João Manuel Trigueiro Costa

The role of calpains on TrkB and Gephyrin cleavage under excitotoxic conditions: Characterization and functional implications

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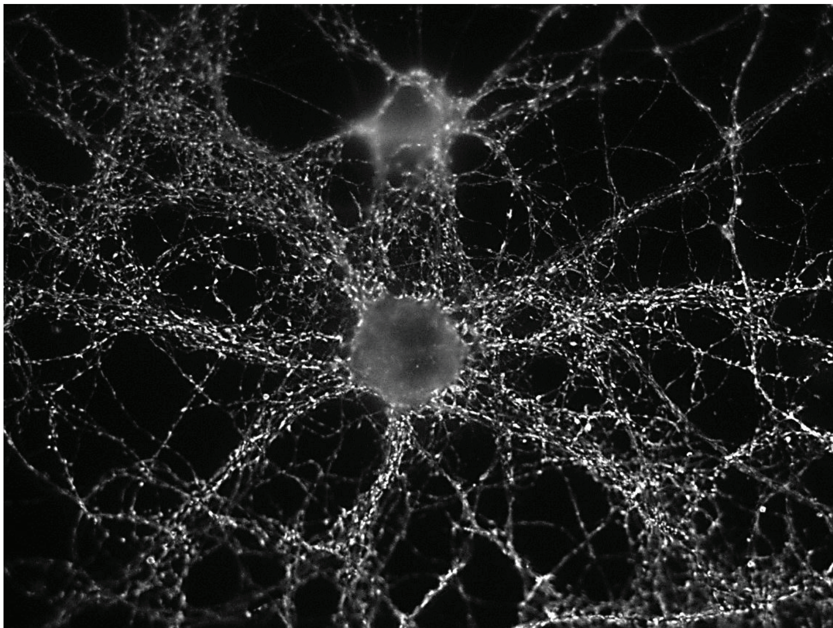
Universidade de Coimbra

About the cover: The front cover shows an hippocampal GABAergic neuron observed in a immunocytochemistry experiment performed in this thesis with an antibody against gephyrin (red), VGAT as GABAergic presynaptic marker (green) and MAP2 (blue). In this thesis I describe a new truncated form of gephyrin which may influence GABAergic neurotransmission in various neurologic conditions, and changes in TrkB receptors that affect intercellular signaling by BDNF.

The Role of calpains on TrkB and Gephyrin cleavage under excitotoxic conditions: characterization and functional implications



João Manuel Trigueiro Costa

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Abbreviations

ActD	Actinomycin D
AD	Alzheimer's disease
Aniso	Anisomycin
AIF	Apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
$[Ca^{2+}]_i$	Intracellular free Ca^{2+}concentration
cDNA	Complementary DNA
CIP	Intestinal calf alkaline phosphatase
CLAP	Chymostatin, leupeptin, antipain and pepstatin
CNS	Central nervous system
DA	Dopamine
DIV	Days in vitro
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, TTP)
DOC	Deoxycholic acid
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
GABA	γ-aminobutyric acid
GABAR	γ-aminobutyric acid receptor
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide-exchange factor
GFP	Green fluorescent protein
Glu	Glutamate
GPCR	G protein-coupled receptor
GST	Glutathione S-transferase
hCAST	Human calpastatin
htt	huntingtin protein
HD	Huntington's disease
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
KA	kainic acid
KCC2	K^+-Cl^-cotransporter

LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
mRNA	Messenger RNA
NGF	Nerve growth factor
NKCC1	Na^{+}-K^{+}-Cl^{-}cotransporter
NMDA	N-methyl-D-aspartate
OGD	Oxygen/glucose deprivation
$p75^{NTR}$	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PC-12	Pheochromocytoma cell line
PCR	Polymerase chain reaction
PSD	Postsynaptic density
PD	Parkinson’s disease
PEST	Sequence rich in the amino acids: proline, glutamate, serine and threonine
PI3-K	Phosphatidylinositol 3-kinase
PLC-γ	Phospholipase C-γ
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RhoA	Ras homolog GTPase, member A
RhoGDI 1	Rho guanine nucleotide dissociation inhibitor 1
RNA	Ribonucleic acid
SDS-PAGE	SDS-polyacrylamide gel
SOS	Son of Sevenless
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
TrkB.FL	Tropomyosin-related kinase B, full length
TrkB.T	Truncated tyrosine kinase receptor, truncated
TTC	2,3,5-Triphenyltetrazolium chloride
Geph.T	Truncated gephyrin
VGAT	Vesicular GABA transporter (= VIAAT)
VGCC	Voltage-gated Ca^{2+}channels
VGLUT 1	Vesicular glutamate transporter 1

Resumo

Os acidentes vasculares cerebrais (AVC) são causados principalmente por um bloqueio das artérias responsáveis pela irrigação do cérebro, como por exemplo a artéria cerebral média, levando à interrupção da circulação sanguínea. Os AVC podem provocar danos neurológicos significativos e são uma das principais causas de morte na Europa Ocidental, incluindo em Portugal. Os AVC isquémicos desencadeiam uma cascata de eventos ao longo do tempo: activação excessiva de receptores do glutamato com consequentes alterações metabólicas (excitotoxicidade), despolarização da membrana plasmática, inflamação e morte celular. A excitotoxicidade é um dos primeiros passos dos AVC e contribui também para a morte neuronal em outras patologias do sistema nervoso. Os mecanismos de excitotoxicidade podem levar á morte neuronal de forma aguda (necrose) ou desencadear eventos moleculares que levam a uma morte celular retardada, como a apoptose. A excitotoxicidade é ainda considerada como um dos principais alvos para a terapia do AVC, embora nenhum fármaco tenha sido ainda encontrado com eficácia nestes pacientes. A excitotoxicidade também desempenha um papel importante na morte celular em resposta a lesões da espinhal medula, traumatismos cranianos e em doenças neurodegenerativas.

As neurotrofinas desempenham um papel importante na sobrevivência e na diferenciação nuronal, tanto no sistema nervoso central como no sistema nervoso periférico. Em particular, os efeitos neuroprotectores do BDNF têm sido largamente investigados em condições de isquémia, trauma e em diversas doenças neurodegenerativas. O BDNF desempenha um papel importante na sobrevivência neuronal no Sistema Nervoso Central através da activação dos receptores TrkB. O gene *trkB* codifica os receptores com actividade de tirosina cinase (TrkB.FL) e as isoformas truncadas (T1 e T2) sem actividade catalítica. Neste trabalho, investigámos as alterações nos níveis de proteína de TrkB e na sua actividade de sinalização, em condições de excitotoxicidade. Verificou-se que a estimulação excitotóxica dos neurónios do hipocampo e do estriado em cultura provoca a diminuição dos níveis de TrkB.FL e aumenta os níveis das suas formas truncadas. A diminuição dos níveis de TrkB.FL foi mediado pela actividade de calpaínas, enquanto que o aumento dos níveis de TrkB.T foi eliminado na presença de inibidores da transcrição e da tradução.A diminuição dos níveis proteicos de TrkB.FL em neurónios do hipocampo sujeitos a condições excitotóxicas correlacionou-se com a diminuição da actividade da cascata de sinalização Ras/ERK induzida por BDNF. No entanto, a inibição de calpaínas de modo a impedir a degradação dos receptores TrkB.FL não permitiu manter a activação da via da Ras/ERK em resposta à estimulação com BDNF.Pelo contrário, a inibição da transcrição com anisomicina, de modo a impedir o aumento do número de receptores TrkB.T em neurónios sujeitos a condições de excitotoxicidade, assegurou a

manutençãoda actividade de sinalização intracelular induzida por estimulação com BDNF. Estes resultados sugerem que os receptores TrkB.T formados em condições de excitotoxicidade actuam como dominantes negativos. O aumento dos níveis de TrkB.T em condições de excitotoxicidade resultou também num aumento da sua actividade de sinalização como inibidor da RhoA. Os resultados obtidos sugerem que o efeito de dominante negativo resultante do aumento dos níveis de TrkB.T relativamente ao número de receptores TrkB.FL pode contribuir para a neurodegeneração em condições excitotóxicas, através da diminuição dos efeitos tróficos do BDNF. Por outro lado, na presença de BDNF os receptores TrkB.T parecem ser neuroprotectores ao inibirem a actividade da RhoA e a cascata de morte celular que resulta da actividade desta proteína. Os resultados obtidos neste trabalho indicam que as calpainas desempenham um importante papel na desregulação neuronal que sucede a uma lesão excitotóxica, afectando os efeitos tróficos do BDNF mediados pelos receptores TrkB.FL.

A investigação em isquémia cerebral e o estudo dos danos neuronais resultante da excitotoxicidade têm sido focados principalmente nos efeitos excitotóxicos e muito menos é sabido acerca das alterações ocorridas na actividade GABAérgica, a qual é responsável pela maioria dos efeitos inibitórios no Sistema Nervoso Central. A libertação de GABA na zona do cérebro que sofre a lesão isquémica e a consequente activação dos receptores $GABA_A$ pode ter efeitos neuroprotectores através da redução da despolarização da membrana. A gefirina é uma proteína responsável pelo tráfego e estabilização dos receptores GABAérgicos e Glicinérgicos na sinapse. Consequentemente, alterações na expressão da gefirina e na sua oligomerização podem causar alterações na actividade das sinapses inibitórias. Neste trabalho foram investigadas as alterações ao nível da expressão da gefirina durante insultos excitotóxicos ou isquémicos, as quais podem ter um efeito significativo na organização desta proteína na sinapse. A estimulação excitotóxica com glutamato induziu a clivagem da gefirina, originando um produto de clivagem de 47 kDa, em culturas de neurónios do hipocampo e do estriado. A clivagem da gefirina foi também observada em situação de oclusão da artéria média cerebral (MCAO) em murganhos (um modelo de isquémia cerebral) assim como após a injecção de cainato no hipocampo de murganhos. No entanto, neste último modelo, a clivagem da gefirina foi atenuada em animais transgénicos que sobrexpressam a calpastatina, um inibidor endógeno da calpaína. De acordo com estas observações, a clivagem da gefirina não foi observada em neurónios do hipocampo em cultura submetidos a um estímulo excitotóxico com glutamato na presença dos inibidores das calpaínas ALLN ou MDL28170. No conjunto estes resultados mostram que as calpaínas são responsáveis pela clivagem da gefirina em condições de excitotoxicidade. A colibistina é outro importante interactor da gefirina e dos receptores $GABA_A$ nestas sinapses, e verificámos que esta proteína também é clivada pelas calpaínas em condições de excitotoxicidade. Em resumo, os resultados obtidos nesta tese sugerem que as calpaínas desempenham um papel

importante na desregulação da transmissão GABAérgica e nas alterações dos efeitos tróficos do BDNF em condições de excitotoxicidade.

Abstract

Stroke is a cerebrovascular incident mainly caused by the blockage (ischemia) of an artery responsible for the blood supply to the brain (e.g. middle cerebral artery), causing a significant neurological impairment. In the Western European countries, including Portugal, stroke is one of the main causes of death. The occlusion of blood vessels is followed by a cascade of events that take place over time: overactivation of glutamate receptors causing metabolic anomalies (excitotoxicity), perinfarct depolarization, inflammation and cell death. Excitotoxicity is one of the first steps of ischemic stroke and is also an important trigger and executioner in other neurological disorders. Excitotoxic mechanisms can cause acute cell death (necrosis) or initiate molecular events that lead to a delayed type of cell death, such as apoptosis. Excitotoxicity is still considered a prime target for stroke therapy although no clinical drug has reached the bedside of patients. It has also been shown to be a key player in spinal cord injury, traumatic brain injury and more recently in neurodegenetative diseases.

Neurotrophins have proven to support survival and differentiation of neuronal populations both in the central and peripheral nervous system and in particular Brain-derived neurotrophic factor (BDNF) has been shown to provide neuroprotection in different brain regions. BDNF rescues different types of neurons from various injuries, such as ischemia, trauma, and in several neurodegenerative disorders. In addition to the effects observed upon addition of exogenous BDNF the endogenous neurotrophin also provides neuroprotection to neuronal networks. BDNF plays an important role in neuronal survival in the CNS through activation of TrkB receptors. The *trkB* gene encodes full-length tyrosine kinase receptors (TrkB.FL) and truncated (T1 and T2) isoforms. In this work we investigated the changes in TrkB protein levels and signaling activity under excitotoxic conditions. We found that excitotoxic stimulation of cultured hippocampal or striatal neurons downregulates the TrkB.FL and upregulates a truncated form of the receptor. Downregulation of TrkB.FL was sensitive to calpain inhibitors whereas the increase in TrkB.T protein levels required de novo transcription and translation. The down-regulation of TrkB.FL receptors in hippocampal neurons subjected to excitotoxic conditions correlated with a decrease in BDNF-induced activation of the Ras/ERK pathway but inhibition of calpain, which prevents TrkB.FL degradation, did not affect its signaling activity. In contrast, incubation with anisomycin, to prevent the upregulation of TrkB.T, protected to a large extent the TrkB.FL signaling activity, suggesting that the truncated receptors act as a dominant negative. The upregulation of TrkB.T under excitotoxic conditions was correlated with an increased signaling activity as RhoA inhibitor. In the presence of BDNF the TrkB.T receptors may provide neuroprotection by inhibiting RhoA and the downstream neurotoxic cascade. Altogether the results obtained in this work further indicate that

calpains play an important role in neuronal deregulation following excitotoxic injury, affecting the TrkB-mediated trophic support by BDNF.

