Functions of JNK stresskinases
in neuronal apoptosis and differentiation

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1. Introduction

The present study investigated the role of c-Jun-N-terminal kinases (JNKs) in cell death and in differentiation. In PC12 cells, 6-hydroxy-dopamine (6-OHDA) induced death was attenuated by the JNK-inhibitor SP600125 and transfection of dominant negative JNK2. The JNK2, but not JNK1 isoform, translocated to the nucleus and the mitochondria after 6-OHDA treatment where it exerted its apoptotic functions. Besides PC12 cells, the function of JNKs was also investigated in primary hippocampal and cortical neurons, where JNKs have both apoptotic and physiological functions.

1.1 Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are important mediators for intracellular signalling. MAPK cascades are evolutionarily conserved in all eukaryotic cells and play a key role in the regulation of gene expression as well as cytoplasmic activities [1]. To date four major types of MAPK pathways have been defined in mammalian cells. These include the (i) extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade which preferentially regulates cell growth and differentiation, (ii,iii) the c-Jun N-terminal kinase (JNK) and p38 MAPK cascades, which function mainly in stress responses like inflammation and apoptosis, and finally (iv) the ERK5 cascade, which is activated by environmental stress and osmotic shock, but not by vasoactive peptides or inflammatory cytokines [1-3]. All MAP kinases are serine/threonine kinases that are activated in response to their phosphorylation on invariant threonine (Thr) and tyrosine (Tyr) residues within a Thr-X-Tyr motif (where X is any amino acid). This phosphorylation is catalysed by MAPK kinases (MAP2Ks, MEKs, MKKs), which in turn, are activated by MAPK kinase kinases (MAP3Ks) via serine/threonine phosphorylation. MAP3Ks receive signals from cell surface receptors through a variety of intermediates, including other protein kinases and small GTP binding proteins (reviewed by [3-6]) (Fig. 1.1). Importantly activation of MAPKs leads to phosphorylation of other kinases or transcription factors that activate a variety of response genes [3].

Among the four types of MAPK pathways, the present thesis focused at the c-Jun N-terminal kinase (JNK) pathway.

1.2. The organization of c-Jun N-terminal kinases (JNKs)

Ten isoforms of JNK are expressed in human adult brain. These ten isoforms are encoded by three genes: *jnk1*, *jnk2* and *jnk3*. The *jnk3* gene is preferentially expressed in the heart, pancreas, brain and testis, while *jnk1* and *jnk2* are expressed in many tissues [7-10]. Alternative splicing of the genes yields four JNK1 isoforms, four JNK2 isoforms and two JNK3 isoforms. The protein products of JNK isoforms have molecular weights of 46 kDa and 55 kDa. The 55 kDa JNK isoforms contain a C-terminal extension, the result of alternative splicing, which distinguishes them from 46 kDa isoforms. No apparent functional differences have been ascribed to these isoforms. An additional alternative splicing exists in the kinase domains of JNK1 and JNK2 but not in JNK3. JNK1 and JNK2 have significant functional differences particularly in regard to substrate binding [7].

The JNK pathway is one of the known cellular signalling pathways that respond to much diverse stimuli. JNK is activated by mitogenic signals such as epidermal growth factor, lymphocyte activation signals and oncogenic Ras, pro-inflammatory cytokines such as TNF-α and IL-1, lipopolysaccharide, osmotic stress, shear stress and protein synthesis inhibitors. JNK is also activated by apoptotic stimuli such as growth factor withdrawal, ischemia, ceramides, UV light, oxidative stress and DNA damaging agents [3, 7, 10-12].

The JNKs are phosphorylated and activated by MAP2Ks, MKK4 and MKK7, which phosphorylate JNK1 and JNK2 on their Thr 183 and Tyr 185 residues and JNK3 on Thr 221 and Tyr 223 residues. These kinases in turn are activated by MAP3Ks, of which several examples have been identified such as, MEKK1-4, ASK1, TAK1, MLKs or the GTP-binding proteins of the Rho family, Rac and cdc42 [3, 6, 13]. JNK activities can be down-regulated by protein phosphatases, including M3/6 and MKP-1. These phosphatases display selectivity toward JNK family members [14, 15]. An additional level of control of the JNK signalling pathway is provided by JNK-interacting proteins (JIPs). These are scaffold proteins that mediate the signal transduction through MLK, MKK and JNK pathways. The JIPs can antagonize the JNK actions by recruiting the MAPK phosphatase MKP7 with subsequent dephosphorylation and inactivation of JNKs [16] or by retention of JNKs in the cytoplasm [17].

Many substrates are phosphorylated by the JNKs including the transcription factors c-Jun, ATF2, Elk-1, NFAT, as well as tumour suppressor p53 and a cell death domain protein, MADD. Additionally, cytoplasmic targets such as the neurofilament heavy unit, Bcl-2 and tau serve as JNK substrates [6, 18, 19].
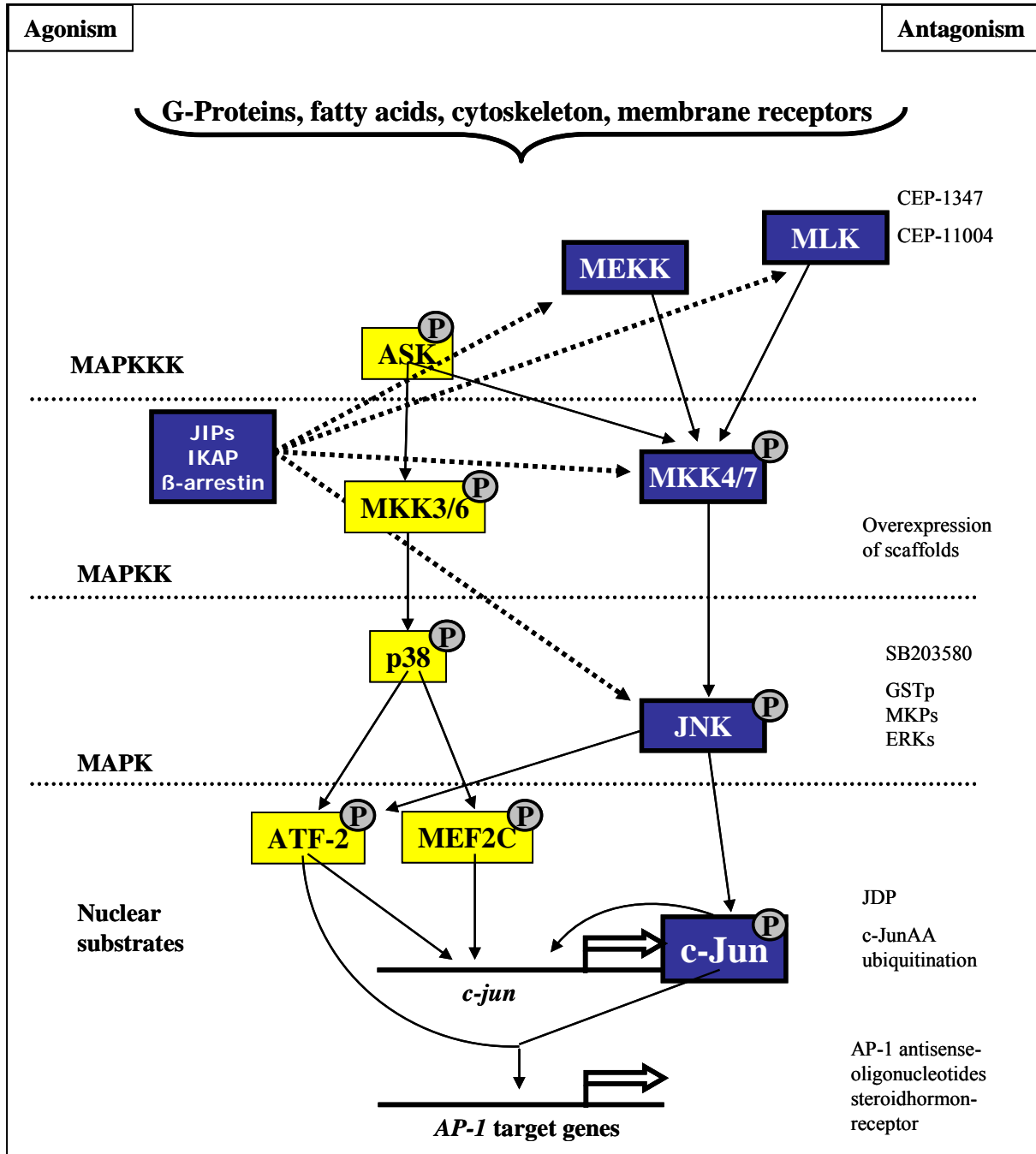
Fig. 1.1. The organization of the p38 and JNK system of MAP kinases

1.3. JNKs as mediators of neurodegeneration

Numerous experimental data suggest a pro-degenerative or apoptotic function for the JNKs [20-22]. JNKs trigger neuropathological events, *e.g.* after trophic support withdrawal in sympathetic neurons [23-25] or cerebellar neurons [26, 27], delayed ischemic cell death [28, 29], excitotoxicity [30] or in models for Alzheimer's disease [31] and Parkinson's disease [32-36].

1.3.1 Mitochondria-induced death

Mitochondrial dysfunctions are central to the pathogenesis of neurodegenerative diseases such as Alzheimer's, Parkinson's or Huntington's diseases [37], and are important for neuronal excitotoxicity [38]. JNKs translocate and associate with the mitochondria [39] where they inactivate anti-apoptotic and promote pro-apoptotic proteins of the Bcl-2 family such as Bcl-2, Bcl-xL, Bad, Bim or Dp5 [40-44]. Moreover JNKs release inhibitors of anti-apoptotic proteins, such as Smac/Diablo from the mitochondria [45]. In fibroblasts, UV irradiation provoked apoptosis, caspase-3 activation and cytochrome c release from mitochondria due to JNK presence, whereas in *jnk1-/- jnk2-/-* fibroblasts, UV irradiation did not cause cytochrome c release and caspase-3 activation [46]. When the constitutively active form of apoptosis signal-regulated kinase 1 (ASK1, JNK activating kinase) was transiently expressed *in vitro*, it induced apoptotic cell death [47] and cytochrome c was released from mitochondria [48]. These results demonstrate that JNKs are involved in the mitochondrial apoptotic pathways. However, it remains to be elucidated which JNK isoform(s) mediate(s) the mitochondrial pathology.

1.3.2. Induction of death genes

Besides mitochondrial apoptotic pathways, JNKs activate transcription factors in the nucleus, such as c-Jun, ATF-2 or Elk-1. In the nervous system, JNKs are involved in the transcription of pro-apoptotic target genes coding for Fas-ligand, TNFα or Bim [41, 49]. The activation of transcription factors might be exerted by JNK2 and JNK3 which rapidly translocate into the nucleus whereas the basal constitutive presence of activated JNK1 in the nucleus is not effective in phosphorylation of transcription factor such as c-Jun [26, 27, 50].

JNK3 deficient mice (JNK3 -/-), but not JNK1 -/- and JNK2 -/-, and mutation of the serine residues 63 and 73 into non-phosphorylable alanins (c-JunAA mutant) show increased resistance to kainic acid induced seizures and to death of hippocampal neurones [30, 51]. JNK3 mutant mice also show a severe defect in phosphorylation of c-Jun and the transcriptional activity of the AP-1 transcription factor complex [30]. Moreover, in culture, functional inhibition of c-Jun by microinjection of antibodies or dominant negative c-Jun protects neonatal sympathetic neurons from NGF withdrawal-mediated neuronal cell death [52, 53]. These results suggest that JNK and c-Jun transduce the cell death signal at least partially through transcription-dependent machinery in neurons.

1.3.3. Degeneration of dopaminergic neurons

JNKs are also essential mediators of neurodegeneration provoked by oxidative and/or radical stressors *e.g.* MPTP or 6-OHDA, which are hallmarks of Parkinson's disease. MPTP induced neuronal damage is accompanied by activation of JNKs and MKK4 in the substantia nigra compacta [54]. Importantly, MPTP-induced cell death and JNK activation in dopaminergic neurons can be effectively reduced in rats by administration of the MLK-antagonist and indirect JNK-inhibitor, CEP-1347 [36] and in mice by administration of specific JNK-inhibitor SP600125 [55]. Furthermore, expression of the JNK-binding domain of JIP-1 or of a dominant-negative MKK4 attenuated both, the dopaminergic neuronal death and the activation of JNK and c-Jun following MPTP exposure [56]. However, all JNK isoforms do not contribute equally to stress-induced dopaminergic cell death. Deletion of *jnk2/jnk3,* but not *jnk1,* genes suppressed MPTP-induced phosphorylation of c-Jun and attenuated MPTP-induced dopaminergic cell death *in vivo* [34]. Similarly, our group showed that JNK3 ko and c-JunAA mutation conferred cell-specific protection of dopaminergic neurons whereas JNK1 ko enhanced the damage following ischemia [57].

1.4. Physiological functions of JNKs

Expression and activation of JNK and c-Jun in the mammalian adult brain in the absence of any harmful stimulus or in neuronal cell cultures during differentiation suggest important physiological/protective roles for JNK and c-Jun (reviewed by [21]). To understand the physiological functions of JNKs in the nervous system, their roles in development, neuroplasticity and neurorepair has to be explained.

JNKs are imperative for the physiological normal development of the nervous system. Thus, double knock out of JNK1 + JNK2 results in embryonic lethality due to failed closure of the neuronal tube [58]. Moreover, JNK1-/- JNK2 -/+ or JNK1 -/+ JNK2 -/- mice display distinct malformations in the brain [59]. The activities of JNKs and MKK4/7 in the forebrain rise after birth, indicating a role of JNKs in postnatal maturation [26]. Importantly, JNK1 is required for the maintenance of neuronal microtubules, and JNK1-/- mice display anatomical malformations and neurodegeneration such as disrupted formation of the anterior tract commissure which becomes evident within the first weeks after birth [60]. The truncated JNK3 (trJNK3, i.e. the terminal 273 amino acids) as the only identified genetic alteration occurs in children with early severe neurological symptoms such as drug-resistant seizures, cognitive deficits and neuronal degeneration (Kalscheuer and Ropers, oral communication). In addition, deficiency of the JNK target c-Jun resulted in a severe proliferation defect in fibroblasts and /or in hepatoblasts of c-Jun knockout mice [61, 62]. Thus, at least in cultured fibroblasts and hepatoblasts, c-Jun acts as a positive regulator of cell growth. Moreover, mice lacking c-Jun survive only to embryonic day 12 and die due to massive hemorrhage in the liver, suggesting physiological roles of the JNK/c-Jun axis (reviewed by [63]).

Besides the embryonic development, JNKs exert important functions on the re-arrangements of neuronal cytoskeleton such as differentiation, neuronal plasticity and neuroregeneration. JNKs can phosphorylate actin, tau, neurofilament, MAP-1 or MAP-2, and thereby alter the cytoskeletal functions [60, 64, 65]. The functional deletion of JNK1, but not JNK2, provokes a progressive loss of microtubules in axons and dendrites [60]. JNKs have also essential functions for the differentiation of neurons. MKK7 and JNK activity increase during neuritogenesis in primary cerebellar granule neurons [26] and inhibition of JNKs dramatically reduced axonal outgrowth in explanted or dissociated ganglia [66]. Similarly, c-Jun and JNKs may also contribute to NGF induced neurite outgrowth in PC12 cells [67].

In addition to cytoskeletal functions and differentiation JNKs play important roles in neuroregeneration and neuroprotection. JNK activation is sustained in dorsal root ganglia after axotomy in regenerating neurons and returns to basal levels after the regeneration process has been completed [68, 69]. JNKs and their nuclear substrate c-Jun are considered important mediators of the regenerative cell body response [54, 70, 71]. Besides neuroregenerative effects, JNKs may promote cell survival. Immature thymocytes and peripheral mature T cells deficient in *jnkk1 (sek1/mkk4)* were highly susceptible to Fas/CD95- and CD3-mediated apoptosis suggesting that JNKK1 may play an anti-apoptotic role in T cells [72]. Biochemical analysis also revealed that activation of JNKs may be involved in suppression of apoptosis in B lymphocytes through phosphorylation and inactivation of the pro-apoptotic molecule Bad [73].

1.5. Inhibition of JNKs as a therapeutic strategy

JNKs play an integral role in neuronal death and this pathway might be operative in several diseases of the central nervous system. Neuroprotection by inhibition of JNKs may offer an attractive strategy for prevention of the neuronal death. However, the potentially relevant physiological functions of the JNKs might limit the use of antagonists as a novel strategy for therapy. For example, given the important role of JNK1 in the maintenance of neuronal microtubules or plasticity [60], it is important to avoid complete JNK inhibition. Due to the high similarity of JNK isoforms, the possibility of developing isoform-specific inhibitors is difficult. Moreover, inhibition of all or one JNK isoform(s) is not restricted to neurons, but may also affect other cells which contribute to brain integrity such as microglia [74].

The indirect JNK-inhibitor CEP-1347 originally called KT7515 [75] (3,9 bis [(ethylthio)methyl]-K252a), which primarily acts as a MLK inhibitor, has already entered phase II-III trials for treatment of Parkinson's disease (www.parkinson-study-group.org). The mixed JNK-p38 inhibitor CNI-1493 which is currently applied in the inflammatory Cohn's disease [76], attenuates neuroinflammation in the rat nervous system following neuropathic pain [77]. SP600125 (anthrax [1,9-cd] pyrazol-6(2H)-one), another direct JNK-inhibitor, also displays an effective anti-inflammatory potential in rheumatoid arthritis in rats [78] and in primary rat microglia [74]. This anti-inflammatory activity will enlarge the operative range of JNK-inhibitors in the nervous system providing further protection e.g. against Alzheimer's and Parkinson's disease [79].

In addition, *jnk1-/-* mice have been shown to be significantly protected from obesity-induced insulin resistance ([80], reviewed by [81]) suggesting that JNK is a potential therapeutic target for obesity and type-2 diabetes. The JNK-inhibitor SP600125 reduces bronchoalveolar accumulation of eosinophiles and lymphocytes in animals subjected to repeated allergen exposure, and reduces serum immunoglobulin E levels, indicating its possible use in treatment of asthma [82]. Finally, JNK pathway may provide tumour suppression in mice and human [83-85].

Because of the cross-talk within MAPK signalling cascade, as well as its cell-type and response-specific modulation, it is difficult to predict the potential adverse effects that might arise from the inhibition of the pathway. Numerous experiments are needed to achieve specific therapeutic outcomes without substantial side-effects.

Fig. 1.2. Chemical inhibitors of the JNK pathway
A. CEP-1347, originally called KT7515 (or 3,9 bis [(ethylthio)methyl]-K252a), was identified in a medicinal chemistry approach directed to retain the desirable properties of the naturally occurring compound K252a whilst minimising any undesirable effects. **B.** SP600125, (anthrax [1,9-cd] pyrazol-6(2H)-one], was defined by screening of a proprietary diversity library. SP600125, acted as a reversible ATP-competitive inhibitor of equal potency towards three JNK gene products, JNK1, JNK2 and JNK3.

1.5. Scientific aims of the present study

The present thesis studied neurodegenerative and physiological functions of JNK isoforms in PC12 cells and in primary neurons. For degeneration, 6-OHDA was used as inductor of neuronal death and experimental model for the degeneration of dopaminergic neurons underlying Parkinson's disease. This study investigated the following questions:

1. How does 6-OHDA affect the cell survival, the activation of JNK and the activation of its nuclear transcription factor c-Jun?

2. Does the specific JNK-inhibitor SP600125, protect PC12 cells from 6-OHDA-induced death?

3. Which JNK isoform(s) is/are important for the 6-OHDA-induced death in PC12 cells?

4. Does 6-OHDA change the association of JNK with mitochondria?
   Which JNK isoform(s) do(es) mediate the mitochondrial-triggered death following 6-OHDA including cytochrome c release and caspase-3 activation?

5. Which upstream kinases are involved in the pro-degenerative role of JNK at the mitochondria?

6. The JNK/c-Jun axis plays an important role in neuronal excitotoxicity. Primary hippocampal and cortical neurons were analyzed for the excitotoxicity of glutamate and 6-OHDA, respectively. Do JNKs mediate the excitotoxicity-induced death in primary neurons?

7. Does inhibition of JNKs also interfere with the neurite outgrowth in primary neurons, as basic and relevant function for the neuronal differentiation and development of the nervous system?

2. Material and methods

2.1. Materials

Unless otherwise indicated, all solutions and dilutions were prepared in double-distilled water (DDW). All chemicals were *pro analysis (p.a.)*. Apart from antibodies Table 2.1 gives all materials used in this study.

Table 2.1. Materials

Material	Manufacturer / Supplier
ABC kit	Alexis; Grünberg, Germany
Acrylamide / bis-acrylamide solution 29:1	Bio-Rad; München, Germany
Agarose SeaKem LE	Biozym; Oldendorf, Germany
6-Aminocaproic acid	Merck-Schuchardt; Hohenbrunn, Germany
Ammonium persulphate (APS)	Merck; Darmstadt, Germany
Albumin, bovine fraction	Sigma ;München, Germany
Boric acid	Merck; Darmstadt, Germany
Bovine serum albumin (BSA)	Boehringer; Mannheim, Germany
Bromophenol blue	Merck; Darmstadt , Germany
B-27 Supplement	Invitrogen/Gibco; Karlsruhe, Germany
Calcium chloride	Merck; Darmstadt, Germany
Cell culture plates (3.5 cm)	Sarstedt; Nümbrecht, Germany
Cell culture plates (10 cm, 6 wells, 24 wells)	Nunc; Karlsruhe, Germany
Chamber slides	Nunc; Karlsruhe, Germany
Cover glasses	Nunc; Karlsruhe, Germany
Cryotubes (2 ml)	Greiner; Frickenhausen, Germany
Cytosine-ß-D-arabinofuranoside	Sigma; München, Germany
Cytotoxicity detection kit (LDH)	Roche; Mannheim, Germany
DAB tablets	Sigma; München, Germany
Dimethylsulfoxide (DMSO)	Merck; Darmstadt, Germany
DNAse	Sigma; München, Germany
dNTP set (10 mM solutions)	Invitrogen/Gibco; Karlsruhe, Germany
DTT 0.1 M	Invitrogen/Gibco; Karlsruhe, Germany
Dye Reagent	Biorad; München, Germany

ECL Plus	Amersham Biosciences; Freiburg, Germany
EDTA	Merck; Darmstadt, Germany
EGTA	Merck; Darmstadt, Germany
Ethanol *p. a.*	Merck; Darmstadt, Germany
Ethanol, technical (denatured)	Bundesmonopol für Branntwein (BfB); Offenbach, Germany
Ethidium bromide solution (10 mg/ml)	Invitrogen; Karlsruhe, Germany
Fetal calf serum (FCS)	Bio Whittaker; Vervriers, Belgium
Filter paper	Whatman; Maidstone, UK
Filter unit 0.22 μm, syringe-driven	Qualilab; Bruchsal, Germany
Formaldehyde	Merck; Darmstadt, Germany
Gentamycin	Invitrogen/Gibco; Karlsruhe, Germany
G 418, sulphate (solution)	Stratagene; Amsterdam, The Netherlands
Glucose	Merck; Darmstadt, Germany
Glutamate	Sigma; München, Germany
Glutamax	Invitrogen/Gibco; Karlsruhe, Germany
Glycerol	Merck; Darmstadt, Germany
Glycine	Merck; Darmstadt, Germany
Hank's balanced salts	Sigma; München, Germany
HEPES	Merck; Darmstadt, Germany
Hoechst 33258	Sigma; München, Germany
Horse serum	Invitrogen/Gibco; Karlsruhe, German
Hyperfilm ECL	Amersham Biosciences; Freiburg, Germany
Immobilon P 1500	Millipore; Eschborn, Germany
Insulin	Sigma; München, Germany
Kaiser's glycerol gelatine	Merck; Darmstadt, Germany
Light antifade kit	Molecular probes; Oregon, USA
Magnesium chloride (PCR)	Invitrogen/Gibco; Karlsruhe, Germany
Magnesium chloride	Merck; Darmstadt, Germany
Magnesium sulphate	Sigma; München, Germany
2-Mercaptoethanol	Sigma; München, Germany
Methanol	Merck; Darmstadt, Germany
Microtiter plates, 96-well, round bottom, lid	Nunc; Karlsruhe, Germany
Minimum essential medium (MEM)	Sigma; München, Germany
NGF (2.5 S)	Alomone labs; Jerusalem, Israel

Non-fat dry milk	Uelzena; Uelzen, Germany
Nonidet P-40	Fluka Chemie; Buchs, Switzerland
Oligo(dT)-Primer	Invitrogen/Gibco; Karlsruhe, Germany
Paraformaldehyde	Merck; Darmstadt, Germany
PBS (w/o Ca^{2+} and Mg^{2+})	Invitrogen/Gibco; Karlsruhe, Germany
PCR buffer (10 x)	Invitrogen/Gibco; Karlsruhe, Germany
pEGFP-C3	Clontech; Heidelberg, Germany
Penicillin/Streptomycin solution (10,000 IU / 10,000 μg/ml)	Invitrogen/Gibco; Karlsruhe, Germany
pGEMT Easy	Promega; Mannheim, Germany
Phosphatase Inhibitor Cocktail II	Sigma; München, Germany
Pipettes (serological, sterile; 5 / 10 / 25 ml)	Sarstedt; Nümbrecht, Germany
Pipette tips (10 / 200 / 1,000 μl)	Sarstedt; Nümbrecht, Germany
PMSF	Sigma; München, Germany
Poly-l-Lysine	Sigma; München, Germany
Ponceau S	Sigma; München, Germany
Potassium chloride	Merck; Darmstadt, Germany
Potassium dihydrogen phosphate	Merck; Darmstadt, Germany
Potassium disulphate	Merck; Darmstadt, Germany
Protease Inhibitor (Complete)	Roche; Mannheim, Germany
Protein marker, prestained, broad range	New England Biolabs; Frankfurt, Germany
Total RNA isolation kit	NucleoSpin; Germany
RNAse H	Invitrogen/Gibco; Karlsruhe, Germany
RPMI-1640 medium	Invitrogen/Gibco; Karlsruhe, Germany
SDS-PAGE standards, prestained, broad range	Bio-Rad; München, Germany
Sodium bicarbonate	Sigma; München, Germany
Sodium carbonate	Sigma; München, Germany
Sodium chloride	Merck; Darmstadt, Germany
Sodium dodecyl sulphate	Merck; Darmstadt, Germany
Sodium hydrogen carbonate	Sigma; München, Germany
di-Sodium hydrogen phosphate	Merck; Darmstadt, Germany
Sodium hydroxide	Merck; Darmstadt, Germany
SP600125	Alexis; Grünberg, Germany
SuperScript	Invitrogen/Gibco; Karlsruhe, Germany

Sucrose	Sigma; München, Germany
TEMED	Carl Roth; Karlsruhe, Germany
TdT enzyme	Roche; Mannheim, Germany
Thermanox coverslips	Nunc; Karlsruhe, Germany
TransFast	Promega; Mannheim, Germany
Transferrin	Calbiochem; Schwalbach, Germany
Tris	Merck; Darmstadt, Germany
Triton X-100	Merck; Darmstadt, Germany
Trypan blue solution, cell culture tested	Sigma; München, Germany
Trypsin	Sigma; München, Germany
Trypsin inhibitor	Sigma; München, Germany
Tubes (0.5 / 1.5 / 2.0 ml)	Sarstedt; Nümbrecht, Germany
Tubes for PCR	Sarstedt; Nümbrecht, Germany
Tubes, sterile (15 / 50ml)	Sarstedt; Nümbrecht, Germany
Tween-20	Calbiochem; Schwalbach, Germany
Ultrapure water	Biochrom; Berlin, Germany
6-hydroxy-dopamine	Sigma; München, Germany

2.2. Laboratory Equipment

Table 2.2. Equipments

Equipment	Manufacturer / Supplier
Agarose gel electrophoresis	Bio-Rad; München, Germany
AnalySIS software	Soft Imaging System; Münster, Germany
Autoclave DS 202	Webeco; Bad Schwartau, Germany
Centrifuge; Biofuge fresco, Labofuge GL, Megafuge 1.0 R	Heraeus; Hanau, Germany
Centrifuge; Mikrofuge	Neolab; Heidelberg, Germany
Cell incubator	Heraeus; Osterode, Germany
DMR microscope	Leica; Solms, Germany
Electrophoresis power supply	Invitrogen/Gibco; Karlsruhe, Germany
Film processor	AGFA; Mortsel, Belgium
Fluorescence microscope	Leica; Solms, Germany
Hemocytomer twin chamber	Omnilab; Hamburg, Germany
Heating block (Thermomixer 543)	Eppendorf; Hamburg, Germany

Incubator (Innova 4000)	New Brunswick Scientific; Amsterdam, The Netherlands
LeicaQwin software	Leica; Solms, Germany
Microplate reader 680	Bio-Rad; München, Germany
MiniProtean II Vertical PAGE chamber	Bio-Rad; München, Germany
Olympus CK 2 microscope	Olympus; Hamburg, Germany
pH meter	WTW; Weilheim, Germany
Rotator (Polymax 2040)	Heidolph; Kehlheim, Germany
Semi-dry transfer unit(Pegasus)	Phase; Lübeck, Germany
Sonicator (Sonopuls GM 70)	Bandelin; Berlin, Germany
Spectrophotometer (U-2000)	Hitachi; Wiesbaden, Germany
Thermocycler (Personal Cycler)	Biometra; Göttingen, Germany
UV light (Image Master VDS)	Bio-Rad; München, Germany
Water bath	Heraeus; Osterode, Germany

2.3. Culture, staining and stimulation of PC12 cells

2.3.1. Standard cell culture

Medium

RPMI-1640 medium

Horse serum (HS, inactivated at 56° C for 30 min)   10%

Fetal calf serum (FCS, inactivated at 56° C for 30 min)   5%

Penicillin/Streptomycin   1%

The rat PC12 pheochromocytoma cell line derives from a transplantable rat pheochromocytoma [86]. PC12 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in medium described above. The penicillin/streptomycin solution, horse serum and FCS were stored at –20° C, and the RPMI-1640 medium was stored in a cooling chamber (4° C). The cells were cultured in 10 cm or 20 cm plates according to the experiments. Twenty cm plates were used for mitochondrial extractions and 10 cm plates were used for the other experiments. Ten cm plates were filled with 7 ml medium and 20 cm plates were filled with 14 ml medium. The cells were fed every 2-3 days.

