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Neuronal cell death induced by antidepressants: lack of correlation with Egr-1, NF-κB and extracellular signal-regulated protein kinase activation

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Abstract

The tricyclic antidepressants (TCA) amitriptyline and desipramine and the serotonin reuptake inhibitor fluoxetine induce, at μM concentrations, cell death in HT22 immortalized hippocampal neurons and PC12 pheochromocytoma cells. Here, we show that these neurotoxic effects are accompanied by a selective activation of extracellular signal-regulated protein kinase (ERK), the biosynthesis of the transcription factor Egr-1 and an increase in the transcriptional activity of NF-κB. However, an impairment of both ERK activation and Egr-1 biosynthesis by the MAP kinase kinase-1 (MEK-1) inhibitor PD98059 did not block cell death. Moreover, stimulation of ERK phosphorylation and Egr-1 biosynthesis by sphingosine-1-phosphate did not induce cell death, indicating that stimulation of the ERK signaling pathway and Egr-1 biosynthesis are not required for neuronal cell death induced by antidepressants. Likewise, attenuation of antidepressant-induced NF-κB activity by elevation of the intracellular cAMP concentration or by retroviral driven expression of the non-degradable superrepressor IκBαS32A/S36A demonstrated that the elevation of NF-κB activity by amitriptyline, desipramine and fluoxetine is not an integral part of the apoptotic signaling cascade triggered by these compounds.

Keywords: Antidepressants; Egr-1; Extracellular signal-regulated kinase; Neuronal cell death; NF-κB; Sphingosine-1-phosphate

1. Introduction

TCA have been used since 40 years for the treatment of major depression. The initial action of these compounds is an immediate inhibition of norepinephrine and, in part, serotonin, reuptake into the nerve endings. Desipramine functions as the most selective norepinephrine-reuptake inhibitor among the TCA [1]. Thus, noradrenergic and, in part serotonergic, functions in the brain are elevated as a result of TCA treatment. In contrast, compounds such as fluoxetine are selective inhibitors of the active reuptake of serotonin and they are therefore termed ''selective serotonin reuptake inhibitors'' (SSRIs).

The TCA drugs exhibit, in addition to their primary action of inhibiting norepinephrine and serotonin transport, many other neuropharmacological reactions. Thus, the neuropharmacology of TCA cannot be reduced simply to the initial inhibition of norepinephrine and serotonin reuptake into the synapse. Likewise, the SSRIs have been shown to trigger a complex set of secondary reactions that follow the initial blockade of neuronal transport of serotonin. Chronic administration of antidepressants has been shown, for example, to increase the expression of cAMP-specific phosphodiesterases and upregulate transcription mediated *via* the cAMP-responsive element [2,3].

Here, we have studied the cellular effects of μM concentrations of amitriptyline, desipramine and fluoxetine in the neuronal cell lines HT22 and PC12. Amitriptyline and desipramine belong to the group of TCA drugs. Amitriptyline is used for the treatment of spontaneous endogenous depression and exhibits an extreme sedative effect (http://www.psyweb/com/Drughtm/amitri.html). Amitriptyline is

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Abbreviations: dbcAMP, *N*,2′-*O*-dibutyryladenosine-3:5-cyclic monophosphate; Egr-1, early growth response 1; ERK, extracellular signal-regulated protein kinase; IBMX, isobutyl-1-methylxanthine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCA, tricyclic antidepressants; TUNEL, terminal deoxynucleotidyl transferase fluorescein-dUTP 3′-end labeling.

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still the ''leading antidepressant'' after 40 years of research [4]. Desipramine is used for the treatment of endogenous depression and is also known to reduce the craving for cocaine (http://www.psyweb/com/Drughtm/desipr.html). Fluoxetine (Prozac) is a serotonin-specific drug used in the treatment of depression. Fluoxetine is often administrated to patients not responding to heterocyclic antidepressants.

Recently, tricyclic antidepressants and selective serotonin uptake inhibitors were reported to induce apoptosis in neuronal and glioma cells and in lymphocytes [5–7]. It has been proposed that those compounds generate oxidative stress and a subsequent activation of the transcription factor NF-κB, that finally leads to cell death [7]. NF-κB has been proposed to function either as a pro-apoptotic or as an anti-apoptotic protein, determined by the cell type and the apoptotic inducers. Recently, we generated a PC12 pheochromocytoma cell line expressing an NF-κB-responsive reporter gene. Additionally, a cell line was engineered that expresses—together with the NF-κB-responsive reporter gene—a non-degradable form of IκB that impairs NF-κB activation by retaining NF-κB in the cytoplasma (Erlandsson, Baumann, Rössler, Kaufmann, Giehl, Wirth and Thiel, manuscript submitted). The fact that the activation of NF-κB was proposed to be responsible for the neurotoxic effects of μM concentration of antidepressants [7] prompted us to investigate the relationship between NF-κB activity and neuronal cell death using these PC12 cell lines as tools. Moreover, we have studied the functional role of ERK in amitriptyline, desipramine or fluoxetine treated HT22 immortalized hippocampal cells, because in these cells, a persistent activation of ERK has been reported to be associated with glutamate-induced oxidative toxicity [8], suggesting that antidepressant-induced cell death may require an activation of ERK. The results show that μM concentrations of antidepressants induce a specific signaling cascade in neuronal cells involving an activation of ERK, synthesis of the zinc finger protein Egr-1, and stimulation of NF-κB. However, we provide evidence that activation of ERK and NF-κB as well as the synthesis of Egr-1 are not required for the cell damage induced by amitriptyline, desipramine or fluoxetine.

2. Materials and methods

2.1. Chemicals

Amitriptyline, desipramine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluoxetine, isobutyl-1-methylxanthine (IBMX), *N*,2′-*O*-dibutyryladenosine-3:5-cyclic monophosphate (dbcAMP), and puromycin were purchased from Sigma–Aldrich Chemie GmbH. G418, neurobasal medium and N_2-supplement were from Invitrogen GmbH. The MEK inhibitor PD98059 was purchased from Calbiochem (# S513000).

2.2. Cell culture

The HT22 immortalized hippocampal cell line was a kind gift of David Schubert, The Salk Institute, La Jolla, CA [9]. The cells were cultured in DMEM medium supplemented with 10% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine. The PC12κBluc cell line is an engineered subclone of PC12 pheochromocytoma cells containing an integrated κB-dependent luciferase reporter transcription unit. Cells were maintained in RPMI medium supplemented with 10% heat inactivated horse serum, 5% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, and 2.0 μg/mL puromycin at 37° in 5% CO_2. PC12κBluc-IκBαS32A/S36A cells and PC12κBluc-neo cells that express the non-degradable *IκBαS32A/S36A* mutant or neomycin acetyltransferase, respectively, were additionally cultured in 0.6 mg/mL G418. The generation of these PC12 cell lines were described recently (Erlandsson, Baumann, Rössler, Kaufmann, Giehl, Wirth and Thiel, manuscript submitted).