The research in cerebral ischemia and excitotoxic neuronal damage has been focused mainly on the excitatory mediators and much less is known regarding the changes in GABAergic activity, which accounts for most of the inhibitory effects in the CNS. The release of GABA in the ischemic brain and the consequent activation of $GABA_A$ receptors may be neuroprotective through reduction of membrane depolarization. Gephyrin is a scaffold protein responsible for the traffic and anchoring of Glycine and $GABA_A$ receptors, and changes in the expression and oligomerization of this protein may produce alterations in the activity of GABAergic synapses. In this work we investigated the changes in gephyrin protein levels during ischemia and in excitotoxic conditions, which may affect its oligomerization. We found that gephyrin is cleaved following excitotoxic stimulation of hippocampal or striatal neurons with glutamate, giving rise to a cleavage product with about 47 kDa. Gephyrin cleavage was also observed after transient middle cerebral artery occlusion (MCAO) in mice, a model of focal cerebral ischemia, and following intrahippocampal injection of kainate. The latter effect was not observed in transgenic mice overexpressing calpastatin, an endogenous inhibitor of calpain, indicating that calpains mediate gephyrin cleavage under excitotoxic conditions. Accordingly, the calpain chemical inhibitors MDL28170 and ALLN prevented gephyrin cleavage in hippocampal neurons subjected to excitotoxic stimulation with glutamate. Collybistin is another important partner of gephyrin and $GABA_A$Rs at the synapse, and we showed that this protein is also cleaved under excitotoxic conditions by calpains. Altogether these results suggest that calpains may play an important rolein the deregulation of GABAergic synaptic transmission in brain ischemia.

Chapter 1

Introduction

1.1. Cerebral Ischemia

1.1.1. Stroke/Excitotoxicity

Stroke is a complex and devastating disease, being the second leading cause of death worldwide. It is the major causeof acquired disability in adults, and is a considerable social burden due to excessive costs of long hospitalizations, and rehabilitation (Brouns *et al.,* 2010; Moskowitz *et al.,* 2010). In Portugal, stroke is also one of the main causes of death, and statistics indicate that the country has the highest stroke mortality rate in Western Europe (Correia *et al.,* 2004). Stroke can be subdivided into ischemic and hemorrhagic (Doyle *et al.,* 2008b). Ischemic strokes are more frequent and can be caused by a thrombosis, an embolism or a general hypo-perfusion, all of which result in a constraint of blood flow to the brain (Dirnagl *et al.,* 1999; Doyle *et al.,* 2008a). Symptoms of stroke include impaired vision and speech, dizziness, muscular weakness or numbness and severe headache (Candelario-Jalil, 2009; Moskowitz *et al.,* 2010). The incidence of stroke is also predicted to increase with an aging population (Hachinski *et al.,* 2010; Moskowitz *et al.,* 2010).

Because of its high metabolic activity, together with large concentrations of glutamate (Choi, 1992), the brain is particularly vulnerable to ischemic insults. These can occur as a consequence of thrombosis or an embolic occlusion of a cerebral blood vessel, more frequently the middle and anterior cerebral arteries (Candelario-Jalil, 2009; Durukan *et al.*, 2009; Moskowitz *et al.,* 2010). This occlusion is usually due to a blood clot. Brain tissue has a relatively high consumption of oxygen and glucose, and depends almost exclusively on oxidative phosphorylation for energy production (Verweij *et al.,* 2007). Therefore, the brain is particularly sensitive to ischemia. With the interruption of the supply of glucose and oxygen the energetics required to maintain ionic gradients fail (Martin *et al.,* 1994), inducing a loss of membrane potential in neurons and glia. This depolarization leads to the release of glutamate from the excitatory synapses, and the lack of energy prevents the reuptake of the excitatory amino acids at the synapse, leading to an accumulation of glutamate in the extracellular space (Rossi *et al.,* 2000). Under these conditions, there is an overactivation of synaptic and extrasynaptic glutamate receptors (NMDA, AMPA and kainate), resulting in an overload of Ca^{2+},

Na^+ and Cl^- in neurons, in addition to a less significant efflux of K^+ (Choi, 1992; Arundine and Tymianski, 2003). The increased influx of cations into the cells is accompanied by the passive entry of water, resulting in edema in the infarct zone. Brain edema also affects the surrounding areas due to the increased intracranial pressure and vascular compression (Siesjo, 2008).

The neuronal damage observed in the ischemic brain also results from the rise in the intracellular Ca^{2+} which overactivates several intracellular proteases. Calpains are a group of Ca^{2+}-dependent proteases that cleave the cytoskeletal proteins like actin and spectrin, in addition to other substrates, such as calcium channels, turning them more permeable to calcium or avoiding them to extrude calcium (L-type Ca^{2+} channel and Na^+/Ca^{2+} exchanger, respectively) (Hell *et al.,* 1996; Bano *et al.,* 2005; Araujo *et al.,* 2007; Araujo *et al.,* 2008; Bevers *et al.*, 2008). Lipases are also activated by calcium, further increasing the production of free radical species (Farooqui and Horrocks, 1998). The neuronal isoform of nitricoxide synthase (NOS) is also activated by Ca^{2+}, further increasing the production of free radical species.

Mitochondria are disrupted by NO produced by the cytosolic and mitochondrial forms of NOS, downregulating the production of ATP and inducing the release of proteins that contribute to cell death and membrane leakage (Bolanos *et al.,* 1997; Beal, 1998; Heales *et al.,* 1999). Furthermore, Ca^{2+} accumulates into mitochondria through the proton electrochemical gradient generated by the electron transport chain, which creates a mitochondrial membrane potential. The influx of Ca^{2+} decreases mitochondrial electrochemical gradient, thereby reducing ATP synthesis (Pepe, 2000). At the same time, and in response to elevated $[Ca^{2+}]_i$, cells direct ATP spending to drive plasma membrane pumps to expel Ca^{2+}. Together, the accumulation of intramitochondrial Ca^{2+}, reduced ATP synthesis and increased ATP usage were suggested to be a cause of cell death (Schinder *et al.,* 1996; Racay *et al.,* 2009).

The release of cytochrome C from the mitochondria activates other class of intracellular proteases, the caspases, which initiate a cascade that ultimately causes cell death by apoptosis. Release of apoptogenic factors from mitochondria plays a critical role in mediating neuronal cell death after cerebral ischemia (Fujimura *et al.,* 1999). Activation of the mitochondrial-signaling cascade can activate both caspase-dependent and caspase-independent cell death-execution pathways (Graham and

Chen, 2001). In the caspase independent-mechanism, loss of mitochondrial membrane integrity leads to the release of apoptosis-inducing-factor (AIF), which kills cells by activating the machinery responsible for the degradation of the nuclear genome without requiring caspase activity (Susin *et al.,* 1999). Recent studies indicate that AIF is truncated by calpain, and this is followed by translocation of the protein to the nucleus where it promotes chromatin condensation and cell death (Cao *et al.,* 2003; Culmsee *et al.,* 2005; Cao *et al.,* 2007). This caspase-independent mechanism appears to be particularly relevant to adult brain injury, because AIF is expressed abundantly in the adult brain (Cao *et al.,* 2003), whereas the expression of caspases and the caspase dependence of ischemic neuronal death markedly declines with brain maturation (Yakovlev *et al.,* 2001). Moreover, in the last years several studies have shown that blocking the AIF signaling pathway, through different strategies, is neuroprotective (Culmsee *et al.,* 2005; Gao *et al.,* 2010).

Although there are different types of cell death leading to apoptosis in cerebral ischemia, a clear cross-talk between them has been demonstrated (Nakka *et al.,* 2008; Sun *et al.,* 2008).

In addition to calcium dysregulation, there are other important ionic imbalances after ischemia. Large amounts of zinc are stored in synaptic vesicles of excitatory neurons and are co-released upon depolarization (Frederickson, 1989). In vitro, excessive zinc is neurotoxic (Weiss *et al.,* 1993). The presynaptic release of ionic zinc (Zn^{2+}), followed by translocation and accumulation of Zn^{2+} ions in postsynaptic neurons is also an important mechanism of excitotoxic neuronal injury (Lo *et al.,* 2003; Suh *et al.,* 2006).

The haemodynamic, metabolic and ionic changes described above do not affect the ischemic territory homogeneously. In the center or core of the perfusion deficit, the blood flow is reduced to about 20%, or less (Hossmann, 1994). In this region the cells die rapidly by lipolysis and proteolysis, followed by a total bioenergetic failure and breakdown of ion homeostasis, in a type of cell death denominated necrosis (Ferrer, 2006). Between the core and the well irrigated tissue lies the penumbra, where there is some constraint in the supply of oxygen and nutrients but the cellular energetics is still preserved. In this region cells die in a more controlled, apoptotic way, described above (Candelario-Jalil, 2009).

Depending on the duration of the vessel occlusion, severeness of the core and the time period before clinical intervention, the penumbra can progress to an infarct zone similar to the core. Excitotoxicity expands from the core to the rest of the penumbra through the spreading of plasma membrane depolarization (peri-infarct depolarization), inflammation and apoptosis (Figure 1) (Umegaki *et al.,* 2005).

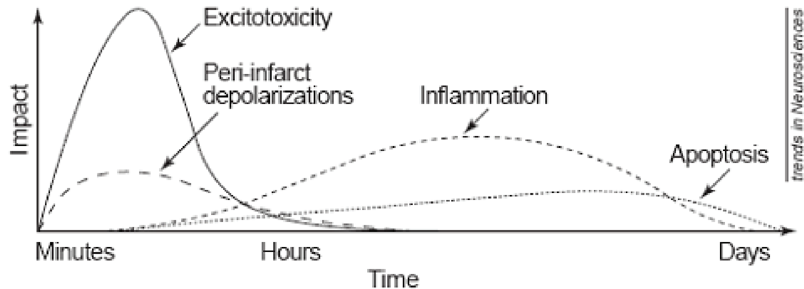

Figure 1.Cascade of damaging events in Ischemic Stroke. The x-axis reflects the evolution of the cascade over time, while the y-axis illustrates the expected impact of each component of the cascade on the final outcome (Dirnagl *et al.,* 1999).

Excitotoxicity is well established as an important trigger and executioner of tissue damage in focal cerebral ischemia. The overactivation of Ca^{2+}-permeable glutamate receptors can cause acute cell death (necrosis), but it may also initiate molecular events that lead to a delayed type of cell death or apoptosis (Ankarcrona *et al.,* 1995; Dirnagl *et al.,* 1999). Therefore, understanding the pathways involved in excitotoxic cell death is of critical importance to the development of new therapeutic strategies for brain ischemia.

Presently, excitotoxicity is considered a prime target for stroke therapy although no clinical drug has reached the bedside of patients (Dirnagl, 2006). The toxic effects resulting from overactivation of glutamate receptors are also characteristic of other neurologic disorders, including spinal cord injury, traumatic brain injury and neurodegenerative pathologies with distinct genetic etiologies. In Huntington disease (HD), the polyQ expansion in htt (huntingtin) is associated with an increase in excitotoxic cell death mediated by glutamate receptors of the NMDA subtype, containing GluN1A/GluN2B subunits. Full-length mutant htt enhances the expression of GluN1A/GluN2B subunits, the predominant NMDAR subtype in neostriatal medium-

size spiny neurons (Zeron *et al.,* 2001), and this may account for the sensitivity of this cell type in the HD brain. Excitotoxic neuronal death mediated by persistent Ca^{2+} influx through NMDARs in specific brain regions is also believed to be one of the major causes of neurodegeneration in Alzheimer's disease (AD) (Parameshwaran *et al.,* 2008). Parkinson's disease (PD) is a neurological disorder that is caused by the degeneration of nigral dopaminergic neurons and the consequent massive drop of dopamine (DA) content in the striatum. Knockdown of endogenous parkin or expression of PD-linked parkin mutants profoundly enhances synaptic efficacy and triggers a proliferation of glutamatergic synapses. This proliferation is associated with increased vulnerability to synaptic excitotoxicity. The resulting excessive glutamatergic drive could be a source of excitotoxicity in the substantia nigra (Helton *et al.,* 2008).