PC12 cells, like many other adherent cells, need a structured substrate for growing. Therefore, all plates used for cultivation and experiments were coated with collagen (0.1 mg/ml in PBS). The bottom of the cell culture plates was covered with a thin layer of collagen solution, placed in the incubator for 1 h and then washed with PBS to remove collagen that had not adhered to the plate. Air-dried plates were stored under sterile conditions.

2.3.2. Passaging of PC 12cells

Passage-EDTA

PBS	1x
EDTA	0.5 mM

Store at 4°C.

Since PC12 cells grow adherent, they were treated with passage EDTA before passaging to reduce cell-substrate adhesion.

Passaging of PC12 cells was maintained according to the following procedure:
1. The cells were washed with PBS (37° C).
2. The cells were rinsed with 2 ml passage-EDTA (37° C) and placed for 2 min in the incubator (37° C, 5% CO_2).
3. 5 ml of medium was added (37° C).
4. The cells were scraped off the cell culture plate and transferred into a 15 ml tube.
5. The cells were centrifuged for 10 min; (1000 xg; r.t.).
6. The pellets were first resuspended with 2 ml of medium and then resuspended with 5 ml of medium.
7. The cells were aliquoted into new cell culture plates according to the diameter of the cell culture plate and the density.

2.3.3. Freezing and thawing of PC12 cells

Freezing medium

RPMI-1640 supplemented with HS, FCS and pen/strep. (see 2.3.1)	80%
FCS	10%
DMSO	10%

PC12 cells were stored in aliquots at -80°C. When the cells had been passaged 20 times, the cells were discarded and a stock aliquot was quickly thawed in a 37° C water bath. The cell solution was transferred to a 10 cm plate with medium. Since DMSO is toxic for the cells, the medium had to be changed within the first 24 hours. After that, the cells were fed every 2-3 days, passaged and used for the experiments.

For production of PC12 stock aliquots, cells were harvested and pelleted as described in section 2.3.2 and resuspended in 1 ml freezing medium. The cell solution was aliquoted in 2 ml cryotubes and incubated 10 min at r.t., transferred to ice for 35 min, incubated at -20° C for 45 min and finally stored at -80° C.

2.3.4. Differentiation of PC12 cells

(-) Serum Medium
RPMI-1640 medium

FCS	0.5%
Penicillin/streptomycin	1%

In response to NGF, growth arrest is induced and PC12 cells differentiate into neuron-like cells with formation and elongation of neurites.

PC12 cells were passaged as described (See 2.3.2) and cultured in growth medium. After 24 h, the cells were fed for 72 h with [-] serum medium to synchronize the cell cycle activity of the cells. After 72 h, the medium was changed and the cells were incubated with 50 ng/ml of NGF. The differentiating cells were fed with fresh medium containing NGF every 2–3 days. After 7 days of NGF treatment the cells were used for the experiments.

2.3.5. Stimulation of PC12 cells

In this study 6-OHDA was used for oxidative stress. 6-OHDA (10, 25, 50, 100 μM) was freshly prepared from 10 mM stock solution which was prepared in ascorbic acid solution to prevent oxidation. The cells were incubated with the JNK-inhibitor SP600125 (1, 2 μM) 30 min before addition of 6-OHDA. After the respective incubation periods, RNA or proteins were extracted.

2.3.6. Trypan blue viability staining

Various manipulations of cells, including passaging, freezing and stimulations may provoke cell death. To determine the number of surviving cells in a given population, exclusion of the dye trypan blue was used. Healthy cells are able to exclude this dye for a certain time, but trypan blue will quickly diffuse into the cells which have lost their membrane integrity.
Protocol:

1. The cells were washed with PBS (37° C).
2. The cells were rinsed with 2 ml passage EDTA (37° C) and placed for 2 min in the incubator (37° C, 5% CO_2).
3. 5 ml of medium was added (37° C).
4. The cells were scraped off the cell culture plate and transferred into a 15 ml tube.
5. The cells were centrifuged for 10 min; (1000 xg; r.t.).
6. After centrifugation, the pellet was thoroughly resuspended in an appropriate amount of PBS.
7. Twenty µl of the cell suspension were mixed with 20 µl of trypan blue solution and transferred to a hemocytometer twin chamber. Living cells in the 16 squares of both chambers were counted, and the percentage of viable cells was determined.

2.3.7. Transfection

Transfection is a process in which the gene of interest is introduced to eukaryotic cells by biochemical or physical methods. The choice of a particular expression vector and the transfection methods depend on the objectives of the study. In this study, an enhanced green fluorescent protein (EGFP)-tagged vector was used so that transfected cells could easily be identified due to their green fluorescence. To deliver the nucleic acids into PC12 cells, a mixture of synthetic cationic lipids and neutral lipids (TransFast liposomes) was used. The surface of these liposomes is positively charged and attracted electrostatically to the negatively charged DNA. So the liposome complex neutralizes the negative charge of the DNA, allowing an association of the complex with the negatively charged cell membrane. The entry into the cell may occur by endocytosis or fusion of the plasma membrane. The neutral lipids disrupt endosomes and the DNA is set free into the cytoplasm. The exact mechanism by which the DNA enters the nucleus is still unclear.

In this study, stable cell clones were established. PC12 cells were plated in 6-well plates and cultivated until they were 90% confluent. Plasmid DNA (3.75 µg) was dissolved in 988.75 µl of medium (without antibiotics and serum) in a 1.5 ml tube. Subsequently, 11.25 µl of TransFast Reagent (suspended at least 24 h before transfection) was added and the mixture was vortexed briefly. The DNA/TransFast reagent mixture was incubated at r.t. for 10–15 min. After removing the growth medium from the cells, the transfection mixture (1 ml) was added to each well and the plates were returned to the incubator for 1-4 h. Subsequently, 1 ml of medium (without antibiotics, but supplemented with 10% horse serum and 5% FCS) was added to each well. After 24 h, the transfection medium was replaced by growth medium and after another 24 h, the antibiotic G 418 (500 µg/ml) was added to select the transfected cells. The selection of cell clones that stably expressed the desired protein usually took 4–6 weeks. Several clones were tested for expression of the respective plasmid and the clones expressing the highest amounts of the recombinant protein were used for experiments.

For activation, JNKs are phosphorylated on Thr^{183} and Tyr^{185} in the conserved Thr-X-Tyr MAPK activation motif. In this study both phosphorylation sites of JNK1 and JNK2 were mutated to inhibit phosphorylation and thereby activation of recombinant protein. So, PC12 cells were transfected with these mutated JNK1 and JNK2 (dominant-negative JNK1 and JNK2). PC12 cells were also transfected with JNK3-EGFP to characterize the functional potential of JNK3 in 6-OHDA-induced toxicity.

2.4. Denaturing protein extraction and protein quantification

2.4.1. Harvest of cells

1. The medium of the cells was discarded and the cells were washed with PBS.
2. PC12 cells were rinsed again with 800 µl PBS and scraped off the plates.
3. The cell suspension was transferred into a 1.5 ml tube and centrifuged.
4. The supernatant was removed and the pellets were used for protein extraction.

2.4.2. Denaturing extraction of whole cell proteins

Denaturing lysis buffer (DLB-buffer)

Tris	10 mM
SDS	1%
Phosphatase inhibitor Cocktail II	1%

Denatured protein extracts were prepared using a Tris-buffered sodium dodecyl sulphate (SDS) lysis buffer (denaturing lysis buffer).

1. The cell pellet in a 1.5 ml tube (see 2.4.1) was resuspended in 50–300 µl of lysis buffer (depending on the size of the pellet).

2. The cell solution was incubated for 5 min at 95° C in a heating block.

3. The samples were sonicated twice for 5 s to disrupt the cells by cresting vibrations which cause mechanical shearing of the cell wall.

4. Insoluble material was removed by centrifugation (15 min; 13,000 $\times g$; 4° C).

5. The supernatant was transferred into a new 1.5 ml tube and stored at -80° C.

2.4.3. Denaturing extraction of nuclear proteins

Lysis buffer without Nonidet

HEPES	10 mM
KCl	10 mM
MgCl$_2$	1.5 mM

Lysis buffer with Nonidet

HEPES	10 mM
KCl	10 mM
MgCl$_2$	1.5 mM
Nonidet	0.1% v/v

To analyse nuclear proteins or the translocation of cytoplasmic proteins to the nucleus, cytoplasmic and nuclear proteins were extracted separately.

1. PC12 cells were harvested (see 2.4.1.) and washed in lysis buffer without Nonidet.

2. The cell pellets were resuspended in lysis buffer supplemented with Nonidet depending on the size of the pellet (80-250 µl) and placed on ice for 10 min.

3. The cell solution was centrifuged for 5 min (11,700 $\times g$; 4° C).

4. The supernatants, containing the cytoplasmic proteins, were transferred into a 1.5 ml tube. And 10% SDS solution was added to a final concentration of 1% SDS.

5. Pellets were resuspended in denaturing lysis buffer to extract nuclear proteins (see 2.4.2.).

6. Cytoplasmic and nuclear extracts were boiled for 5 min at 95° C.

7. Nuclear extracts were sonicated twice for 5 s as described (see 2.4.2.) and centrifuged (15 min; 13,000 xg; 4° C).

8. Cytoplasmic and nuclear extracts were stored at -80° C.

2.4.4. Preparation of mitochondrial extracts

<u>Sucrose buffer</u>

HEPES	20 mM	DTT	1 mM
KCl	10 mM	EGTA	1 mM
MgCl$_2$	1.5 mM	Sucrose	250 mM
PMSF	0.1 mM	EDTA	1 mM

Complete protease inhibitor

For preparation of mitochondrial extracts, all steps were conducted at 4° C.

1. Cells plated on 20 cm cell culture plates were washed twice with PBS and harvested.
2. Cells were centrifuged for 6 min (1000 xg; 4° C).
3. The pellets were resuspended in 4 ml of PBS and the living cells were counted with trypan blue (see 2.3.6.).
4. Cells were centrifuged again; the pellets were resuspended in sucrose buffer (for 5x10^6 cells 100 µl sucrose buffer) and transferred into 1.5 ml tubes. The cell solution was stored for 1 h on ice.
5. After 1 h the cells were lysed by aspiration through a 27 gauge syringe (25-30 times).
6. The lysates were centrifuged for 5 min (750 xg; 4° C).
7. The supernatants were collected into a sterile 1.5 ml tube and centrifuged for 15 min (10,000 xg; 4°C).
9. The supernatants containing the cytoplasmic proteins were transferred into a sterile 1.5 ml tube and SDS from a 10% stock solution was added to a final concentration of 1% SDS.
10. The pellets containing mitochondrial extracts were washed twice in sucrose buffer and lysed in DLB-Buffer (see 2.4.2).
11. Cytoplasmic and mitochondrial extracts were boiled for 5 min at 95° C.
12. Mitochondrial extracts were sonicated twice for 5 s as described (see 2.4.2.) and centrifuged (15 min; 13,000 xg; 4° C) to remove insoluble materials.
13. Cytoplasmic and mitochondrial extracts were stored at -80° C.

2.4.5. Determination of protein concentrations

Protein concentrations were determined using Dye Reagent, a variant of Bradford's colorimetric assay. To prepare working solution, the Dye Reagent stock solution was diluted 1:5 with DDW.

To determine protein concentrations, bovine serum albumin BSA was used as protein standard. Serial dilution of a 1.2 mg/ml BSA stock solution in autoclaved DDW (0.1 to 0.6 mg/ml) was produced. Samples were diluted 1:30 or 1:50 in autoclaved DDW. Twenty µl of sample, standard and DDW (as blank) were pipetted into the disposable plastic cuvettes. One ml of the working solution described above was added to each cuvette. All cuvettes were vortexed to start the reaction and incubated at r.t. for 10 min. The absorbance at 595 nm (A_{595}) was measured using a spectrophotometer. The concentration of proteins was calculated as:

$$\frac{[\text{absorbance of sample}] \times [\text{concentration of standard}]}{[\text{absorbance of standard}]} \times \text{dilution factor}$$

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

2.5.1. SDS-PAGE

Resolving Buffer			Stacking Buffer		
Tris, pH 8.8	1.5 M		Tris, pH 6.8	0.5 M	
SDS	0.4%		SDS	0.4%	
Store at 4° C			Store at 4° C		

Acrylamide/bis-acrylamide solution			Electrophorese Buffer (10x)		
Acrylamide	30%		Tris, pH 8.3	0.25 M	
Bis-acrylamide	0.8%		Glycine	1.92 M	
Stored at 4° C in the dark			SDS	1%	
			Stored at r.t.		

<u>Sample Buffer (5x)</u>

Tris, pH 6.8	312.5 mM
SDS	10%
ß-Mercaptoethanol	10%
Glycerol	50%

Stored at 4° C.

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) is a low-cost, reproducible and rapid method for quantifying, comparing and characterizing proteins. This method separates proteins based on their molecular weights. SDS binds along the polypeptide chain and the length of the reduced SDS-protein complex is proportional to its molecular weight.

Polyacrylamide gels were prepared by co-polymerization of acrylamide monomers with the cross-linker bis-acrylamide. The reaction was catalyzed by ammonium persulphate (APS) and initiated by N,N,N',N'-tetramethylethylenediamine (TEMED). For better resolution, a short stacking gel was set on top of the main resolving gel. Differences in composition between these two gels resulted in concentration of the protein samples into narrow bands in the stacking gel and separation of the bands according to their size in the resolving gel. Table 2.3 and 2.4 provides the composition of stacking and resolving gels. For preparation of the resolving gel different percentages of acrylamide were used, depending on the molecular weight of protein to be analyzed. A 15% resolving gel was used to detect cytochrome c, caspase-8, cleaved caspase-3 and caspase-3 proteins, whereas a 10% resolving gel was used for PARP-1 protein. The remaining proteins were analyzed in a 12% resolving gel. (Table 2.5.).

Table 2.3. Composition of separating gels for SDS-PAGE

Target protein size	< 30 kDa	30-60 kDa	60–100 kDa
Components	15% gel	12% gel	10% gel
Acrylamide	6.7 ml	4 ml	3.3 ml
4 × resolving buffer	2.3 ml	2.5 ml	2.5 ml
Autoclaved DDW	2.3 ml	3.4 ml	4.1 ml
TEMED	10 µl	10 µl	10 µl
10% APS	100 µl	100 µl	100 µl

Table 2.4. Composition of the 3% stacking gel for SDS-PAGE

Component	Quantity
Acrylamide	0.6 ml
4 × stacking buffer	1.5 ml
Autoclaved DDW	3.9 ml
TEMED	6 µl
10% APS	60 µl

Preparation of polyacrylamide gels

Glass plates from a MiniProtean II Vertical PAGE chamber were cleaned with 70% ethanol, clamped together and placed in a vertical position on the bench top. A mark on the glass plate 1 cm below the teeth of the comb was placed to determine the level of resolving gel which had to be poured. Resolving gel monomer solution combining all reagents containing TEMED and APS was prepared. The solution was pipetted between the gel plates to the mark. The solution was carefully introduced to minimize possibility of air bubbles trapped within the gel. When the appropriate resolving gel solution was added, the gel was over-layed with DDW to keep the gel surface flat and allowed to polymerize for 10-30 min at r.t. After polymerization, a distinct interface appeared between the separating gel and DDW which has to be removed.

Subsequently, the stacking gel (Table 2.4) was prepared and pipetted on top of the polymerized resolving gel until the solution reached top of front plate. Immediately, a 15-well comb was inserted into the gel plates. It is important to be sure that bubbles were not trapped on the ends of teeth. The stacking gel was allowed to polymerize within 30 min at r.t.

After the stacking gel had polymerized the comb was removed carefully and the gel plates were placed into electrophoresis chamber. The chamber was filled with 1x electrophoresis buffer and the wells were cleaned from residual gel particles.

Preparation of protein samples

Protein samples, used for Western blot were diluted with autoclaved DDW in order to obtain 20 µg of total protein in a volume of 8 µl. Two µl of 5 × SDS sample buffer were added, and

the samples were heated to 95° C for 5 min and loaded onto the gel. To estimate the molecular weights of bands detected by Western blotting, broad range prestained protein marker was used.

Electrophoresis

The samples and the protein marker were carefully loaded into the cleaned wells and run through the stacking and resolving gels at a constant current of 30 mA. Usually, gels were run for 30 min after the tracking dye had passed the end of the gel. Finally, gels were removed from the gel plates, the stacking gel was discarded, and the resolving gel was used for Western blotting (see 2.5.2).

2.5.2. Western blot

TBS (10x)		TTBS		Blocking Solution
Tris	200 mM	TBS	1x	4% non fat dry milk
NaCl	1.37 mM	Tween-20	1 ml/ l	in TTBS
Store at 4° C		Store at 4° C		Store at 4° C

Anode Buffer I		Anode Buffer II	
Tris	30 mM	Tris	300 mM
Methanol	20%	Methanol	20%

Cathode Buffer	
Tris	25 mM
Methanol	20%
6-aminocaproic acid	40 mM
Store at r.t.	

In Western blotting experiments proteins are transferred from a SDS-PAGE gel to a synthetic membrane and then the proteins are detected. Once the proteins have been transferred from the polyacrylamide gel to the synthetic membrane, specific proteins were detected by specific antibodies. Prior to addition of antibodies, the membrane is coated with blocking solution e.g. non-fat milk or a BSA solution. Blocking the membrane is very important so that antibodies

do not bind non-specifically to the membrane. The primary IgG (*e.g.* produced in mice) antibody recognizes the protein of interest while the secondary antibody recognizes the Fc portion of the first antibody. This secondary antibody is coupled to an enzyme, *e.g.* a horseradish peroxidase (HRP), which converts a chemiluminescence substrate.

Blotting

After the proteins were separated by SDS-PAGE, they were transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry blotting using an electroblotter. Three different transfer buffers were used (anode buffer I and II, cathode buffer). The PVDF membrane which was cut to the size of gel (9 x 6 cm) was activated in 100% methanol for 3 min. Subsequently, the membrane was rinsed in DDW for 2 min and equilibrated in anode buffer I until use. For each gel, 15 pieces of blotting paper were cut to the size of the gel (9 × 6 cm). Six pieces of them were pre-soaked in anode buffer II and placed on a glass plate. Three pieces of blotting paper were pre-soaked in anode buffer I and placed over the anode buffer II-soaked filter paper. The equilibrated membrane was then placed over the filter paper and the gel was placed in close contact with membrane after pre-soaked in cathode buffer. The 'sandwich' was completed by stacking remaining 6 pieces of filter paper pre-soaked in cathode buffer. Finally, the blotting 'sandwich' was turned around and placed into the semi-dry transfer unit in which the bottom is the cathode.

Proteins were transferred to the PVDF membrane using a constant current of 0.8 mA/cm^2 for 1 h (protein size 30–80 kDa) or 1.5 h (> 80 kDa). After transferring the protein to the membrane, the membrane was washed for 20 min in 1 x TTBS buffer and blocked with blocking solution for 60 min. Subsequently, the membrane was incubated with the first antibody at 4° C overnight.

On the next day, the primary antibody solution was discarded and the unbound antibody was removed by washing the membranes once for 15 min and twice for 5 min each with TTBS. Usually the membrane was incubated with a dilution of 1:4000 goat-anti-rabbit secondary antibody or with a dilution of 1:3000 goat-anti-mouse secondary antibody for 30 min at r.t. After discarding the secondary antibody solution, the membrane was washed once for 15 min and three times for 5 min each with TTBS.

Table 2.5. Primary and secondary antibodies for Western blots

Antibody	Dilution and Buffer	Second antibody	Manufacturer
Caspase-3	1:1000 in blocking solution	α-rabbit	Santa Cruz
Caspase-8	1:500 in blocking solution	α-rabbit	Santa Cruz
Cleaved caspase-3	1:1000 in blocking solution	α-rabbit	CST
Cytochrome c	1:5000 in blocking solution	α-mouse	BD
Grp-75	1:5000 in TTBS	α-mouse	Stressgen
JNK1	1:1000 in TTBS	α-mouse	Pharmingen
JNK2	1:1000 in blocking solution	α-mouse	Santa Cruz
JNK3	1:1000 in TTBS	α-rabbit	Alexis
JIP-1	1:1000 in blocking solution	α-rabbit	Santa Cruz
MKK4	1:500 in TTBS	α-rabbit	Santa Cruz
MKK7	1:1000 in blocking solution	α-rabbit	Santa Cruz
MKP 7	1:2000 in TTBS	α-rabbit	generous gift from Dr. A. Whitmarsh
phospho-c-Jun	1:1000 in TTBS	α-rabbit	CST
phospho-JNK	1:2500 in blocking solution	α-rabbit	Promega
phospho-MKK4	1:500 in TTBS	α-rabbit	Santa Cruz
PARP-1	1:2000 in TTBS	α-mouse	Alexis
total JNK	1:1000 in TTBS	α-rabbit	CST
ß-Actin	1:5000 in TTBS	α-mouse	Sigma

ECL-Reaction

After the last washing step, the membrane was placed with the protein side up on a glass plate. For a 6 × 9 cm (standard sized) membrane, 1 ml of ECL Plus HRP substrate was prepared immediately prior to use by mixing 0.975 ml of ECL Plus Reagent A with 25 µl of ECL Plus Reagent B. The membrane was carefully covered with the HRP substrate solution and incubated for 3 min. Subsequently, all the HRP substrate was allowed to drip off the membrane and the membrane was placed inside the plastic pocket of a film cassette. The chemiluminescence on the membranes was detected by exposing the membranes to Hyperfilm ECL films in a darkroom. Films were developed and fixed by a film processor.

2.5.3. Stripping of Western blot membranes

Stripping solution

Tris	62.5 mM
SDS	2%
ß-Mercaptoethanol	100 mM

Store at 4° C.

After Western blotting and detection of the protein, the membrane can be used to detect another protein. For stripping, the membrane was incubated for 30 min at 50° C and 25 rpm in an incubator. After stripping, the membrane was washed twice for 10 min each with TTBS and blocked with blocking solution for 1h. Subsequently, the membrane was incubated with primary antibody.

2.5.4. Ponceau S staining of Western blot membranes

Ponceau S is the only staining method which is completely compatible with all procedures of immunological probing, because the stain is transient and can be washed away so that it does not interfere with subsequent detection of antigens. After the ECL reaction, membranes were washed twice with TTBS and then stained with Ponceau S for 20 min. The staining solution was re-used several times. Stained membranes were washed twice with DDW for 5 min each before air-drying.

2.6. Isolation of RNA

2.6.1 RNA extraction

During RNA extraction it is very important not to have any contamination with RNAse. So all of the equipments used were autoclaved and the working place was cleaned with 70% ethanol. For the isolation of total RNA extraction NucleoSpin® RNA II isolation kit was used. The cells were harvested as described in section 2.4.1. The pellet was re-suspended in lysis buffer (350 µl RA1 buffer with 3.5 µl ß-mercaptoethanol per sample). The cell solution was applied to a NucleoSpin® Filter unit (violet) and centrifuged for 1 min (11,000 xg; r.t.). The filter unit was discarded, 350 µl ethanol (70%) was added to the homogenized lysate and mixed. The

lysate was loaded to the NucleoSpin® RNA II column and centrifuged for 30 s (8,000 xg; r.t.). The column was placed in a new collection tube, 350 µl MDB (Membrane Desalting Buffer) was added and centrifuged for 1 min (11,000 xg; r.t.) to dry the membrane. Subsequently, 95 µl DNAse reaction mixture (10 µl DNAse I + 90 µl DNAse reaction buffer) was added directly onto the center of the membrane and incubated at r.t. for 15 min. To inactivate DNAse, 200 µl RA2 buffer was added to each column and centrifuged for 30 s (8,000xg; r.t.). After washing the pellet twice with RA3 buffer, the column was placed into a nuclease-free 1.5 ml tube and RNA was eluted in 60 µl RNAse-free water. A 5 µl aliquot of the RNA solution was removed for quantification and quality validation. The remaining RNA was stored at -80° C.

2.6.2. RNA quantification and quality control

RNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. RNA samples were diluted 1:200 in RNAse-free water and pipetted into quartz cuvettes. Absorbance of RNAse-free water was measured as blank. An absorbance of 1 unit at 260 nm corresponds to 40 µg/ml of RNA in water. RNA concentration could be determined as;

$$C_{RNA} = A_{260} \text{ x dilution factor x } 40$$

To detect protein contaminants, the absorbance at 280 nm was also measured. The ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) gives us the purity of RNA. A ratio of 1.6–2.0 indicates pure RNA (in water).

2.7. Polymerase chain reaction (PCR)

2.7.1. Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a method which combines complementary DNA (cDNA) synthesis from RNA templates with using a RNA-dependent DNA polymerase (the reverse transcriptase, RT). The RT can start from oligo (dT) primers binding to the polyadenylated tail of the mRNAs. In this study reverse transcription is carried out with the SuperScript First-Strand Synthesis System for RT-PCR.

For the reverse transcription, 5 μg of RNA were diluted in a volume of 11 μl DEPC-DDW. One μl of the oligo(dT) solution was added to each sample and incubated in a thermocycler at 70° C for 10 min to denature the samples. Subsequently the temperature was decreased to 4° C and during this time the RT master mix was prepared on ice.

Table 2.6. Composition of RT the master mix

Component	Quantity
10 x PCR Buffer	2 μl
MgCl$_2$ (25 mM)	2 μl
dNTP mix (10 mM)	1 μl
DTT (0.1 mM)	2 μl

Seven μl of the RT master mix was added to the each sample and incubated at 42° C for 5 min for annealing of the primer. Subsequently, 1 μl SuperScript II RT was added to each sample and incubated at 42° C for 50 min and followed by 70° C for 15 min to denaturate RNA-cDNA hybrids. To stop the reaction 1 μl RNAse H was added to each sample and incubated at 37° C for 20 min. cDNA samples were used immediately for PCR or stored at -20° C.

