The Pro-Apoptotic Substance Thapsigargin Selectively Stimulates Re-Growth of Brain Capillaries

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Abstract

Thapsigargin is a pro-apoptotic chemical, which has been shown to be useful to study cell death of cholinergic or dopaminergic neurons, or cells, which degenerate in Alzheime's disease or Parkinso's disease, respectively. The aim of the present work was to study the effects of thapsigargin in the well established organotypic brain co-slice model composed of the basal nucleus of Meynert (nBM), ventral mesencephalon (vMes), dorsal striatum (dStr) and parietal cortex (Ctx). Cholinergic acetyltransferase-positive neurons in the nBM and dStr and dopaminergic tyrosine hydroxylase-positive neurons in the vMes survived, when cultured for 4 weeks with nerve growth factor and glial cell line-derived neurotrophic factor. Nerve fibers of cholinergic nBM neurons grew into the cortex and dopaminergic nerve fibers sprouted into dopamine D2 receptor-positive dStr. The whole co-slice contained a dense laminin-positive capillary network. Treatment of co-cultures with 3 μM thapsigargin for 24 hr significantly decreased the number of cholinergic neurons and dopaminergic neurons. This cell death displayed apoptotic DAPI-positive malformed nuclei and enhanced TUNEL-positive cells. Thapsigargin selectively stimulated the laminin-positive capillary growth between the nBM and Ctx. In conclusion, the induced cell death of cholinergic and dopaminergic neurons may be accompanied by enhanced angiogenic activity.

Keywords

Angiogenesis; vascularization; *in vitro* model; neuronal cell death

INTRODUCTION

Neurodegeneration in the central nervous system is a common characteristic of several brain disorders. Alzheime's disease (AD) is a chronic progressive disease, accompanied by cell death of cholinergic neurons of the basal nucleus of Meynert (nBM) and septum. A lack of acetylcholine in the cortex and hippocampus directly correlates with cognitive decline and memory dysfunction (Davies and Maloney, 1976; Whitehouse *et al.*, 1982; Wilcock *et al.*, 1982; Winkler *et al.*, 1998). In Parkinso's disease (PD) neurons of the ventral

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mesencephalon (vMes) (esp. of the substantia nigra pars compacta (SNc)) degenerate and a lack of dopamine in the dorsal striatum (dStr) causes motor dysfunction in humans (Ganten *et al.*, 2004; Olanow *et al.*, 1999). The causes for cell death of cholinergic and dopaminergic neurons in AD or PD, respectively are not known. Several cell death inducing stimuli are discussed, such as oxidative stress (Zhu *et al.*, 2004; Markesbery *et al.*, 1997; Jenner, 2003; Zhang *et al.*, 2000), glutamate-induced cell death (Blandini *et al.*, 1996; Masliah *et al.*, 1996), toxic compounds (Olanow *et al.*, 1999; Dauer *et al.*, 2003), proinflammatory cytokines (Mrak *et al.*, 1995; Vila *et al.*, 2001) or pro-apoptotic stimuli (Honig *et al.*, 2000; Takadera *et al.*, 2007). Thapsigargin is one of these cell death inducing stimuli, which have been discussed to be involved in the onset of AD and PD. As an inhibitor of the Ca^{2+}-ATPase in the sarcoplasmic (SR) and endoplasmic reticula (ER), thapsigargin is able to induce ER stress, which leads to accumulation of unfolded proteins and to apoptosis of neurons (Takadera *et al.*, 2007; Lindholm *et al.*, 2006; Chin *et al.*, 2007; Ferri and Kroemer, 2001). Some studies additionally indicate an influence of thapsigargin on the vascular system (Shukla *et al.*, 1997, 2001; Birket *et al.*, 1999; George *et al.*, 1997).

The aim of the present study was to explore the effects of thapsigargin on cholinergic and dopaminergic neurons and vascularization in an organotypic brain slice model. The organotypic brain slice model has been originally developed by Crain *et al.* (1982) using spinal cord-dorsal root ganglia, was modified by Gähwiler and colleagues (Gähwiler *et al.*, 1997) and further improved by Stoppini and colleagues (Stoppini *et al.*, 1991). In slices individual cells are in close contact and do not lose density dependent regulatory mechanisms, three dimensional architecture as well as tissue specific transport and diffusion probabilities. Organotypic brain slices are well established in our research group and we have extensively studied the survival of cholinergic neurons in the presence of NGF (Weis *et al.*, 2001) or of dopaminergic neurons in the presence of GDNF (Schatz *et al.*, 1999). In addition the combination of two closely related brain areas is well established, such as e.g. septum - hippocampus (Gähwiler and Hefti, 1984; Keller *et al.*, 1983), or vMes - dStr (Schatz *et al.*, 1999; Franke *et al.*, 2003) or nBM - cortex (Humpel and Weis, 2002).

In the present study we aim to culture four brain slices together to study interactions between brain areas, their axonal growth as well as angiogenesis due to thapsigargin treatment.

METHODS

Organotypic Brain Slice Cultures

Organotypic brain slice cultures were established as described by us in detail (Weis *et al.*, 2001; Humpel and Weis, 2002; Schatz *et al.*, 2000). Briefly, the nBM, vMes, parietal cortex and dorsal striatum of postnatal day 7-9 (P7-9) Sprague Dawley rats was dissected under aseptic conditions, 400 μm slices were cut with a tissue chopper (McIlwain, USA), and the 4 slices were placed on a 30 mm diameter Millicell-CM 0.4 μm membrane insert (Millipore, Austria), where they become attached to the membrane after 2 weeks of incubation. It is important to note, that the ipsilateral as well as contralateral brain areas were dissected at the same time, cut on the tissue chopper and all slices pooled in medium. Slices were cultured in 6-well plates (Greiner) at 37°C and 5% CO_2 with 1.2 ml/well of the following culture

medium: 50% MEM/HEPES (Gibco), 25% heat inactivated horse serum (Gibco/Lifetech, Austria), 25% Hank' solution (Gibco), 2 mM $NaHCO_3$ (Merck, Austria), 6.5 mg/ml glucose (Merck, Germany), 2 mM glutamine (Merck, Germany), pH 7.2. Medium was changed twice a week. Brain slice cultures were incubated with or without 10 ng/ml nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) for 4 weeks. To analyze the cell death of neurons, four weeks old cultures were incubated for 3 days without growth factors, treated afterwards with or without 3 μM thapsigargin in medium for 24 hr and then incubated for further 3 days in medium without growth factors. At the end of the experiment, slices were fixed for 3 hr at 4°C in 4% paraformaldehyde/10 mM phosphate buffered saline (PBS) and then stored at 4°C in PBS until use. All experiments conformed to Austrian guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering.