2.3. Reporter gene assays

To measure NF-κB activities in PC12κBluc cells, cells were seeded at the density of 2×10^5 cells per well into 12-well plates in RPMI medium supplemented with 10% heat inactivated horse serum, 5% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine, and incubated overnight. The next day, the medium was changed to neurobasal medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine and 1% N_2-supplement. The cells were incubated for a further 24 hr period, the medium was renewed and the drugs were applied to the cells as indicated. Cells were harvested in cold PBS, washed twice and lysed using reporter lysis buffer (# 397A) from Promega GmbH. Luciferase activities were measured as described [10]. Protein concentrations were determined using the BCA assay (Pierce). Luciferase activities were expressed as luciferase light units/μg protein. The experiments were done at least twice with similar results obtained each time.

2.4. Survival assays and detection of apoptotic markers

Cells were seeded in quadruplicate at a density of 2.5×10^3 (HT22 cells) or 1×10^4 cells per well (PC12κBluc cells) in 96-well plates in serum-containing medium. For HT22, the serum concentration was reduced to 5%. 24 hr later, the medium was renewed for HT22 cells and the drugs were added. For PC12κBluc cells, the medium was replaced by neurobasal medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine and 1% N_2-supplement. The cells were incubated for 24 hr and the medium was renewed before the application of the compounds. The mitochondrial reduction capacities were

determined by quantification of the levels of MTT reduction to formazan dye crystals. MTT solution (0.5 mg/mL final concentration per well, dissolved in PBS) was added to cultures and incubated for 4 hr at 37° in 5% CO_2. Cells were solubilized in 10 mM HCl containing 10% SDS, and the plates were incubated overnight at 37°. Absorbance was quantified on a BioRad Model 550 microplate reader, using a test wavelength of 595 nm. MTT-reduction was expressed as a percentage of controls.

Apoptotic DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated 3′-tailing with TMR red-labeled dUTP (TUNEL) using the "*in situ* cell death detection kit" (Roche Diagnostics) according to the manufacturers protocol.

2.5. Preparation of nuclear extracts and Egr-1 immunoblot analysis

Preparation of whole cell extracts and nuclear extracts and detection of phosphorylated ERK and Egr-1 immunoreactivities by Western blotting were performed as previously described [11].

3. Results

3.1. Induction of neuronal cell death by amitriptyline, desipramine, or fluoxetine

The cytotoxic effects of μM concentrations of the antidepressants amitriptyline, desipramine, and fluoxetine on neuronal cell survival was tested in two neuronal cell lines, that have been frequently used to monitor neurocytotoxic effects. HT22 cells are immortalized hippocampal cells that are very susceptible to oxidative stress [9]. The PC12 pheochromocytoma cell line is a well known model for the study of neuronal differentiation, signaling and cell death [12]. Here, we used a subclone of PC12 cells containing an integrated NF-κB responsive reporter gene (see below). To study the effects of antidepressants on cell survival, HT22 and PC12κBluc cells were treated with different concentrations of amitriptyline, desipramine, or fluoxetine, respectively. Cells were incubated for 24 hr with the indicated concentrations of these compounds and the mitochondrial reduction capacities were measured using the reduction of MTT to formazan dye crystals. In this assay, colorless tetrazolium salts are reduced by mitochondrial NADP(H)-dependent dehydrogenases to form a colored product termed formazan that was subsequently detected spectrophotometrically. The data are presented in comparison to the amount of formazan formed in the absence of the cytotoxic compound. Fig. 1 (left panels) shows that concentrations of 50 μM amitriptyline, 10 μM desipramine, and 20 μM fluoxetine were neurotoxic for HT22 cells. For PC12κBluc cells, slightly higher concentrations were necessary to exhibit similar effects. As shown

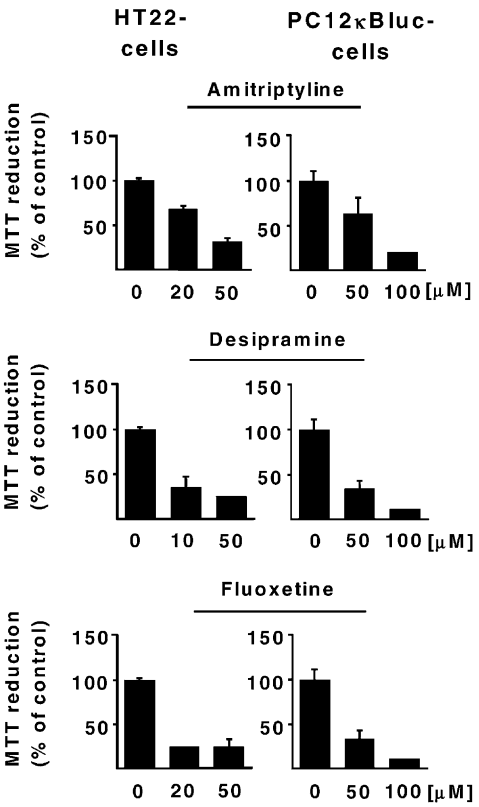

Fig. 1. Induction of cell death in HT22 and PC12κBluc cells by antidepressants. HT22 and PC12κBluc cells were exposed for 24 hr to different concentrations of amitriptyline, desipramine, or fluoxetine as indicated. The cytotoxic effects of the applied compounds were determined spectrophotometrically by the MTT method.

in Fig. 1 (right panels), concentrations of 100 μM amitriptyline, 50 μM desipramine, and 50 μM fluoxetine were neurotoxic for PC12κBluc cells. Please note that these concentrations are usually not found in patients treated with antidepressants (usual dose: amitriptyline, 100–200 mg per day; desipramine, 100–200 mg per day; fluoxetine, 20–40 mg per day [1]). For amitriptyline and desipramine, a typical concentration of 100–250 ng/mL is found in the serum, whereas toxic effects can be expected at serum concentrations above 500 ng/mL [1].

3.2. Induction of ERK phosphorylation and Egr-1 biosynthesis by antidepressants in HT22 cells

The activation of ERK is often associated with a mitogenic response that counteracts cell death-inducing signals. Surprisingly, a persistent activation of ERK has been reported to be associated with glutamate-induced oxidative toxicity in HT22 cells. Likewise, inhibition of the ERK activation protected HT22 cells from glutamate toxicity [8]. Therefore, we tested whether the antidepressants amitriptyline, desipramine, and fluoxetine function as activators of ERK. HT22 cells were cultured in medium containing 5% fetal calf serum. Cells were treated with 50 μM amitriptyline, 10 μM desipramine, or 20 μM fluoxetine for 2, 5 and

10 min. Whole cell extracts were prepared and ERK activation monitored by Western blotting using an antibody against the phosphorylated form of the kinase. The antibody mainly detected the activated p42 isoform of ERK termed ERK2. Fig. 2A shows that administration of amitriptyline, desipramine, or fluoxetine triggered a rapid phosphorylation, i.e. activation of ERK2 in HT22 cells.