2.7.2. PCR

The purpose of a PCR (polymerized chain reaction) is to make a huge number of copies of a gene. There are three basic steps in PCR. First, the target genetic material must be denatured, i.e. the strands of its helix must be unwound and separated by heating to 90-96° C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the single-stranded DNA. Annealing temperature chosen for a PCR depends directly on length and composition of the primers and calculated using the following formula:

T_M = 4 x (number of G + C) + 2 x (number of A + T).

The third step is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly. This results in two new helixes in place of the first, each composed of one of the original strands plus its newly assembled complementary strand. Repeating these cycles results in generating millions of copies of DNA strands.

The cDNA produced in the RT reaction was amplified in a total volume of 50 µl in a thermocycler. Two µl of cDNA sample were pipetted into single PCR tubes and placed on ice. The PCR master mix was prepared on ice and 48 µl of master mix was added to each sample.

Table 2.7. The master mix for PCR

Component	Quantity (for 48 µl per sample)
10 x PCR Buffer	5 µl
MgCl$_2$ (50 mM)	2 µl
dNTP mix (10 mM)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase	0.25 µl
Autoclaved DDW	37.5 µl

After addition of the PCR master mix, samples were placed in a thermocycler and the PCR program was started.

PCR-Program

1. 94°C,   5 min
2. 94°C,   1 min
3. 53°C,   1 min (annealing temperature)   25x
4. 72°C,   30 s
5. 72°C,   10 min
6. 4°C,   ∞

In this study primers for Bim and for the *house-keeping*-gen H2.Az were used. All primers were designed using the software Primer 3 Software Distribution (Whitehead Institute/Howard Hughes Medical Institute; Boston, MA, USA). The specificity of the primer pairs was verified by comparative alignment using the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) at the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA).

Table 2.8. Primer pairs and reaction conditions for PCR amplications

Gene	Primers	Product (bp)	Annealing temp. (°C)	Number of cycles
Bim	fw: CAACACAAACCCCAAGTCCT rv: TCTTCCGCCTCTCGGTAAT	182	55	25
H2A.z	fw: CGTATTCATCGACACCTGAAA rv: CTGTTGTCCTTTCTTCCCAAT	282	55	25

After the PCR, the products were aliquoted and stored at −20° C. An aliquot of the sample was used to visualize by agarose gel electrophoresis.

2.7.3. Detection and analysis of the reaction product

10x TBE-Buffer (Tris-boric acid-EDTA buffer) 10x Loading Buffer

Tris   9 M   Bromophenol blue   0.25%

Boric acid   9 M   Glycerol   30%

EDTA   0.2 M

Store at r.t.   Store at 4° C.

Agarose

Agarose (dissolved in TBE buffer)   x %

Ethidium bromide   5 µg/ml

Prepared freshly.

The PCR product should be a fragment or fragments of defined DNA lengths. The simplest way to check for the presence of these fragments is to load a sample taken from the reaction product, along with appropriate molecular-weight markers onto an agarose gel. One % or 1.5% agarose gels were prepared with 1x Tris-buffer boric acid-EDTA. The 1x TBE buffer was set up by diluting 10x TBE buffer with DDW. The required amount of agarose was dissolved in 1x TBE buffer by heating in a microwave oven. 5 µl/100 ml of a 10 mg/ml ethidium bromide stock solution was added to warm agarose solution and mixed well. For agarose gel electrophorese a horizontal gel system was used. The agarose solution with ethidium bromide was pipetted to this gel system and allowed to polymerize for 20-30 min.

When the gel had polymerized, it was transferred to an electrophorese chamber filled with 1x TBE buffer. Twenty µl of DNA sample were mixed with 5 µl 10 x Loading buffer in 1.5 ml tubes. At the same time 1 µl DNA-Ladder was mixed with 5 µl 10 x Loading buffer and with 19 µl TBE buffer, resulting in a total volume of 25 µl. The DNA-Ladder and the samples were loaded into wells and the gel was run at 80 mV for ca. 60 min. Bands were visualized with 312 nm UV light.

2.8. Culture, staining and stimulation of primary cells

2.8.1 Solutions for primary cultures

Hanks Solution		Dissection Solution	
Pulver media	w/o Mg^{2+} and Ca^{2+}	Hanks solution	
$NaHCO_3$	35 mg/ml	Albumin	3 mg/ml
HEPES	10 mM	$MgSO_4$	1.4 mg/ml
D-Glucose	6 mg/ml		
Gentamycin	5 µg/ml		

Digestion Solution		Culture Media	
NaCl	8 mg/ml	MEM	
KCl	0.37 mg/ml	D-glucose	5mg/ml
Na_2HPO_4	0.99 mg/ml	Transferrin	0.1 mg/ml
HEPES	5.95 mg/ml	Insulin	25 µg/ml
$NaHCO_3$	0.35 mg/ml	Glutamax	2 mM
		Gentamycin	5 µg/ml

1st day culture media		3rd day culture media	
Culture media		Culture media	
FCS / HS	10%	FCS / HS	5%
		B 27 supplement	2%
		AraC	5 µM

All of the solutions are at pH 7.4 and stored at 4° C

2.8.2. Coating of the plates

For providing cell attachment and growth, 4-well plates and culture dishes (35 mm) have to be coated. Cover glasses or plastic cover slips were placed in 4-well plates in the LFU. Plastic culture dishes and 4-well plates were coated with poly-l-Lysine, dissolved in ultrapure water (100 µg/ml). To coat the culture dishes or 4-well plates, the bottom of the slides was covered with a thin layer of poly-l-Lysine, and placed in the incubator overnight. The next day, slides were washed two times with DDW. Subsequently, 1st day culture medium was added to the plates and placed in the incubator until use. Cover glasses used in this study were not sterile. So they were autoclaved before use and stored under sterile conditions.

2.8.3. Culturing of primary rat neurons

Hippocampal or cortical cultures were obtained from the newborn rats (up to 24 h postnatal). All the culturing procedure was performed on ice. Rat puppies were decapitated and the brains were dissected under sterile conditions. Brains were transferred into petri-dishes which contain ice-cold dissection solution. Cortex and hippocampus were dissected and cleaned from blood vessels and meninges. Subsequently, they were transferred into different petri-dishes and cut into 1 mm^3 pieces and finally transferred into 15 ml tubes. After washing 4 times with 3 ml dissection solution and treating with 5 ml Hanks solution, hippocampal and cortical slices were warmed for 1 min in digestion solution, mixed with trypsin (3.3 mg/ml), DNAse (0.83 mg/ml), and treated with the same solution for 5 min at r.t. The hippocampal and cortical slices were then incubated in dissection solution with 0.6 mg/ml trypsin inhibitor to inhibit trypsin activity for 5 and 3 min, respectively, and finally with fetal calf serum (200 µl/ml in dissection solution) for 10 min. Hippocampal and cortical slices were washed 4 times with dissection solution and homogenized in dissection solution, mixed with 0.4 mg/ml DNAse, with three different diameters of fire polished Pasteur pipettes. Five ml of dissection solution were added and the cells were centrifuged (15 min; 800 xg; 4° C). To determine the number of surviving cells in cell suspension, cells were counted with trypan blue (see 2.2.5.). Subsequently, cells were plated on 4 well plates or 35 mm plastic culture dishes containing 1st day culture media (Culture Media, 10% FCS). After two days half of the culture media was replaced with 3rd day media and every 2nd –3rd day half of the media was replaced with fresh culture medium.

This culturing procedure results in a mixed neuronal culture containing 70-80% neurons. The remaining cells were identified as astrocytes.

2.8.4. Culturing of primary murine neurons

Cortical cultures from newborn mice (up to 24 h) were obtained as described above (see 2.8.3). However, instead of 10% FCS, 10% horse serum was used for culturing and feeding of the primary murine neurons.

2.8.5. Incubation with inhibitors and other substances

Five days old hippocampal cultures were treated with 250 µM glutamate freshly made from a 5 mM stock solution, for 4 or 24 h in the culture medium without serum and B27 supplement. Five days old cortical cells were treated with 25 µM, 50 µM, and 100 µM 6-OHDA freshly made from 100 mM stock solution in ascorbic acid solution to prevent oxidation. The JNK-inhibitor SP600125 (2 µM) was given to the both cultures 30 min before the addition of glutamate or 6-OHDA.

2.9. Immunocytochemistry

Immunocytochemistry bases on the use of a primary antibody directed against the cellular target(s) and a secondary antibody which is directed against the primary antibody and labelled with an enzyme. The most commonly used enzymes are peroxidase and alkaline phosphatase. Peroxidase activity is most frequently detected using 3,3'-Diaminobenzidine (DAB) as the electron acceptor with hydrogen peroxide serving as the substrate. The reaction product is observed as a brown precipitate at the site of the enzyme activity. To enhance the signal, the secondary antibody is biotinylated and in consequence bound by avidin coupled to a complex containing the enzyme (avidin-biotin-complex coupled with a peroxidase for the substrate reaction, *i.e.* the ABC complex). The immunocytochemical procedure involves fixation, permeabilization, blocking sites that are prone to unspecific interactions, labelling with the primary and secondary antibodies, incubation with the ABC complex and finally the substrate reaction.

Staining was performed using the following protocol:

1. The cells were incubated with pre-warmed para-formaldehyde (4% in PBS, 37° C) at r.t. for 30 min.

2. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) at r.t. for 2 min.

3. The cells were blocked in 5% normal goat serum, diluted in PBS.

4. The cells were washed in 1% normal goat serum, diluted in PBS, two times for 3 min each.

5. The cells were incubated with primary antibody (overnight at 4° C).

6. The cells were washed in PBS, three times for 3 min each.

7. The cells were incubated with secondary antibody for 1 h (37° C).

8. The cells were washed in PBS, three times for 3 min each.

9. The cells were incubated with ABC complex for 1 h. (37° C).

ABC solution: Vectastain® Elite ABC Reagent (Vector). Two drops of reagent A were added to 5 ml of PBS. After the addition of two drops of reagent B the solution was mixed immediately. The solution was ready to use after 30 min.

10. The cells were washed in PBS, two times for 3 min each.

11. The cells were incubated in DAB solution until staining was optimal as determined by light microscopic examination (5-10 min at r.t.).

DAB Reagent: 3,3`-diaminobenzidine tablet sets (Sigma). When dissolved in 5 ml DDW each Sigma Fast DAB tablet sets contains O9292 DAB: 0.7 mg/ml; U1380 Urea Hydrogen Peroxide: 0.2 mg/ml and Tris buffer 0.06 M.

12. The cells were washed in DDW two times each for 5 min.

The stained cells were mountained and analyzed using a DMR microscope with a camera system and the software LeicaQwin.

2.9.1. Double staining

The cells were fixed, permeabilized, incubated with the primary and the secondary antibody as described above. After incubation with ABC solution and DAB reagent, the cells were incubated with another primary antibody overnight at 4° C. After that, the cells were incubated with secondary antibody against the second primary antibody. After ABC incubation, the cells were stained with Vector® SG substrate kit which gives a blue-grey signal. The stained cells were analyzed as described above.

Vector® SG substrate kit: Three drops of chromogen were added to 5 ml of PBS. After the addition of three drops of hydrogen peroxide, the solution was mixed immediately.

2.9.2. Immunofluorescence staining

The cells were fixed, permeabilized and incubated with the primary antibody as described above. The next day after removing the unbound antibody, the cells were incubated with a fluorescein (FITC)-conjugated secondary antibody against the primary antibody. The cells were mountained with an anti-fade kit and visualized under fluorescence microscope using a camera system and a software program.

Table 2.9. Primary and secondary antibodies for immunocytochemistry and immunofluorescence

First Antibody	Manufacturer	Second antibody for immunocytochemistry	Second antibody for immunofluorescence
GFAP	Santa Cruz	Biotinylated anti-rabbit IgG	FITC-conjugated anti-rabbit IgG
JNK1	Pharmingen	Biotinylated anti-mouse IgG	FITC-conjugated anti-mouse IgG
JNK2	Santa Cruz	Biotinylated anti-mouse IgG	FITC-conjugated anti-mouse IgG
MAP-2	Chemicon	Biotinylated anti-mouse IgG	FITC-conjugated anti-mouse IgG
p-c-Jun	CST	Biotinylated anti-rabbit IgG	FITC-conjugated anti-rabbit IgG
p-JNK	Promega	Biotinylated anti-rabbit IgG	FITC-conjugated anti-rabbit IgG

2.10. LDH Assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is present in all cells. An increase in the amount of cell death or plasma membrane damaged cells results in an increase of the LDH enzyme activation in the culture supernatant which directly correlates to the amount of formazan formed during a limited time period. Therefore, the amount of colour formed in the assay is proportional to the number of lysed cells.

For the experiments a kit from Roche was used and the experiments were performed according to manufacturer's instruction. The culture supernatant is collected in a 1.5 ml tube and centrifuged (2000 xg; 10 min; 15-20° C). Subsequently, a 96-well tissue culture plate was filled with 100 µl supernatants. To determine LDH activity in the supernatants 100 µl reaction mixture was added to each well and incubated 30 min at 15-25° C. During this incubation period the 96-well plates was protected from light. Finally, the absorbance of the samples was measured at 490 nm using an ELISA reader.

Reaction Mixture: The reaction mixture should be prepared freshly. For 100 tests 250 µl of solution I was mixed with 11.25 ml of solution II before use.

2.11. Staining of the cells with Hoechst Dye

For evaluation of apoptotic cells Hoechst 33258 (Sigma) was used. Hoechst 33258 binds all DNA and stains nuclear material allowing the visualization of apoptotic nuclei. For the staining, untreated and stimulated cells were fixed with 4% paraformaldehyde for 30 min at r.t. and permeabilized with 0.2% Triton X-100 in PBS. After washing the cells in PBS three times, the cells were incubated with Hoechst 33258 for 15 min at r.t. (5 µg/ml in PBS).

The stained cells were analyzed using a fluorescence microscope with a camera system and the software Analysis.

2.12. Statistical analysis

Unless otherwise indicated, all experiments were carried out independently 3-6 times. Statistical analysis was performed with GraphPrism software (www.graphpad.com) using one-way analysis of variance with repeated measures. Means were compared using the post-hoc Bonferroni test, and significance was defined for $p \leq 0.05$. Data were expressed as mean ± standard deviation.

3. Results

3.1. Function of JNK isoforms following 6-hydroxydopamine-(6-OHDA-) induced oxidative stress

The first aim of this study was to investigate the specific actions of JNK isoforms in oxidative stress that is connected with mitochondrial dysfunction in PC12 cells.

3.1.1. 6-OHDA-induced cell death in PC12 cells

To examine the 6-OHDA-induced toxicity, PC12 cells were treated with different concentrations of 6-OHDA for 24 h. Cell death was detected by trypan blue exclusion assays. Twenty-five μM, 50 μM and 100 μM 6-OHDA induced significant cell death in PC12 cells (Fig. 3.1).
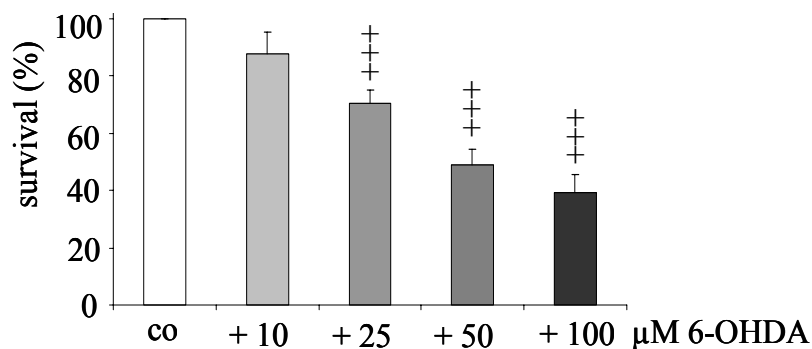
Fig. 3.1. Cell death in response to 6-OHDA
The diagram shows the survival of cells in response to 6-OHDA after 24 h. Cell death was determined by trypan blue exclusion assay. n = 4, +++ gives the significance compared with controls for $p \leq 0.001$.

3.1.2. Activation of JNK and c-Jun

6-OHDA dose-dependently induced a distinct phosphorylation of JNKs in the cytoplasm and the nucleus. Concomitantly, the N-terminal phosphorylation of c-Jun dose-dependently increased in the nucleus (Fig. 3.2 A and B). 6-OHDA induced JNK activation after 4 h in the cytoplasm and the nucleus. According to this time course the majority of experiments were

performed at the time point of maximal JNK activity, i.e. 4 h after 6-OHDA stimulation (Fig. 3.2 A). The distribution of JNK isoforms changed depending on the concentration of 6-OHDA. JNK1 and JNK2 did not change in the cytoplasm following 6-OHDA, while the amount of JNK2 increased in the nucleus between 10 μM and 50 μM 6-OHDA. JNK1 levels did not change in the nucleus after different concentrations of 6-OHDA (Fig. 3.2 B). Thus, the augmentation in total phospho-JNK in the nucleus paralleled the increase of JNK2.
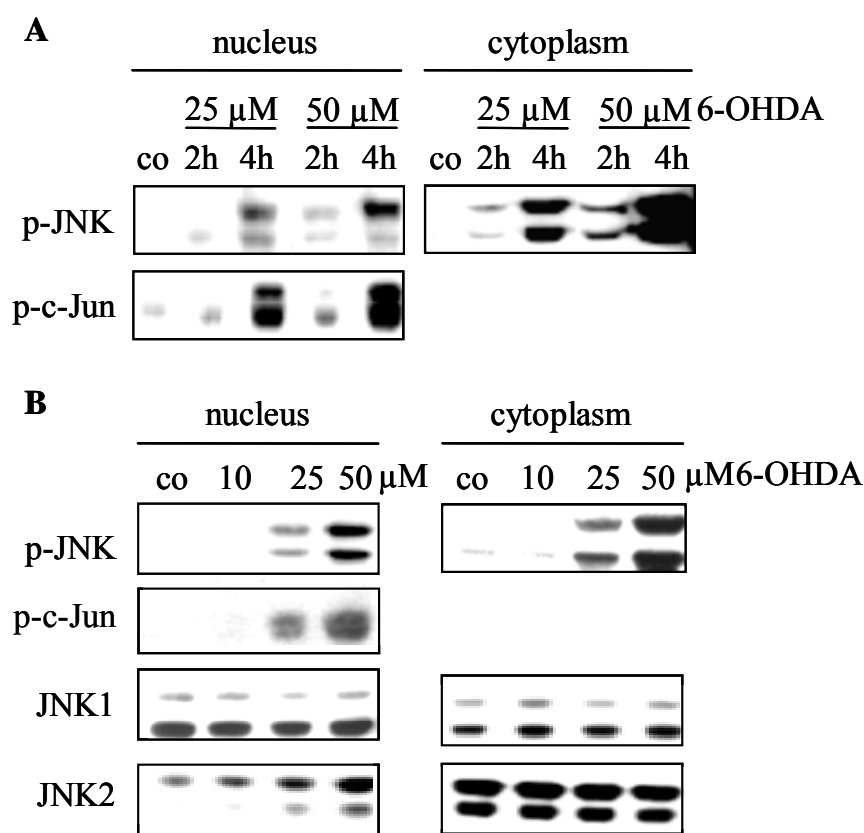
Fig. 3.2. Phosphorylation of JNKs and c-Jun in response to 6-OHDA
A. JNKs were dose dependently phosphorylated in the cytoplasm and the nucleus 4 h after 6-OHDA treatment as compared to control groups (co). **B.** JNKs were phosphorylated in the nucleus and the cytoplasm after 4 h of 6-OHDA stimulation, but only JNK2 was translocated to the nucleus in response to 6-OHDA. Neither the expression of JNK1 nor JNK2 displayed any changes in the cytoplasm. n = 4.

3.1.3. Protection by SP600125 and dnJNK2

To investigate the role of JNKs in the 6-OHDA-induced cell death, the direct and specific JNK-inhibitor SP600125 was used. As analyzed in trypan blue exclusion assay, addition of 25 μM 6-OHDA caused substantial cell death after 24 h. The pre-incubation with 1 μM or 2 μM SP600125 rescued 51% and 68% of the otherwise dying PC12 cells (Fig. 3.3).
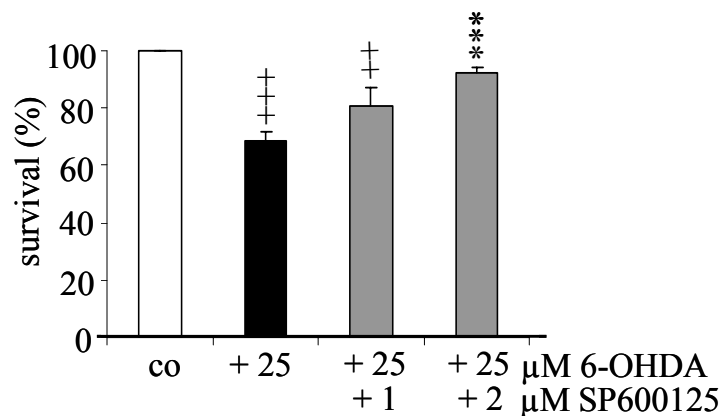
Fig. 3.3. Protection of PC12 cells by the direct JNK-inhibitor SP600125
Pre-incubation with 1 μM or 2 μM SP600125 (30 min) significantly protected PC12 cells from 6-OHDA-induced cell death. n = 4. Controls = 100%. +++ ($p \leq 0.001$) and ++ ($p \leq 0.01$) refer to 6-OHDA vs. controls, *** ($p \leq 0.001$) indicates significant differences between 6-OHDA vs. SP600125 and 6-OHDA.

The inhibition of JNKs, however, does not provide a permanent protection. Forty-seven and seventy-four hours after 25 μM 6-OHDA (without change of the medium), the survival rates did not differ significantly between the SP600125-treated cells and controls (Fig. 3.4 B). This fading protection of JNK inhibition suggests a switch from an early JNK-dependent to a late JNK-independent pathway to cell death. However, SP600125 did not protect against 50 μM 6-OHDA-induced cell death at any time point (Fig. 3.4 C). The time window for SP600125-mediated protection is smaller than 4 h, since JNK inhibition was still effective when applied 2 h but not 4 h after 6-OHDA (Fig. 3.4 A). Thus, the protective time window of SP600125 can be correlated with the onset of JNK and c-Jun activation beginning between 2 h and 4 h post-stimulus.
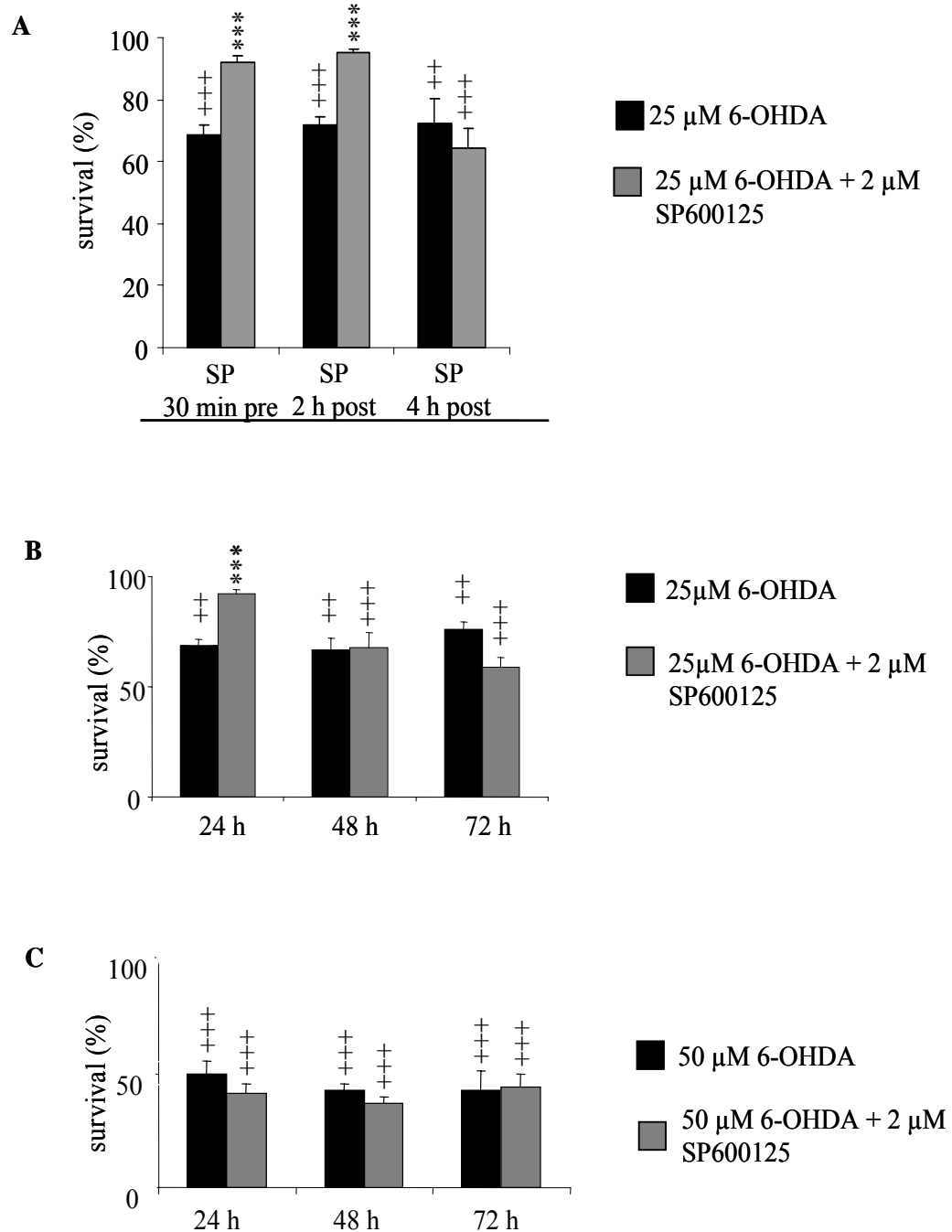
Fig. 3.4. Protection of PC12 cells from 6-OHDA-induced death is time dependent
A. Survival of PC12 cells (Trypan blue exclusion assay) 24 h following 25 μM 6-OHDA and 2 μM SP600125 either 30 min prior or 2 h and 4 h after the onset of 6-OHDA treatment. SP600125 conferred its protection when applied 30 min prior or up to 2 h after stimulation with 6-OHDA. **B.** SP600125 was not protective after 48 h and 72 h. **C.** SP600125 had no protective effects against 50 μM 6-OHDA at any time point.
n = 4. Controls = 100%. +++ (p ≤ 0.001) and ++ (p ≤ 0.01) refer to 6-OHDA vs. controls, *** (p ≤ 0.001) indicates significant differences between 6-OHDA vs. SP600125 and 6-OHDA.

