Research Report

Effect of 3,4-methylenedioxyamphetamine on dendritic spine dynamics in rat neocortical neurons — Involvement of heat shock protein 27

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ABSTRACT

Along with chronic neurotoxic effects, the long-term consumption of amphetamines has been associated to psychiatric symptoms and memory disturbances. Dendritic spine dynamics have been discussed as a possible morphological correlate. However, the underlying mechanisms are still elusive. 3,4-Methylenedioxyamphetamine (MDA), a major drug of abuse and a main metabolite after 3,4-methylenedioxymethamphetamine (MDMA) intake, provokes a loss of dendritic spine-like protrusions in primary cultures of rat cortical neurons. 3,4-Methylenedioxyamphetamine also induced a rapid activation of the p38 mitogen activated protein kinase (p38 MAPK) pathway and phosphorylation of heat shock protein 27 (hsp27) indicative for its decreased chaperone activity. Concurrent pharmacological inhibition of the p38 MAPK by SB203580 abolished hsp27 phosphorylation and diminished the loss of dendritic spine-like protrusions. Moreover, upon MDA treatment dendritic spine-like protrusions were stabilized in neurons constitutively expressing hsp27. In parallel experiments we observed a robust activation of the heat shock transcription factor 1 (HSF-1) and a subsequent increase of hsp27 and hsp70. The regulation of small heat shock proteins corroborates the existence of a neuronal stress response after MDA treatment. Pharmacological targeting of small heat shock protein phosphorylation may provide a new strategy to preserve spine integrity after amphetamine exposure.

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1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy), methamphetamine (speed), and the MDMA metabolite 3,4-methylenedioxyamphetamine (MDA) are addictive drugs, whose consumption has been increasingly associated to neurological disorders (Rothwell and Grant, 1993; Bitsch et al., 1996) and psychiatric complications, such as psychosis, hallucinations or agitations, and memory disturbances (Karlsen et al., 2008). Neurotoxicity has been demonstrated for a variety of...
Amphetamine derivates and their tested metabolites, with graduations in their toxicity profile (Capela et al., 2006a, 2007; Cadet et al., 2007; Tata and Yamamoto, 2007). However, the formation of neurotoxic MDMA metabolites differs among species with strong limitations to extrapolate experimental data to the human situation (de la Torre and Farre, 2004; de la Torre et al., 2009). In rats, N-demethylation of MDMA to MDA is one of the main metabolic pathways (Chu et al., 1996). 3,4-Methylenedioxyamphetamine and its metabolites mainly affect the monoaminergic system characterized by a long-term degeneration of serotonergic axon terminals (O’Hearn et al., 1988) and decreased brain serotonin concentrations (Miller et al., 1997). Recently, we have demonstrated that monoamine oxidation by monoaminooxidase B with subsequent mitochondrial damage might be an important contributing factor for MDMA-induced neurotoxicity (Alves et al., 2007).

Amphetamine induced structural degeneration of serotonergic and dopaminergic nerve terminals suggests changes of cytoskeletal proteins (O’Hearn et al., 1988; Ricaurte et al., 1982). Abstinence from D-amphetamine self-administration induces the expression of CapZb (Freeman et al., 2005), a protein which decreases actin polymerization by blocking actin barbed ends (Yamashita et al., 2003). In contrast, the expression of the actin assembling proteins ARP3 and coronin 1A were decreased specifically during abstinence (Freeman et al., 2005). In addition, it has been shown that amphetamine sensitized nonhuman primates exhibit reduced dendritic branching and spine densities of pyramidal neurons (Selemon et al., 2007). After 6-hydroxydopamine injection, similar results were obtained in rat matured neurons of the ipsilateral caudo-putamen showing a reduced length of dendrites, neuronal branches, and a reduced density of dendritic spines (Solis et al., 2007).

Morphological changes might be due to direct cytotoxicity or hyperthermia, a common side effect of amphetamines attributed to an interaction between the hypothalamic–pituitary–thyroid axis and the sympathetic nervous system (Sprague et al., 2003). Presumably as a consequence of increased body temperature, liver and brain levels of hsp70 are elevated after amphetamine treatment (Nowak, 1988). Moreover, upregulation of hsp27 and hsp70 in the rat liver in combination with amphetamine pre-treatment protected the rat liver against an acute dose of hepatotoxic drugs probably related to the activation of heat shock proteins (Salminen et al., 1997). Heat shock preconditioning mediated by hsp70 also protected neurons against MDMA toxicity (Escobedo et al., 2007).