Immunohistochemistry

Immunohistochemistry was performed similarly as previously described (Weis *et al.*, 2001). All incubations were performed free floating for 2 days including 0.1% Triton, such that the antibodies can penetrate from both sides of the slices, which allows good penetration of the antibody into the brain slices. Slices were washed 30 min with 0.1% Triton/PBS (T-PBS) at room temperature and pre-treated 20 min with 20% methanol/1% H_2O_2/PBS (only for 3,3′-diaminobenzidine labeling). After thorough rinsing, the slices were blocked with 20% horse serum/0.2% BSA/T-PBS and then incubated for 2 days at 4°C with primary antibodies against choline acetyltransferase (ChAT, 1:750, Chemicon), tyrosine hydroxylase (TH, 1:500, Chemicon), dopamine D2 receptor (D2R, 1:250, Santa Cruz), glial fibrillary acidic protein (GFAP, 1:500, Chemicon) or laminin (1:500, Sigma). Then the slices were washed again with PBS and incubated with secondary biotinylated anti-goat (ChAT), anti-chicken (GFAP), anti-rabbit (laminin, TH), or anti-mouse (D2R) antibodies (1:200, Vector Lab., USA) for 1 hr at room temperature. Following further washing steps with PBS, slices were incubated in an avidin-biotin complex solution (ABC-Elite Vectastain reagent Vector Lab.) for 1 hr. After being washed with 50 mM *Tris*-buffered saline (TBS), the signal was detected by using 0.5 mg/ml 3,3′-diaminobenzidine (DAB) including 0.003% H_2O_2 as a substrate in Tris-buffered saline. The slices were mounted on glass slides, air dried and coverslipped with Entellan (Merck, Germany). Unspecific staining was defined by omitting the primary antibody. For fluorescence immunohistochemistry the methanol pre-treatment was omitted and as secondary antibodies Alexa-488 (Invitrogen, 1:400) was used. To label nuclei, slices were incubated with 4,6-diamidino-2-phenylindole (DAPI, 1:10000; Sigma) for 30 min. Certain sections were stained with cresyl violet for 10 min and rinsed afterwards for further 5 min in a.d. Immunolabeling was visualized with a Leica DMIRB fluorescence inverse microscope equipped with an Apple computer.

Acetylcholinesterase Histochemistry

Acetylcholinesterase (AChE) histochemistry was performed as described by us (Humpel & Weis, 2002). The slices were fixed with 4% paraformaldehyde for 3 hr at 4°C. After being washed with 100 mM maleate buffer (pH 6.0) slices were incubated in a fresh solution of 0.036 mM acetylthiocholine iodide (Sigma), 0.005 mM $K_3Fe(CN)_6$(III) (Merck), 0.03 mM $CuSO_4$ (Merck), 0.05 mM sodium citrate (Merck), 19.77 mM ethopropazine (Aldrich) in

100 mM maleate buffer (pH 6.0) for 1.5 hr at room temperature. Thereupon, slices were rinsed in PBS buffer (pH 7.4) and Tris buffer (pH 7.6). Subsequently, slices were developed in a 0.04% DAB/0.3% nickel ammonium sulfate/0.003% H_2O_2/*Tris* pH 7.6 solution.

DNA Nick-End Labeling (TUNEL Staining)

The TUNEL reaction was performed as described earlier (Schatz *et al.*, 1999). Fresh co-slices were carefully transferred to glass slides, frozen on a CO_2 snow, and stored at $-20°C$ until use. Co-slices were then air dried, and incubated with 150 U/ml terminal transferase (Roche) and 10 nmol/ml biotinylated 16-dUTP (Roche) in terminal transferase buffer (Roche) at 37°C for 2 hr. Afterwards, co-slices were fixed with 4% paraformaldehyd/10 mM PBS for 30 min and then washed in PBS. Co-slices were incubated with ABC reagent (Vectastain) for 30 min at room temperature, then washed in 50 mM *Tris* buffer pH 7.6 (3×10 min) and the staining was performed in *Tris* pH 7.6 with 0.5 mg/ml DAB as a substrate, including 0.003% H_2O_2 and 0.4% $NiCl_2$. Sections were extensively washed in a.d., air dried, and covered with Entellan.

Analysis, Quantification and Statistics

The number of microscopically detectable immunoreactive neurons was counted in the whole slice visualized under a 20× objective. The areas were identified by the respective immunohistochemial stainings. The crossings of laminin-positive capillaries, between the respective brain areas were counted in the whole slice under the microscope. The number of crossings were normalized to crossings per cm. Cell counting was performed for DAPI-positive malformed and TUNEL-positive nuclei on a Leica DMIRB fluorescence inverse microscope equipped with an Apple computer and Improvision Openlab Darkroom software. Cell counting was performed on a random field (260 μm × 190 μm) per slice in the vMes and nBM. Multistatistical analysis was obtained by one way ANOVA, followed by Fisher PLSD posthoc test by comparing controls against the respective treatments, where $p < 0.05$ represents statistical significance.