The translocation of JNK2 into the nucleus, which paralleled the increase of JNK activity, raised the question if JNK2 is responsible for 6-OHDA-mediated death rather than the more ´physiological` or even ´protective` JNK1 [60, 87]. For this purpose, PC12 cells were stably transfected with EGFP-tagged dnJNK1 (dominant-negative) and dnJNK2 (see 2.3.7). DnJNK2, but not dnJNK1, protected PC12 cells from cell death induced by 25 µM 6-OHDA (Fig. 3.5 A). Similar to SP600125, dnJNK2 was not protective after 48 h and 72 h (data not shown). It did not protect against 50 µM 6-OHDA-induced cell death (Fig. 3.5 A). Interestingly, pre-incubation of dnJNK2 with SP600125 did not protect cells from 25 µM 6-OHDA-induced cell death. Moreover, with SP600125 treatment, the protection of dnJNK2 was lost (Fig. 3.5 B).
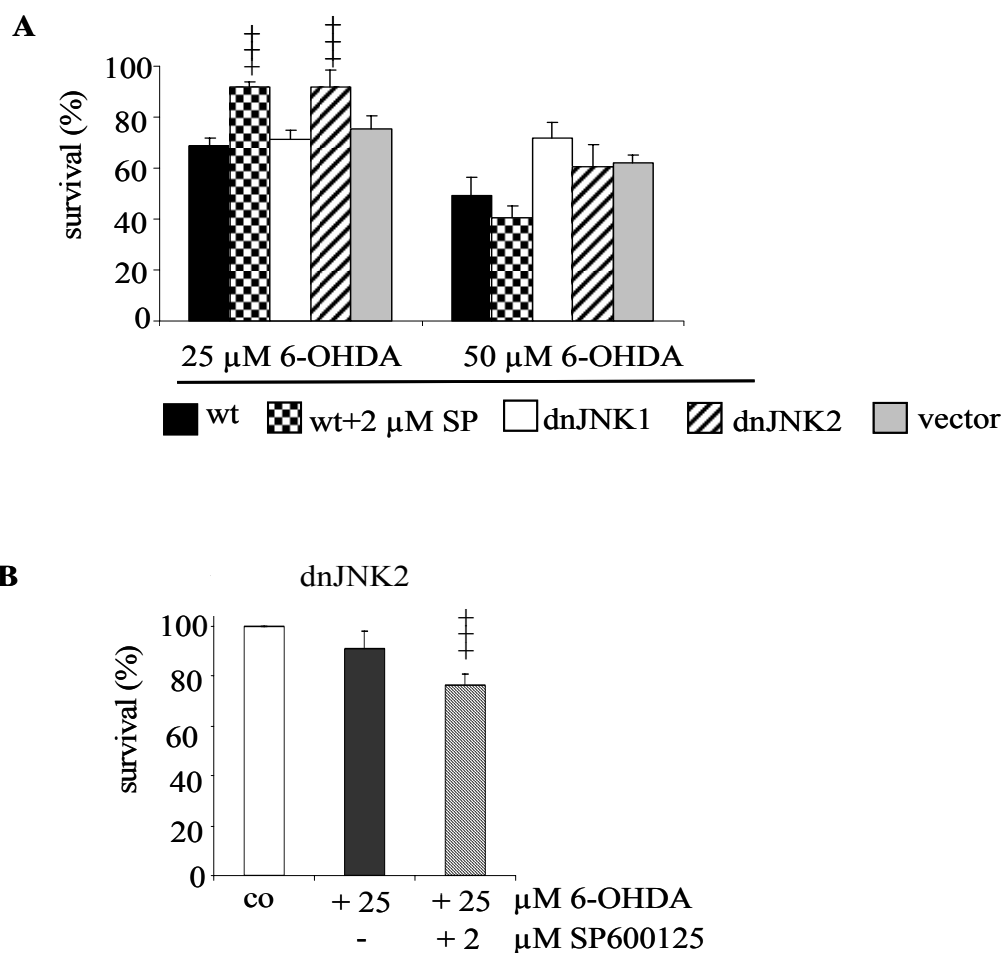
Fig. 3.5. Protection of PC12 cells from 6-OHDA-induced death is dose dependent
A. Trypan blue exclusion assays 24 h after stimulation showed that JNK-inhibition – either by pre-treatment with 2 µM SP600125 (30 min) or transfection with dnJNK2 – significantly reduced cell death in response to 25 µM 6-OHDA whereas dnJNK1 had no protective effects. Cell death triggered by 50 µM 6-OHDA, however, could not be prevented by JNK-inhibition. **B.** Pre-incubation of dnJNK2 with SP600125 did not protect the cells from death induced by 25 µM 6-OHDA.
n = 4. +++ (p ≤ 0.001) indicates significant differences between 6-OHDA vs. JNK inhibition. co, unstimulated.
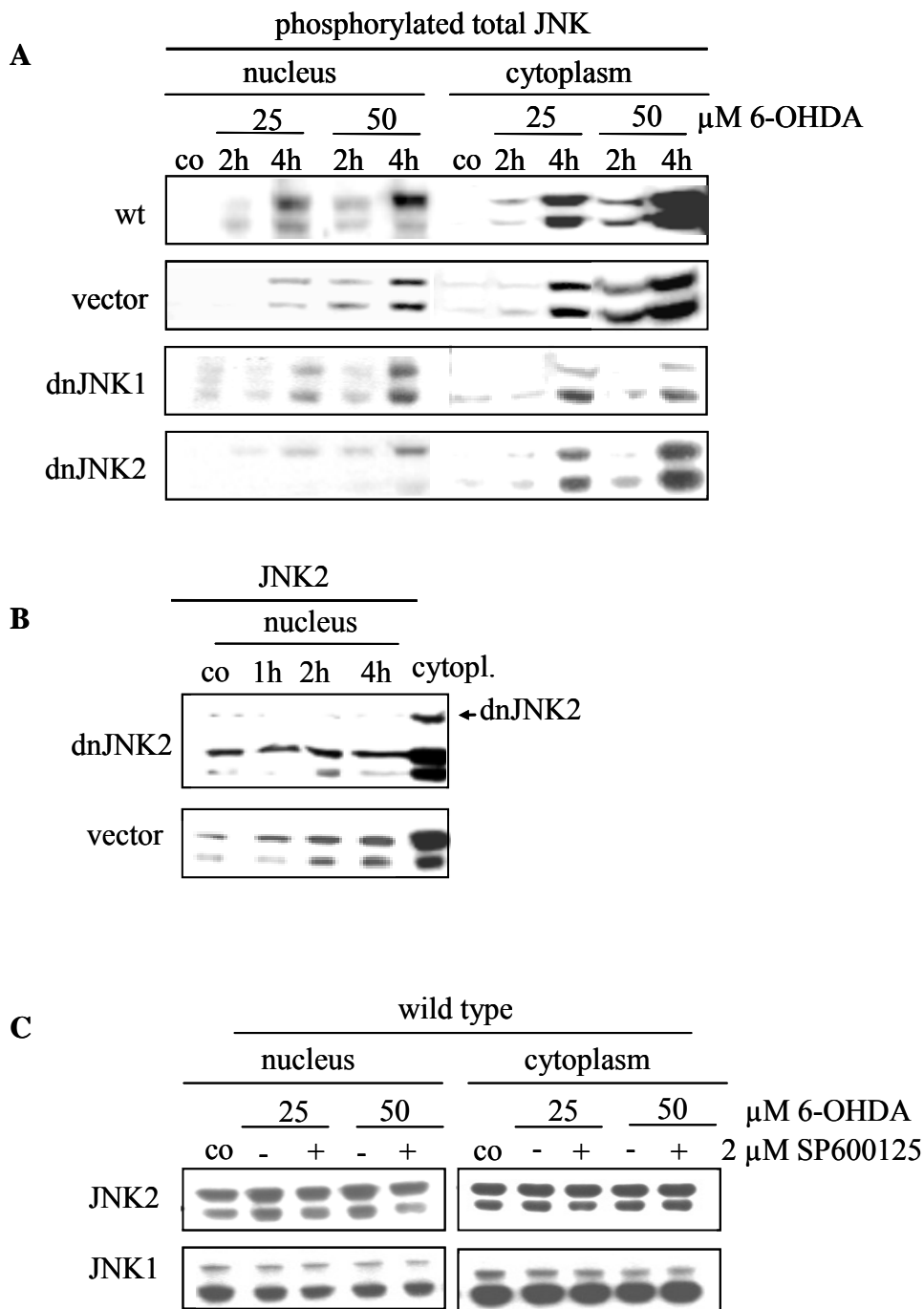
Fig. 3.6. Effects of dnJNK2 on the activation and localization of JNKs
 A. After stimulation with 25 µM and 50 µM 6-OHDA JNKs are activated in nucleus and cytoplasm. Only transfection with dnJNK2 reduced JNK activity. **B.** Twenty-five µM 6-OHDA increased the translocation of JNK2 into the nucleus in vector controls. Translocation of endogenous JNK2 into the nucleus was inhibited by transfection of dnJNK2. **C.** Pre-incubation of PC12 cells with SP600125 inhibited the translocation of JNK2, but not JNK1 into the nucleus.
n = 4. co, unstimulated.

Transfection of dnJNK2 interfered with the activation of JNKs. Importantly, dnJNK2 reduced the pool of phosphorylated total JNK in the nucleus after stimulation with 6-OHDA to a much higher extent than in the cytoplasm, while dnJNK1 had not such effect (Fig 3.6 A). In contrast to wild type cells, transfection with dnJNK2 strongly reduced the 6-OHDA-induced translocation of endogenous JNK2 to the nucleus (Fig. 3.6 B). The construct itself was almost completely excluded from the nucleus since it was barely detectable in nuclear preparations, but clearly visible in the cytoplasm (Fig.3.6 B). Similar to dnJNK2, SP600125 inhibited the translocation of JNK2, but not JNK1 to the nucleus (Fig. 3.6 C).

3.1.4. Translocation of JNK2, but not JNK1 to the mitochondria

Translocation of JNK2 into the nucleus after 6-OHDA treatment raised the question whether JNK2 also translocates to the mitochondria, the central target organelles of 6-OHDA-mediated pathology. One and 2 h after 6-OHDA treatment, the amount of JNK2 present at the mitochondria increased and reached its maximal level at 4 h (Fig. 3.7 A). Dominant-negative JNK2 could not be detected at the mitochondria, as it was seen in the nucleus. Moreover, dnJNK2 did not interfere with the increase of endogenous JNK2 at the mitochondria following 25 μM 6-OHDA (Fig. 3.7 A) or with the mitochondrial pool of JNK1 (Fig. 3.7 B).

In contrast to JNK2, a substantial amount of JNK1 was present at the mitochondria of untreated PC12 cells and this pool did not change following 6-OHDA treatment. Interestingly, translocation of dnJNK1 to the mitochondria after addition of 6-OHDA was observed (Fig. 3.7 B). Similar to nucleus, inhibition of JNK with SP600125 prevented the translocation of JNK2 to the mitochondria, but had no effect on the constitutive presence of JNK1 (Fig. 3.7 C). Consequently, the pool of activated JNK at the mitochondria was determined. Under basal conditions, hardly any phosphorylated JNK could be detected at the mitochondria. After stimulation with 25 μM 6-OHDA, the amount of phosphorylated JNK increased at the mitochondria within 4 h and returned to basal levels until 48 h after stimulation (Fig. 3.7 D).
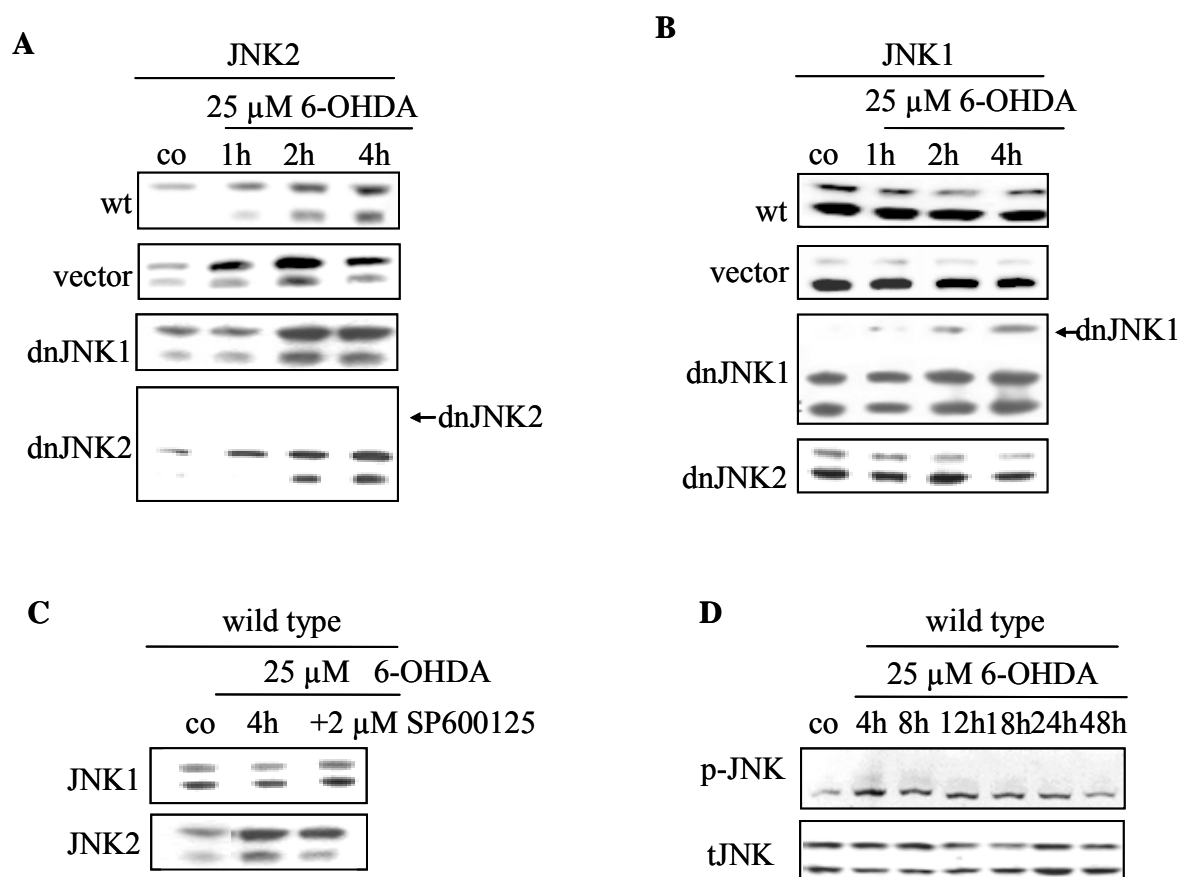
Fig. 3.7. Translocation of JNKs into the mitochondria
A. JNK2 translocated to the mitochondria after 6-OHDA treatment under all experimental conditions. The EGFP-tagged dnJNK2 was not present at the mitochondria. **B.** In contrast to increasing levels of JNK2, JNK1 levels did not change after addition of 6-OHDA at the mitochondria. **C.** Pre-incubation of PC12 cells with SP600125 inhibited the translocation of JNK2, but not JNK1, into the mitochondria. **D.** Levels of phosphorylated JNK (p-JNK) were increased in mitochondrial preparations between 4 h and 24 h after 6-OHDA stimulation while total JNK protein (tJNK) did not change. n = 4. co, unstimulated. wt, wild type

3.1.5. Upstream kinases and scaffolds of JNKs at the mitochondria

A substantial amount of MKK4 was detected at the mitochondria from untreated cells. Stimulation of the cells with 25 μM 6-OHDA activated the mitochondrial MKK4 pool within 1 h and its activation reached its maximal levels between 2 h and 4 h. Transfection of the cells with dnJNK2 abrogated the phosphorylation, but not the presence of MKK4, while transfected dnJNK1 had no effect (Fig. 3.8 A). Additionally the JNK-inhibitor SP600125 prevented the phosphorylation of MKK4 at the mitochondria (Fig. 3.8 B). In contrast to MKK4, MKK7 was not detected at the mitochondria, while it was detectable in cytoplasmic extracts (Fig. 3.8 C). The JNK scaffold protein JIP-1 was present in mitochondrial

preparations and its expression did not change after stimulation with 6-OHDA (Fig. 3.8 C) underlining the specificity and selectivity of the observed localization and activation of JNK signalosome components. The presence of only weakly phosphorylated JNK at the mitochondria suggests the presence of mitochondrial phosphatases which de-activate JNKs. Therefore, MAP kinase phosphates 7 (MKP7) which was recently identified as a major and JNK-specific phosphatase was investigated [88]. Indeed, MKP7 was detectable at the mitochondria and in the cytoplasm and its expression did not change after 6-OHDA (Fig. 3.8 C).
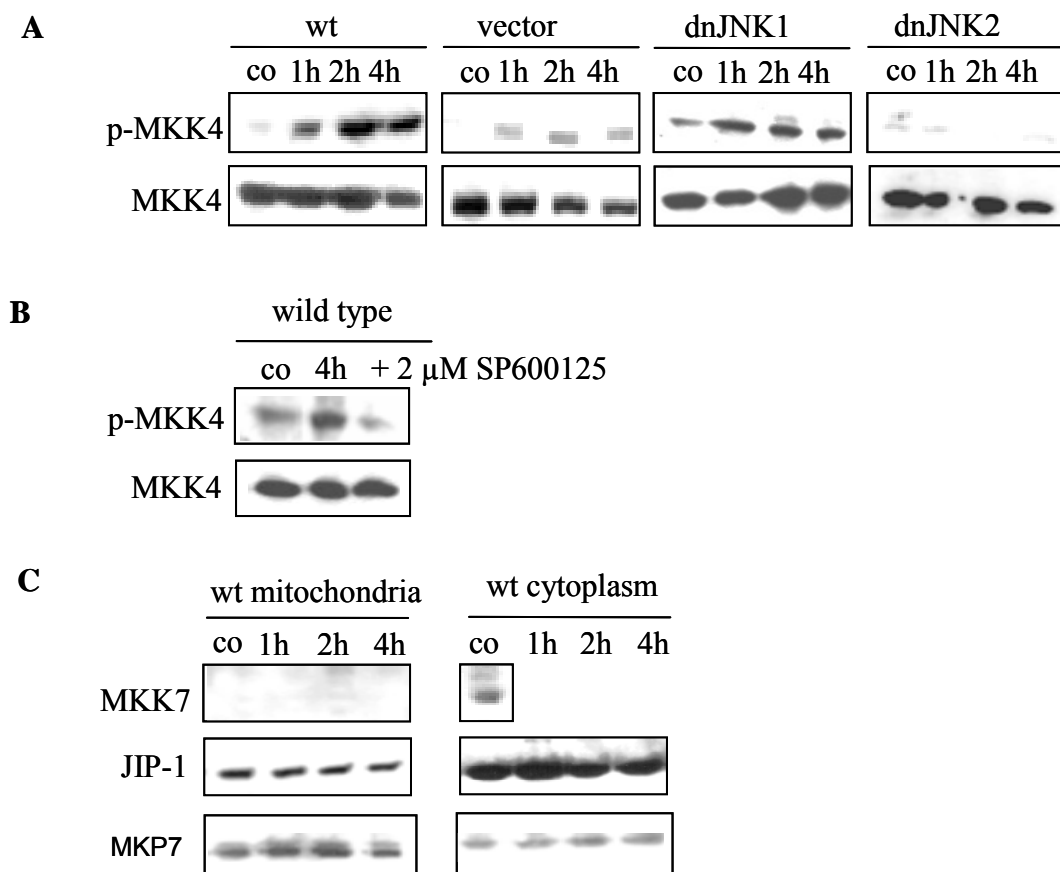
Fig. 3.8. Upstream kinases and the scaffolds of JNKs at the mitochondria
A. In mitochondrial preparations, MKK4 protein was detected in all cells which was phosphorylated in response to 25 μM 6-OHDA. However, dnJNK2 inhibited this phosphorylation. **B.** Similar to dnJNK2, pre-incubation with SP600125 blocked MKK4 phosphorylation. **C.** MKK7 was not present at the mitochondria, while JIP-1 and MKP7 were present at the mitochondria.
n = 4. co, unstimulated.

3.1.6. Cytochrome c release, caspase-3 activation and regulation of *bim*

Twenty-five µM 6-OHDA caused a strong depletion of mitochondrial cytochrome c which was inhibited by pre-incubation with 2 µM SP600125 in wild type and vector controls. Similar to SP600125, transfection of the cells with dnJNK2 reduced cytochrome c release in response to 25 µM 6-OHDA. However, dnJNK1 had no effect on cytochrome c release (Fig. 3.9).
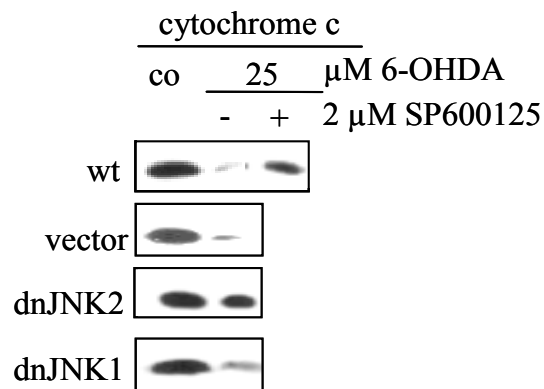

Fig. 3.9. Release of cytochrome c
Twenty-five µM 6-OHDA induced cytochrome c release from mitochondria. Pre-incubation with SP600125 inhibited the release of cytochrome c. DnJNK2 had similar effects as SP600125 while dnJNK1 had no effect. co, unstimulated. wt, wild type.

To investigate the function of JNK2 in apoptosis induced by 6-OHDA, caspase-3, PARP-1 and caspase-8 cleavage was examined. 6-OHDA induced caspase-3 and PARP-1 cleavage, which was inhibited by incubation with SP600125 (Fig. 3.10 A) or transfection with dnJNK2 (Fig. 3.10 B). As expected dnJNK1 had no effect on caspase-3 or PARP-1 cleavage (Fig. 3.10 C). Caspase-8 cleavage was not seen either in wild type or in dnJNK2 cells (Fig. 3.10 A and B), indicating the importance of the mitochondrial pathway in 6-OHDA-induced apoptosis.
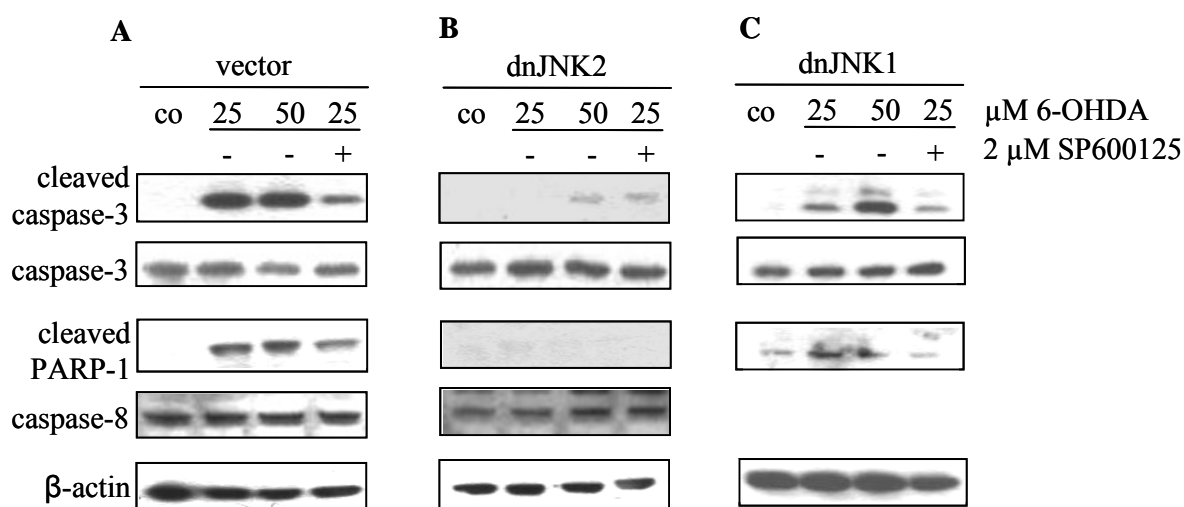
Fig. 3.10. Activation of apoptotic mediators
A. Twelve hours after 6-OHDA stimulation caspase-3 and PARP-1 cleavage was seen in vector controls. This activation was attenuated by SP600125 **B.** The cleavage was blocked by dnJNK2, **C.** but not with dnJNK1. Caspase-8 cleavage was not detected under any conditions, although a strong expression was detected. co, unstimulated.

Finally, in order to investigate *bim* regulation in response to 6-OHDA-induced apoptosis, *bim* mRNA levels were analyzed by semi-quantitative RT-PCR. Eighteen and 24 h after 25 µM 6-OHDA stimulation, *bim* did not increase beyond/over basal level. However, dnJNK2 markedly reduced *bim* mRNA after 18 h and 24 h 6-OHDA stimulation (Fig. 3.11).
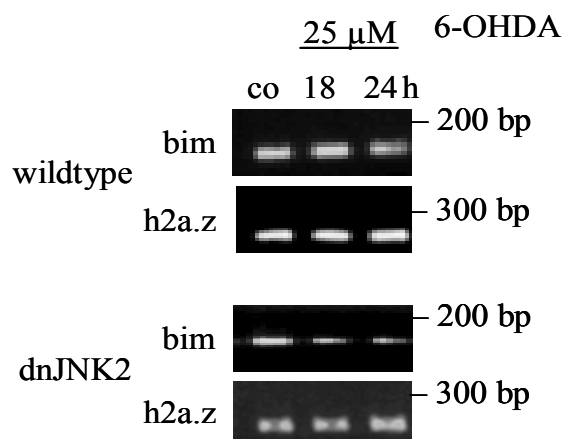
Fig. 3.11. Regulation of *bim*
Bim expression was not induced within 24 h. DnJNK2 reduced *bim* expression between 18 h and 24 h. h2a.z, histone 2A as control to normalize *bim* measurement. co, control.

3.1.7. The effect of JNK3 on 6-OHDA-induced toxicity

PC12 cells only express JNK1 and JNK2 [22, 89] and this property renders PC12 cells a well-defined cell culture system for the analysis of a single transfected JNK3 isoform. Therefore, PC12 cells were transfected with JNK3-EGFP and the functional potential of JNK3 in 6-OHDA-induced toxicity was characterized.

To examine if JNK3 had any influence on 6-OHDA-induced cell death, JNK3-transfected PC12 cells were stimulated with 25 μM and 50 μM 6-OHDA for 24 h. There was no increase in cell death of JNK3-transfected cells after treatment with 6-OHDA compared to wild type and vector-transfected cells (Fig. 3.12).
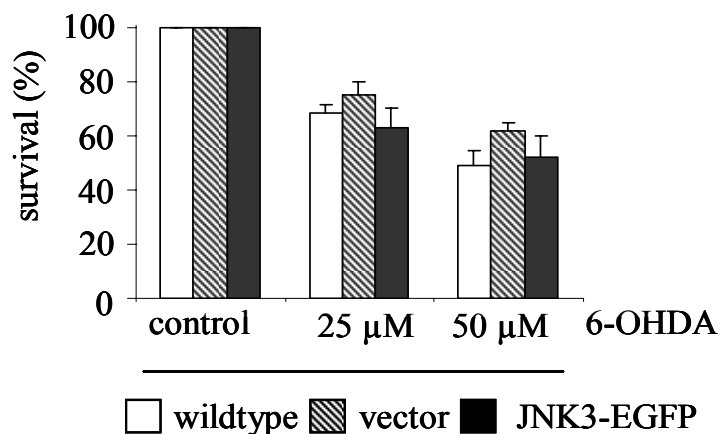
Fig. 3.12. Survival of wild type, vector-transfected and JNK3-transfected cells after stimulation with 6-OHDA
In response to 6-OHDA survival of PC12 cells was reduced as compared to controls. The survival of JNK3-transfected cells was not significantly different from wild type or vector-transfected cells. n = 6.

