 h after treatment with glutamate (excitatory neurotransmitter in the brain, involved in stroke). To find a time-window for potential intervention the neurons were transfected with gfp-tagged expressor plasmids for NGFI-B and RXR. The default localization of NGFI-Bgfp and RXRgfp was nuclear, however, translocation out of the nucleus was observed 2–3 h after glutamate treatment. We therefore hypothesized that the time-window between treatment and translocation would allow late protection against neuronal death. The RXR ligand 9-*cis* retinoic acid was used to arrest NGFI-B and RXR in the nucleus. Addition of 9-*cis* retinoic acid 1 h after treatment with glutamate reduced the cytosolic translocation of NGFI-B and RXRα, the cytosolic translocation of NGFI-Bgfp observed in live neurons, as well as the neuronal death. However, the reduced translocation and the reduced cell death were not observed when 9-*cis* retinoic acid was added after 3 h. Thus, late protection from glutamate induced death by addition of 9-*cis* retinoic acid is possible in a time-window after apoptosis induction.

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## 1. Introduction

NGFI-B (nerve-growth-factor-induced clone B, also known as Nur77, TR3 or NR4A1), an immediate early gene of the nuclear receptor superfamily [1], has been shown to play a key role in regulation of apoptosis, cell growth and transformation, inflammation and angiogenesis [2,3]. It has been shown that the NGFI-B level is high in several cancer cells, NGFI-B translocates from nuclei to mitochondria upon exposure of various cancer cells to different apoptotic stimuli [4–7], and it induces caspase 3 dependent apoptosis [4]. NGFI-B translocates to the mitochondria as a heterodimer with RXR in the LNCaP cell line [8]. A high affinity ligand for RXRs, 9-*cis* retinoic acid (9cRA) [9], suppresses a nuclear export sequence (NES) activity present in the carboxyl terminus of RXRα by inducing RXRα homodimerization or altering NGFI-B/RXR heterodimerization [8]. Consequently, RXR ligands were shown to inhibit mitochondrial targeting of NGFI-B/RXR heterodimers as well as the ability to induce apoptosis in cancer cells [8]. Several brain regions express the NGFI-B gene [10], however, far less is known about the role of NGFI-B in neuronal apoptosis. Cerebellar granule neurons express high basal levels of the NGFI-B protein, which is associated with mitochondria in neuronal apoptosis induced by excessive glutamate stimulation [11]. Glutamate is the major excitatory neurotransmitter in the mammalian CNS. When glutamate receptors are over-stimulated, e.g. during stroke, a mixture of apoptosis and necrosis is induced [12].

To evaluate if intervention with NGFI-B/RXR translocation out of the nucleus is a possible target for therapy aimed at reducing neuronal cell death following excitotoxic stimuli, further knowledge about the translocation process is needed. Here, we report that delayed (hours) translocation of NGFI-B/RXR out of the nucleus is associated with apoptosis induced by glutamate in cerebellar granule neurons, and that arresting NGFI-B in the nucleus by late addition of the RXRα ligand 9cRA reduces neuronal death.

## 2. Materials and methods

### 2.1. Materials

Rats were obtained from Harlan (Horst, The Netherlands). Basal Eagle's medium (BME) and fetal calf serum were from Gibco (Paisley,

*Abbreviations:* 9cRA, 9-*cis* retinoic acid; gfp, green fluorescent protein; glu, glutamate; NGFI-B, nerve-growth-factor-induced clone B; NMDA, *N*-methyl-D-aspartate; RXR, retinoid X receptor.

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UK). BCA protein assay kit and HRP-conjugated goat anti-rabbit antibody were from Pierce (Rockford, USA). RXRα and α-tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). Nur77 (NGFI-B) antibody was from Cell Signaling (Danvers, USA). Hoechst 33342 was obtained from Invitrogen (Oregon, USA). Pure nitrocellulose membrane was from Bio-Rad (Hercules, USA). SYBR green was obtained from Applied Biosystems (Warrington, UK). Caspase 3 inhibitor Z-DEVD-cmk was obtained from Bachem AG (Bubendorf, Switzerland). All other reagents were from Sigma (St. Louis, USA).

