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Nitric oxide donors induce neurotrophin-like survival signaling and protect neurons against apoptosis

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Running title: NO-donors mimic growth factor survival signaling

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Abbreviations:

BDNF: brain-derived growth factor; cGMP: cyclic 3’5’ guanosine monophosphate; CREB: cAMP-response element-binding protein; DPN: Ethyl-3,4-dephostatin; EGF: epidermal growth factor; IGF: insulin-like growth factor; MAPK: mitogen-activated protein kinase; Me-DPN: 4-O-Methyl-ethyl-3,4-dephostatin; NMDA: N-methyl-D-aspartic acid; ODQ: 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; PDGF: platelet-derived growth factor; PI3-K: phosphoinositide-3-kinase; PTP: protein tyrosine phosphatase; RTK: receptor tyrosine kinase; sGC: soluble guanylyl cyclase; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SHP-1: src homology-2-containing protein tyrosine phosphatase-1; SNAP: S-nitroso-N-acetylpenicillamine; Trk: tropomyosine-related kinase;

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Abstract

Our previous results showed that inhibition of protein tyrosine phosphatases (PTP) by orthovanadate is an appropriate strategy to mimic nerve growth factor (NGF) effects in neurons including enhanced phosphorylation of TrkA, stimulation of downstream survival signaling pathways, and protection against apoptotic stress. Here, we wanted to trigger such NGF-like survival signaling in primary hippocampal neurons with the more specific PTP inhibitors Et-3,4-dephostatin (DPN), 4-O-Me-Et-3,4-dephostatin (Me-DPN) and methoxime-3,4-dephostatin (methoxime-DPN). Strikingly, only the nitric oxide (NO)-releasing dephostatin analogues DPN and Me-DPN, but not the nitrosamine-free methoxime derivative (which did not release NO) enhanced TrkA phosphorylation and protected the neurons against staurosporine (STS)-induced apoptosis. The established NO-donor S-nitroso-N-acetylpenicillamine (SNAP) also enhanced TrkA phosphorylation and prevented apoptosis similar to DPN and Me-DPN. Analysis of the major signaling pathways downstream TrkA revealed that both SNAP and DPN enhanced phosphorylation of Akt and the mitogen-activated kinases (MAPK) Erk1/2. Blocking these signaling pathways by the PI3-K inhibitor wortmannin or the MAPK-kinase (MEK) inhibitor UO126 equally abolished the neuroprotective effect of the NO-donors. Strikingly, inhibition of the soluble guanylyl cyclase (sGC) by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or protein kinase g (PKG) inhibition by KT 5823 also blocked the neuroprotective effect of the NO-donors, and ODQ clearly attenuated SNAP-induced phosphorylation of TrkA, Akt and MAPK. In conclusion, NO release by the dephostatin derivatives and subsequent stimulation of sGC and PKG is essential for their neuroprotective effects. In primary neurons such NO-activated survival signaling involves NGF-like effects including enhanced phosphorylation of TrkA and activation of PI3-K/Akt and MAPK pathways.

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Nitric oxide is an endogenous regulator of different cellular functions, including vascular tone (Furchgott and Zawadtzki, 1980; Gruetter et al., 1979), neurotransmission (Garthwaite and Boulton, 1995; Vincent, 1994), inflammation (Nathan and Shiloh, 2000), and cellular signaling cascades (Dawson et al., 1992). In neurons, NO has been found a janus-faced molecule that can mediate survival signaling (Troy et al., 2000; Contestabile and Ciani, 2004) but may on the other hand contribute to neuronal death and brain damage in neurological diseases, e.g. stroke (Zhang et al., 1996), Alzheimer's disease (Lee et al., 1999) amyotrophic lateral sclerosis (Urushitani and Shimohama, 2001), or Parkinson's disease (Liberatore et al., 1999). In the brain, NO can be synthesized from L-arginine by three isoforms of NO synthase (NOS), neuronal NOS (nNOS), inducible NOS (iNOS) in microglia, and endothelial NOS (eNOS) (Wendland et al., 1994; Alderton et al., 2001). Previous studies indicated that NO produced by eNOS mediated cerebroprotection (Huang et al., 1996; Endres et al., 1998), whereas activation of nNOS or iNOS rather accelerated neuronal damage (Eliasson et al., 1999; Iadecola et al., 1995).

The physiological actions of NO are primarily mediated through stimulation of soluble guanylate cyclase (sGC) which results in accumulation of cyclic 3'5' guanosine monophosphate (cGMP) and subsequent activation of the protein kinase G (PKG) (for review, see Schlossmann et al., 2003). Such NO-induced cGMP signaling can prevent apoptosis via activation of the phosphoinositide-3-kinase (PI3-K)/protein kinase B (PKB/Akt) pathway (Ha et al., 2003) or by stimulation of the transcription factor cAMP-response element-binding protein (CREB; Ciani et al., 2002). Moreover, activation of the mitogen-activated protein kinase (MAPK) cascade through NO/cGMP signaling has been found in PC12 cells (Kim et al., 2003). In addition, NO has been reported an endogenous inhibitor of protein tyrosine phosphatases (PTP) (Callsen et al., 1999; Caselli et al., 1994, 1995). Therefore, NO-donors may stimulate growth factor receptor tyrosine kinases (RTK) and downstream survival

signaling pathways through inhibition of PTP and independent of cGMP signaling, similar to effects of the PTP inhibitor orthovanadate.

Previous studies in our laboratories and by others suggested inhibition of PTP as a promising strategy to enhance tyrosine phosphorylation of RTK and to stimulate downstream growth factor signaling pathways (Fujiwara et al., 1997; Gerling et al., 2004; Lu et al., 2002). In these studies, the broad spectrum PTP inhibitor orthovanadate has been applied in cultured neurons or via intracerebral injection in vivo to demonstrate protective effects against apoptotic stress or ischemic brain damage, respectively. In our previous study we demonstrated in primary rat neurons that PTP inhibition by orthovanadate could mimic nerve growth factor (NGF)-induced tyrosine phosphorylation of tropomyosine-related kinase A (TrkA) and enhanced downstream neurotrophin-like survival signaling cascades involving Akt and MAPK (Gerling et al., 2004).

The applicability of orthovanadate as a neuroprotectant, however, is limited because of its low stability in aqueous solutions and in biological systems (Morinville et al., 1998). Moreover, orthovanadate unselectively inhibits a broad range of PTP, which limits specificity and safety, and even neurotoxic effects have been reported at high doses (Figiel and Kaczmarek, 1997; Gerling et al., 2004). Therefore, recent approaches aimed at the development of stable, specific and safe PTP inhibitors that could serve as useful tools to study the role of PTP in disease and therapeutic approaches. Umezawa and colleagues identified dephostatin as a naturally occuring PTP inhibitor and developed stable analogues such as Ethyl-3,4-dephostatin (DPN) and 4-O-Methyl-ethyl-3,4-dephostatin (Me-DPN) (Fig. 1A) which were found to inhibit PTP-1B and src homology-2-containing protein tyrosine phosphatase-1 (SHP-1) selectively (Umezawa et al., 2003). DPN increased the tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 in the presence or

absence of insulin in mouse adipocytes (Suzuki et al., 2001). Since DPN contains a nitrosamine moiety which potentially release nitric oxide (NO), nitrosamine-free dephostatin analogues such as methoxime-3,4-dephostatin (methoxime-DPN) were designed (Fig. 1A) to exclude the involvement of NO-mediated signaling in the observed insulin-like effects (Hiroki et al., 2002).

The aim of the present study was to investigate whether the PTP-inhibiting dephostatin-derivatives DPN and Me-DPN could induce neurotrophin-like effects in neurons, including enhanced TrkA phosphorylation, activation of downstream survival signaling pathways, and protection against STS-induced apoptosis. In order to find out whether such neuroprotective effects depend on NO released by these dephostatin derivatives we also included the nitrosamine-free methoxime-DPN and the established NO-donors S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprussid (SNP) in our experiments. In addition, we used inhibitors of sGC and PKG to clarify whether the observed activation of neurotrophin-like survival signaling required activation of cGMP pathways or rather resulted from NO-mediated PTP inhibition.

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Materials and Methods

Materials

The dephostatin analogues DPN, Me-DPN, and methoxime-DPN (Fig. 1A) were synthesized as described previously (Umezawa et al., 2003). The DNA fluorochrome Hoechst 33258, bovine serum albumine, staurosporine (STS), dimethyl sulfoxide, monoclonal anti-α-tubulin, and monoclonal anti-β-actin antibodies and Kodak film were purchased from Sigma (Taufkirchen, Germany). Wortmannin was obtained from Calbiochem (Schwalbach, Germany) and UO126 from Cell Signaling (Beverly, USA). The sGC inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and SNAP were received from Tocris Cookson Ltd. (Bristol, UK). The mouse monoclonal-anti-phospho-TrkA (Tyr^{490}) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA), polyclonal anti-phospho-Akt (Ser^{473}), polyclonal anti-Akt, and polyclonal anti-phospho-Erk1/2 (Thr^{202}/Tyr^{204}) antibodies were purchased from New England Biolabs (Beverly, USA). The chemiluminescence reagent and the anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Freiburg, Germany). Biotinylated anti-mouse and biotinylated anti-rabbit antibodies were from Vector Laboratories (Burlingame, USA), and streptavidin oregon green from Molecular Probes (Oregon, USA).

Measurement of NO-release

Nitric oxide release from DPN, Me-DPN, and methoxime-DPN was quantified based on a gas-phase chemiluminescent reaction between NO and ozone using the Nitric Oxide Analyzer NOA 280^{TM} (Sievers Instruments, Boulder, USA). Reactions were carried out at 37 °C at pH 7.4 under anaerobic conditions and continuous stirring for 20 h. The incubation mixture contained PBS with or without the oxygen donor iodosylbenzene (IOPh) prepared

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according to the literature (Lucas et al., 1995) and the dephostatin analogues (0.6 mM in ethanol). A defined volume of the gas phase above this solution was collected with a gastight syringe and transferred “into the purge and trap reaction vessel” of the NO-analyzer for nitric oxide measurement.