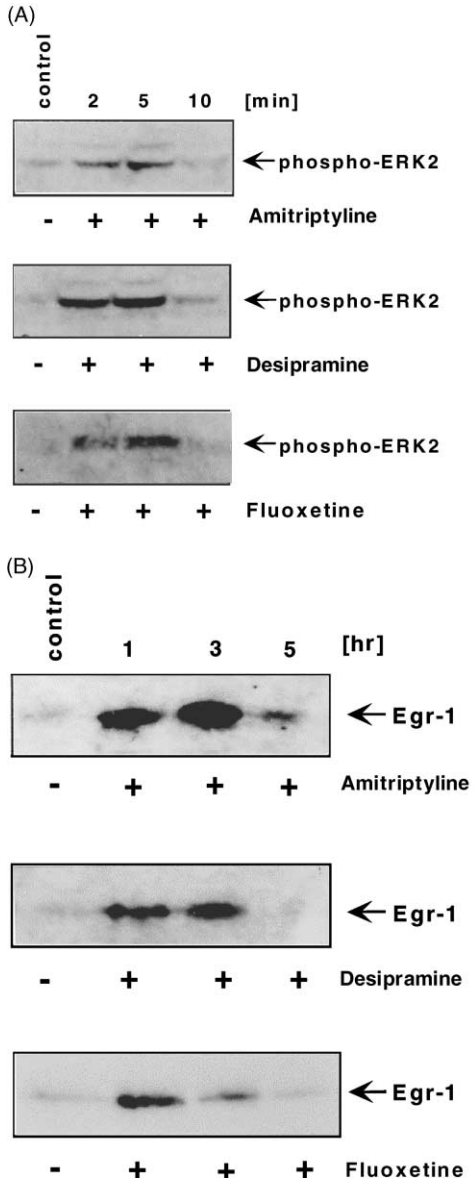

Fig. 2. Phosphorylation and activation of extracellular signal-regulated protein kinase and stimulation of Egr-1 biosynthesis in HT22 cells following administration of antidepressants. (A) HT22 cells were cultured in medium containing 5% serum for 24 hr, treated with 50 μM amitriptyline, 10 μM desipramine, or 20 μM fluoxetine for 2, 5 and 10 min or left untreated (control). Whole cell extracts were prepared and subjected to Western blot analysis. The blot was incubated with an affinity purified rabbit antibody directed against the phosphorylated active form of p42 (ERK2). The antibody reacts weakly with p44 (ERK1). (B) HT22 cells were treated with 50 μM amitriptyline, 10 μM desipramine, or 20 μM fluoxetine for 1, 3 and 5 hr or left untreated (control). Nuclear extracts were prepared and subjected to Western blot analysis with an antibody recognizing Egr-1.

This activation was of transient nature, because the levels of phosphorylated ERK2 decreased already 10 min later.

Activation of ERK is frequently coupled to an induction of Egr-1 transcription, due to the phosphorylation and activation of the ternary complex factor Elk1 [11,13]. Egr-1 is a zinc finger transcriptional regulator connecting signaling pathways to changes of the gene expression program. Interestingly, it has been proposed that Egr-1 may function as a pro-apoptotic protein [14]. To test the effect of antidepressants on Egr-1 biosynthesis, HT22 cells were cultured in medium containing 5% serum for 24 hr. Cells were incubated with 50 μM amitriptyline, 10 μM desipramine, or 20 μM fluoxetine for 1, 3 and 5 hr. Nuclear extracts from drug and vehicle treated cells were prepared and analyzed for Egr-1 immunoreactivity. Weak Egr-1 immunoreactivity was observed in the absence of the compounds. However, amitriptyline, desipramine, and fluoxetine strikingly induced Egr-1 biosynthesis, with highest amounts observed after 1–3 hr of stimulation (Fig. 2B). This induction of Egr-1 biosynthesis was transient, as we observed a decrease in Egr-1 immunoreactivity after 5 hr of incubation with the drugs.

3.3. Effects of ERK inhibition on neuronal cell death of HT22 cells

To verify the role of ERK and Egr-1 in antidepressant-induced cell death, we preincubated HT22 cells with PD98059. This compound inhibits phosphorylation of the MEK, thus blocking the activation of ERK [15]. As shown in Fig. 3A, PD98059 blocked efficiently the induction of Egr-1 synthesis by amitriptyline, desipramine, or fluoxetine, indicating that the activation of ERK is required for *Egr-1* gene transcription. Next, we tested the cytotoxic activity of amitriptyline, desipramine, and fluoxetine under conditions where the ERK signaling pathway was blocked by PD98059. HT22 cells were preincubated with PD98059 and than challenged with either amitriptyline, desipramine, or fluoxetine. The cytotoxic effects were determined by the MTT assay. Fig. 3B shows that incubation with PD98059 caused a slight decrease in the mitochondrial reduction capacities. However, additional challenge with either amitriptyline, desipramine, and fluoxetine strikingly decreased the mitochondrial reduction capacities of HT22 cells, in the presence or absence of PD98059. We conclude that the activation of ERK and the biosynthesis of Egr-1 are not required for antidepressant-induced neuronal cell death.

3.4. Effects of sphingosine-1-phosphat-induced activation of ERK phosphorylation and Egr-1 biosynthesis on the mitochondrial reduction capacities in HT22 cells

The lipid messenger sphingosine-1-phosphate has been shown to activate ERK and Egr-1 biosynthesis in astrocytes [16,17]. To further elucidate whether both ERK activation and Egr-1 biosynthesis are linked to neuronal cell death, we