### 2.2. Cell culture and treatments

Cerebellar granule neurons were obtained from 7–8-day old albino rats [13,14]. The cells were seeded on plastic dishes coated with poly-L-lysine and cultured in basal Eagle's medium supplemented with 2 mM glutamine, 100 μg/ml gentamicin, 10% heat inactivated fetal calf serum, and 25 mM KCl [14]. To prevent growth of non-neuronal cells, cytosine arabinofuranoside (10 μM final concentration) was added to the cultures 16–18 h after seeding. Glutamate treatment of the cells day 6–7 *in vitro*: cultures were preincubated with physiological buffer (in mM; NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 2.3, glucose 5.6, HEPES 5, pH 7.4) in the presence or absence of 9cRA (1 μM) or caspase 3 inhibitor (1 μM) for 15 min before they were exposed to 100 μM glutamate (in the presence of 10 μM glycine as coagonist) in physiological buffer for 15 min at room temperature. Control cultures were either untreated or exposed to physiological buffer in the absence of glutamate but in the presence of 1.2 mM $MgCl_2$, a concentration that has been shown to block the NMDA receptor-induced toxicity [15]. At the end of glutamate or buffer exposure, cultures were washed and incubated in serum-free basal medium (25 mM KCl). The cells were harvested for Western blotting (after 2 h) or real-time polymerase chain reaction (after 0.5, 1, 2, or 3 h), or cell death was measured by trypan blue exclusion (after 24 h), a method suitable for primary neurons since it offers visual control of cell morphology and cell type. Animals used for cell cultures were handled in accordance with the Norwegian Animal Welfare Act and the EU directive 2010763/EU for animal experiments.

### 2.3. Western blot analysis

The cells were washed twice with ice-cold phosphate-buffered saline. Whole cell lysates were harvested in 2% SDS. For cytosol enriched fractions cells were harvested in 0.25 M sucrose buffered with 100 mM Hepes pH 7.4, centrifuged at 3500 rpm for 15 min at 4 °C, and 10 μl 20% SDS was added to the supernatant. Equal amounts of proteins were subjected to electrophoresis on a 10% polyacrylamide gel. Proteins were transferred onto pure nitrocellulose membranes. After protein transfer, membranes were blocked with 5% fat free dry milk in TBS (Tris buffered saline) for 1 h before addition of anti NGFI-B (1:1000), RXRα (1:200), lamin A/C (1:1000), or α-tubulin (1:1000) antibodies and incubated at 8 °C overnight. The membranes were then incubated for 1 h at room temperature in TBS-T containing horseradish peroxidase-linked secondary antibody (1:10,000). Immunoreactive products were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Western blotting detection system (Pierce, USA). Band intensities were normalized to α-tubulin. The specificity of the NGFI-B antibody was demonstrated by transfection of CV1 cells with gfp-tagged expressor plasmids for the three proteins in the NR4A nuclear receptor subfamily (NGFI-Bgfp, Nurr1gfp, or NOR-1gfp). Similar gfp-levels were detected with Western blotting for all the three proteins using a gfp antibody (1:5000, Clontech (California, USA)) whereas the NGFI-B antibody only detected NGFI-Bgfp (results not shown).