Embryonic hippocampal cultures

Hippocampi were removed from embryonic day 18 Sprague-Dawley rats (Charles River, Sulzfeld, Germany) and dissociated by mild trypsination and trituration as described previously (Culmsee et al., 2002). They were then seeded onto polyethylenimine-coated 35 mm culture dishes (for survival analysis), glass coverslip containing 35 mm culture dishes (for immunocytochemistry) or 60 mm culture dishes (for immunoblot analysis) containing Eagle`s Minimum Essential Medium (GIBCO Life Technologies, Eggenstein, Germany) supplemented with 1 mM HEPES, 26 mM $NaHCO_3$, 40 mM glucose, 20 mM KCl, 1 mM sodium pyruvate, 1.2 mM l-glutamine, 10% (v/v) heat-inactivated fetal bovine serum and 10 mg/l gentamicin sulfate. After a 6 h incubation period the medium was replaced with Neurobasal medium with B27 supplements (GIBCO Life Technologies, Eggenstein, Germany), 4.6 mM HEPES, 1.2 mM l-glutamine and 10 mg/l gentamicin sulfate. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C and medium was exchanged after 5 d in culture. Experimental treatments were performed with 7-8 d old cultures in Neurobasal medium with B27 supplements.

Apoptosis was induced by STS (200 nM) in Neurobasal medium or N-methyl-D-aspartate (NMDA, 10µM) in Locke’s solution (NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2.3 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 3.6 mM; glucose, 5 mM; HEPES, 5 mM; pH 7.2) and quantified 24 h later after fixing the cells for 30 min in methanol and staining with 10 µg/ml of the DNA fluorochrome Hoechst 33258 in methanol for 15 min. Nuclear morphology was analyzed under a fluorescence microscope. Neurons exhibiting reduced nuclear size (pycnotic

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nuclei), chromatin condensation (visible as an intense fluorescence) and nuclear fragmentation were considered apoptotic. About 250 cells per culture in at least five separate cultures per treatment condition were counted, and the percentage of apoptotic neurons was determined and expressed as the percent ratio of neurons with apoptotic nuclei of the total number of cells. Experiments were repeated at least three times and analyses were performed without knowledge of the treatment history of the cultures.

Immunoblotting

For Western blot analysis cells were lyzed in ice cold homogenization buffer (150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EGTA, and 1 mM sodium orthovanadate) supplemented with 0.1 mM phenylmethanesulfonyl fluoride, 5 µg/ml trypsin inhibitor, 5 µg/ml aprotinin, and 5 µg/ml leupeptin. The protein content in the lysates was determined using the Pierce BCA kit (Perbio Science, Bonn, Germany). Lysates containing equal amounts of total protein were incubated for 5 min at 95 °C after adding 1/6 (v/v) loading buffer (130 mM Tris pH 6.8, 10% sodium dodecylsulfate, 10% 2-mercaptoethanol, 20% glycerol, and 0.06% bromophenol blue) and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Afterwards, the proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). The membrane was then incubated in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% non-fat milk for 1 h at room temperature (RT) and further exposed to monoclonal anti-phospho-TrkA (1:1,000), polyclonal anti-phospho-Akt (1:1,000), polyclonal anti-Akt (1:1,000), monoclonal anti-α-tubulin (1:10,000), or anti-β-actin (1:10,000) antibodies overnight at 4 °C. The blots were washed several times with TBST, incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 1 h and then detected on Kodak film after exposure to a chemiluminescence reagent (Amersham Biosciences, Freiburg, Germany). Densitometric analysis of protein phosphorylation was performed after scanning of the immunoblots from at

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least 3 different experiments by using Scion Image for Windows (Scion Corporation, Frederick, MD, USA). Grey values of the specific bands of the phosphorylated proteins were corrected for the background signal and normalized to the signal of the individual loading control, i.e. the respecive unphosphorylated protein, α-Tubulin or β-actin.

Immunocytochemistry

The methods were similar to those described previously (Culmsee et al., 2002). Cells were fixed in 4% paraformaldehyde in PBS for 30 min and then permeabilized by exposure to Triton X-100 (0.2% in PBS) for 5 min. After blocking with 5% horse or goat serum in PBS for 30 min the cells were exposed to the respective primary antibodies (1:100) overnight at 4 °C. After washing with PBS, cells were incubated with the appropriate biotinylated secondary antibodies (1:200) for 1 h at RT and then exposed to streptavidin oregon green for 45 min at RT in the dark. Alternatively, a secondary antibody labeled with Texas Red was used. Images were acquired using a confocal laser scanning microscope (LSM 510, Zeiss, Germany) with a 40x oil immersion objective. All images are representative for at least 3 independent experiments and were acquired using the same laser intensity and photodetector gain to allow comparisons of relative levels of immunoreactivity between cultures. The respective primary antibody was omitted in negative controls. The specificity of the monoclonal phospho-TrkA antibody was demonstrated previously by Western blot analysis with protein extracts from wildtype PC12 cells and PC12nnr5 cells lacking TrkA (Culmsee et al., 2002; Gerling et al., 2004).

Statistical analysis

All values were given as means ± S.D. For all data one-way analysis of variance (ANOVA) with subsequent Scheffé test was employed.

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Results

The dephostatin analogues DPN and Me-DPN but not methoxime-DPN release NO.

We measured NO-release by the PTP inhibitors DPN, Me-DPN and methoxime-DPN (Fig. 1A) using the chemiluminescence analyzer NOA 280. As demonstrated in Fig. 1B and 1C, only DPN and Me-DPN released NO, whereas no NO-release was detectable with the nitrosamine-free methoxime-DPN. The NO-release from DPN increased 3.7-fold (Fig. 1B) and from Me-DPN 4.2-fold (Fig. 1C) after co-incubation with the oxygen donor IOPh. In contrast, incubation of methoxime-DPN with IOPh did not result in any NO release.

DPN and Me-DPN, but not nitrosamine-free methoxime-DPN enhance TrkA phosphorylation and protect hippocampal neurons against apoptotic stress.

Since dephostatin analogues have been developed as PTP inhibitors with the potential to enhance receptor tyrosine kinase (RTK) signaling, we evaluated the effect of DPN, MeDPN, and methoxime-DPN on TrkA phosphorylation in embryonic rat hippocampal neurons. Immunostaining revealed an increase in phospho-TrkA immunoreactivity in cultured neurons after treatment with DPN (1 μM) within 5 min and up to 60 min after exposure (Fig. 2A). This observation was confirmed by immunoblot analysis (Fig 2B), and quantification of the immunoblot signals revealed an significant increase of pTrkA phosphorylation by DPN 5 min and 10 min after exposure (3.1 ± 0.27-, and 3.7 ± 0.11-fold increase as compared to controls, $p<0.001$) Such a transient increase in TrkA phosphorylation was also observed with Me-DPN (Fig. 3A; 1.8 ± 0.16, 2.9 ± 0.8, and 2.5 ± 0.57-fold increase at 5, 10, 30 min as compared to controls, $p<0.001$), but not with methoxime-DPN (Fig. 4A,B).

Further experiments in neurons exposed to apoptotic stress also revealed differences in the neuroprotective potential of DPN and Me-DPN versus methoxime-DPN. As shown in Figure 2C, Figure 3B, both DPN and Me-DPN attenuated STS-induced apoptosis in a

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concentration-dependent manner with the most pronounced protection achieved at 1-10 µM of the dephostatin analogues. The nitrosamine-free dephostatin analogue methoxime-DPN, however, did not protect the neurons against STS-induced apoptosis (Fig. 4C). Similar results were obtained when apoptosis was induced by 10 µM NMDA in Locke's medium. DPN (0.1-10 µM) protected hippocampal neurons against NMDA-induced apoptosis, whereas methoxime-DPN (0.01-100 µM) did not attenuate cell death in this paradigm (Fig. 5). Notably, methoxime-DPN exerted pronounced neurotoxic effects at concentrations of 100 µM in Locke's solution (Fig. 5B).

The NO-donor SNAP enhances TrkA phosphorylation and protects hippocampal neurons against STS-induced apoptosis.

The results obtained with the dephostatin derivatives indicated that NO release is required for the enhanced TrkA phosphorylation and the associated neuroprotective effects. Therefore, we included the established NO-donors SNAP and SNP in the following experiments. As shown by immunocytochemistry and Western blot analysis the NO-donor SNAP increased levels of phosphorylated TrkA in cultured neurons within 10 min of exposure, and this effect persisted for at least 180 min (Fig. 6A,B). Densitometric analysis of the western blots confirmed a pronounced SNAP-induced TrkA phosporylation in neurons that peaked at a 7.5 ± 0.24-fold increase over control signals at 60 min ($p<0.001$). In line with the previous results with DPN and Me-DPN, 6 h pretreatment with SNAP (0.01-10 µM) protected hippocampal neurons against STS- (Fig. 6C) and NMDA-induced apoptosis (10µM in Locke's solution, data not shown). Notably, SNAP induced neuronal damage at concentrations of 100 µM, which most likely reflected the reported neurotoxic effects of NO at high concentrations. Similar to SNAP the NO-donor SNP (0.1-1 µM) also protected hippocampal neurons against STS- or NMDA-induced apoptosis (data not shown).

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Overall, these results revealed that NO released by the dephostatin derivatives DPN and Me-DPN or the NO-donor SNAP enhanced TrkA phosphorylation and exerted anti-apoptotic effects in hippocampal neurons in a similar pattern as previously documented for NGF or the PTP inhibitor orthovanadate (Culmsee et al., 2002; Gerling et al., 2004).

Activated PI3-K/Akt and MAPK pathways mediate DPN- and SNAP-induced neuroprotection in embryonic hippocampal neurons.