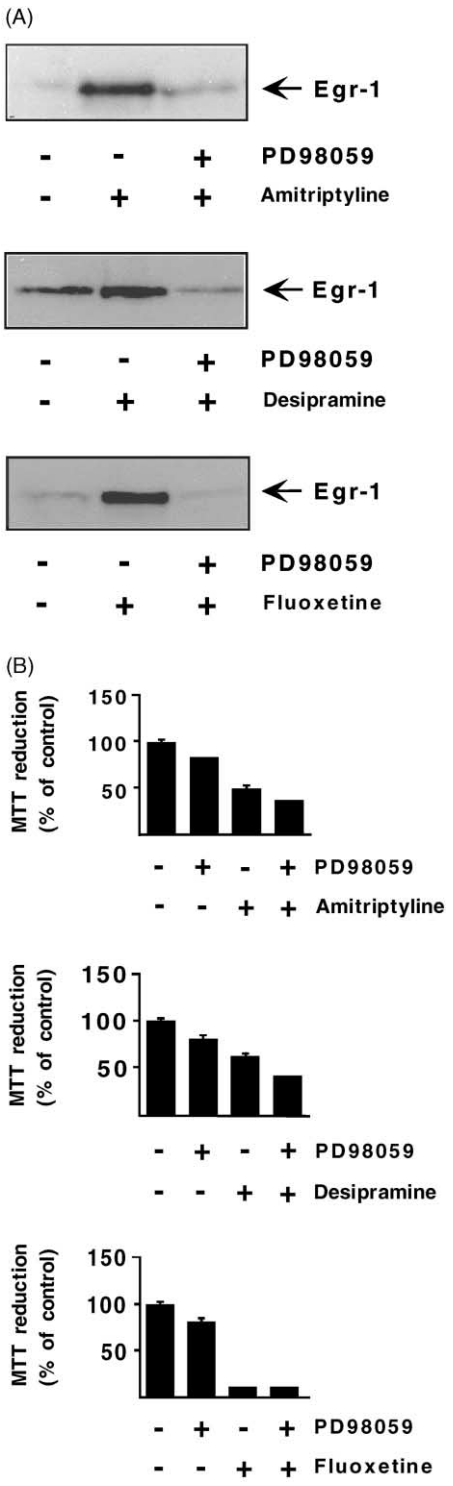

Fig. 3. Effects of PD98059 on Egr-1 biosynthesis and cell death in HT22 cells. (A) HT22 cells were preincubated for 20 hr with the MEK inhibitor PD98059 and then stimulated with 50 μM amitriptyline, 10 μM desipramine, or 20 μM fluoxetine for 1 hr. As a control, cells were incubated solely with vehicle. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. (B) HT22 cells were preincubated for 20 hr with PD98059 and then stimulated with 50 μM amitriptyline, 10 μM desipramine, or 20 μM fluoxetine for 24 hr. As a control, cells were incubated solely with vehicle. The cytotoxic effects of the applied compounds were determined spectrophotometrically by the MTT method.

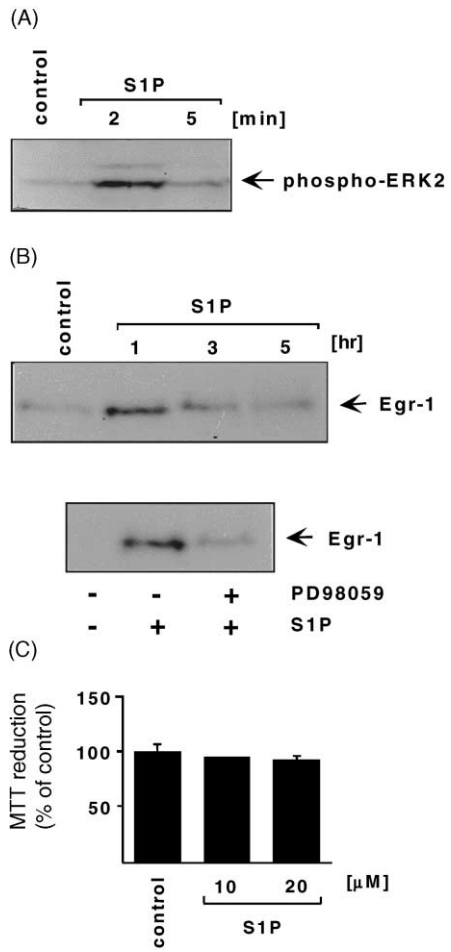

Fig. 4. Activation of ERK and Egr-1 biosynthesis by sphingosine-1-phosphate does not induce cell death in HT22 cells. (A) HT22 cells were treated with 10 μM sphingosine-1-phosphate for 0, 2 and 5 min. Whole cell extracts were prepared and subjected to Western blot analysis. The blot was incubated with an affinity purified rabbit antibody directed against the phosphorylated active form p42 (ERK2). (B) Upper panel: HT22 cells were incubated with 10 μM sphingosine-1-phoshate for 1, 3 and 5 hr or left untreated (control). Nuclear extracts were prepared and subjected to Western blot analysis with an antibody recognizing Egr-1. Lower panel: HT22 cells were preincubated for 20 hr with PD98059 and then stimulated with 10 μM sphingosine-1-phosphate. Nuclear extracts were prepared and subjected to Western blot analysis with an antibody recognizing Egr-1. (C) HT22 cells were treated with 10–20 μM of sphingosine-1-phoshate for 24 hr. The cytotoxic effects of the applied compounds were determined spectrophotometrically by the MTT method.

incubated HT22 cells with sphingosine-1-phosphate. Fig. 4 shows that under these conditions, ERK is transiently activated (A). Likewise, sphingosine-1-phosphate activated the biosynthesis of Egr-1 *via* the ERK signaling pathway (B). However, sphingosine-1-phosphate did not trigger neuronal cell death (C), indicating that solely an activation of ERK and a subsequent synthesis of Egr-1 is not toxic for the cells.

3.5. Activation of NF-κB by antidepressants in PC12κBluc cells

It has been reported that the antidepressants amitriptyline, desipramine, and fluoxetine cause a moderate activa-

tion of NF-κB in HT22 cells [7] suggesting that NF-κB may play a role in the execution of the cytotoxic/apoptotic activities of those compounds [7]. To elucidate the relationship between antidepressant-induced cell death and NF-κB activity, we employed a recently engineered PC12 cell line (PC12κBluc) in the analysis containing an integrated NF-κB-responsive luciferase gene (Erlandsson, Baumann, Rössler, Kaufmann, Giehl, Wirth and Thiel, manuscript submitted). Enhanced reporter gene transcription in these cells functions as a direct indicator for an activation of NF-κB. In addition, we had already shown in Fig. 1 that amitriptyline, desipramine, and fluoxetine are cytotoxic for this PC12 cell clone. PC12κBluc cells were seeded in serum-containing medium, then cultured in a defined medium (neurobasal plus N_2-supplement) for 24 hr, and challenged with amitriptyline, desipramine, or fluoxetine for 8 hr. Cell extracts were prepared and the protein concentrations and luciferase activities determined. Luciferase activities were normalized to the protein concentration of the extracts and expressed as light units/μg protein. Fig. 5 shows the antidepressants triggered a concentration-dependent increase in the NF-κB activity in PC12κBluc cells. The stimulation of NF-κB activity by 100 μM amitriptyline, 100 μM desipramine, and 100 μM fluoxetine was in the order of 44-, 50-, and 10-fold, respectively.

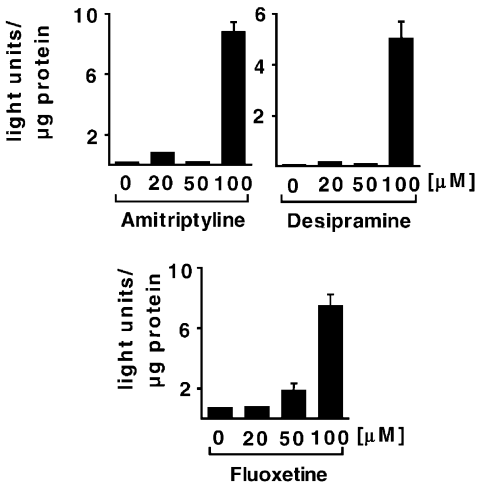

Fig. 5. Activation of NF-κB by amitriptyline, desipramine, and fluoxetine in PC12κBluc cells. PC12κBluc cells were seeded in serum-containing medium and incubated overnight. The medium was replaced by neurobasal medium containing N_2-supplement and the cells were incubated for 24 hr. The neurobasal medium was renewed and the cells were treated for 8 hr with the indicated concentrations of amitriptyline, desipramine, or fluoxetine. Cell extracts were prepared and the protein concentrations and luciferase activities determined. Luciferase activities were normalized to the protein concentration of the extracts and expressed as light units/μg protein.

