Fange es an was du dir erträumst,

denn der Kühnheit

wohnen Genie, Macht und Zauber inne.

Goethe

Aus der
Klinik und Poliklinik für Neurologie
des Neurozentrums
des Universitätsklinikums Hamburg-Eppendorf
Direktor: Prof. Dr. med. Cornelius Weiller

Identification of transcripts regulated by ischemic preconditioning in a rat primary cortical cell culture model

Dissertation

zur Erlangung des Grades eines Doktors
der Medizin

dem Fachbereich Medizin der Universität Hamburg vorgelegt von

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aus Maastricht

Hamburg
2005

Angenommen vom Fachbereich Medizin
der Universität Hamburg am:

Veröffentlicht mit Genehmigung des Fachbereiches
Medizin der Universität Hamburg

Prüfungsausschuss, der/die Vorsitzende:

Prüfungsausschuss: 2. Gutachter/in:

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Abbreviations

AA	arachidonic acid
ATP	adenosine triphosphate
bp	base pair
BSS	buffered saline solution
cDNA	copy deoxyribonucleic acid
CNS	central nervous system
DAG	diacylglycerole
dC	deoxycytidine
DD	differential display
DMF	dimethylformamide
DIV	day in vitro
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dT	deoxytymidine
ER	endoplasmic reticulum
EST	expressed sequence tag
FCS	fetal calf serum
FS	forward subtracted
GAS	growth arrest
GFAP	glial fibrillary acidic protein
HSP	heat shock protein
IP_3	inositol 3-phosphate
kB	kilobase
LD PCR	long distance polymerase chain reaction
MCAO	middle cerebral artery occlusion
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
NeuN	neuronal Nuclei

OGD	oxygen and glucose deprivation
on	over night
PBS	phosphate buffered saline
PKC	protein kinase C
PUFA	polyunsaturated fatty acids
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RS	reverse subtracted
RT	room temperature
RT PCR	reverse transcriptase polymerase chain reaction
S1P	sphingosine 1-phosphate
SAGE	serial analysis of gene expression
SDS	sodium dodecyle sulfate
SMART	switching mechanism at 5’ end of RNA template
SMVT	sodium dependent multi vitamin transporter
SSC	sodium sodium citrate
ss	single stranded
SSH	suppression subtractive hybridization
tRNA	transfer ribonucleic acid
US	unsubtracted
UTR	untranslated region
vNB	virtual Northern blot

1. Introduction

1.1 Ischemic Preconditioning

1.1.1 The phenomenon of Ischemic Preconditioning

Over the past two decades the pathophysiological consequences of myocardial ischemia have received much attention. With the description of myocardial stunning and the hibernating myocardium, it has been assumed, until recently that intermittent episodes of ischemia lead to cumulative myocardial damage. Theoretically this seems logical. As the life expectancy of early man was short, ischemia was not a major problem and selection bias to prevent ischemic diseases was therefore not present. However, researchers have demonstrated that an endogenous myocardial protection mechanism does exist (Murry, Jennings et al. 1986). Brief ischemic episodes, followed by reperfusion, increase the resistance to further ischemic damage. This protective response is called ischemic preconditioning (IP). Subsequently IP was shown to have protective effects in many other tissues, such as brain (Khaspekov, Shamloo et al. 1998), spinal cord (Matsuyama, Chiba et al. 1997), retina (Larsen and Osborne 1996), liver (Lloris-Carsi, Cejalvo et al. 1993), lung (Du, Hicks et al. 1996), kidney (Islam, Mathie et al. 1997) and skeletal muscle (Pang, Yang et al. 1995). The time required to induce IP is tissue and species specific. (Murry, Jennings et al. 1986; Liu and Downey 1992), as is the duration of protection.

IP occurs in two different time windows for which different mechanisms of protection have been proposed (Hawaleshka and Jacobsohn 1998). The first phase is called the immediate protection that occurs within minutes or hours after IP. The second phase is called the delayed phase and exerts protection after one to three days.

1.1.2 Mediators of ischemic preconditioning

In the beginning, mechanisms such as collateral perfusion and myocardial stunning were believed to play a role in the immediate protection phase, but these models had to be abandoned. Instead molecular mechanisms entered the center stage.

As in any biochemical model the molecular basis must consist of an ordered series of events. In response to the stimulus a signal must be generated, transduced into an intracellular message and amplified to influence effector mechanisms. Various players in this cascade could be identified and therefore IP is likely to be a multifactorial process that requires a well-ordered combination of many factors.

It has been shown that adenosine, an endogenous nucleotide produced through ATP decay, limits ischemic injury (Downey, Liu et al. 1993; Thornton, Thornton et al. 1993; Tsuchida, Liu et al. 1993). The accumulating adenosine exerts its functions during ischemia via the G-protein-coupled A_1–receptor. Other substances such as bradykinin (Goto, Liu et al. 1995) and catecholamines (Toombs, Wiltse et al. 1993; Hu and Nattel 1995), which also act through G-protein-coupled receptors, demonstrated protective abilities. These G-proteins increase the activity of phospholipases C and D, which then stimulate diacylglycerol (DAG) and inositol triphosphate (IP_3) liberation from membrane phospholipids (Sugden and Bogoyevitch 1995).

DAG activates protein kinase C (PKC), an enzyme that was shown to play a key role in IP through the phosphorylation of effector molecules (Eskildsen-Helmond, Gho et al. 1996). In contrast, other studies called the role of PKC into question, demonstrating that PKC inhibition is not associated with a loss of protection (Vahlhaus, Schulz et al. 1996). Taken together the results suggest that PKC is involved, but not essential in IP.

Acute protection was also demonstrated to require calcium influx (Smith, Stefenelli et al. 1996; Miyawaki and Ashraf 1997). This effect can be mimicked by exposure to high calcium levels (Node, Kitakaze et al. 1997) and is accompanied by the activation of mitogen-activated protein kinase (MAPK) in the heart (Maulik, Watanabe et al. 1996).

ATP dependent K^+ channels were demonstrated to be effectors of PKC in the immediate phase (Van Winkle, Chien et al. 1994; Yao and Gross 1994). PKC phosphorylation of the channels is believed to increase their “open” probability. These channels open when ATP concentrations decrease. The hyperpolarized membrane potential shortens the action potential, decreases the calcium influx and reduces the ATP consumption of the cell (Noma 1983; Cason, Gordon et al. 1995). These effects may be protective in the heart.

New gene expression is believed to play a key role in the delayed phase of protection. Development of delayed protection is dependent on protein synthesis (Barone, White et al. 1998) and takes at least one day to be established. Since PKC translocates to the nucleus, it has been proposed that the transcriptional activation after IP could at least in part be mediated by PKC in the heart.

Heat shock proteins (HSP) have been implicated in the delayed phase of IP. Increased expression of HSPs 70, 27 and 32 in the brain has been extensively documented in association with a variety of insults, including ischemia, and may play a role in cell survival and recovery after injury (Sharp, Massa et al. 1999; Rajdev, Hara et al. 2000; Xanthoudakis and Nicholson 2000).

A number of stresses, including ischemia can increase the activity of antioxidant enzymes (Hoshida, Kuzuya et al. 1993). These enzymes might be beneficial in limiting reperfusion injury due to reactive oxygen species.

Antiapoptotic proteins, such as Bcl2, have also been shown to be involved in the establishment of protection. Bcl2 action was shown to be associated with reduced cytochrome c release from the mitochondria, an activation of mitochondrial ATP sensitive K^+ channels and reduced association of the proapoptotic gene Bax with the mitochondrial membranes (Liu, Lu et al. 2002; Liu and Downey 1992). Taken together these data prove that Bcl2 has a strong anti-apoptotic influence in ischemic preconditioning.

The example of PKC shows that, although some interesting mediators of IP have been identified, there are many more factors likely to be involved.

1.1.3 IP in nervous tissue

To mimic IP in nervous tissue, *in vivo* and *in vitro* models can be used. The middle cerebral artery occlusion model (MCAO) is a model of focal ischemia (Longa, Weinstein et al. 1989), whereas the 4-vessel occlusion model mimics global ischemia (Pulsinelli and Brierley 1979; Pulsinelli and Buchan 1988).

In contrast to *in vivo* models, *in vitro* models have the advantage that factors which may vary in a living animal, such as blood pressure, collateral perfusion and body temperature are easy to control. Moreover, the experimental procedures do not require vascular surgery on the small arteries of a mouse or a rat. *In vitro* IP experiments were shown to be successful using brain slice preparations (Badar-Goffer, Thatcher et al. 1993; Gage and Stanton 1996) and primary neuronal cultures (Bruer, Weih et al. 1997; Bossenmeyer-Pourie and Daval 1998; Khaspekov, Shamloo et al. 1998). In *in vitro* models, ischemia is usually mimicked by oxygen and glucose deprivation (OGD) and reperfusion by keeping cultures at normal conditions for 24 hours.

Interestingly, there are significant differences regarding the mediators of IP in heart and brain. PKC activation, MAPK signaling and calcium influx via AMPA receptors (Gage and Stanton 1996), all important mechanisms in the immediate phase in the heart, were shown to have no impact on IP in nervous tissue (Tauskela, Chakravarthy et al. 1999).

The mechanisms implicated in delayed protection, and the genes involved, are more consistent between different tissues. As in the heart, heat shock proteins, (Xanthoudakis and Nicholson 2000), antioxidative enzymes and antiapoptotic genes are mediators of protection in the brain (Liu, Lu et al. 2002).

Despite the efforts made over the last years a breakthrough in finding the key players or regulating elements of IP in nervous tissue has not yet been made. As the development of tolerance is dependent on protein synthesis (Barone, White et al. 1998), it seems reasonable that preconditioning of neuronal cells leads to upregulation and translation of protective genes. Therefore, gene expression analysis of preconditioned cells should lead to the identification of novel neuroprotective proteins.

1.2 Differential gene expression methodologies

Differentially expressed genes are believed to play important roles when cells have to adjust to a new environment. The past decade has been marked by a dramatic increase in the availability of techniques to identify and clone genes that are up or down regulated (differentially expressed) in disease states as compared to normal tissue. The applications of such techniques to problems in medicine are manifold and the implications of discovering novel or known genes that are perturbed in neurological disorders are profound (Strakhova and Skolnick 2001). While there are success stories, it is becoming ever more apparent that each of these techniques has its limitations and pitfalls, particularly when applied to the complexity of the central nervous system. Given that these methods are labor-intensive, some very expensive or time-consuming, it is important to understand the limitations and advantages of the different approaches. Moreover, both, the functional and morphological organization of the central nervous system present challenges that may not be encountered in other systems. In the following, the advantages and disadvantages of some of these techniques with respect to a possible application in IP will be considered.

1.2.1 DNA chips

DNA chips have become the most *en vogue* of the gene expression profiling techniques. Recent success in the Human Genome Project made a large amount of genetic information available to the scientific community. Most sequences of the human genome have already been deposited at GenBank. This abundance of genetic information led to the development of microarray technology. Characteristic 25-mer oligonucleotides of 10,000 to 30,000 different genes can be applied to defined spots on one single array. Total or messenger RNAs from two tissues to be compared with each other are reverse-transcribed in the presence of a labeled nucleotide. Subsequently, the prepared probes are hybridized to two identical arrays and signal patterns are analyzed. If a gene is upregulated in tissue A the spot on the array corresponding to that particular gene gives a more intens signal than the same spot on the array hybridized with tissue B. Employing high resolution manufacturing techniques allows for each gene (or EST) to be represented by 16-20 pairs of specific 25-mer oligonucleotides. Sequences are carefully chosen from unique parts of each gene to eliminate cross-hybridizations among genes from the same family. To differentiate true signals from the background each spot on the chip contains a perfect match and mismatched oligonucleotides. Hybridization of probes to mismatched oligonucleotides allows for efficient subtraction of non-specific hybridization and background signals.

GeneChip arrays are able to detect changes in mRNA expression of more than twofold, however only genes of medium to high abundance give sufficient signals. Furthermore, gene chips are restricted to already known genes. While this technology presents an extremely attractive alternative to labor- and time-consuming differential-display techniques, the cost of this technology remains prohibitive for many laboratories. Its application in IP is still untested.

1.2.2 Serial analysis of gene expression (SAGE)

SAGE (Velculescu, Zhang et al. 1995) yields information about absolute transcript numbers of many, if not all, genes expressed in a given tissue and therefore allows for the identification of differentially expressed genes when applied to tissues in different conditions. SAGE is based on the reduction of each expressed transcript to short (14–15 bp), yet representative, sequences which are ligated into concatamers and cloned. The sequencing of these molecules reveals the identity of multiple

transcripts simultaneously. The number of times a particular gene (tag) is detected in a SAGE library therefore provides a quantitative and digital measure of gene expression. However, sequencing of 30,000 to 60,000 SAGE tags per tissue, which is the recommended number of tags, only enables statistically significant detection of medium and highly abundant transcripts (> 50 per cell).

1.2.3 Differential display (DD)

Differential display was the first among the high throughput screening methods for the identification of differentially expressed genes. Since this method was introduced by Liang and Pardee (Liang and Pardee 1992), differential display has been used to characterize hundreds of genes involved in development, cell differentiation, and pathogenesis. mRNA differential display is PCR-based, and offers a rapid and comprehensive analysis of differences in gene expression (Livesey and Hunt 1996). In this technique, total RNA from two or more tissues under study are reverse transcribed using an anchored oligo(dT) primer with either G, C, or A on its 3´ end. By using anchored rather that ordinary oligo(dT) primers, all mRNA species may be separated into three buckets, depending on the nucleotide immediately preceding its poly(A) tail. This separation allows for better resolution by limiting the number of bands that are present in each lane when PCR´s are subsequently loaded and run on a gel. Arbitrary, rather than specific primers are used for this PCR in order to amplify as many different mRNA species as possible. To allow for the subsequent visualization of PCR products, the reaction mixture incorporates one radio-labeled dNTP. Matching reactions are run side-by-side on a gel. The gel is exposed to X-ray film and the band patterns are analyzed. Bands exhibiting different intensities between compared reactions represent candidate genes with altered expression levels. Corresponding bands are cut out and DNA is eluted. The cDNAs are sequenced and compared to GenBank for identification. The obtained cDNAs can be used to hybridize Northern blots to verify the regulation.

The differential-display procedure is relatively straightforward, and potentially results in the rapid identification of genes of interest. However, while mRNA differential display has been successfully used to identify targets of potential interest in the CNS (Huang, Strakhova et al. 1997; Liu, Clemens et al. 1999) the application of this method has proved to be more challenging than might have been anticipated. mRNA differential display may be, as all the other so far discussed techniques, biased

towards highly abundant mRNAs, and characterized by a high rate of false-positives (i.e. changes in cDNA species that cannot not be confirmed by subsequent Northern blot analysis). The proportion of such false signals can be as high as 95% (Sompayrac, Jane et al. 1995). This makes validation of candidate cDNAs by far the most laborious and time-consuming element of the differential display procedure. Methods to overcome the problem of false-positives are discussed in 1.2.5.

1.2.4 Suppression subtractive hybridization (SSH)

Suppression-subtractive hybridization is another method that has recently become available for high-throughput screening of differentially expressed genes (Diatchenko, Lau et al. 1996; Leypoldt, Lewerenz et al. 2001; von Stein, Thies et al. 1997; Diatchenko, Lukyanov et al. 1999). Details of this method are outlined in 2.2.4.
As opposed to conventional subtractive library screening, suppression subtractive hybridization allows for the identification of both up- and downregulated messages. In contrast to differential display, which is biased toward abundant mRNAs, suppression subtractive hybridization may provide an up to 1000-fold enrichment of differentially expressed transcripts, making this method very attractive for examination of potential differences in frequent and rare mRNA species. Considering the complexity of the central nervous system, it seems logical that changes in expression of the many low abundant genes may have dramatic results. Therefore, when studying the CNS, SSH seems to be an appropriate technique. One principal disadvantage of suppression subtractive hybridization, as well as the earlier discussed differential display technique, is the high rate of false positive results (up to 95%). This is especially true if two conditions that only differ in the metabolic state are compared. Therefore an effective screening of candidate clones is necessary.

1.2.5 Elimination of false-positive clones in DD and SSH

Elimination of false positive clones can be achieved by means of conventional Northern blot analysis. However, in most cases this approach is not practical, since it requires relatively large quantities of RNA (which in most cases is limiting) and is extremely laborious. In the past few years, methods that permit a more economical use of experimental recources and time have been developed. In many cases, selection of the appropriate clones is achieved by the so-called “reverse Northern blot” (Corton and Gustafsson 1997; Huang, Strakhova et al. 1997). In this procedure, colonies from the cloned reactions are randomly picked. The inserts from these

colonies are amplified, by using primers flanking the cloning site. PCR reactions that contain inserts of the expected size are then dot-blotted on duplicate nylon membranes. Probes for hybridization are prepared by reverse transcription of each of the original RNA samples in the presence of a radiolabeled nucleotide. Each of the two different probes is hybridized to identical membranes and the results are compared. Those dot-blotted PCR fragments that produce signals of considerably different intensities on the two identical blots, hybridized with the two different radiolabeled cDNAs, represent transcripts that may be truly differentially expressed between the two groups. Since equal amounts of DNA are applied to the duplicate membranes, differences in intensity can only result from differences in the amount of hybridized molecules in the two experimental RNA pools. Colonies containing truly differentially expressed cDNAs are sequenced. Inserts can subsequently be used as hybridization probes for Northern blots or virtual Northern blots (Endege, Steinmann et al. 1999; Spirin, Saghizadeh et al. 1999). Quantitative RT-PCR (Gomi, Sun et al. 1999; Leypoldt, Lewerenz et al. 2001) is another commonly used, but more laborious strategy to verify up-regulation. Regardless of the approach, validation of gene candidates is time consuming and is usually a much longer process than the differential gene expression method itself.

1.3 Purpose of this study

Short episodes of ischemia followed by reperfusion protect mammalian cells and tissues against a subsequent lethal ischemia and reperfusion. This phenomenon is called ischemic preconditioning (IP).

As the development of tolerance depends on protein synthesis (Barone, White et al. 1998), it seems reasonable to speculate that preconditioning of neuronal cells leads to upregulation and translation of protective genes. Therefore, the transcriptional analysis of preconditioned cells should lead to the identification of novel neuroprotective proteins. A very efficient method for the identification of differentially expressed genes is the suppression subtractive hybridization technique (SSH).

The purpose of this study was to identify uncharacterized transcripts and known genes upregulated in rat primary cortical cultures preconditioned by oxygen glucose deprivation (OGD). Although other groups used differential gene expression strategies in the paradigms of global and focal ischemia (Wang, Zhan et al. 2001; Yokota, Uchijima et al. 2001; Trendelenburg, Prass et al. 2002), this is the first report describing transcriptional differences in the clearly protective paradigm of ischemic preconditioning.

2. Materials and Methods

2.1 Cell biology

2.1.1 Materials

Phosphate Buffered Saline (PBS)

NaCl	8 g
$Na_2HPO_4 \cdot (7\ H_2O)$	1.44 g
KH_2HPO_4	0.24 g
KC	0.2 g

Adjust to pH 7.4 with NaOH

Final volume 800 ml

EDTA

0.002% w/v EDTA (Sigma)

Trypsin

0.005% w/v EDTA (Sigma)

HEPES

1 M HEPES pH 8.0

2.1.2 Primary neuronal cultures

Cortical neurons were prepared from whole cerebral cortices of fetal Wistar rats (E16-18) as described previously (Bruer, Weih et al. 1997). After removing the meninges the tissue was minced and digested with trypsin (15 min, 37°C) followed by mechanical dissociation. $2x10^6$ cells were seeded in 32 mm culture dishes (Nunc, Wiesbaden, Germany) in plating medium consisting of neurobasal medium with B-27 serum-free supplement, 100 U/ml penicillin and 100 µg/ml streptomycin, 2.5 mM glutamate and 2 mM L-glutamine (all Gibco BRL, Division of Invitrogen, Groningen, Netherlands). Culture dishes were precoated with 10 µg/ml poly-L-lysine (Biochrom, Germany) and 10 µg/ml collagen G (Biochrom, Germany). For immunocytochemical analysis, $3x10^5$ cells were plated on similarly coated 10 mm cover slips. The rat

cortical cultures were preparated by Susanne Thomsen, and handed over after preparation. After four days, medium was changed to neurobasal medium supplemented with B-27 only.

2.1.3 Oxygen-glucose deprivation (OGD)

On DIV8 (day in vitro) 2% fetal calf serum (FCS, Linaris, Germany) was added to the cultures. For Ischemic Preconditioning (IP), maintenance medium was collected and stored. On DIV9, cultures were washed twice with PBS. Experimental cultures were then subjected to the deoxygenated OGD Buffer containing NaCl (115 mM), KCl (5.4 mM), $MgSO_4$ (0.8 mM), CaCl (1.8 mM), HEPES-NaOH (20 mM), 2-deoxy-D-glucose (20 mM), and 2% FCS. Cultures were then placed in an airtight plastic chamber (figure 2.1), flooded with argon, and kept at 37°C for the times corresponding to experimental paradigms.

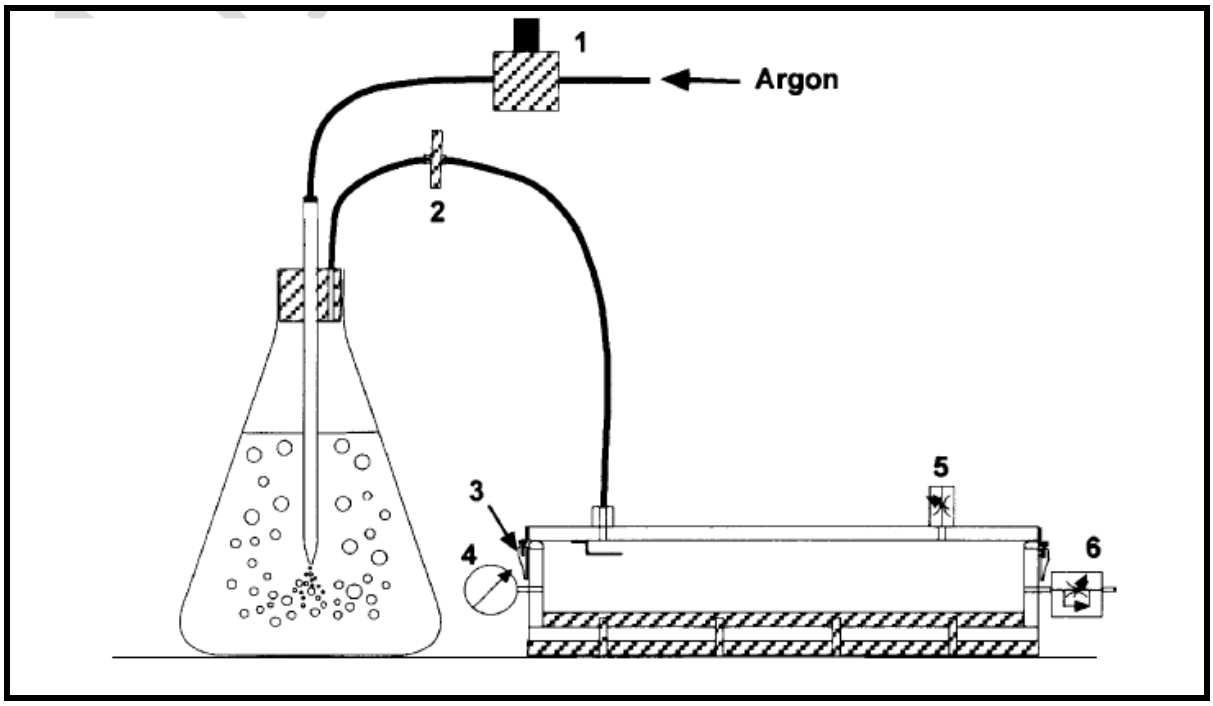

Figure 2.1. Scheme of the ischemia chamber used. Pressure of argon entering the gas-washing flask is controlled by a one-way fow control valve (1). The humidified gas is sterile-filtered (2). The box is constructed of Makrolon plates with two aluminum plates on the bottom (striped); it can be tightly closed by toggle latches (3), displays a pressure gauge (4), a safety valve (5) and a quick coupling socket (6) (Lewerenz, Thomsen et al. 2004).