The PI3-K/Akt pathway and the MAPK pathway are the most prominent signaling pathways downstream of TrkA activation (Sofroniew et al., 2001), and these were also involved in neuroprotective signaling stimulated by the PTP inhibitor orthovanadate (Gerling et al., 2004). Therefore, we further investigated the potential of the NO-donors DPN and SNAP to activate these neuroprotective signaling pathways. Immunoblot analysis of protein extracts from rat hippocampal cultures revealed enhanced Akt phosphorylation after 5 min (2.2 ± 0.7-fold increase) and up to 60 min (2.0 ± 0.3-fold increase) following treatment with the PTP inhibitor and NO-donor DPN (1 μM; Fig. 7A). Notably, the increase in Akt phosphorylation induced by the dephostatin analogue was suppressed by preincubation (60 min) with the PI3-K inhibitor wortmannin (20 nM). Levels of total Akt were not affected after treatment with DPN. Similar results were obtained with SNAP which induced a transient phosphorylation of Akt that also peaked after 60 min of exposure (2.6 ± 0.58-fold increase; $p<0.01$) (Fig. 7C). Importantly, preincubation with the PI3-K inhibitor wortmannin (20 nM) abolished the neuroprotective effects of DPN (Fig. 7B) and SNAP (Fig. 7D). These findings clearly point at an involvement of the PI3-K/Akt pathway in the underlying mechanisms of NO-donor-mediated neuroprotection.

Next, we investigated effects of DPN and SNAP on the MAPK pathway in rat neurons. Immunostaining followed by confocal laser scanning microscope analysis revealed

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that DPN (1 μM) enhanced phosphorylation of Erk1/2 in hippocampal neurons within 10 min after exposure (Fig. 8A). The enhanced phosphorylation of Erk1/2 was sustained for 3 h and declined to basal levels after 6 h. Immunocytochemistry (not shown) and Western blot analysis (Figure 9B) revealed that treatment with SNAP caused an increase in Erk1/2 phosphorylation (1.7 ± 0.30-fold and 2.3 ± 1.0-fold of Erk1 and Erk2 after 60 min of SNAP-exposure, respectively) in a similar pattern as DPN. The importance of the MAPK pathway activation was further confirmed by pretreatment of hippocampal cultures with the MAPK-kinase (MEK) inhibitor UO126 (20 μM) which significantly blocked the antiapoptotic effect of DPN (Fig. 8B) and SNAP (Fig. 8C). Overall, these data implicated that both the PI3-K/Akt pathway and the MAPK pathway were involved with equal importance in DPN- and SNAP-mediated neuroprotective signaling pathways in neurons.

NO-induced cGMP signaling is involved in DPN- and SNAP-mediated neuroprotective signaling.

Neuroprotective signaling by NO has been linked to the established cGMP-dependent signal transduction cascade which involves the activation of sGC with subsequent synthesis of cGMP and activation of PKG (Thippeswamy and Morris, 1997). In order to evaluate the involvement of sGC in the neuroprotective signaling mediated by the NO-releasing dephostatin derivatives and SNAP we preincubated rat embryonic hippocampal neuronal cultures with the sGC inhibitor ODQ (20 μM, Schrammel et al., 1996). Strikingly, the sGC inhibitor blocked SNAP-induced phosphorylation of TrkA, Akt (Fig. 9A) and Erk1/2 (Fig. 9B) in cultured neurons. Moreover, ODQ blocked the neuroprotective effects of DPN (Fig 10A), Me-DPN (not shown) and SNAP (Fig. 10B) against STS-induced apoptosis. Furthermore, neuroprotection by the NO-donors DPN and SNAP was significantly suppressed by the PKG inhibitor KT5823 as presented in Fig. 11. Altogether, these findings indicate that

enhanced TrkA phosphorylation and the induction of survival signaling through PI3-K/Akt and MAPK pathways by the NO-donors as well as the associated anti-apoptotic effects require activation of sGC and PKG. Therefore, it is suggested that NO release and subsequent induction of cGMP signaling pathways rather than inhibition of PTP-1B or SHP-1 were involved in neuroprotection by DPN, Me-DPN and SNAP.

The Trk-inhibitor K252a does not affect NO-donor mediated neuroprotection.

Finally, we wanted to know, whether the observed phosphorylation of TrkA is necessery to contribute to the antiapoptotic effects of the NO donors, or if the observed cGMP- and neurotrophin-related survival signaling pathways could mediate neuroprotection independent of TrkA activity. Therefore, the Trk inhibitor K252a was applied to block TrkA phosphorylation (Culmsee et al., 2002). This inhibitor did not affect the protective effect of SNAP (1-10 µM) against STS-induced apoptosis in the cultured neurons (Figure 12). These results suggest that activation of the neurotrophin receptor was not required for induction of the downstream neurotrophin-related survival pathways further strengthening the finding that the cGMP/PKG-pathway predominantely mediated neuroprotection by the NO-donors.

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Discussion

The results of the present study in primary neurons indicate that NO release is a major requirement for activation of neurotrophin-like signaling pathways and neuroprotection mediated by the PTP inhibitors DPN and Me-DPN. Similar to the NO-donors SNAP or SNP, NO-releasing dephostatin derivatives enhanced levels of phosphorylated TrkA, Akt and Erk1/2, and such neuroprotective signaling required sGC and PKG activity. By contrast, the nitrosamine-free methoxime-DPN failed to enhance TrkA phosphorylation and did not exert protection against staurosporine-induced apoptosis.

In accordance with previous reports, the NO donors used in the present study were neuroprotective at low concentrations (1-10 μM) while higher concentrations (> 100 μM) were inactive or even neurotoxic depending on treatment conditions (Figuera et al., 2005). The observed activation of neurotrophin-like survival signaling by NO can be explained by distinct mechanisms. A large body of evidence suggests that survival signaling in neurons can be mediated through NO-dependent activation of sGC with subsequent cGMP synthesis and activation of PKG (for review see Schlossmann et al., 2003; Hanafy et al., 2001). In addition, it has been proposed that NO acts as a PTP inhibitor (Hanafy et al., 2001; Monteiro, 2002). This may result in reduced RTK dephosphorylation thereby accelerating neurotrophin-like neuroprotective signaling similar to our previous results obtained with the PTP inhibitor orthovanadate (Gerling et al., 2004). The present results demonstrate a similar potential for the NO-releasing dephostatin analogues DPN, Me-DPN and the established NO-donor SNAP to enhance tyrosine phosphorylation of TrkA and phosphorylation of Erk1/2 and Akt. These results are in line with recent data showing decreased PTP activity after incubation with NO-donors (Caselli et al., 1994; 1995) with subsequent activation of growth factor receptors (Callsen et al., 1999) and tyrosine kinases such as focal adhesion kinase (FAK), src-kinase, and MAP-kinases (Monteiro et al., 2000). Our results showing that the TrkA inhibitor K252a

did not block neuroprotection by the NO donor SNAP suggested that TrkA activation is dispensible for the activation of survival signaling pathways. This exclude, however, that PTP inhibition and subsequent activation of RTK or other tyrosine-phosphorylation-dependent survival signaling pathways still contributed to the observed protection by NO-donors. Notably, it has been shown that NO can enhance tyrosine phosphorylation of other growth factor receptors like the receptor for platelet-derived growth factor (PDGF) (Callsen et al., 1999). Therefore, we assume that NO can further induce phosphorylation of other RTK in neurons including RTK receptors for PDGF, EGF or IGF-1, or other neurotrophin receptors like TrkB or TrkC.

Inhibition of PTP has been introduced as a promising strategy to sustain accelerated RTK phosphorylation in order to mimic the effects of growth factor or insulin signaling (Suzuki et al., 2001; Hiroki et al., 2002). In neurons, we recently demonstrated that protection against apoptotic stress by the PTP inhibitor orthovanadate was associated with enhanced TrkA phosphorylation and activation of downstream survival signaling even in the absence of NGF (Gerling et al., 2004). In the present study, the activation of TrkA and downstream signaling pathways by DPN and Me-DPN could be mediated through NO-dependent inhibition of a –yet undefined- PTP that usually dephosphorylates TrkA. According to the literature, potential PTP candidates directly involved in TrkA regulation are PTP-1B and src homology-2-containing protein tyrosine phosphatase-1 (SHP-1) (Haj et al., 2003; Marsh et al., 2003). PTP-1B is a known regulator of PDGF (Markova et al., 2003), insulin and IGF-I receptors (Kenner et al., 1996; Buckley et al., 2002) and affects downstream signaling through the Ras-MAPK cascade (Zhang et al., 2002), suggesting that PTP-1B may as well be involved in the regulation of other RTK such as TrkA as well. More recently, SHP-1 has been identified as a TrkA phosphatase that controls both basal and NGF-regulated levels of TrkA

activity in neurons (Marsh et al., 2003). Notably, inhibition of PTP-1B and SHP-1 has been identified as the underlying mechanism of DPN- and Me-DPN-mediated activation of the insulin-related signaling in cultured 3T3-L1 adipocytes, including enhanced tyrosine phosphorylation of the insulin receptor (Hiroki et al., 2002; Suzuki et al., 2001). However, similar acceleration of insulin receptor signaling in vitro and antidiabetic effects in vivo were also obtained with the nitrosamine-free dephostatin analogue and PTP-1B inhibitor methoxime-DPN (Hiroki et al., 2002). In the present study, the PTP-1B inhibitor methoxime-DPN neither enhanced TrkA phosphorylation nor protected neurons against STS- or NMDA-induced apoptosis. Therefore, it is unlikely that inhibition of PTP-1B was involved in neuroprotection by the NO-donors.

Apart from PTP inhibition, NO-induced RTK phosphorylation and activation of downstream survival signaling may be mediated through activation of sGC and the resulting cGMP-dependent signaling. For example, recent data showed that NO induced phosphorylation of the EGF receptor and subsequent activation of the Ras-MAPK pathway was mediated through cGMP (Oliveira et al., 2003). Our results strongly suggest, that indeed activation of the NO/cGMP signaling pathway plays a major role in the regulation of TrkA phosphorylation and the associated downstream survival signaling pathways by NO-donors in neurons. Strikingly, enhanced phosphorylation of TrkA as well as phosphorylation of Akt and Erk1/2 induced by the NO donor SNAP was blocked by the sGC inhibitor ODQ. Consequently, the neuroprotective effects of SNAP and the NO-releasing DPN were clearly blocked by ODQ or the PKG inhibitor KT5823. These findings are in line with neuroprotective effects of NO in cultured neurons (Lipton, 1999; Vidwans et al., 1999) and in vivo (Gidday et al., 1999; Laufs et al., 2000). These and other studies indicated that NO-mediated activation of the cGMP signaling cascade prevents apoptotic neuronal death through

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activation of PKG (Farinelli et al., 1996; Kim et al., 1999; Shen et al., 1998). Such cGMP-dependent neuroprotection was associated with reduced mitochondrial cytochrome c release and activation of caspases (Kim et al., 1997), and preserved high levels of antiapoptotic Bcl-2 (Kim et al., 1998).