1.1.2. Experimental models of ischemia

A number of experimental models are currently used to study brain ischemia. In this work we used an *in vivo* model of focal cerebral ischemia, and an *in vitro* model of global ischemia.

Oxygen and glucose deprivation (OGD) is an established *in vitro* model of transient global ischemia (Goldberg *et al*., 1993; Ying *et al.,*1997; Hertz, 2008; Liu *et al.,* 2008). *In vitro* models require longer periods of OGD to induce cell death, and ATP levels do not fall as much as in *in vivo* models. The absence of blood vessels and blood flow simplifies interpretation of the results. We used this model to induce transient global ischemia in primary cultures of hippocampal neurons of embryonic rats. At 14 DIV, the culture medium was exchanged with a deoxygenated, glucose-free salt solution to induce OGD (Liu *et al.,* 2008) for different periods of time.

Focal ischemia is the animal model closer to stroke in humans (Longa *et al.,* 1989), and is conducted experimentally by occlusion of one major cerebral blood vessel such as the middle cerebral artery (MCA). MCA occlusion (MCAO) reduces the cerebral blood flow in both striatum and cortex, but the degree and distribution of the effect depends on the duration of insult, the site of occlusion in the MCA and the amount of collateral blood flow into the MCA territory (Traystman, 2003). Two surgical approaches are

used to access the cerebral vasculature to induce focal ischemia: i) the first group requires opening of the skull to allow direct access to the cerebral arteries; ii) to avoid opening the skull, a second group of methods uses intra-arterial access to occlude cerebral arteries. The most commonly used of these is thread occlusion of the MCA (Howells *et al.,* 2010). This method was first described by Koizumi et al. (Koizumi *et al.,* 1986) and modified by Longa et al. (Longa *et al.,* 1989). It involves introducing an occluding thread into the extracranial internal carotid artery and advancing until the tip occludes the origin of the MCA. This method is most frequently applied in rats and mice (Howells *et al.,* 2010). The great advantage of these techniques is that the thread can either be left in place for permanent occlusion or withdrawn at any time to permit controlled reperfusion, and is characterized by the presence of a significant ischemic penumbra. In this work, we used the method described in (Nygren and Wieloch, 2005), in which transient focal ischemia was induced using the intraluminal filament placement technique.

We also used intrahippocampal injection of kainic acid (KA) as a model to induce excitotoxicity. KA is an agonist for two subtypes of ionotropic glutamate receptors AMPA and kainate receptors, and administration of KA has been shown to increase production of ROS, mitochondrial dysfunction, and apoptotic-like cell death in neurons in many regions of the brain, particularly in the hippocampal subregions CA1, CA3 and dentate gyrus. Therefore, KA-injection is often used as a model to study excitotoxic cell death(Tomioka *et al.,* 2002; Wang *et al.,* 2005).

1.1.3. Calpains

Calpainsare one of the most important groups of proteases in the body, including the nervous system (Wu and Lynch, 2006). This family of proteases requires calcium and is defined by the sensitivity to specific inhibitors, but their substrate specificity defies complete classification. Calpains cleave at preferred sequences in association with preferred tertiary structures of substrates, but without known absolute rules (Tompa *et al.,* 2004). Most protease substrates arenot destroyed, but instead gain new functions and lose others (Lynch *et al.,* 2007). Proteolysis by calpain is a

posttranslational modification which can change the integrity, localization and activity of their targets. The numerous calpain targets that have been identified explain the diversity of roles played by these proteasesin physiological and pathological conditions, including in cell adhesion, cell division and long-term synaptic potentiation.In the nervous system calpain has been shown to participate in the control of neuronal excitability, neurotransmitter release, synaptic plasticity, signal transduction, vesicular trafficking, structural stabilization and gene expression (Scholzke *et al.,* 2003; Wu *et al.,* 2006; Franekova *et al.,* 2008; Grumelli *et al.,* 2008; Rao *et al.,* 2008; Khoutorsky and Spira, 2009; Granic *et al.,* 2010). Calpains are Ca^{2+}-dependent cysteine proteases that are regulated by at least three major factors: Ca^{2+}, redox state and by the endogenous calpain inhibitor protein, calpastatin (Goll *et al.,* 2003; Croall *et al.,* 2007). While many isoforms of calpain (15 identified) are present throughout the human body (Saido *et al.,* 1994; Sorimachi *et al.*, 2001; Bevers *et al.,* 2008), and virtually in all vertebrate cells (Saido *et al.,* 1994), only the ubiquitous forms, termed calpain I (µ-calpain) and calpain II (m-calpain), seem to be found in neurons and glia in the brain. These two isoforms are heterodimers with 80 kDa catalytic subunits and a shared 30 KDa regulatory subunit. During activation, the 30 kDa subunit is cleaved to yield a final 17 KDa form, while the 80 KDa subunit is converted to a 76 kDa enzymatically active form (Yamashima, 2004). The two neuronal calpain isoforms are located throughout the neuron, both in the somatodendritic regions and in the axons (Yamashima, 2004), and have similar physiological functions and pathological actions (Kuchay and Chishti, 2007).

Calpastatin is the endogenous inhibitor of calpain, which blocks the substrate binding site of calpain in a use dependent manner, since its interaction with calpain is Ca^{2+}-dependent. Calpastatin is composed of an N-terminal domain followed by four repeated calpain inhibitory domains. Each of the four inhibitory domains can inhibit one molecule of calpain (Guttmann, 2007), which accounts for the strong inhibitory effects of calpastatin. Although calpain and calpastatin are both found in the soluble fraction of the cell, they seem to have different intracellular localizations, being calpain diffusely located in the cytosol and calpastatin associated to the nuclear membrane invaginations (De Tullio *et al.,* 1999). Following an elevation of intracellular calcium,

calpastatin diffuses into the cytosol and colocalizes with calpain, thereby modulating calpain activity. The activity of calpain can also be modulated with posttranslational modifications of the substrates, such as phosphorylation (Rong *et al.,* 2001).

Calpains cleave their substrates at preferred sequences in association with favorite tertiary structures, but without known unequivocal rules. According to the PEST hypothesis, calpains cleave target proteins preferentially near PEST sequences, which are enriched in Pro, Glu/Asp, and Ser/Thr,and flanked by Arg/Lys residues, being related with a short lifetime of proteins (Rogers *et al.,* 1986; Rechsteiner and Rogers, 1996). This negatively charged region is thought to bind Ca^{2+} and deliver peptide bonds for calpain activity (Tompa *et al.,* 2004). However, whether the presence of PEST sequences provides a signal for calpain cleavage is not entirely consensual, since mutations of these sequences did not abolish calpain cleavage of some substrates (Molinari *et al.,* 1995, Carillo *et al.,* 1996), and there are some calpain substrates that lack PEST sequences (Carillo *et al.,* 1996).

1.1.3.1 Calpain in physiological functions

All calpains can act in two modes: under physiological conditions they undergo controlled activation (involving only a few molecules of calpain), whereas during sustained calcium overload under pathological conditions they are hyperactivated (involving most available calpain molecules) (Liu *et al.,* 2008). Although several different calpain substrates have been identified, calpain 1 and 2 are somewhat selective regarding substrate selection, truncating or degrading only 5% of the proteins, even under extreme conditions (Wang *et al.,* 1989).

Calpain mediated spectrin cleavage is implicated in dendritic spine changes associated to LTP induction (Vanderklish and Bahr, 2000). LTP also requires rapid insertion of AMPARs in the postsynaptic membrane, and these receptors are stabilized in the postsynaptic density (PSD) through interaction with anchoring proteins (Shi *et al.,* 1999; Kessels *et al.,* 2009). Calpain cleaves the glutamate receptor interacting protein (GRIP), disrupting its interaction with GluA2, and causing rapid insertion of AMPARs into postsynaptic membranes (Lu *et al.,* 2001; Zadran *et al.,* 2010). Furthermore,

calpain 2 cleaves PSD95, which induces changes in the organization of the PSD, modifying the anchoring of NMDARs and enhancing the efficacy of synaptic activity (Vinade *et al.,* 2001). Activation of calpains by BDNF in dendrites and dendritic spines changes actin polymerization locally and may contribute to the role of BDNF in LTP (Zadran *et al.,* 2010). The decrease of calpain 1 activity of about 50% also reduced the incidence and magnitude of LTP (Vanderklish *et al.,* 1996), further suggesting a key role for calpains in this form of synaptic plasticity.

Calpain is also important in dopaminergic transmission, because its activation in response to Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (VGCCs) cleaves protein kinase C (PKC), releasing the constitutively active protein kinase M (PKM) fragment, which regulates the maintenance of burst firing, a process that enhances synaptic dopamine release (Tobler *et al.,* 2005; Liu *et al.,* 2007). PKM is also implicated in addiction and motivation, and is associated with learning and memory, suggesting that calpains could contribute to these processes independently of LTP mechanisms (Osten *et al.,* 1996; Steketee *et al.,* 1998).

Under physiological conditions, activation of calpain is temporary, since the rise in intracellular Ca^{2+} is transient, returning rapidly to basal levels. Once the resting conditions are established, calpain molecules return to their inactive state.

1.1.3.2. Calpains in CNS pathological conditions

Calpains are associated to several cell death processes and disease. Hyperactivation of calpains in response to sustained increase in $[Ca^{2+}]_i$ is related with severe cellular damage caused by physical trauma or ischemic insults, and was implicated in various neurodegenerative disorders, including Alzheimer's disease (AD) (Liang *et al.,* 2010), Parkinson's disease (PD) (Esteves *et al.,* 2010) and Huntington's disease (HD) (Reijonen *et al.,* 2010).

Calpain activation following ischemic injury leads to the cleavage of proteins in several cellular compartments, including synapse, plasma membrane, ER, mitochondria and nucleus. The calpain targets indentified under these conditions belong to various functional categories, especially neurotransmitter receptors, postsynaptic structural

proteins, calcium regulatory proteins and signaling proteins (Bevers and Neumar, 2008).

A critical component of the postischemic injury is the plasma membrane depolarization as a consequence of ATP reduction, which results in the opening of voltage-gated Na^+ and Ca^{2+} channels and the release of glutamate. The excessive glutamate release causes an overactivation of glutamate receptors, further contributing to alterations in ion homeostasis. Calpain itself may promote the sustained calcium overload by cleaving and altering the function of several proteins involved in $[Ca^{2+}]_i$ homeostasis (Neumar, 2000). Some examples are L-type calcium channels (Hell *et al.,* 1996), the NCX (Bano *et al.,* 2005) and the plasma membrane Ca^{2+} ATPase (Pottorf *et al.,* 2006). Cleavage of these proteins reduces the cell ability to maintain a low $[Ca^{2+}]_i$, contributing to cytosolic calcium overload.

The excessive activation of glutamate receptors following physical and ischemic insults leads to calpain activation, exacerbating cell death and injury. For example, calpain cleaves the C-terminal of mGluR1α preventing the coupling of the receptor to Akt activation; the consequent downregulation of Akt contributes to cell death (Xu *et al.,* 2007). Several subunits of the ionotropic NMDA, AMPA receptors undergo calpain-mediated truncation under excitotoxic conditions. These truncations further enhance calcium entry, increasing the proteolytic activity and cell death (Guttmann *et al.,* 2001; Chen *et al.,* 2009; Doshi and Lynch, 2009). Calpain has also been implicated in several chronic neurodegenerative conditions. The Huntingtin protein (Htt), for example, is cleaved by calpain generating fragments that are smaller than those generated by caspase cleavage. These Htt fragments translocate to the nucleus, because of their small size, and are toxic to cells (Hackam *et al.,* 1998). Inhibition of calpains also prevents neuronal and behavioral deficits in a mouse model of Parkinson disease (PD) (Crocker *et al.,* 2003). Given the role of calpains in neuronal death selective and potent calpain inhibitors might have therapeutic potential in the treatment of diseases of the nervous system involving calpain activation.

1.2. Neurotrophins and Signaling

1.2.1 BDNF and TrkB

The neurotrophins are a family of proteins that are essential for the development of the vertebrate nervous system (Chao, 2003; Cohen-Cory *et al.,* 2010). The initial studies in the field led to the discovery of nerve growth factor (NGF) by (Levimontalcini *et al.,* 1995), and since then neurotrophins have been shown to play a role not only in developmental biology, with a key role in neuronal cell survival, but also as regulators of axonal and dendritic growth and guidance, synaptic structure, and synaptic plasticity (McAllister *et al.,* 1999; Poo, 2001; Arevalo, 2006; Skaper, 2012). This group of low-molecular weight proteins includes mainly the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophins-3 and -4/5 (NT-3 and NT-4/5). The actions of neurotrophins depend on two different transmembrane-receptor signaling systems – the Trk receptor tyrosine kinases, which includes the TrkA, TrkB and TrkC receptors, and the p75 neurotrophin receptor ($p75^{NTR}$), member of the tumour necrosis factor (TNF) receptor family (Chao, 2003, Huang and Reichardt, 2003; Barker, 2004; Teng and Hempstead, 2004; Reichardt, 2006; Schecterson and Bothwell, 2010). The initial studies suggested that the effect of neurotrophins in promoting cell survival was mediated by Trk receptors, and the activation of $p75^{NTR}$ was related with neuronal cell death (Kalb, 2005). However, more recent studies have shown that $p75^{NTR}$ can also promote cell survival, and Trk receptors can adversely affect neuron health (Longo *et al.*, 2008; Moises*et al.,* 2009).