3.6. Effects of attenuation of antidepressant-induced NF-κB activity by elevated cAMP concentrations on the mitochondrial reduction capacities

Recently, we showed that an elevation of the intracellular cAMP concentration impaired tumor necrosis factor α or phorbol ester triggered activation of NF-κB in PC12κBluc cells (Erlandsson, Baumann, Rössler, Kaufmann, Giehl, Wirth and Thiel, manuscript submitted). We therefore tested whether an increase in the intracellular cAMP concentration also blocked antidepressant-induced NF-κB activation. Cells were preincubated with the cAMP analogue dibutyryl cAMP and the phosphodiesterase inhibitor IBMX for 48 hr and then stimulated with either amitriptyline, desipramine, or fluoxetine. Cells were harvested, cell extracts prepared and the relative luciferase activities determined. Induction of NF-κB by either amitriptyline, desipramine, or fluoxetine was efficiently inhibited by an increase in the intracellular cAMP concentration (Fig. 6A). Next, we tested the neuro-

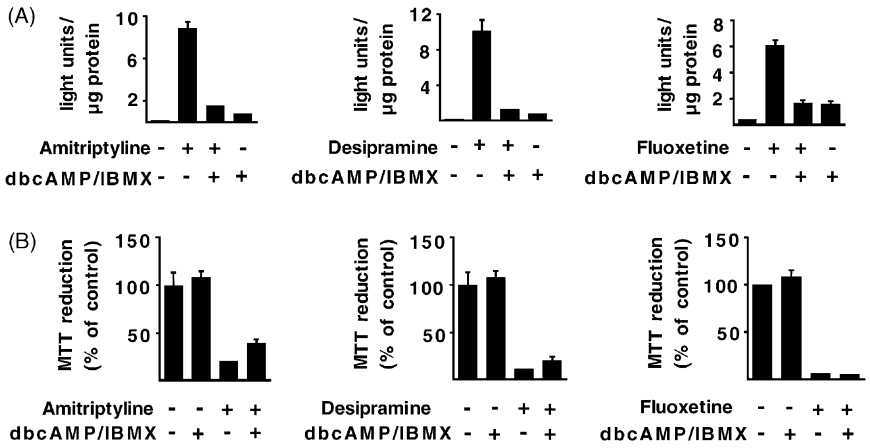

Fig. 6. Pharmacological inhibition of NF-κB has no impact on the survival of PC12κBluc cells. (A) PC12κBluc cells were cultured in neurobasal medium containing N_2-supplement. Cells were preincubated for 48 hr with 0.2 mM dbcAMP and 0.1 mM IBMX. The stimulation was performed with 100 μM of amitriptyline, desipramine, or fluoxetine for 8 hr. Cell extracts were prepared, the protein concentrations and luciferase activities determined, and the relative luciferase activities calculated. (B) PC12κBluc cells were cultured in neurobasal medium containing N_2-supplement. Cells were preincubated for 48 hr with 0.2 M dbcAMP and 0.1 M IBMX and then exposed to 100 μM of amitriptyline, desipramine, or fluoxetine for 24 hr. Cytotoxicity was determined spectrophotometrically by the MTT method.

toxic activity of amitriptyline, desipramine, and fluoxetine under conditions of suppressed NF-κB. PC12κBluc cells were incubated with dbcAMP and IBMX for 48 hr and then challenged with amitriptyline, desipramine, and fluoxetine for 24 hr. The mitochondrial reduction capacities were measured using the MTT assay. Fig. 6B reveales that an impairment of NF-κB activation had at the time points analyzed no impact on cell survival.

3.7. Effects of attenuation of antidepressant-induced NF-κB activity by expression of the superrepressor IκBαS32A/S36A on the mitochondrial reduction capacities

The activation process of NF-κB requires phosphorylation and subsequent degradation of IκB. Thus, expression of a non-degradable form of IκB impairs NF-κB activity by retaining NF-κB in the cytoplasma. Recently, we generated PC12κBluc cells expressing a non-degradable *IκBαS32A/S36A* mutant that contains alanine residues instead of the critical serine residues (PC12κBluc-IκBαS32A/S36A cells). As a control, we generated PC12κBluc cells expressing solely the neomycin resistance gene (PC12κBluc-neo cells). The biological effect of IκBαS32A/S36A was tested in cells challenged with amitriptyline, desipramine, and fluoxetine. Fig. 7A (right panel) shows that expression of the superrepressor efficiently attenuated the antidepressant-triggered elevation of NF-κB activity. In contrast, PC12κBluc cells expressing neomycin acetyltransferase (PC12κBluc-neo) showed a strong induction of NF-κB following stimulation of the cells with either amitriptyline, desipramine, or fluoxetine (Fig. 7A, left panel). We further tested the neurotoxic activity of amitriptyline, desipramine, and fluoxetine in PC12κBluc-IκBαS32A/S36A and PC12κBluc-neo cells. The data show that the mitochondrial reduction capacities were reduced in IκBαS32A/S36A expressing cells as a result of antidepressant treatment (Fig. 7B, right panel). We did not observe significant differences in the neurotoxic activities of amitriptyline, desipramine, and fluoxetine in PC12 cells expressing either neomycin acetyltransferase or the IκBα superrepressor IκBαS32A/S36A (compare Fig. 7B, left and right panels). These results indicate that NF-κB is not involved in the signaling pathway initiated by amitriptyline, desipramine, and fluoxetine that leads to neuronal cell death.