Matched sister cultures were treated with control buffer containing D-glucose (15 mM) instead of the glucose antimetabolite that was used for OGD. Control cultures (referred to as BSS for buffered saline solution) were placed in a CO_2 incubator for corresponding times.

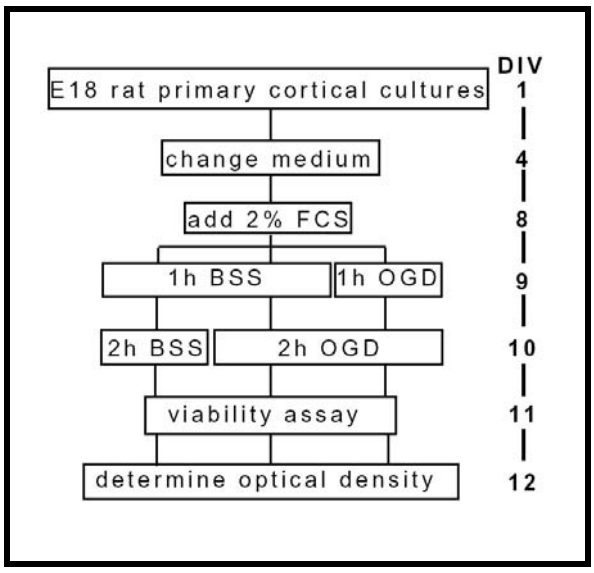

Figure 2.2: Flowchart of experimental procedures. After preparation of E18 cortical cultures medium was changed and FCS added as indicated. Sister cultures were subjected to OGD or BSS treatment. 24 hours after experiments viability of cultures was assessed by MTT assay. E18, embryonic day 18; DIV, day in vitro.

After OGD, experimental and BSS cultures were washed twice with PBS, conditioned medium was added, and cultures maintained in the CO_2 incubator for 24 hours. On DIV10, either cell viability was assessed or cultures were subjected to lethal OGD or BSS treatment using the same procedure as described above. The full preconditioning procedure is depicted in the flow chart in figure 2.2.

2.1.4 Phase contrast microscopy

Cell damage was estimated in all experiments qualitatively by examination of the cultures under phase contrast microscopy (Axiovert 25, Carl Zeiss, Germany) at 200-fold and 320-fold magnification.

2.1.5 Viability assay

Cell survival was measured 24 hours after OGD or BSS by the MTT assay (Carmichael, DeGraff et al. 1987). In this assay the amount of blue formazan produced by viable cells from a tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTT, Sigma) is proportional to the number of viable cells (Mosmann 1983). Briefly, MTT diluted in PBS was added to the culture media in a final concentration of 1 mg/ml and incubated at 37°C. After 2 hours, cells were lysed for 24 hours with a buffer containing 50% DMF, 20% SDS, 2.5% acetic acid and 2.5% 1 M HCl (pH 4.7). Finally 100 µl of the lysate was removed and the formation of formazan monitored by reading optical density at 550 nm using a microplate reader (SLT Labinstruments, Crailsheim, Germany).

2.1.6 PC12 cells

56.7 cm^2 cell culture dishes (Nunc, Wiesbaden, Germany) were precoated with Collagen (Roche Diagnostics, Mannheim, Germany, final concentration: 5 $\mu g/cm^2$). RPMI 1640 medium (BioWhittaker Molecular Applications, USA) was supplemented with 10% horse serum (Seratec, Goettingen, Germany), 5% FCS, Penicillin-Streptomycin (final concentration 100 units/ml Penicillin and 100 µg/ml Streptomycin), 1 mM Glutamine, 2 mM Na Pyruvate (all Gibco BRL, Division of Invitrogen, Groningen, Netherlands), and HEPES pH 7.2 (10 mM). Frozen PC12 cells were generously provided by the lab of Prof. Dr. Melitta Schachner-Camartin and thawed in a 37°C water bath for 30 s. As soon as the frozen nucleus was detached from the wall of the cryotube the whole content was poured into a cell culture dish containing 12 ml medium. To dispense the cells, the dishes were moved in the shape of an eight before placing them in the 37°C CO_2 incubator. Medium was replaced with 8 ml of fresh medium every 3 days. Confluent cells were detached from the dish by repetitive pipetting and 2 ml of the suspension were added to 10 ml of fresh medium. To differentiate PC12 cells Nerve Growth Factor (Upstate Biotechnologies, USA) was added in a concentration of 50 ng/ml. After about 7 days most of the cells had differentiated (Greene and Tischler 1976).

2.2 Molecular biology

2.2.1 Materials

20 x SSC

3 M NaCl

0.3 M Trinatriumcitrat

pH 7.0

DENAT

1.5 M NaCl

0.5 M NaOH

10 x MOPS

0.2 M MOPS pH 7

0.5 M NaAc pH 7

0.01 M EDTA pH 8

50 x TAE

2 M Tris

1 M Acetic Acid

0.05 M EDTA pH 8

Bromphenole blue sample buffer

10 mM NaOH

10% Glycerin

0.01% Bromophenole blue

Chemicals:

Chloroform p.a.

Ethanol p.a.

Methanol p.a.

Isopropanol p.a.

Formaldehyde 37% p.a.

Acetone p.a. (all Merck, Darmstadt, Germany)

Formamide (Fluka, Buchs, Switzerland)

All other chemicals were purchased form Merck (Darmstadt, Germany) Sigma (St. Louis, USA) or Fluka (Buchs, Switzerland).

2.2.2 RNA preparation

About $2x10^7$ IP and BSS treated cells that were stored at –70°C as a dry pellet were thawed in 1 ml of TRIzol Reagent (GibcoBRL). Cells were lysed by repetitive pipetting and RNA prepared as described in the TRIzol protocol. Briefly, 0.2 ml Chloroform was added, tubes were incubated to allow complete dissociation and then centrifuged to separate the aqueous and the organic phases. The aqueous phase containing the RNA was carefully removed from the tube, and the remainder

stored for protein or DNA isolation (see Protein biochemistry). 0.5 ml isopropanol was added to the RNA solution, incubated and centrifuged. In order to wash the RNA the supernatant was decanted and 1 ml of 75% ethanol added to the pellet. The pellet was vortexed, centrifuged and the supernatant decanted. The RNA was allowed to dry at room temperature until the whitish pellet had a gel like consistence and color. 20 to 50 µl of RNAse free water was added and the pellet dissolved by vortexing and shaking in an incubator at 50°C. To determine RNA quality 1 µl RNA supplemented with 1 µl sample buffer (50 µl 5x Bromphenole blue sample buffer and 0.7 µl 1000x Ethidiumbromide) was electrophorezed (Mini Sub Cell GT, Sub Cell GT, PowerPac 300, Bio-Rad Laboratories, Germany) on a 1% weight per volume (w/v) agarose (Sea Kem LE Agarose, BioWhittaker Molecular Applications, USA) RNA gel at 20 V/cm for 10 minutes in a cold water bath to minimize RNA degradation and analyzed. If the 28S and 18S RNA bands were visible in an intensity of roughly 3:1 and the tRNA band was visible as well, the quality of the RNA was believed to be sufficient for further experiments. RNA was quantified photometrically (UV 160 A, Shimadzu, Japan) by diluting 1 µl of RNA into 100 µl of water, and, after calibration of the photometer, the probe was measured against water at 260 and 280 nm. The amount of RNA obtained can be calculated by applying the following formula: OD_{260} x dilution factor x total amount of RNA [µl] / 0.025 = amount of RNA [ng/µl]. Typically 50 to 100 µg total RNA could be obtained from the above mentioned amount of cells. The OD_{260}/OD_{280} quotient indicates the purity of RNA and was believed to be sufficient when higher than 1.65.

The Dynalbeads Oligo $(dT)_{25}$ kit (Dynal, Norway) was used to purify mRNA from 100 µg of total RNA following the manufacturer`s instructions. Briefly, the RNA was heated to dissolve any secondary structures. Then the RNA was mixed with the Dynalbeads Oligo $(dT)_{25}$ which contain a magnetic particle with an Oligo $(dT)_{25}$ tail to which only the polyA tail of the mRNA anneals. Then the tube is placed in a magnetic field which pulls the particles, with the attached mRNA, towards the wall of the tube. While in the magnetic field all other components of the solution can be collected from the bottom of the tube. After washing once again in the same manner, pure mRNA can be eluted from the magnetic beats. The amount of mRNA, normally only 1-2% of the total RNA was calculated from the previous amount of total RNA.

2.2.3 SMART PCR cDNA Synthesis

cDNA for the following subtraction experiment was generated using the SMART PCR cDNA Synthesis Kit (Contech, USA). This system was chosen because of the limited amount of starting material (preconditioned cells) that could be produced. It allows for the amplification of first strand cDNA by PCR. A schematic overview of this method is depicted in figure 2.3. Generally, all procedures were performed as described in the protocol with a few adjustments. Briefly 5 µl polyA$^+$ RNA (0.5 µg) were used for first strand cDNA synthesis. Therefore the amount of buffer was increased to 2.5 µl. SMART cDNA synthesis comprises a few specialties which should be mentioned. The Oligo dT primer (CDS primer) used for the reaction contains a primer binding site which allows for subsequent PCR amplification. The other primer binding site is located on the SMART II A oligonucleotide. The SMART II A oligonucleotide anneals to the 3´end of the first strand after the polymerase has added a few cytidine residues (dC tailing) to the end of the first strand. After annealing of the SMART II A oligonucleotide the polymerase extends the first strand to the end of the oligonucleotide. The SMART PCR is the step where the single stranded (ss) SMART cDNA is amplified using SMART cDNA as template for PCR with the respective primers. 17 cycles of PCR were sufficient to obtain enough amplified cDNA from IP RNA whereas 18 cycles were required for BSS RNA. After PCR the samples were purified according to the protocol. To quantify the amount of cDNA after purification the DNA Dipstick Kit (Invitrogen, Groningen, Netherlands) was used.

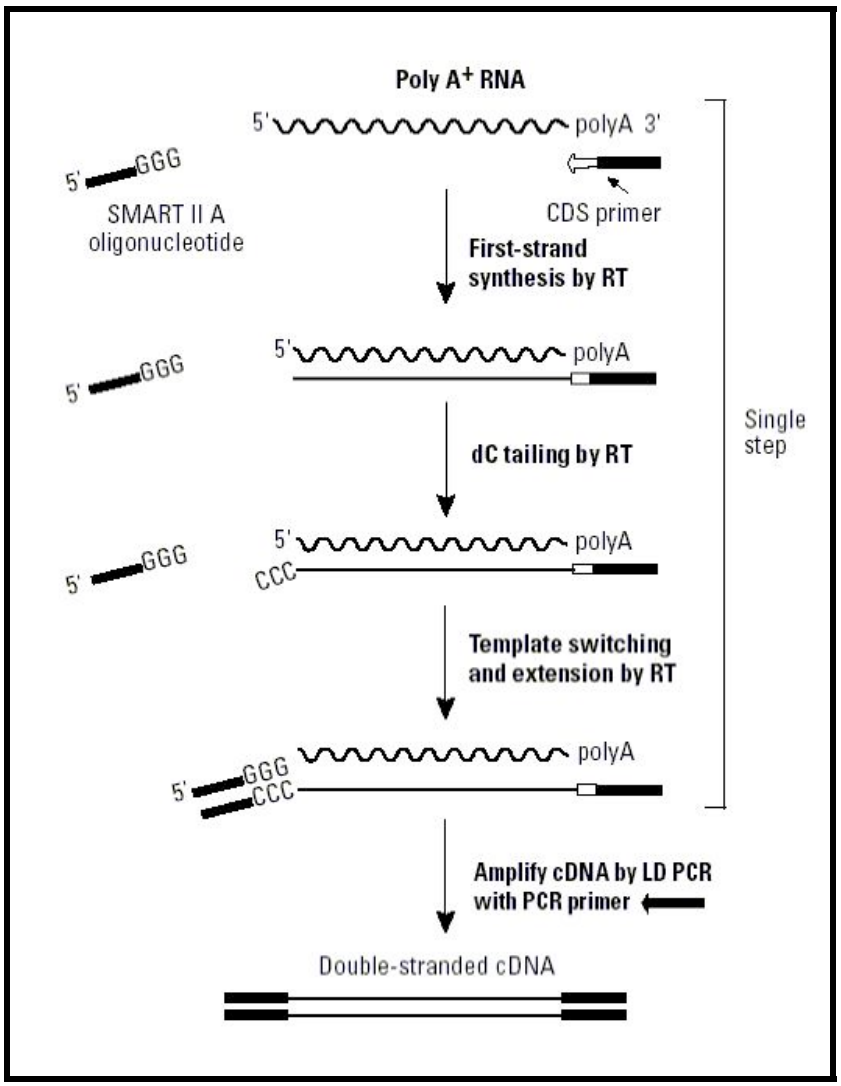

Figure 2.3: Schematic overview of SMART PCR cDNA synthesis. CDS primer, cDNA synthesis primer; dC, deoxycytidine; RT, reverse transcriptase; Adapted from the SMART PCR cDNA synthesis kit user manual (Clontech).

2.2.4 Subtractive Suppression Hybridization (SSH)

SSH was used to identify genes upregulated by IP. SSH is a combination of a classical hybridization with a PCR based suppression step. One characteristic of this method is the normalization of high and low expressed sequences during hybridization. This means that not only upregulated genes with a high number of copies within the cell can be detected, but also genes with a few copies per cell. In the following, the principles of SSH, as developed by Diatchenko (Diatchenko, Lau et al. 1996; Diatchenko, Lukyanov et al. 1999), will be explained. A schematic overview is depicted in figure 2.4. Genes upregulated by IP (cDNA pool A), by definition, should not, or to a much lesser extent, be present in the BSS sample (cDNA pool B). At first, both cDNAs were digested with a restriction enzyme with a high probability of cutting the cDNA (e.g. RSA I, 4-base cutter, statistically cuts every 256 base pairs). cDNA pool A is then divided into two equal portions. One part is ligated to adaptor 1, and the other part to adaptor 2. SSH comprises two hybridization steps. For the first

hybridization, all cDNAs are denatured and an excess of cDNA B is added to the two portions of cDNA A. In this step, such cDNA species which are present in both pools (not differentially expressed) form hybrids with partners from the other pool (c-type molecules). Therefore, these cDNA species will be subtracted during suppression PCR, because only a-type molecules will later be amplified. The first hybridization is also the step where normalization of high and low expressed differential transcripts takes place. Differentially expressed transcripts do not find hybridization partners within the cDNA pool B. Therefore they hybridize, with respect to their abundance with themselves (b-type molecules). Since frequent transcripts hybridize faster with each other than non-frequent transcripts, the normalization takes place, because as mentioned previously, only a-type molecules will later be amplified in the suppression PCR.

For the second hybridization the two portions are mixed with an excess of cDNA B without previous denaturation.

Since only a-type molecules remain single stranded, only they hybridize. By hybridizing with cDNAs from pool B (red) the reaction is further enriched for differentially expressed transcripts. A-type molecules hybridizing to the a-type molecules ligated to the other adaptor form e-type molecules.

After generating the complementary strands to the adaptors suppression PCR is performed with primers binding to the complementary sequence in the outer (green) regions of the adaptors. A- and d-type molecules are not amplified because they either do not have a complementary strand or do not have the adaptors with the primer binding sites, respectively. C-type molecules contain adaptors on one side only and can therefore not be amplified exponentially. B-type molecules form pan-like structures after denaturation. Therefore amplification is effectively inhibited. These pan-like structures are formed because intramolecular annealing between the two adaptors is energetically more stable and kinetically faster than the intermolecular annealing between primers and the adaptor (Siebert, Chenchik et al. 1995).

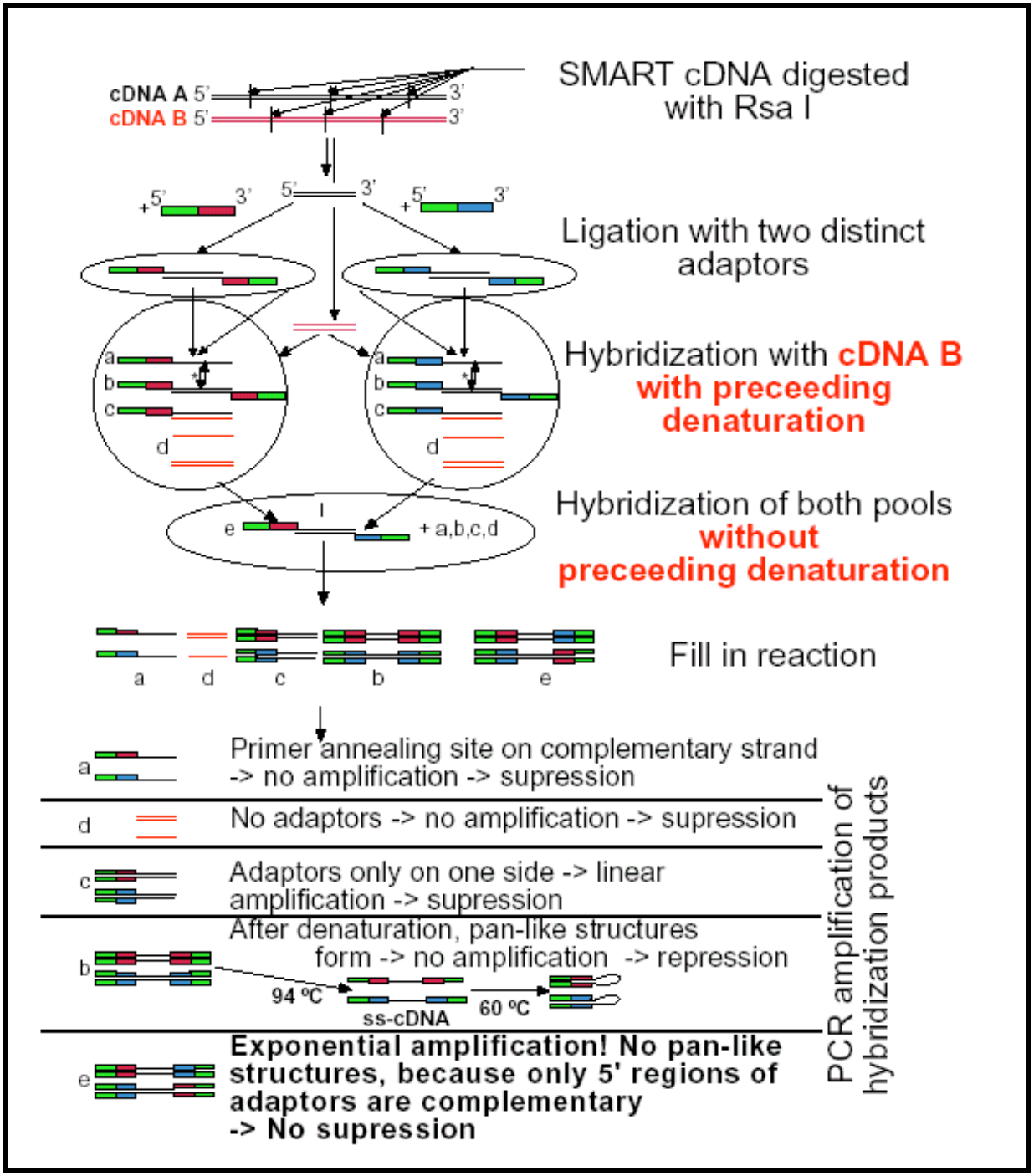

Figure 2.4: SSH overview

Because of the two different adaptors only e-type molecules can be amplified exponentially. Therefore this step is called suppression PCR. A mathematical simulation of these processes is described in Gurskaya, Diatchenko et al. 1996.

Subtraction was performed in both directions, resulting in two libraries. The first library, IP minus BSS represents genes which are upregulated by ischemic preconditioning and the second library (BSS – IP) represents genes which are downregulated by IP.

Generally, SSH was performed as described in the protocol with only minor adjustments. Briefly, the RSA I digestion was performed over night (ON) at 37°C. The buffer contained 1 mM Bis-Tris-Propan-HCl, pH 7.0; 1 mM $MgCl_2$; 0.1 mM dithiothreitol. 0.3 units/µl RSA I (New England Biolabs, Beverly, USA) and 10 ng/µl bovine serum albumine (BSA, New England Biolabs) was added. A small portion of the undigested cDNA was saved and analysed alongside the digested cDNA on a 1% Agarose gel to check for restriction efficiency.

For ligation, concentrated T4 DNA ligase (final concentration 200 units/µl; New England Biolabs, USA) with the corresponding buffer (final concentration 50 mM TrisCl pH 7.5, 10 mM $MgCl_2$, 10 mM Dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumine; New England Biolabs, USA) was used to achieve high ligation efficiency, which is critical for all following experiments. Ligation efficiency was checked by PCR reactions as suggested in the SSH protocol. Briefly, the housekeeping gene β-Actin, which is known to have a RSA I restriction-site, was chosen and specific primers were constructed for this gene using the OLIGO software (Molecular Biology Insights Inc, Cascade, USA). The sequences of all primers and probes, as well as the annealing temperatures and cycle conditions can be obtained from table 2.1. The control experiment for ligation efficiency generally comprises two types of PCR reactions. The first is a reaction with the specific primers which results in a PCR product of the corresponding size. In the second reaction, only one of the specific primers is used and one of the adaptor primers is used as a second primer resulting in a longer PCR product. If the specific and the longer PCR products were visible in the gel after roughly the same cycle numbers, ligation was believed to be successful. In this way, both cDNA species were checked for ligation efficiency to both adaptors. First and second hybridization were performed according to the manufacturer's protocol. Primary PCR (suppression PCR) was performed with 27 and 30 cycles and secondary PCR with 12 cycles to amplify the transcripts to a level sufficient for cloning. Subtraction control experiments were performed as described in the SSH manual.