### 2.4. Reverse transcription followed by real-time polymerase chain reaction (PCR)

Total RNA extraction was performed using Agilent Total RNA Isolation Mini Kit (50) (Aligent Technologies Inc., USA). Total RNA was reversely transcribed with hexamer primers using a Perkin–Elmer Thermal Cycler 9600 (25 °C for 10 min, 37 °C for 1 h, 99 °C for 5 min), and a TaqMan reverse-transcription reagent kit (Applied Biosystems, UK). Real-time PCR was performed using an ABI PRISM 7000 Detection System (Applied Biosystems). DNA expression was determined by SYBR Green. Primers were designed using Primer Express (Applied Biosystems): NGFI-B (U17254) F-CATCTTCTTCCTCGTCCTCG, R-AACTGCTCAGTCCATACCCG; β-actin (NM_031144) F-GATGACGATATCGCTGCGCTC, R-GTCAGGATGCCT CTCTTGCTC. Each target was quantified in duplicate or triplicate for each experiment (3–9) according to the protocol of Applied Biosystems. The transcription levels were normalized to the housekeeping control gene β-actin.

### 2.5. Transient transfection

Rat cerebellar granule neurons were cultured for 4–5 days before transfection. Transient transfection of the expressor plasmid NGFI-Bgfp [11] or RXRgfp (kind gift from Dr. Noa Noy, Department of Pharmacology, Case Western Reserve University, Ohio) into the neurons was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, USA). Cells were transfected with 1 μg of NGFI-Bgfp or RXRgfp with the use of 6 μl lipofectamine™ transfection reagent per 35 mm dish (Ibidi). The medium was removed before addition of the transfection mixture and incubation for 5 h, and the medium was then put back. Two days after transfection neurons were treated with buffer or glutamate. Cells were stained with Hoechst 33342 (0.1 μg/ml for 30 min) before the cells were examined with a Nikon TE2000-E microscope configured with a C1 Nikon confocal unit run by EZ-C1 software.

### 2.6. Statistical analysis

The variation in the results from the dishes treated the same way was comparable for dishes from the same experiment and dishes from independent experiments. Therefore, all the dishes were considered independent, and values are given as mean ± S.E. of 3–19 dishes from 2 to 7 experiments. Multiple comparisons were analyzed by one-way ANOVA, followed by Dunn's or Dunnett's post hoc test (SigmaStat software). When two groups were compared student's *t*-test or Mann–Whitney Rank Sum Test was used. A $p$-value of $<0.05$ was considered significant.

## 3. Results

### 3.1. NGFI-B and RXR translocates out of the nucleus following treatment with glutamate

To make sure that sufficient NGFI-B protein was present to be a part of the apoptosis process whole cell lysates were harvested 2 h after initiation of the different treatments and Western blots were analyzed. Glutamate treatment involves the replacement of medium with a physiological buffer (for 30 min). Therefore, the cells were also treated with buffer alone as a control to evaluate the effect of this procedure. The results showed a maintained NGFI-B level 2 h after initiation of treatment with buffer or glutamate (Fig. 1A) as well as after 6 h (results not shown). It has previously been shown in PC12 cells that NGFI-B is rapidly posttranslationally modified [16]. In accordance with this all the treatments induced a modification of the NGFI-B protein resulting in increased molecular