As shown in section 3.1.2, 6-OHDA induced a dose-dependent phosphorylation of total JNK in wild type and vector-transfected cells. Following JNK3 transfection, 6-OHDA caused a similar activation of JNK3 in cytoplasmic extracts. Interestingly, JNK3 was not activated in nuclear extracts while JNK1 and JNK2 were activated. However, JNK3 was present in the nucleus in untreated and 6-OHDA-stimulated cells (Fig. 3.13 A). Similar to wild type cells, 6-OHDA induced translocation of JNK2, but not JNK1, into the nucleus in JNK3-transfected PC12 cells. Transfection with JNK3 did not enhance c-Jun phosphorylation compared to wild type after 6-OHDA stimulation (Fig. 3.13 B).
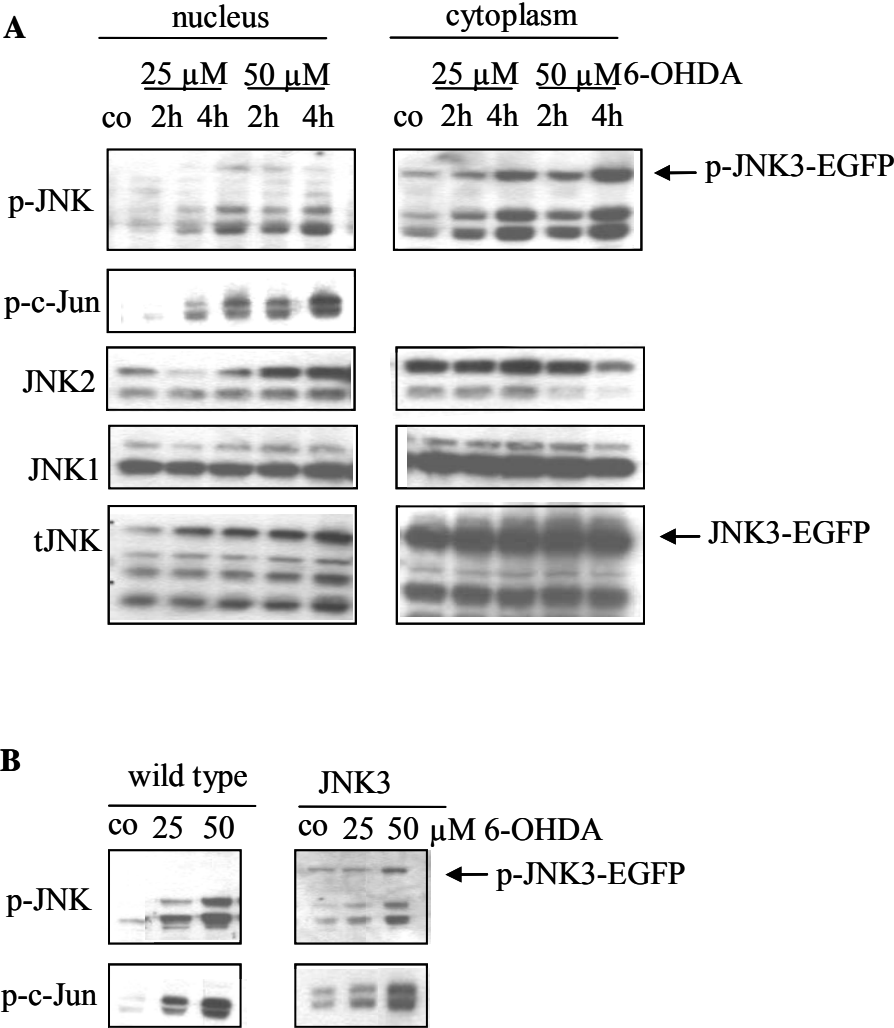
A

nucleus   cytoplasm

<u>25 µM</u> <u>50 µM</u>   <u>25 µM</u> <u>50 µM</u>6-OHDA

co 2h 4h 2h 4h   co 2h 4h 2h 4h

p-JNK   ← p-JNK3-EGFP

p-c-Jun

JNK2

JNK1

tJNK   ← JNK3-EGFP

B

wild type   JNK3

co 25 50   co 25 50 µM 6-OHDA

p-JNK   ← p-JNK3-EGFP

p-c-Jun

Fig. 3.13: Activation of JNK3 in response to 6-OHDA.
A. Transfected JNK3 was activated after 6-OHDA treatment in the cytoplasm. JNK3 was not activated in the nucleus although it was translocated to the nucleus. **B.** Both wild type JNK1/2 and transfected JNK3 were activated in response to 6-OHDA in whole cell extracts, but transfection with JNK3 did not enhance c-Jun phosphorylation compared to wild type after 6-OHDA stimulation.

3.1.8. Differentiation protects PC12 cells against 6-OHDA-induced death

When treated with NGF, PC12 cells differentiate into neuron like cells [86]. To investigate the influence of 6-OHDA on differentiated PC12 cells, they were treated with 50 ng/ml NGF for 7 days and stimulated with different concentrations of 6-OHDA for 24 h. In contrast to naïve PC12 cells, differentiated PC12 cells were resistant to 6-OHDA-toxicity. Twenty-five µM and 50 µM 6-OHDA did not provoke cell death in differentiated PC12 cells (Fig. 3.14 A). Interestingly, in contrast to its protective effects in naive cells, the specific JNK-inhibitor SP600125 induced cell death either in unstimulated (controls) or stimulated differentiated PC12 cells (Fig. 3.14 B).
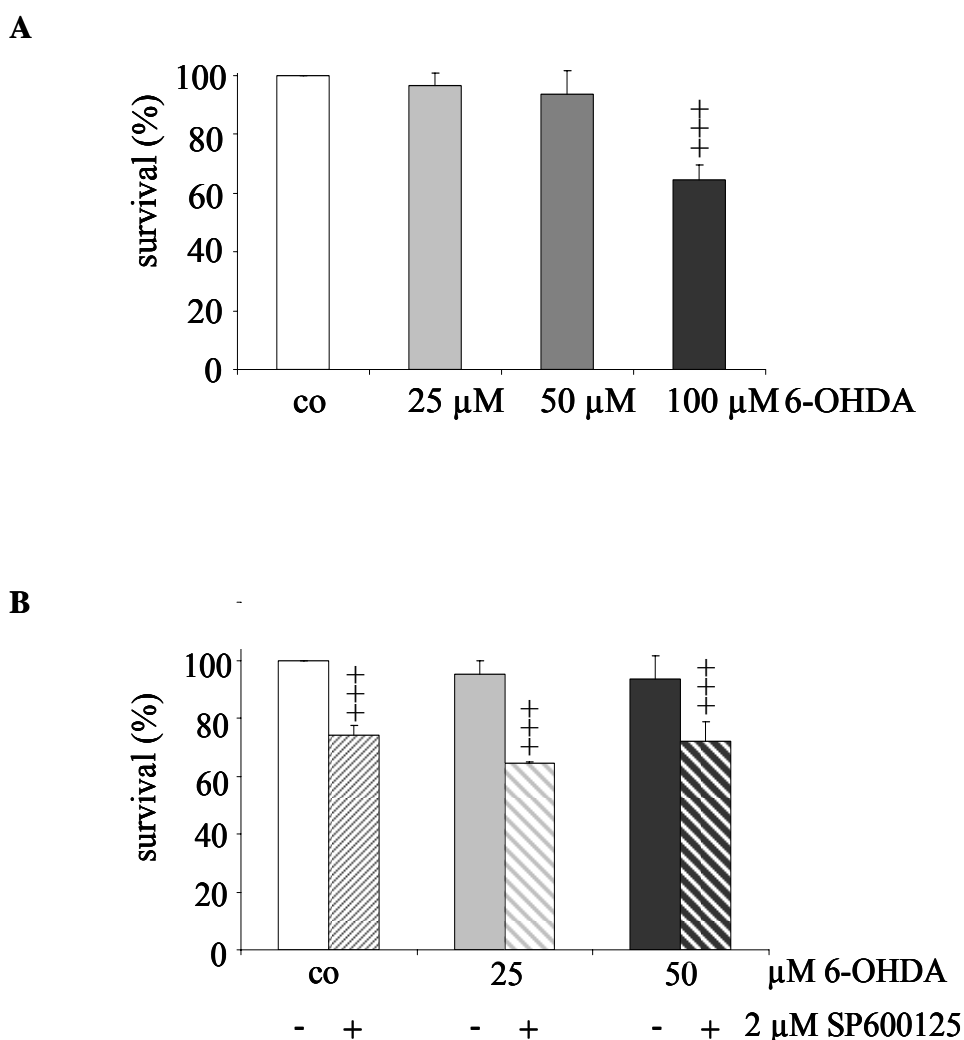
Fig. 3.14. 6-OHDA did not induce cell death in differentiated PC12 cells
A. PC12 cell were treated with 50 ng/ml NGF and stimulated with different concentrations of 6-OHDA. Only 100 µM 6-OHDA induced significant cell death. **B**. Stimulation of differentiated PC12 cells with 2 µM SP600125 induced cell death either in controls or in 6-OHDA-stimulated cells.
+++ gives the significance of changes compared with controls for $p \leq 0.001$. co, unstimulated. n = 3.

3.2. JNKs and death of primary neuronal cells

The aim of the second part of this study was to investigate the role of JNKs in the death of primary neurons. Two kinds of primary neuronal cultures, hippocampal and cortical cells, were grown from newborn rats and mice.

3.2.1. Characterization of hippocampal and cortical neurons

The hippocampal and cortical neurons were cultured for six days. The culture condition resulted in a mixed cell population with less than 20% astroglia cells and more than 80% neurons. Neurons were identified with the neuronal marker MAP-2 (microtubule associated protein 2) staining. In undifferentiated neurons MAP-2 is present in the axons, dendrites and the cell body. With subsequent development, MAP-2 vanishes from the axons, but remains in the dendrites and the cell body [90]. Astrocytes were identified with the astrocyte marker GFAP (glial fibrillary acidic protein) staining. Double staining of the cell cultures with MAP-2 and GFAP showed the interaction between astrocytes and neurons (Fig.3.15).
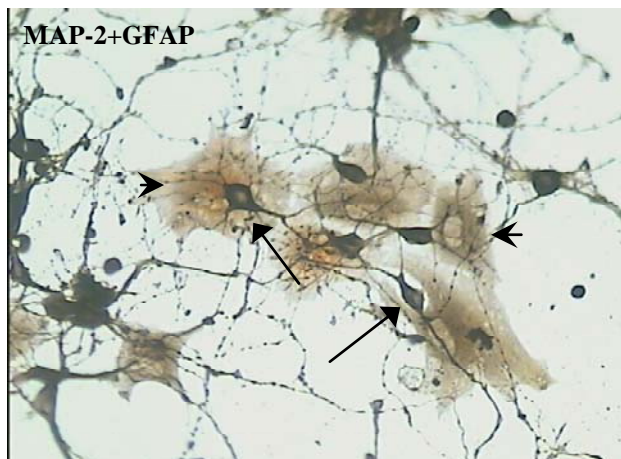

Fig. 3.15. Double staining of the cells with MAP-2 and GFAP
Arrows indicate neurons (blue) and arrowheads indicate astrocytes (brown). Magnification 200X.

The following figures display hippocampal and cortical neurons and astrocytes in the culture. Immunofluorescence staining showed the typical characteristics of astrocytic and neuronal nuclei which are bigger in astrocytes and smaller in neurons. Nuclei were stained with Hoechst dye (Fig. 3.16).

A

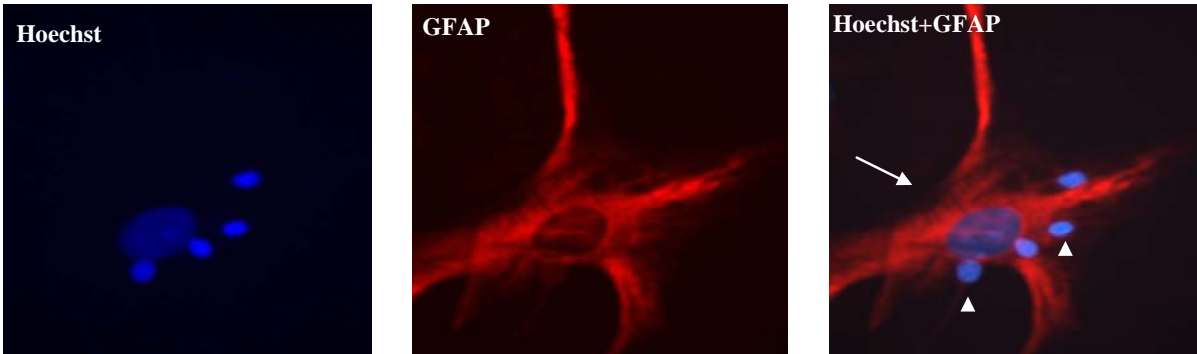Hippocampal neurons
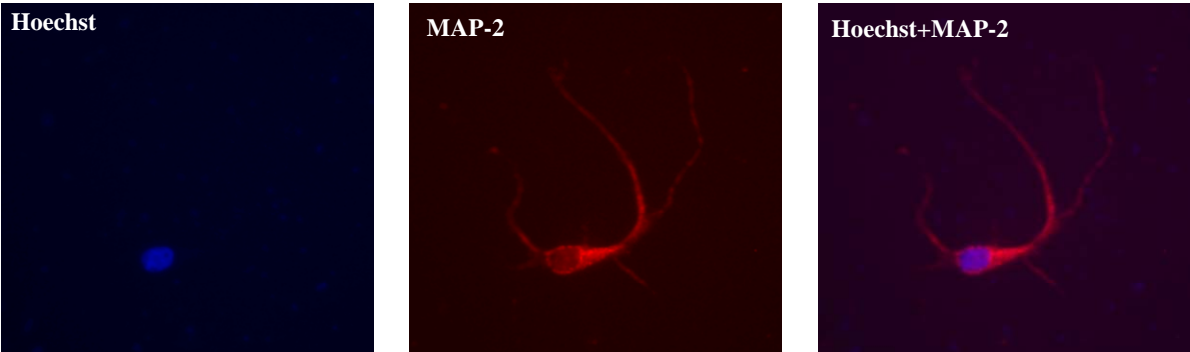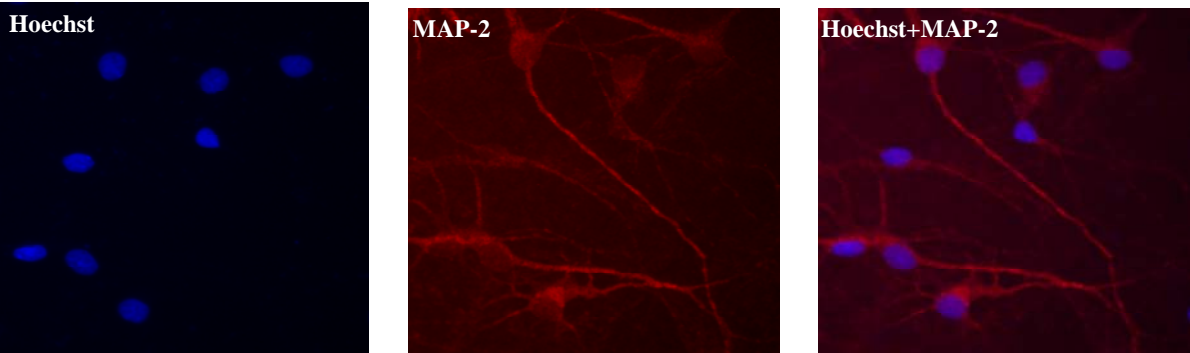
Cortical neurons

B

Fig. 3.16. Hippocampal and cortical neurons in the culture
A. Hippocampal and cortical neurons in the culture. Nuclei were stained with Hoechst dye (blue) and neurons were stained with MAP-2 (red). **B.** Astrocytes were stained with GFAP (red). Arrow indicates astrocytes in the culture and arrowheads indicate nuclei of hippocampal neurons. Magnification 200X.

3.2.2. Glutamate-induced death of primary hippocampal neurons

L-glutamic acid is the principal excitatory neurotransmitter in the mammalian central nervous system and plays a crucial role in plasticity and toxicity of certain neuronal cells [91]. To examine whether JNKs are involved in the glutamate-induced excitotoxicity of neuronal cells, primary hippocampal cells from newborn rats (up to 24 hours) were isolated.

Five days after plating the cells (DIV 5), the mixed neuronal cultures were stimulated with glutamate for 24 h and then stained with MAP-2. The density of neurons in untreated cells was counted and set as 100%. The neurons lost their MAP-2 immunoreactivity (IR) and the number significantly decreased to 66% after glutamate treatment. To investigate the role of JNKs on glutamate-induced cell death, mixed hippocampal cultures were pre-incubated with SP600125, the specific JNK- inhibitor, 30 min before glutamate treatment which rescued 56% of the otherwise dying neurons. This suggests that JNK pathway could be involved in glutamate-induced neuronal death (Fig. 3.17).
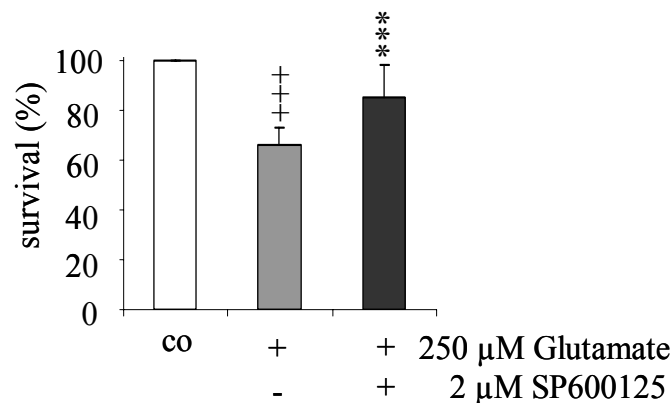

Fig. 3.17. Protection of hippocampal neurons by SP600125 against glutamate
Glutamate caused significant cell death in neurons as compared to unstimulated controls (co) after 24 h stimulation. Loss of neurons was indicated by the decrease of MAP-2 immunoreactivity. Pre-incubation of mixed hippocampal cultures with 2 µM SP600125 significantly protected neurons from glutamate toxicity. Controls were defined as 100%. +++ gives the significance of changes compared with controls for $p \leq 0.01$. *** gives the significance of changes by SP600125 compared with 250 µM glutamate-treated cells for $p \leq 0.05$. n = 6.

As described above, only 66% neurons were alive after 24 h glutamate exposure. However, these surviving neurons exhibited morphological signs of severe cell stress such as short dendrites and vacuolated cytoplasm in comparison with untreated cultures (Fig. 3.18).
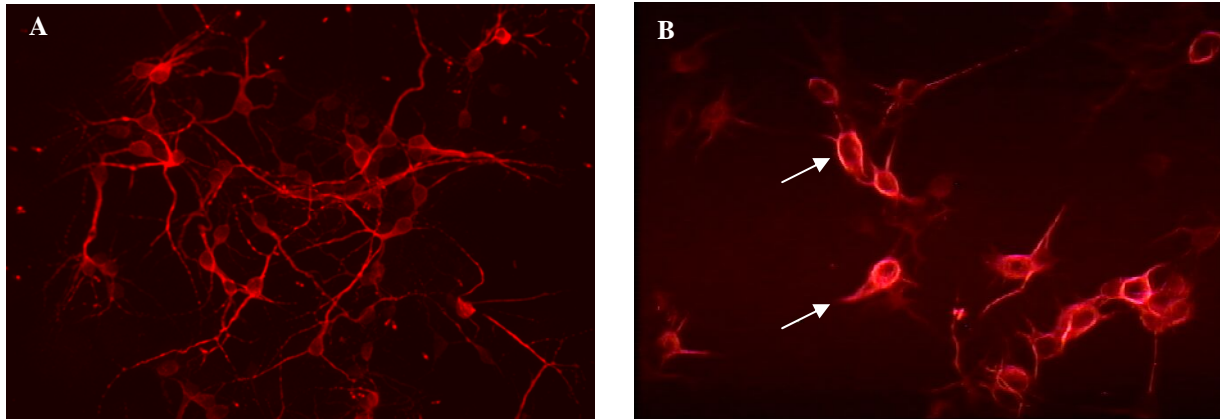

Fig. 3.18. Morphology of degenerating neurons
A. Untreated hippocampal neurons with intact continuous neurites and round smooth cell bodies. **B.** 24 h after glutamate-treatment, neuronal morphology with short dendrites and vacuolated cytoplasm was seen (arrow). Magnification 200X.

To determine whether glutamate induces apoptosis in hippocampal neurons, neurons were stained with the DNA dye Hoechst 33258 to visualize nuclear morphology. In the absence of glutamate, the cultured neurons exhibited normal cellular morphology with evenly stained nuclei (Fig. 3.19 A). Glutamate caused morphological changes that are characteristic for apoptosis, including fragmentation and condensation of nuclei (Fig. 3.19 B). However, it had no effect on astrocytes.

Caspase is considered to play an essential role in the execution stage of apoptosis [92]. Activation of caspase-3 requires cleavage of its inactive form (32 kDa) into active p17 and p12 subunits [93]. To investigate the influence of glutamate on caspase-3 activation, Western blot analysis was performed using a cleaved-caspase-3 specific antibody that recognizes the p17 subunit of activated caspase-3. After determining cleaved-caspase-3, blot membranes were stripped and rehybrized with antibody against caspase-3 to normalize for protein contents. Glutamate stimulation induced caspase-3 activation which was inhibited after pre-incubation with JNK-inhibitor SP600125 (Fig. 3.19 C).
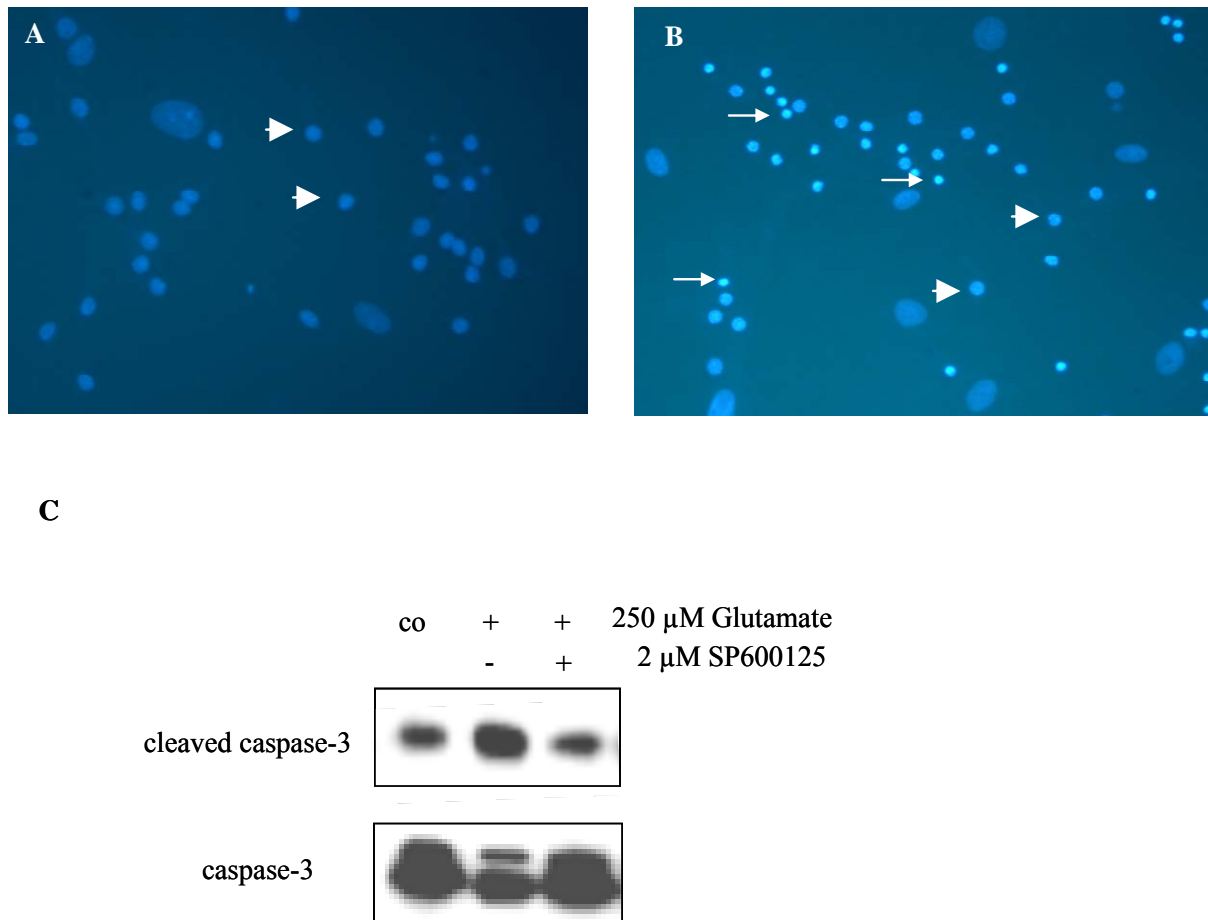
Fig. 3.19. Death of hippocampal neurons induced by glutamate is apoptotic
Hoechst staining visualizes nuclear morphology. **A**. Untreated cells. **B.** Cells treated with 250 μM glutamate for 12 h. Arrowheads indicate healthy cells with uniformly stained nuclei. Arrows mark neurons with apoptotic morphology including condensed or fragmented nuclei. Magnification 200X. **C.** Glutamate induced caspase-3 activation in mixed hippocampal cultures. Pre-incubation with SP600125 (30 min) inhibited the activation of caspase-3. Western immunoblot probed with antibodies to cleaved caspase-3 (top) and caspase-3 (bottom). n = 2.

Regulation of JNK activity in glutamate treated neurons

JNK pathways are essential for apoptosis [58]. To examine the role of the JNK pathways in glutamate toxicity, the cells were treated with 250 μM glutamate for 4 h. Immunocytochemistry was performed using a specific anti-phospho-JNK and anti-phospho-c-Jun antibody. In untreated cells, phosphorylated-JNK was concentrated in the nucleus and the varicosities of the neurons and astrocytes. After glutamate treatment the phosphorylated-JNK immunoreactivity faded out in the varicosities and the nucleus of the neurons while the JNK activity persisted in astrocytes (Fig. 3.20 A and B). However, in untreated cells,

phoshporylated-c-Jun was hardly seen, but it increased slightly after glutamate treatment. (Fig. 3.20 C and D).

For Western blot experiments nuclear extracts were obtained as described in methods. Equal amounts of protein (10 μg) extracts were separated by SDS-PAGE, and detected by anti-p-JNK and anti-p-c-Jun antibodies. A basal JNK activity was seen in the untreated primary cell nucleus while this activation was suppressed after glutamate treatment which was also seen by staining of the cells with a specific anti-p-JNK antibody. In untreated cells, c-Jun had little or no activity which was increased in the nuclear extracts of primary neurons following 4 h glutamate stimulation. Pre-incubation of the neurons with 2 μM SP600125 reduced this activation (Fig. 3.20 E).
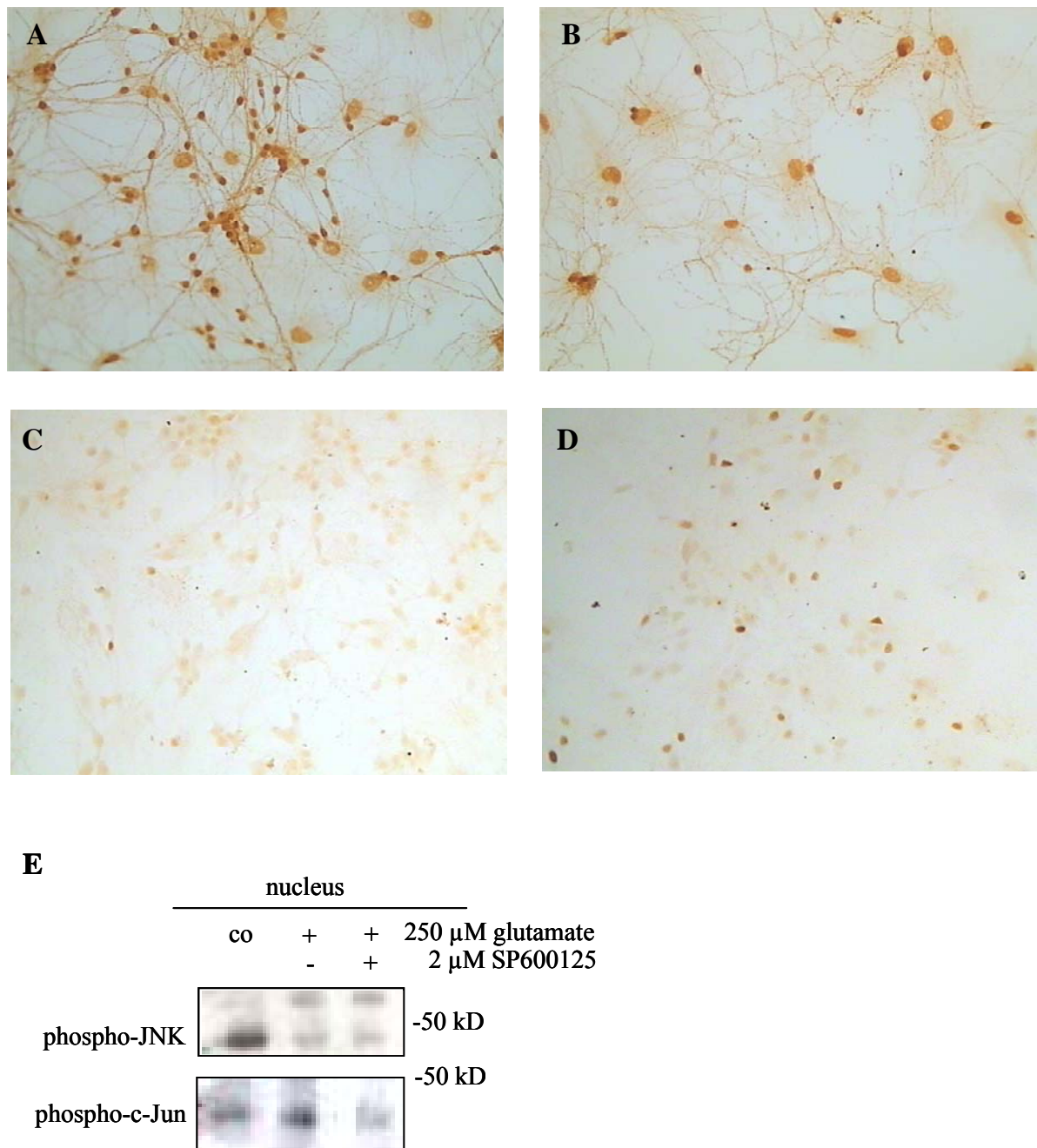
Fig. 3.20. Regulation of JNKs after glutamate treatment
A. Phosphorylated-JNK was concentrated in the nucleus and the varicosities of the neurons and the nucleus of astrocytes. **B.** Phosphorylated-JNK immunoreactivity faded out in varicosities and the nucleus of neurons but persisted in astrocytes in cultures stimulated with 250 μM glutamate for 4 h. **C.** Phospho-c-Jun could not be detected in untreated cells. **D.** Glutamate stimulation increased c-Jun phosphorylation. Magnification 200X. **E.** Equal amounts of protein (10 μg) from mixed hippocampal neuron extracts were electrophoresed and immunoblotted with anti-p-JNK and anti-p-c-Jun antibodies. JNK had a basal activity in untreated primary cell nucleus while this activation was attenuated after glutamate treatment. However c-Jun had a weak basal activation which was increased in the nucleus of primary neurons after glutamate stimulation. Pre-incubation of the neurons with 2 μM SP600125 reduced this activation.
n = 2. co, unstimulated.