2.2.5 Cloning of the subtracted libraries

Amplified and subtracted libraries were ligated into the pGEM-T vector (Promega, Madison, Wisconsin, USA). This vector possesses a 3´ polyA overlap which is complementary to the 3´ polyT overlap that most Taq DNA polymerases add to the 3´

end of the PCR product. Furthermore this vector contains a multiple cloning site within the coding region of the β-galactosidase gene and an ampicillin resistance gene. When a DNA fragment is successfully ligated into the vector, the β-galactosidase gene is disrupted and the resulting colonies appear white instead of blue in the presence of 30 µl of 100 mM Isopropyl-1-Thio-β-D-Galactosid (IPTG) und 30 µl of 50 mg/ml X-Gal (both Biomol, Hamburg, Germany).

Vectors were transformed into competent DH5α E. coli bacteria using a standard protocol. Bacteria were plated on ampicillin containing agar plates and incubated overnight at 37°C. 350 white clones were randomly picked and grown overnight in 96 deep well plates (Advanced Biotechnologies LTD, Epsom, UK) containing 1.25 ml LB medium supplemented with 100 µg/ml ampicillin (Gibco BRL).

2.2.6 Differential Screening

The subtracted libraries still contain false positive clones. To eliminate these false positive clones, a differential screening was performed, generally as described in the PCR select differential screening kit (Contech, La Jolla, USA). At first, the presumed regulated transcripts were amplified from bacterial suspension by colony PCR. This was possible using the adaptor primers np1 and np2r (for sequences see table 2.1) since the cloned fragments still contain adaptors that were ligated to both ends of the fragments. The following PCR conditions were used: 10 mM TrisCl pH 8.8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.4 mM of the oligonucleotides np1 and np2r; 0.4 mM of each deoxynucleotide (dATP, dCTP, cGTP, dTTP); 25 units/µl Taq Polymerase (Eppendorf, Hamburg, Germany); 0.5 µl bacterial solution in 30 µl H_2O). Bacterial suspension was transferred to the PCR tubes using a 96 pin dot blot tool (V&P Scientific INC, San Diego, CA, USA. Cycle conditions were 94°C for 1 minute, and 30 cycles 94°C for 10 s, 65°C for 30 s und 72°C for 90 s in 96-well PCR plates (Advanced Biotechnologies, Surrey, Great Britain) in a cycler (TGradient, Biometra, Göttingen, Germany). 5 µl of the reaction product were analyzed on a 2% agarose gel (0.5 µg/ml ethidiumbromide) (Mini Sub Cell GT, Sub Cell GT, PowerPac 300, Bio-Rad Laboratories, München, Germany). PCR reactions where no reaction product was visible were used as negative controls for the later hybridization experiments or substituted with positive controls such as GAPDH or β-Actin PCR products which were used to normalize dot blots. Of the colony PCR products 0.5 µl were blotted onto a nylon membrane (Porablot; Marchery & Nagel GmbH, Düren Germany) using

the same dot blot device. Two identical replicas were produced for every single dot to control for internal errors. Four identical replicas of each blot were produced. The four membranes represent identical copies of the 350 clones that were picked from the forward subtracted (IP-BSS) library. PCR products were denatured by soaking the whole blot in DENAT for 5 minutes and crosslinked using a UV stratalinker (Stratagene, La Jolla, USA) after allowing the blot to dry.

The four blots were then hybridized with radiolabeled cDNAs from the IP and BSS samples, or hybridized with radiolabeled cDNAs from the two subtracted libraries. SMART amplified cDNA from an independent SMART PCR was random labeled (Megaprime DNA Labeling System, Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers instructions using 50 µCi α-^{32}P dATP (Amersham Pharmacia Biotech, Uppsala, Sweden). Generally, during random labeling, random nonameres are used as primers and Klenow polymerase is used to extend the strand and incorporate the radioactive and normal deoxynucleotides into the growing chain. All reaction products were purified using G-50 Sephadex Quick Spin Columns (Boehringer Mannheim Corporation, Indianapolis, USA). The gel matrix eliminates all fragments up to a size of 72 bp as well as the non incorporated radioactivity. Specific activity was measured using a Beckman counter (LS 6000IC, Beckman Instruments, Fullerton, USA). If the specific activity was lower than 4 x 10^8 counts per µg DNA, labeling experiments were repeated with an increased amount of template since this appeared to be critical.

The cDNAs from the forward and reverse subtracted library were labeled by radioactive linear PCR. Reaction conditions were the following: 20 mM TrisCl pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 1 mM np1 primer; 20 µM of the deoxynucleotides dCTP, cGTP, dTTP; 1.3 µM of the deoxynucleotide dATP; 7 µM (≈ 50 µCi) α-^{32}P dATP; 0.1 units/µl Platinum Hot Start Taq polymerase (Life Technologies, Division of Invitrogen, Groningen, Netherlands); 1 µl (25 ng) of the purified PCR product from the secondary PCR was used as template in a final volume of 25 µl. PCR parameters were 94°C for 2 minutes, 65°C for 2 minutes und 72°C for 5 minutes for a total number of 30 cycles. Reaction products were again purified and specific activity measured as described for random labeling. As for random labeling experiments, reactions were repeated if specific activity was lower than 4 x 10^8 counts per µg DNA.

The four blots were prehybridized in hybridization solution (ExpressHyb, Clontech, Palo Alto, California, USA) to reduce background. Then, each of the four identical

blots was hybridized with one of the labeled cDNAs using 2 x 10^6 counts per ml hybridization solution overnight at 68°C (OV 5, Biometra, Göttingen, Germany) under continuous rotation. The membranes were washed twice in 2 x SSC, 0.1% (v/v) SDS (sodium dodecyl sulfate) and twice in 0.1 x SSC, 1% SDS for 15 min. at 68°C. Autoradiography was performed using a phospho-imaging system (Phosphoimaging device BAS2000; Fuji Photo Film GmbH, Düsseldorf, Germany) for various exposure times. The digital picture was analyzed using the Visual Grid Software (GPC Biotech, Martinsried, Germany) which can be downloaded from the manufacturer`s homepage http://www.gpc-biotech.com. With this software the mean from two identical replicas of each dot was calculated and the background subtracted. The local background was calculated for each dot by defining two dots in the vicinity of two identical replicas. Clones hybridizing to SMART cDNA from IP cells, but not to BSS samples, and clones hybridizing to the forward subtracted library but not to the reverse subtracted library, or clones showing an at least twofold higher intensity in both screens were considered to be differentially expressed and sequenced.

2.2.7 DNA Sequencing and gene identification

cDNA inserts of presumed differentially expressed clones were amplified by colony PCR as described above and sequenced by the dideoxy chain termination method (Sanger, Nicklen et al. 1977) using an Applied Biosystems automated DNA sequencer (Applied Biosystems, Foster City, California, USA). The sequences were compared to the publicly accessible databases by Advanced Blast 2.0 (Altschul, Gish et al. 1990) at http://www.ncbi.nlm.nih.gov/blast and relevant publications were obtained from the PubMed database at http://www.ncbi.nlm.nih.gov/pubmed. Information on the tissue distribution was obtained by using Bodymap at http://bodymap.ims.u-tokyo.ac.jp. For clones showing homologies to expressed sequence tags, but not to known mammalian genes, tentative human consensus sequences were created by assembling overlapping ESTs into a virtual transcript. This was done using the DNASTAR software. For some genes, information on the tissue distribution and their relevance to disease, could be obtained by comparing the sequences to the SAGE database (serial analysis of gene expression) (Velculescu, Zhang et al. 1995) at http://www.ncbi.nlm.nih.gov/SAGE/. We preferentially analyzed clones that showed a mostly neuronal expression pattern and omitted those with no homology to any known sequences.

2.2.8 Virtual Northern blots

Virtual Northern blots were performed to verify the upregulation of the identified sequences. The advantage of the virtual Northern blot is that PCR amplified cDNA is used instead of RNA. This method can therefore be of great value if RNA is limited. At first, new and independent SMART PCRs were performed from the IP and BSS samples. Reaction products were then separated on a 1.2% (w/v) agarose gel and analyzed under UV light. To denature the reaction products the gel was submerged in DENAT for 20 min. A large glass dish was filled with DENAT and a glass plate was placed on the dish so that the plate formed a bridge over the dish. One large sheet of Whatman Paper (Schleicher & Schuell, Dassel, Germany) was wetted in DENAT and placed on the glass bridge so that the two ends of the paper dipped into the DENAT in the glass dish. Three sheets of Whatman paper were cut to the size of the gel, wetted in DENAT and placed on the large paper. The gel was placed on the three Whatman papers upside down. A Nylon membrane was cut to the size of the gel and allowed to submerge in DENAT for 5 min and placed on the gel. Positions of the slots were indicated with a pencil and the lower right corner was marked to indicate the orientation of the membrane. An additional three sheets of Whatman papers were cut, wetted and placed on the blot. Each time air bubbles were squeezed out using a pipette. The blot was surrounded with parafilm and covered with a stack of paper towels. A weight was added to the top of the blot and left overnight. The next day the blot was carefully disassembled and the membrane allowed to dry completely before UV crosslinking.

Radioactive probes were generated by linear radioactive PCR as described in chapter 2.2.6. Nested primer np1 was used for linear amplification of 25 to 50 ng of purified colony PCR products. Blots were hybridized to the probes, washed and autoradiography performed as described in 2.2.6.

2.2.9 Quantitative Polymerase Chain Reaction (qPCR)

qPCR experiments were performed using the Platinum Hot Start Taq Polymerase with the corresponding buffer (Life Technologies, Division of Invitrogen, Groningen, Netherlands). Forward and reverse primers were designed for the detected EST, Bax Inhibitor-1, ABC 50 and β-Actin and synthesized by MWG Biotech (Ebersberg, Germany). The original, unamplified, single stranded cDNA of IP and BSS cells was used as template. cDNA was normalized to β-Actin expression by PCR. In order to

estimate the exponential phase of amplification, aliquots of the PCR reaction were removed after 15, 20, 25, and 30 cycles, and analyzed on ethidiumbromide stained agarose gels. PCR reactions were performed with either β-Actin or gene specific primer pairs (see table 2.1). All results were reproduced at least three times. 3 µl aliquots of the reaction were removed quickly after every second or third cycle and dot blotted onto a nylon membrane with the blotting procedure described above. Blots were denatured, crosslinked and hybridized with β-Actin or gene specific end-labeled probes. Probes were generated using the Megaprime DNA labeling system (Amersham Pharmacia Biotech, Little Chalfont, UK) and 50 µCi [γ-^{32}P]-ATP. Approximately 10^7 cpm/ml hybridization solution (0.6 M NaCl, 120 mM Tris pH 8, 4 mM EDTA pH8, 0.2% SDS, 50 µg/mL heparin) was used and membranes incubated overnight at 42°C. Membranes were washed twice in 2 x SSC, 0.1% SDS and twice in 0.5 x SSC, 0.5% SDS at 42°C for 15 min. Autoradiography was performed using a phospho-imaging system (Fujix) for various exposure times. Autoradiographies were analyzed using Tina 2.10 h (Raytest Isotopenmessgeräte GmbH, Straubenhard, Germany). The density of each spot on the blot was determined (with the background subtracted) and plotted on a semi logarithmic scale against the cycle number for each reaction. PCR reactions using the same primer pairs on normalized cDNA from IP and BSS cells were compared. All values were presumed to be valid if compared curves were parallel at a given cycle number, indicating both reactions were in the exponential phase of amplification. Valid data was normalized to β-Actin optical density. The mean, standard deviation, and standard error of the mean were calculated from the normalized optical densities of amplification products from the two templates and Student's ratio paired t-test performed to determine the statistical significance.

Realtime quantitative PCR. A Mouse Multiple Tissue cDNA panel (MTC™, Clontech) served as template in real time PCR performed on a ABI PRISM 7900HT real-time PCR cycler (Applied Biosystems) using the qPCR™ Core Kit for Sybr™ Green I (Eurogentec) according to the manual. PCR was done in triplicates with primers and conditions given in table 2.1. Primers used with the cDNA panel are based on a mouse sequence tag located downstream of the TrkB open reading frame (Image clone ID 30622250) showing 88% identity with BU198076 (BLAST p-value 5.1e-157). A standard dilution series of TrkB.T1 vector DNA was supplied as template to calculate amplification efficiencies, perform absolute quantification and

test for differences in efficiency between 96-well plates. All runs were analyzed using the SDS 2.1 software (Applied Biosystems). Baseline and threshold was optimized empirically, PCR efficiency was calculated using the slope of the regression curve fitted to the standard dilution C_T values. Relative regulation χ normalized to mean housekeeping gene regulation was calculated using $x = \frac{E_{TrkB}^{\Delta C_T}}{\frac{1}{2}\left(E_{gapdh}^{\Delta C_T} + E_{\beta-actin}^{\Delta C_T}\right)}$ with E amplification efficiency, ΔC_T difference of C_T values between two different samples. Mean and standard error was calculated using Prism software (Graphpad).

2.2.10 Northern blotting

A Northern blot using rat cortical RNA was performed to obtain information on the total length of the truncated TrkB isoform.

Two grams of agarose were dissolved in 144 ml of distilled and RNAse free water heated and cooled to 60°C in a water bath. Subsequently the flask was placed in a fume hood and 20 ml of 10 x MOPS and 36 ml of 37% formaldehyde were added. The gel was poured and allowed to set. The comb was removed and the gel placed in a gel tank filled with 1 x MOPS.

10 µg of total RNA were adjusted to a volume of 5.5 µl. 2.5 µl of 10 x MOPS, 4.5 µl of 37% formaldehyde and 12.5 µl of formamide were added. The sample was vortexed and heated to 55°C for 10 min. 5 µl of loading buffer containing 10 mM sodium phosphate (pH 7.0) / 1.1 M formaldehyde was added to the sample and 30 µl (10 µg RNA) were loaded into each of the wells. The samples were run on the gel at 2.5 V/cm alongside 10 µg of a RNA size marker (Promega) until the bromphenol blue had migrated 2/3 of the length of the gel.

To partially hydrolyze the RNA, the gel was placed in an RNAse free dish filled with 0.05 M NaOH / 1.5 M NaCl for 20 min followed by neutralizing the gel in 0.5 M Tris (pH 7.4) for 30 min. The gel was placed in 20 x SSC for 45 min. A large glass dish was filled with 20 x SSC and a glass plate was placed on the dish so that the plate formed a bridge over the dish. One large sheet of Whatman paper was wetted in 20 x SSC and placed on the glass bridge so that the two ends of the paper dipped into the 20 x SSC in the glass dish. Three sheets of Whatman paper were cut to the size of the gel, wetted in 20 x SSC and placed on the large paper. The gel was placed on the three Whatman papers upside down. A Nylon membrane was cut to the size of the gel and allowed to submerge in RNAse free water for 5 min and placed on the

gel. Positions of the slots were indicated with a pencil and the lower right corner was marked to indicate the orientation of the membrane. Again three sheets of Whatman papers were cut, wetted and placed on the blot. Air bubbles were squeezed out using a pipette. The blot was surrounded with parafilm and covered with a stack of paper towels. A weight was added on the top of the blot and the blot was left overnight. The next day the blot was carefully disassembled and the membrane allowed to dry completely before it was baked at 80°C for 2 hr. To check for equal loading and integrity of the RNA the blot was stained with methylene blue (0.03% Methylene Blue, 0.3% Ammonium Acetate pH 5.2) for 45 s and destained in RNAse free water for 4 min. The blots were kept at RT until hybridization experiments were performed.
Radioactive probes were generated by linear radioactive PCR as described in chapter 2.2.6. For the TrkB Northern Blot a specific primer (see table 2.1) was used and 25 to 50 ng of a purified specific PCR product were used as template. Blots were hybridized to the probes and washed as described previously. Autoradiography was performed on the blots as described in 2.2.6.

2.2.11 Long Distance PCR and sequencing of truncated TrkB

Long distance PCR was performed using the primers shown in table 2.1 and the PfuTurbo Hotstart DNA Polymerase system (Stratagene, La Jolla, USA). Both primers were designed to include an EcoRI restriction site to allow for subsequent ligation of the digested PCR product into a predigested EcoR I vector. Cycle parameters were 95°C for 2 min, 57°C for 30 s and 72°C for 4 min. For cycles 10 to 30 an additional 5 seconds were added to the extension time. The PCR product was analyzed on an ethidium bromide stained agarose gel. The 5.3 kB band was excised from the gel, purified, EcoR I digested and ligated into a predigested pSK+ vector. Bacteria were transformed and 96 clones picked and screened for inserts by restriction enzyme digestion and ethidium bromide stained agarose gel analysis. Clones corresponding to all predicted insert sizes were found and sequenced. The obtained sequence data was compared to the EST data and submitted to Genebank. Sequence data is available under the accession number AF508964.

2.3 Protein Biochemistry

2.3.1 Materials

TBS

25 mM Tris

137 mM NaCl

2.7 mM KCl

Chemicals

Tween 20 (Feinbiochemica, Heidelberg, Germany)

SDS (Sodiumdodecyle Sulphate, Merck)

Paraformaldehyde (Merck, Darmstadt, Germany)

2.3.2 Sample preparation and western blotting

RNA and DNA were isolated according to the TRIzol protocol and the supernatant containing the proteins precipitated by the addition of 3 volumes acetone followed by centrifugation at 5000 x g for 2 minutes. After centrifugation the pellet was dispersed by sonication in 100 µl Tris-HCl (pH 8.5) and the yield of proteins assessed using the BCA reagent (Pierce, Rockford, USA).

After separation by reducing SDS-PAGE of the lysates, proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, USA) and blocked with 5% non fat milk in 0.1% Tween 20/TBS (TBS-T). The membranes were incubated with the respective primary antibodies in blocking solution overnight at 4°C. The following primary antibodies were used:

- rabbit polyclonal anti-TrkB (C-13, 1:200, Santa Cruz Biotechnology, CA, USA).
- rabbit polyclonal affinity isolated β-Actin (1:1000 Sigma-Aldrich).
- goat polyclonal anti-GFAP (1:500; Santa Cruz Biotechnology, Santa Cruz, USA).
- mouse monoclonal anti-Neuronal Nuclei (NeuN) (1:500; Chemicon, Temecula, USA).

Blots were subsequently washed in TBS-T and incubated for 1 h with

- goat anti-mouse (diluted 1:30.000; Sigma, St. Louis, USA)
- rabbit anti-goat (diluted 1:30.000;Sigma, St. Louis, USA).

- goat anti-rabbit antibodies (1:7000 Promega).

Blots were again washed as described above and labeled proteins were detected using the ECL-reagent (Lumi-Phos WB; Pierce, Rockford, USA) for various exposure times.

2.3.3 Immunocytochemistry

Cultures plated and grown on coverslips were subjected to the same experimental protocol as the others and immunolabeled using a polyclonal rabbit anti–glial fibrillary acidic protein (GFAP) antibody (1:50, Sigma) and a monoclonal mouse anti-neuronal nuclei (NeuN) IgG antibody (1:100, Chemicon, Temecula, USA). Briefly, cultures were fixed with 4% paraformaldehyde, preincubated for 1 hour with PBS containing 10% horse serum and 0.2% BSA and then incubated overnight at 4°C with the primary antibodies in PBS containing 1% horse serum and 0.2% BSA. After washes, cultures were incubated for 2 hours at room temperature with an Alexa Fluor 488 dye-conjugated goat anti-mouse IgG (MoBiTec, USA, 1:2000) and a Cy3-labelled goat anti-rabbit IgG (Amersham, Germany, 1:3000) as secondary antibodies. Double labeling was visualized by confocal microscopy (Leica DM IRBE, Wetzlar, Germany).

Name	Forward Primer 5´-3´	Reverse Primer 5´-3´	T_{anneal}	Cycles
Gapdh	ACCACAGTCCATGCCATCAG	TCCACCACCCTGTTGCTGTA	65°C	18-33
β-actin #1	CGGGACCTGACAGACTACCTCA	GGCCATCTCTTGCTCGAAG	61°C	18-24
β-actin #2	AGGTCATCACTATTGGCAACGA	TTGGCATAGAGGTCTTTACGGA	60°C	18-25
NP1/NP2r	TCGAGCGGCCGCCCGGGCAGGT	AGCGTGGTCGCGGCCGAGGT	68°C	10-12
Abc 50	GCCCACCGAGTACCTGC	CCTCCAACACCTCTCGCTTGT	57°C	18-24
BI-1	GGCCTATGTCCATGTGGTCAC	GGCTCATGGCTGACATCAAGA	61°C	18-24
TrkB T1 Rat	GGGTCCAAATCCATCTGCTG	TTGAAAACCCAGAATATTATA	53°C	20-25
TrkBT1 Mouse	TCCCAAACCCACACTACACC	TCTCCCTCCCAAGAACACTAA	60°C	20-30
LD PCR	AAACCGGAATTCCCCTGAAGTCC	TGATGGAATTCCGCAAGTACA	57°C	30

Table 2.1: Sequences, annealing temperatures and cycle numbers of PCR primers.

3. Results

3.1 Ischemic Preconditioning

3.1.1 Oxygen glucose deprivation harms primary neuronal cultures and is modulated by FCS

We prepared cortical cultures from embryonal rats (E16-18) and cultivated them under serum-free conditions to avoid cytostatic, and potentially neurotoxic, antiproliferative substances otherwise required to prevent glial overgrowth (Tomkins, Edwards et al. 1994; Dessi, Pollard et al. 1995; Sanz-Rodriguez, Boix et al. 1997). Medium conditions were the same as evaluated by Brewer et al., who optimized for plating efficiency (Brewer, Torricelli et al. 1993; Brewer 1995).

Cortical preparations, which are frequently used in oxygen glucose deprivation (OGD) experiments (Goldberg and Choi 1993; Kusumoto, Dux et al. 1996), as they contain more neurons per animal than hippocampal cultures were chosen. For OGD experiments neurons have to express ionotropic glutamate receptors especially of the N-methyl-D-aspartate (NMDA) subtype to be susceptible to excitotoxicity (Abdel-Hamid and Tymianski 1997). In vitro, primary cortical cultures show a developmental increase in NMDA receptor expression (Li, Wang et al. 1998; Schubert and Piasecki 2001). Expression levels high enough to induce substantial excitotoxic cell death appear after seven to eight days in vitro (DIV) (Keilhoff and Erdo 1991, Schubert and Piasecki 2001). At DIV9 a two hour OGD reduced the viability of our cultures initially only about 20% as compared to control cultures (named BSS in the following for treatment with buffered saline solution) (fig. 3.1, middle group). In part, this was caused by a significant cell death after a two hour BSS treatment of approximately 40% as revealed by control experiments with similarly handled cultures exposed to medium instead of BSS (data not shown). We hypothesized that this demise of control cultures was provoked by growth factor-deprivation during BSS exposure and supplemented the buffers, used for BSS and OGD experiments with 2% fetal calf serum (FCS). Cultures were accustomed to serum-derived substances by adding the same concentration to the culture medium 24 hours prior to the experiments. The same medium was reconstituted after OGD. Addition of FCS resulted in a cell death reduction of 20% associated with BSS exposure in one and two hour experiments. In contrast, the difference in viability in serum-treated and untreated cultures

undergoing OGD was only significant in the two hour group. This means that OGD-specific cell death, defined as the difference between BSS and OGD-treated cultures, increased from 14 to 23% in the two hour group (fig 3.1).