The classic view of cGMP as the exclusive mediator of NO activity has been recently supplemented with possible direct interactions of NO with protein factors involved in cell signaling (Davis et al., 2001). Such post-translational modifications may include direct inhibition of caspases by S-nitrosylation of cysteine present in the active site of all caspase enzymes (Haendeler et al., 1997; Kim et al., 1997; Li et al., 1997). For S-nitrosylation, however, higher concentrations of NO are required than for activation of sGC (Ahern et al., 2002; Davis et al., 2001).

Although our data clearly show that NO release and subsequent cGMP signaling pathways are prerequisites for neuroprotection by NO-donors, an involvement of PTP inhibition in the observed induction of neurotrophin-like signaling cannot be excluded. It has been shown that neurotrophins can enhance NO synthesis (Holtzmann et al., 1994) thereby activating subsequent cGMP pathways which essentially contribute to various neurotrophin actions including neuronal outgrowth and neuroprotection (Ha et al., 2003; Hindley et al., 1997). In addition to direct actions of NO, inhibition of PTP by the NO-donors may thus mimic neurotrophin actions, including a sustained elevation of NO synthesis and related cGMP signaling in a positive feedback loop. A similar positive feedback loop has been recently proposed for NO and BDNF in neural progenitor cells (Cheng et al., 2003). Whether or not PTP inhibition is required for neuroprotective signaling induced by NO-donors and which PTP could be involved in the presented activation of NGF-like signaling in differentiated neurons remains to be clarified.

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Neuroprotection by DPN or SNAP was blocked by the PI3-K inhibitor wortmannin or the MEK inhibitor UO126, providing evidence that the PI-3K/Akt and MAPK pathways were equally involved in the underlying mechanism. Enhanced PI3-K activity and subsequent phosphorylation and activation of Akt are key elements in many growth factor or cytokine signaling pathways and promote survival of many different cell types including neurons (Philpott et al., 1997; Crowder and Freeman, 1998). In line with our previous findings in hippocampal neurons exposed to NGF or the PTP inhibitor orthovanadate (Culmsee et al., 2002; Gerling et al., 2004), the present results also support a crucial role for the PI-3K/Akt pathway in NO-mediated neurotrophin-like survival signaling. A large number of substrates have been identified for the serine/threonine kinase Akt that may block cell death by both impinging on the cytoplasmatic cell death machinery and by regulating the expression of factors involved in cell death and survival (reviewed in Brunet et al., 2001).

In addition to the PI3-K/Akt pathway, growth factors can activate the MAPK cascade that mediates differentiation, proliferation, and survival in various cell types including neurons (Gómez and Cohen, 1991; Xia et al., 1995; Zhu et al., 2002). Our results regarding the involvement of enhanced MAPK activity in the neuroprotective effect of DPN and SNAP are in line with previous findings in neurons demonstrating an activation of Ras-MAPK by NO (Gonzalez-Zulueta et al., 2000; Yun et al., 1998).

Overall, our findings suggest that NO is involved in the protective effect by DPN-derivatives and that neuroprotection by NO is associated with phosphorylation and activation of TrkA and downstream survival signaling through PI-3K/Akt and MAPK pathways. Our findings imply that treatment with NO-donors is an appropriate strategy to trigger neurotrophin-like survival signaling pathways to protect neurons against apoptotic stress.

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Figure legends

Figure 1. DPN and Me-DPN, but not methoxime-DPN release NO. (**A**) Molecular structures of Ethyl-3,4-dephostatin (DPN) (**1**), 4-O-Methyl-ethyl-3,4-dephostatin (Me-DPN) (**2**), methoxime-3,4-dephostatin (methoxime-DPN) (**3**). (**B,C**) The release of NO was measured under anaerobic conditions using a chemiluminescence analyzer. In the presence of the oxygen donor iodosylbenzene (IOPh) the NO-release from DPN was increased 3.7-fold (**B**) and from Me-DPN 4.2-fold (**C**) as compared to measurements under anaerobic conditions. NO-release was not detectable with methoxime-DPN, neither under anaerobic conditions nor in the presence of IOPh (**C**). Values are the mean ± S.D. of 4 measurements in each group. Different from measurements of NO-release without IOPh: ***$p<0.001$ (ANOVA; Scheffé's test).

Figure 2. DPN enhances TrkA phosphorylation and attenuates STS-induced apoptosis in hippocampal neurons. Ethyl-3,4-dephostatin (DPN, 1μM) induced a transient phosphorylation of TrkA in embryonic rat hippocampal neurons as demonstrated by immunocytochemistry (**A**) and immunoblot analysis (**B**). Enhanced phospho-TrkA levels were detectable within 5 and up to 60 min after exposure to DPN. Controls received vehicle (DMSO) instead of DPN. (**C)** Hippocampal neurons were incubated with DPN (0.01-10 μM) 6 h before and during exposure to staurosporine (STS, 200 nM). Twenty-four h later the percentage of apoptotic neurons was evaluated by nuclear staining with the DNA-fluorochrome Hoechst 33258. Percentages of apoptotic nuclei were given as means ± S.D. from 5 dishes/group. Different from vehicle-treated cells: ***$p<0.001$; different from vehicle-treated, STS-exposed cultures: $^{###}p<0.001$ (ANOVA, Scheffé's test).

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Figure 3. Me-DPN enhances TrkA phosphorylation and reduces STS-induced apoptosis in hippocampal neurons. (**A**) 4-O-Methyl-ethyl-3,4-dephostatin (Me-DPN) induced phosphorylation of TrkA in embryonic rat hippocampal neurons within 5-30 min of exposure as demonstrated by immunoblot analysis (**B**) Hippocampal neurons were exposed to Me-DPN 6 h before induction of apoptosis by staurosporine (STS; 200 nM). Twenty-four hours later apoptotic cell death was determined after nuclei staining with Hoechst 33258. Percentages of apoptotic nuclei are given as means and S.D. from 5 dishes/group. ***$p<0.001$ compared to vehicle-treated controls, $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ compared to STS-exposed controls (ANOVA, Scheffé's test).

Figure 4. Methoxime-DPN neither affects TrkA phosphorylation nor STS-induced apoptosis in hippocampal neurons. Methoxime-3,4-dephostatin (methoxime-DPN, 1 µM) did not change phosphorylation of TrkA in embryonic rat hippocampal neurons as demonstrated by immunocytochemistry (**A**) and immunoblot analysis (**B**). (**C)** Hippocampal neurons were incubated with Ethyl-3,4-dephostatin (DPN, 1 µM) or methoxime-DPN (0.01-100 µM) 6 h before and during exposure to staurosporine (STS, 200 nM). Twenty-four h later the percentage of apoptotic neurons was evaluated by nuclear staining with the DNA-fluorochrome Hoechst 33258. Percentages of apoptotic nuclei were given as means ± S.D. from 5 dishes/group. Different from vehicle-treated cells: ***$p<0.001$; different from vehicle-treated, STS-exposed cultures: $^{\#\#\#}p<0.001$ (ANOVA, Scheffé's test). Note, that in contrast to DPN, methoxime-DPN did not attenuate STS-induced apoptosis at any concentration tested.

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Figure 5. DPN but not methoxime-DPN reduces NMDA-induced apoptosis in hippocampal neurons. Hippocampal neurons were incubated with the dephostatin derivatives Ethyl-3,4-dephostatin (DPN, 0.1-10 µM) (**A**) or methoxime-3,4-dephostatin (0.01-100 µM) (**B**) 6 h before and during exposure to N-methyl-D-aspartat (10 µM) in Locke's solution. Twenty-four hours later, apoptotic cell death was determined after nuclei staining with Hoechst 33258. Percentages of apoptotic nuclei are given as means and S.D. from 5 dishes/group. ***$p<0.001$ compared to vehicle-treated controls, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to NMDA-exposed controls (ANOVA, Scheffé's test). In contrast to DPN, methoxime-DPN did not attenuate NMDA-induced apoptosis at 0.01 and 1 µM. Note the pronounced toxicity of methoxime-DPN at concentrations of 100 µM in Locke's solution.

Figure 6. The NO-donor SNAP increases the phosphorylation level of TrkA and protects hippocampal neurons against apoptosis. S-nitroso-N-penicillamin (SNAP, 1 µM) enhanced phosphorylation of TrkA in primary rat neurons as detected by confocal laser scanning microscopy after immunostaining with an anti-phospho-TrkA antibody (**A**) and by Western blot analysis (**B**). Note, that enhanced phospho-TrkA level were detectable within 10-180 min after exposure to SNAP. **(C**) Hippocampal neurons were incubated with SNAP (0.01-100 µM) 6 h before and during exposure to staurosporine (STS, 200 nM). Twenty-four hours later the percentage of apoptotic neurons was evaluated by nuclear staining with Hoechst 33258. Percentages of apoptotic nuclei were given as means ± S.D. from 5 dishes/group. ***$p<0.001$ compared to vehicle-treated cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$ compared to vehicle-treated, STS-exposed cultures: (ANOVA, Scheffé's test). Note, that 100 µM SNAP increased the number of apoptotic cells under control conditions and did not attenuate apoptosis in neurons exposed to STS.