3.2.3. 6-OHDA-induced death of primary cortical cells

6-OHDA induced a substantial death in PC12 cells. To investigate the effects of this substance on primary neuronal cells, cortical neurons were prepared from newborn rats as hippocampal cells. Five days after (DIV 5) plating the cells, cortical cells were stimulated with different concentrations of 6-OHDA (25 μM, 50 μM, 100 μM) for 24 h and cell death was analysed by measurement of LDH activity. After 24 hours of stimulation 25 μM 6-OHDA weakly increased the LDH release but the difference was not significant compared to untreated cells. Fifty and 100 μM 6-OHDA induced significant cell death after 24 hours of stimulation (Fig. 3.21).
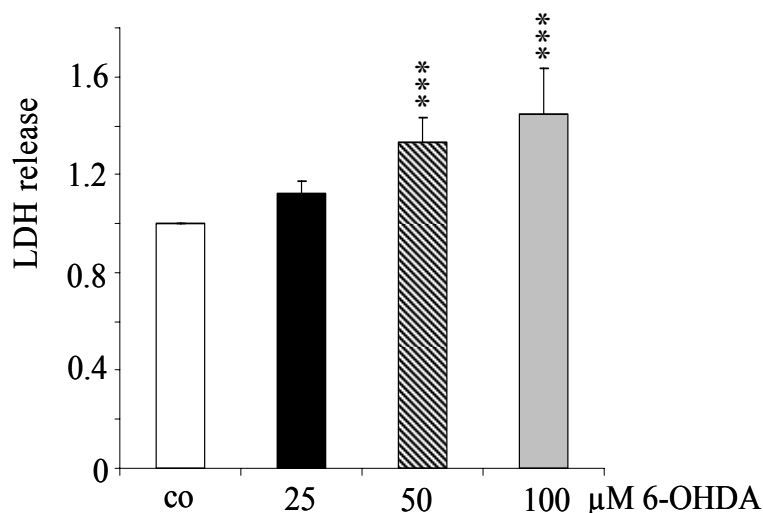

Fig. 3.21. 6-OHDA-induced death
Neuronal death induced by different concentrations of 6-OHDA in mixed cortical neuronal culture was analysed 24 h later by measurement of LDH released. Untreated cells (co) were set as 1.0. *** gives the significance of changes compared with the controls for $p \leq 0.001$. n = 6.

To determine whether 6-OHDA induces apoptosis in cortical neurons, neurons were stained with the DNA dye Hoechst 33258 to visualize nuclear morphology. In the absence of 6-OHDA the cultured neurons exhibited normal cellular morphology with evenly stained nuclei which were scored as healthy, viable neurons. 6-OHDA caused morphological changes characteristic of apoptosis, including fragmentation and condensation of nuclei. However, 13% apoptotic nuclei were also seen in untreated neurons, indicating dissociation and cultivation may cause DNA fragmentation in neurons (Fig. 3.22).
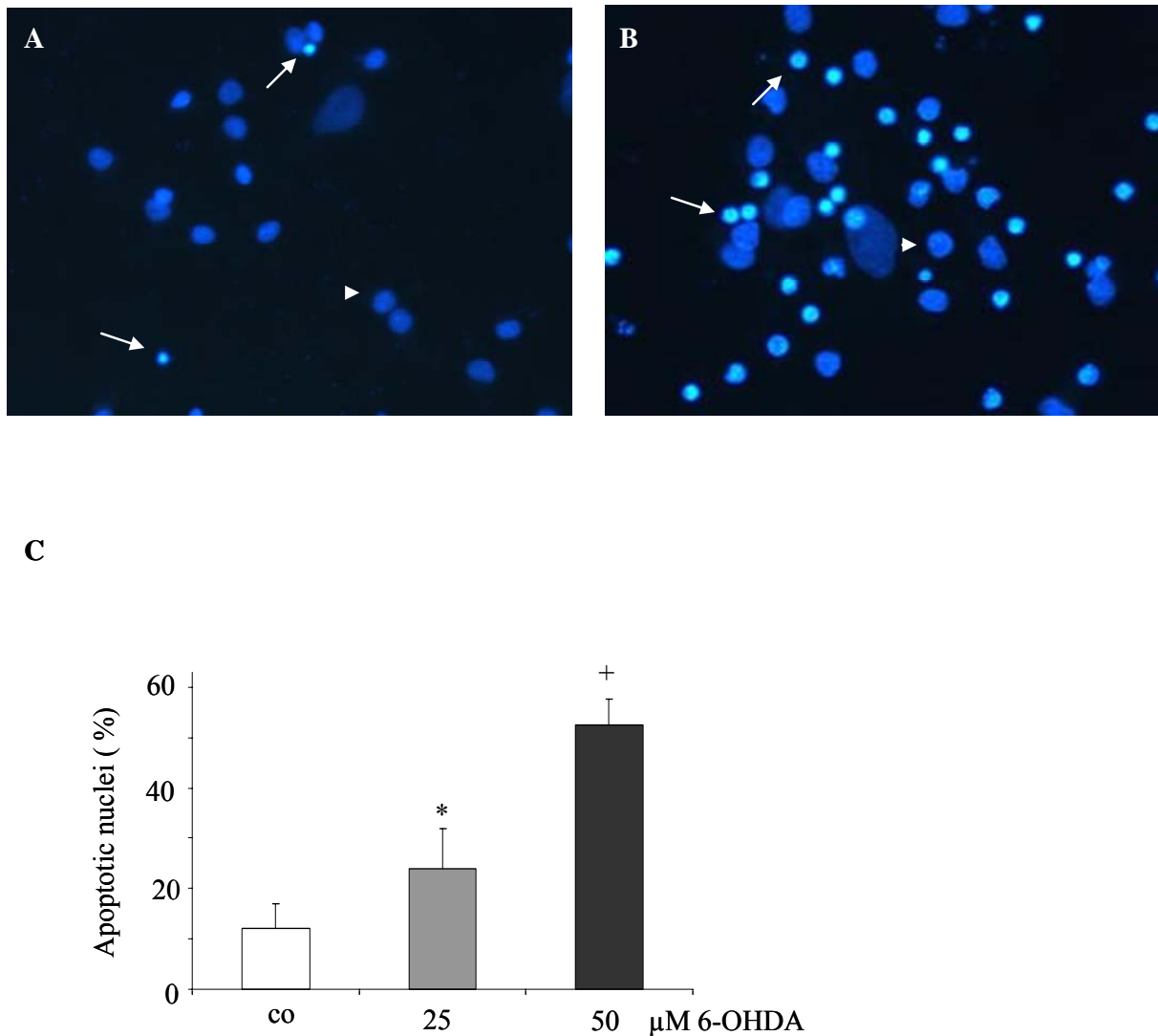
C

Fig. 3.22. Nuclear pyknotic morphology in mixed cortical neuronal cultures treated with 6-OHDA for 24 h
Neurons 5 days in vitro (DIV 5) were stimulated with 6-OHDA for 24 h and stained with Hoechst 33258 to show nuclear pyknotic morphology. **A**. Untreated (control) cultures. **B.** Cultures exposed to 50 μM 6-OHDA for 24 hours. Note the typical apoptotic morphology in neurons (nuclear condensation). Arrows indicate neurons with normal morphology whereas arrowheads indicate apoptotic neurons. Magnification 200X. **C.** The proportion of apoptotic nuclei is shown. In untreated cultures 13% of cells have apoptotic nuclei. * gives the significance of change compared with controls for $p \leq 0.05$. + gives the significance of change compared with controls for $p \leq 0.001$. $n = 5$.

Regulation of JNKs after 6-OHDA treatment

Glutamate altered the distribution of JNK activity in hippocampal neurons. Does this also hold true for cortical neurons following 6-OHDA? Western blot experiments demonstrated a distinct basal JNK activity in the cytoplasm and nucleus of untreated cortical neurons. Similar to hippocampal neurons, JNK activity of cortical neurons was reduced in the nucleus

following 6-OHDA while it did not change in the cytoplasm. Which JNK isoforms are responsible for this attenuation of nuclear JNK activity? Western blotting demonstrated the translocation of JNK2 into the nucleus of cortical neurons between 1 h and 2 h following 6-OHDA and the parallel decrease between 2 h and 4 h in the cytoplasm, whereas the JNK1-IR faded out in the nuclei. At the same time, 6-OHDA induced the activation of c-Jun and the maximal phosphorylation at 2 h coincided with the maximal presence of JNK2 and the decrease of JNK1 (Fig. 3.23).
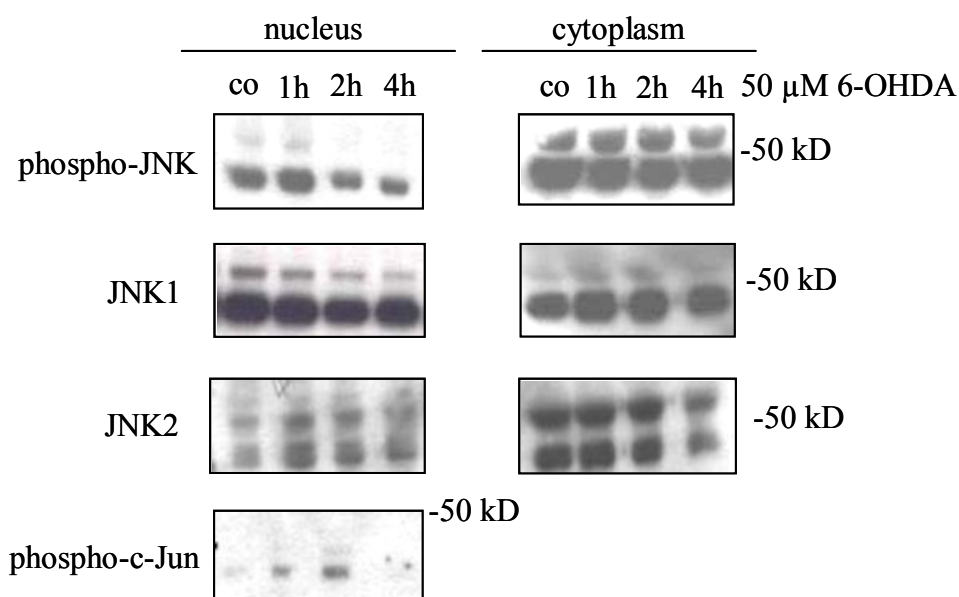

Fig. 3.23. JNKs after 6-OHDA treatment
Western blot experiments against phospho-JNK, phospho-c-Jun, JNK1 and JNK2 after 50 µM 6-OHDA in nuclear and cytoplasmic extracts. In the nucleus, 6-OHDA induced a distinct phosphorylation of c-Jun whereas the pool of the upstream JNK kinases decreased. Analysis of the individual JNK isoforms demonstrated that JNK1 slightly declined and JNK2 clearly increased in the nucleus. In parallel, JNK2 weakly faded out in the cytoplasm, whereas JNK1 did not change.

SP600125 protects cortical neurons from 6-OHDA-induced death

Previous studies demonstrate that JNKs play an important role in neuronal apoptosis. To investigate the role of JNKs in 6-OHDA-induced cortical cell death, the specific JNK-inhibitor SP600125 was used. Pre-incubation of cortical neurons with the JNK inhibitor SP600125 significantly decreased LDH release and diminished the proportion of apoptotic nuclei by 40% (Fig. 3.24 A-B).

Finally, the influence of 6-OHDA and SP600125 on caspase-3 activation was also investigated. The cortical neuronal cultures were pre-incubated with 2μM SP600125 (30 min) and stimulated with 50 μM 6-OHDA. Western blot analysis was performed using a cleaved-caspase-3 specific antibody that recognizes the p17 subunit of activated caspase-3 as hippocampal neuronal cultures. Caspase-3 was activated after 12 h of 6-OHDA treatment and this activation was attenuated with SP600125 (Fig. 3.24 C).
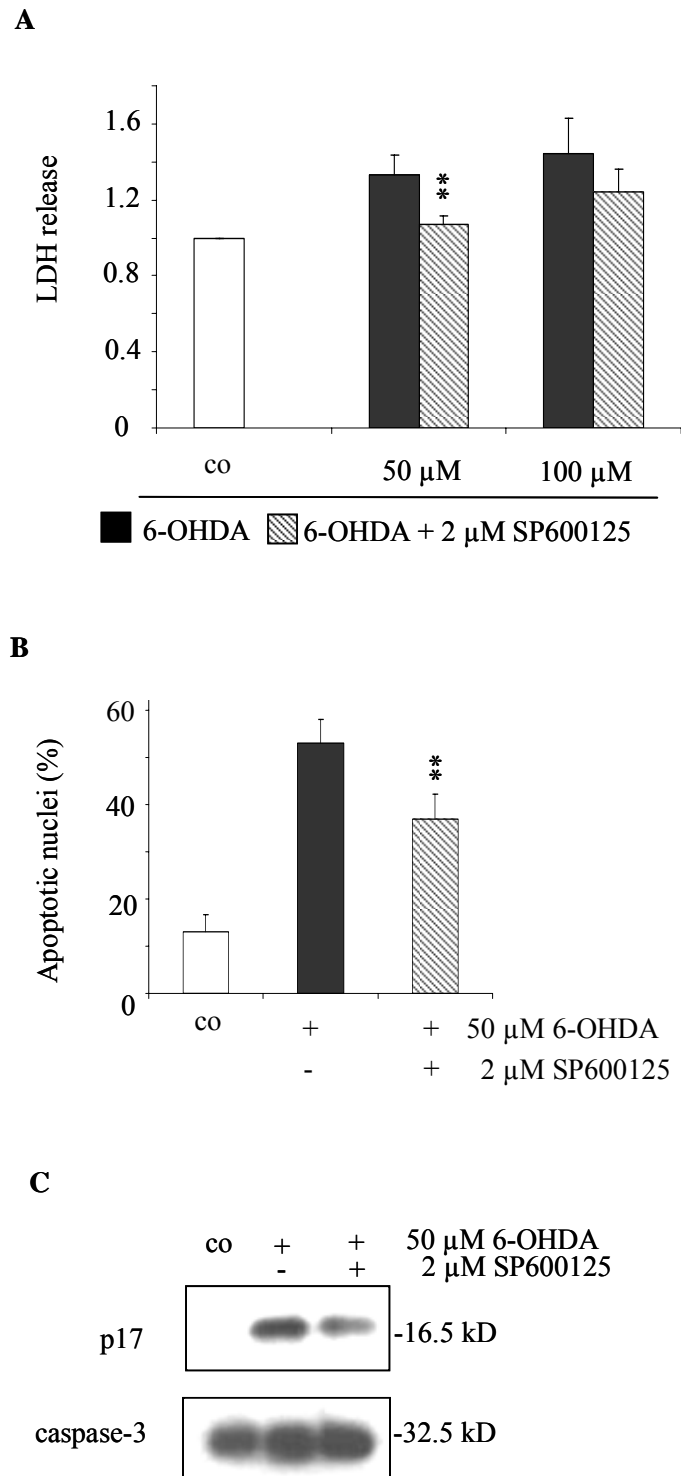
A

B

C

Fig. 3.24. Protection of cortical neuronal cells from 6-OHDA-induced death
A. Pre-incubation with 2 μM SP600125, protected neurons from 50 μM 6-OHDA-induced death. Neuronal death was analysed by LDH measurement. n = 6. **B.** JNK-inhibitor SP600125 diminished 6-OHDA-induced apoptotic nuclei. Apoptotic nuclei were visualized by Hoechst 33258 test. n = 5. **C.** 6-OHDA induced caspase-3 activation following 50 μM 6-OHDA treatment for 12 hours in mixed cortical neuronal cultures. The JNK-inhibitor SP600125 inhibited the activation of caspase-3. Western blots probed with antibodies against cleaved caspase-3 (top) and caspase-3 (bottom). n = 3.
** (p ≤ 0.01) indicates significant difference between 6-OHDA vs. SP600125 and 6-OHDA

Effects of JNK isoforms on 6-OHDA-induced apoptosis

JNKs play an important role in 6-OHDA-induced cell death in primary cortical neurons. To examine which JNK isoform is responsible from this cell death, primary neurons from JNK1 and JNK3 ko mice were used. Cell death was measured with LDH assay. Analysis showed that 25 and 50 µM 6-OHDA induced death in mouse cortical cells, however there was no significant difference between cortical neurons of wt, JNK3 ko and JNK1 ko mice, suggesting all JNK isoforms are involved in 6-OHDA-induced neuronal death, however, experiments with JNK2 ko have to be performed (Fig. 3.25).
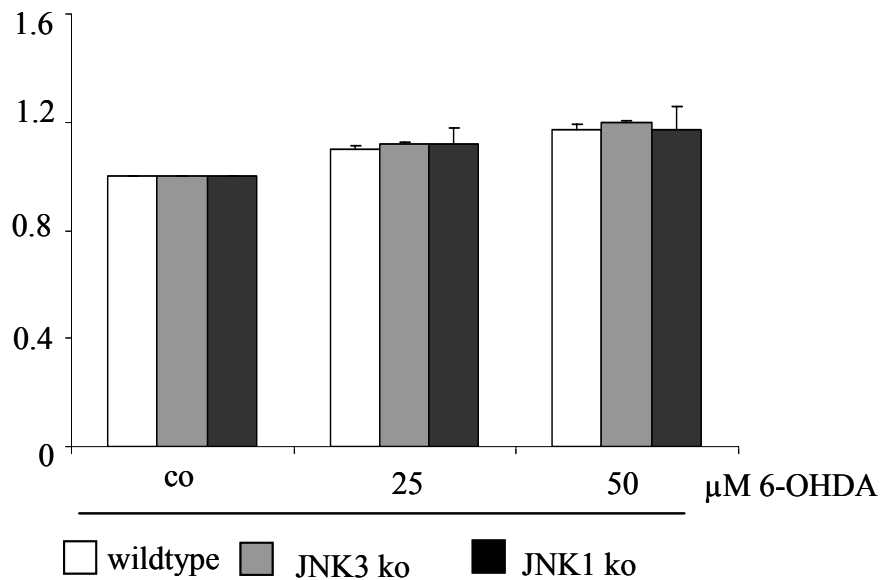
Fig.3.25 Effect of JNK isoforms on 6-OHDA-induced cell death
LDH assay showed that there was no significant change between wt, JNK3 ko and JNK1 ko isoforms after 25 and 50 µM 6-OHDA treatment.

3.3. Function of JNKs in differentiation of primary hippocampal and cortical neurons

Besides their apoptotic functions, JNKs are important for differentiation of neurons and the development of the brain [26]. The aim of the third part of this study was to investigate the physiological role of JNKs in neuronal differentiation as visualised by neurite extention.

3.3.1. Effects of the JNK-inhibitor SP600125 on neurite elongation

JNKs are essential for the formation and elongation of neurites in PC12 cells [57, 94, 95]. Does this also hold true for primary neurons in which it has been demonstrated a most effective execution of apoptosis by JNK? Hippocampal and cortical cultures were exposed to 2 µM SP600125 at the 2nd, 3rd or 5th day *in vitro* (DIV) i.e. following explantation. At DIV 6, the cultures were stained with MAP-2 antibody, the lengths of neurites were measured and classified in 4 groups (<40, <80, <120 and >120 µm). The inhibition of JNK between DIV 2 and DIV 3 effectively prevented the neurite elongation in primary hippocampal neurons as shown by MAP-2 immunocytochemistry (Fig. 3.26 A-B). Measurement of the neurite length demonstrated a significant shift from long to short neurites with 89% and 57% of <40 µm neurites when SP600125 was applied within the first 48 h or 72 h respectively. (Fig. 3.26 C). To examine If JNK inhibitor SP600125 has some effect on neuronal survival, the hippocampal cultures were stimulated with 2 and 10 µM SP600125 at the 2nd day and the cells were stained with the DNA dye Hoechst 33258 to visualize nuclear morphology at the 6th day. Analysis showed that 2 µM SP600125 had not any effect on neuronal survival and JNK inhibition has a specific effect on neuritogenesis. However, 10 µM SP600125 raised the apoptotic nuclei slightly, indicating that higher concentrations of SP600125 may have toxic effects (Fig.3.26 D).

In contrast to rat hippocampal neurons, JNK inhibition by SP600125 did not significantly affect neurite elongation in cortical neurons apart from an increased proportion of short neurites after application at DIV3 (Fig. 3.27).
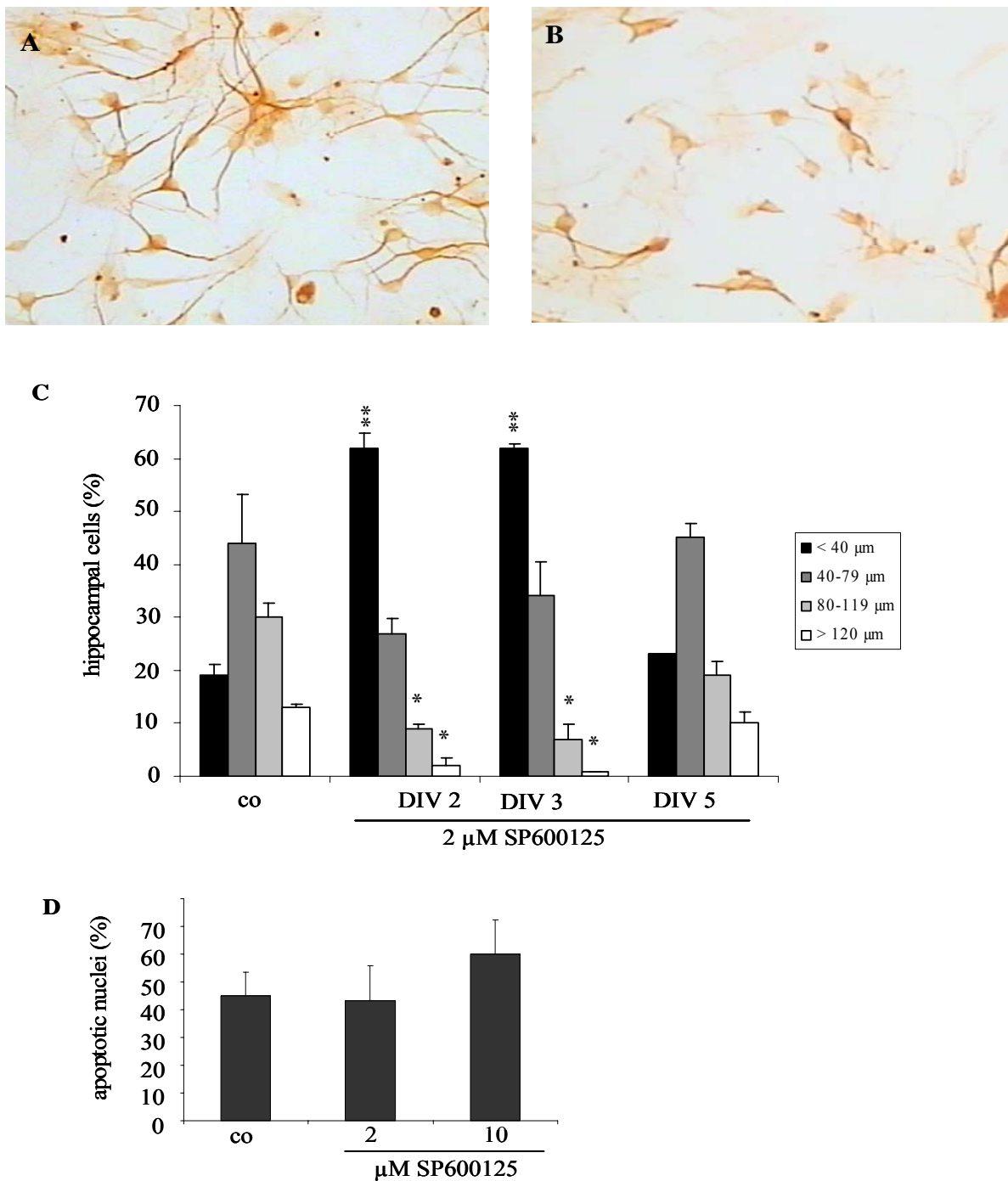
Fig. 3.26. Effects of JNK-inhibitor, SP600125 on neurite elongation in hippocampal neurons
Hippocampal rat neurons were exposed to 2 µM SP600125 at 2nd, 3rd or 5th day in vitro (DIV). At DIV 6, cultures were stained with MAP-2 antibody. **A.** Untreated cultures and **B.** cultures exposed to SP600125 at DIV 2 with dramatically abrogated neurites. Magnification 200X.
C. The distribution of the neurite length (µm) after SP600125 incubation revealed that SP600125 significantly attenuated the neurite elongation. **D. T**he hippocampal cultures were stimulated with 2 and 10µM SP600125 at the 2nd day and the cells were stained with the DNA dye Hoechst 33258 to visualize nuclear morphology at the 6th day. 2µM SP600125 had not any effect on neuronal survival.
*, ** give the significant changes compared with the controls for $p \leq 0.01$ and $p \leq 0.001$, respectively (each n=3). co = unstimulated control after cultivation for 6 days.
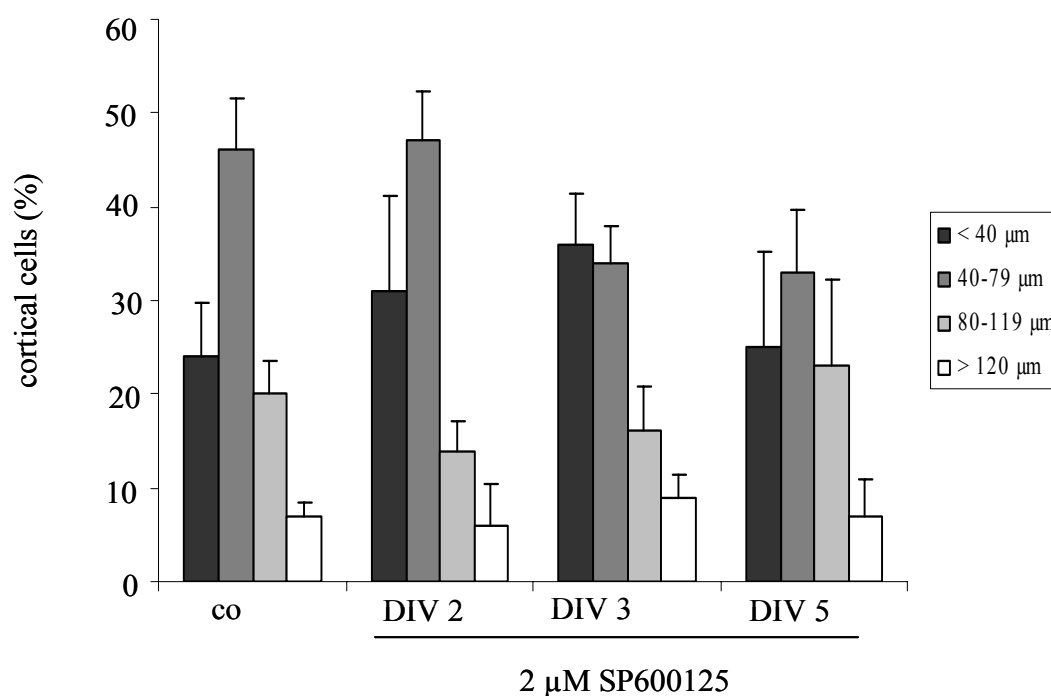
Fig. 3.27. Effects of JNK-inhibitor, SP600125 on neurite elongation in hippocampal neurons
The effect of the JNK inhibitor SP600125 on neurite elongation of rat cortical neurons was analysed by MAP-2 immunocytochemistry. Cultures were exposed to 2 μM SP600125 at 2nd, 3rd or 5th day (DIV2, 3, 6) (each n=3). At DIV 6, cultures were fixed and the neurite lengths were measured. SP600125 did not affect neurite elongation in neonatal rat cortical cultures.