RESULTS

Cholinergic and Dopaminergic Neurons in Co-Cultures

Cholinergic neurons in co-slices strongly stained by immunohistochemistry for ChAT (Fig. 1A). Approximately 120 neurons per slice of ChAT-positive neurons (Table 1) were found in the nBM slice (Fig. 1A), and approximately 50 neurons per slice (Table 1) were found in the dStr slice (Fig. 1A), when the co-slice was incubated for 4 weeks with NGF and GDNF. No ChAT-positive neurons were found in the cortex (Fig. 1A) or vMes (Fig. 1A). Cholinergic nerve fibers grew from the nBM to the cortex slice (Fig. 2D), as shown by a strong staining of acetylcholinesterase-positive nerve fibers. Dopaminergic neurons in co-cultures were stained by immunohistochemistry for tyrosine hydroxylase (Fig. 1B). In a co-slice incubated for 4 weeks with NGF and GDNF approximately 80 TH-positive neurons per slice (Table 1) were found exclusively in the vMes (Fig. 1B). When slices were incubated without growth factors the number of cholinergic and dopaminergic neurons was significantly reduced (data not shown). Co-slices composed of four brain regions grew together and formed a large slice after the cultivation of four weeks. At the borders between

the brain areas a weaker stained layer of cresyl violet was seen (Fig. 2A). GFAP-staining was low over the entire co-slice, whereas the connective regions between the brain slices displayed a decreased staining for GFAP (Fig. 2B). Immunohistochemistry for D2R revealed several strongly stained cells in the dStr (Fig. 2E). TH-positive nerve fibers (Fig. 2F) from the vMes grew into the dStr as identified by D2R staining in co-labelled sections (Fig. 2G).

Laminin-Positive Capillary Network

When co-slices composed of four brain regions were cultured for 4 weeks with growth factors, a very dense laminin-positive capillary network was seen over all 4 brain areas (Fig. 3). The laminin-positive capillaries grew within the brain areas and most of the new connections were found between the nBM and cortex (Fig. 3B). In some slices the brain capillaries were broken (Fig. 3B) or did not pass the border between the brain areas (Fig. 3C). The laminin-positive capillaries crossed the borders between cortex and nBM (Table 2) and to a lesser extent between vMes and cortex or dStr (Table 2) or nBM and dStr (Table 2), while no crossings were found between dStr and cortex (Table 2).

Effects of Thapsigargin in Co-Cultures

The number of cholinergic and dopaminergic neurons in co-cultures composed of four brain regions was significantly decreased after the incubation with 3 μM thapsigargin (Fig. 4; Table 1). Thapsigargin had a significant toxic effect on cholinergic neurons in the nBM and dStr and on the dopaminergic neurons in the vMes (Table 1). The remaining cholinergic and dopaminergic neurons (Fig. 4B) in co-cultures after thapsigargin treatment displayed a shrunken and smaller shape compared to untreated co-slices (Fig. 4A). Furthermore, the treatment of co-cultures with thapsigargin enhanced the number of DAPI-positive malformed nuclei in the vMes and nBM (Table 1). Healthy nuclei appeared round, well-formed with an intensive staining (data not shown). Whereas, malformed nuclei emerged in spindle, enlarged or granular form, with a weaker DAPI-labeling (Fig. 4D, indicated by arrows). The incubation of co-cultures with thapsigargin (Fig. 4C) revealed a significantly increased number of TUNEL-positive nuclei (Table 1), compared to untreated co-slices (Table 1). Co-localization studies of TH-/ChAT-immunoreactivity and DAPI staining displayed that DAPI-positive malformed nuclei were not related to TH (Fig. 4D) or ChAT-positive immunoreactive neurons (data not shown). Treatment of co-cultures with thapsigargin exhibited a significantly enhanced laminin-positive capillary network between cortex and nBM (Table 2; Fig. 3D, E). Capillaries at the border between nBM and cortex displayed enhanced processes (Fig. 3D) after thapsigargin treatment compared to capillaries in untreated co-slices (Fig. 3B, C). However, the angiogenic effect of thapsigargin emerged exclusively between nBM and cortex, but not between the borders of the other brain regions (Table 2).

DISCUSSION

The present study demonstrates, that the pro-apoptotic chemical thapsigargin exhibited angiogenic activity between functional related brain areas and induced cell death of cholinergic and dopaminergic neurons in organotypic brain co-cultures.