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Figure 7. Activation of the PI3-K/Akt pathway mediates neuroprotection by DPN and SNAP. A. Immunoblot analysis of protein extracts from rat hippocampal cultures revealed enhanced Akt phosphorylation after 5 min and up to 60 min after treatment with Ethyl-3,4-dephostatin (DPN, 1 µM). Note, that the increase in Akt phosphorylation by the NO-donor was suppressed by preincubation (1 h) with the PI3-K inhibitor wortmannin (WM, 20 nM). Levels of total Akt were not different in the cultures and remained unchanged after treatment with DPN. Anti-α-tubulin immunostaining confirmed equal protein loading in each lane. **(B**) The PI3-K inhibitor wortmannin (WM, 20 nM) was added to embryonic rat hippocampal neurons 1 h prior to exposure to Et-3,4-dephostatin (1µM, D). Six hours later staurosporine (STS, 200 nM) was added to the cultures and 24 h later the percentage of apoptotic neurons was evaluated after nuclear staining with Hoechst 33258. Values are the mean ± S.D. of 5 dishes in each group. ***$p<0.001$ compared to vehicle-treated controls; $^{\#\#}p<0.01$ compared to STS-treated cultures, and $^{+++}p<0.01$ compared to DPN-treated cultures exposed to STS (ANOVA, Scheffé's test). **C.** Enhanced phospho-Akt immunoreactivity was detected by immunoblot analysis after treatment with S-nitroso-N-acetyl-penicillamin (SNAP, 1 µM) in rat hippocampal neurons at 7 d in culture. Note, that enhanced phospho-Akt levels were detectable within 30-180 min after exposure to SNAP. **D.** The PI3-K inhibitor wortmannin (WM, 20 nM) was added to embryonic rat hippocampal neurons 1 h prior to exposure to SNAP (1 µM). Six h later the cultures were exposed to staurosporine (STS, 200 nM) for 24 h and then the percentage of apoptotic neurons was evaluated after nuclear staining with Hoechst 33258. Values are the mean ± S.D. of 5 dishes in each group. ***$p<0.001$ compared to vehicle-treated controls; $^{\#\#\#}p<0.001$ compared to STS-treated cultures; and $^{+++}p<0.01$ compared to SNAP-treated cultures exposed to STS (ANOVA, Scheffé's test).

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Figure 8. Involvement of the MAPK pathway in NO-mediated neuroprotection in hippocampal neurons. (**A**) Enhanced phospho-Erk1/2 immunoreactivity was detected by confocal fluorescence laser scanning microscopy after treatment with Ethyl-3,4-dephostatin (DPN, 1 µM) in rat hippocampal neurons at 7 d in culture. Enhanced phospho-Erk1/2 levels were detectable within 10-60 min after exposure to DPN. The increase in phospho-Erk1/2 immunoreactivity declined to basal levels within 6 h (not shown). (**B,C**) The MAPK kinase inhibitor UO126 (20 µM) was added to embryonic rat hippocampal neurons 1 h prior to exposure to DPN (1 µM, **B**) or S-nitroso-penicillamin (SNAP, 1 µM, **C**). Six h later the cultures were incubated with staurosporine (STS, 200 nM), and percentage of apoptotic neurons was evaluated by nuclear staining with Hoechst 33258 24 h after exposure to STS. Values are the mean ± S.D. of 5 dishes in each group. ***$p<0.001$ compared to vehicle-treated controls; $^{###}p<0.001$ compared to STS-treated cultures; $^{+++}p<0.001$ compared to DPN- or SNAP-treated cultures exposed to STS: (ANOVA, Scheffé's test).

Figure 9. Induction of neurotrophin-like signaling by the NO-donor SNAP depends on sGC activity. One hour after incubation with the inhibitor of the soluble guanylyl cyclase (sGC) 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 20 µM) the NO-donor S-nitroso-acetylpenicillamin (SNAP, 1 µM) was added to primary neuronal cultures. Controls were incubated with vehicle (DMSO), and the other cultures received ODQ or SNAP alone as indicated. Thirty minutes after onset of SNAP treatment the cells were harvested for protein extraction. Western blot analyses of these protein extracts show that SNAP enhanced phosphorylation levels of TrkA, Akt (**A**) and Erk2 (**B**). The sGC inhibitor ODQ blocked the SNAP-mediated enhanced phosphorylation of the respective factors. Content of unphosphorylated factors was controlled by exposure of the immunoblots to anti-Akt and anti-Erk antibodies, and protein loading was controlled by anti-ß-actin antibodies.

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Figure 10. The sGC inhibitor ODQ blocks the neuroprotective effects of DPN and SNAP. One hour after preincubation with the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 20 µM) Ethyl-3,4-dephostatin (DPN, 1 µM, **A**) or S-nitroso-penicillamin (SNAP, 1-10 µM, **B**) were added to embryonic hippocampal cultures. Six h later the cultures were exposed to staurosporine (STS, 200 nM) for 24 h and the percentage of apoptotic neurons was evaluated after nuclear staining with Hoechst 33258. Percentages of apoptotic nuclei are given as means ± S.D. from 5 dishes/group. ***$p<0.001$ compared to vehicle-treated cells; $^{###}p<0.001$ compared to vehicle-treated, STS-exposed cultures; $^{+++}p<0.001$ compared to DPN- or SNAP-treated cultures exposed to STS (ANOVA, Scheffé's test).

Figure 11. Neuroprotection by DPN or SNAP requires PKG activity. Ethyl-3,4-dephostatin (DPN, 1-10 µM, **A**) or S-nitroso-penicillamin (SNAP, 1-10 µM, **B**) were added to embryonic rat hippocampal cultures 1 h after pretreatment with the protein kinase G (PKG) inhibitor KT5823 (20 µM) and 6 h before incubation with staurosporine (STS, 200 nM). Twenty-four h later the percentage of apoptotic neurons was evaluated after nuclear staining with the DNA-fluorochrome Hoechst 33258. Percentages of apoptotic nuclei are given as means ± S.D. from 5 dishes/group. Different from vehicle-treated cells: ***$p<0.001$; different from vehicle-treated, STS-exposed cultures: $^{###}p<0.001$; $^{+++}p<0.001$ compared to DPN- or SNAP-treated cultures exposed to STS (ANOVA, Scheffé's test).

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Figure 12. Neuroprotection by SNAP is not affected by the Trk-inhibitor K252a. . S-nitroso-penicillamin (SNAP, 1 µM, **A** or 10 µM, **B**) was added to embryonic rat hippocampal cultures 1 h after pretreatment with the Trk-inhibitor K252a (200 µM) and 6 h before incubation with staurosporine (STS, 200 nM). Twenty-four h later the percentage of apoptotic neurons was evaluated after nuclear staining with the DNA-fluorochrome Hoechst 33258. Percentages of apoptotic nuclei are given as means ± S.D. from 5 dishes/group. Different from vehicle-treated cells: ***$p<0.01$; different from vehicle-treated, STS- or STS+K252a-exposed cultures: $^{\#\#}p<0.001$ (ANOVA, Scheffé's test).