NGF binds to TrkA receptors, BDNF and NT-4/5 to TrkB receptors, and NT-3 to TrkC; TrkB receptors may also be activated by NT-3. In contrast with the specificity displayed by the Trk family of receptors, the $p75^{NTR}$ bind both the mature form of the neurotrophins and their uncleaved (precursor) forms (proneurotrophins) (Lee *et al.,* 2001, Underwood *et al.,* 2008). Neurotrophins are synthesized in a precursor form (proneurotrophins) and proteolytically processed to generate the mature neurotrophin. The cleavage of the neurotrophins may take place during the traffic in

the Golgi apparatus and/or by extracellular proteases after secretion (Bronfman *et al.,* 2007). BDNF was suggested to be released mainly in the precursor form and cleaved by tPA (Kuczewski *et al.,* 2009,; Lessmann *et al.,* 2009). Both the proneurotrophin and the mature form have biological activity (Lee *et al.,* 2001). Proneurotrophins tend to have higher affinity for $p75^{NTR}$, whereas the responses induced by the mature forms of neurotrophins are mediated by Trk receptors (Kalb, 2005).

The effects of BDNF in neuronal survival have been extensively investigated, both in the central and peripheral nervous system. Addition of exogenous BDNF has been shown to rescue different types of neurons form various injuries, such as in brain ischemia and trauma, and in several neurodegenerative pathologies (Almeida *et al.,* 2005; Almeida *et al.,* 2009; Jourdi *et al.,* 2009; Gyarfas*et al.,* 2010). The release of endogenous BDNF also provides protection to neuronal networks against injuries (von Bartheld *et al.,* 2001). Furthermore, changes in the abundance of BDNF are thought to be involved in so many diverse pathologies as depression (Castren and Rantamaki, 2010), dementia, diabetes and in body weight control (Pedersen *et al.,* 2009). Exercise as a mean of keeping cognitive function in healthy humans seems to involve the regulation of BDNF production, indicating that BDNF is also likely to mediate some of the beneficial effects of exercise (Stranahan *et al.,* 2009). Although a significant progress has been made in the understanding of the physiological roles of BDNF, additional work is required to unravel all of its actions (Schecterson and Bothwell, 2010). The presented thesis presents one more detail for this story.

The receptors of BDNF, TrkB receptors are highly expressed in the nervous system (Klein *et al.,* 1993; Kumanogoh *et al.,* 2008). The extracellular region of the receptor consists of a signal peptide, two cysteine-rich domains, a cluster of three leucine-rich motifs, and immunoglobulin-like domains, while its intracellular region contains the tyrosine kinase domain (Figure 2). The *trkB* gene also gives rise to truncated TrkB receptors, without tyrosine kinase activity. These receptors are formed through alternative splicing from the same *trkB* gene (Middlemas *et al.,* 1991). They lack the intracellular tyrosine kynase domain and have distinct short C-terminal amino acid sequences (T1, 11 residues; T2, 9 residues). Recent studies suggested that additional TrkB splicing variants may exist, mainly truncated forms of TrkB (Kumanogoh *et al.,* 2008, Luberg *et al.,* 2010).

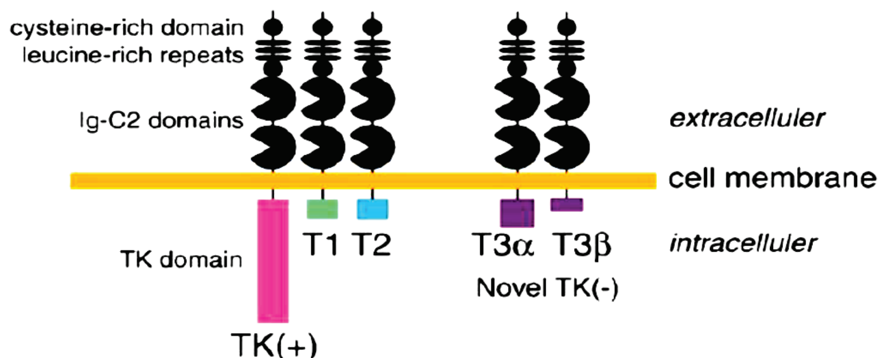

Figure 2.Structure of TrkB receptors. Protein structure of the full length (TK(+)) and truncated T1 and T2 receptors, and the new TrkB splicing variants (T3) (Kumanogoh *et al.,* 2008).

1.2.2. TrkB signaling pathways

Binding of neurotrophins to the Trk receptors promotes their dimerization (Jing *et al.,* 1992)and, in this form, the receptors phosphorylate each other on specific tyrosine residues, in the cytoplasmic domain. This creates docking sites for different adaptor proteins and enzymes, leading to the activation of various parallel signal transduction cascades, with distinct functions (Atwal *et al.,* 2000; Huang *et al.,* 2003; Reichardt, 2006). Given the high homology in the intracellular region of Trk receptors it is not surprising that their phosphorylation sites and the nature of the pathways activated are highly conserved (Atwal *et al.,* 2000). Three main cascades are activated by neurotrophins Trk receptors: the Ras/ERK pathway, the PI3-K/Akt pathway and the phospholipase C-γ (PLC-γ) pathway (Chao, 2003; Huang *et al.,* 2003; Reichardt, 2006; Numakawa *et al.,* 2010). Interestingly, the Ras/ERK pathway appears to be preferentially activated by the TrkA receptors, whereas TrkC receptors are preferentially coupled to the activation of Akt (Markus *et al.,* 2002; Jin *et al.,* 2010).

1.2.2.1. PI3-Kinase pathway

Production of P3-phosphorylated phosphoinositides is critical in mediating survival and neuroprotection of many populations of neurons (Brunet *et al.,* 2001), as well as in the

regulation of vesicular trafficking (Chen *et al.,*2001; Krag *et al.,* 2010). These inositol phospholipids generated by PI3-Kinase are targeted by proteins containinga pleckstrin homology (PH) domain, such as PKB/Akt, which are translocated to the membrane. After translocation to the membane PKB/Akt is activated by phosphorylation by upstream kinases, including PDK1 (phosphoinositide-dependent protein kinase 1) and PDK2 [possibly the rictor-mTOR complex; (Sarbassov *et al.,* 2005)].PKB/Akt phosphorylates several proteins that are important in the promotion of cell survival (Brunet *et al.,* 2001), most of them regulators of apoptosis (Yuan and Yankner, 2000). Bad, for example, is a Bcl-2 family member that promotes apoptosis through sequestration of Bcl-XL, which otherwise would inhibit Bax, a proapoptotic protein (Datta *et al.,* 1997). Phosphorylation of Bad by PKB/Akt sequesters the protein in the cytosol, bound to the protein 14-3-3, thereby avoiding its interaction with Bcl-XL (Dummler and Hemmings, 2007). Many other proteins in the apoptotic pathway have consensus sequences for Akt phosphorylation but, up to now, no data have provedthat they are Akt substrates (Dummler and Hemmings, 2007; Mullonkal and Toledo-Pereyra, 2007; Parcellier *et al.,* 2008; Rane *et al.*, 2009). Glycogen synthase kinase 3-β (GSK3β) is also phosphorylated by Akt, preventing its pro-apoptotic actions (Hetman *et al.,* 1999; Arboleda *et al.,* 2010). Furthermore, Akt interferes with the nuclear factor-κB (NF-κB) pathway, by phosphorylating and thus promoting the degradation of IκB, the inhibitory partner of this pathway. This results in the liberation of active NF-κB that promotes gene transcription, associated with neuronal survival (Foehr *et al.,* 2000; Wooten *et al.,* 2001; Bai *et al.,* 2009).

1.2.2.2. Ras/Erk pathway

Activation of the small GTPase Ras in response to neurotrophins has been connected to signaling and transcriptional regulation implicated in neuronal survival and differentiation (Arevalo, 2006). Since Ras proteins cycle between active GTP-bound and inactive GDP-bound states, the biological activity of these GTPases is controlled by guanine nucleotide exchange factors (GEFs) and guanosine triphosphatase activating proteins (GAPs). TrkA receptor stimulation by NGF engages Shc and Grb2 to activate the GEF SOS, which then activates Ras (Figure 3) (Stephens *et al.,* 1994). Raf-1 and B-

Raf activation downstream of Ras subsequently triggers the activation of extracellular signal-regulated kinases/mitogen-activated protein kinases (Erk/MAPK) (Troppmair *et al.,* 1992). Stimulation of Ras through this pathway promotes only transient activation of MAPK (Marshall, 1995). Modulation of Ras signaling can be achieve by a negative feedback loop, as activation of MAPK can stop the signaling of this pathway by phosphorylating SOS to disrupt the Grb2-SOS complex (Kao *et al.,* 2001).

Neurotrophins stimulate two different phases of MAPK activation. A transient one, described above, involves Shc-Grb2-SOS-Ras-B-Raf/Raf1-ERK, while a prolonged MAPK activation requires the adaptor CrkII/CrkL, the GEF C3G, the small GTPase Rap1, and B-Raf (Wu *et al.,* 2001). NGF activation of TrkA leads to activation of C3G by CrkII/CrkL.C3G then activates Rap1 to signal further downstream through B-Raf, resulting in the sustained activation of ERK. This pathway requires internalization of the Trk receptor into the endosomal compartment (York *et al.,* 2000).

1.2.2.3. Phospholipase C-γ

Trk receptor-mediated phosphorylation of PLCγ at Y785 leads to PLCγ recruitment and activation, which results in the hydrolysis of PtdIns(4,5)P_2 to generate inositol trisphosphate (IP3) and diacylglycerol (DAG) (Obermeier *et al.,* 1993). DAG activates different PKC isoforms, acting together with Ca^{2+} released from IP3-sensitive stores. PKC subsequently activates the Erk1 signalling pathway viaRaf (Corbit *et al.,* 1999). The release of Ca^{2+} from internal stores also stimulates Ca^{2+}-calmodulin-regulated protein kinases (CaM kinases) (Figure 3).

PLCγ activation in response to neurotrophins has been implicated in growth cone chemotropism and in the potentiation of thermal sensitivity by VR1, a heat activated ion channel on sensory neurons (Ming *et al.,* 1999; Chuang *et al.,* 2001). Furthermore, PLCγ signaling may be important for TrkB signaling in response to BDNF that is involved in synaptic plasticity in the hippocampus (Kang *et al.,* 1995; Patterson *et al.,* 1996; Minichiello *et al.,* 1999). The physiological roles of TrkB-mediated PLCγ signaling wasfurther investigated in vivo, by mutating the recruitment site, Y816, and showed a key role in the mechanisms of synaptic plasticity (Minichiello *et al.,* 2002). The Trk

stimulation of PLCγ pathway is also known to activate the transient receptor potential cation channels (TRPC) (Clapham, 2003). Two members of the TRPC subfamily, TRPC3 and 6, were shown to play a key role in the protection by BDNF of cerebellar granule neurons subjected to deprivation of trophic factors (Jia *et al.,* 2007).

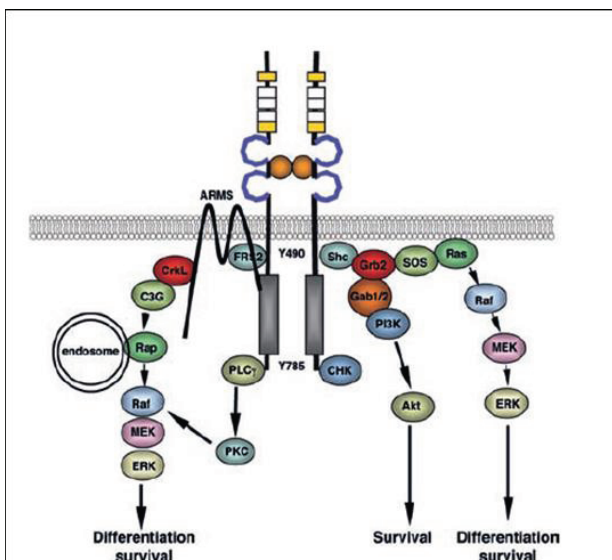

Figure 3.Trk receptor-mediated signaling pathways.Neurotrophin binding to Trk receptors leads to their activation to the recruitment of different proteins that associate with specific phosphotyrosine residues in the cytoplasmic domain of Trk receptors. These interactions trigger the activation of various signaling pathways, such as the Ras, Rap, PI3K, and PLCγ pathways, which result in survival, neurite outgrowth, gene expression, and synaptic plasticity (Arevalo, 2006).