In summary we hypothesize, that stress in control cultures should be kept to a minimum when studying ischemic preconditioning. Therefore all further preconditioning experiments were performed adding 2% FCS on DIV8.

Times required to stress the primary neuronal cultures were determined in the same experiments (fig. 3.1, 1/2/3h groups). The results demonstrate that OGD harms cortical cultures in a time dependent manner. One hour OGD (2% FCS) reduced the viability to 90%, two hours to 60% and three hours to 40%. All experiments were done in triplicates and repeated at least three times.

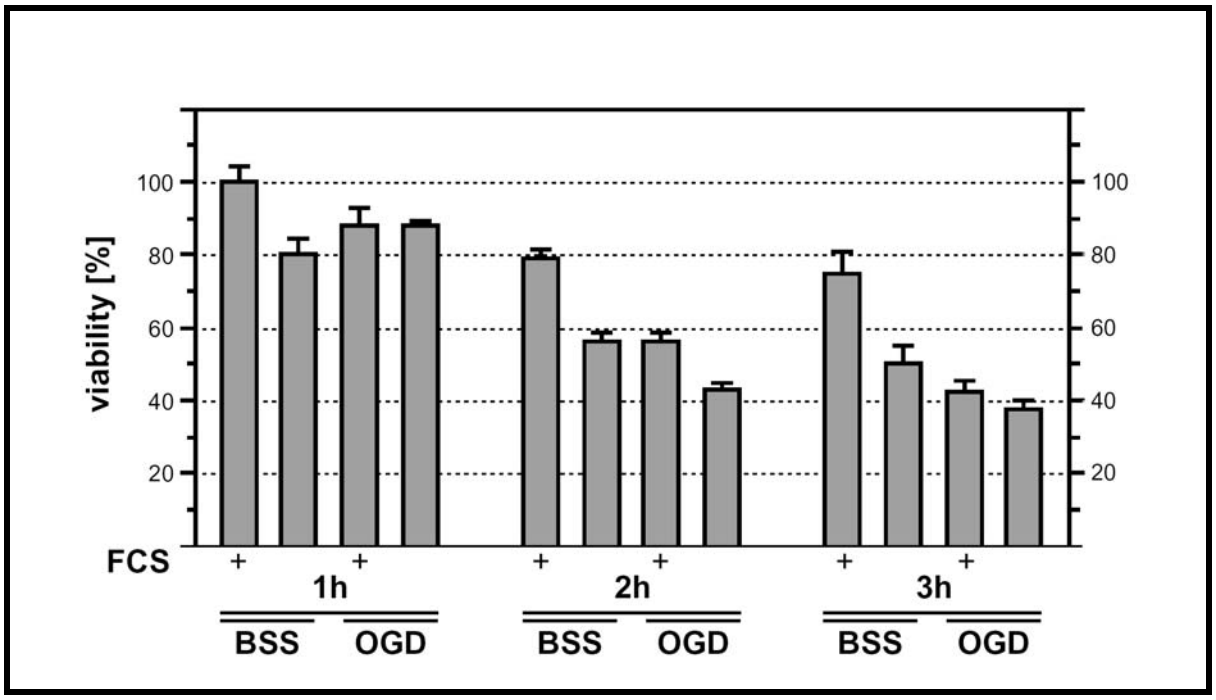

Figure 3.1: Viability of cultures after 1/2/3 h of BSS/OGD treatment with and without FCS as determined by MTT testing. Addition of 2% FCS one day prior to experiments resulted in an enhanced survival especially in control (BSS) cultures and thereby increased OGD specific cell death. Error bars, SEM.

Using this information, a preconditioning paradigm was established. For the non-lethal insult (preconditioning phase) cultures were subjected to one hour of OGD on DIV 9. This resulted in a slight (12%) but significant reduction of cell viability (compare first and third bar of figure 3.1), but did not cause morphological changes to the cultures (fig. 3.2). After 120 min of OGD fragmentation of neuronal processes and disruption of cells could be observed (fig. 3.2).

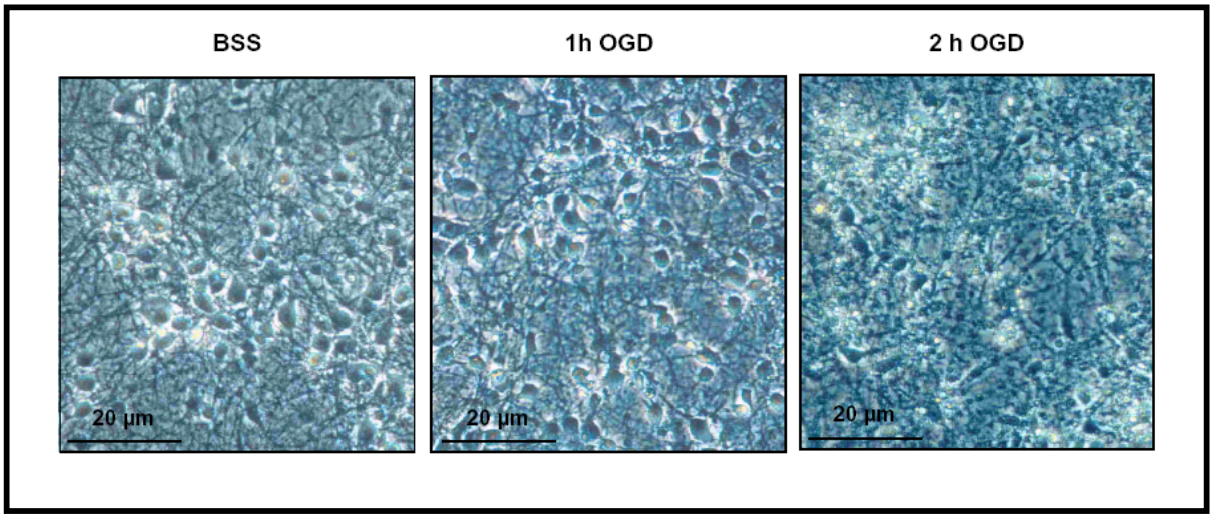

Figure 3.2: Phase contrast photomicrographs of BSS and OGD treated cultures. No morphologic changes were seen after preconditioning (1h OGD) whereas 2h OGD treatment caused fragmentation of neuronal processes and disruption of cells.

It is also important to examine the cultures after one hour of OGD for their content of neuronal and glial cells, especially to exclude glial proliferation after preconditioning which could contribute to an improved survival after the following two hour OGD. This was achieved by staining Western blots for the neuronal marker NeuN and the astrocyte marker GFAP. Results (fig. 3.3) show an unaltered intensity of the mentioned proteins before and after preconditioning.

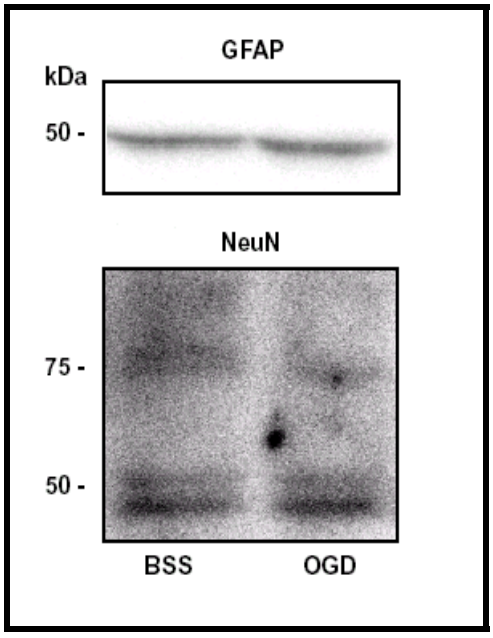

Figure 3.3: Western blots stained for NeuN and GFAP after 1h BSS/OGD show an unaltered intensity of these neuronal and glial markers.

It could be observed that few of the more susceptible neurons were already harmed by the preconditioning and that no reactive gliosis, which could mimic an enhanced survival in the viability assay, was produced by one hour of OGD.

3.1.2 IP is neuro-protective

To examine the protective effect, preconditioned cultures were treated by two hours of OGD or BSS the next day. As shown in figure 3.4 54% of the cells that would otherwise die from two hours of OGD can be rescued by IP. The achieved protection is statistically highly significant ($p < 0.001$) as determined by the Student's t-test.

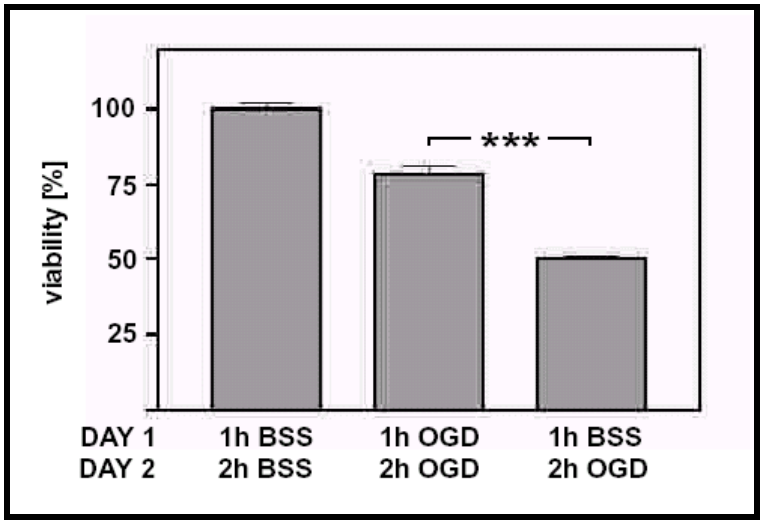

Figure 3.4: Viability of cultures after preconditioning experiments as determined by MTT testing. 54% of cells that would normally die after 2h of OGD (bar 3) can be saved by pretreatment with 1h OGD (bar 2).

To further investigate whether the observed survival benefit in the preconditioned cultures is due to enhanced glial or neuronal survival, cultures were plated and grown on coverslips and treated according to the experimental protocol. After preconditioning experiments, cells grown on coverslips were double-flourescence immunolabeled. To selectively stain neurons and glia, antibodies recognizing the protein neuronal nuclei (NeuN) and the glial fibrillary acidic protein (GFAP) were used respectively. The results demonstrated in figure 3.5 show that the survival benefit of preconditioning, seen in figure 3.4, is mainly due to an enhanced survival of neurons.

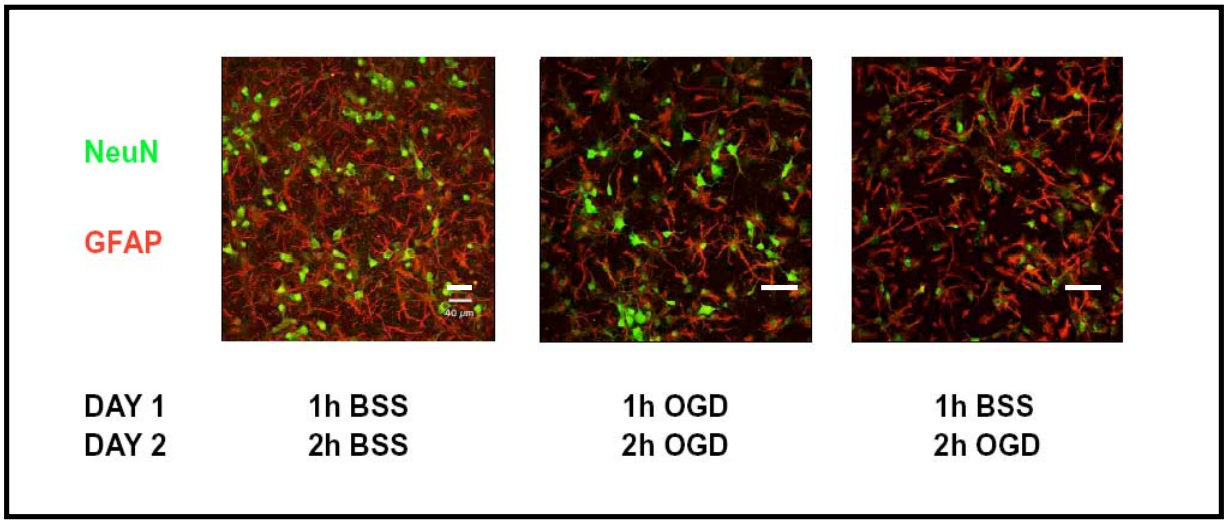

Fig 3.5: Double-flourescence immunolabeled cultures after preconditioning experiments. Preconditioned cultures (middle) display a much higher amount of neurons after a 2h OGD as compared to non-preconditioned cultures (right). Therefore IP is demonstrated to be neuroprotective. Scale bar 40 µm.

3.2 Identification of regulated transcripts

3.2.1 SMART PCR cDNA was synthesized

RNA. About 100 µg total RNA was purified from approximately 2 x 10^7 preconditioned and control cells. The integrity of the RNA was monitored on an ethidium bromide stained agarose gel.

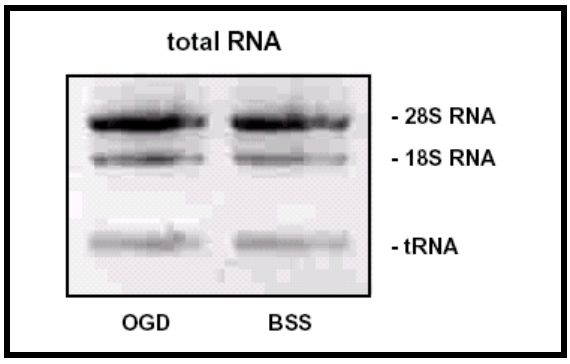

Figure 3.6: Integrity of RNA samples is sufficient as seen on an ethidium bromide stained agarose gel.

Figure 3.6 shows three bands typical for the isolation of total RNA. The two larger bands correspond to the 28S and 18S ribosomal RNAs whereas the smallest band represents tRNA. RNA quality is believed to be sufficient when intensities of 28S to 18S bands show a ratio of about 2.5:1 and if the tRNA band is visible as observed here.

cDNA. PolyA RNA was purified from total RNA, single stranded cDNA was synthesized and this cDNA used as template for SMART PCR. 17 cycles of SMART PCR (IP sample) produced a typical smear of cDNAs ranging from 0.5 kb to 12 kB

with more pronounced bands becoming visible at 0.9, 1.2, 1.8 and 2.6 kB. For the BSS sample 18 cycles of SMART PCR were required to reach an intensity comparable to the IP sample, when both specimen were analyzed alongside on an ethidium bromide stained agarose gel (fig. 3.7A). Please note that despite time consuming optimization of SMART PCR parameters equal amounts of cDNAs could only be produced for cDNA sizes up to 4kB.

RSA I digestion. cDNAs were digested and purified. RSA I digestion (fig. 3.7B) cut cDNAs to fragments ranging from 0.1 to 2kB.

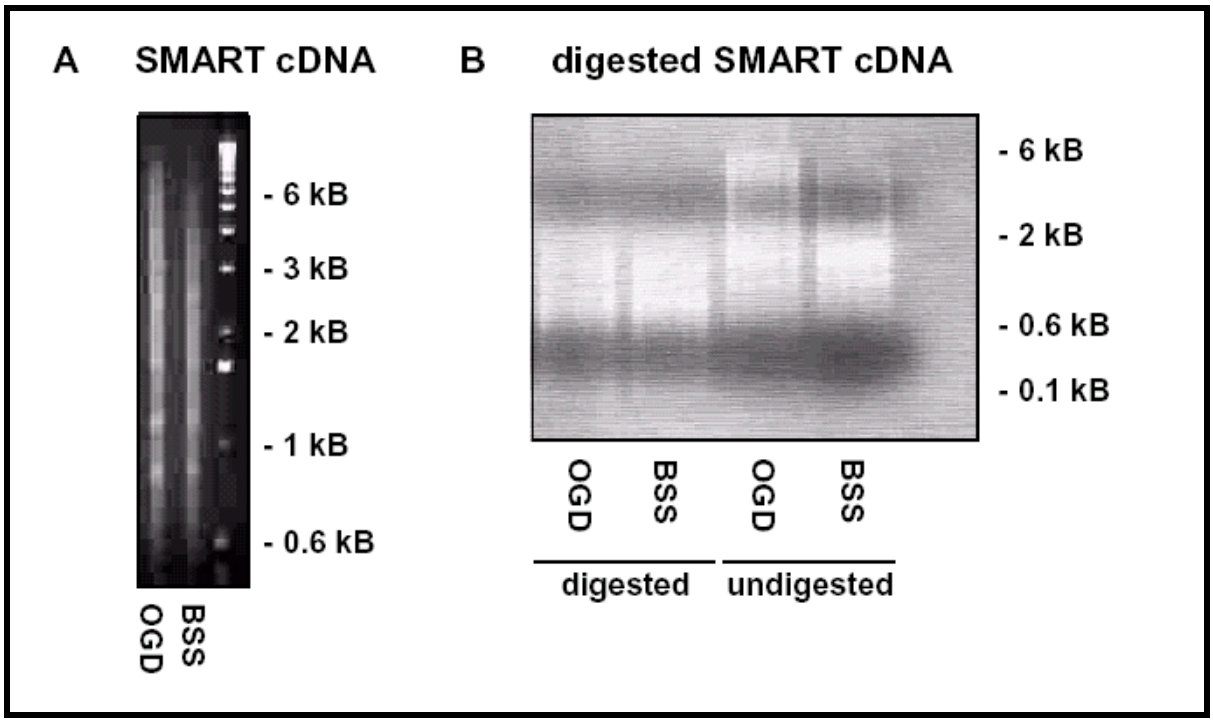

Figure 3.7: Ethidium bromide stained agarose gels showing the full size range (0.5 to 12 kB) of SMART amplified cDNAs in A). Note that equal amounts of cDNA could not be produced for cDNAs larger than 4 kB. Successful size reduction by RSA I digestion is seen in B).

3.2.2 SSH yielded differentially expressed sequences

Adaptor ligation. The digested and purified cDNA was ligated to the adaptors provided in the PCR select kit. Since adaptor ligation efficiency is one of the critical steps during SSH it has to be controlled. Therefore, a ligation control experiment was performed as follows. PCR reactions using the adaptor ligated cDNA as template and either gene specific primer pairs (Actin3´ vs. Actin5´) or one gene specific primer and one adaptor primer (Actin3´ vs. P1) were used (fig. 3.8A). If adaptor ligation was successful, meaning that most RSA I fragments were ligated to an adaptor, the longer (specific vs. adaptor primer) PCR band should appear at about the same cycle number in the gel as the PCR band generated using both specific primers (fig. 3.8B).

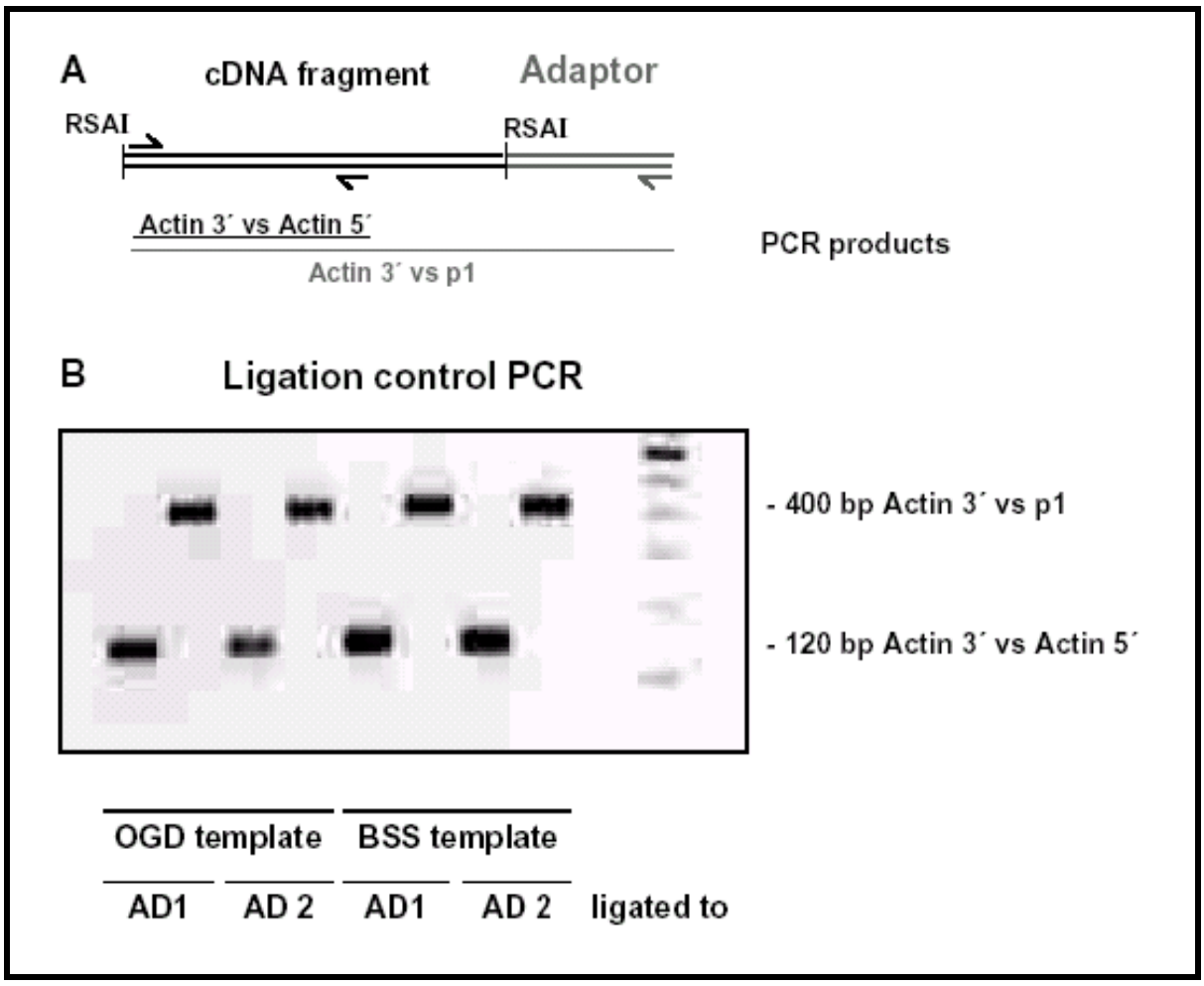

Figure 3.8: A) Scheme of adaptor ligation control experiment. Intensities of PCR bands from one reaction using two specific primers (shorter black PCR product) and another reaction using one specific and one adaptor primer (longer grey PCR product) are compared. B) PCR reactions on an ethidium bromide stained agarose gel demonstrate equal band intensities and thus successful adaptor ligation. The two different adapters needed for subsequent suppression PCR were ligated to BSS and OGD samples. AD, adaptor.