3.3.2. Time course of JNK and c-Jun activation during differentiation

From the very beginning of regrowth, JNKs are mandatory for the elongation of neurites. Does the time course of JNK and c-Jun activation parallel this function? Whole cell extracts from mixed rat hippocampal cultures at DIV 2, DIV 3 and DIV 6 were screened by Western blotting. The pool of activated JNKs substantially and continuously raised with a maximum at DIV 6. Furthermore, the use of individual JNK antisera demonstrated that all three JNK1, 2 and 3 isoforms moderately increased. In contrast to the increase in JNK activity, the pool of phosphorylated c-Jun declined from its maximal level at DIV 2 and faded out at DIV 6 (Fig. 3.28 A). Thus, the increase of activated JNKs is not automatically indicative for the phosphorylation of their nuclear high affinity substrate c-Jun.

JNKs are not mandatory for the neurite elongation of cortical neurons following explantation from neonatal rat brain. Is this lack of efficacy reflected by the absence of activated JNKs and/or c-Jun? Whole cell extracts from cortical cultures were lysed at DIV 2, DIV 3 and DIV 6 and screened for total JNK, phosphorylated JNK and phosporylated c-Jun by Western blotting. Unexpectedly, the pattern of JNK activation resembled that of hippocampal neurons

with a moderate increase in total JNK and a substantial increase in phosphorylated JNK. The time course of phosphorylated c-Jun, however, revealed some difference. In contrast to its distinct presence after 3 and 6 days in hippocampal neurons (Fig.3.28 A), phosphorylated c-Jun was completely absent during this observation period (Fig. 3. 28 B). Again, the activation of the upstream JNK kinases in whole cell extracts does not automatically indicate the phosphorylation of their high affinity substrates such as the nuclear c-Jun.
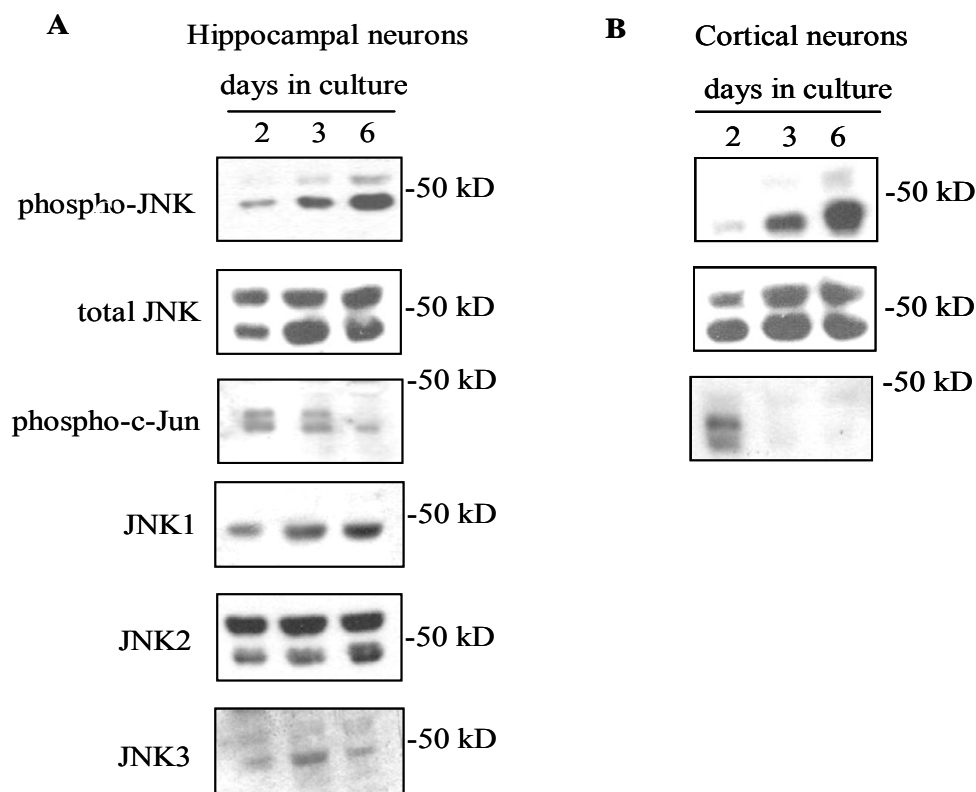
Fig. 3.28. Regulation of JNKs during differentiation
Time course of activated JNKs and c-Jun during the differentiation of hippocampal and cortical neurons. **A.** Whole cell extracts from cell cultures at 2, 3 or 6 days in culture were prepared for Western blotting for phospho-c-Jun, phospho-JNK, total JNK, JNK1, JNK2 and JNK3. The activated phospho-c-Jun declined during the observation period whereas the pool of activated upstream JNK kinases was strongly enhanced. This increase was carried by all three JNK isoforms, but in particular by JNK1 and JNK2. **B.** As in neonatal hippocampal neurons, the activation of c-Jun activation decreased and in parallel the activation of JNK increased during the differentiation of cortical neurons.

4. Discussion

The aims of the present study were to determine the physiological and pathological roles of JNK in PC12 cells and in primary neurons by characterizing its activity and expression as well as the phosphorylation of its substrate c-Jun. Moreover, I determined the effect of its inhibition on cell survival and on neurite outgrowth. Finally, isoform-specific functions of JNKs in the mitochondrial apoptosis pathway in PC12 cells were elucidated.

4.1. The cell culture system

In this study, PC12 cells, primary hippocampal and cortical neurons were used for the determination of the role of JNKs in physiological or pathological conditions.

The PC12 cell line was cloned in 1976 from a transplantable rat pheochromocytoma [86]. In general, PC12 cells exhibit many of the phenotypical properties associated with pheochromocytoma cells and their non-neoplastic counterparts, adrenal chromaffine cells. Additionally, PC12 cells respond to NGF and, in its presence undergo a dramatic change in phenotype wherein they acquire many properties of sympathetic neurons [86]. Importantly for this thesis, PC12 cells have fairly well been characterized with regard to their biochemical properties. They express only JNK1 and JNK2 isoforms, but not JNK3 isoform [89, 96]. These qualities render PC12 cells a well-defined cell culture system for the analysis of the pathological role of individual JNK isoforms at the mitochondria and the mode of JNK-induced release of cytochrome c.

However, some different properties between proliferating and differentiated permanent neuronal cells raise the need for the use of primary neuronal cells in this study. Therefore, primary hippocampal and cortical cultures were also used. The hippocampus is a source of neurons with well-characterized properties typically for central nervous system neurons in general [97] and suitable for dissociated cell culture.

Immunocytochemical analysis showed that hippocampal and cortical cultures revealed after six day in culture the characteristic properties of the corresponding neurons. Staining with the neuronal marker MAP-2 (microtubule associated protein-2) and the astrocyte marker GFAP (glial fibrillary acidic protein) identified more than 80% of the cells as neurons in the culture.

Very few cells being neither positive for MAP-2 nor for GFAP, were other non-neuronal cells such as fibroblasts and endothelial cells. Proliferation of these cells and astrocytes were inhibited with cytosine arabinoside (AraC).

In undifferentiated hippocampal or cortical neurons MAP-2 is present in the axons, dendrites and the cell body. With subsequent development, MAP-2 vanishes from axons, but remains in dendrites and the cell body [90], and this was also characteristic for the development of hippocampal neurons in vitro [98].

4.2. Role of JNK isoforms following 6-OHDA-induced oxidative stress in PC12 cells

The results of the present thesis show that the neurotoxin 6-OHDA, a generator of reactive oxygen species (ROS) and activator of mitochondrial stress [99], kills a high proportion of PC12 cells within 24 h. JNK2, but not JNK1, was translocated to the nucleus and to the mitochondria upon 6-OHDA challenge and mediates 6-OHDA-induced apoptosis. Inhibition of JNK activity by SP600125 or transfection with the dominant-negative JNK2 (dnJNK2), but not dominant-negative JNK1 (dnJNK1), substantially attenuated 6-OHDA-induced cell death, release of cytochrome c and caspase-3 activation. Surprisingly, transfection of PC12 cells with EGFP-JNK3 did not enhance cell death induced by 6-OHDA. Interestingly, JNK3 was not activated in nuclear extracts while JNK1 and JNK2 were activated. However, JNK3 was present in the nucleus of untreated and 6-OHDA-stimulated cells. In contrast to naïve PC12 cells, 25 and 50 μM 6-OHDA did not induce any cell death in NGF-differentiated PC12 cells, and the JNK-inhibitor SP600125 even induced the death of non-stimulated differentiated PC12.

4.2.1. JNKs are mediators of 6-OHDA-induced cell death in PC12 cells

The neurotoxin 6-OHDA is a widely used experimental stimulus for the degeneration of (dopaminergic) neurons in vivo and in vitro. It mediates cell death via several different mechanisms. It is a strong producer of oxidative stress with all its deleterious consequences for the cell, e.g. impairing intracellular redox potential regulation, lipid peroxidation, DNA strand break (reviewed by [100, 101]). Independent of ROS formation, 6-OHDA interacts with and inhibits the complex-I in isolated brain mitochondria [102-104] but the relevance of complex-I inhibition for cell death has not yet been proven [105]. Closely related to the

mitochondrial pathology is the 6-OHDA-mediated cytochrome c release with subsequent activation of caspase-9 and caspase-3, PARP-1 cleavage and DNA fragmentation [106-108] (reviewed by [101, 109]). Moreover, protection against 6-OHDA toxicity by NO is mediated by the inhibition of cytochrome c release [110]. Beyond generation of ROS and mitochondrial dysfunction, 6-OHDA is a strong activator of JNKs [106], but the mode of JNK activation by 6-OHDA remains to be elucidated.

JNK-mediated neuronal death occurs in response to various stimuli, e.g. growth factor withdrawal [111], excitotoxicity [30] or neurotoxins such as MPTP [54, 56]. 6-OHDA caused cell death in PC12 cells. Inhibition of JNK activity by 2 μM SP600125 rescued 68% of the otherwise dying neurons, and a similar protective effect was reached by dnJNK2, but not by dnJNK1. The rescue by dnJNK2 demonstrates that one individual JNK isoform rather than the total pool of JNKs is responsible for cell death as already shown for JNK3 [30, 57] and JNK2 [112]. However, inhibition of JNKs only provided a transient protection against 25 μM 6-OHDA since SP600125 and dnJNK2 were no longer protective after 48 h and 72 h. These data were confirmed by recent findings that the inactivation of JNK3 and the N-terminal phosphorylation domains of c-Jun conferred significant but only transient protection against 6-OHDA-mediated degeneration of dopaminergic neurons in adult mice [57]. Transient protection against 6-OHDA was also reported after inhibition of caspases [102]. The pathway of cell death induced by 6-OHDA is highly dependent on the concentration of 6-OHDA used. At 50 μM 6-OHDA, JNK inhibition either by SP600125 or by transfection with dnJNK2 was no longer able to protect the cells at any time point, indicating a shift in the cellular death pathway(s). Recently, the role of JNKs for 6-OHDA-mediated death was examined with regard to cell death caused by ER stress [107] in response to 40-100 μM 6-OHDA. At higher concentrations, 6-OHDA induces a different, i.e. accelerated (necrotic) cell death [113] which confirms the findings of the present study. The overload of mitochondrial calcium during excitotoxicity can result in spill-over of calcium to the ER with subsequent ER stress-mediated death [45, 114].

JNK was activated in response to 6-OHDA within 2 h and reached its maximal levels at 4 h in the cytoplasm, nucleus and at the mitochondria. This activation was accompanied by a translocation of JNK2 into the nucleus and to the mitochondria, whereas the constitutive presence of JNK1 did not change in these compartments. The differential translocation of JNK isoforms is a critical element for the understanding of isoform-specific JNK actions. For

instance, the nuclear translocation of activated JNK2 and JNK3, but not JNK1, is essential for the c-Jun/AP-1 mediated transcription [27]. It is also conceivable that JNK2 translocation into the nucleus leading to an increased nuclear JNK activity and the subsequent c-Jun phosphorylation enhances the expression of Fas-ligand, which in turn facilitates apoptosis [113]. Transfection of PC12 cells with dnJNK2 reduced the pool of phosphorylated JNK in the nucleus after stimulation with 6-OHDA and strongly reduced the 6-OHDA-induced translocation of endogenous JNK2 to the nucleus while dnJNK1 had not such effect. Similarly, JNK-inhibitor SP600125 attenuated the translocation of JNK2, but not JNK1 into the nucleus following 6-OHDA treatment. These findings suggest that JNK2 is the active JNK isoform which mediates the neurodegenerative effects of 6-OHDA. However, the additional JNK inhibition via SP600125 in dominant negative JNK2 cells completely abolished the protection obtained by either dominant negative JNK2 or SP600125. This suggests the physiological role of JNK1 for the survival of PC12. Indeed, even an anti-apoptotic function of JNK1 in non-neuronal cells has been suggested, further complicating the different aspects of JNK signalling [87].

4.2.2. The JNK signalosome at the mitochondria

The specific mitochondrial translocation of JNK2, but not JNK1, raises the question which signalling pathways control the JNK translocation to and/or the JNK activation at the mitochondria. Phosphorylation of JNKs is a prerequisite for translocation. On the other hand, the presence of non-phosphorylated JNK1 and JNK2 in untreated cells argues against phosphorylation as an absolute precondition for translocation. Similarly, the inhibition of MKK4 phosphorylation by dnJNK2 or SP600125 did not reduce the amount of MKK4 at the mitochondria indicating that translocation and phosphorylation can be separate events. The unexpected inhibitory effect of dnJNK2 on MKK4 phosphorylation is a novel observation which warrants further investigation. However, SP600125 may have some ability to inhibit upstream kinases in the JNK pathway [115]. It is well conceivable, and supported by the findings so far, that a constitutive pool of MKK4 is present at the mitochondria, part of which is activated or replaced with activated MKK4 from the cytoplasmic environment upon 6-OHDA stimulation. Overexpression of dnJNK2 or treatment with SP600125 could shift the equilibrium between active and inactive MKK4 by recruiting an excessive amount of active MKK4 from the mitochondria-associated pool after 6-OHDA stimulation, which may result in

the observed suppression of the mitochondrial phospho-MKK4 signal in dnJNK2-transfected or SP600125 treated cells.

It was shown that MKK4 and JIP-1 are localized at the mitochondria, whereas MKK7 was not detectable in the mitochondrial fraction. This suggests a specific JNK pathway for mitochondrial pathology. MKK4 was previously found in mitochondrial fractions from cardiomyocytes [116], whereas the presence of JIP-1 and the absence of MKK7 are completely novel observations. The absence of MKK7 in mitochondrial homogenates is also conceivable with the findings, that the MKK7/JNK pathway is responsible for neuritogenesis and neurite regrowth after injury [26, 50]. Furthermore, the co-localization of activated JNK with the JNK-binding protein Sab [39] points to specific mitochondrial components of the JNK pathway. Sab strongly associated with the smaller JNK splice variants [39], which is in agreement with the present finding that activated JNK was almost exclusively found in the 46 kD band.

4.2.3. JNK2 controls the release of cytochrome c, expression of Bim and activation of apoptotic proteins

Cytochrome c released from the mitochondrial transition pore is indispensable for the generation of the apoptosome with the subsequent activation of downstream caspases (reviewed by [117]). Therefore, the control of cytochrome c release is central to anti-apoptotic and apoptotic cellular pathways (reviewed by [118]). JNKs can activate pro-apoptotic mediators of the transition pore such as Bax, BAD, BIM, BID or Dp5 [40-42, 46, 118] and de-activate anti-apoptotic proteins such as Bcl-2 [39, 43, 118]. Moreover, JNKs mobilize Smac/Diablo [45] which is released from mitochondria and inhibits anti-apoptotic proteins in the cytoplasm. The release of cytochrome c is tightly linked to the presence and activation of JNKs [40, 111], and the JNK-mediated cytochrome c release can involve the nuclear activation of c-Jun [25] with the subsequent expression of the pro-apoptotic BH3-only protein BIM [25, 46].

JNK inhibition prevented ischemia-induced mitochondrial translocation of Bax and Bim, release of cytochrome c and Smac, and activation of caspase-9 and caspase-3 [119-121]. However, the issue of the JNK isoform(s) responsible for cytochrome c release in neuronal cells has not been clarified. It was shown in this study that dnJNK2, but not dnJNK1, antagonises 6-OHDA-induced cell death, cytochrome c release, caspase-3 and PARP-1

activation to a similar, or even stronger, extent as the JNK-inhibitor SP600125. The translocation of JNK2 to the nucleus and mitochondria supports the notion that the assembly of a selective JNK2 signalosome propagates the mitochondrial pathology (Fig. 4.1). A central role of JNK2, but not JNK1, for cellular degeneration was also seen in fibroblasts where JNK2 mediates tumor necrosis factor-α-induced cell death via caspase and cathepsine signalling [56, 122] and the role of JNK2/3 for neuronal stress in primary cerebellar granule neurons was examined [27]. Recently, it was shown that in various cell types including fibroblasts, erythroblasts and hepotocytes, JNK2 deficiency leads to increased cellular proliferation [123]. However, the role of JNK2 is controversially discussed. JNK2 has been implicated in tumor growth and cell proliferation [124, 125], and in response to neurotrophic factors [50, 126]. Importantly, SP600125, which prevents the stress-induced alterations in the membrane potential [87], blocks the mitochondrial translocation of JNK2, but not JNK1. This finding suggests that the intracellular distribution of JNK2 depends on its activation and, by a positive feedback mechanism, on activated upstream kinases [127]. It remains to be clarified whether the apparently moderate mobility of JNK1 might be due to the absence of this feedback loop.

The induction of apoptosis and the activation of caspase-3 by 6-OHDA has been examined in several studies [128, 129]. Especially in PC12 cells, it has been shown that 25 μM 6-OHDA caused apoptosis, whereas the application of 50 μM 6-OHDA caused necrotic cell death [107, 130]. Activation of mitochondria independent caspase-8 after addition of 6-OHDA was not detected despite a strong expression and this underlines the importance of the mitochondrial pathway. In fact, the 6-OHDA-induced activation of caspase-8 seems to be a cell type-specific event since it has only been described to occur in MN9β cells and mesencephalic neurons [131]. The crucial involvement of JNKs in the mitochondrial stress pathway after stimulation with 6-OHDA and its effect on the activation of caspase-3 and the cleavage of PARP-1 has not been examined before. However, it is known that JNKs play a role in mitochondrial signalling in the context of oxidative stress, where JNK inhibition reduced caspase-9 activity [31].

In addition to the phosphorylation of mitochondria-associated substrates, nuclear JNK2 joins the nuclear pool of activated JNKs and subsequently contributes to the transcription of c-Jun/AP-1 controlled target genes such as BIM [25, 41].On the other hand, SP600125 or dnJNK2 distinctly reduce nuclear levels of activated JNKs. Dominant-negative JNK2

interfered with the transcription of *bim* at a delay between 18 h and 24 h, but not between 4 h and 12 h. The role of the constitutively present JNK1 for mitochondrial functions remains to be defined. JNK1 was recently found in the anti-apoptotic XIAP-TAK1-cascade [87], but the link to mitochondria-associated protection is speculative. Moreover, the clear distinction between anti-apoptotic JNK1 and apoptotic JNK2 is not a general principle since JNK1 can also confer apoptosis by Bcl-2 phosphorylation in immune cells [132].
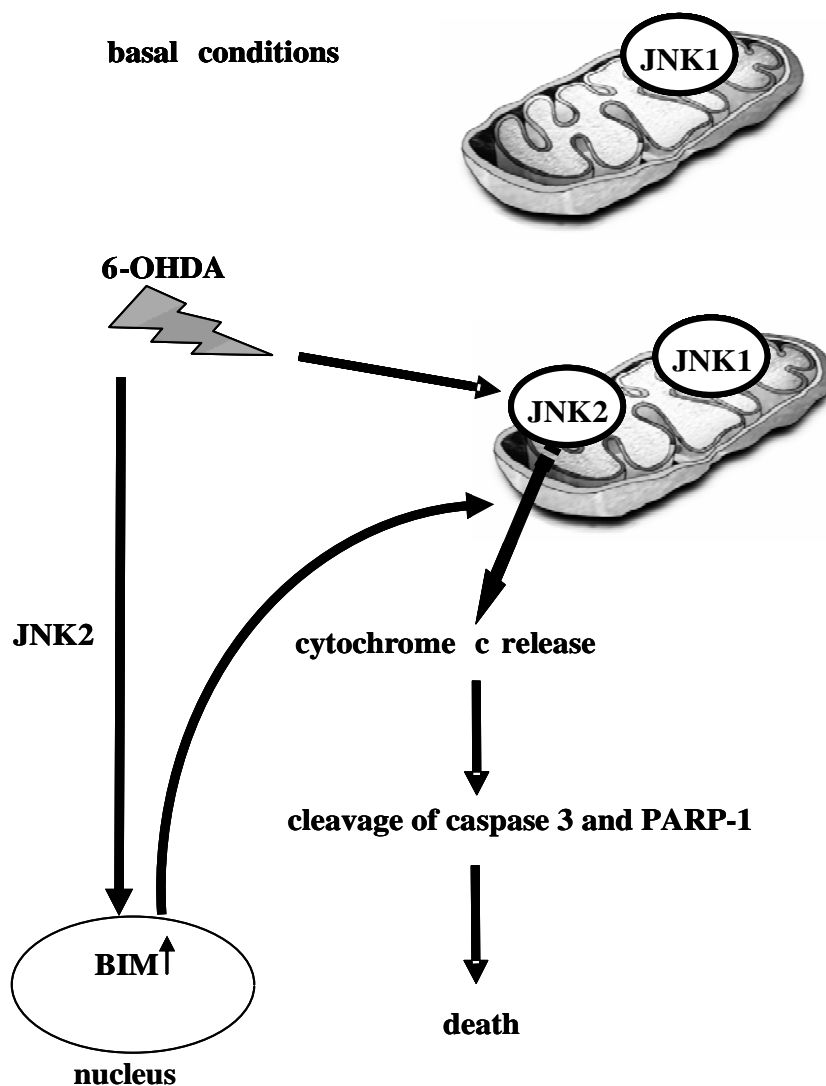

Fig. 4.1. Effects of 6-OHDA on JNK isoforms at the mitochondria
The JNK2, but not the JNK1 isoform, mediates the death of PC12 following 25 µM 6-hydoxy-dopamine. Following translocation to the mitochondria, JNK2 mediates cytochrome c release, activation of caspase-3 and PARP-1.

4.2.4. The effect of JNK3 on 6-OHDA-induced cell death

From all JNK isoforms, JNK3 in particular has been associated with apoptosis in pathological contents, and due to its defined expression, predominantly in neuronal degeneration. Deletion of the mouse JNK3 gene caused resistance to excitotoxic glutamate receptor agonist kainic acid with reduction in seizure activity and apoptosis of hippocampal neurons [30] as well as the resistance to 6-OHDA and nerve fiber injury [57]. JNK3 is likely to contribute to ischemic death in prenatal and adult rats [133, 134]. In cortical and cerebellar neurons JNKs hold a central role in apoptosis caused by UV irradiation, growth factor withdrawal, ß-amyloid and sodium arsenide [135-138], however, it has a minor role in cell death following DNA damage [139]. Phosphorylation of c-Jun and activation of JNK3 is important in cell death induced by *Jnk3* gene [30].

In the present study, PC12 cells which normally only express JNK1 and JNK2, were additionally transfected with JNK3. In these cells 6-OHDA did not enhance cell death and it did not increase c-Jun phosphorylation compared with vector transfected cells. JNKs realize their degenerative effects at least partially in the nucleus and at the mitochondria. However, after transfection with JNK3, 6-OHDA caused a similar activation of JNK3 in cytoplasmic extracts, but not in the nuclear and mitochondrial ectracts which could explain the absence of enhanced cell death in JNK3 transfected cells. Absence of JNK3 activation in the nucleus did not induce influence increase in c-Jun activation indicating that there is a correlation between JNK3 activation and c-Jun phosphorylation and also JNK3-mediated degeneration. It was recently shown that JNK3-deficiency reduced the increase of c-Jun phosphorylation and apoptosis after stressful stimulus [30, 137]. The findings in this study suggest that JNK3 is not involved in 6-OHDA-induced death, at least in PC12 cells. The missing apoptotic action of JNK3 in PC12 cells not necessarily means that JNK3 is without any function in PC12 cells. It was shown that transfected JNK3 significantly enhanced NGF-induced neurite outgrowth in PC12 cells [67].

4.2.5. Differentiation protects PC12 cells against 6-OHDA-induced death

When treated with NGF, PC12 cells differentiate into neuron like cells [86]. In contrast to naïve PC12 cells, differentiated PC12 cells did not die following 25 µM and 50 µM 6-OHDA. It was recently shown that NGF prevented PC12 cells from MPTP induced cell death by suppressing caspase-3 activity [140] and protected PC12 cells against 6-OHDA-induced

oxidative stress [141], indicating that NGF has protective effect against excitotoxic stimulus. However 6-OHDA induced death also in differentiated PC12 cells at higher concentrations such as 100 μM [142] which is in line with this study.

Inhibition of JNKs with SP600125 induced cell death in either unstimulated or stimulated differentiated PC12 cells suggesting that JNKs have different functions in differentiated PC12 cells than in naïve cells. JNKs may have functions in cytoskeletal regulation. Indeed, JNKs are relevant for both, the integrity and/or function of the cytoskeleton and the reception of signal emanating from the cytoskeleton. Several cytoskeletal components are JNK substrates such as neurofilaments and microtubule-associated proteins (MAPs). MAP-2 and MAP-1B polypeptides are hypophosphorylated in Jnk1-/- brains, and this compromised the ability to bind microtubules and promote their assembly. These results suggest that JNK1 is required for maintaining the cytoskeletal integrity of neuronal cells and is a critical regulator of MAP activity and microtubule assembly [60]. In cultured neurons from the intermediate zone, activated JNK was detected along microtubules in the neurite processes. Application of a JNK-inhibitor caused irregular morphology and increased stable microtubules in processes, and decreased phosphorylation of microtubule associated protein 1B, raising a possibility of the involvement of JNK in controlling tubulin dynamics in migrating neurons [143]. Additionally, it is known that JNKs have also anti-apoptotic functions [87]. It appears that the JNK pathways function in a cell-type and stimulus dependent manner, i.e. their different components and compositions can play opposite roles e.g. in apoptosis or in differentiation.