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Figure 1

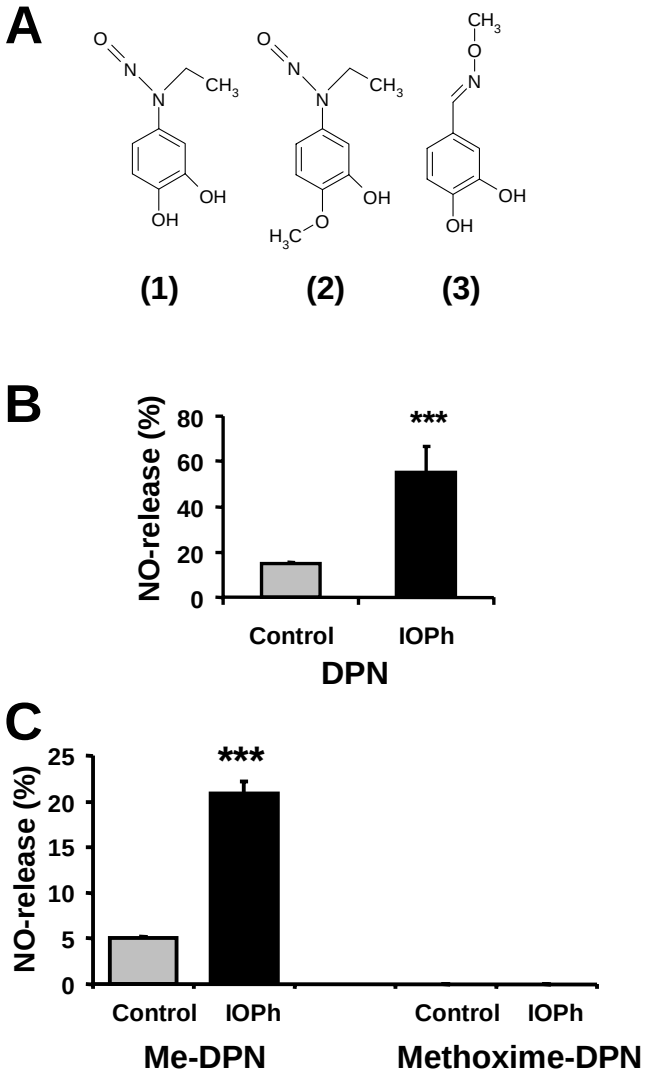

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Figure 2

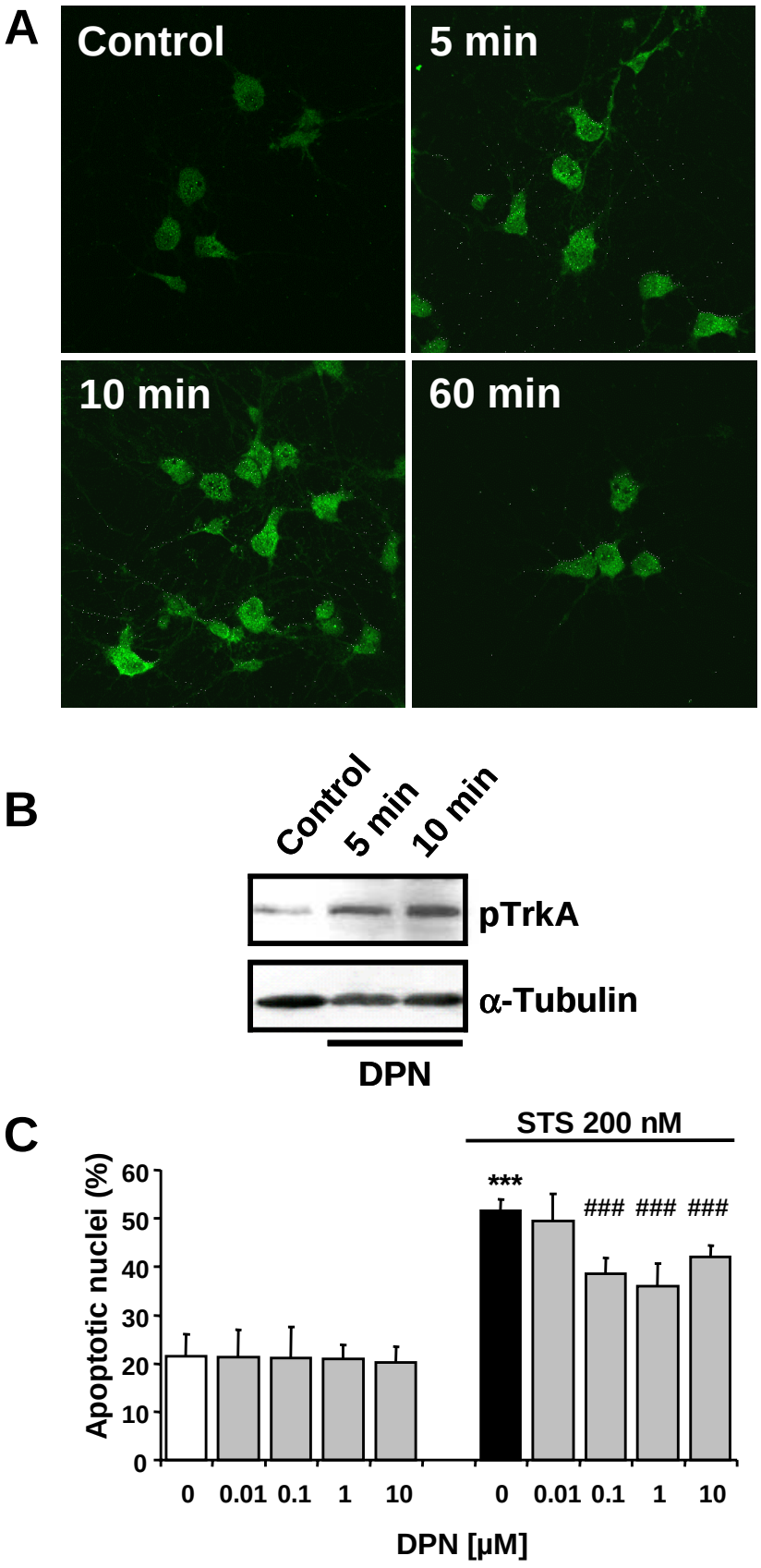

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Figure 3

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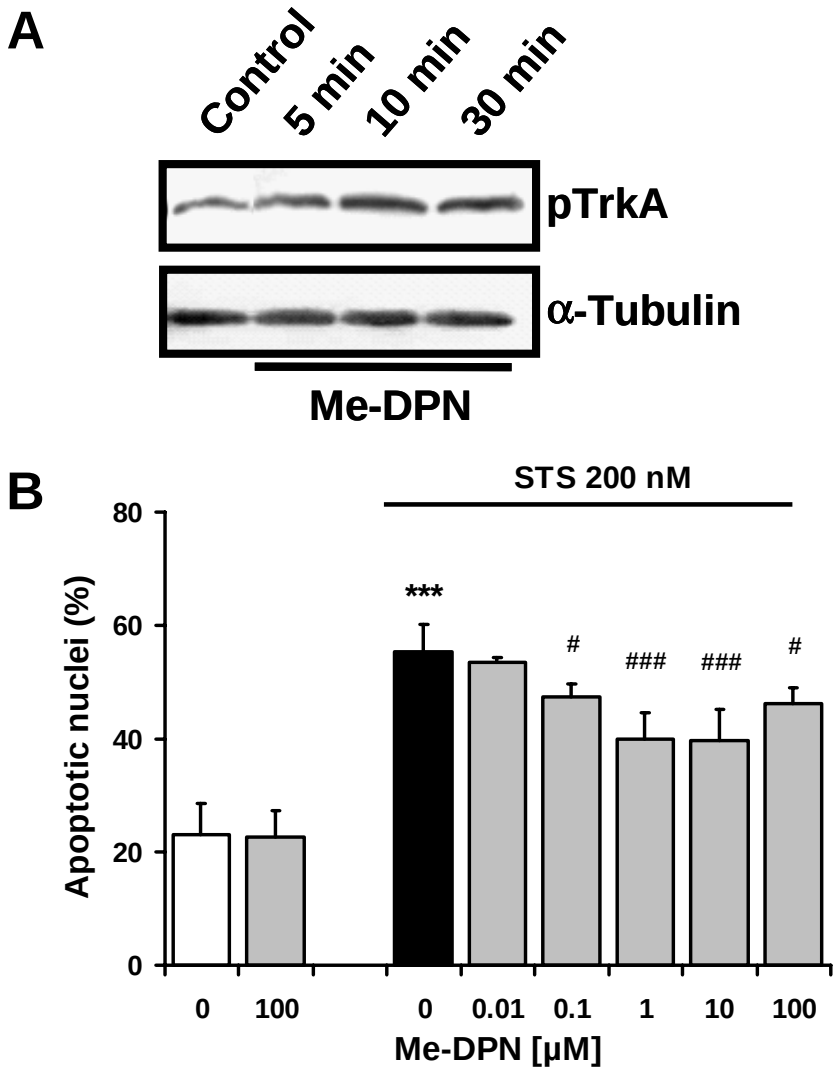

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Figure 4

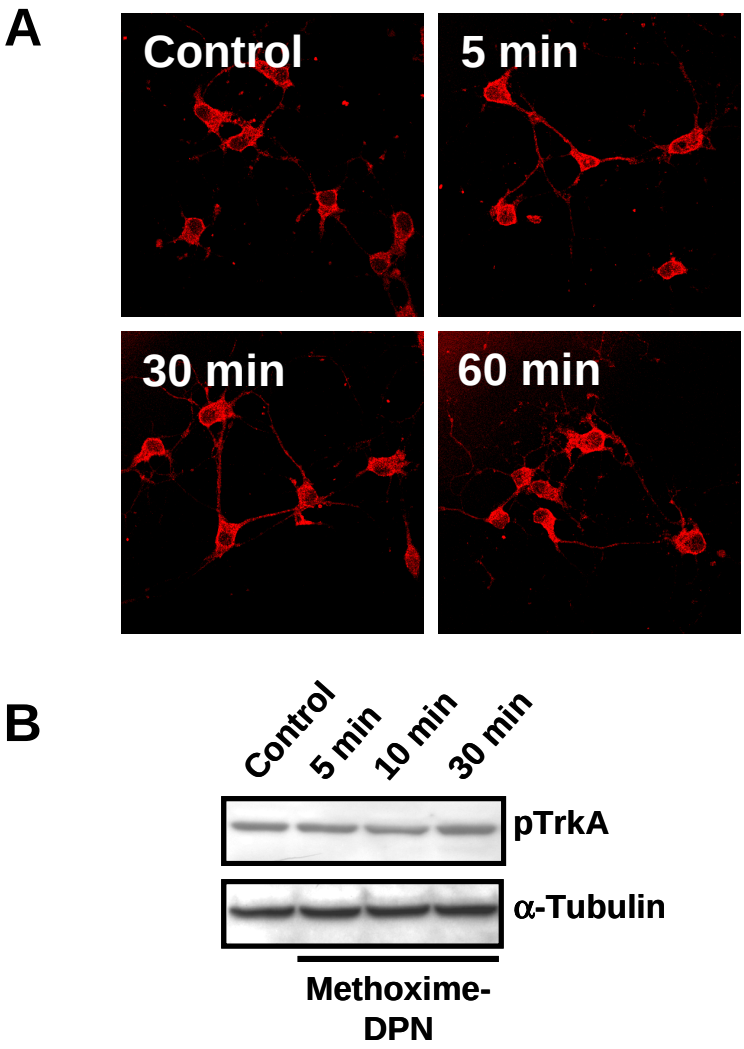

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Figure 5

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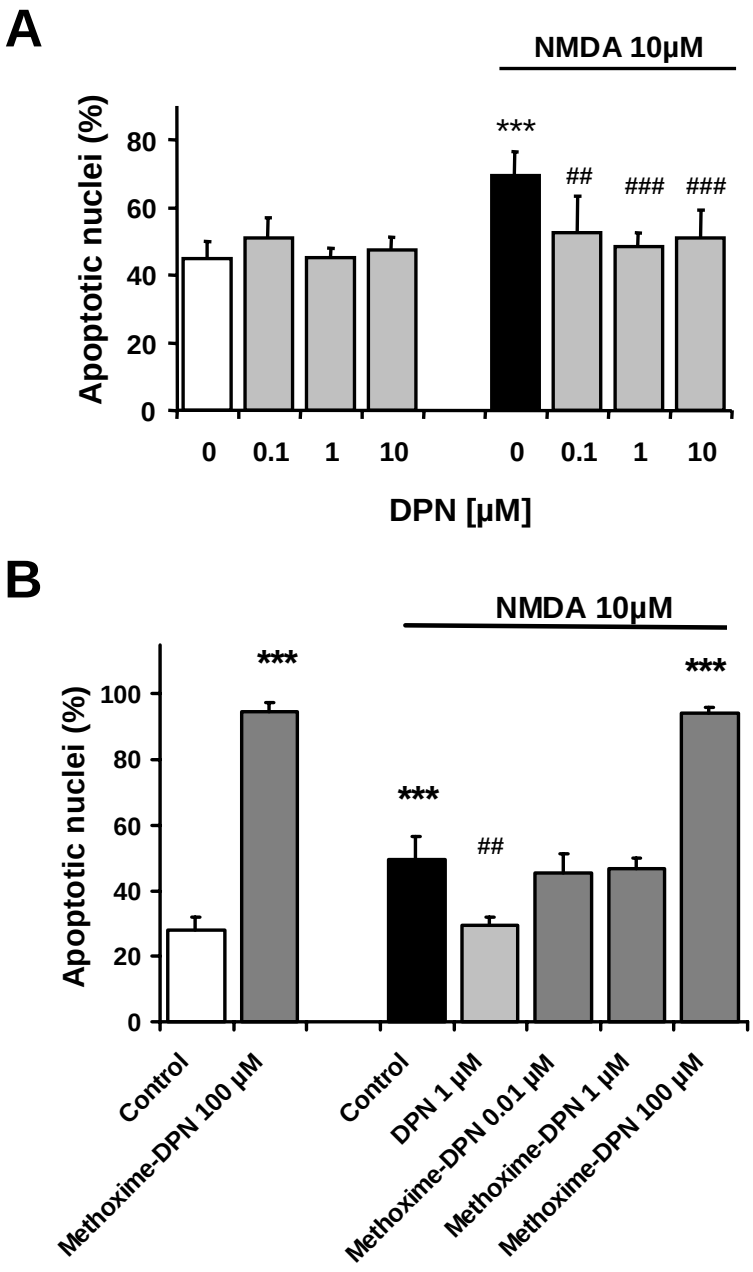