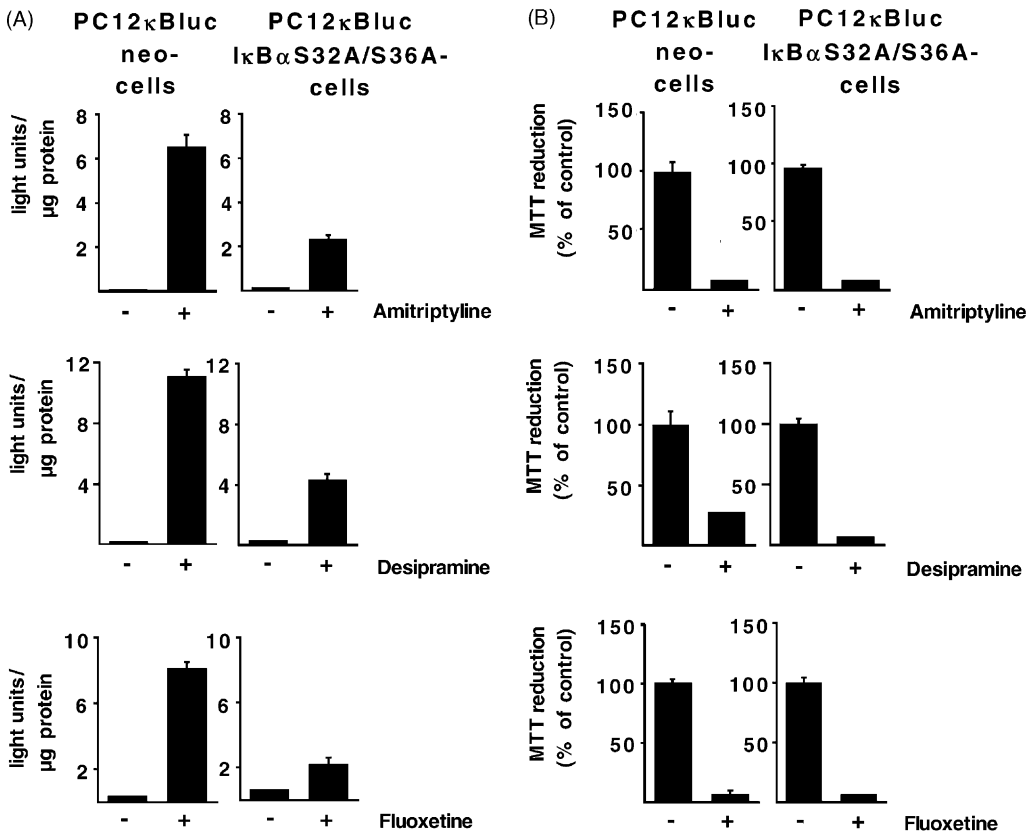

Fig. 7. Biological activity of the superrepressor IκBαS32A/S36A upon antidepressant-induced NF-κB activity and mitochondrial reduction capacity in PC12 cells. (A) PC12κBluc-neo (left panel) and PC12κBluc-IκBαS32A/S36A cells (right panel) were cultured in neurobasal medium containing N_2-supplement for 24 hr and stimulated with 100 µM of amitriptyline, desipramine, or fluoxetine for 8 hr. Cell extracts were prepared, the protein concentrations and luciferase activities determined, and the relative luciferase activities calculated. (B) PC12κBluc-neo (left panel) and PC12κBluc-IκBαS32A/S36A cells (right panel) were exposed to 100 µM of amitriptyline, desipramine, or fluoxetine for 24 hr. Cytotoxicity was determined spectrophotometrically by the MTT method.

3.8. Role of NF-κB activation for antidepressant-induced apoptotic cell death

To confirm these data, we analyzed the fragmentation of chromatin as a result of the neurotoxic challenge with antidepressants using the terminal deoxynucleotidyl transferase-mediated dUTP 3′-end-labeling (TUNEL) technique. Cells were preincubated with dbcAMP and IBMX for 48 hr and then exposed to amitriptyline, desipramine, or fluoxetine for 15 hr. Fig. 8 shows the morphology of PC12κB cells in the presence or absence of the drugs. It is clearly visible that μM concentrations of antidepressants killed the cells. The right panels depicts the TUNEL analysis of PC12κB cells. No chromatin fragmentation was detected in the absence of the cytotoxic stimuli. TUNEL-positive cells emerged as a result of drug-treatment, indicating that these compounds initiated apoptotic cell death. The preincubation of PC12κBluc cells with dbcAMP and IBMX, in order to attenuate NF-κB activity, did not reduce the amount of TUNEL positive cells, indicating that the level of NF-κB activity is not of any importance in drug-induced cell death. These data were confirmed in PC12κBluc cells expressing the IκBαS32A/S36A superrepressor. PC12κBluc cells died as a result of amitriptyline, desipramine, or fluoxetine treatment, despite the expression of IκBαS32A/S36A (Fig. 9, right panels). We did not observe a difference in either IκBαS32A/S36A or neomycin acetyltransferase expressing PC12κBluc cells. Taken together, these data show that the elevation of NF-κB activity by amitriptyline, desipramine, or fluoxetine is not required for the apoptotic cell death of PC12κBluc cells.

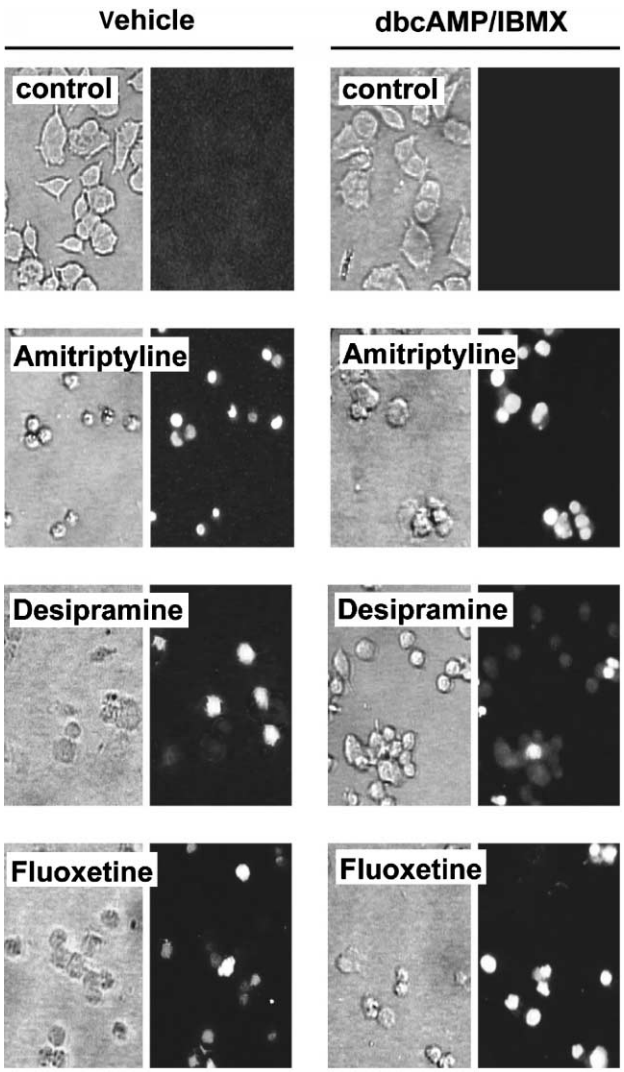

Fig. 8. Attenuation of NF-κB activity in antidepressant-treated PC12κBluc cells by elevated cAMP concentration does not block programmed cell death. PC12κBluc cells were cultured in neurobasal medium containing N_2-supplement. Cells were preincubated for 48 hr with vehicle (left panels) or 0.2 mM dbcAMP and 0.1 mM IBMX (right panels) and then exposed to 100 μM of amitriptyline, desipramine, or fluoxetine for 15 hr. Control cells are shown on top. Cells were analyzed using the TUNEL technique with TMR red-labeled UTP. Phase contrast and fluorescence micrographs of the same locations are depicted.