This study was conducted to investigate different aspects of the neuronal response to MDA treatment (Fig. 1). In particular, to study the molecular mechanisms involved in the loss of dendritic spines after amphetamine treatment, we investigated the effect of MDA on the dynamics of dendritic spine-like protrusions in primary cultures of cortical neurons. We observed increased levels of phosphorylated hsp27 accompanied with a reduced number of dendritic spine-like protrusions after MDA treatment. In addition, we also found an upregulation of hsp27 and hsp70 protein most likely attributed to a delayed general neuronal stress response.

2. Results

2.1. **MDA provokes delayed cell death in cortical neurons**

Matured cortical neurons are integrated in an intact network with continuous branchings and dendritic spine-like protrusions determined by beta III tubulin immunoreactivity (Fig. 2A). Upon MDA treatment (200 μM), disintegration of the neuronal branches was observed showing fragmented neurites and also a reduction of dendritic arborization (Fig. 2B) earliest at 48 h of treatment. To correlate morphological changes with cell death supernatant culture medium was collected from untreated and MDA (200 μM) treated cultures at the indicated times (Figs. 2C and D). In contrast to

![Fig. 1](image-url)  
**Fig. 1** – Effects of MDA on neocortical neurons. (A) Experimental design to study morphological changes and viability of neurons after MDA treatment. At the indicated times lactate dehydrogenase activity was measured in the supernatant cell culture medium directly correlating with neuronal cell death. (B) Experimental design to study the involvement of hsp27 phosphorylation in the dynamics of dendritic spine-like protrusions. (C) Experimental design to study the expression of heat shock proteins.
other MDMA metabolites (Capela et al., 2006a), MDA applied into the culture medium did not induce cell death within the first 48 h assessed by measurement of LDH release into the supernatant medium. A significant increase of LDH activity was found in cultures stimulated with 200 μM DAF or 96 h (166.0±23.7 U/mL, control: 118.1±31.9 U/mL). Due to limitation of the cell culture system, partial cell death also was observed in PBS treated control cultures at this time. We also tested the viability of cultures treated with 100, 400, 800 μM and 1.6 mM MDA (data not shown). The lower concentration (100 μM) did not affect morphological changes and neuronal viability compared with control treated cultures at this time. We also tested the viability of cultures treated with 100, 400, 800 μM and 1.6 mM MDA (data not shown). The lower concentration (100 μM) did not affect morphological changes and neuronal viability compared with control treated cultures (LDH activity at 96 h 131.5±33.6 U/mL) and cultures exposed to higher concentrations (800 μM and 1600 μM) showed rarefaction of neuronal branches and neuronal death at early time points (6 and 12 h) corresponding with an increase of cell death (i.e. 800 μM at 12 h, 170.8±40.3 U/mL). Therefore, concentrations between 100 and 400 μM were chosen for subsequent experiments.

2.2. Hsp27 is rapidly phosphorylated by the p38 MAPK pathway in MDA treated neurons

Upon cellular stress conditions, the small heat shock protein hsp27 binds to actin filaments and it is involved in stress fiber formation and recovery in a phosphorylation dependent manner regulated by the p38 MAPK (Landry and Huot, 1995; Schneider et al., 1998). Assuming fast p38 MAPK activation neurons were treated with MDA (100–400 μM) for 30 min. Basal phosphorylation was found in vehicle treated neurons which was further elevated by MDA treatment in a concentration-dependent manner (Fig. 3A). MDA did not affect p38 MAPK protein levels at all concentrations tested (Fig. 3A). Results show that p38 MAPK activation might be involved in the early stress response after MDA challenge and mediate hsp27 phosphorylation. As shown in Fig. 3B, increased hsp27 phosphorylation was detected in neurons dependent on the MDA concentration applied. Maximum phosphorylation was found in neurons treated with 400 μM MDA. Total hsp27 protein levels remained stable after MDA treatment (Fig. 3B). In conclusion, results show an immediate activation of the p38 MAPK pathway resulting in hsp27 phosphorylation in response to MDA.