Organotypic Brain Slice Model

The organotypic brain slice model is able to maintain the survival of different cell types, the cytoarchitecture of the tissue, the connections between cells and neuronal properties. The basic cellular and connective organization of the donor brain regions are well preserved, thus the slice cultures provide an easily accessible experimental model for studies of toxic, degenerative and plastic regenerative or developmental changes in the brain (Zimmer *et al.*, 2000). In slices, the individual cells are arranged in close contact and do not lose density-dependent regulatory mechanisms, three-dimensional architecture as well as tissue-specific transport and diffusion probabilities. Organotypic brain slices were first introduced by Gähwiler and colleagues (Gähwiler and Hefti, 1984, 1997) as roller tube cultures. The technique was modified (Stoppini *et al.*, 1991; Buchs *et al.*, 1993) and is meanwhile used by several research groups (Ostergaard *et al.*, 1990 and 1993; Gähwiler *et al.*, 1990; Robertson *et al.*, 1997). The culturing of slices on membranes is well established in our research group (Humpel *et al.*, 1995; Schatz *et al.*, 1999; Weis *et al.*, 2001; Humpel and Weis, 2002). Furthermore, we have shown in previous studies, that the addition of growth factors into the culture medium prevents the neurons from cell death (Weis *et al.*, 2001; Humpel und Weis, 2002; Schatz *et al.*, 1999). This is necessary, because slices do not produce enough endogenous growth factors to support the survival of the cholinergic or dopaminergic neurons. It is well established, that NGF prevents the loss of cholinergic neurons *in vitro* and *in vivo* (Hagg *et al.*, 1990; Hefti *et al.*, 1985; Kew *et al.*, 1996; Svendsen *et al.*, 1994; Van der Zee *et al.*, 1996; Connor *et al.*, 2001). In fact we have previously shown that slices contain less than 3 pg NGF/mg tissue, which is insufficient to support the cholinergic phenotype (Weis *et al.*, 2001). Whereas, dopaminergic neurons are protected and stimulated very potently by GDNF (Airaksinen *et al.*, 2002; Henderson *et al.*, 1994; Arenas *et al.*, 1995; Gill *et al.*, 2003), which have been additionally shown in previous studies in organotypic brain slices by Schatz *et al.* (1999). Moreover, co-cultures of the nBM together with the cortex (Humpel and Weis, 2002) and the vMes together with the dStr (Schatz *et al.*, 1999) provide an excellent system to study interaction between two brain regions. In the present study, we used a co-culture system composed of four brain regions, the nBM, vMes, dStr and cortex. The slices for these co-cultures derive from early postnatal day 7-9 rat brains and neurons at this developmental stage are very resistant for explantation, the cytoarchitecture is already established and the tissue has the property to flatten. This flattening to about 100-200 m is also an internal mean for a good preparation and dissection, whereas it is not completely clear why the slices become thinner. Our data show, that the four different brain areas (nBM, Ctx, vMes, dStr) grew together and formed a single large organotypic slice. The astroglial GFAP-staining was moderate to low in all slices, indicating that no reactive gliosis occurred, being in line with previous experiments in our group (Schmidt-Kastner *et al.*, 2002).

Cholinergic and Dopaminergic Neurons in Co-Cultures

Cell death of cholinergic and dopaminergic neurons are the central hallmark in AD and PD. Our brain slice model allows to study survival and neurodegeneration of the cholinergic and dopaminergic neurons as reported in several recent papers by us (Humpel and Weis, 2002; Zassler *et al.*, 2003, 2005; Weis *et al.*, 2001; Humpel *et al.*, 1995; Schatz *et al.*, 1999). The

enzyme ChAT can be used as a key enzyme to label cholinergic neurons and the number of ChAT-positive neurons strongly correlate with the survival rate. We have previously shown, that cholinergic neurons survive in co-slices of the nBM and cortex and cholinergic nerve fibers grew into the cortex slice (Humpel and Weis, 2002). Our present study shows that cholinergic ChAT-positive nBM neurons survive in a co-slice composed of four brain areas. In addition, also a high number of smaller ChAT-positive interneurons survived in the dStr, while in the cortex and vMes no ChAT-positive neurons were seen. Similarly to ChAT, we have used the key enzyme TH to label dopaminergic neurons, which is well established (Schatz *et al.*, 1999). Likewise to cholinergic neurons, dopaminergic neurons grew into the functional target, the dStr. Again, these nerve fibers can be regarded as new innervation from the ventral mesencephalon, because all nerve fibers in the axotomized dorsal striatum degenerate. Our present model shows further survival of TH-positive dopaminergic neurons exclusively into the dStr, D2 receptor staining in striatum and vMes and selective ingrowth of TH-positive fibers.

Effects of Thapsigargin on Cholinergic and Dopaminergic Cell Death

Thapsigargin is an effective inhibitor of the Ca^{2+}-ATPase in the sarcoplasmic and endoplasmic reticulum and activates plasma membrane calcium channels (see scheme Fig. 5), resulting in enhanced influx of calcium into the cell (Takadera *et al.*, 2007; Thastrup *et al.*, 1990; Treimann *et al.*, 1998; Chin *et al.*, 2007; Ferri and Kroemer, 200; Dahmer, 2005; Deniaud *et al.*, 2008). It is well known that thapsigargin has a dual effect on cells: it can induce apoptosis but also inhibit apoptosis (see Fig. 5), dependent on the cellular co-factors. (1) Thapsigargin exhibits apoptotic cell death in various cell types, e.g. cortical cells, hypothalamic cell lines or muscle cells (Takadera *et al.*, 2007; Thastrup *et al.*, 1990; Treimann *et al.*, 1998; Wei *et al.*, 1998; Mattson *et al.*, 1998; Linford *et al.*, 2002). Two major pathways have been shown to be responsible for this apoptotic cell death: the extrinsic receptor-mediated and the intrinsic signaling pathways (Chin *et al.*, 2007). Recent studies have shown, that thapsigargin treatment stimulates the proteolytic processing of caspase-2, -3 and -7, which are involved in the intrinsic ER-stress mediated apoptosis (Dahmer, 2005; Deniaud *et al.*, 2008; Kass and Orrenius, 1999; Chin *et al.*, 2007; Ferri and Kroemer, 2001). (2) On the other hand it has been shown that thapsigargin can also inhibit apoptosis via activation of the bcl-2 cascade. Chin and colleagues (2007) demonstrated, that Ca^{2+}-sensitive mitogen-activated protein kinase (MAPK) phosphorylation was induced by thapsigargin and acted as a downstream effector of phosphatidylinositol 3-kinase (PI 3-kinase), which is involved in the inhibition of apoptosis (Downward, 2004). Nevertheless, it is suggested that ER stress and mitochondrial dysfunction leads to an activation of Bcl-2 or other additional anti-apoptotic substances (Ferrari *et al.*, 2002; Giacomello *et al.*, 2007).