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Figure 6

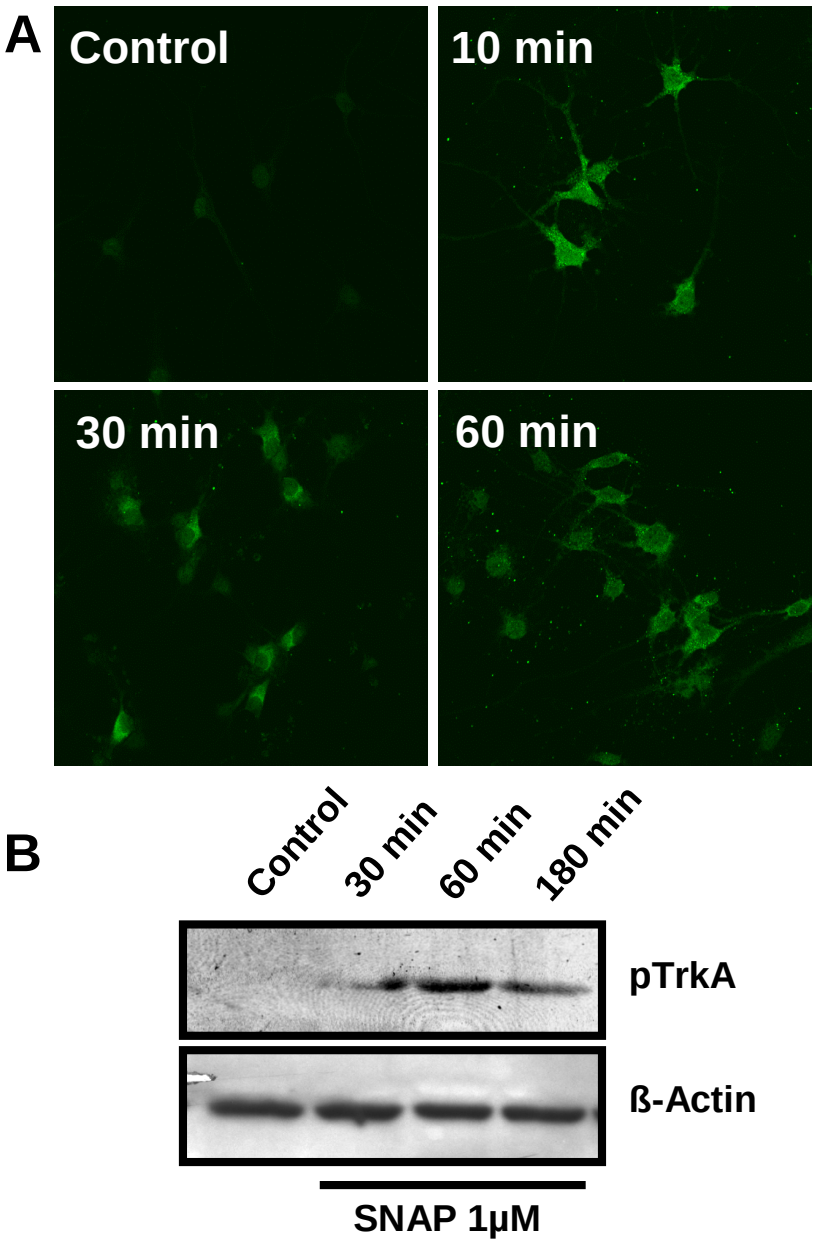

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Figure 7

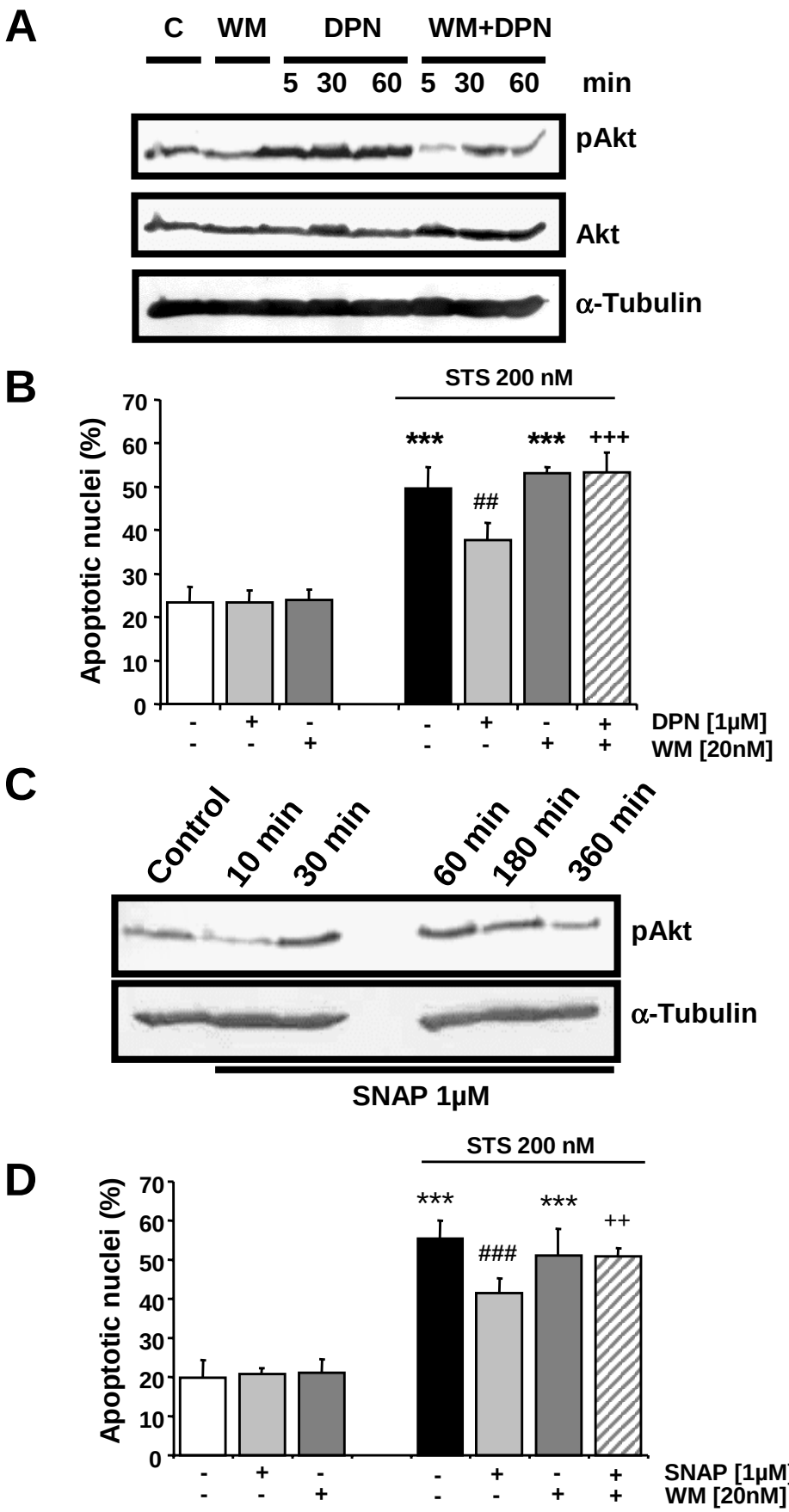

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Figure 8

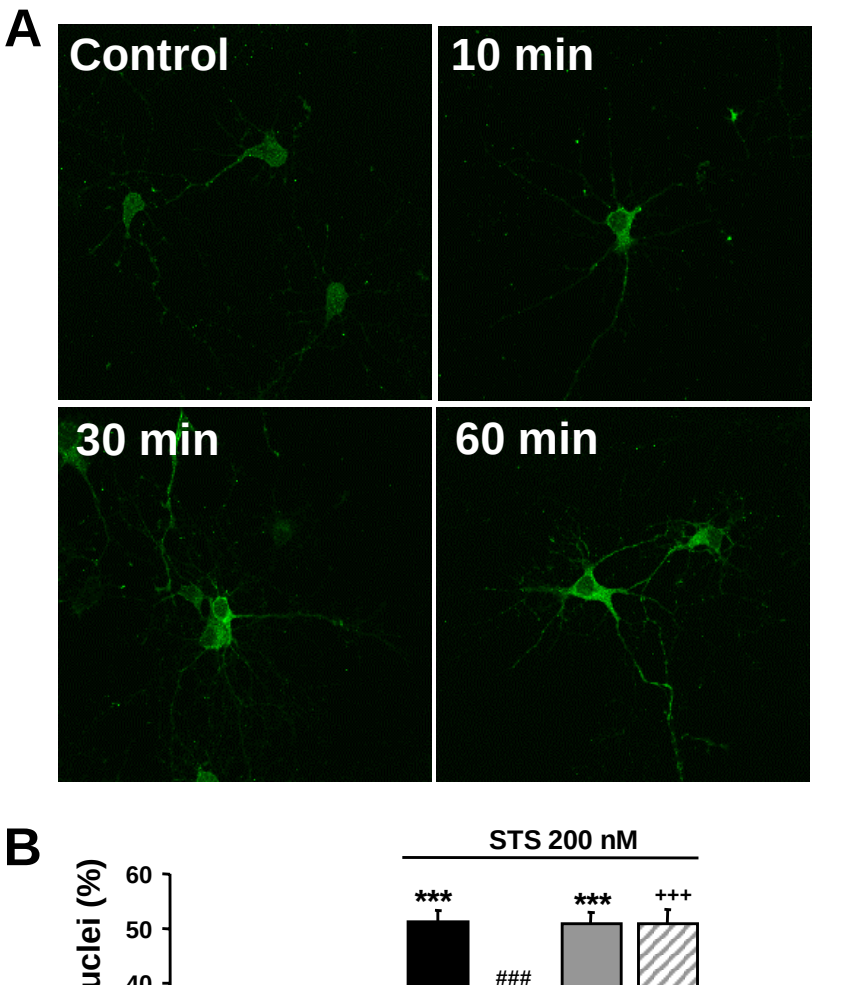

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Figure 9

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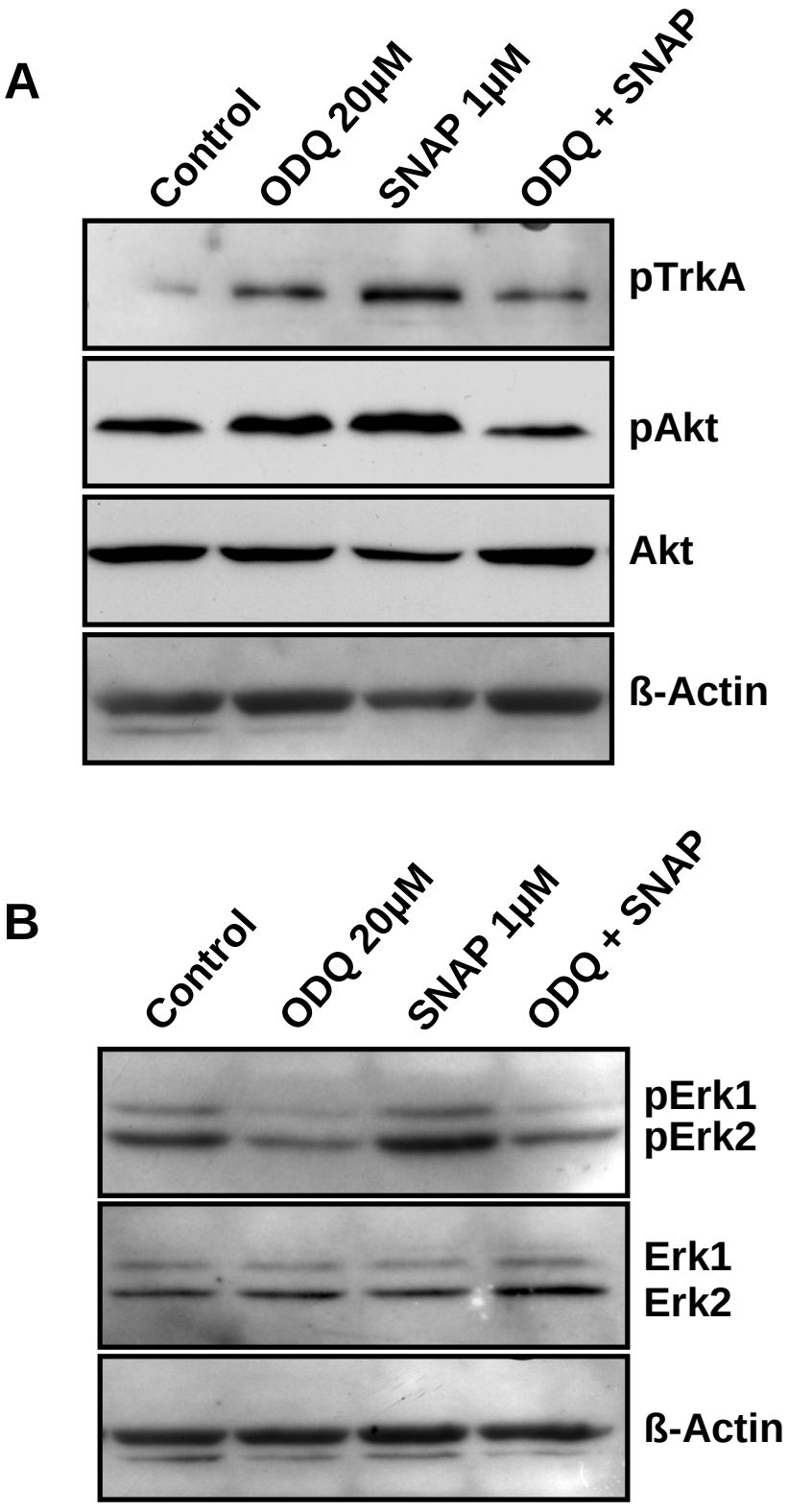