2.3. MDA induced loss of spine-like protrusions is mediated by hsp27 phosphorylation

Hsp27 chaperone activity is strongly diminished by phosphorylation (Rogalla et al., 1999). To study if hsp27 phosphorylation has an impact on the dynamics of dendritic spine-like protrusions, neurons were transfected with a plasmid expressing eGFP-actin and treated with MDA. Different morphologies

Fig. 2 – MDA causes disintegration of neuronal branches and a delayed cell death in neurons. Beta III tubulin immunocytochemistry from neurons treated with vehicle (A) or 200 μM MDA (B) for 48 h. Scale bars 10 μm. (C) Lactate dehydrogenase activity measured in the supernatant medium from (C) vehicle and (D) MDA (200 μM) treated neurons at the indicated time points. Data are presented as means±SD. For statistical analysis multiple comparison (Dunn’s method) was performed after Kruskal–Wallis one-way ANOVA on ranks (p<0.01 vs control).
of dendritic spine-like protrusions (mushroom like, stubby) were observed before stimulation and were included in subsequent analysis. After vehicle treatment, no changes in the number of dendritic spine-like protrusions were observed (contr, Fig. 4A). In contrast, treatment with MDA significantly reduced the number of dendritic spine-like protrusions (Fig. 4A). After 24 h, only blunt ended branches and filopodia like structures were detected on dendrites. These results corroborate our initial findings showing a reduction of neuronal branches at 48 h implicating dendritic spine loss at an earlier time upon MDA exposure.

To evaluate the involvement of p38 MAPK activation on dendritic spine-like protrusion dynamics after MDA challenge we co-applied the specific p38 MAPK inhibitor SB203580 (final concentration 25 μM) (Tong et al., 1997) in the culture medium. As shown in Fig. 4A, dendritic spine-like protrusions were partially preserved in MDA/SB203580 treated neurons. Co-application of SB203580 and MDA abolished hsp27 phosphorylation in parallel cultures suggestive for an involvement of hsp27 phosphorylation in destabilization of dendritic spine-like protrusions (Fig. 4B). Confocal micrographs of individual neuronal branches were captured and the number of spine-like protrusions were counted and calculated per 100 μm length of the dendritic branch. As shown in Fig. 4C, the number of spine-like protrusions was significantly decreased to 5.5 (25th percentile: 0, 75th percentile: 12) after MDA treatment compared to 38.5 spine-like protrusions (25th percentile: 32.75, 75th percentile: 44.25) found in vehicle treated neurons. The number of spine-like protrusions was partially preserved by concurrent treatment with SB203580 (median 22.5; 25th percentile: 17.5, 75th percentile: 30.25). Sole application of SB203580 had no effect on spine density (median 38.5; 25th percentile: 33.0, 75th percentile: 46.0). Together, data confirm a possible involvement of hsp27 phosphorylation in dendritic spine stability following MDA treatment. To test if constitutive overexpression of hsp27 may stabilize dendritic spine-like protrusions neurons were transfected with an eGFP-HA27 construct expressing a functional active eGFP-hsp27 fusion protein (Williams et al., 2006) and treated with 200 μM MDA for 24 h. Dendritic spine-like protrusions were partially preserved in transfected neurons (median 22.5; 25th percentile: 17.5, 75th percentile: 30.25, vehicle treated cultures: median 38.5, 25th percentile: 32.75, 75th percentile: 44.25) (Fig. 4C). From these experiments we conclude that hsp27 contributes in stabilization of dendritic spine-like protrusions after MDA treatment.