1.2.3. Intracellular signaling by truncated Trk receptors

The truncated Trk receptors found in developing and mature neurons and astrocytes can inhibit signaling by full-length receptors (Eide *et al.,* 1996), an effect that may be mediated, at least in part, by a reduction in the cell surface expression of the full-length receptor (Haapasalo *et al.,* 2002). Although the truncated TrkB receptors lack tyrosine kinase activity they are still capable of inducing multiple signaling responses. Activation of the truncated TrkB receptor can induce the release of Ca^{2+} from Ins(1,4,5)P3-sensitive intracellular stores in glial cells (Rose *et al.,* 2003), and this effect may result from the release of a messenger from neurons that activates glial receptors, thereby regulating the release of glutamate from these cells. This regulatory process

may be propagated through many astroglia as calcium waves, which are able to pass through gap junctions.

Truncated TrkB receptors also regulate glial cell morphology via Rho GDP dissociation inhibitor (Ohira *et al.,* 2005; Ohira *et al.,* 2007) (Figure 4), and a similar role may be played by truncated TrkC receptors through a mechanism involving the activation of ADP-ribosylation factor 6 (Arf6) and the Rac1 GTPase (Esteban *et al.,* 2006). BDNF causes Rho GDI1 to dissociate from TrkB.T1 receptors, which acts negatively on Rho and Erk signaling cascades in a BDNF-dependent manner, and that TrkB.T1-Rho GDI1 signaling regulates the cellular morphology of glioma cells via remodeling of the actin cytoskeleton (Ohira *et al.,* 2007).

Heterologous expression studies examining coexpression of truncated and full-length trkB cDNAs in *Xenopus laevis* embryos (Eide *et al.,* 1996) and sympathetic neurons (Ninkina *et al.,* 1996) have shown that coexpression of truncated TrkB receptors with TrkB.FL reduced the ability of TrkB.FL to induce particular BDNF-dependent events in a truncated TrkB:TrB.FL ratio dependent manner. Taken together with evidences showing that the two TrkB isoforms are coexpressed in a least some neurons, the results suggest that TrkB.T receptors can act as naturally occurring dominant negative elements in cells coexpressing TrkB.FL receptors.

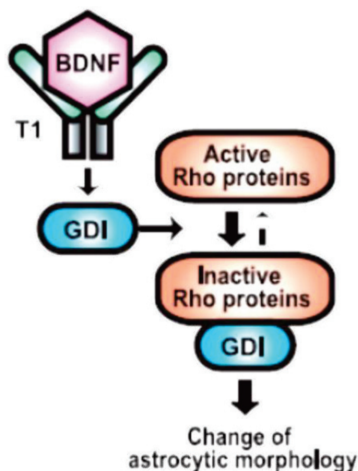

Figure 4.Truncated TrkB receptors signaling. In astrocytes, TrkB.T1 acts as a negative inhibitor of the Rho GTPases in a BDNF-dependent manner, changing the morphology of astrocytes (Ohira *et al.,* 2005).

1.2.4. TrkB receptor role in ischemia

Administration of mature neurotrophins to specific brain regions and to cultured neurons has been shown to prevent cell loss associated with excitotoxic events that take place upon chemical or mechanical insults, and in cerebral ischemia. Due to the very short half-life of neurotrophins in the plasma (Hetman *et al.,* 1999) and their poor blood-brain barrier permeability, modified neurotrophins have been used in some in vivo studies, in order to overcome those limitations (Gozes, 2001; Wu, 2005; Emerich and Thanos, 2008; Madduri and Gander, 2010). Application of BDNF, NT-3 and NT-4 in the brain all decreased infarct volume in models of cerebral ischemia (Beck, 1994; Chan *et al.,* 1996; Yamamoto *et al.,* 1997; Shi *et al.,* 1999; Chung *et al.,* 2009) and, interestingly, mice lacking NT-4 or deficient in BDNF develop significantly bigger infarcts then their wild-type littermates (Larsson *et al.,* 1999; Endres *et al.,* 2000), suggesting that endogenous neurotrophins also play a neuroprotective role under these conditions. The expression of BDNF increases in the ischemic penumbra (Kinoshita *et al.,* 2001; Lee *et al.,* 2001; Lee *et al.,* 2002; Miyake *et al.,* 2002; Rickhag *et al.,* 2006) due to glutamate receptor activation (Zafra *et al.,* 1990), constituting a feed forward mechanism that allows sustaining the neuroprotective BDNF signaling activity.

Neuronal injury in brain ischemia is primarily due to metabolic deprivation and excessive release of glutamate, leading to overactivation of glutamate receptors (excitotoxicity; see section 1.1.1) (Choi, 1988). This can be mimicked, to some extent, by stimulating cultured neurons with toxic concentrations of glutamate or glutamate receptor agonists, which cause cell death by an apoptotic-like mechanism or by necrosis, depending on the magnitude of the insult (Ankarcrona *et al.,* 1995). Neurotrophins, particularly BDNF and NT-3, have been shown to protect cultured hippocampal and cerebrocortical neurons from cell death evoked by glutamate (Mattson *et al.,* 1995; Wu *et al.,* 2004; Almeida *et al.,* 2005; Jiang *et al.,* 2005) or metabolic insults (Cheng and Mattson, 1991, 1994; Kim *et al.,* 2004), in agreement with the *in vivo* findings.

Activation of neurotrophin Trk receptors triggers parallel signaling pathways, including the PI3-K pathway, the Ras/ERK pathway and PLCγ (see section 1.2.2). The PI3-K pathway, and to some extent the Ras/ERK pathway, play a key role in neuroprotection

by neurotrophins and other neurotrophic factors, but their relative contribution has been shown to depend on the cell type and the survival factor. Neurotrophic signaling may result from the activation of receptors located in the cell body or in the neurites. The retrograde neurotrophic signaling, originated in distal axons, has been studied in neurons of the PNS, and is triggered by ligand-gated and Pincher-mediated internalization of the Trk receptors (Valdez *et al.,* 2005). According to one of the current models, the internalized receptors are incorporated into 'signaling endosomes' that convey the trophic effect to the cell body, along the microtubules using dynein as a motor (Howe *et al.*, 2005; Zweifel *et al.,* 2005; Cosker *et al.,* 2008; Wu *et al.,* 2009; Perlson *et al.,* 2010).

The PI3-K pathway suppresses cell death mainly by inhibiting the apoptotic effects of the forkhead transcription factor and Bcl-2-associated death protein (Bad) (Brunet *et al.,* 2001; Downward, 2004). In contrast with this pathway, the major role of the Ras/ERK pathway is to protect neurons from death due to injury or toxicity (e.g. excitotoxicity, calcium overload, oxidative injury, hypoxia), rather than from growth factor withdrawal. This pathway acts mainly by stimulating the activity and/or expression of anti-apoptotic proteins (e.g. Bcl-2) (Peng *et al.,* 2008) and the transcription factor CREB (Hetman *et al.,* 1999; Watson *et al.,* 2001), but a down-regulation in pro-apoptotic proteins may also be involved (Biswas *et al.,* 2002). Thus, in cortical neurons, inhibition of MEK prevented BDNF-induced protection from campothecin-induced apoptosis, but this was without effect on the trophic effects of the neurotrophin in serum-free medium (Hetman *et al.,* 1999; Hetman *et al.,* 2000).

The overlap in the contribution of the PI3-K and Ras/ERK pathways in neuroprotection by neurotrophins observed in some experimental paradigms [e.g. (Nakazawa *et al.,* 2002; Almeida *et al.,* 2005; Kaur *et al.,* 2007; Sun *et al.,* 2008)] may be due to cross-talk between the PI3-K and the Ras/ERK pathways. Depending on the cellular background, the PI3-K has been shown to stimulate or inhibit the activity of ERK (Moelling *et al.,* 2002; Sato *et al.,* 2004; Almeida *et al.,* 2005). In cultured hippocampal neurons, where ERK stimulation by BDNF requires PI3-K activity, the neurotrophin has a neuroprotective effect under excitotoxic conditions that is mediated by the PI3-K and the Ras/ERK pathways (Almeida *et al.,* 2005). The mechanisms acting in

neuroprotection by neurotrophins downstream of the ERK and PI3-K pathways may also differ depending on the insult. While the upregulation of anti-apoptotic proteins (e.g. Bcl-2) by neurotrophins and inhibition of caspase activity account for neuroprotection under conditions where cell death is typically apoptotic (Michaelidis *et al.,* 1996; Aloyz*et al.,* 1998; Madeddu *et al.,* 2004; Lesne *et al.,* 2005) other mechanisms may be involved in protection from insults where caspase activation plays a minor role in the demise process. Although calpains rather than caspases play a major role in excitotoxic cell death (Higuchi *et al.,* 2005; Wu *et al.,* 2007; Saatman *et al.,* 2010), neurotrophins have been shown to play a protective role under these conditions, both in cultured neurons and in vivo (see references above). Therefore, the activation of a complex program of changes in gene expression by Trk receptors (Schulte *et al.,* 2005; Schulte *et al.,* 2008) is expected to cause multiple changes in the proteome, rendering the cells better prepared against a wide range of insults.

As mentioned before two additional TrkB splicing isoforms are expressed in the brain, TrkB.T1 and TrkB.T2, containing unique short C-terminal amino acid sequences (T1, 11 residues; T2, 9 residues) and lacking tyrosine kinase activity (Manadas *et al.,* 2007). New truncated isoforms were recently described (Kumanogoh *et al.,* 2008), but the overall physiological role of these receptors remains obscure. The truncated TrkB receptors were shown to behave as dominant negative inhibitors of TrkB-FL in the regulation of cell survival (Steinbeck *et al.,* 2005, De Wit *et al.,* 2006, Dorsey*et al.,* 2006, Turner*et al.,* 2006) but they may also induce their own signaling cascades independent of full length TrkB (Manadas *et al.,* 2007; Carim-Todd*et al.,* 2009). Important studies reported that TrkB.T1 receptors are coupled to the inhibition of RhoA (Ohira *et al.,* 2005), a small GTPase that mediates the Ca^{2+}-dependent activation of p38MAPK which is followed by neuronal death under excitotoxic conditions (Semenova *et al.,* 2007). This regulation is made through the Rho guanine nucleotide dissociation inhibitor (Rho GDI) 1 that binds the C-terminal of T1 (Ohira *et al.,* 2005; Ohira *et al.,* 2007). The binding of BDNF to T1 causes the Rho GDI 1 dissociation from the C-terminal of T1. Once dissociated Rho GDI 1 inhibits the activation of RhoA by forming a complex with the GDP-bound form of the Rho (Gorovoy *et al.,* 2007). This mechanism has been shown to alter astrocytic morphology, but it has not yet been demonstrated in neurons.

The TrkB isoforms are differentially regulated under pathological conditions (Checa *et al.,* 2001; Asai *et al.,* 2007; Manadas *et al.,* 2007). Injection of kainate in the hippocampus of the adult rat increased BDNF protein levels and the truncated TrkB receptor, whereas the full length receptor levels did not change significantly (Rudge *et al.,* 1998). The TrkB and BDNF immunoreactivity also increased in the penumbra region in a rat model of stroke (middle cerebral artery occusion) (Ferrer *et al.,* 2001). In this study the truncated TrkB was also increased in astrocytes surrounding the infarct area. The impact of the expression of truncated TrkB receptors in neurons in bain ischemia was investigated using a transgenic mouse line overexpressing the truncated TrkB.T1 in postnatal cortical neurons. The expression of TrkB.T1 was found to increase the rate of neuronal death after transient focal cerebral ischemia when compared with wild type mice (Saarelainen *et al.,* 2000). Furthermore, preconditioning ischemia was found to downregulate TrkB.T1 protein levels,therebypromoting BDNF-mediated protective signaling via the full-length receptor and thus contribuing to ischemic preconditioning tolerance (Steinbeck and Methner, 2005). Results from our laboratory also showed an upregulation of TrkB.T and a down-regulation of TrkB.FL in cultured hippocampal neurons exposed to excitotoxic stimulation with glutamate (Chapter 2).

1.3. GABAergic neurotransmission in physiological and pathological conditions

The amino acid γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS), being present in a large percentage of the synapses in the brain. GABA is synthesized by decarboxylation of glutamate by the enzyme glutamic acid decarboxylase (GAD). This inhibitory neurotransmitter also exists in plants and bacteria, where it serves a metabolic role in the Krebs cycle (Streeter and Thompson, 1972; Jorgensen, 2005; Panagiotou *et al.,* 2005). Although the expression of GABA in the nervous system was first described in 1950 by Eugene Roberts and Jorge Awapara, it was only accepted as a neurotransmitter more than 10 years later (Roberts and Frankel, 1950; Del Castillo *et al.,* 1964). The difficulty in the identification of GABA as a neurotransmitter came from its enormous abundance in the vertebrate brain, more than 1000 fold higher than monoamine transmitters, its simple structure

and the role in Krebs cycle, suggesting that it was likely to be involved in metabolism rather than in intercellular signaling (Schuske *et al.,* 2004; Jorgensen, 2005). GABA was only proposed as a truly neurotransmitter after the studies conducted in nematodes, especially in C. elegans (Del Castillo *et al.,* 1964; Edwards*et al.,* 1999).