Little is known about the effects of thapsigargin in inducing cell death of cholinergic and dopaminergic CNS neurons. Recent studies indicate, that ER stress in conjugation with abnormal protein degradation can contribute to the pathophysiology of PD, but also AD (Lindholm *et al.*, 2006). Our data show a significant decline of ChAT-positive nBM neurons, TH-positive vMes neurons and ChAT-positive interneurons in the dStr after thapsigargin treatment. The data suggest, that the inhibition of the Ca^{2+}-ATPase in the sarcoplasmic and endoplasmic reticula is a vulnerable target for cholinergic as well as

dopaminergic neurons in the brain. The increased TUNEL-positive staining in the nBM and vMes in our co-cultures indicates that apoptotic cell death occurred after thapsigargin application. In parallel, many DAPI-positive malformed nuclei were seen after treatment with thapsigargin. The number of TUNEL- and malformed DAPI-positive cells was many times higher than the number of cholinergic and dopaminergic neurons. In fact, co-localization studies previously showed that dopaminergic neurons did not co-localize with TUNEL-positive nuclei after 6-OHDA lesions (Schatz *et al.* 1999). The present study is in line with our previous data, showing that DAPI-positive malformed nuclei were not related to dopaminergic or cholinergic neurons. The data may indicate that astroglia undergo apoptotic cell death, which may result in neurodegeneration of dopaminergic and cholinergic neurons. It needs to be pointed out, if thapsigargin can induce DAPI- or TUNEL-positive staining in purified astroglial cell culture. In addition, more advanced methods need to be performed, such as caspase assays, to detect the responsible mechanisms, which are involved in the neurodegeneration of cholinergic and dopaminergic neurons due to thapsigargin treament.

Angiogenic Activity of Thapsigargin in Brain Slices

Brain capillaries constitute the blood-brain barrier and innervate all areas of the brain. We have previously shown, that organotypic brain slices contain a strong network of laminin- or RECA-1 positive brain capillaries (Moser *et al.*, 2004, 2003). Laminin is a well-established basement membrane marker and stains excellently the vascular structures of the brain (Eriksdotter-Nilsson *et al.*, 1986; Jucker *et al.*, 1996; Sixt *et al.*, 2001). Our present study is in full agreement with our previous ones and shows a strong network of laminin-positive brain capillaries all over the four brain slices. Furthermore we show, that brain capillaries grow from one side to another and connect the brain slices. Such a growth between two brain slices was most prominent between the nBM and cortex and to a lesser extent between vMes and dStr. Thus it is suggested, that the co-slice model represents a useful *in vitro* system, where capillary re-growth is specifically observed between two functionally related brain areas, such as the nBM - cortical and meso-striatal system. On the other hand, no connections were observed between the dorsal striatum and cortex. This region-specific effect is of importance and points to a positive influence of the neocortex to the nBM, suggesting that the nBM cholinergic input to the neocortex provides a vascular rather than a neuronal modulation, being in line with Kurosawa *et al.* (1989).

Thus, we demonstrated for the first time, that thapsigargin possesses pro-angiogenic activity in the brain by increasing the capillary growth between the nBM and cortex. It is known that thapsigargin has anti-angiogenic activity in vascular smooth muscle cells (VSMC) (Shukla *et al.*, 2001; Birket *et al.*, 1999; George *et al.*, 1997; Shukla *et al.*, 1997), but it is fully unknown how thapsigargin may exhibit this angiogenic activity, specifically on nBM - Ctx capillaries. It seems possible, that thapsigargin-induced Ca^{2+} release stimulates vascular endothelial growth factor (VEGF) production (Josko *et al.*, 2000) and subsequently angiogenesis (Fig. 5). In fact, it is well established that thapsigargin increases the expression and release of VEGF (Koyama *et al.*, 2008; Marjon *et al.*, 2004), which is one of the most potent angiogenic factors and exerts its effect by binding to its specific receptors Flt-1 and Flk-1 in endothelial cells (Yancopoulos *et al.*, 2000). Both VEGF receptors Flk-1 and Flt-1

are expressed in the cortex (Yang *et al.*, 2003). Furthermore, recent studies demonstrated that VEGF and GDNF use the same signaling pathway in ureteric bud cells (Tufro *et al.*, 2007). Such a direct interaction between VEGF and GDNF has not been demonstrated in the brain, however, it cannot be excluded that GDNF may stimulate VEGF in the brain too. Alternatively, it seems possible that the neurodegeneration of the neurons and/or the apoptotic cell death of e.g. glial cells may secrete chemokines or cytokines, which stimulate capillary growth. And finally, a putative anti-apoptotic effect of thapsigargin on brain capillary endothelial cells may contribute to the angiogenetic responses. This angiogenic sprouting may play a role in tumor growth and metastasis (Liekens *et al.*, 2001), because a direct correlation between the tumor suppressor p53 and cells with a dysfunctional ER has been postulated recently (Pluquet *et al.*, 2005). Further studies will be necessary to elucidate the cellular mechanisms of thaspigargin in the brain, especially of VEGF and p53 involvement.

In summary we demonstrated a pro-angiogenic effect of thapsigargin between functional related brain areas in an *in vitro* co-culture system composed of four brain regions. Furthermore we have shown, that dopaminergic as well as cholinergic neurons degenerate after ER-induced calcium stress. It can be emphasized, that the dysregulation of the ER may play a role in initiation of early PD or AD and possibly the vascular pathology in that neurodegenerative disease.