2.4. MDA induces a delayed heat shock response in cortical neurons

Amphetamines induce an acute increase in core body temperature in vivo accompanied by a heat shock response (Miller et al., 1991). However, the neuronal heat shock response strongly depends on the type of neuron and the experimental model (Batulan et al., 2003; Sato and Matsuki, 2002; Lowenstein et al., 1991). We studied if MDA directly affects the expression of small
heat shock proteins in cortical neurons. Activation of the transcription factor HSF-1 was observed 60 min after MDA application. Compared to control treated cells, HSF-1 was activated in neurons stimulated with a concentration of 100 μM (Fig. 5A) and was further elevated in neurons stimulated with 400 μM (Fig. 5A). Co-application of an unspecific or specific competitor as well as an antibody against HSF-1 determined the specificity of HSF-1 binding activity. While HSF-1 binding was partially inhibited by the unspecific competitor, the specific competitor abolished HSF-1 binding (Fig. 5A). Addition of an anti-HSF-1 antibody together with an unspecific competitor further diminished the specific HSF-1 signal compared to the unspecific competitor alone (Fig. 5A). The levels of hsp27 and hsp70 were evaluated in neurons stimulated with different MDA concentrations (100–400 μM, Fig. 5B). After 24 h, baseline expression of hsp70 was found in vehicle (saline) treated cells. Treatment with MDA (100–400 μM) showed a concentration-dependent increase of hsp70 level (Fig. 5B). Similarly, the hsp27 level was upregulated at 24 h after MDA treatment (Fig. 5B). Together, results support the notion of a reactive upregulation of small heat shock proteins which may be involved in neuronal stress mechanisms.

3. Discussion

The present study shows that neuronal heat shock stress response is correlated with changes of dendritic spine-like protrusions, both affected by MDA. Moreover, we show that hsp27 undergoes fast phosphorylation by p38 MAPK activation and, thereby, it might be involved in loss of dendritic spine-like protrusions after MDA treatment. The stress response is accompanied by the activation of the transcription factor HSF-1 and a delayed increase of hsp27 and hsp70 protein levels in cortical neurons.

For the present study, MDA was chosen for the following reasons: 1. It causes direct toxic effects. 2. MDA represents one of the major metabolites of MDMA generated via the CYP450 1A2 and 2D6 system (Maurer et al., 2000). 3. N-demethylation of MDMA to MDA is the main metabolic pathway in rats (Chu et al., 1996). We found that MDA induces morphological changes in cortical neurons. In contrast, MDA did not provoke a fast neuronal cell death as observed for other amphetamine metabolites (Capela et al., 2006a,b). Neurotoxicity might rely on a necessary O-demethylation of the drug to obtain the more toxic α-methyldopamine by CYP2D6 (Capela et al., 2006a). However, MDA metabolism remains complex and achieving higher levels of neurotoxicity may need further conversion into more potent metabolites by oxidation to the corresponding ortho-quinones, alkylation of ortho-quinones and their conjugation with glutathione (Erives et al., 2007). Hence, we have found a decrease of glutathione (GSH, reduced form) in cortical neurons after MDA application supporting the hypothesis that MDA toxicity is mediated by the conversion of α-methyldopamine-O-quinone to 5-(glutathion-S-yl)-α-methyldopamine in neurons. Our results also confirm previous findings showing that amphetamines induce cell death not only in dopaminergic and serotonergic neurons (Lotharius and O’Malley, 2001; Ricaurte et al., 2000) but also in neocortical neurons (Schmued, 2003; Stumm et al., 1999). Nevertheless, MDA neurotoxicity is accompanied by substantial morphological changes in neurons and may involve loss of dendritic spine-like protrusions and dendritic branches.
Our study shows a reduction of dendritic spine-like protrusions after MDA treatment. These changes may reflect an early response of neurons in culture and the immediate response of prefrontal cortical pyramidal neurons in the brain is persistent for weeks after amphetamine sensitization (Selemon et al., 2007). Our results are not in contradiction to previous studies showing an increase, a decrease or no changes of dendritic spines months after repetitive amphetamine injections (Robinson and Kolb, 1999; Crombag et al., 2005). Long-term and persistent changes of dendritic spines are strongly dependent on the experimental model, the type of neuron and brain region (Robinson and Kolb, 2004). Thus, amphetamine sensitization might contain an early reduction and a secondary formation of new dendritic spines.

We found a robust concentration-dependent phosphorylation of the p38 MAPK after MDA treatment. The p38 MAPK is involved in various cellular stress conditions upon a wide range of triggers (Cowan and Storey, 2003; Waxman and Lynch, 2005; Hu et al., 2009).