Subtraction. After hybridizations, subtraction PCRs were performed (fig. 3.9). For the primary PCR, 27 and 30 cycles were used. These reactions were diluted and used for the secondary PCR with 12 cycles. In lanes labeled FS (forward subtraction refers to OGD – BSS) and RS (reverse subtraction refers to BSS – OGD) distinct bands became visible after reactions. These bands should correspond to the up or down regulated genes. This is in contrast to the unsubtracted (US) control reactions where no distinct bands can be seen. These results hint towards a successful subtraction.

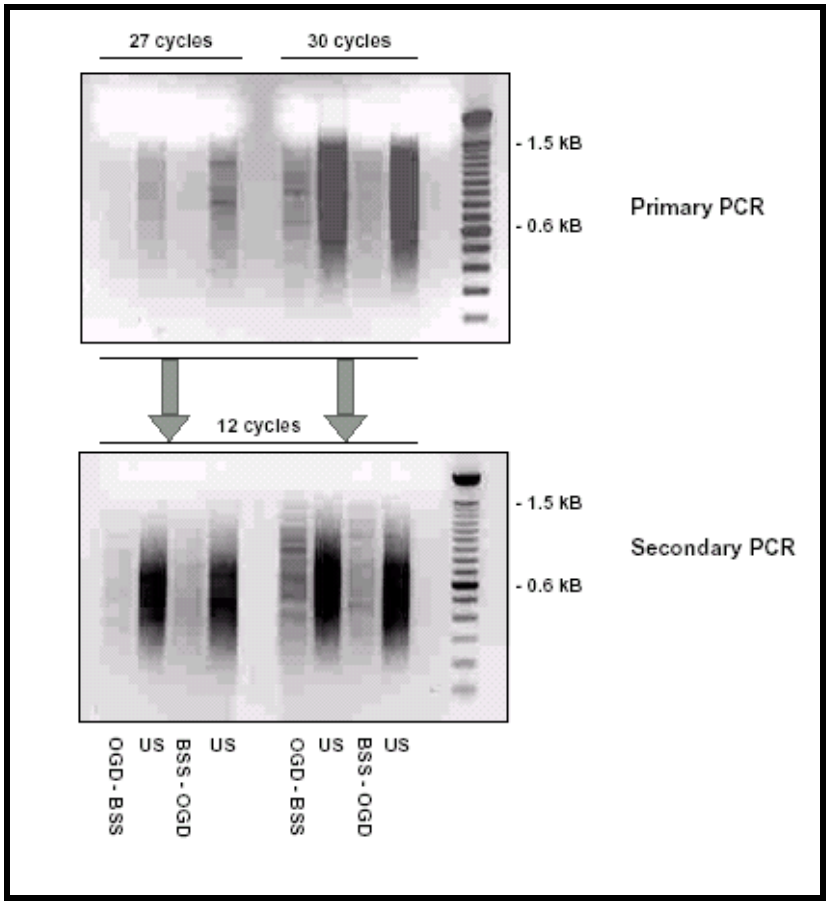

Figure 3.9: Ethidium bromide stained agarose gels of primary and secondary PCRs. Distinct bands, resembling most likely differentially expressed sequences appear after subtraction PCRs. Combination of 30 cycles primary PCR with 12 cycles secondary PCR produced most distinct results. US, PCR reactions using un-subtracted control templates.

Subtraction control. To further control for the subtraction efficiency a subtraction control experiment was performed. The forward subtracted PCR product from the secondary PCR (30 + 12 cycles) and the corresponding unsubtracted PCR products were diluted and used as template for β-Actin and GAPDH PCRs.

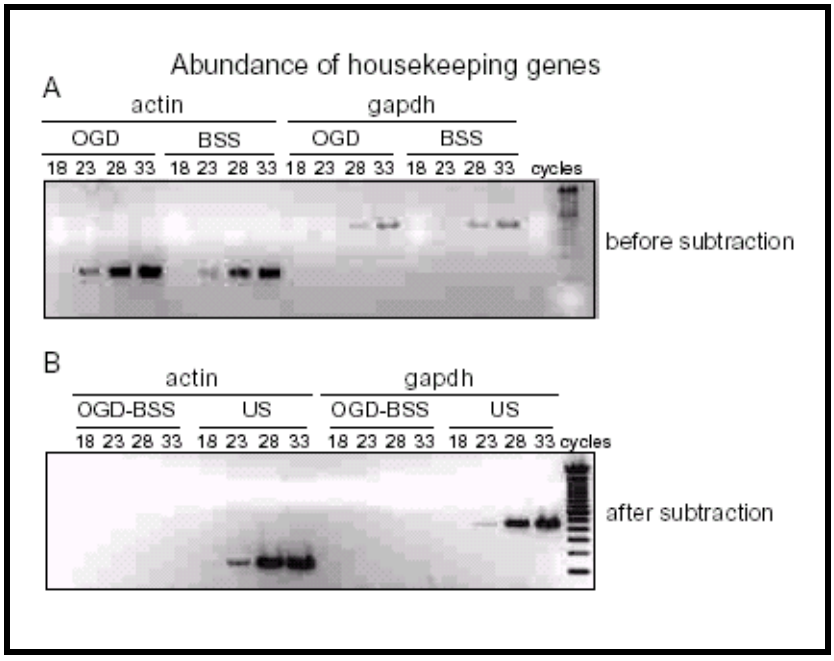

Figure 3.10: Subtraction control experiment. Quantitative PCR experiment visualized on etidium bromide stained agarose gels. Housekeeping genes β-Actin and GAPDH have an equal expression in original cDNA (A, but can not be amplified from subtracted reactions after 33 cycles of PCR as opposed to unsubtracted controls, US.

The results of this experiment demonstrate that the housekeeping genes β-Actin and GAPDH have an equal expression in the original cDNA (fig. 3.10A) but can not be

amplified from the subtracted reactions after 33 cycles of PCR whereas they can still be amplified from unsubtracted controls (fig. 3.10 B). This suggests that an efficient subtraction was achieved.

Cloning. The forward subtractions (27 + 12 cycles and 30 + 12 cycles) were directly ligated into the plasmid vector p-GEM-T and transformed into competent DH5α E. coli bacteria. Cloning of these reactions yielded about 600 clones. 350 clones were randomly chosen and amplified by colony PCR. The results of an average colony PCR are shown in figure 3.11. Fifty-two colony PCR reactions yielded no amplification product and the average size of products from the remaining clones was 500 bp.

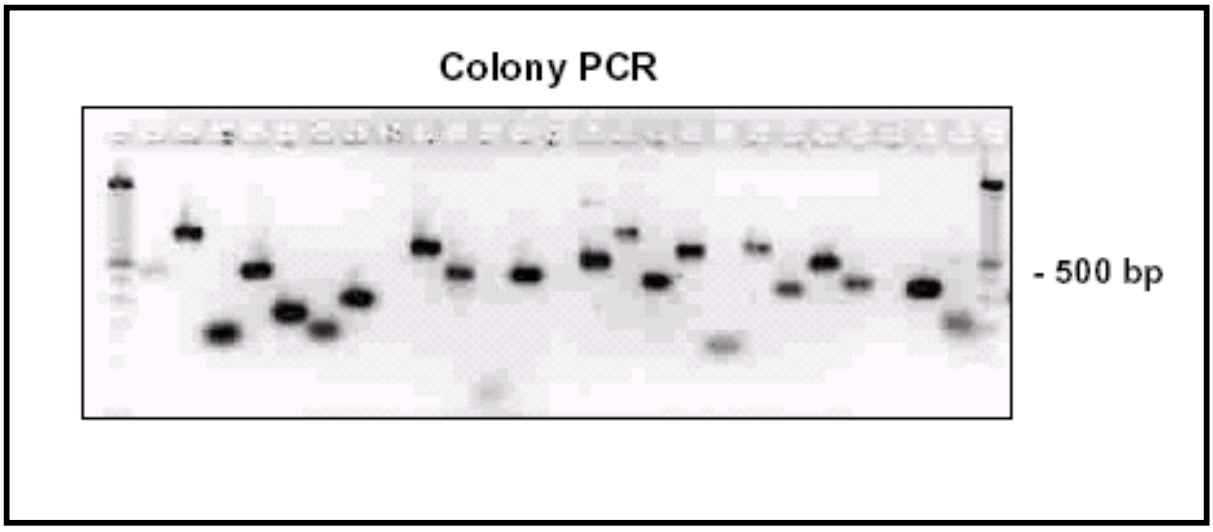

Figure 3.11: Results of 25 colony PCR reactions on an ethidium bromide stained agarose gel. The average size of colony PCR products was 500 bp. 14 % of reactions showed no amplification product.

Differential screening. The colony PCR products were then used to produce four identical dot blots. All four dot blots were hybridized with radiolabeled cDNAs.

Figure 3.12A shows the first 96 clones (from a total of 350) on the four blots. Those blots named OGD and BSS were probed with the original radiolabeled SMART amplified cDNA, whereas the blots named OGD-BSS and BSS–OGD were hybridized with radiolabeled PCR products from the forward and reverse subtracted reactions. The two dots in the boxes represent two identical replicas of one transcript to control for blotting artifacts. Comparison of dot intensities between the OGD and the BSS hybridized blot reveal upregulation of corresponding transcripts in preconditioned cultures as compared to control cultures. The same can be seen when dots in the boxes on the OGD–BSS and BSS–OGD blots are compared. All spot intensities were determined, the background subtracted, and normalized to β-

Actin expression. Next the ratio of spot intensities was calculated from the two pairs of dotblots.

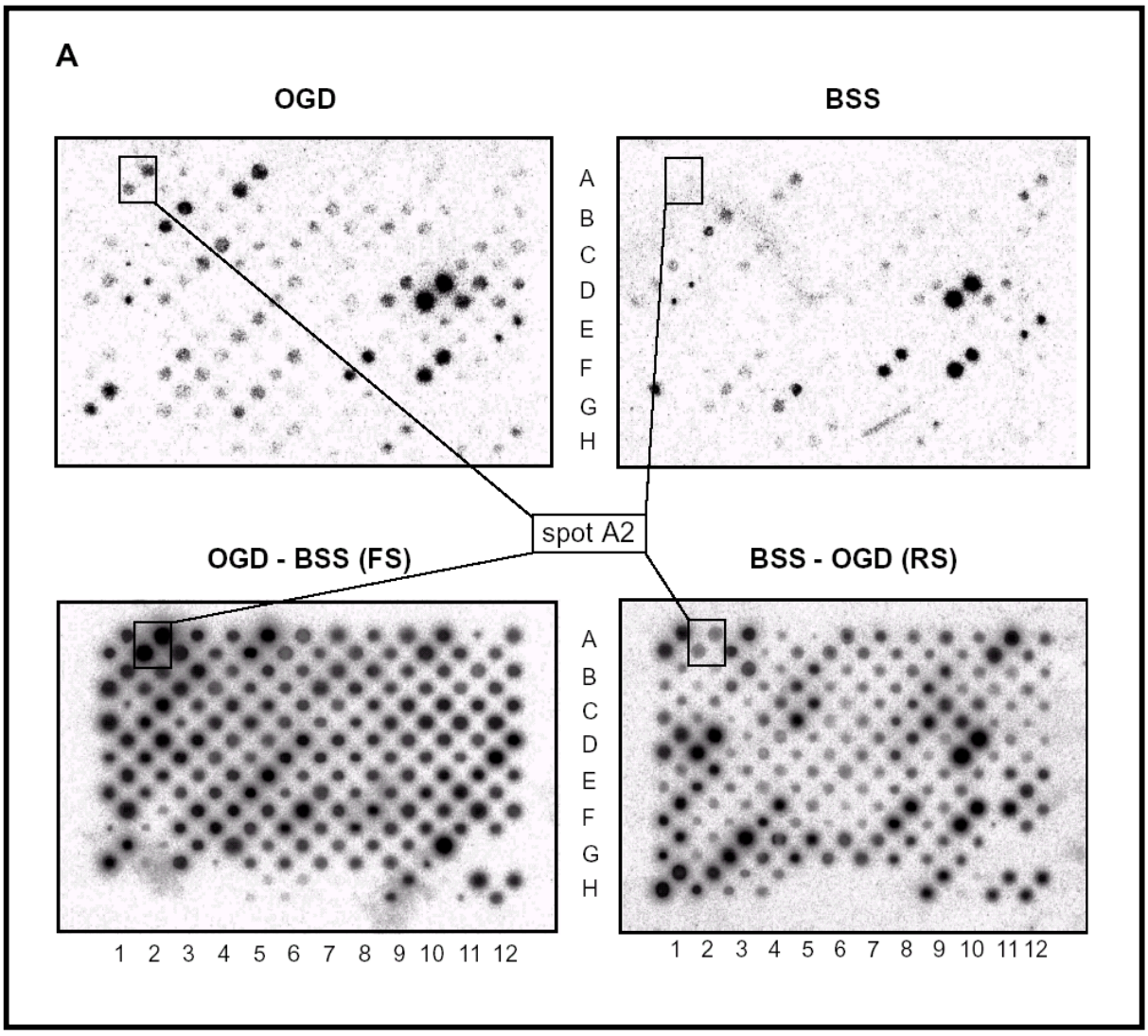

Figure 3.12A: Identical dot blots of first 96 of 350 clones hybridized with different radiolabeled cDNAs as indicated above the blots. Differences in spot intensity reveal putative upregulation. Spot A2 resembles a differentially expressed (upregulated) gene.

This revealed the degree of putative regulation. Spot intensity ratios of all spots are depicted in figure 3.12B. Those genes, showing a more than fivefold regulation in one screen or a more than twofold regulation in both screens were believed to be strong candidates for upregulation to be confirmed by SMART independent methods. In the diagram, the dots representing these genes lie to the right and to the top of the black line. Thirty-eight clones (12.7%) showed such a positive hybridization signal on dot blots and were sequenced. This resulted in the identification of 18 different known genes. The names of these genes, the putative regulation in the two screens and the position on the blot are shown in table 3.1. Two genes were found three times and four genes were found twice. The spot in the box (A2) from fig. 3.12A, for example,

was thereby identified as rat ribosomal protein. Using this dot blot screen it was determined that this gene is regulated 4.9 fold in the cDNA screen and 4.5 fold in the screen using the subtracted libraries. The dot in the box in diagram 3.12B shows the putative regulation for this gene. Moreover, the dot blot screen identified ten expressed sequence tags as candidates for upregulation.

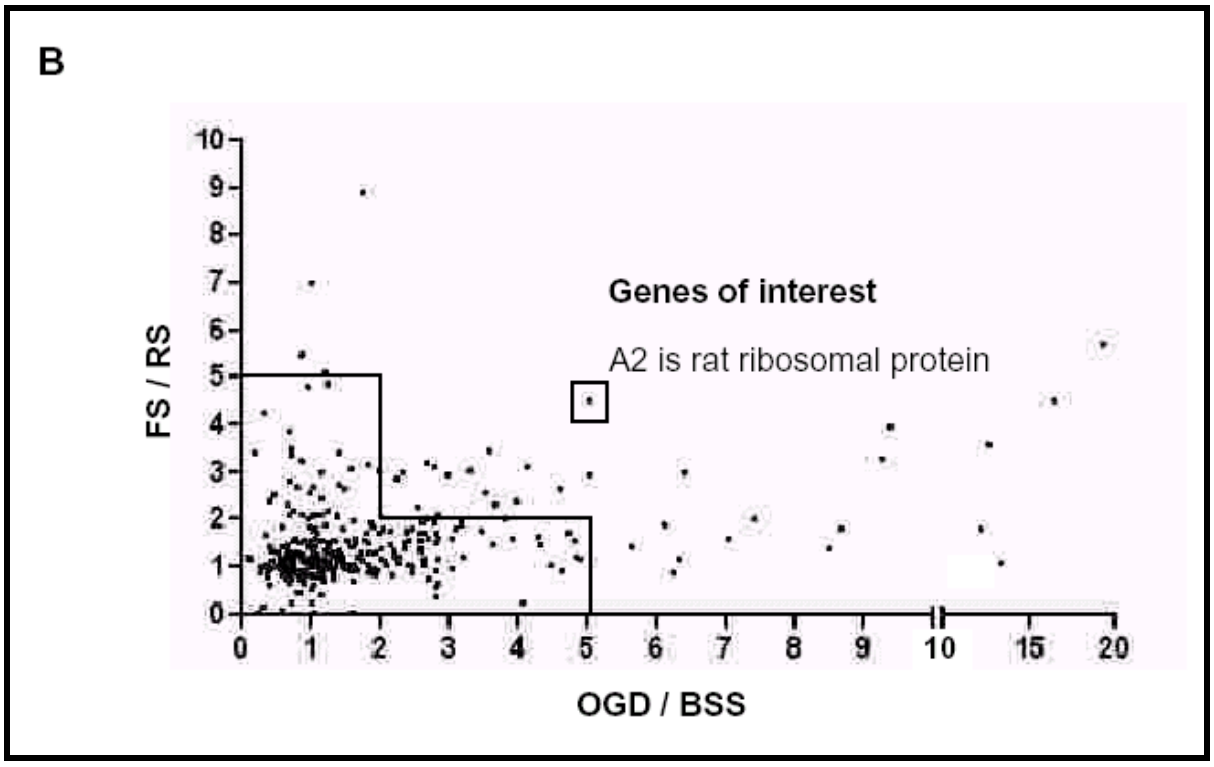

Figure 3.12B: Spot intensity ratios from the blots in fig. 3.12A. Genes of interest (upregulated genes) lie to the right and upper side of the black line.

#	Gene name	Accession #	Description	Fold regulation		
				Dot blots hybridized w/ cDNA		vNB
Expressed sequence tags			**Human ortholog**	**SMART**	**OGD-BSS**	
E1		**BU198069**	n.i.	2.2	4.0	-
E2		**BU198070**	n.i.	2.0	2.1	-
E3		**BU198071**	n.i.	2.7	2.0	-
E4		**BU198072**	n.i.	2.8	2.1	-
E5		**BU198073**	n.i.	5.0	2.9	-
E6		**BU198074**	BM477764 on 1q21.1	4.6	2.3	8.4
E7		**BU198075**	BF224045 on 6p22	6.2	0.9	3.9
E8	TrkB.T1	**BU198076**	AA779107	6.1	3.0	9.5
E9		**BU198077**	n.i.	2.7	3.0	-
E10		**BU198078**	n.i.	4.1	2.0	-
Characterized genes						
Genes related to transcription and translation						
N1	ABC50	**AF293383**	ATP sens. transcription enhancer	3.2	2.3	3.8
N2	Rat ribosomal protein	**XM234569**	Ribosomal protein	2.8	1.5	6.5
N3	Transcr. adaptor 2 like	**XM220793**	Transcription factor	5.6	1.4	-
N4	Spl. factor 3b subunit 3	**XM214697**	Splicing factor	1.8	5.5	1.5
Cytoskeleton and intracellular transport						
N5	β-Tubulin	**NM139245**	Microtubule	3.2	3.1	4.1
N6	Tau	**NM017212**	Neurofilament	8.1	3.6	11.3
N7	AP50	**M23674**	Adaptor protein	3.9	4.5	2.1
N8	TMP 21-I	**NM054367**	Vesicle trafficking	3.0	2.4	1.3
Metabolism						
N9	Glycogen phosphorylase	**XM215893**	Pacemaker of glycogenolysis	7.0	1.6	5.1
N10	Steaoryl CoA Desaturase	**NM139192**	Lipid metabolism	8.7	6.9	7.8
N11	SMVT	**AF081204**	Multivitamin transporter	8.6	5.1	5.6
Cell fate						
N12	FATSO	**AJ237917**	Proapoptotic gene	3.5	2.6	3.9
N13	Cathepsin D	**NM134334**	Lysosomal protease	9.3	3.3	4.6
N14	Bax-Inhibitor 1	**AF118564**	Inhibitor of apoptosis	3.3	1.4	6.5
N15	CCTh-Gene	**AA900460**	Cytosolic chaperone	8.1	1.3	10.7
N16	P4HB / PDI	**NM012998**	Chaperone	6.3	3.1	5.1
N17	S1P lyase 1	**XM228274**	Sphingosine-1-phosphate decay	11.0	8.9	8.1
N18	GAS7	**NM053484**	Growth arrest gene	6.1	1.9	2.5

Table 3.1: Names, description, accession numbers and regulation of all identified transcripts. vNB, virtual Northern blot; n.i. not identified;

3.3 Classification of regulated genes

The up-regulated transcripts were assigned to different groups according to their function, if known. Fourteen percent of the genes are related to transcription or translation, 14% are cytoskeletal genes or are related to intracellular transport, 10% are metabolic genes, 27% are genes related to cell fate, of which 10% are deleterious, 10% are protective and 7% remain ambiguous. 35% of the transcripts corresponded to ESTs. This demonstrates the high percentage of still unclassified genes and ESTs.

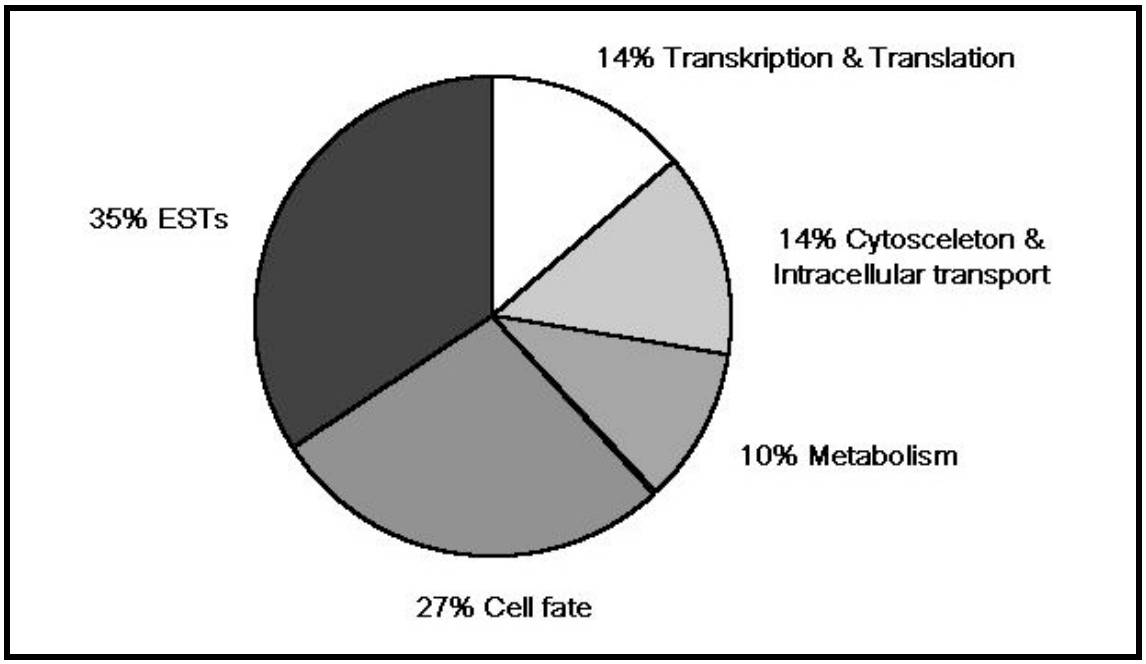

Figure 3.13: Classification of regulated transcripts

3.3.1 Transcription and translation

Since the phenomenon of Ischemic Preconditioning is dependent on the synthesis of new proteins (Barone, White et al. 1998), it was expected that genes exerting their function in this field would be among the candidates.