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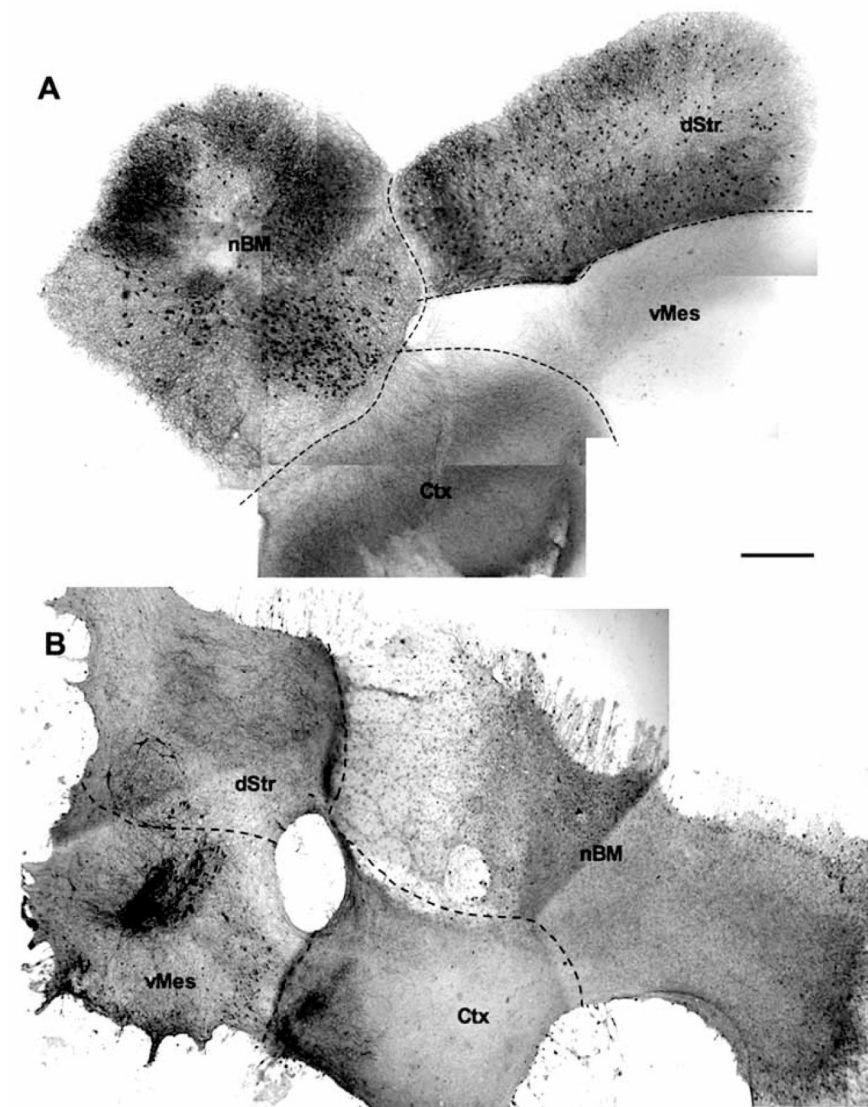
Fig. (1).

Immunohistochemistry for cholinergic (**A**) or dopaminergic (**B**) neurons *in vitro*. Co-slices composed of four brain regions, the basal nucleus of Meynert (nBM), dorsal striatum (dStr), ventral mesencephalon (vMes) and cortex (Ctx) were incubated for 4 weeks with nerve growth factor and glial cell line-derived neurotrophic factor and then stained for choline acetyltransferase (ChAT) (**A**) or tyrosine hydroxylase (TH) (**B**). The different brain areas are indicated by dashed lines. Scale bar in A = 550 m (**A, B**).
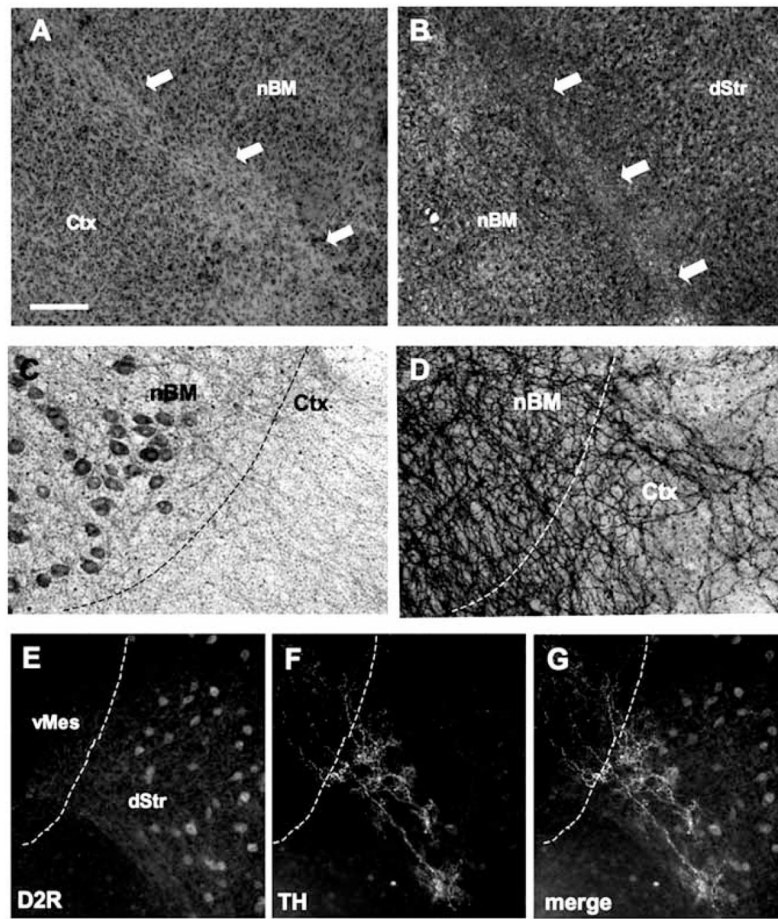
Fig. (2).
Borders between the brain areas in co-cultures. Ventral mesencephalon (vMes) Fig. (**A**) shows a weaker band of cresyl violet staining between the basal nucelus of Meynert (nBM) and cortex (Ctx) (arrows). Fig. (**B**) displays weaker glial-fibrillary acidic protein (GFAP)-like immunoreactivity between nBM and dorsal striatum (dStr) (arrows). Fig. (**C**), choline acetyltransferase (ChAT)-positive neurons in the nBM give rise to strong acetylcholinesterase-positive nerve fibers which cross the border between the nBM and Ctx (**D**). In the dStr dopamine D2 receptor-(D2R) neurons are expressed (**E**), and TH-positive nerve fibers (**F**) grow into the dStr (**G**, merged). The different brain areas are indicated by dashed lines. Scale bar in A = 250 μm (**A**, **B**), 100 μm (**C**-**D**), 200 μm (**E**-**G**).
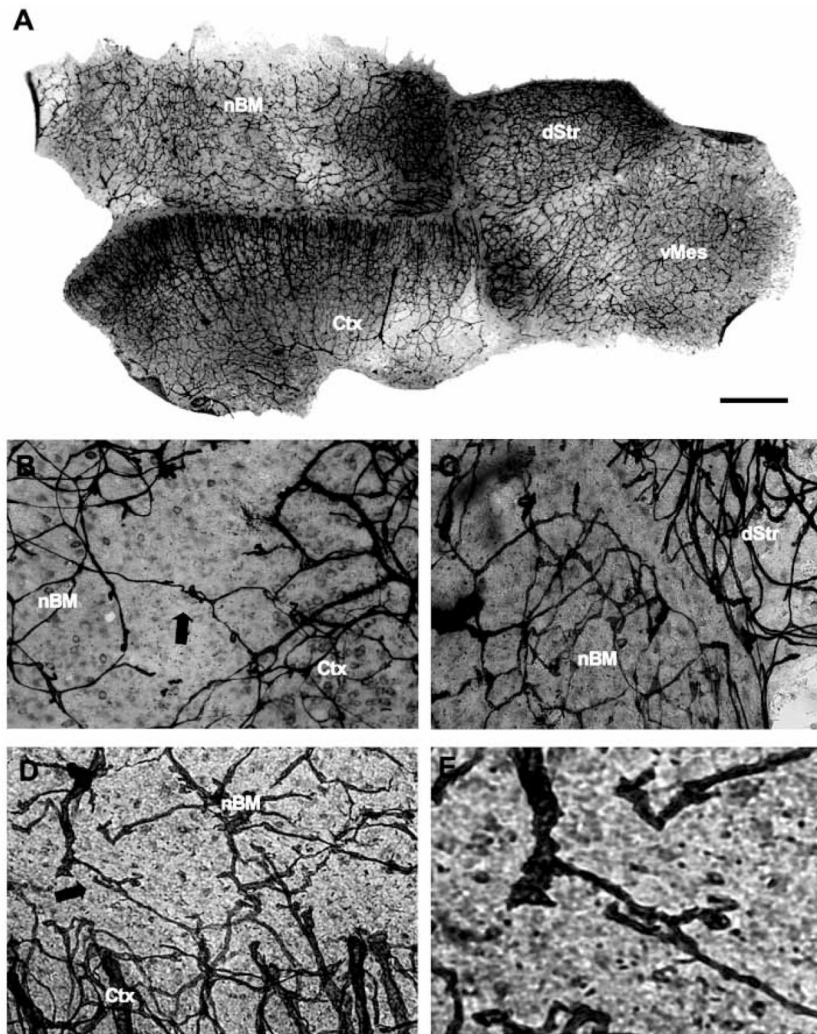
Fig. (3).