To block p38 MAPK activity we used the specific inhibitor SB203580 with an IC50 for the SAPK2a/p38 and SAPK2b/p38β in the range of 50 to 500 nM. Those values were obtained in in vitro systems using purified enzymes (Eyers et al., 1998) and further in vitro studies have shown that 10 μM SB203580 leads to a specific reduction of p38 MAPK activity without affecting the activity of other kinases (Davies et al., 2000). The use of SB203580 in more complex cell culture systems requires a higher concentration since the compound has to penetrate biological membranes. In addition, a portion of SB203580 may bind unspecifically to proteins (i.e. albumin) in the cell culture medium without a biological effect. Thereby only free SB203580 can enter the cell. We performed the experiments with a SB203580 concentration that was used successfully in a variety of studies (i.e. Guay et al., 1997; Junger et al., 2003; Stringaris et al., 2002). Importantly, the more complex a cell culture system the higher the concentration of SB203580 which is necessary to achieve inhibition of the p38 MAPK (Ohnishi et al., 2010; Greenwood and Bushell, 2010). Therefore, the concentration of 25 μM for SB203580 was also chosen to assure that the p38 MAPK is sufficiently inhibited to achieve reduced phosphorylation of hsp27 without effects on the activity of other kinases and cell death.

Although hsp27 contains more than one phosphorylation site we have studied only phosphorylation at Ser82. Hence, once the protein is phosphorylated at only one phosphorylation site, all three serine residues are phosphorylated (Ser15,
Ser78 and Ser82) resulting in a decreased chaperone activity (Rogalla et al., 1999). Indeed, we found a significant loss of dendritic spine-like protrusions suggestive of a decreased chaperone activity. Our findings are in line with studies showing that actin filaments are modulated by hsp27 through activation of the p38 MAPK pathway (Landry and Huot, 1995).

Activated hsp27 acts as a capping protein limiting actin stress fiber and subsequent focal adhesion formation (Schneider et al., 1998). Thus, phosphorylated hsp27 binds to denatured actin monomers or short oligomers dissociated from actin filaments. Thereby, it protects these molecules from aggregation by forming small and highly soluble complexes (Pivovarova et al., 2007). We also observed small aggregations of eGFP-actin in MDA stimulated neurons (data not shown). However, further studies are needed to clarify the exact kinetics of intracellular actin aggregation and disaggregation in neurons upon MDA treatment.

We found that dendritic spine-like protrusions were preserved by specific inhibition of the p38 MAPK in MDA treated neurons. This result suggests an involvement of the p38 MAPK pathway in the arrangement of actin filaments. Hence, the regulation of the p38 MAPK and hsp27 are essential components in mechanisms of neurite outgrowth and formation and/or retraction of dendritic spines (Williams et al., 2006; Yasaki et al., 2007). Moreover, preservation of dendritic spine-like protrusions in neurons constitutively expressing hsp27 also corroborates the notion that hsp27 is involved in maintenance of spine integrity.

Amphetamine and methamphetamine induced stress response further involves the delayed de-novo synthesis of small heat shock proteins in neurons (Escobedo et al., 2007). However, the neuronal heat shock response strongly depends on the type of neuron and the experimental model (Batulan et al., 2003; Sato and Matsuki, 2002; Lowenstein et al., 1991). We, therefore, aimed at evaluating if MDA directly induces a heat shock response in cortical neurons. We observed a rapid and specific increase of HSF-1 DNA binding activity in MDA treated neurons. This finding is in contrast to previous studies showing that HSF-1 is mainly activated in glial cells of the neocortex (Batulan et al., 2003; Marcuccilli et al., 1996). Our data corroborate an HSF-1 in cortical neurons. However, the discrepancies between our and previous studies might be caused by the type of treatment and different experimental conditions. As proposed previously, a paracrine transmission of heat shock proteins by astroglia has to be elucidated, but seems unlikely (Batulan et al., 2003) since our cultures contain less than 10% astroglial cells (Batulan et al., 2002). Concomitantly, we observed a delayed upregulation of hsp27 and hsp70 after MDA treatment strengthens a neuronal expression.