The release of GABA by neurons can be mediated by several different mechanisms (Saransaari and Oja, 1992; Ge *et al.,* 2007): (1) Amino acid molecules may simple leak through plasma membranes; (2) Reversal of the plasma membrane GABA transporters (due to changes in the electrochemical gradients) (Conti *et al.,* 2004); (3) Ion channels in the membranes may also mediate GABA release despite the size of the molecule (Chen *et al.,* 2004) ; (4) Finally, GABA can be extruded from the cells by exocytosis of synaptic vesicles (the more physiological and most common mechanism under normal physiological conditions. The release of GABA in extrasynaptic regions leads to the activation of non-synaptic $GABA_A$ receptors to generate tonic inhibitory currents. These synaptic and extrasynaptic modes of GABA action have been termed phasic and tonic effects, respectively, and control neuronal excitability in a different manner (Mody and Pearce, 2004; Farrant and Nusser, 2005).

Inhibitory neurotransmission in the adult nervous system is primarily mediated by the exocytosis of synaptic vesicles containing GABA and glycine. GABAergic inhibition predominates in the brain, whereas both glycine and GABA act as the primary inhibitory neurotransmitter in the spinal cord and brainstem. There are three types of GABA receptors, named $GABA_A$, $GABA_B$ and $GABA_C$ receptors. These GABA receptor subtypes were originally characterized based on their pharmacological properties. $GABA_A$ receptors are activated by muscimol and blocked by bicuculline, whereas $GABA_B$ receptors are activated by baclofen and blocked by saclofen or phaclofen (Guillon *et al.,* 1999). The fast inhibitory actions of GABA are mediated by the activation of $GABA_A$ receptors in the brain, and a similar role is played by $GABA_C$ receptors in the retina. The slow prolonged actions of GABA are mediated by metabotropic G-protein-coupled $GABA_B$ receptors (Jacob *et al.,* 2008).

$GABA_A$ receptors are ionotropic chloride channels, carrying primarily Cl^- in or out of the cell depending on the electrochemical gradient of the anion. Since chloride ions are pumped out of the cells in adult neurons the activation of $GABA_A$ receptors allows the influx of chloride, hyperpolarizing the membrane and decreasing the excitability of the

cell. This type of inhibition is called hyperpolarizing inhibition. In some cells, particularly in embryonic neurons, the intracellular chloride concentration is higher than the extracellular concentration. Activation of $GABA_A$ receptors under the latter conditions causes an efflux of Cl^-, depolarizing the membrane (Ge *et al.,* 2007). The chloride homeostasis in neurons is determined by two major transporters, the Na^+-K^+-Cl^- co-transporter, NKCC1 (a Cl^- accumulator), and the K^+-Cl^- co-transporter KCC2 (a Cl^- exporter) (Ben-Ari, 2002; Owens and Kriegstein, 2002). During embryonic development, NKCC1 is expressed in immature neurons, whereas little KCC2 is expressed, resulting in a high $[Cl^-]_i$. During embryonic development and maturation, neurons downregulate NKCC1 expression and upregulate KCC2 expression, resulting in a lower $[Cl^-]_i$ in most mature neurons.

Unlike $GABA_A$ receptors that form ion channels, $GABA_B$ receptors are coupled to the regulation of second messenger systems through the binding and activation of guanine nucleotide-binding proteins (G proteins) (Campbell *et al.,* 1993; Pinard *et al.,* 2010). $GABA_B$ receptors predominantly couple to Giα- and Goα-type G proteins (Pinard *et al.,* 2010). It is now well established that presynaptic $GABA_B$ receptors repress Ca^{2+} influx by inhibiting Ca^{2+} channels in a membrane-delimited manner via the Gβγ subunits. Postsynaptic $GABA_B$ receptors trigger the opening of K^+ channels, again through the Gβγ subunits (Bettler and Tiao, 2006). These results are a hyperpolarization of the postsynaptic neuron that underlies the late phase of inhibitory postsynaptic potentials (IPSPs) (Luscher *et al.,* 1997). Besides modulating ion channels through Gβγ, $GABA_B$ receptors activate and inhibit adenylyl cyclase via the Giα/Goα and Gβγ subunits.

Many studies have reported that $GABA_B$ receptors inhibit forskolin-stimulated cAMP formation, but others also observed a stimulation of cAMP production (Bowery *et al.,* 2002; Calver *et al.,* 2002). Giα and Goα proteins inhibit adenylyl cyclase types I, III, V, and VI, while Gβγ stimulates adenylyl cyclase types II, IV, and VII. This stimulation depends on the presence of Gsα, which results from the activation of other GPCRs by, e.g., norepinephrine, isoprenaline, histamine, or vasoactive intestinal polypeptide (Tang and Gilman, 1991; Simonds, 1999). Therefore, the stimulatory action of $GABA_B$ receptors on cAMP levels is a consequence of G protein crosstalk and depends on the expression of specific adenylyl cyclase isoforms together with $GABA_B$receptors and

Gsα-coupled GPCRs. The activity of $GABA_B$ receptors on adenylyl cyclase may modulate neuronal function on a longer time scale (Bettler *et al.,* 2004).

The activity of GABAergic synapses is important in the regulation of the overall activity of neuronal networks, refraining the effects resulting from the activity of excitatory synapses. Thus, in the cerebral cortex there are two main classes of neurons, pyramidal cells and interneurons, which use glutamate and γ-aminobutyric acid (GABA) as main neurotransmitters, respectively. Therefore, in the adult cerebral cortex the pyramidal cells are excitatory while the GABAergic interneurons are typically inhibitory.Some evidences suggest that disruption of the excitatory–inhibitory balance maintained by pyramidal cells and interneurons is linked to the etiology of multiple neuropsychiatric and ischemic conditions (Obrenovitch, 2008; Xu *et al.,* 2008; Gogolla *et al.,* 2009; Haberg *et al.,* 2009). The generation of GABAergic synapses during development precedes the establishment of functional glutamatergic inputs (Chen *et al.,* 1995), and the initially excitatory action of GABA during development may therefore be a self-limiting process that establishes early network activity and serves as a mechanism to avoid excitotoxicity (Ben-Ari, 2002; Wojcik *et al.,* 2006).

Excitotoxicity, which is mediated by an overactivation of glutamate receptors, plays a key role in neuronal death characteristic of different disorders of the nervous system, such as ischemia, epilepsy and neurodegenerative diseases (Szydlowska and Tymianski, 2010). The research in cerebral ischemia and excitotoxic neuronal damage has been mainly explored from the point of view of the excitatory mediators, and much less is known regarding the changes in GABAergic activity (Schwartz-Bloom and Sah, 2001). The release of GABA in the ischemic brain and the consequent activation of $GABA_A$ receptors may be neuroprotective through reduction of membrane depolarization. However, Cl^- entry through $GABA_A$ receptors in association with overactivation of glutamate receptors may further increase the influx of water and cell swelling. The former mechanisms may be dominant since neuroprotective strategies to increase GABAergic neurotransmission, targeting both sides of the synapse, have been tested, and some of them were found to be quite efficacious in animal models of ischemia (Schwartz-Bloom and Sah, 2001).

The impairment of GABAergic synaptic transmission in brain ischemia is partly due to a down-regulation of synaptic $GABA_A$ receptors, which can contribute to the ongoing

neuronal excitability and possibly to neuronal death (Schwartz-Bloom and Sah, 2001). The alterations in GABAergic synaptic transmission were investigated in hippocampal slices exposed to oxygen- and glucose-deprivation (OGD), and the results showed an early release of GABA by exocytosis, followed by a delayed phase of neurotransmitter release mediated by reversal of the plasma membrane transporter (Allen *et al.,* 2004). Furthermore, OGD induced a decline in amplitude of miniature inhibitory post-synaptic potentials, showing a significant decrease in functional synaptic $GABA_A$ receptors (Kelley *et al.,* 2008). A down-regulation of a subset of $GABA_A$ receptor subunits expressed in cultured mouse cerebellar granule cells was also observed after excitotoxic stimulation witn kainate (Uusi-Oukari *et al.,* 2004). Moreover, a recent study showed a depletion of synaptic $GABA_A$ receptors containing β3 subunits in hippocampal neurons subjected to oxygen and glucose deprivation, by a mechanism dependent on a motif containing three arginine residues (405RRR407), present in the intracellular domain of this subunit, which is responsible for the interaction with AP2 adaptor for clathrin-mediated endocytosis (Smith *et al.,* 2012). Thus, interventions to prevent ischemia-induced decline in synaptic $GABA_A$ receptors may represent a novel neuroprotective strategy.

1.3.1. The role of gephyrin at GABAergic synapses

Gephyrin was originally identified as a peripheral membrane protein that copurifies with the mammalian GlyR (Graham *et al.,* 1985; Prior *et al.,* 1992). Additional studies showed a role for gephyrin as a scaffold protein essential for stabilizing glycine and $GABA_A$ receptors at inhibitory synapses (Kirsch and Betz, 1998; Kralic *et al.,* 2006). Gephyrin consists of three major domains, two of which were named the G and E domains because of sequence similarities with the bacterial Moco-synthesizing enzymes MogA and MoeA. The 20 kDa N-terminal G domain and the 43 kDa C-terminal E domain in gephyrin are linked by a central domain (C domain; also referred to as the linker region) of 18-21kDa (Smolinsky *et al.*, 2008) (Figure 5).

The interactions between gephyrin and glycine receptors, as well as gephyrin oligomerization, have been characterized to a great extent. The isolated G domain

forms stable trimers that are also found in purified, nonaggregated hologephyrin. G domain trimerization depends on identified residues (Saiyed *et al.,* 2007). In contrast, the isolated E domain forms dimers in solution and contains the high-affinity binding site for the M3-M4 intracellular loop of the GlyR β subunit (β loop). The E domain crystal structure bindsto the GlyR β loop at the dimerization interface, with residues F330, Y673 and P713 being crucial for the formation of a high-affinity binding site (Schrader *et al.,* 2004). On the other hand, the C domain contains binding sites for several gephyrin-interacting proteins such as Pin 1, dynein light chain 1 and 2 and collybistin (Figure 6) (Sola *et al.,* 2004).

Figure 5.Structure of gephyrin.Gephyrin has threedomains: G domain, C domain or linker region and E domain.

Upon overexpression, recombinant gephyrin forms intracellular aggregates in various cell lines. The current model of gephyrin aggregation, derived from the structure of the isolated G and E domains, postulates the formation of a regular hexagonal lattice by G domain trimerization and E domain dimerization (Saiyed *et al.,* 2007). A major open question is whether gephyrin aggregation (the capacity to oligomerize and form intracellular aggregates) and synaptic clustering represent two facets of the same process. The selective postsynaptic localization of gephyrin clusters in neurons suggests that specific mechanisms prevent gephyrin aggregation before targeting synaptic sites. Thus, gephyrin clustering in neurons is likely to be regulated by numerous factors, including posttranslational modification, binding of interacting proteins and possibly activation of GlyRs (Kirsch and Betz, 1998).

In the CNS, gephyrin clusters are selectively found at postsynaptic sites of glycinergic, GABAergic and mixed glycinergic/GABAergic synapses. In primary neuronal cultures, gephyrin likewise clusters at postsynaptic sites, although in the absence of appropriate presynaptic input these clusters can also face glutamatergic terminals (Brunig *et al.,* 2002; Christie *et al.,* 2002). However, in these cultures, gephyrin and PSD-95 appear to

be mutually exclusive, suggesting that these proteins play a role in determining the molecular composition of the postsynaptic density.

It is well accepted that postsynaptic clustering of GlyR is strictly dependent on gephyrin clustering. Introduction of the gephyrin-binding motif of the GlyR β loop into other membrane proteins is sufficient for co-clustering with gephyrin (Meyer *et al.,* 1995). Yet, the interaction between gephyrin and the GlyR β loop has not been fully clarified. Initially, it was suggested that gephyrin aggregation might be directly linked to GlyR dynamics and activity, because the GlyR β loop binds at the E domain dimerization interface (Bedet *et al.,* 2006). However, both GlyR and gephyrin were found to be co-transported intracellularly to the plasma membrane, suggesting binding of nonclustered gephyrin to the receptor (Hanus *et al.,* 2004). Finally, the binding affinity of gephyrin to the GlyR β loop is modulated by a conformational change induced by phosphorylation-dependent binding of Pin1 to the C domain of gephyrin (Zita *et al.,* 2007), suggesting a role for specific protein kinases in regulating gephyrin and/or GlyR clustering.