Laminin-positive capillaries in a co-slice. Co-slices composed of four brain regions, the basal nucleus of Meynert (nBM), dorsal striatum (dStr), ventral mesencephalon (vMes) and cortex (Ctx) were incubated for 4 weeks with nerve growth factor and glial cell line-derived neurotrophic factor and then stained for laminin (**A-E**). Higher magnifications of the borders between two brain regions are shown in Fig. (**B-E**). Note that laminin-positive capillaries cross the border between nBM and Ctx (**B**, arrow), or are damaged (**B**). A laminin-negative area between the nBM and dStr is displayed in (**C**). Co-cultures after thapsigargin treatment displayed an enhanced crossing of laminin-positive capillaries between the nBM and Ctx (**D**, **E**, arrow). Fig. (**E**) showed a higher magnification of vascular growth in Fig. (**D**). Scale bar in A = 900 μm (**A**), 300 μm (**B-D**), 100 μm (**E**).
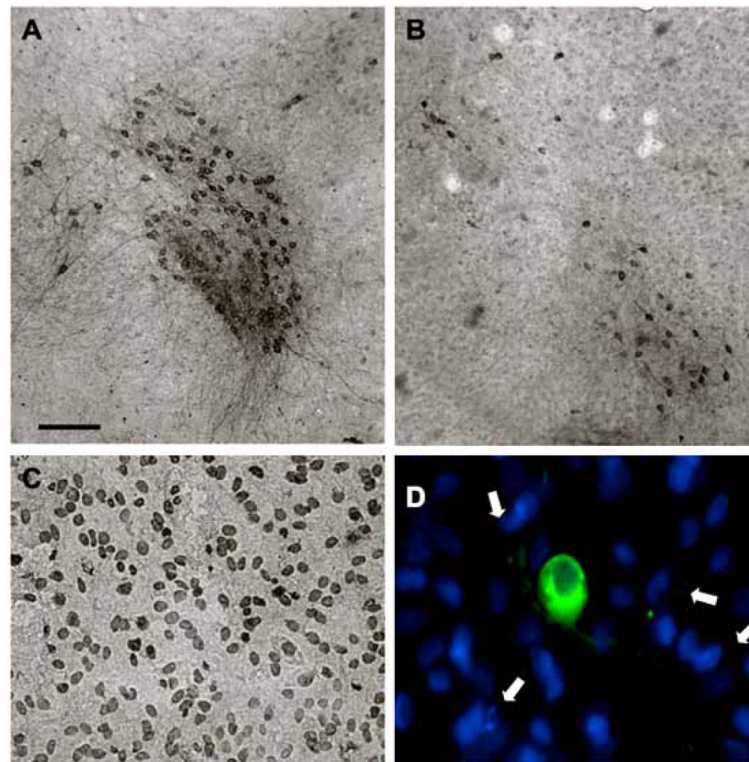
Fig. (4).