In conclusion, we show that hsp27 is involved in dendritic spine-like protrusion stability after MDA treatment. Our results suggest that hsp27 chaperone activity is markedly diminished by phosphorylation and thereby it may contribute to a reduction of dendritic spine-like protrusions. Delayed upregulation of hsp27 and hsp70 might be integrated in a general neuronal stress response upon MDA treatment. Pharmacological targeting of small heat shock protein activation may provide a new strategy to maintain dendritic spine integrity to overcome amphetamine associated long-term complications.

4. Experimental procedures

4.1. Cortical neuronal cell cultures

All experiments were carried out with the approval of the Malmö-Lund ethical committee. Primary cultures of neocortical neurons were obtained from embryos (E16–E18) of Wistar rats (Harlan, The Netherlands). Cultures were prepared as described previously (Ruscher et al., 2002). Cortices were dissected, meninges were removed and tissue was incubated for 15 min in trypsin/EDTA (0.05/0.02% w/v in PBS) at 37 °C, rinsed twice with PBS and once with dissociation medium (modified Eagle's medium with 10% fetal calf serum, 44 mM glucose, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, 100 IE insulin/L), dissociated by Pasteur pipette in dissociation medium, pelleted by centrifugation (210 × g for 2 min at 21 °C), resuspended in starting medium (Neurobasal medium™ supplemented with B27™ (further referred as NBM), Invitrogen, Paisley, UK, 100 U/mL penicillin/streptomycin, 0.5 mM L-glutamine, 25 μM glutamate), and plated in 24-well or 6-well plates in a density of 200,000 cells per square centimeter. Wells were pretreated by incubation with poly-L-lysine (0.5% w/v in PBS) for 1 h at room temperature, then rinsed with PBS, followed by incubation with coating medium (dissociation medium with 0.03% w/v collagen G) for 1 h at 37 °C, rinsed twice with PBS before cells were seeded out in starting medium. Cultures were kept at 36.5 °C and 5% CO2 and fed beginning from the 4th day in vitro (div) with cultivating medium (starter medium without glutamate) by replacing half of the medium twice a week. The cultures were used for experiments after 8 div containing less than 10% astroglial cells.

4.2. Treatment protocols

 Matured neurons were treated with 100, 200, or 400 μM of MDA (Sigma-Aldrich, Munich, Germany) for different intervals as indicated in the figures Capela et al., 2006a. Stock solutions were prepared in NBM and were used as 1:100 dilutions in all experiments. For control experiments, the same volume of NBM (referred to as vehicle) was added to the culture medium. The specific p38 MAPK inhibitor SB203580 (Tocris Bioscience, Avonmouth, UK) was dissolved in DMSO and was used as 1:100 dilutions in a final concentration of 25 μM.

4.3. Neuronal transfection

Neurons were plated on poly-L-lysine coated 13 mm2 glass coverslips in a density of 200,000 cells/cm2. After 5 div, cells were transfected with 1 μg pCAG-EGFPActin-IP vector DNA (kindly provided by Dr. Hitoshi Niwa) expressing an eGFP-actin fusion protein or with 1 μg pIRE2-eGFP-Ha27 (kindly provided by Dr. K. Maerow) (Williams et al., 2006) expressing an eGFP-hsp27 fusion protein using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, USA), according to the directions of the manufacturer. Forty-eight hours later, cells were stimulated with MDA as indicated in the figure legends and fixed with 4% PFA for 10 min. Micrographs were taken using a Zeiss LSM510 confocal laser scanning microscope system (Zeiss, Jena, Germany). Transfection experiments were performed in triplicate out of three independent experiments.
4.4. Fluorescent gel shift assays