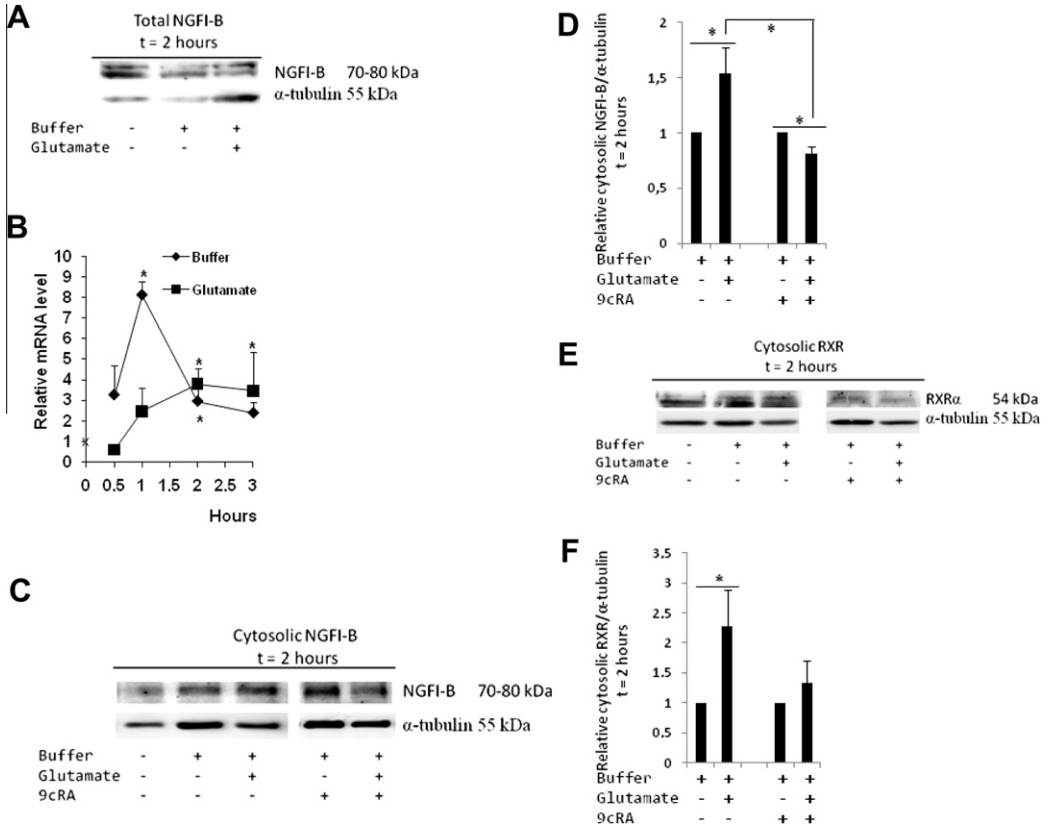


**Fig. 1.** The increased cytosolic NGFI-B and RXR levels after initiation of glutamate treatment are inhibited by addition of 9cRA. Neurons were treated with buffer or glutamate (100 μM for 15 min). (A) Cerebellar granule neurons were harvested in 2% SDS 2 h after initiation of treatment. Western blot analysis was done with NGFI-B and α-tubulin primary antibodies, and horseradish peroxidase-linked secondary antibody. One representative of three different experiments is shown. (B) The expression of NGFI-B was analyzed using reverse transcription followed by real-time polymerase chain reaction and the transcription level was normalized to β-actin. Neurons were harvested at different time-points after initiation of treatment. The *X* on the *y*-axis is the NGFI-B mRNA level in the untreated cells. Results are mean ± SE from 3–9 independent experiments with duplicates. $^{*}p < 0.05$ versus untreated cells, Dunn's post hoc test. (C–F) The RXR ligand 9cRA (1 μM) was added 1 h after initiation of treatment. Neurons were harvested 2 h after initiation of treatment in 0.25 M sucrose buffered with 100 mM Hepes, and fractionated as described in Section 2. Western blot analysis was done with NGFI-B, RXRα, or α-tubulin primary antibodies, and horseradish peroxidase-linked secondary antibody. (C) Western blot shows the cytosolic NGFI-B level at 2 h after initiation of the treatments. One representative of 4 different blots is shown. (D) Cytosolic NGFI-B in glutamate exposed neurons harvested 2 h after initiation of the treatments was quantified and presented as fraction of respective buffer treatment. Results are mean ± SE from four independent experiments. $^{*}p < 0.05$ Mann Whitney Rank Sum Test. (E) Western blot shows the cytosolic RXRα level at 2 h. One representative blot of 3 is shown. (F) Total cytosolic RXRα was quantified and presented as fraction of RXRα in the buffer treated neurons. Results are mean ± SE from 4–5 dishes from 3 independent experiments. $^{*}p < 0.05$ versus glutamate exposed cells, Mann Whitney Rank Sum Test. 9cRA = 9-*cis* retinoic acid.