ABC50. Currently 30 human ABC proteins are represented by full sequences in various databases. ABC proteins are composed of transmembrane domains, and nucleotide binding domains. Based on the arrangement of these domains and sequence similarity scores, the members of the human ABC protein family can be grouped into eight subfamilies (Klein, Sarkadi et al. 1999). ABC 50 is a member of the GCN20 subfamily. Unlike the majority of ABC proteins, which are membrane-associated transporters, ABC50 associates with the ribosome (Tyzack, Wang et al. 2000) and was shown to enhance translation in an ATP dependent manner and to

associate with the Eukaryotic initiation factor 2. Since ATP levels decrease during ischemia, ABC50 might play a role in regulating the translation efficiency of protective and deleterious genes. Interestingly ABC50 was first identified in mammals in a candidate approach for genes regulated by TNF-alpha (Richard, Drouin et al. 1998). And moreover TNF-alpha is known to protect cells from ischemia induced damage (Ginis, Jaiswal et al. 2002). Thus, one may speculate that ABC50 is an effector of TNF-alpha induced neuroprotection. Human ABC proteins are also known to be mutated in inherited diseases.

Ribosomal Protein S18. The 30S ribosomal subunit assembles *in vitro* through the hierarchical binding of 21 ribosomal proteins to the 16S rRNA. The central domain of the 16S rRNA becomes the platform of the 30S subunit upon binding of ribosomal proteins S6, S8, S11, S15, S18 and S21. The ribosomal protein S18 is thus an important part in the translation machinery (Recht and Williamson 2001).

Transcriptional adaptor 2, ADA2. Transcriptional adaptors are non-DNA binding proteins that bridge, or stabilize, the interaction between upstream enhancer elements and the promoter of a gene which is located more adjacent to the coding region. ADA2 is specifically required for the interaction between acidic transcriptional activators which bind to the enhancer, and the general transcription factors binding to the promoter (Berger, Pina et al. 1992). Thus an upregulation of ADA2 may have a positive transcriptional influence on specific gene expression.

Splicing factor 3b subunit 3. The splicosome is a multicomponent ribonucleoprotein complex that is used to remove the introns from pre-mRNAs. One of the components of the splicosome is the Splicing factor 3b that comprises at least four subunits (Will and Luhrmann 1997). I found subunit 3 to be upregulated.

Taken together, four genes were found to be regulated that exert their function at different stages in the synthesis of new proteins, ranging from transcriptional activation, to splicing and finally to the assembly of the ribosome and translational initiation. These results demonstrate the importance of protein synthesis for ischemic preconditioning.

3.3.2 Cytoskeleton and intracellular transport

β-Tubulin and Tau. Ischemia-induced neuronal injury is thought to result at least in part from an excessive release of excitatory amino acids and the subsequent activation of their postsynaptic receptors. This excitotoxic pathway may also

contribute to brain injury after acute insults (Nicole, Ali et al. 2001). The first step in the excitotoxic cascade is mediated by the activation of ionotropic glutamate receptors, especially NMDA receptors, probably due to their high calcium permeability (Choi, Chung et al. 1995). The subsequent calcium influx and activation of cytoplasmatic Ca^{2+}-dependent enzymes has been proposed to mediate final excitotoxicity. The elevated intracellular calcium level has a short term negative effect on the dynamic stability of microtubules consisting heterodimers of α- and β-Tubulin. Calcium levels therefore influence the short term dynamic stability of microtubules.

In contrast, the long-term stability of microtubules is controlled by microtubule associated proteins, such as MAP2 and Tau (Delacourte and Buee 1997).

The cytosolic chaperone CCT, whose subunit CCTh was found to be upregulated in this study, has been shown to facilitate the folding of β-Tubulin (Kubota, Hynes et al. 1995) and other cytoskeletal proteins. The data from the cross hybridization experiments (chapter 3.6) suggested a rather neuronal expression pattern for CCTh, which is consistent with the above cited articles since all the discussed processes play predominant roles in the widely ramified dendrites and axons of neuronal cells.

β-Tubulin as a part of the microtubules itself, CCTh which assists its folding, and, the microtubule stabilizing protein Tau were observed to be upregulated in this study. The identification of these three genes demonstrates the important role of cellular integrity for ischemic preconditioning. Interestingly, the regulation occurred at a time point where fragmentation of neuronal processes was not visible yet, indicating that protective mechanisms are initiated before damage becomes visible.

TMP-21 and AP50. Microtubule are not only important for the structural integrity of the cell, they also serve as a scaffold for the intracellular transport of numerous molecules (Presley, Cole et al. 1997). For example all newly synthesized proteins have to be directed from their place of synthesis, usually the Endoplasmatic Reticulum, first towards the Golgi apparatus for posttranslational modification and then to their place of action (Palade 1975). Many different types of molecules have been implicated in the facilitation of this trafficking, and it is imaginable that under changing circumstances, when the cell has to adjust to a new environment, these processes play a critical role. Two genes that are involved in intracellular transport were found in this study. Tmp-21 is localized to Golgi cisternae (Sohn, Orci et al. 1996) and to the ER (Blum, Feick et al. 1996). It is furthermore concentrated in specific vesicles that facilitate transportation from the ER to the Golgi apparatus

(Blum, Pfeiffer et al. 1999). Tmp-21 is an integral part of the membrane of these vesicles and has a conserved cytoplasmatic tail. This tail contains an ER retrieval signal (Blum, Feick et al. 1996) that has been shown to redirect vesicles carrying this protein to the ER. Thus Tmp-21 is a trafficking molecule that contains targeting information for the transported molecules.

Clathrin coated vesicles are also important in the intracellular transport and endocytosis of membrane proteins (Robinson 1992). Coated vesicles that bud from the plasmamembrane contain the adaptor complex AP1, whereas those that bud from the Golgi apparatus contain the adaptor complex AP2. AP50 is a constituent of AP1. There is evidence that the adaptor complexes bind to the cytoplasmatic domains of membrane proteins such as the receptors for LDL, transferrin and EGF at the plasma membrane and the mannose-6-phosphate receptor at the Golgi apparatus (Pearse 1988; Glickman, Conibear et al. 1989; Sorkin and Waters 1993; Sosa, Schmidt et al. 1993; Sorkin, McKinsey et al. 1995) to become concentrated for subsequent transport to another compartment of the cell.

3.3.3 Intermediary metabolism

Glycogen phosphorylase. Glucose is the most important molecule of the intermediary metabolism in the brain since most neuronal cells are not capable of utilizing other substrates for ATP synthesis, such as fatty acids. Glucose can be stored in the brain, mainly in the form of glycogen in astrocytes (Reinhart, Pfeiffer et al. 1990). Glycogen break down, which is facilitated by the enzyme glycogen phosphorylase, is activated upon various stimuli such as noradrenergic activation, cAMP signaling, glucose deprivation and ATP depletion (Magistretti, Sorg et al. 1994; Poblete and Azmitia 1995; Sorg, Pellerin et al. 1995). Glucose-1-phosphate, the product of glycogenolysis can then be utilized as substrate for glycolysis, not only in astrocytes, but also in the surrounding neurons. Thus, the activation of this gene is most likely a cellular response to the demands of OGD or hypoxia.

Steaoryl-CoA desaturase. The Stearoyl-CoA desaturase is the key enzyme involved in the biosynthesis of unsaturated fatty acids, as well as in the regulation of this process (Ntambi 1995). One of the most important functions of this gene is the synthesis of oleic acid in the brain. Myelin is comprised to about 80% of fatty acids (DeWille and Farmer 1992). One of the predominant fatty acids is oleic acid (Garbay,

Boiron-Sargueil et al. 1998). Thus the up-regulation of Steaoryl-CoA desaturase might serve to prevent damage to the myelination of neurons.

Polyunsaturated fatty acids (PUFAs) such as linolenate and linoleate are essential fatty acids that cannot be synthesized by mammals, and therefore, must be obtained from plant sources. The third PUFA of importance is Arachidonic acid (AA). Although seen as an essential fatty acid, AA can be synthesized in mammals by a combination of desaturation and chain extension from linoleate. Interestingly, PUFAs play a regulatory role in the coordination of the intermediary metabolism. These fatty acids coordinately repress the expression of genes encoding lipogenic, glycolytic, and cholesterogenic enzymes while they concomitantly increase expression of genes encoding enzymes involved in hepatic and skeletal muscle β-oxidation (Jump, Clarke et al. 1994; Clarke, Baillie et al. 1997; Kim and Ntambi 1999). The immediate outcome of these events is a decrease in lipogenesis and an increase in fatty acid oxidation and ketogenesis (Ntambi and Bene 2001). Thus, one might speculate that the PUFAs contribute to the adjustment of the intermediary metabolism to OGD where utilization of ketone bodies as a response to glucose deprivation seems reasonable. Interestingly, polyunsaturated fatty acids, particularly linolenic acid (present in vegetable oils), can provide a potent tolerance against neurodegeneration (Blondeau, Widmann et al. 2002). The transcriptional inhibition of Bax, a death-related gene is associated with the protective effect of PUFAs.

Sodium dependent multi vitamin transporter (SMVT). Vitamins are required for essential metabolic processes in all mammalian cells. Such cells have developed intrinsic mechanisms for active accumulation of essential vitamins. The small intestine and the kidney, tissues that are important for the absorption of vitamins, possess active transport mechanisms that mediate the transcellular transfer of these essential nutrients. Many other tissues, including the brain have been shown to express SMVT as well (Prasad, Wang et al. 1998). The active transport system responsible for the uptake of the water-soluble vitamins pantothenate and biotin, and the essential metabolite lipoate, is driven by a transmembrane Na^+ gradient. The up-regulation of this gene might serve to draw the attention to the special metabolic state of the cell under OGD conditions, for which vitamins are necessary.

3.3.4 Cell fate, destructive

Neuronal loss is common to all neurodegenerative diseases. Although necrosis is a common histopathologic feature observed in neuropathologic conditions, increasing evidence suggests that apoptosis significantly contributes to neuronal demise. The prevalence of either type of cell death, apoptosis or necrosis, and the relevance for the progression of disease is still unclear. The debate over the occurrence and prevalence of one or the other type of death in pathologic conditions, such as stroke, may in part be resolved by the proposal that different types of cell death within the same tissue reflect different susceptibilities, metabolic states and localizations within the infarct. Apoptosis is an active process of cell destruction that requires gene expression. It is morphologically characterized by cell shrinkage, chromatin aggregation with extensive genomic fragmentation, and nuclear pyknosis. In contrast, necrosis is characterized by cell swelling, linked to rapid energy loss, and generalized disruption of ionic and internal homeostasis. This swiftly leads to membrane lysis, release of intracellular constituents that evoke a local inflammatory reaction, edema, and injury to the surrounding tissue (Linnik, Zobrist et al. 1993; Nicotera, Leist et al. 1999). After brain ischemia, necrosis is believed to play an essential role in the center of the infarct, whereas apoptosis, or delayed cell death plays a major role at the border of the infarct, the so-called penumbra.

Oxygen-glucose deprivation is rather a model for processes at the border of the infarct, where hypoxic stress is not massive enough to kill neurons in a necrotic way. Since one hour of OGD already resulted in a slight decrease of viability, it was clear that the preconditioned cultures suffer hypoxic stress. Therefore, it was expected that genes involved in the active proapoptotic process would be up-regulated.

Cathepsin D. Cathepsin D is a lysosomal aspartaic protease present in practically all animal cells. The enzyme degrades proteins at low pH and is believed to play important roles in protein catabolism, antigen processing and protein targeting (Peters, Neefjes et al. 1991; Cantor, Baranski et al. 1992). Cathepsin D is translocated from lysosomal structures to the cytosol after exposure to oxidative stress in cells undergoing apoptosis. Cathepsin D translocation precedes cytochrome c release from the mitochondria. Induction of apoptosis by oxidative stress can be prevented using a cathepsin D inhibitor. This points to an essential role of cathepsin D early in apoptosis induced by oxidative stress (Kagedal, Johansson et al. 2001; Roberg 2001).

FATSO. FATSO is a gene involved in processes such as development and programmed cell death. It was shown that mice, lacking the gene suffer from partial syndactyly of forelimbs and massive thymic hyperplasia indicating that programmed cell death is affected (Peters, Ausmeier et al. 1999). FATSO was also found to be up-regulated after optic nerve injury (Wang, Chen et al. 2002).Therefore, this candidate plays a rather proapoptotic role in ischemic preconditioning.

3.3.5 Cell fate, protective

Efficient folding of many newly synthesized proteins depends on assistance from molecular chaperones, which prevent protein misfolding and aggregation within the cell (Hartl and Hayer-Hartl 2002). But chaperones are also important for the correct refolding of proteins misfolded during cellular stress. Increased expression of heat shock proteins 70, 27 and 32 in the brain has been extensively documented in association with a variety of insults, including ischemia, and may play a role in cell survival and recovery after injury (Sharp, Massa et al. 1999; Rajdev, Hara et al. 2000; Xanthoudakis and Nicholson 2000; Hartl and Hayer-Hartl 2002). Two chaperones were found to be up-regulated in this study.

CCTh gene. CCTh belongs to a cytosolic, hetero-oligomeric molecular chaperone assisting the folding of proteins such as actin and tubulin. Its function is discussed in chapter 3.2.6 together with the other genes involved in cytoskeletal synthesis.

Protein disulfide isomerase (PDI). The gene encoding PDI has a variety of biological functions. In its monomeric form the PDI is a 55kDa protein that resides in the lumen of the ER. It catalyzes the formation and rearrangement of disulfide bonds (Puig and Gilbert 1994). However there is also evidence that PDI even facilitates the folding of proteins without disulfide bonds, suggesting a role as a classical chaperone (Cai, Wang et al. 1994). In addition the PDI protein can also form homodimers and serve as the beta subunit of the prolyl-4-hydroxylase tetramer, an essential enzyme in collagen synthesis. The third function ascribed to this gene is as thyroid hormone binding protein (Pajunen, Jones et al. 1991). Whereas the chaperone activity seems to be beneficial to cells suffering from hypoxic stress, the relevance of the two other functions for IP remain elusive.

Bax inhibitor-1. One of the upregulated transcripts coded for a six-transmembrane protein known to suppress apoptosis induced by Bax, etoposide, staurosporine and growth factor deprivation, which made it a reasonable candidate to be involved in the

observed protection mediated by preconditioning. The role of BI-1 is further discussed in chapter 4.

3.3.6 Cell fate, ambiguous

Sphingosine-1-phosphate lyase gene 1 (S1P lyase). Sphingosine-1-phosphate is released from platelets as well as from other cell types in response to activation. S1P is a ligand for the EDG family of G-protein coupled receptors and can function as a paracrine/autocrine signal to regulate cell survival, proliferation and differentiation. S1P lyase is an important enzyme in S1P decay, thus regulating the above mentioned effects on tissues (Pyne and Pyne 2000).

GAS7. Growth arrest-specific (GAS) genes are expressed preferentially in cultured cells that enter a quiescent state following serum deprivation or growth to confluence (Brenner, Lin-Chao et al. 1989). *In vivo*, GAS gene expression has been observed during the growth arrest that accompanies terminal differentiation of cells during development of peripheral nerves. GAS7 expression occurs primarily *in vivo* in terminally differentiated brain cells and prominently in mature cerebellar Purkinje neurons. In contrast, interference with GAS7 production in cultured embryonic cerebellar cells by GAS7-specific antisense oligonucleotides inhibits neurite formation normally observed during development of these neurons. Furthermore overexpression of GAS7 in cultured mouse neuroblastoma cells induces neurite-like outgrowth. These data suggest that GAS7 expression may not only cause growth arrest, but may also have a role in promoting neurite outgrowth in maturing neurons (Ju, Chang et al. 1998). Since the two described functions of GAS7 are contradictory, it is not possible to attribute the gene to the clearly protective or deleterious group. Growth arrest could have negative consequences for the adjustment of the cell to OGD, whereas the stimulation of neurite outgrowth could serve to prevent or repair OGD induced damages.

3.3.7 ESTs and unknown sequences.

Expressed sequence tags are mRNA sequences that could not yet be assigned to a known gene. Since most researchers are interested in finding genes that have not previously been linked to the field of examination, the analysis of ESTs promises to be a straight forward method in this purpose. Identification of the complete sequences of the ESTs and unknown sequences may lead to the identification of genes relevant for stroke pathophysiology. Since ESTs were submitted to the public

database these results were made accessible to the scientific community and may benefit other groups.

The amount of ESTs and unknown sequences found in this study (35% of all positive clones) is comparable with other results in this field (Trendelenburg, Prass et al. 2002). This also indicates that the various genome projects, human, mouse, rat and many more are still far from completion.

3.4 Verification of regulation

3.4.1 Virtual Northern blots of the known genes

To verify the upregulation of these candidates virtual Northern blots were performed. For the known genes the results are shown in figure 3.14.

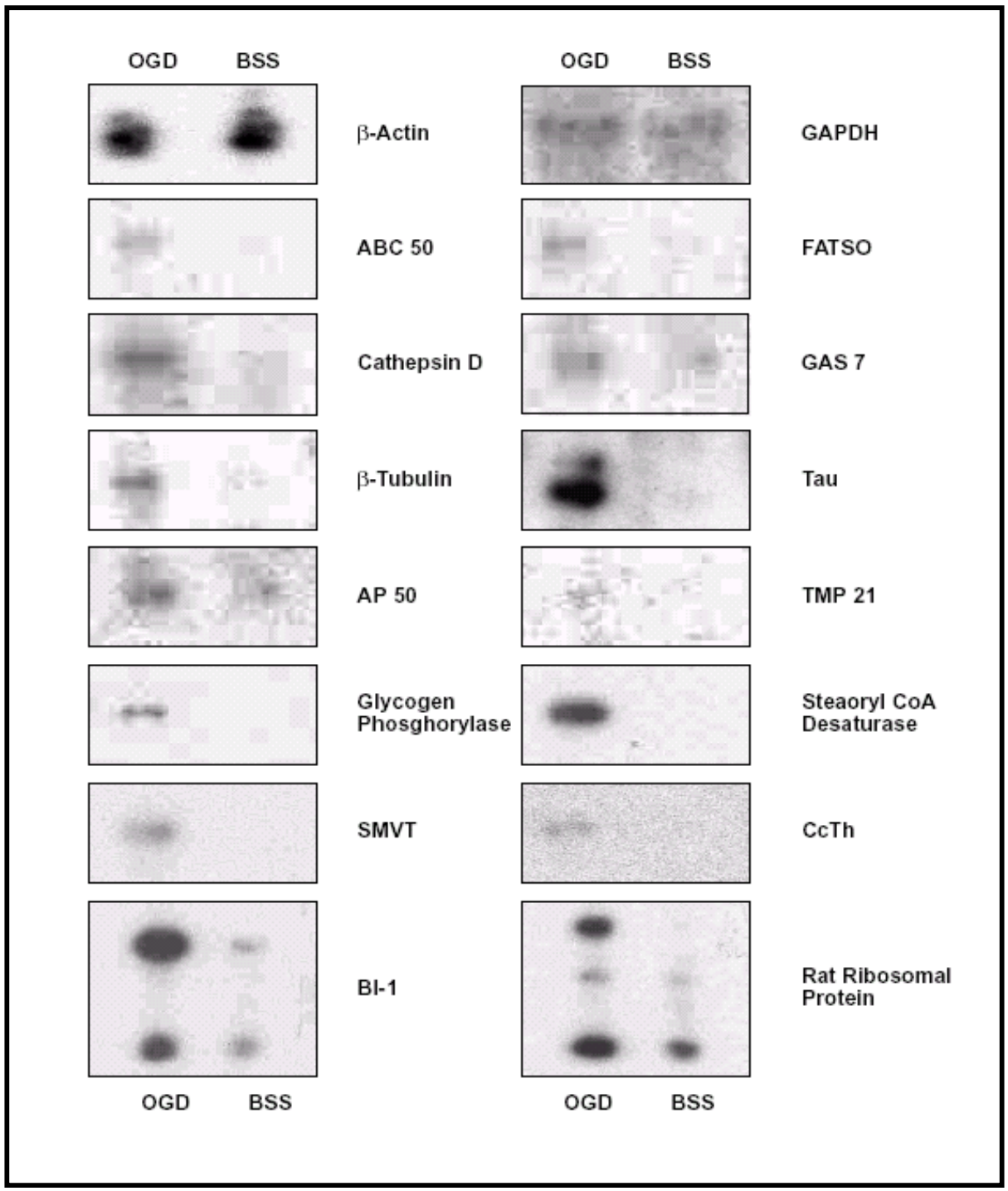

Figure 3.14: Virtual Northern blots (Southern blots of size-separated, SMART amplified cDNA) were hybridized with specific radiolabeled probes. Upregulation of candidate genes can be calculated when compared to housekeeping genes β-Actin and GAPDH.

Virtual Northern blots are supposed to correlate with results obtained by traditional Northern blotting (Endege, Steinmann et al. 1999) .

3.4.2 Expression Sequence Tags are upregulated

Figure 3.15A shows the putative regulation for the 10 identified ESTs (named E1 to E10) as determined from dot blot experiments. The corresponding dots from the screen using original cDNA are depicted in fig 3.15B (E6 to E10). Again virtual Northern blotting confirmed the upregulation for E6, E7, E8 and E9 (fig. 3.14C).