Nuclear extracts were prepared as described previously (Ruscher et al., 2000). One picomole of Cy5 labeled specific probe was incubated with 25 μg protein of nuclear extracts in binding buffer BBN (20 mM HEPES, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 25 ng poly(dI)-poly(dC), 10% glycerol) for 60 min on ice. The following Cy5 labeled specific double-stranded probe was used for HSF-1 gel shift assays: 5′-Cy5-GATCTCGAAATGTTCCGAAAG-3′. Specificity was confirmed by addition of a 50-fold excess of either unlabeled specific competitor (specific probe without Cy5 labeling) or unlabeled nonspecific competitor (5′-GAGCTATGAGTCAGTCCA-3′). For HSF-1 supershifts, nuclear extracts were incubated with a monoclonal antibody against HSF-1 (Santa Cruz Biotechnology, Freiburg, Germany) for 15 min at room temperature prior to addition of the Cy5 conjugated specific probe. Ten microliters of the mixture were separated on a native 5% polyacrylamide gel at 4 °C in 1×TBE (90 mM Tris borate, 2 mM EDTA, pH 8.3) using an external temperature regulated ALF-Express™ DNA-sequencer. Gels were analyzed directly by ALFwin™ and Allelix™ software (GE Healthcare, Amersham, Freiburg, Germany).

4.5. Western blotting

Cells were harvested in cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin, 1 mM PMSF). After incubation on ice for 15 min and centrifugation at 18,000 × g at 4 °C for 10 min, whole protein concentrations were determined by the BCA assay (Pierce, IL) using bovine serum albumin as a standard. Cell lysates were diluted in SDS sample buffer (final concentrations: 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromphenol blue) and the mixture was boiled for 5 min. Thirty micrograms of protein were separated on a 10% SDS polyacrylamide gel. Blocking was performed onto polyvinylidifluoride membranes using blocking buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin, 1 mM PMSF). After incubation on ice for 15 min and centrifugation at 18,000 × g at 4 °C for 10 min, whole protein concentrations were determined by the BCA assay (Pierce, IL) using bovine serum albumin as a standard. Cell lysates were diluted in SDS sample buffer (final concentrations: 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromphenol blue) and the mixture was boiled for 5 min. Thirty micrograms of protein were separated on a 10% SDS polyacrylamide gel. Blocking was performed onto polyvinylidifluoride membranes using blocking buffer (20 mM Tris, 136 mM NaCl, pH 7.6, 0.1% Tween 20, 5% nonfat dry milk), and detected using primary polyclonal antibodies against p38 MAPK, phosphorylated (Pi-) p38 MAPK (Thr180/Tyr182, New England Biolabs, Frankfurt/M, Germany, 1:2000), hsp27, Pi-hsp27 (Santa Cruz Biotech, 1:1000), tubulin (clone B-5-1-2, Sigma, and diluted 1:4000). After densitometric scan, proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk, incubated with primary antibodies for 1 h at room temperature, washed three times with Tris-buffered saline (TBS), and incubated with respective Cy3 conjugated secondary antibody (1:300; Jackson ImmunoResearch Europe, Suffolk, UK). Omission of the primary antibody served as a negative control.

4.6. Immunocytochemistry

Cells were gently washed with PBS (without Ca2+/Mg2+), fixed with 4% paraformaldehyde in PBS at room temperature, rinsed with PBS, and incubated with a monoclonal anti-III tubulin (1:500, Millipore, UK) at 4 °C overnight, followed by incubation with respective Cy3 conjugated secondary antibody (1:300; Jackson ImmunoResearch Europe, Suffolk, UK). Omission of the primary antibody served as a negative control.

4.7. Cell death assessment

Cell death was assessed by measurement of lactate dehydrogenase (LDH) activity in the supernatant medium. In neuronal cultures, LDH release into the medium reliably correlates with the number of damaged cells (Ruscher et al., 2007).

4.8. Dendritic spine quantification

Confocal micrographs (pinhole>1.5 μm) were taken by a person blinded to the experiments from branches of eGFP-actin or eGFP-Ha27 transfected neurons. The number of dendritic spines was counted on the length of a dendritic branch and was extrapolated to a length of 100 μm using the following equation: number of counted spines/length of dendritic branch ×100 μm.

4.9. Statistics

Experiments were performed in triplicate with 16 to 20 cell cultures each. Data are presented as means ± SD. The number of spine-like protrusions are presented as medians ± the 25th and the 75th percentile. Statistical analyses were performed using the Kruskal–Wallis one-way ANOVA on ranks followed by the Dunn’s method for multiple comparisons.

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Dunn’s method for multiple comparisons.

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