weight (Fig. 1A). To further examine the presence of endogenous NGFI-B during the treatment time, neurons were transfected with a plasmid containing the NGFI-B response element (NBRE) linked to a reporter gene (luciferase). The neurons were harvested 6 h subsequent to treatment, and luciferase was measured. There was no significant difference between the luciferase level in neurons treated with glutamate and their controls (results not shown), supporting the presence of endogenous NGFI-B in the cells during the treatment time. Both glutamate and buffer induced the expression of *NGFI-B* (Fig. 1B), further supporting the presence of endogenous NGFI-B. The expression pattern differed, showing more long lasting NGFI-B mRNA after glutamate treatment compared to buffer. To evaluate the localization of endogenous NGFI-B, cytosol enriched fractions were prepared. Western blotting analyses showed that the cytosolic fraction contained approximately 65% of the total cellular tubulin and 32% of the total cellular lamin A/C (results not shown). The results showed an increase in the cytosolic NGFI-B in glutamate treated neurons 2 h after initiation of treatment compared to buffer (Fig. 1C and D). It has been reported that NGFI-B translocates as a heterodimer with RXR [8]. Therefore, the cytosolic level of RXRα 2 h after glutamate treatment was also evaluated. The cytosolic RXRα level was increased in glutamate treated neurons compared to buffer treated neurons (Fig. 1E and F). These results indicate the presence of an apoptosis-inducing signal in glutamate treated neurons, whereas in the absence of an apoptosis signal endogenous NGFI-B and RXR remained nuclear. Addition of the RXR ligand 9cRA arrests NGFI-B/RXR in the nucleus [8]. In accordance with this we found that addition of 9cRA resulted in a reduction in the cytosolic level of NGFI-B (Fig. 1C and D) and a tendency for reduction of the cytosolic level of RXRα (Fig. 1E and F).

### *3.2. NGFI-Bgfp and RXRgfp translocate out of the nucleus following treatment with glutamate*

The neurons were transfected with either NGFI-Bgfp or RXRgfp expressor plasmids, and the localization of the gfp-tagged proteins was visualized live 1–9 h after initiation of the different treatments. Both NGFI-Bgfp and RXRgfp localized to the nucleus in the controls whereas it also localized to the thin rim of cytosol surrounding the nucleus as well as some neurites in glutamate treated neurons (Fig. 2A–D). Translocated NGFI-Bgfp and RXRgfp remained cytosolic during the observation time. The RXR ligand 9cRA was added to the NGFI-Bgfp transfected neurons 1 or 3 h after glutamate treatment to evaluate if this would arrest NGFI-Bgfp in the nucleus. The addition at 1 h reduced the translocation of NGFI-Bgfp out of the nucleus in response to glutamate,