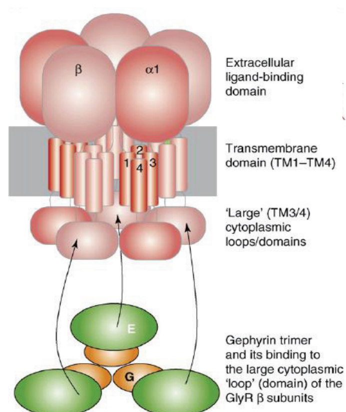

Figure 6.Model of gephyrin structure and postsynaptic targeting and clustering with GlyR and GABA$_A$ receptors. Cartoon of the pentameric GlyR with its three functional domains, the extracellular ligand-binding, the transmembrane and the cytoplasmic (loop) domains.Interaction with the gephyrin trimer is depicted (Fritschy *et al.,* 2008).

The dependence of GlyR on gephyrin for clustering does not imply that glycinergic postsynaptic sites are static structures. Rather, GlyR binding to gephyrin might be relevant forintracellular transport (Fuhrmann *et al.,* 2002; Hanus *et al.,* 2004) and might regulate the lateral mobility of receptors in the cell membrane (Dahan *et al.,* 2003; Levi *et al.,* 2004; Hanus *et al.,* 2006; Ehrensperger *et al.,* 2007) as well as their interaction with the cytoskeleton (Charrier et al., 2006). Taken together, the currently

available experimental evidences showing the intracellular co-transport of GlyR and gephyrin suggests that receptor mobility is crucially dependent on interactions of gephyrin with specific regulatory proteins and/or posttranslational control mechanisms.

The interactions between gephyrin and $GABA_A$receptors are more complex and the specific role of scaffold proteins at GABAergic synapses is not well understood. It is conceivable that postsynaptic clustering may be limited to specific gephyrin conformations or posttranslational modifications, or requires the presence of additional molecules. In support of this hypothesis, gephyrin forms intracellular aggregates, but not postsynaptic clusters, in neurons lacking certain $GABA_A$ receptor subunits in vivo (Kralic *et al.,* 2006; Studer *et al.,* 2006). Furthermore, a chimeric gephyrin protein with two E domains, designed to impair intermolecular dimerization, was shown to preferentially form intracellular aggregates and affect endogenous postsynaptic gephyrin clustering (Lardi-Studler *et al.,* 2007). This mutant gephyrin aggregates with endogenous gephyrin molecules, but it is rarely found at postsynaptic sites. These results strongly suggest that formation of gephyrin aggregates is differentially regulated (Kralic *et al.,* 2006).

Although removal of gephyrin by gene targeting or small interference RNA expression strongly affects $GABA_A$ receptor clustering (Yu *et al.,* 2007), neurons lacking gephyrin may still exhibit $GABA_A$ receptor clustering indicating tha additional factors are also involved (Fischer *et al.,* 2000; Levi *et al.,* 2004). In fact, the subsynaptic localization of gephyrin in GABAergic synapses also depends on $GABA_A$ receptor clustering (Alldred *et al.,* 2005). Thus, when $GABA_A$ receptor clusteringis disrupted by targeted deletion of the gene encoding the γ2 subunit, gephyrin clusters disappear and the receptors disperse in the cell membrane, suggesting an important role for gephyrin in the $GABA_A$ receptors anchoring (Schweizer *et al.,* 2003; Li *et al.,* 2005). Likewise, when $GABA_A$ receptors are lacking owing to targeted deletion of a α subunit isoform, gephyrin forms intracellular aggregates, indicating disruption or prevention of postsynaptic targeting and localization (Kralic *et al.,* 2006; Studer *et al.,* 2006). In contrast with GlyR, it is still unclear whether gephyrin interacts with $GABA_A$ receptors intracellularly and contributes to their trafficking.

$GABA_A$ receptors might be trafficked to the plasma membrane by a mechanism dependent on palmitoylation (Keller *et al.,* 2004; Rathenberg *et al.,* 2004; Fang *et al.,* 2006), suggesting that interaction with gephyrin occurs mainly at the membrane and regulates cell-surface dynamics. Inhibition of gephyrin expression did not modify the total number of $GABA_A$ receptors expressed on the neuronal cell surface but significantly decreased the number of receptor clusters. These clusters that formed in the absence of gephyrin were significantly more mobile compared with control neurons. Together, these results suggest a specific role for gephyrin in reducing the diffusion of $GABA_A$ receptors, facilitating their accumulation at inhibitory synapses (Luscher *et al.,* 2004; Yu *et al.,* 2007).

An important unresolved question is how the subcellular targeting of distinct $GABA_A$ receptor subtypes (differing in subunit composition) to specific synaptic or extrasynaptic sites is regulated. Some $GABA_A$ receptor subtypes localizing extrasynaptically, such as those containing the α4 subunit, largely fail to form postsynaptic clusters with gephyrin, as shown in thalamic neurons (Kralic *et al.,* 2006). With regard to synaptic receptors, interactions of gephyrin with extracellular matrix proteins have been implicated in the targeting of GABAergic terminals to the axon initial segment of pyramidal cells (Burkarth *et al.,* 2007).

Regulation of gephyrin-$GABA_A$ receptor subunit interactions possibly represents a key difference when compared with GlyR-gephyrin interactions, because different mechanisms clearly control the postsynaptic localization of these two receptor types. It is conceivable, for example, that $GABA_A$ receptor binding to gephyrin occurs cooperatively, involving more than one subunit type, or that it is more stringently dependent on specific gephyrin conformations, splice variants and/or posttranslational modifications. In contrast to GlyR, none of the many different $GABA_A$ receptor subunits is present with three copies in a given receptor subtype, suggesting overlapping binding sites for the gephyrin trimer (Fritschy *et al.,* 2008). The heterogeneity of $GABA_A$ receptor subtypes needs to be considered, as their specific location in distinct synapses within a given neuron hardly appears compatible with high-affinity binding of a ubiquitous $GABA_A$ receptor subunit to gephyrin (Fritschy *et al.,* 2008). The $GABA_A$ receptor α2 subunit has a hydrophobic motif in the intracellular loop, which binds

directly to gephyrin (Tretter *et al.,* 2008), but recent studiesshowed that gephyrin binds preferentially to α1 and α3 (Maric *et al.,* 2011). Furthermore, the binding sites to $GABA_A$ or glycine receptors are mutually exclusive and are formed by aromatic residues; in particular, hydrophobic interactions play a key role in gephyrin binding to $GABA_A$ receptors (Maric *et al.,* 2011).

The mechanisms of gephyrin trafficking to subsynaptic sites have been investigated in detail following the identification of proteins which regulate these processes (see Table 1). Some of these interactors are currently seen as key molecules contributing to gephyrin transport and clustering, such as dynein light chain (Dlc) 1 and 2, which bind to the gephyrin C domain (linker region) (Fuhrmann *et al.,* 2002) and are components of motor proteins likely to contribute to gephyrin transport along microtubules (Maas *et al.,* 2006).

Table 1. Gephyrin interactors(Adapted fromFritshy *et al.,*2008).

Interactor	Synaptic location	Interaction site	Biological role and key references
Collybistin/hPEM2	Unknown	C domain	Collybistin is a RhoGEF for Cdc42 that controls submembrane accumulation and synaptic localization of gephyrin in cellular and neuronal models. However, knockout mice reveal a region-specific loss of gephyrin and $GABA_A$ receptor clusters in hippocampus and amygdala, suggesting that other gephyrin clustering factors exist [28,30–32].
Dlc1/2	Yes	C domain	Dlc1 and Dlc2 are cargo-binding components of cytoplasmic dynein and myosin–Va complexes and mediate the retrograde transport of gephyrin [23,24,70].
GABARAP	No	C domain	GABARAP interacts with the $GABA_A$ receptor γ subunits, gephyrin, microtubules and other proteins implicated in $GABA_A$ receptor trafficking (PRIP-1/2, NSF); knockout mice suggest that GABARAP is not essential for trafficking of $GABA_A$ receptors and gephyrin (for a review, see Refs [52,71]).
$GABA_A$ receptor α2 subunit	Yes	Unknown	Binding of gephyrin to a hydrophobic motif of the α2 subunit intracellular loop regulates the postsynaptic localization of α2-$GABA_A$ receptors in cultured cortical neurons [58].
GlyR β subunit	Yes	E domain	GlyRs are clustered at synapses by gephyrin via a direct interaction with the β subunit. Gephyrin is crucial for the intracellular transport of GlyRs and regulation of the lateral mobility of GlyRs in the cell membrane (for a review, see Ref. [4]).
Mena/VASP (*m*ammalian *ena*bled/*vas*odilator *s*timulated *p*hosphoprotein)	Yes	E domain	Mena/VASP increases the efficiency of actin polymerization by recruiting profilin and G-actin and is involved in actin filament formation and bundling. Direct interaction of Mena/VASP with gephyrin might mediate or regulate gephyrin–microfilament interactions [26,27].
Pin1 (peptidyl-prolyl isomerase NIMA interacting protein 1)	Unknown	C domain	Pin1 is a peptidyl-prolyl isomerase that interacts with gephyrin in a phosphorylation-dependent manner, triggering conformational changes in gephyrin that enhance the binding of the GlyR β subunit [36].
Profilin 1 and profilin 2	Yes	E domain	Profilins regulate actin polymerization in a PIP_2-dependent manner. Direct interaction of profilins with gephyrin might mediate or regulate gephyrin–microfilament interactions [26,72].
Protein kinase(s)	Unknown	C domain and unknown	Gephyrin is phosphorylated on serine and threonine residues by a protein kinase that utilizes ATP, but not GTP, as phosphate donor. The activity of this kinase is not affected by various activators and/or inhibitors of cyclic nucleotide-dependent kinases, calcium/calmodulin-dependent kinases or protein kinase C [73].
RAFT1 (rapamycin and FKBP12 target (FRAP/mTOR)	Unknown	C domain or E domain?	RAFT1 mediates drug actions of the immunosuppressant rapamycin and affects protein translation by phosphorylating the 70 kDa S6 kinase (pp70S6k), which in turn phosphorylates 4E-BP1 (a repressor of protein translation initiation) and the ribosomal S6 protein. RAFT1 mutants that no longer interact with gephyrin do not signal to these downstream molecules, suggesting that gephyrin might act as a hub for signal transduction pathways involving translational control at synapses [74].
Tubulin	Unknown	C domain?	Gephyrin binds with high affinity to polymerized tubulin *in vitro*, possibly via a motif in the C domain which displays high sequence similarity to the core repeats of the microtubule-binding domains of MAP2 and tau [68].

The main partner of gephyrin in its clustering at inhibitory synapses is collybistin, a protein capable of translocating gephyrin from cytoplasmatic aggregates to a

submembranous compartment (Kins *et al.,* 2000), contributing to the formation ofthe hexameric structure characteristic of gephyrin clusters (Tretter *et al.,* 2008) (Figure 7). Collybistin belongs to the Dbl family of guanine nucleotide exchange factors (RhoGEFs) and is specific for the small GTPase Cdc42 (Reid *et al.,* 1999). It has an N-terminal src homology (SH3) domain, a catalytic RhoGEF domain and a pleckstrin homology (PH) domain, but several variants encoding different N- and C-terminally modified forms are generated by alternative splicing (Kins *et al.,* 2000; Harvey *et al.,* 2004). The SH3 domain regulates collybistin activity and gephyrin cluster size, whereas the phosphoinositide-binding PH domain is required for submembrane translocation of collybistin and gephyrin *in vitro* (Harvey *et al.,* 2004).

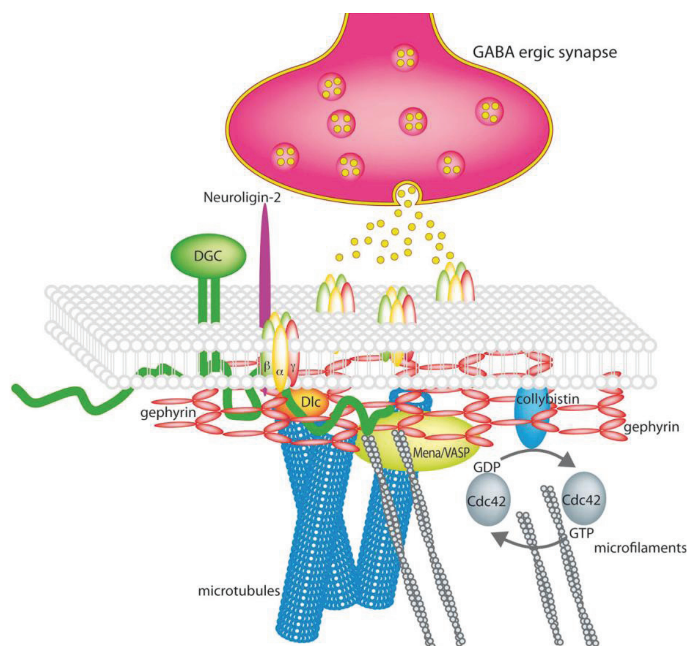

Figure 7.Gephyrin hexameric structure at GABAergic synapses. The interaction between collybistin, gephyrin and dynein, showing the scaffold structure at GABAergic synapses (Tretter *et al.,* 2008).