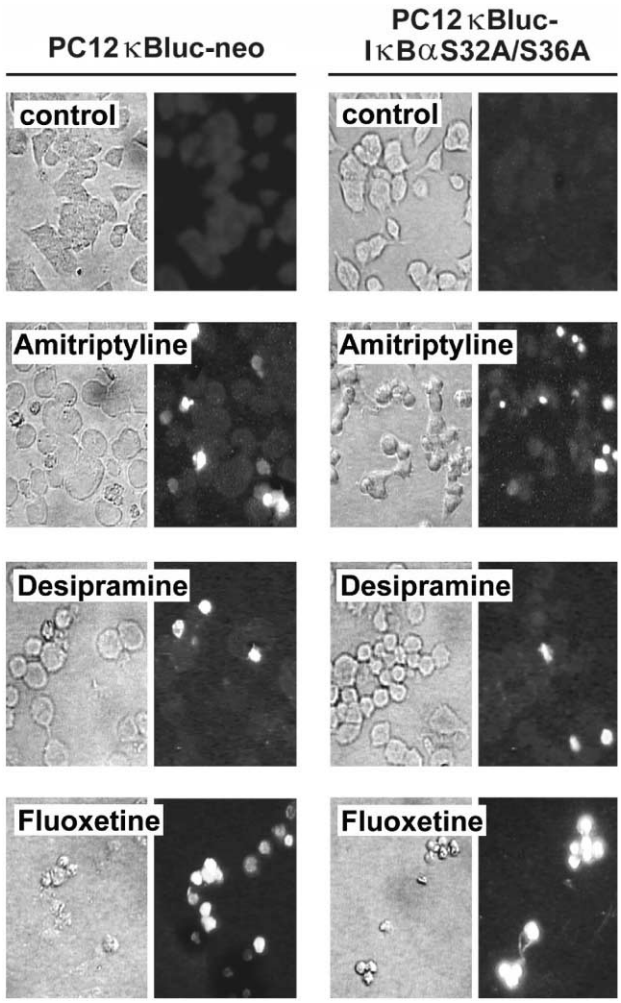

Fig. 9. Attenuation of NF-κB activity in antidepressant-treated cells *via* expression of the superrepressor IκBαS32A/S36A does not block programmed cell death. PC12κBluc-neo (left panels) and PC12κBluc-IκBαS32A/S36A cells (right panels) were cultured in neurobasal medium containing N_2-supplement for 24 hr and exposed to vehicle (top), 100 μM of amitriptyline, desipramine, or fluoxetine for 15 hr. Cells were analyzed using the TUNEL technique with TMR red-labeled UTP. Phase contrast and fluorescence micrographs of the same locations are depicted.

4. Discussion

We have analyzed the signaling pathways induced by μM concentrations of the tricyclic antidepressants amitriptyline and desipramine and the serotonine reuptake inhibitor fluoxetine. It is well known that antidepressants exhibit a variety of secondary neuropharmacological reactions, in addition to their primary activity, the inhibition of the norepinephrine and/or serotonin transporter. Here, we showed that amitriptyline, desipramine and fluoxetine are very active signaling molecules, leading to an elevation of ERK and NF-κB activities and Egr-1 concentrations. Moreover, we confirmed previous observations [4–6] that these antidepressants—when applied at μM concentrations—triggered apoptotic cell death. Amitriptyline, desipramine and fluoxetine are thought to increase the generation of reactive oxygen species and trigger a reduction of intracellular glutathione levels [7]. Accordingly, the HT22 cells were more sensitive to these drugs than PC12 cells, confirming the observation that HT22 cells are particularly sensitive to oxidative stress [18].

Recently, a persistent activation of the ERK was proposed to contribute to glutamate-induced oxidative toxicity in HT22 cells [8]. Accordingly, inhibition of the ERK activating kinase MEK-1/2 protected HT22 cells from glutamate toxicity [8]. In contrast, activation of ERK has been connected with enhanced survival in cortical neurons and PC12 cells [19,20]. Here, we observed a transient activation of ERK following exposure of HT22 cells to elevated concentrations of amitriptyline, desipramine and fluoxetine. The activation of ERK was the result of a specific activation of the MAP kinase signaling pathway, as shown by the effects of PD98059, a specific MEK inhibitor. However, this activation of ERK was not required for the cytotoxic effects of the drugs since inhibition of ERK activation by PD98059 did not block antidepressant-induced cell death. Moreover, incubation of HT22 cells with PD98059 slightly reduced cell survival, indicating that the ERK signaling pathway has, at least in part, a protective effect for the cells. Further investigations are required to test whether a persistent activation of ERK promotes cell survival in antidepressant-treated HT22 cells. Recently, an activation of the MEK/ERK signaling pathway by the chemotherapeutic drug paclitaxel has been reported [21]. According to our observation, inhibition of MEK did not alter the cell death induced by paclitaxel [21].

One of the targets of the ERK signaling pathway is the gene encoding the transcriptional regulator Egr-1 [13]. A variety of environmental signals including growth factors induce *Egr-1* gene expression suggesting that Egr-1 couples extracellular signals to long-term responses by altering expression of Egr-1-regulated target genes. Since the discovery of the *Egr-1* gene as an "early growth response gene" [22], research was directed towards a function of Egr-1 in growth and proliferation. However, Egr-1 activation has also been linked to cell death [14]. In the nervous system, an enhanced expression of Egr-1 has been connected with neuronal apoptosis of cerebellar granule cells [23]. Here, we have shown that cell death induced by elevated concentrations of amitriptyline, desipramine, or fluoxetine is accompanied by a rapid synthesis of Egr-1. However, we demonstrated that an impairment of Egr-1 biosynthesis has no effect upon HT22 cell survival. These data indicate, that Egr-1 plays under these circumstances no substantial role in the apoptotic signaling cascade triggered by antidepressants in HT22 cells.

NF-κB has been proposed to function either as a pro-apoptotic or as an anti-apoptotic protein, determined by the cell type and the apoptotic inducers. The protective effect of NF-κB was demonstrated by p65 knock-out mice that died at day 14/15 of embryonic development, due to massive apoptosis of hepatocytes [24]. In the nervous system, the activation of NF-κB has been connected with an enhanced neuronal survival [25] but also with an induction of cell death [26,27]. Accordingly, NF-κB has been suggested to be crucial for the neurotoxic effects of antidepressants [7]. Using a PC12 cell clone with an integrated NF-κB responsive transcription unit, we confirmed that amitriptyline, desipramine, and fluoxetine activated the transcriptional activity of NF-κB when applied to the cells in μM concentrations. This effect was specific, because expression of a non-degradable *IκBα* mutant attenuated antidepressant-induced stimulation of NF-κB, indicating that those compounds activate the IκB kinase complex. Furthermore, no NF-κB activation was detected following incubation of the cells with toxic concentrations of H_2O_2, $K_2Cr_2O_7$, $MnCl_2$, or C_2-ceramide (Erlandsson, Baumann, Rössler, Kaufmann, Giehl, Wirth and Thiel, manuscript submitted). However, inhibition of NF-κB activity by either an elevation of the intracellular cAMP concentration or by expression of a non-degradable IκB superrepressor demonstrated that an induction of NF-κB is not the molecular mechanism underlying antidepressant-stimulated neuronal cell death.