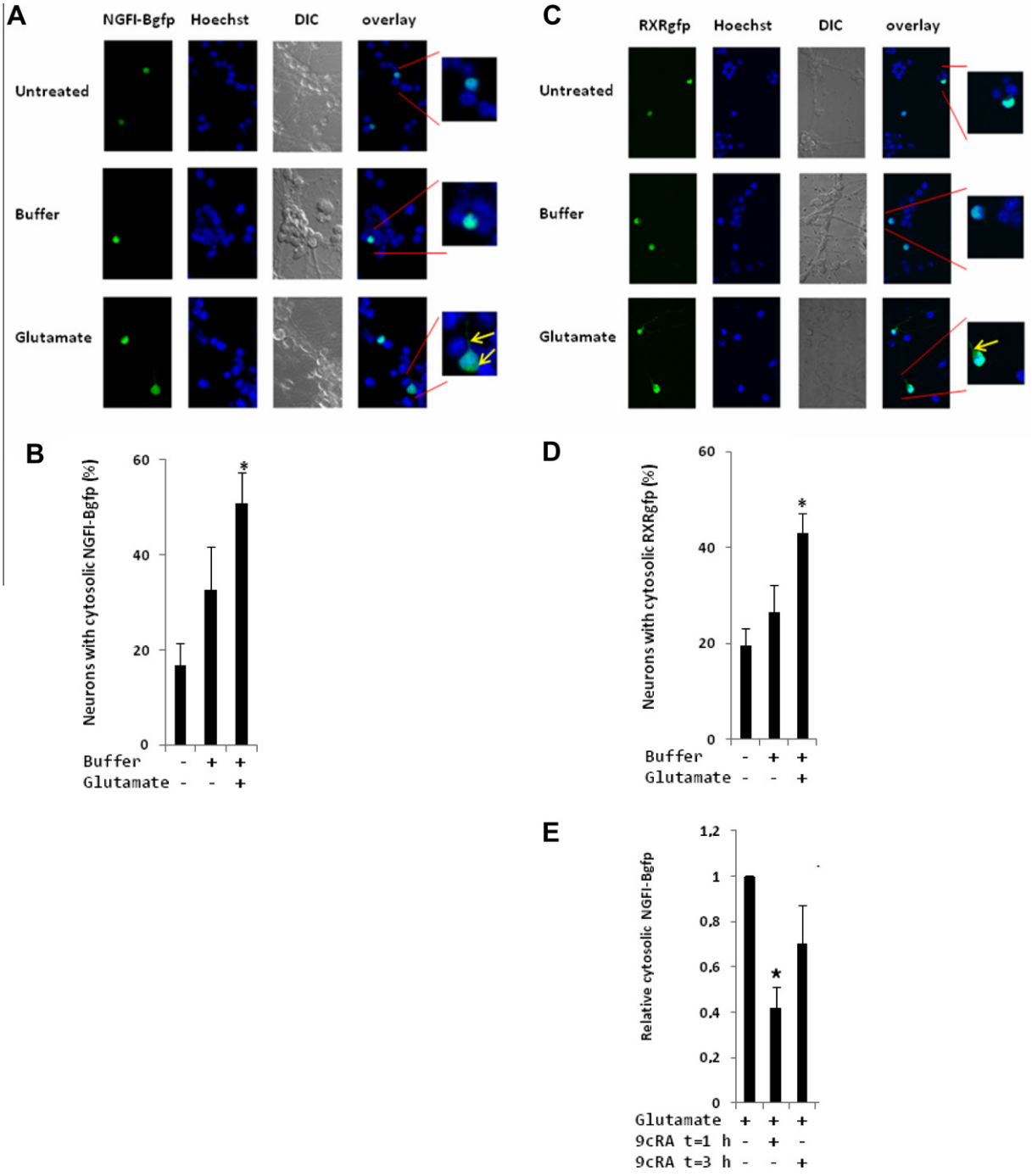


**Fig. 2.** Glutamate induces translocation of NGFI-Bgfp and RXRgfp out of the nucleus. Neurons were transfected with NGFI-Bgfp or RXRgfp. The cells were treated with glutamate (100 μM for 15 min) and stained with Hoechst (0.1 μg/ml for 30 min) before investigation of the localization of NGFI-Bgfp or RXRgfp 1–9 h after initiation of treatment. Images from live neurons were taken 3–9 h after initiation of the treatment. Arrows show cytosolic localization of NGFI-Bgfp (A) or RXRgfp (C), that is, regions of green fluorescence which are not overlapping with blue fluorescence (Hoechst). The number of neurons with cytosolic localization of NGFI-Bgfp (B) or RXRgfp (D) was quantified. (B) Results are mean ± SE from 3–5 dishes from 2–3 independent experiments. $^{*}p < 0.05$ versus untreated cells, Dunnett's post hoc test. (D) Results are mean ± SE from 6–10 dishes from two independent experiments. $^{*}p < 0.05$ versus untreated cells, Dunnett's post hoc test. (E) 9cRA (1 μM) was added 1 or 3 h after initiation of treatment. Results are mean ± SE from 5 dishes from two independent experiments. $^{*}p < 0.05$ versus glutamate treated cells, Dunn's post hoc test. 9cRA = 9-*cis* retinoic acid.

whereas the addition at 3 h did not (Fig. 2E). The glutamate induced translocation of NGFI-Bgfp and RXRgfp out of the nucleus was delayed and could first be observed 2 h after initiation of the cell death stimulus. These results support the presence of an apoptosis-inducing signal in glutamate treated neurons. As a control cells were transfected with gfp. Gfp localized to nucleus, cytosol, and neurites, and did not translocate after any of the treatments (results not shown).