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Figure 10

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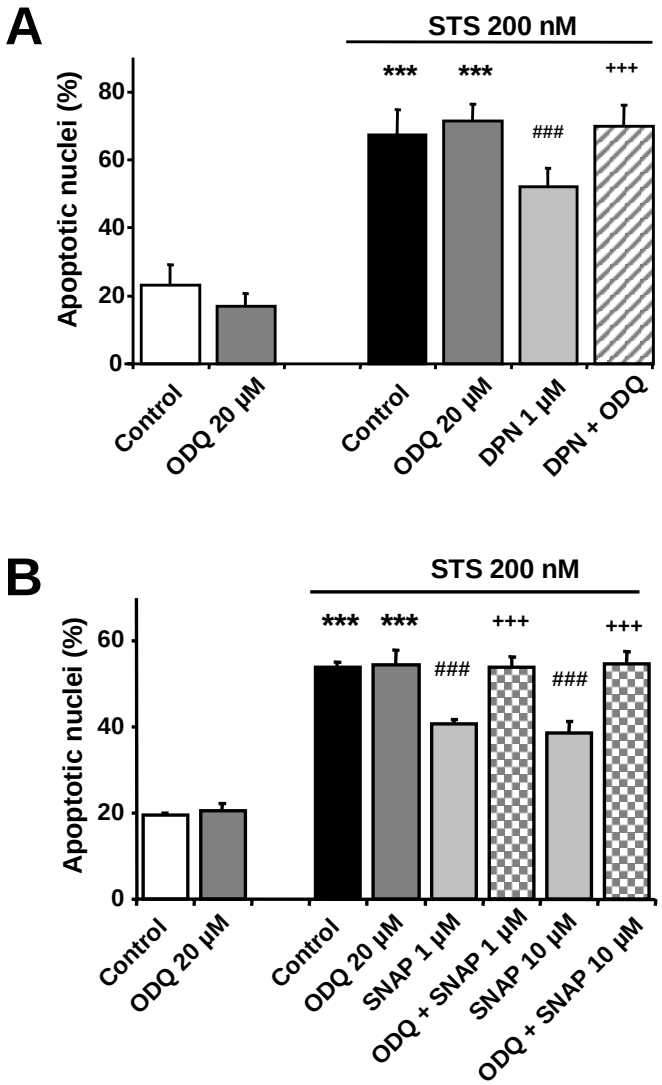

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Figure 11

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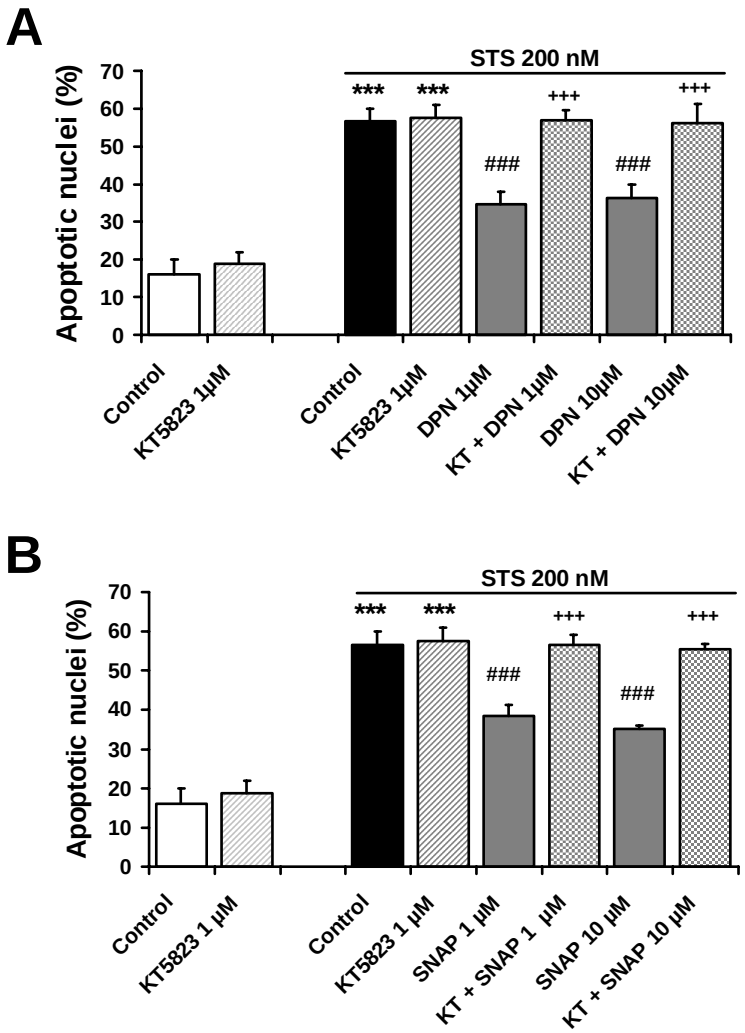

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Figure 12