Here, we have shown that amitriptyline, desipramine and fluoxetine induced signaling pathways in neuronal cells leading to an activation of ERK and NF-κB as well as to enhanced Egr-1 synthesis. However, the activation of ERK and NF-κB is not required for the apoptotic signaling cascade induced by amitriptyline, desipramine, or fluoxetine. Furthermore, an impairment of the Egr-1 biosynthesis did not rescue the cells. Recently, the generation of reactive oxygen intermediates has been described as a result of antidepressant-treatment of HT22 cells, followed by a concomitant reduction of the intracellular glutathione concentrations [7]. Neuronal cell death may thus be the result of oxidative stress and the reduction of the cellular antioxidative capacity. Reactive oxygen intermediates are known to function as messengers in the activation of NF-κB [28]. Furthermore, the transcription factor Elk1 has

been identified to be responsible for the serum-response element regulated transcription of the *c-fos* gene [29]. The Egr-1 5′-flanking region contains five serum response elements, suggesting that reactive oxygen intermediates activate Egr-1 transcription *via* these genetic elements. Moreover, activation of Elk1 by reactive oxygen intermediates was dependent upon the integrity of ERK2 [29]. Taken together, we propose that oxidative stress induced by μM concentrations of amitriptyline, desipramine or fluoxetine is responsible for the cell damage as well as for the activation of ERK, NF-κB and the synthesis of Egr-1. However, ERK, Egr-1 and NF-κB are not responsible for the cytotoxic effects of these drugs. The fact that antidepressant-induced neuronal death is thought to change the cellular antioxidative capacity may give an indication where future studies should be directed.

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References

[1] Baldessarini RJ. Drugs and the treatment of psychiatric disorders. In: Goodman LS, Gilman A, editors. The pharmacological basis of therapeutics. New York: Macmillan, 2001. p. 447–83.

[2] Takahashi M, Terwilliger R, Lane C, Mezes PS, Conti M, Duman RS. Chronic antidepressant administration increases the expression of cAMP-specific phosphodiesterases 4A and 4B isoforms. J Neurosci 1999;19:610–8.

[3] Thome J, Sakai N, Shin K-H, Steffen C, Zhang Y-J, Impey S, Storm D, Duman RS. cAMP responsive element-mediated gene transcription is upregulated by chronic antidepressant treatment. J Neurosci 2000;20:4030–6.

[4] Barbui C, Hotopf M. Amitriptyline versus the rest: still the leading antidepressant after 40 years of randomised controlled trials. Br J Psychiatr 2001;178:129–44.

[5] Spanová A, Kováru H, Lisá V, Lukásová E, Rittich B. Estimation of apoptosis in C6 glioma cells treated with antidepressants. Physiol Rev 1997;46:161–4.

[6] Xia Z, DePierre JW, Nässberger L. Modulation of apoptosis induced by tricyclic antidepressants in human peripheral lymphocytes. J Biochem Toxicol 1998;12:115–23.

[7] Post A, Crochemore C, Uhr M, Holsboer F, Behl C. Differential induction of NF-κB activity and neuronal cell death by antidepressants *in vitro*. Eur J Neurosci 2000;12:4331–7.

[8] Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MR, Johnson JW, DeFranco DB. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem 2000;275:12200–6.

[9] Maher P, Schubert D. Signaling by reactive oxygen species in the nervous system. Cell Mol Life Sci 2000;57:1287–305.

[10] Thiel G, Kaufmann K, Magin A, Lietz M, Bach K, Cramer M. The human transcriptional repressor protein NAB1: expression and biological activity. Biochim Biophys Acta 2000;1493:289–301.

[11] Kaufmann K, Thiel G. Epidermal growth factor and platelet-derived growth factor induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cells. J Neurol Sci 2001;189:83–91.

[12] Valavanis C, Hu Y, Yang Y, Osborne BA, Chouaib S, Greene L, Ashwell JD, Schwartz LM. Model cell lines for the study of apoptosis *in vitro*. Meth Cell Biol 2001;66:418–36.

[13] Kaufmann K, Bach K, Thiel G. Extracellular signal-regulated protein kinases Erk1/Erk2 stimulate expression and biological activity of the transcriptional regulator Egr-1. Biol Chem 2001;382:1077–81.

[14] Liu C, Rangnekar VM, Adamson E, Mercola D. Suppression of growth and transformation and induction of apoptosis by Egr-1. Cancer Gene Ther 1998;5:3–28.

[15] Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 1995;92:7686–9.

[16] Sato K, Ishikawa K, Ui M, Okajima F. Sphingosine-1-phosphate induces expression of early growth response-1 and fibroblast growth factor-2 through mechanism involving extracellular signal-regulated kinase in astroglial cells. Mol Brain Res 1999;74:182–9.

[17] Pébay A, Toutant M, Prémont J, Calvo C-F, Venance L, Cordier J, Glowinski J, Tencé M. Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. Eur J Neurosci 2001;13:2067–76.

[18] Tan S, Wood M, Maher P. Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem 1998;71:95–105.

[19] Xia Z, Dickens M, Raingeaud J, Davies RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.

[20] Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem 1999;274:22569–80.

[21] Okano J-I, Rustgi AK. Paclitaxel induces prolonged activation of the Ras/MEK/ERK pathway independently of activating the programmed cell death machinery. J Biol Chem 2001;276:19555–64.

[22] Sukhatme VP, Cao X, Chang LC, Tsai-Morris C-H, Stamenkovich D, Ferreira PCP, Cohen DR, Edwards SA, Shows TB, Curran T, Le Beau MM, Adamson ED. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 1988;53:37–43.

[23] Catania MV, Copani A, Calogero A, Ragonese GI, Condorelli DF, Nicoletti F. An enhanced expression of the immediate early gene, *Egr-1*, is associated with neuronal apoptosis in culture. Neuroscience 1999;91:1529–38.

[24] Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature 1995;376:167–70.

[25] Mattson MP, Culmsee C, Yu ZF, Camandola S. Roles of nuclear factor κB in neuronal survival and plasticity. J Neurochem 2000;74:443–56.

[26] Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. NF-κB is activated and promotes cell death in focal cerebral ischemia. Nature Med 1999;5:554–9.

[27] Denk A, Wirth T, Baumann B. NF-κB transcription factors: critical regulators of hematopoiesis and neuronal survival. Cytokine Growth Factor Rev 2000;11:303–20.

[28] Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 1991;10:2247–58.

[29] Müller JM, Cahill MA, Rupec RA, Baeuerle PA, Nordheim A. Antioxidants as well as oxidants activate c-fos *via* Ras-dependent activation of extracellar signal-regulated kinase 2 and Elk-1. Eur J Biochem 1997;244:45–52.