### 3.3. RXR ligand 9cRA reduces apoptosis induced by glutamate

Since it was possible to inhibit the NGFI-B/RXR translocation by 9cRA added one, but not three, hours after initiation of treatment, we wanted to investigate the effect of 9cRA addition on the cell death induced by glutamate. First, the cell death induced by glutamate was investigated in the presence or absence of caspase 3 inhibitor since activated caspase is downstream of NGFI-B in the apoptosis process.

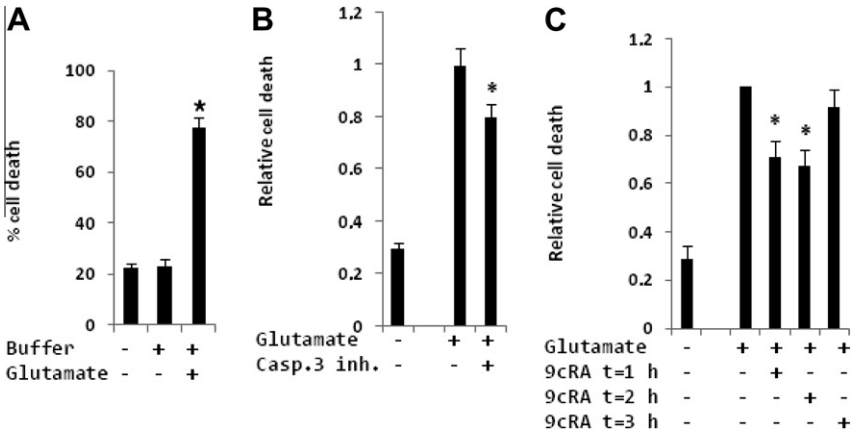


**Fig. 3.** 9cRA reduces glutamate induced caspase 3 dependent apoptosis. Neurons were treated with buffer (15 min) or glutamate (100 μM for 15 min), and cell death was measured by trypan blue assay 24 h after initiation of treatment. (A) Results are mean ± SE from 5–19 dishes from 3–7 independent experiments. $^{*}p < 0.05$ versus untreated cells, Dunnett's post hoc test. (B) Caspase 3 inhibitor was added 15 min before and during treatment with glutamate. Results are mean ± SE from 5–7 dishes from 3 independent experiments. $^{*}p < 0.05$ comparing presence or absence of caspase 3 inhibitor, *t*-test. (C) The RXR ligand 9cRA (1 μM) was added 1, 2, or 3 h after initiation of treatment. Results are mean ± SE from 4–6 dishes from 2–6 independent experiments. $^{*}p < 0.05$ versus glutamate treated neurons, Dunn's post hoc test. Casp. 3 inh. = caspase 3 inhibitor; 9cRA = 9-*cis* retinoic acid.

The results showed that glutamate (100 μM for 15 min in $Mg^{2+}$ free buffer) induced neuronal death (Fig. 3A), and that pretreatment with caspase 3 inhibitor (1 μM) partly reduced the neuronal death induced by glutamate (Fig. 3B). This confirms that glutamate induces apoptotic cell death, consistent with the observed translocation of NGFI-B and RXR induced by these treatments. Since there was a delay between the initiation of the treatment with glutamate and the translocation of NGFI-Bgfp, 9cRA was added at different time-points after the initiation of the treatment to evaluate if late protection was possible. The addition of 9cRA at 1 or 2 h after exposure to glutamate reduced the neuronal death whereas addition at 3 h had no effect on the cell death (Fig. 3C). The RAR specific ligand TTNPB (ethyl-*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]-benzoic acid) did not affect the cell death (not shown). Thus, the reduction in the cell death after addition of 9cRA was due to an interaction with RXR and not RAR, in support of the proposed mechanism for NGFI-B/RXR cotransport. Thus, the effect of 9cRA protection against cell death mirrors its effect on NGFI-B/RXR translocation.