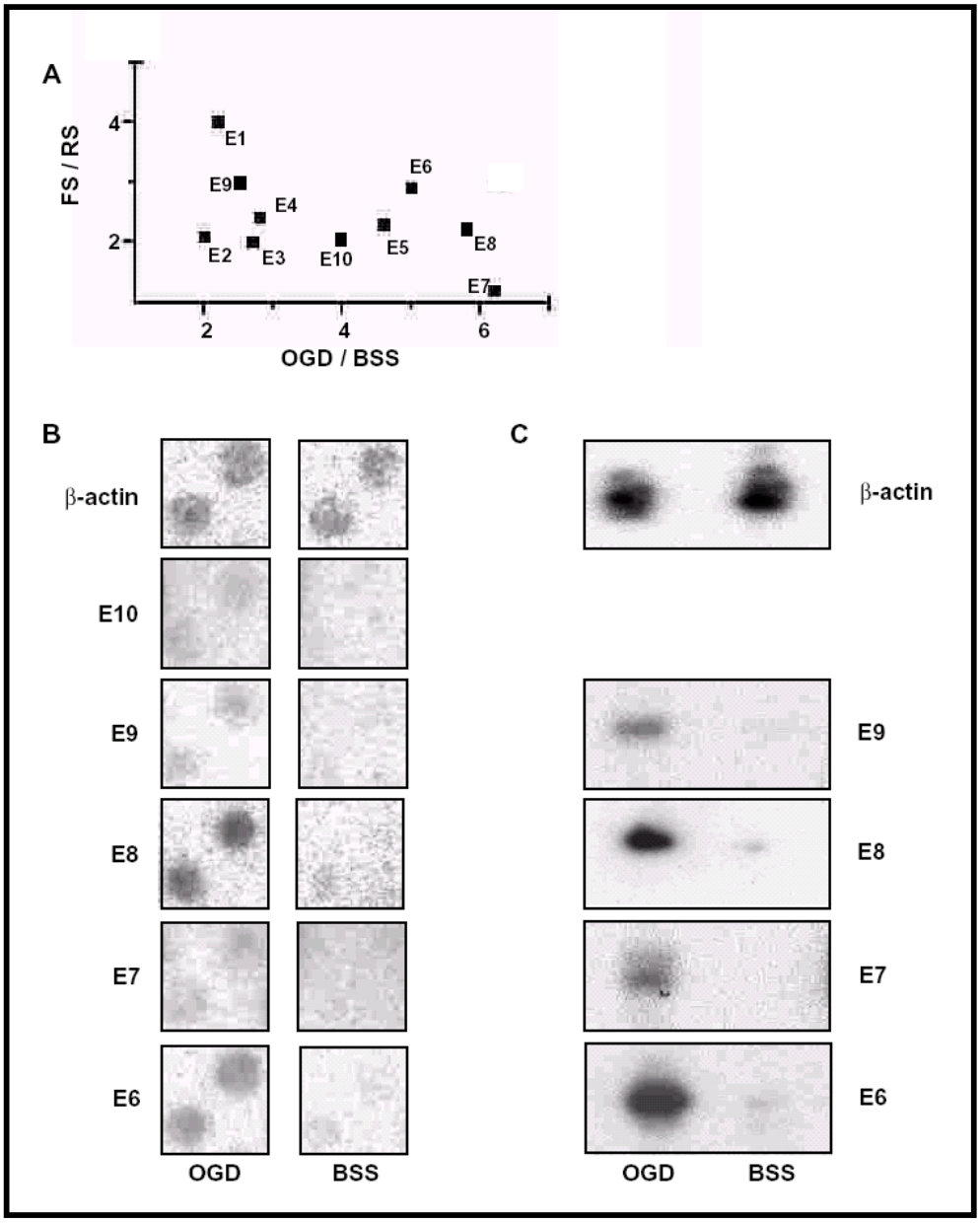

Figure 3.15: Upregulated ESTs. In A) Spot intensity ratios from the dot blots are depicted as in fig. 3.12B but for the identified ESTs only. In B) the dot blots for ESTs E6 to E10 are shown as compared to the housekeeping gene β-Actin. Virtual Northerns blots as shown in C) demonstrate upregulation of ESTs E6 to E9 as compared to β-Actin housekeeping gene expression.

3.4.3 Quantative PCR

qPCR experiments were performed to confirm the upregulation of three genes in a SMART independent method. Figure 3.16A shows the results of one representative qPCR experiment for these three genes. As seen, the three differentially expressed genes can be detected at an earlier cycle number in OGD than in BSS reactions, whereas β-Actin occurs at the same cycle number in both reactions.

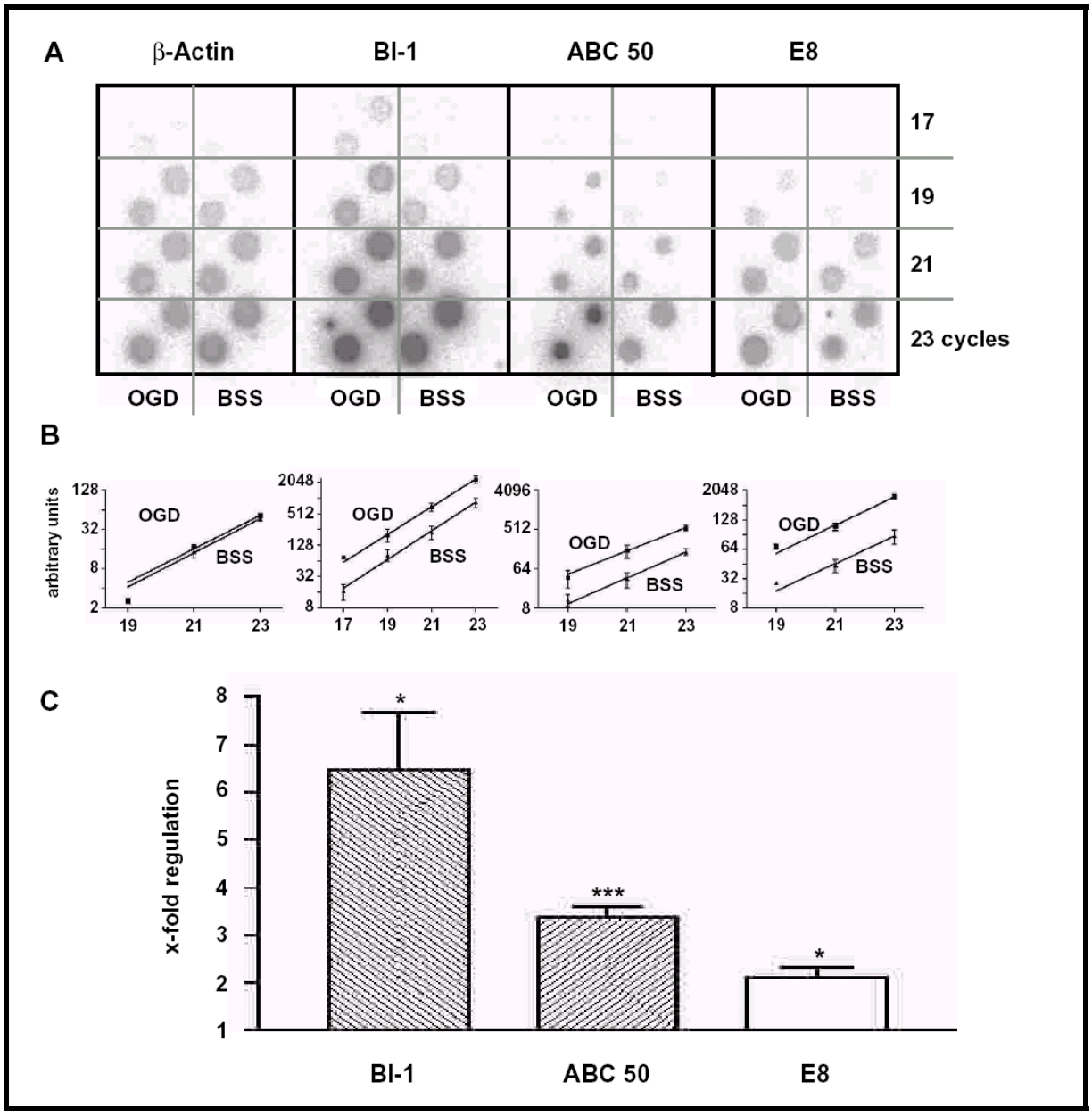

Figure 3.16: Results of qPCR experiments. A) In OGD upregulated genes give earlier or stronger signals at the same cycle number. PCR products are dotblotted and hybridized to a gene specific probe and compared to BSS. Housekeeping gene β-Actin shows equal expression in OGD and BSS. In B the data from A is plotted in a semilogarithmic diagram against the cycle number. C) For further statistical analysis only the exponential phase of amplification was used. Three way ANOVA reveals significant upregulation (mean +/- SEM, n=3, ***, p<0.001; *, p<0.05) for all three genes as compared to β-Actin expression.

In figure 3.16B this data is plotted in a semi logarithmic diagram against the cycle number. The curves from the OGD reactions reside above the curves from BSS reactions. Bar graph 3.16C contains the results of three independent experiments. BI-1 was found to be upregulated 6.4-fold ($p<0.05$, SEM 2.06, n=3), ABC 50 3.8-fold ($p<0.001$, SEM 0.38, n=3) and E8 2.1-fold ($p<0.05$, SEM 0.257) in cDNA from preconditioned cultures as compared to β-Actin expression, although virtual Northern blotting and the dot blot analysis hinted at a stronger regulation.

3.5 Identity of the Expressed Sequence Tags (ESTs)

3.5.1 ESTs belong to different genes

Since ten ESTs were identified among the regulated transcripts I was interested to determine which genes the ESTs might be derived from. Therefore we used two different *in silico* strategies. First I tried to search for human ortholog ESTs, and if successful, the human ortholog was blasted against the human genome to find the corresponding gene. This was successful for the ESTs E6 and E7. All sequences were submitted to GenBank and are publicly available under the accession numbers given in table 3.1. The gene or chromosome to which orthologs map are as well shown in table 3.1.

3.5.2 The EST E8

For EST E8 I had to use a different approach, since the search for a human ortholog at first yielded inconclusive results. We first assembled 136 overlapping ESTs to one virtual transcript. 67% of these sequences were originally obtained from neuronal tissues. This assembly resulted in a virtual 7kb message with homology to the human truncated TrkB tyrosine kinase receptor (tTrkB) at the 5’ end (figure 3.17A). Long distance PCR using cDNA from human NT2 cells with a forward primer situated in the extracellular domain of the human TrkB gene and the reverse primer in the later identified EST ortholog yielded a product of the expected size (fig.3.17B) which was cloned and sequenced. The full-length cDNA message was deposited at GenBank with the accession number AF508964. A rat brain Northern blot (fig. 3.17C) hybridized with E8 labeled a singular band of 7kB. This corresponds to the largest truncated TrkB band shown in a Northern Blot by Middlemas et al. (Middlemas, Lindberg et al. 1991).

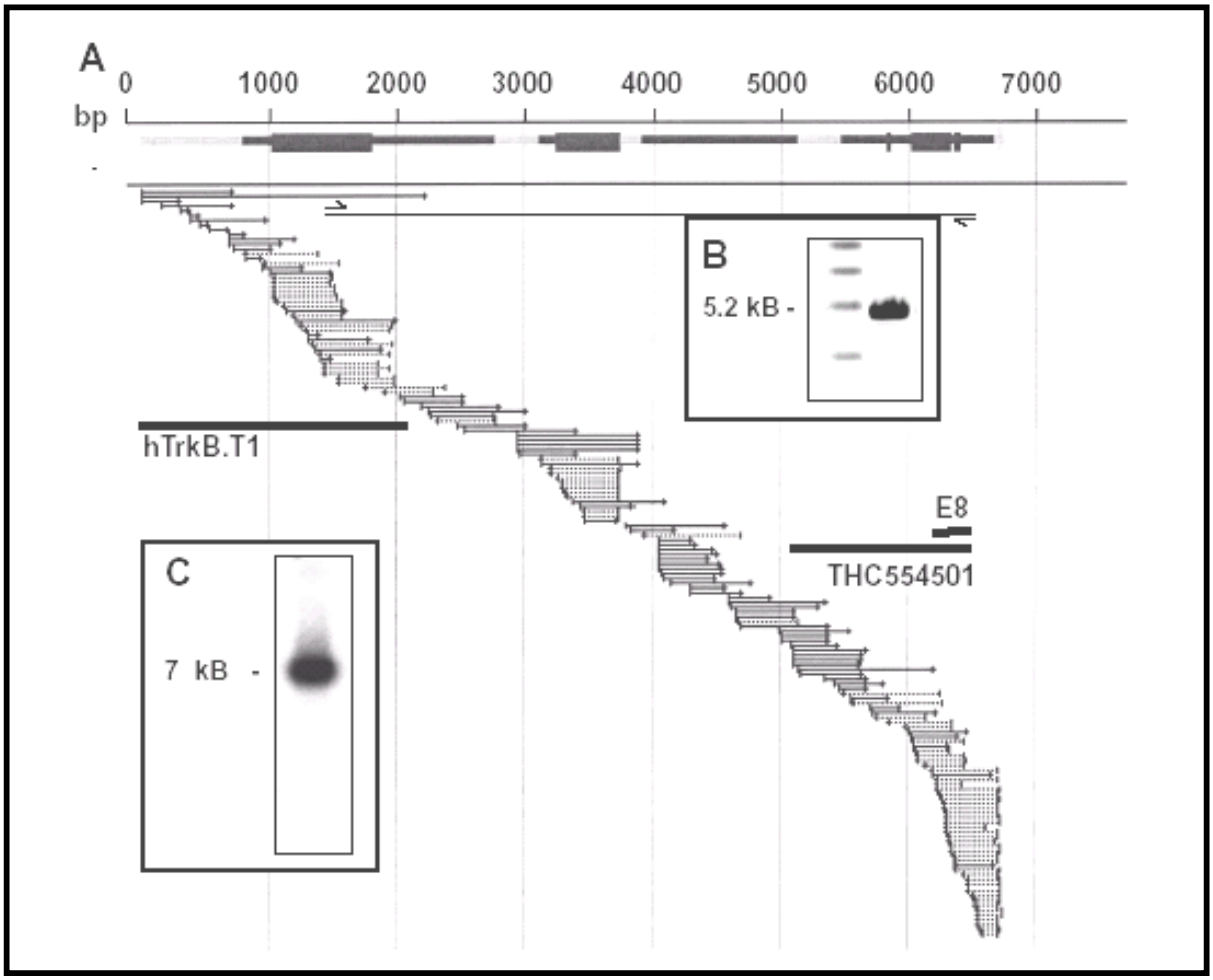

Figure 3.17: Clone E8 is part of the 3' untranslated region of the growth factor receptor TrkB.T1 A) All available overlapping ESTs were assembled and thereby a human ortholog identified. The relative position of human E8, the previously known sequence of TrkB.T1 and the long distance PCR primers are indicated. B) Long distance PCR product using human NT2 cell cDNA and the above primers separated by agarose gel electrophoresis. Size is indicated. C) Northern blot loaded with 10µg total RNA hybridized with labeled rat E8.

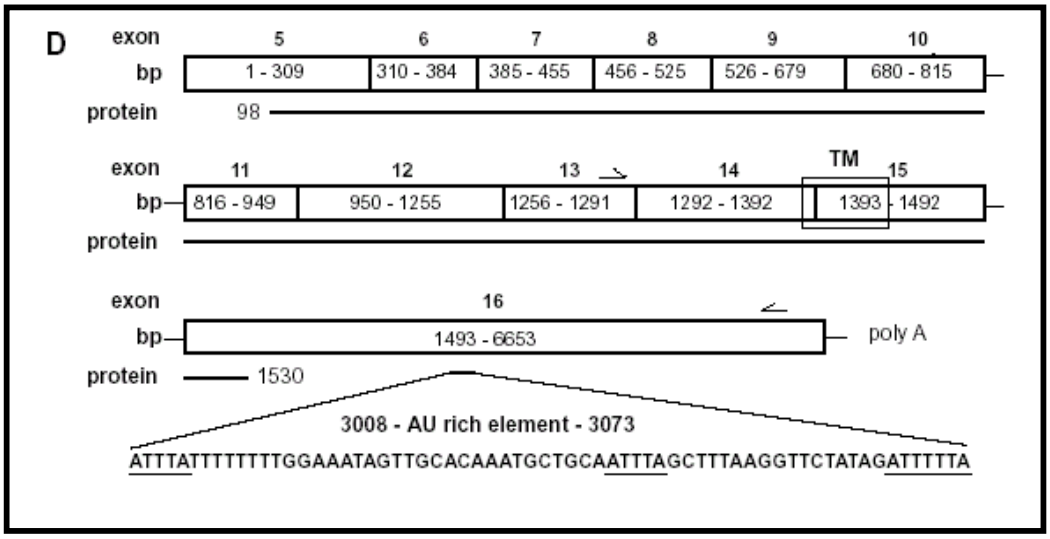

Figure 3.17D: Schematic organization of the human TrkB.T1 mRNA. Exons, coding and transmembrane region as well as the location and sequence of an AU-rich element is indicated.

The schematic organization of the transcript is shown in figure 3.17D with exon numbers according to Stoilov *et al*. (Stoilov, Castren et al. 2002). Large regions of the 3´UTR of the transcript were found to be very AT rich. Within this region we found an element with two ATTTA pentamers and an ATTTTTA extended pentamer within 66 nucleotides that has a strong likelihood to be an AU-rich element (ARE). This region has a similarity to the 3´UTR of the EGF receptor, another growth factor receptor.

E8 is downregulated at the protein level. Because of the different degrees of regulation obtained by virtual Northern blotting and quantitative PCR (compare figs. 3.15 and 3.16) I decided to examine the degree of regulation at protein level. A specific antibody generated against the intracellular part that differentiates TrkB.T1 from other TrkB variants was applied. The antibody labeled a singular band of the correct size of 95 kD in lysates from rat primary cultures subjected to one hour OGD or BSS. Results could be reproduced in three independent samples. Truncated TrkB immunoreactivity was, to our surprise more prominent in BSS-treated cultures, especially when compared to the simultaneously stained β-Actin control (fig 3.18). Therefore, TrkB is downregulated at the protein level in preconditioned cultures probably due to increased degradation of the protein in preconditioned cells or other posttranslational mechanisms.

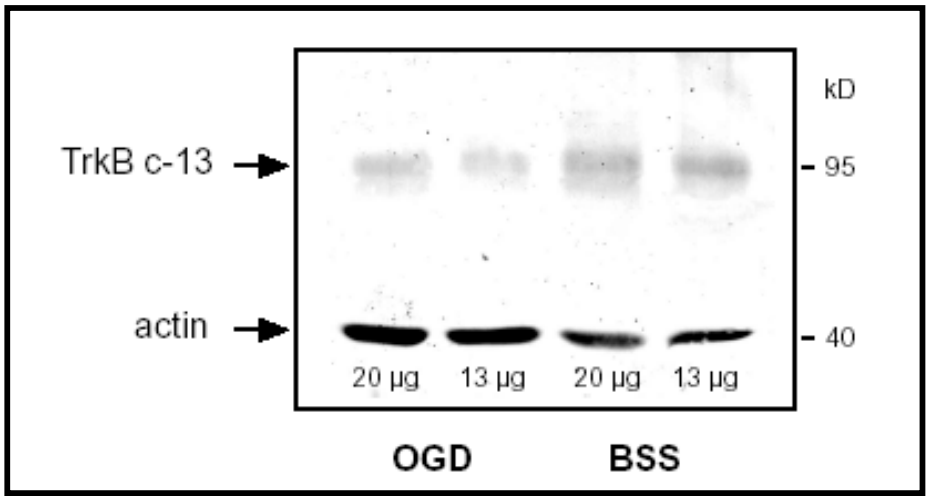

Figure 3.18: TrkB.T1 is downregulated at the protein-level. A Western blot containing the indicated amount of protein from preconditioned and control rat primary cultures DIV9 was stained simultaneously with antibodies directed against β-Actin and TrkB.T1.

Tissue distribution of TrkB. The tissue distribution of TrkB.T1 has not been investigated before. We therefore employed a commercial cDNA panel normalized for the expression of twelve different housekeeping genes to investigate its expression pattern by quantitative PCR. TrkB.T1 was most prominently expressed in

adult brain followed by lung, skeletal muscle and late embryonic tissue. Testis, liver, heart, spleen, kidney and early embryonic tissue, in contrast, only gave week signals (fig. 3.19, note the exponential scale). The major postnatal upregulation in brain is in concordance with a previous report, where a sensitive ribonuclease-protection assay was used to study the developmental regulation of different TrkB isoforms in the rat forebrain (Fryer, Kaplan et al. 1996) which further proves that BU198076 corresponds to TrkB.T1

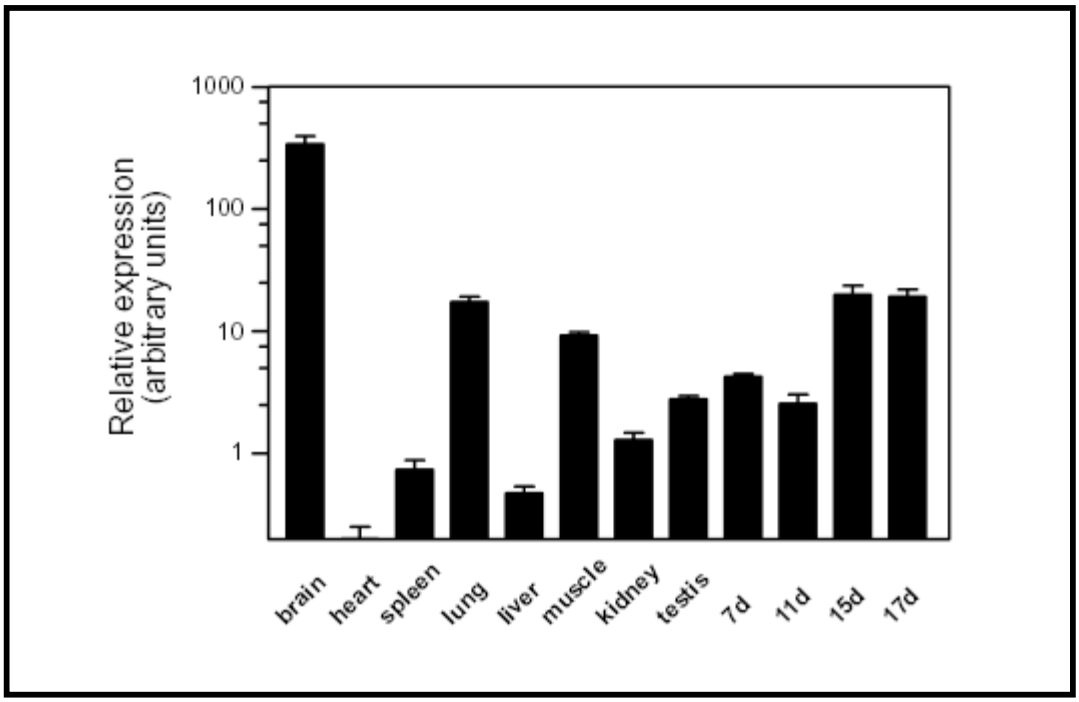

Figure 3.19: TrkB.T1 tissue expression as x-fold regulation over heart expression as determined by real-time quantitative PCR. Bar graphs represent mean ± SEM of two experiments done in triplicate. cDNA is normalized to the expression level of 12 different housekeeping genes.

In Silico expression of E8. We then subjected the sequence to a SAGE database search that compares ischemic to normal brain (Trendelenburg, Prass et al. 2002). The tag occurred twice in cDNA from ischemic tissues and not in normal brain tissue. The sequence was also subjected to *in silico* SAGE analysis at NCBI, which revealed that the tag is upregulated in well differentiated astrocytomas (283/million), has an expression of 95/million in anaplastic astrocytomas and is finally downregulated to 15-46 tags per million in the glioblastoma multiforme.