Effects of thapsigargin on cell death. Co-cultures were treated overnight without (control, **A**) or with 3 μM thapsigargin (**B**-**D**). After 3 days, slices were immunohistochemically stained against tyrosine hydroxylase (**A**, **B**), which stains dopaminergic neurons in the vMes. In situ labelling of nuclear DNA fragmentation exhibited an enhanced TUNEL-positive staining in vMes slices after thapsigargin (**C**). Co-localization experiments showed malformed DAPI-positive nuclei (blue, arrows), which did not co-localize with TH-positive neurons (green, Alexa-488) after thapsigargin treatment (**D**). Scale bar in A = 250 μm (**A**, **B**), 100 μm (**C**), 50 μm (**D**).
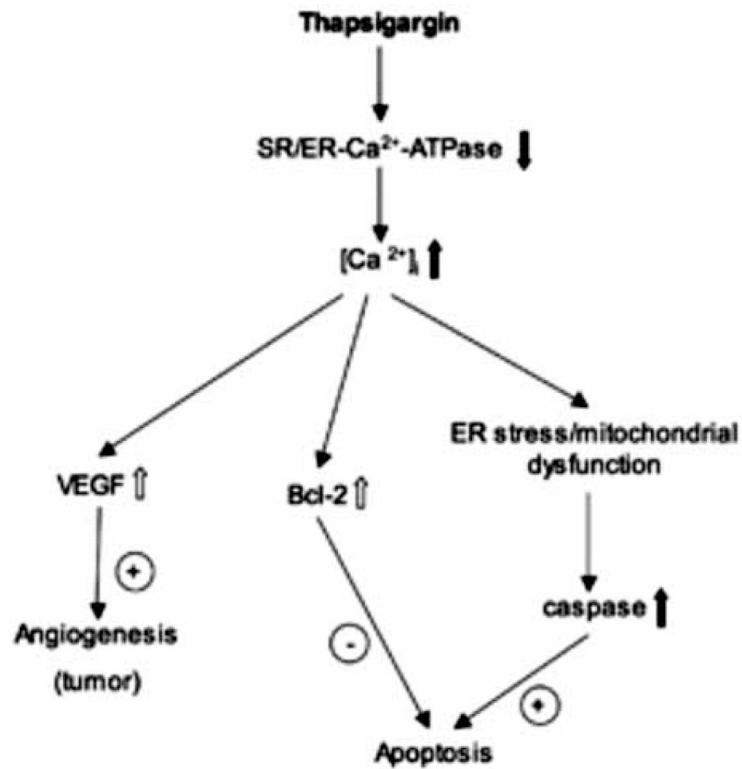
Fig. (5).

Proposed mechanism and effect of thapsigargin. Thapsigargin inhibits the calcium-ATPase of the sarcoplasmic (SR)/endoplasmic reticulum (ER), resulting in enhanced intracellular calcium levels. This enhanced Ca^{2+} can either inhibit apoptosis by activation of anti-apoptotic substances of e.g. the Bcl-2 class or can induce apoptosis by activation of the caspase cascade after ER stress and/or mitochondrial dysfunction. Alternatively, the enhanced production of vascular endothelial growth factor (VEGF) may result in angiogenesis and possibly tumor formation.

Table 1

Effects of Thapsigargin on Neuronal Cell Death

Region		Control	Thapsigargin
ChAT	nBM	124 ± 15 (7) -	60 ± 11 (5) **
	dStr	54 ± 12 (7) -	18 ± 5 (5) **
TH	vMes	81±18 (6) -	18 ± 5 (5) ***
DAPI	nBM	9 ± 1 (7) -	30 ± 2 (5) ***
	vMes	12 ± 2 (7) -	49 ± 3 (5) ***
TUNEL	nBM	45 ± 5 (4) -	392 ± 44 (3) ***
	vMes	53 ± 7 (4) -	313 ± 16 (4) ***

A 4 week old co-culture brain slice, composed of the basal nucleus of Meynert (nBM), the dorsal striatum (dStr), the cortex (Ctx) and the ventral mesencephalon (vMes), was incubated with 3 μM thapsigargin for 24 hr and then for further 3 days in without. Subsequently, the slices were fixed, stained for choline acetyltransferase (ChAT) or tyrosine hydroxylase (TH) or DAPI or DNA fragments were labelled in situ using the TUNEL assay. The number of ChAT/TH or DAPI-positive nuclei with apoptotic morphology or TUNEL-positive nuclei were counted on a random field (260 μm × 190 μm) per slice are given as mean ± S.E.M.; the values in parenthesis give the number of analyzed slices. Statistical analysis was performed by one way ANOVA with a Fisher PLSD posthoc test (*** $p<0.001$; ** $p<0.01$).

Table 2

Effects of Thapsigargin on Re-Growth of Crossing of Laminin-Positive Capillaries

Region	Control	Thapsigargin
Ctx-nBM	19 ± 3 (11) -	38 ± 4 (21) **
vMes-Ctx	6 ± 2 (11) -	6 ± 2 (9) ns
vMes-dStr	10 ± 4 (11) -	7 ± 3 (9) ns
nBM-dStr	5 ± 2 (11) -	2 ± 1 (9) ns
dStr-Ctx	n.c. (11)	n.c. (6)

A 4 week old co-culture brain slice, composed of the basal nucleus of Meynert (nBM), the dorsal striatum (dStr), the cortex (Ctx) and the ventral mesencephalon (vMes), was incubated with 3 μM thapsigargin and then further 3 days without. Then slices were fixed and stained for laminin immunohistochemistry. The number of capillary crossings per cm between the different brain regions was counted under the microscope. Values are given as mean ± S.E.M.; values in parenthesis give the number of analyzed slices. Statistical analysis was performed by one way ANOVA with a Fisher PLSD posthoc test (**$p<0.01$; ns, not significant; n.c., no crossings).