## 4. Discussion

We have previously reported that NGFI-B translocation to the mitochondria is involved in apoptosis induced by glutamate in cerebellar granule neurons [11]. RXRα serves as a shuttle for the mitochondrial targeting of Nur77 (mouse NGFI-B), and Nur77 nuclear export and mitochondrial targeting require RXR heterodimerization [7,8,17]. Therefore, using RXR ligands to interfere with the localization of RXRα may provide a mechanism to modulate the activity of NGFI-B [8]. In this study we show that it is possible to interfere with the translocation of NGFI-B/RXR by addition of RXR ligand the first 2 h after the initiation of the apoptosis stimuli due to a delay between the onset of the stimuli and the translocation and thereby reduce the neuronal death.

Glutamate treatment resulted in cell death which was partially caspase 3 dependent and translocation of NGFI-B out of the nucleus. To evaluate the time-course of the NGFI-B translocation in live neurons, transfection with NGFI-Bgfp and RXRgfp was performed. Translocation of NGFI-Bgfp and RXRgfp out of the nucleus was observed from 2 h after initiation of the apoptosis treatments, comparable to the observed translocation of wild type protein measured at 2 h. Consistent with the delayed NGFI-B/RXR translocation (2 h after treatment) addition of 9cRA at different time-points after treatment with glutamate (1 or 2 h) reduced the cell death whereas later addition did not.

It has been shown in our laboratory that the basal level of the NGFI-B protein in cerebellar granule neurons is sufficient to be used in a cell death pathway (shown by immunostaining of wild type protein) [11], and this was confirmed by Western blotting analysis of total NGFI-B protein 2 h after the onset of the apoptosis stimuli. Thus, there was sufficient endogenous NGFI-B present in the neurons during the period when the translocation of NGFI-Bgfp and RXRgfp was observed.

NGFI-B is often overexpressed in cancer cells (reviewed by [18]). The apoptotic effect of Nur77 (mouse NGFI-B) in cancer cells was discovered during investigation of the apoptotic effect of AHPN (6-(3-(1-adamantyl)-4-hydroxyphenyl)-2-naphta-lenecarboxylic acid), a molecule currently being evaluated in clinical trials as an anticancer agent [19,20]. It has also been reported that Nur77 plays a role in fenretinide-induced apoptosis (a possible therapeutic agent in cancer treatment and prevention) [21]. Nur77-induced apoptosis by AHPN or fenretinide involves the mitochondrial pathway in many cancer cells [21,22]. However, mitochondrial targeting of NGFI-B may also induce neuronal death as shown in the present study and in [11]. Thus, caution needs to be taken to avoid potential neurotoxicity associated with cancer therapeutic agents. A related example is antitumor drug procaspase-3-activating compound 1 (PAC-1), which in high concentrations induces neurotoxicity. By synthesizing a derivative of PAC-1, S-PAC-1, that had lower propensity to cross the blood brain barrier, neurotoxicity was no longer observed [23]. However, targeting NGFI-B to the nucleus in neurons may reduce neuronal death, e.g. following stroke, and late protection by RXR ligands may be possible due to the delay in the translocation of NGFI-B/RXR.

These results support the presence of an apoptosis-inducing signal in glutamate treated neurons that induces a delayed translocation of NGFI-B/RXR out of the nucleus, which can be inhibited by addition of 9cRA within 2 h after initiation of the cell death stimuli. Inhibition of the translocation also reduces the neuronal death, thus, the translocation is a possible target for therapy aimed at neuroprotection.

## Conflict of interest

The authors declare no conflict of interest.

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