3.6 Transcripts are of neuronal and glial origin

3.6.1 Cross Hybridizations

Finally, I wanted to address the question whether the identified clones are upregulated in the neuronal or glial cells of the cortical culture system. Therefore, cDNAs of the 36 different clones were dotblotted and hybridized to radiolabeled cDNAs from neuronal and glial cell lines and signal intensities determined. The phaeochromocytoma cell line PC12 was differentiated by exposure to 100 µg/ml Nerve Growth Factor (NGF) and served as a model for a mature neuronal cell type. After one week exposure to NGF, PC12 cells cease to divide and begin to extend branching varicose processes similar to those produced by sympathetic neurons in primary cell culture (Greene and Tischler 1976) (fig.3.20).

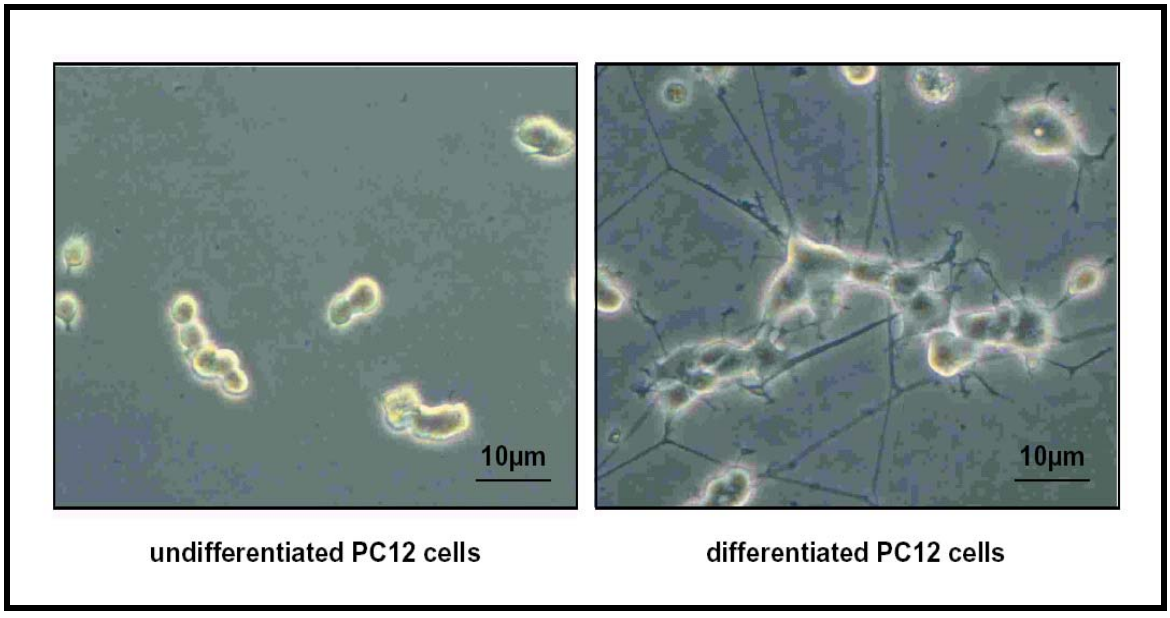

Figure 3.20: Phase contrast photomicrographs of PC12 cells before and after NGF treatment.

C6 glioma cells and primary neuronal cultures after 3 hours of OGD (reactive glia) were used as glial cell lines. Unfortunately, these experiments only yielded conclusive results for 3 genes. For the CCTh gene it could be shown that hybridizations with radiolabeled cDNA from PC12 cells showed the highest intensity, whereas intensities decreased from C6 glioma cells to reactive glia.

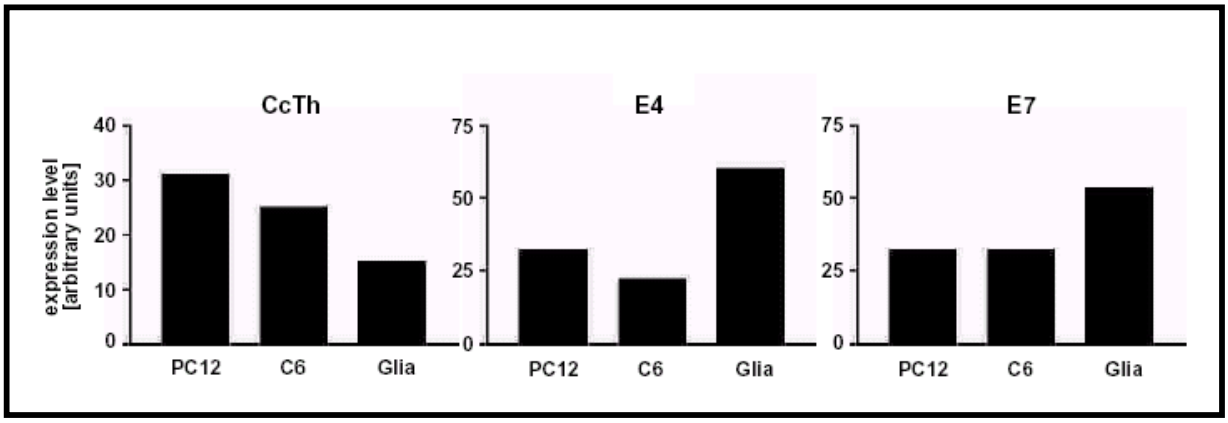

Figure 3.21: Expression level of three upregulated genes in one neuronal (PC12) and two glial cell lines (C6 and reactive Glia). CCTh shows highest expression in the differentiated PC12 cell line whereas ESTs E4 and E7 are more abundant in the glial cell culture models.

Therefore a rather neuronal expression pattern can be assumed for this gene. In contrast, the ESTs E4 and E7 produced a rather glial expression pattern (fig. 3.21) and are likely to be upregulated in glial cells.

4. Discussion

4.1 Subtractive suppression hybridization is successful in the paradigm of ischemic preconditioning

Initially, cortical cultures from embryonal rats were cultivated under serum-free conditions for the total course of experiments to avoid cytostatic, and potentially neurotoxic antiproliferative substances otherwise required to prevent glial overgrowth. In these experiments we observed that a two or three-hour treatment with BSS alone resulted in massive cell death, rendering these conditions unsuitable for experiments investigating the differences between BSS and OGD. Although cell protection assays might still be possible under these conditions, the statistical difference between three hour OGD or BSS treatment was not convincing ($p<0.05$). We hypothesized that growth factor deprivation during BSS treatment contributed to the observed cell loss and therefore supplemented culture medium one day prior to experiments and BSS/OGD buffers with 2% fetal calf serum. Serum supplementation proved to alleviate stress especially during control treatment and therefore increased the OGD specific cell loss in the cultures. This is in analogy to neutrotrophins, which similarly protect cortical cultures against serum deprivation, but increase their susceptibility to OGD (Koh, Gwag et al. 1995).

Using primary cortical cultures with the addition of FCS one day prior to experimental procedures provided an optimal system for the questions to be addressed. Glial overgrowth was prevented, and controls, especially the neurons, stayed as unstressed as possible (Lewerenz, Thomsen et al. 2004). This modifed protocol thus combines the advantages of serum-free and serum-treated culture conditions and is suitable to study the mechanisms of OGD induced neuronal cell death and ischemic preconditioning.

Using the modified protocol rat primary cortical cultures were successfully preconditioned by oxygen-glucose deprivation. In our hands, 60 minutes of preconditioning and 120 minutes of secondary OGD proved to be most effective; a time scale comparable with previous studies (Bruer, Weih et al. 1997; Khaspekov, Shamloo et al. 1998), although the parameters may vary with the equipment used (e.g. the anaerobic chamber) or other parameters such as temperature, density of cultures, or the scientists routine and accuracy in these experiments. By adjusting the

parameters carefully I was able to protect primary neuronal cultures from an otherwise lethal OGD. Performing double-flourescence immunocytochemistry on the cultures further demonstrated that protection is mainly due to enhanced neuronal survival and not caused by glial proliferation after preconditioning.

As the development of tolerance is dependent on protein synthesis (Barone, White et al. 1998), it seemed reasonable to speculate that preconditioning leads to the upregulation of protective transcripts. Considering the vast abundance of low expressed genes in the CNS and the interest to find new genes, subtractive suppression hybridization seemed to be the appropriate method. The large amount of polyA RNA (6µg) required for SSH restricted its application for a long time to cell culture models where large amounts of RNA are easy to obtain. In the pesent study this problem was overcome using the SMART PCR cDNA synthesis kit (Endege, Steinmann et al. 1999). Since the single stranded SMART cDNA contains primer binding sites on both ends it can be amplified before subjecting it to SSH. Thus it is possible to reduce the required amount of mRNA to as little as 0.1 µg. Therefore SMART made the SSH technique available to a wide variety of experimental paradigms. Even tissues from living patients, which are most limited, can now be analyzed (Spirin, Saghizadeh et al. 1999; Tupler, Perini et al. 1999).

The relatively low percentage of regulated genes (12.1%) in our study is comparable to other studies (von Stein, Thies et al. 1997; Hufton, Moerkerk et al. 1999) and strongly suggests that SSH is a powerful tool, but only in combination with an effective screening method. Moreover, the screening method has to allow a high throughput strategy to screen many clones, in the present study more than 300.

SSH was used previously to study the transcriptional differences of ischemic brain in in vivo models of focal stroke (Wang, Zhan et al. 2001) and global ischemia (Yokota, Uchijima et al. 2001). But in contrast to the mentioned studies, I was interested in finding genes upregulated in the more protective paradigm of IP and not by ischemia in general. Therefore only those cultures were used for differential gene expression studies where IP significantly improved viability after the following two hour ODG in sister cultures.

To the best of my knowledge, I present the first expression analysis in the protective model of ischemic preconditioning and give a list of 28 regulated transcripts.

4.2 The inhibitor of apoptosis Bax inhibitor-1 is upregulated

One of the upregulated transcripts coded for a six-transmembrane protein known to suppress apoptosis induced by Bax, etoposide, staurosporine and growth factor deprivation, which made it a reasonable candidate to be involved in the observed protection mediated by preconditioning. BI-1 can physically interact with Bcl-2 and Bcl-XL, two proteins that are also upregulated by ischemic preconditioning in neuronal tissue (Chen, Graham et al. 1997). Bcl-2 appears to be a major determinant of the phenomenon, as its suppression by antisense oligodeoxynucleotides blocks the induction of tolerance induced by 20 minutes of transient focal ischemia in rat brain (Shimizu, Nagayama et al. 2001). Both known transcripts of BI-1 (Walter, Dirks et al. 1994), stemming from alternative polyadenylation sites, were present in the virtual Northern blot and the 2.4 kB transcript seemed to be more prominently upregulated. We verified the observed regulation of BI-1 by quantitative PCR and found an approximately six-fold induction by ischemic preconditioning, confirming the results obtained from virtual Northern blots.

So far, most reports dealing with BI-1 demonstrated protection in various cell death paradigms. However, Yu *et al.* showed that overexpression of BI-1 isolated from *Arabidopsis thaliana* did not protect a human fibrosarcoma cell line from Bax-induced cell death (Yu, Kawai-Yamada et al. 2002) but rather elicited cell death itself. This was attributed to a proposed dominant-negative effect of the plant homologue. Another group reported a toxic effect of human BI-1 when overexpressed in HEK 293 cells (Cowling and Birnboim 1998).

In overexpression experiments carried out in our laboratory, BI-1 protected mammalian cells from OGD. A human HEK 293 cell line that expresses BI-1 under the control of a tetracycline-inducible promoter was constructed, as this allowed the stimulation of preconditioning by turning on expression 24 hours before OGD. Only BI-1 overexpressing cells showed an increased survival in the presence of doxycycline.

Since, in the following work in our laboratory it could be shown that the antiapoptotic gene BI-1 protects from oxygen and glucose deprivation, its upregulation by IP is the major finding of this work, and demonstrates the usefulness of expression profiling strategies in the paradigm of IP.

BI-1 can interact with another antiapoptotic protein, the protooncogene Bcl-2 (Xu and Reed 1998), and both transcripts are rapidly upregulated after OGD, or in the case of

Bcl-2 after brain ischemia (Chen, Graham et al. 1997; Wu, Fujihara et al. 2003). Similar to Bcl-2, BI-1 is expressed in a neuronal pattern as shown by *in situ* hybridization, and could therefore be relevant to the primarily neuronal survival seen in our preconditioning experiments. BI-1 is localized to the endoplasmatic reticulum (ER) (Xu and Reed 1998), similar to Bcl-2, which is primarily localized to the mitochondria and the ER. The antiapoptotic mechanism of Bcl-2 is not yet fully understood but seems to involve, at least in the ER, an alteration of ionic homeostasis. It has been shown previously that Bcl-2 targeted to the ER retains its antiapoptotic properties, meaning that this mechanism is sufficient. Especially alteration of cellular calcium signaling by Bcl-2 has been reported in several studies (Lam, Dubyak et al. 1994; Foyouzi-Youssefi, Arnaudeau et al. 2000; Pinton, Ferrari et al. 2000; Zhang, Sheng et al. 2001) and has been attributed to a possible function of Bcl-2 as an ion channel. Such a function was demonstrated in artificial lipid bilayers and is also supported by the three-dimensional structure of Bcl-2 and related proteins, which is reminiscent of pore-forming bacterial toxins (Foyouzi-Youssefi, Arnaudeau et al. 2000). BI-1, with its six-transmembrane topology, also resembles an ion channel, raising the possibility that BI-1 could participate, or even underlie the changes in calcium homeostasis ascribed to Bcl-2.

In the following work in our laboratory it was demonstrated that similar to Bcl-2, overexpressed BI-1 reduces the agonist-induced cytosolic and mitochondrial calcium rise by a reduction of the amount of calcium stored within the endoplasmic reticulum. A C-terminal deletion mutant lacking a conserved coiled-coil structure does not lower the mitochondrial calcium rise and does not protect from cell death suggesting that the two depend on each other. BI-1 could constitute a channel either alone or with Bcl-2 resulting in a leaky ER. This reduction in ER calcium could reduce the mitochondrial calcium accumulation induced by apoptotic stimuli that precedes cell death (Westphalen, Wessig et al. 2005).

4.3 The EST E8 is the truncated TrkB receptor

We assembled all available human EST sequences to a virtual transcript and used this sequence information to clone and sequence the corresponding full-length gene, the truncated tyrosine receptor kinase TrkB. During the preparation of this manuscript the genomic organization of three Trk B isoforms (Stoilov, Castren et al. 2002) was reported. However, the full-length sequence of human truncated TrkB was not known

hitherto (Steinbeck and Methner 2005). Evidently, the bioinformatic routines used for automatic gene identification are not yet sufficient to assemble all corresponding EST sequences to full-length genes. This could lead to an overestimation of the number of genes in the human genome. Possibly, the extraordinary long 3' UTR presented here contributed to this failure. We found an AU-rich element in this 3' UTR with similarity to the EGF-receptor. For the EGF-receptor the AU-rich element could be shown to regulate mRNA stability and translation efficiency (Balmer, Beveridge et al. 2001). The identification of regulatory sequences in the 3' end will hopefully facilitate further research into TrkB.T1 regulation and the full-length sequence might aid other differential gene expression studies.

TrkB exists as different splice isoforms and the truncated form isolated here lacks tyrosine kinase activity. TrkB is the receptor for the brain derived neurotrophic factor (BDNF) and neurotrophin 4/5. Endogenous BDNF expression is increased by ischemia (Matsushima, Schmidt-Kastner et al. 1998). Elevated BDNF concentrations correlate with reduced vulnerability after ischemia (Boris-Moller, Kamme et al. 1998) and BDNF reduction with increased vulnerability. Infusion of BDNF into the brain during and after ischemia reduces neuronal loss (Beck, Lindholm et al. 1994; Ferrer, Ballabriga et al. 1998) (Schabitz, Schwab et al. 1997; Wu and Pardridge 1999). This suggests that increased endogenous BDNF in response to ischemia protects neurons and it is widely accepted that these BDNF actions are mediated through the catalytic TrkB receptor. TrkB signaling and transphosphorylation is initiated by dimerization of catalytic TrkB. It has been suggested that dimers of catalytic and truncated receptors act as dominant negative inhibitors (Fryer, Kaplan et al. 1997; Haapasalo, Saarelainen et al. 1999). Indeed introduction of truncated TrkB into cells that express the catalytic TrkB isoform inhibits BDNF signaling (Haapasalo, Koponen et al. 2001). Transgenic mice overexpressing truncated TrkB show an increased vulnerability to ischemia than wild type littermates (Saarelainen, Lukkarinen et al. 2000).

However, this is not the only possible function of the truncated receptor. The truncated receptor itself is capable of signal transduction. It was demonstrated that its short intracellular tail is necessary for this signaling capability (Baxter, Radeke et al. 1997). Therefore, a function only as dominant negative inhibitor seems unlikely. A possible intracellular binding partner that might be involved in the signaling cascade was identified (Kryl and Barker 2000) and named TTIP (truncated TrkB interacting

protein). Most importantly, it was shown just recently that astrocytes predominately express TrkB-T1 and respond to brief application of BDNF by releasing calcium from intracellular stores. The calcium transients are insensitive to a tyrosine kinase blocker and persist in mutant mice lacking TrkB(FL). Expression of antisense TrkB messenger RNA strongly reduces BDNF-evoked calcium signals in glia. Thus, TrkB-T1 has a direct signalling role in mediating inositol-1,4,5-trisphosphate-dependent calcium release (Rose, Blum et al. 2003).

Truncated TrkB can also interact with the low affinity neurotrophin receptor p75. Coexpression of p75 with other Trk-receptors changes the subdomain utilization and ligand specific activation of the receptors (Zaccaro, Ivanisevic et al. 2001). Therefore it might be possible that coexpression of p75 also modulates truncated TrkB signaling or vice versa.

Truncated TrkB was found to be upregulated in glial cells in models of CNS injury, e.g. in astrocytes surrounding the area of infarction (Ferrer, Krupinski et al. 2001). *In silico* SAGE analysis revealed a strong upregulation in glial tumors and downregulation in undifferentiated tumors also hinting to a glial expression pattern of truncated TrkB. Astrocytes are capable of mediating the endocytosis of neurotrophins into an acid stable and Triton X-100 resistant intracellular pool that can be functionally released back into the medium (Alderson, Curtis et al. 2000). Therefore, truncated TrkB may play a role in regulating the extracellular level of neurotrophins and may influence or even prolong BDNF mediated protective signaling via the catalytic receptor that is upregulated in neurons at the border of the infarct (Ferrer, Krupinski et al. 2001). Thus the truncated TrkB receptor could have both protective and deleterious qualities depending upon the cell type and interacting molecules. Initially, although with varying degree, truncated TrkB was found to be upregulated at the RNA level. The observed differences in the degree of upregulation between virtual Northern Blots and qPCR might be due to a relative inefficiency of the SMART PCR cDNA amplification with respect to long (>5kB) transcripts. In contrast to results from the RNA level TrkB was found to be downregulated at the protein level. This might be due to enhanced degradation of RNA, possibly through the identified AU rich element in the 3’ UTR. The observed downregulation of truncated TrkB at the protein level might prolong BDNF mediated protective signaling via the catalytic receptor and thus participate in the development of ischemic preconditioning.

4.4. Conclusion

Subtractive suppression hybridization was successfully applied to the paradigm of ischemic preconditioning. Regulated genes and expressed sequence tags so far not implicated in IP were identified. Although other groups applied gene expression profiling strategies to paradigms of global and focal ischemia (Wang, Zhan et al. 2001; Yokota, Uchijima et al. 2001; Trendelenburg, Prass et al. 2002), this is the first report describing transcriptional differences in the clearly protective paradigm of ischemic preconditioning.

Since overexpression studies of the first candidate gene, Bax Inhibitor-1, indeed mediated protection, it was demonstrated that searching for regulated genes in the paradigm of IP results in the identification of novel players with physiological relevance (Steinbeck, Wessig et al. Manuscript in preparation). This provides a hopeful outlook for the overexpression studies that will be undertaken for other candidates. Moreover the putative mechanism through which BI-1 exerts protection was, in the following work, elucidated in our laboratory (Westphalen, Wessig et al. 2005).

Furthermore, the previously unknown 7-kb full-length sequence of an upregulated expressed sequence tag was presented. It was show that it constitutes the 3' end of the large untranslated region of the noncatalytic "truncated" growth factor receptor TrkB.T1. TrkB.T1 was predominantly found to be expressed in the adult brain and was upregulated by ischemic preconditioning at the RNA level. At the protein level, however, TrkB.T1 was clearly downregulated, possibly by increased degradation due to an AU rich, regulating element identified in the 3’ UTR (Steinbeck and Methner 2005).

Taken together the presented study proved to be a worthwhile approach for the identification of novel protective proteins and their mechanisms of action, although only a few mysteries of the complex process of ischemic preconditioning could be resolved.

5. Literature

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6. Appendix

6.1 Danksagung

Besonderer Dank gilt Dr. Axel Methner für die Möglichkeit wissenschaftliches Arbeiten bei ihm erlernen zu dürfen und für die gute Betreuung. Auch Dr. Jan Lewerenz und Dr. Frank Leypoldt haben durch ihre profunden Kenntnisse auf dem Gebiet der differentiellen Genexpression wichtige Beiträge zum gelingen der Arbeit geleistet.
Ebenfalls ganz besonderer Dank gilt Frau Susanne Thomsen für ihre exzellente Arbeit.
Der ganzen Arbeitsgruppe, dazu gehörten zu meiner Zeit insbesondere Dr. Jan Wessig und Dr. Patrick Joost, danke ich für das immer freundliche und persönliche Verhältnis.

Herzlicher Dank gilt auch der Werner Otto Stiftung, die mich zwei Jahre finanziell großzügig unterstützt hat. Dadurch waren die vielen der Freizeit entrissenen abendlichen Stunden im Labor leichter zu verschmerzen.

Ich danke auch Professor Dr. Chica Schaller, in deren Arbeitsgruppe wir sehr gerne zu Gast sind. Durch ihre großzügige materielle und persönliche Unterstützung ist diese Arbeit erst möglich geworden.
Mein Dank gilt auch Ute Süsens, Dr. Uwe Borgmeyer, Dr. Sabine Hoffmeister-Ullerich, Dr. Irm Hermans-Borgmeyer und Dr. Wolfgang Hampe deren langjährige Erfahrung für jeden Doktoranden von unschätzbarem Wert ist.

Zu guter letzt bedanke ich mich von ganzem Herzen bei meinen Eltern, meinen Geschwistern und allen meinen Freunden. Dieser soziale Rückhalt macht selbst Zeiten intensivster Arbeitsbelastung noch lebenswert.

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6.3 Eigene Publikationen, Poster und Vorträge

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6.4 Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Bonn, im September 2005