4.3. Role of JNKs in degeneration of neurons

4.3.1. Glutamate induced cell death in hippocampal neurons

L-Glutamic acid (glutamate) is the principal excitatory neurotransmitter in the mammalian central nervous system. Glutamate does not only mediate excitatory neurotransmission, but is also involved in other phenomena such as neuronal plasticity and cell death [91, 144]. Cell death induced by glutamate is believed to be involved in neuronal loss associated with both acute (e.g. stroke) and chronic (e.g. Alzheimer's disease) neurodegenerative insults [32, 145].

In this study, glutamate induced loss of MAP-2 immunoreactivity about 34% in neurons confirming previous results [30, 146-149]. Staining of the cells with the DNA dye Hoechst

33258 showed that glutamate caused morphological changes characteristic to apoptosis, including fragmentation and condensation of nuclei [150, 151]. The absence of any morphological changes in astrocytes suggested that glutamate had not any effect on astrocytes in concentration which is toxic for neurons.

Caspase is considered to play important roles in the execution stage of apoptosis [92]. Activation of caspase-3 requires cleavage of its inactive form (32 kDa) into active p17 and p12 subunits [93]. In this study caspase-3 activation is involved in cell death in hippocampal mixed neuronal cultures which is in line with the other findings [146, 147, 152, 153]. However, activation of caspases following glutamate treatment is still controversially discussed even in the same type of neurons [153-155]. Postnatal hippocampal cultures consist almost exclusively of neurons and astroglia as seen in this study. Since caspase-3 activation is performed with Western blot experiments, this activation may include both neurons and astrocytes. However, the absence of any morphological changes in astrocytes following glutamate treatment suggests that the caspase-3 activation in response to glutamate is a specific neuronal reaction.

JNK-mediated neuronal death occurs in response to various stimuli, e.g. growth factor withdrawal [111], excitotoxicity [30] or neurotoxins such as MPTP [54, 56]. Inhibition of JNK with SP600125 reduced the MAP-2 loss following glutamate treatment, indicating role of JNKs in glutamate induced-death. Inhibition of caspase-3 activation following SP600125 treatment also demonstrates the JNK-mediated death. However, the involvement of JNKs in glutamate-induced toxicity is controversially discussed. In cerebellar granule neurons glutamate-induced apoptosis is independent of JNKs [149, 155] and inhibition of JNKs had not protective effect [155], whereas JNKs are involved in glutamate-induced toxicity in cortical neurons [156], in striatal neurons [157] and in hippocampal neurons [30]. The basis for this discrepancy is unclear, but presumably reflects differences in the intensity of stimuli and the sensitivity of the cells to glutamate-toxicity.

The presence of high basal JNK activity in hippocampal neurons is in agreement with reports of high basal JNK activity in the brain [158]. Basal JNK activity was primarily present in the cell bodies, processes and the nucleus. After glutamate stimulation, the level of activated JNK dramatically declined from nuclei whereas a proportion of c-Jun was phosphorylated. A similar 'paradox' was reported in embryonic cerebellar neurons [26] and neonatal microglia

[50]. It was recently shown that neuronal stress selectively activates JNK2/3 in the presence of constitutive JNK1 activity and this JNK2/3 activity selectively targets c-Jun, which can be separated from constitutive JNK1 activity [27]. Nuclear JNK activity mediated the apoptotic actions of JNKs [159]. However this does not mean that c-Jun is a mere apoptotic mediator [reviewed by [160]]. Subsequently, the phosphorylation of c-Jun cannot simply be used as a marker for the nuclear activities of either the total nuclear JNK pool or the presence of individual JNK isoforms.

4.3.2. 6-OHDA- induced death in cortical neurons

The neurotoxin 6-OHDA is a widely used experimental stimulus for the death of (dopaminergic) neurons in vivo and in vitro. It mediates cell death via several different mechanisms as described above (see 4.2).

6-OHDA-induced death in cortical neurons resembles that of PC12 cells. It was recently shown that 6-OHDA caused death in cortical neurons [107, 161]. Cell death was measured by LDH release in the culture. Since cortical cultures include around 15% astrocytes, cell death could also occur in astrocytes. However, staining of the cells with DNA dye Hoechst 33258 showed that only neurons displayed morphological changes characteristic to apoptosis, including fragmentation and condensation of nuclei [150, 151] following 6-OHDA treatment. Indeed cortical neurons were found to be more sensitive to oxidative stress than astrocytes [162].

The induction of apoptosis and the activation of caspase-3 by 6-OHDA has been examined in several studies [108, 128, 129, 163, 164]. In cortical neurons, activation of caspase-3 following 6-OHDA was shown before [161, 165] and the results are is in line with those of.

JNK-mediated neuronal death occurs in response to MPTP or 6-OHDA [54-56]. Inhibition of JNK with SP600125 attenuated death following 6-OHDA treatment in cortical neurons. Inhibition of caspase-3 activation following SP600125 treatment also demonstrates the involvement of JNKs in 6-OHDA-mediated death.

Similar as hippocampal neurons, cortical neurons contain high basal JNK activity either in nuclear or in cytoplasmic extracts. Under basal conditions JNK1 was present in the nucleus,

whereas JNK2 was absent in the nucleus. It was already shown that JNK1 is responsible for the high level of basal JNK activity in the brain [27, 58]. The presence of JNK1, but not JNK2 in the nucleus, could explain the absence of c-Jun activation under basal conditions since JNK2 has a higher affinity for c-Jun than JNK1 [7, 8]. 6-OHDA reduces total JNK activity in the nucleus whereas it slightly induced c-Jun phosphorylation within 1 h. The presence of basal JNK1, the enzyme responsible for the basal nuclear JNK phosphorylation, decreased in the nucleus following 6-OHDA whereas JNK2 translocated into the nucleus and activated c-Jun. The presence of a distinct pool of activated JNK in the nucleus suggests that specific compartments of JNK serve different functions in neurons e.g. activation of JNK3, but not JNK1 or JNK2, in cortical neurons [136] and activation of JNK2/3 in the presence of constitutive JNK1 activity in cereballar granule neurons [27] following stress.

It is generally considered that JNK1 has more physiological and anti-apoptotic roles [60, 87, 166] whereas degenerative apoptotic actions are attributed to JNK2 or JNK3 [30, 118]. However 6-OHDA-induced death in cortical neurons in this study, from wildtype, JNK1 ko and JNK3 ko mice were not significantly different suggesting the two isoforms meditate 6-OHDA-induced death not differently. However, further investigations with neurons from JNK2 ko mice are required to decide which JNK isoform is important in 6-OHDA-induced death in cortical neurons.

4.4. The role of JNKs in neurite outgrowth

The family of JNKs has also been implicated in neuronal differentiation and neurodegeneration [29, 34, 57, 58], reviewed by [160, 167]. Several studies have shown an enhanced JNK activation in response to NGF [168, 169] or a decrease in neurite outgrowth following inhibition of JNKs or their upstream kinases [170-172]. Inhibition of total JNK activity by SP600125 almost completely blocked neurite outgrowth and elongation following NGF [94]. JNK inhibition by SP600125 and (D)-JNKI1 dramatically reduced axonal outgrowth in explanted or dissociated ganglia [66]. In primary cultures of dopaminergic neurons, inhibition of JNKs suppressed the total extent of neurites, the primary neurite length and the number of neurites per cell [173]. Similarly, inhibition of JNKs by SP600125 prevented neurite elongation in primary hippocampal neurons in this study. Interestingly, inhibition of JNK by SP600125 did not influence outgrowth of cortical neurons. Some time ago, inhibition of JNK activity by SP600125 was reported to increase neurite outgrowth in

embryonic cerebellar neurons [26]. Thus, JNK exerts different roles in neurite outgrowth *ex vivo* depending on the neuronal phenotype and/or the stage of differentiation. In consequence, the function of JNKs has to be defined for each individual type of neurons and conditions.

During differentiation of hippocampal neurons, total levels of JNK continuously increased up to 6 days, the end of the observation period mainly by elevation of JNK1 and JNK2. In contrast, a very high activation of c-Jun was seen during the early state of differentiation (on DIV 2), however, this activation was reduced at DIV 3. The relatively increased activation of total JNK and the reduced c-Jun activation during differentiation are in agreement with the result of an other study [26]. Increased activation of JNK occurred downstream of activation of MKK7, suggesting an involvement of MKK7/JNK pathway during differentiation rather than MKK4/JNK pathway [26]. It was shown in PC12 cells that JNK2, but not JNK1, was activated above basal levels during NGF-induced neurite regrowth after injury. Transfection of PC12 cells with dnJNK2 reduced neurite outgrowth [94] and also reduced neurite regrowth after injury [174]. Otherwise, treatment of SH-SY5Y cells with retinoic acid induced a strong activation of JNK1 and transfection of dominant-negative SEK-1 (an upstream kinase of JNK1) into the same cells, inhibited retinoic acid induced neurite outgrowth [175] indicating that the effect of JNK isoforms on neurite outgrowth may be cell dependent.

What is the difference between the growth-activities of JNKs in the SP600125-sensitive neonatal hippocampal neurons and SP600125-insensitive cortical neurons? In both cell types, the explantation induced a similar strong and ongoing JNK activation and a moderate increase in total JNK. Thus, the mere activation of JNKs in whole cell extracts is not indicative for its contribution to regrowth. However, the pool of activated c-Jun transcription factor was distinctly smaller in the SP600125-insensitive cortical neurons as compared with the SP600125-sensitive hippocampal neurons. This minor pool of activated c-Jun might underline the independence of JNK since c-Jun is imperative for the regeneration of axons *in vivo* [70] and can be linked to outgrowth of axons, dendrites and neurites [57, 69, 176-178].

4.5. Conclusions

The function of JNK in apoptosis is complex, since JNKs mediate differentiation and apoptosis. It is clear that JNK or at least one of the isoforms activation can contribute to apoptosis induced by certain death insults. A role for JNK in the cell survival signal has also been predicted. The diversity of the roles of JNK in the regulation of cell fate most likely depends on the initiate signalling cascade and/or the protein pattern expressed by JNK.

The present thesis has provided evidence that JNK2, but not JNK1, is a central mediator of cytochrome c release following 6-OHDA-induced death of PC12 cells. JNK2 translocates to the nucleus and the mitochondria where it acts downstream of MKK4. Thus, the difference in activation and translocation of JNK isoforms suggest the existence of separate apoptotic signalosomes. Moreover, this study shows that JNKs are important mediators of neuronal death following excitotoxic stimulus in primary neurons. This thesis also demonstrates that JNKs have physiological roles besides degenerative roles, and they are important for the differentiation of the neurons.

These findings have considerable implications for therapeutic strategies targeting JNK signalling in neurodegenerative diseases. Inhibition of JNK may decrease apoptosis which is contributed to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, however, it could also inhibit the cell-cycle arrest, so might limit the use of antagonists as a novel strategy for the therapeutic benefit.

5. Summary: Functions of JNK stresskinases in neuronal apoptosis and differentiation

The c-Jun N-terminal kinases (JNKs), a subfamily of the mitogen-activated protein kinases (MAPKs), are considered as essential signalling molecules for neurodegeneration in the mammalian brain. However, they also have physiological functions such as in development, differentiation, proliferation and neuroregeneration. The aims of the present study were to determine the physiological and pathological roles of JNK isoforms in PC12 cells and in primary neurons.

6-Hydroxy-dopamine (6-OHDA) causes death of dopaminergic neurons by mitochondrial dysfunction with JNKs as central mediators. The first aim of this study was to analyze the contribution of different JNK isoforms to 6-OHDA-induced death of PC12 cells. 6-OHDA enhanced total JNK activity in the cytoplasm, nucleus and at the mitochondria. Inhibition of JNKs by SP600125 or transfection with dominant negative JNK2 (dnJNK2) substantially attenuated 6-OHDA-induced cell death, whereas transfection with dominant negative JNK1 (dnJNK1) had no protective effect. Following 6-OHDA, JNK2 translocated to the nucleus and to the mitochondria, while JNK1 was constitutively present in the nucleus and at the mitochondria. JNK inhibition by SP600125 or transfection with dnJNK2 reduced the pool of active JNKs in the nucleus, the release of cytochrome c, as well as the cleavage of caspase-3 and its substrate PARP-1. However, transfection with dnJNK1 had no effect on the release of cytochrome c or cleavage of caspase-3 and PARP-1. JNK3 is considered as a stress-specific kinase and is not endogenously expressed in PC12 cells. Surprisingly, transfected JNK3 did not enhance cell death induced by 6-OHDA. Interestingly, JNK3 which was present in nucleus was not activated in nuclear extracts while JNK1 and JNK2 were activated. These results suggest that from all JNK isoforms, JNK2 in particular is associated with 6-OHDA-induced death in PC12 cells. In contrast to naïve PC12 cells, 25 μM and 50 μM 6-OHDA did not induce any cell death in NGF-differentiated PC12 cells. However, inhibition of JNKs with SP600125 induced death either in unstimulated or stimulated differentiated PC12 cells suggesting that JNKs have different functions in differentiated PC12 cells than in naïve cells.

To consider specific neuronal conditions, it was necessary to study with primary neurons. Therefore, the second aim of this study was to determine and compare the role of JNKs in primary hippocampal and cortical neurons following excitotoxic stimulus. Glutamate and

6-OHDA induced death and caspase-3 activation in primary hippocampal and cortical neurons, respectively. Inhibition of JNKs with SP600125 rescued these otherwise dying cells and inhibited caspase-3 activation indicating JNK-mediated death. Western blotting and immunocytochemical experiments revealed that there was a high basal JNK activity in primary hippocampal and cortical neurons which is in contrast with PC12 cells. Following glutamate and 6-OHDA this basal JNK activation declined from nucleus in hippocampal neurons and cortical neurons, respectively. However, stimulus induced c-Jun phosphorylation in these cells.

Besides neurodegenerative and pro-apoptotic functions, the family of JNKs has also been implicated in neuronal differentiation and development. The analysis of the role of JNKs in neuronal differentiation represented the third aim of this study. Hippocampal and cortical cultures were exposed to JNK-inhibitor, SP600125, at the 2nd, 3rd and 5th day in vitro. Inhibition of JNKs by SP600125 prevented neurite elongation in primary hippocampal neurons in this study, whereas cortical neurons were not affected. Increased activation of total JNK during differentiation of hippocampal neurons indicated the importance of JNKs in neuronal development.

Summarizing, these results provide evidence that JNKs have apoptotic effects after an excitotoxic stimulus but in parallel to their apoptotic effects they have also physiological functions on neurite formation and elongation in vitro during neuronal differentiation.

6. Zusammenfassung: Funktionen von c-Jun N-terminalen Stresskinasen in der neuronalen Apoptose und Differenzierung

Die c-Jun N-terminalen Kinasen (JNK), eine Familie der Mitogen-aktivierten Protein Kinasen (MAPK), sind nicht nur wichtige Signalmoleküle der Neurodegeneration im Säugergehirn, sondern sie besitzen auch bedeutende Funktionen in der Entwicklung, Differenzierung und Neuroregeneration. Das Ziel der vorliegenden Arbeit war es, physiologische und pathologische Funktionen der JNK in PC12-Zellen und primären Neuronen in verschiedenen experimentellen Bedingungen zu klären.

6-Hydroxydopamin (6-OHDA) verursacht Zelltod von dopaminergen Neuronen durch mitochondriale Dysfunktion, bei der JNK zentrale Signalmoleküle sind. Die erste Zielsetzung der vorliegenden Arbeit war es, den spezifischen Beitrag der JNK Isoformen beim 6-OHDA-induzierten Zelltod zu analysieren. 6-OHDA verstärkte die Gesamtaktivität der JNKs im Cytoplasma, im Kern und an den Mitochondrien. Der Einsatz der JNK-Hemmstoffe SP600125 oder die Transfektion mit dominant-negativem JNK2 (dnJNK2) verringerte signifikant den Zelltod, während die Transfektion mit dominant-negativem JNK1 (dnJNK1) keinen protektiven Effekt hatte. Nach der Gabe von 6-OHDA verschob JNK2 sowohl in den Kern als auch an die Mitochondrien, JNK1 hingegen war in beiden Kompartimenten konstitutiv präsent. Die Hemmung der JNKs durch SP600125 oder die Transfektion mit dnJNK2 reduzierte außerdem die Menge an phosphorylierten nukleären JNKs, die Freisetzung von Cytochrom c und die Spaltung von Caspase-3 sowie PARP-1. Die Transfektion mit dnJNK1 jedoch hatte keinen Einfluss auf die untersuchten Apoptosemarker. Die generell mit Zellstress assoziierte Isoform JNK3, die nicht endogen in PC12-Zellen exprimiert wird, verstärkte überraschenderweise nicht den 6-OHDA-induzierten Zelltod in diesen Zellen nach Transfektion. Interessanterweise, wurde JNK3 aber in den Kernfraktionen nicht aktiviert, während JNK1 und JNK2 aktiviert wurden. Diese Ergebnisse weisen darauf hin, dass insbesondere JNK2 den Zelltod nach 6-OHDA vermittelt. Im Gegensatz zu naiven PC12-Zellen, konnten unterschiedliche Dosierungen 6-OHDA (25 µM, 50 µM) keinen Zelltod in NGF-differenzierten PC12-Zellen hervorrufen. Allerdings verursachte die JNK-Hemmung mit SP600125 Zelltod in stimulierten und unstimulierten Zellen, was vermuten lässt, dass die JNKs in differenzierten Zellen andere, eher physiologische Funktionen haben als in naiven Zellen.

Um die spezifische neuronale Bedingung zu beacten, war es notwendig, für die weiteren Analysen primäre neuronale Zellen aus dem Hippocampus und dem Kortex zu verwenden. Das zweite Ziel dieser Arbeit war es, die Rolle von JNKs nach exzitotoxischer Stimulation in primären Neuronen zu untersuchen. Dabei riefen Glutamat und 6-OHDA gleichermaßen Zelltod und eine Aktivierung von Caspase-3 hervor. JNK-Hemmmung durch SP600125 hatte einen protektiven Effekt und verhinderte die Aktivierung von Caspase-3. Insgesamt zeigten sowohl Western blots als auch immunozytochemische Färbungen eine hohe Basalaktivierung der JNKs in hippocampalen und kortikalen Neuronen im Vergleich zu PC12-Zellen. Die Stimulation reduzierte die nukleäre Basalaktivität der JNK in hippocampalen und kortikalen Zellen. Allerdings führten beide Stimuli zu einer Phosphorylierung von c-Jun in hippocampalen und kortikalen Zellen.

Neben neurodegenerativem und pro-apoptotischem Potential besitzen die JNKs durchaus eine Bedeutung für die neuronale Differenzierung und Entwicklung. Die Analyse der JNK-Funktionen in der Differenzierung von Neuronen war das dritte Ziel dieser Arbeit. Dabei konnte in hippocampalen Neuronen eine steigende JNK-Aktivierung im Laufe der Kultivierung festgestellt werden. Wurden hippocampale und kortikale Zellen mit dem JNK-Hemmstoff SP600125 am zweiten, dritten und fünften Tag ihrer Kultivierung behandelt, verhinderte die inhibierte JNK-Aktivität das Auswachsen der Neuriten von primären hippocampalen Zellen, während kortikale Neurone sich normal differenzierten.

Zusammenfassend zeigen diese Ergebnisse, dass individuelle JNK Isoformen im Rahmen von Exzitotoxizität pro-apoptotische Signale vermitteln, jedoch auch physiologische Funktionen bei der Neuritogenese in einen Teil der Neuronen wahrnehmen.

7. References

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8. Appendix

8.1. Abbreviations and symbols

A	adenine
ABC	avidin-biotin-complex
AP-1	activator protein-1
ApoER2	apolipoprotein E receptor-2
APS	ammonium persulphate
AraC	cytosine arabinoside (arabinofuranosylcytosine)
ASK1	apoptosis signal-regulating kinase-1
ATF-2	activating transcription factor-2
ATP	adenosine triphosphate
Aß	Beta-amyloid
A_{260}, A_{280}, A_{595}	absorbance at 260 nm, 280 nm or 595 nm, respectively
A_{260}/A_{280}	ratio between the absorbance values at 260 and 280 nm
α	anti- (*e.g.* α-rabbit = anti-rabbit antibody)
Bcl-2	B cell lymphoma-2
Bax	Bcl associated x protein
Bad	BAD, Bcl2/Bcl-xl-antagonist, causing cell death
Bak	Bcl-2 homologous antagonist/ killer
Bid	BH3 interacting death domain agonist
Bim	Bcl-2 Interacting Mediator of Cell Death
bp	base pairs
BSA	bovine serum albumin (albumin fraction V)
cAMP	cyclic adenosine monophospate
caspase	cysteine aspartate-specific protease
cDNA	complementary DNA
cdc42	cell division cycle 42
CD 95	APO-1, Fas
CEP 11004/1347	Cephalon compound
c-Fos	v-fos FBJ murine osteosarcoma viral oncogene homologue
c-Jun	v-jun avian sarcoma virus 17 oncogene homologue
cm	centimeter

c-Myc	v-myc avian myelocytomatosis viral oncogene homologue
CNS	central nervous system
CREB	cyclic adenosine monophospate response element binding protein
d	day(s)
DAB	diamino-benzidine
DDW	double-distilled water
DEPC	diethyl pyrocarbonate
DEPC-DDW	diethyl pyrocarbonate-treated double-distilled water
Diablo	direct IAP binding protein with low pI
DLB	denaturing lysis buffer
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphate
Dp5	neuronal death protein 5
DIV	day *in vitro*
dn	dominant negative
dT	2'-deoxythymidine-5'-triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethylene-bis(oxyethylenenitrilo)tetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
Elk-1	Ets-like gene-1
ERK	extracellular signal-regulated kinase
Fas	fibroblast associated antigen
FCS	fetal calf serum
Fig.	Figure
FITC	Fluorescein-isothiocyanat
g	gram(s)
$\times g$	relative centrifugal force (RCF)
G	guanine
GAP-43	growth-associated protein-43

GFAP	glial fibrially acidic protein
grp-75	glucose regulated protein 75
G protein	guanosine triphosphate binding protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
h	hour(s)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
H2A.z	histone 2A variant z
HS	horse serum
ICE	interleukin-1β converting enzyme (caspase-1)
Ig	immunoglobulin (*e.g.* IgG)
IKAP	inhibitor of NF-κB kinase complex-associated protein
IL	interleukin (*e.g.* IL-1)
imPT	mitochondrial inner-membrane permeability transition
IR	immunoreactivity
IU	international unit(s)
J	Joule
JDP	Jun-dimerizing partners
JIP	c-Jun N-terminal kinase-interacting protein
JNK	c-Jun N-terminal kinase
JNKK	c-Jun N-terminal kinase kinase
JSAP1	Jun N-terminal protein kinase / stress-activated protein kinase-associated protein-1
kb	kilobase
kDa	kilodalton
ko	knock out
l	liter(s)
LDH	lactate dehydrogenase
LFU	laminar flow unit
M	molar (mol/L)
mA	milliamper
MADD	MAP kinase activating death domain protein
MAP	microtubule-associated protein
MAP	mitogen-activated protein

MAPK	mitogen-activated protein kinase
MAPKAPK	mitogen-activated protein kinase-activated protein kinase
MAP2K	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MEF2C	myocyte enhancer factor-2C
MEK	mitogen-activated protein / extracellular signal-regulated kinase kinase
MEKK	mitogen-activated protein / extracellular signal-regulated kinase kinase kinase
MEM	minimum essential medium
mg	milligram(s)
min	minute(s)
MKK	mitogen-activated protein kinase kinase
MKP	mitogen-activated protein kinase phosphatase (*e.g.* MKP-1)
ml	milliliter(s)
MLK	mixed-lineage kinase
mm	millimeter
mM	millimolar (mmol/L)
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
mV	millivolt(s)
μg	microgram(s)
μl	microliter(s)
μm	micrometer(s)
μM	micromolar (μmol/L)
n	number of independent experiments per experimental series
NFAT	nuclear factor of activated T cells
ng	nanogram(s)
NGF	nerve growth factor
nm	nanometer(s)
N-terminal	aminoterminal
p. a.	*pro analysis*
PAGE	polyacrylamide gel electrophoresis
PARP-1	poly(ADP-ribose) polymerase-1
PBS	phosphate-buffered saline
PBST	PBS with Triton X-100

PCR	polymerase chain reaction
PD	Parkinson's disease
pH	potentia hydrogenii (hydrogen ion concentration)
PI3-K	phosphoinositide-3-kinase
PKC	protein kinase C
PMSF	phenyl-methyl sulfonyl fluoride
PNS	peripheral nervous system
Pro	proline
PVDF	polyvinylidene difluoride
p75NTR	p75 (75 kDa) neurotrophin (NGF) receptor
Rac	Ras-related C3 botulinum toxin substrate
Raf	v-raf murine sarcoma 3611 viral oncogene homologue
Ras	rat sarcoma viral oncogene homologue
RCF	relative centrifugal force ($\times g$)
r_{max}	maximum radius (centrifuge parameter)
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rotations per minute (centrifuge parameter)
RPMI-1640	Roswell Park Memorial Institute culture medium 1640
r.t.	room temperature (ca. 20°C)
RT	reverse transcription / reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
s	second(s)
SAPK	stress-activated protein kinase
SB 203580	SmithKline Beecham 203580
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEK	JNK kinase
Ser	serine
Smac	second mitochondria-derived activator of caspase
SP600125	anthra(1,9-cd)pyrazol-6(2H)-one
STAT	signal transducer and activator of transcription
T	thymine
TAK1	transforming growth factor-β-activated kinase-1
Taq	*Thermus aquaticus*

TBE	tris-boric acid-EDTA buffer
TBS	tris-buffered saline
TBST	TBS with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
Thr	threonine
T_M	melting / annealing temperature of primers
TNFα	tumour necrosis factor alpha
Tpl-2	tumour progression locus-2
Tris	tris-(hydroxymethyl)-aminomethane
TrkA	tyrosine receptor kinase A (NGF receptor)
Tyr	tyrosine
U	unit (of enzyme activity)
UV	ultraviolet (light)
v/v	volume per volume
w/o	without
w/v	weight per volume
XIAP	X-linked inhibitor of apoptosis
6-OHDA	6-hydroxydopamine

8.2. Index of figures

8.3. Index of tables

9. Curriculum vitae

PERSONAL INFORMATION

Family name:	Eminel
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10. Declaration (Erklärung)

Apart from the advice of my supervisors, this thesis is my own work. No part of it has been submitted to any other board for another qualification. Parts of the results have already been published in peer reviewed journals and abstract form (see below).

Hiermit erkläre ich, dass diese Dissertation – abgesehen von der Beratung durch meine akademischen Lehrer – nach Inhalt und Form meine eigene Arbeit ist. Sie hat weder im Ganzen noch zum Teil an anderer Stelle im Rahmen eines Promotionsverfahrens vorgelegen. Ein Teil der Ergebnisse dieser Arbeit wurde bereits in Form von Veröffentlichungen und Kongressbeiträgen pupliziert (siehe unten).

Kiel,

Sevgi Eminel

11. Publication list

Peer review publication

Eminel S, Roemer L, Waetzig V and Herdegen T.
c-Jun N-terminal kinases trigger both degeneration and neurite outgrowth in primary hippocampal and cortical neurons.
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* shared first authorship.

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CONGRESS ABSTRACTS

Eminel S, Klettner A, Roemer L, Waetzig V, Herdegen T.
JNK2 translocates to the mitochondria and mediates cytochrome c release in PC12 cells following 6-OHDA.
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