Several studieshave investigated whether collybistin participates actively in membrane targeting of gephyrin or controls gephyrin aggregation, or both. Interestingly, targated disruption of the gene encoding collybistin in mice results in a mild phenotype, unlike mutations altering glycinergic transmission, and did not induce changes on gephyrin or

GlyR clustering at glycinergic synapses (Papadopoulos *et al.,* 2007). However, gephyrin clustering was strongly affected at certain GABAergic synapses, leading to a region-specific loss of $GABA_A$ receptor clusters (Papadopoulos *et al.,* 2007). More recent studies showed a direct interaction between collybistin, gephyrin and $GABA_A$ receptors containing α2 subunit (Saiepour *et al.,* 2010).$GABA_A$R α2 subunits, but not α3, bind to both gephyrin and collybistin using overlapping sites. The reciprocal binding sites on gephyrin for collybistin and $GABA_A$R α2 also overlap at the start of the gephyrin E domain. This suggests that $GABA_A$R α2, collybistin and gephyrin form a trimeric complex and it was proposed that the collybistin-gephyrin complex has an intimate role in the clustering of $GABA_A$Rs containing the α2 subunit. Accordingly, immunocytochemistry experiments showed a preferential co-localization of collybistin with α2-subunit containing $GABA_A$ receptors, but not GlyRs or other $GABA_A$Rs subtypes.

1.4. Objectives

Overactivation of the proteolytic machinery plays a key role in neuronal death under excitotoxic conditions. In particular the $[Ca^{2+}]_i$ overload resulting from the overactivation of glutamate receptors leads to the cleavage of calpain substrates, changing the subcellular localization and/or activity of many proteins in the cell. Thus, in this study we have investigated the effects of excitotoxic stimulation on two key proteins: TrkB, the receptor for BDNF that plays an important role in neuronal survival and Gephyrin, a scaffold protein of $GABA_A$ receptors which is important for clustering and trafficking of these types of receptors at inhibitory synapses.

In the study described in chapter 2, we addressed the changes in TrkB protein expression and activity in cultured hippocampal and striatal neurons under excitotoxic conditions. The TrkB receptors for BDNF play an important role in the survival and differentiation of different neuronal populations, both in the central and peripheral nervous system. Furthermore, TrkB receptors protect different types of neurons from various injuries, including ischemia, trauma, and other pathologies (Almeida *et al.,* 2005; Almeida *et al.,* 2009; Jourdi *et al.,* 2009; Gyarfas *et al.,* 2010). The work performed in this thesis showed a down-regulation of the TrkB.FL receptors following excitotoxic stimulation of cultured hippocampal and striatal neurons, associated with a decrease of the receptor signaling activity. Under the same conditions a transcription-dependent upregulation of truncated TrkB receptors wasinvestigated. The effects on the signaling activity of TrkB.FL receptors were assessed at different levels: i) TrkB.FL phosphorylation, the first step following binding of BDNF to the receptors and their dimerization; ii) PLCγ phosphorylation, which follows the interaction of the enzyme with the active receptor; iii) phosphorylation (presumably activation) of ERK, a downstream mediator of one of the signaling cascades activated by TrkB receptors (Middlemas*et al.,* 1994; Huang and Reichardt, 2003; Manadas *et al.,* 2007). The changes of TrkB.FL protein levels were also studied in a mouse model of transient focal ischemia. The truncated TrkB receptors (splicing isoform of TrkB)upregulated following an excitotoxic injury may have dual effects on cell survival, acting as a dominant negative to inhibit TrkB.FL receptor activity, and/or downregulating the excitotoxic signaling through inhibition of RhoA. The two models were addressed experimentally

in the studies described in Chapter 2. The loss of trophic support by BDNF observed in this study may contribute to neurodegeneration after the ischemic insult and may play an important role in neurodegenerative processes (Drukarch and van Muiswinkel, 2001; Gauthier *et al.,* 2004; Tolosa *et al.,* 2009; Counts and Mufson, 2010). On the other hand, this study aimed at understandingwhether activation of TrkB.T receptors may constitute an endogenous neuroprotective strategy under conditions characterized by excitotoxic cell death,including brain ischemia, cerebral trauma, epileptic seizures and in chronic neurodegenerative disorders.

In the studies described in chapter 3 we investigated postsynaptic changes in GABAergic synapses under excitotoxic conditions, focusing on gephyrin, the protein responsible for anchoring $GABA_A$ receptors at the synapse and for the regulation of their trafficking at cell surface. Considering that gephyrin clustering is required for the stability of GABAergic synapses, we investigated putative changes in the gephyrin protein levels, which can be associated with a disruption of gephyrin submembranous structure formed at GABAergic synapses. To address this question we subjected cultured hippocampal and striatal neurons to excitotoxic stimulation with glutamate, under conditions that induce apoptotic-like death of about 40-50% of the neurons. To some extent these conditions mimic the toxic effects of glutamate in the penumbra region of a stroke in humans. Furthermore, we tested the alterations in gephyrin protein levels in hippocampal neurons subjected to oxygen and glucose deprivation (OGD), an in vitro model of global ischemia, and in the brain of mice subjected to transient focal cerebral ischemia (MCAO). In order to further understand the changes in gephyrin protein levels we investigated the effects of excitotoxicity in gephyrin mRNA in cultured hippocampal neurons, using real-time PCR. The results obtained showed that gephyrin is truncated under excitotoxic conditions and in the ischemic brain, giving rise to a stable cleavage product lacking an amino acid sequence recognized by an antibody against E domain. Therefore, in additional experiments we tested for the role of calpains in gephyrin cleavage under excitotoxic conditions, both in vitro, using chemical inhibitors of calpain (ALLN and MDL28170), and in vivo, using transagenic mice overexpressing calpastatin, an endogenous inhibitor of calpains. Finally, we also studied whether collybistin, an important interactor of gephyrin for $GABA_AR$ $\alpha2$ clustering, is cleaved under excitotoxic conditions and which is the

protease involved in this process. Additional studies are required to determine the functional consequences of gephyrin and collybistin cleavage, particularly the impact on $GABA_A$Rs clustering at GABAergic synapses.

Chapter 2

TrkB signaling under excitotoxic conditions

The results in this chapter werepublishedas:

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[#]J.R.G. and J.T.C. contributed equally to this work;*I have performed the experiments included in Figures 2-5, 8-9, and collaborated in the experiments included in Figure 1.

i

Neurobiology of Disease

Excitotoxicity Downregulates TrkB.FL Signaling and Upregulates the Neuroprotective Truncated TrkB Receptors in Cultured Hippocampal and Striatal Neurons

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Brain-derived neurotrophic factor (BDNF) plays an important role in neuronal survival through activation of TrkB receptors. The *trkB* gene encodes a full-length receptor tyrosine kinase (TrkB.FL) and its truncated (T1/T2) isoforms. We investigated the changes in TrkB protein levels and signaling activity under excitotoxic conditions, which are characteristic of brain ischemia, traumatic brain injury, and neurodegenerative disorders. Excitotoxic stimulation of cultured rat hippocampal or striatal neurons downregulated TrkB.FL and upregulated a truncated form of the receptor (TrkB.T). Downregulation of TrkB.FL was mediated by calpains, whereas the increase in TrkB.T protein levels required transcription and translation activities. Downregulation of TrkB.FL receptors in hippocampal neurons correlated with a decrease in BDNF-induced activation of the Ras/ERK and PLCγ pathways. However, calpain inhibition, which prevents TrkB.FL degradation, did not preclude the decrease in signaling activity of these receptors. On the other hand, incubation with anisomycin, to prevent the upregulation of TrkB.T, protected to a large extent the TrkB.FL signaling activity, suggesting that truncated receptors may act as dominant-negatives. The upregulation of TrkB.T under excitotoxic conditions was correlated with an increase in BDNF-induced inhibition of RhoA, a mediator of excitotoxic neuronal death. BDNF fully protected hippocampal neurons transduced with TrkB.T when present during excitotoxic stimulation with glutamate, in contrast with the partial protection observed in cells overexpressing TrkB.FL or expressing GFP. These results indicate that BDNF protects hippocampal neurons by two distinct mechanisms: through the neurotrophic effects of TrkB.FL receptors and by activation of TrkB.T receptors coupled to inhibition of the excitotoxic signaling.

Introduction

Overactivation of glutamate receptors plays an important role in neuronal damage in brain ischemia, epileptic seizures, and in chronic neurodegenerative disorders (Choi, 1994; Lau and Tymianski, 2010). The resulting $[Ca^{2+}]_i$ overload stimulates calpains, which target numerous substrates within the cell, including cytoskeletal proteins, kinases and phosphatases, membrane receptors, and transporters (Chan and Mattson, 1999; Xu et al., 2007; Gomes et al., 2011).

Impairment of intracellular signaling induced by trophic factors was suggested to play an important role in neurodegenerative processes (Drukarch and van Muiswinkel, 2001; Gauthier et al., 2004; Tolosa et al., 2009; Counts and Mufson, 2010). However, the underlying mechanisms and the interrelationship with excitotoxic injury remain to be established. Brain-derived neurotrophic factor (BDNF) promotes neuronal survival and provides neuroprotection in various brain regions (Lu et al., 2005; Manadas et al., 2007) through activation of tyrosine kinase TrkB receptors (TrkB.FL). Stimulation of these receptors induces their transphosphorylation on tyrosine residues, setting in motion several signaling cascades. Activation of the Ras/ERK and PI3-K (phosphatidylinositol-3-kinase)/Akt pathways accounted for neuroprotection by BDNF under excitotoxic conditions in studies where cultured hippocampal neurons were preincubated with the neurotrophin before the insult (Almeida et al., 2005). The active TrkB-FL receptor also activates phospholipase Cγ (PLCγ) by tyrosine phosphorylation (Reichardt, 2006). Two additional TrkB splicing isoforms are expressed in the brain, TrkB.T1, and TrkB.T2 (Klein et al., 1990; Huang and Reichardt, 2003; Manadas et al., 2007), which may behave as dominant-negative inhibitors of TrkB-FL in the regulation of cell survival (Dorsey et al., 2006), in addition to their own signaling activity (Takai et al., 2001; Ohira et al., 2006; Manadas et al., 2007). Thus, TrkB.T1 receptors are coupled to inhibition of RhoA in astrocytes (Ohira et al.,

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2006). This GTPase mediates Ca^{2+}-dependent activation of p38 MAPK, which is coupled to neuronal death under excitotoxic conditions (Semenova et al., 2007).

The TrkB isoforms are differentially regulated under pathological conditions (Manadas et al., 2007). Injection of kainate in the rat hippocampus increased BDNF and TrkB.T protein levels, whereas the full-length receptor levels did not change significantly (Rudge et al., 1998). In a different study kainate injection upregulated BDNF and TrkB.FL protein levels in the surviving neurons in the CA1 and dentate gyrus subregions of the hippocampus. However, neuronal loss in the damaged areas was preceded by a reduction of BDNF and TrkB.FL immunoreactivity (Goutan et al., 1998). The TrkB.FL and BDNF immunoreactivity was also decreased in the infarct core region in a rat model of stroke (Ferrer et al., 2001).

In this work we investigated the mechanisms contributing to the changes in TrkB.FL and TrkB.T under excitotoxic conditions, the resulting alterations in their signaling activity, and the impact in neuroprotection. The results show that excitotoxicity downregulates TrkB.FL protein levels in cultured hippocampal and striatal neurons, and inhibits their signaling activity by a mechanism that correlates with the upregulation of TrkB.T receptors. This shift in expression of TrkB isoforms may partially alter the role of BDNF, from a neurotrophic to a neuroprotective function.

Materials and Methods

Primary neuronal cultures

Cultures of rat hippocampal neurons were prepared from E18 Wistar embryos as previously described (Almeida et al., 2005; Caldeira et al., 2007; Gomes et al., 2011). Striatal neurons were cultured using the same procedures, from E15–16 Wistar rat embryos (Gomes et al., 2011). Hippocampal cultures were maintained in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), glutamate (25 μM), glutamine (0.5 mM), and gentamicin (0.12 mg/ml). Striatal cultures (15 d *in vitro*; DIV) were maintained in the same medium but in the absence of glutamate. The cells were kept at 37°C in a humidified incubator with 5% CO_2/95% air, for 7 d (hippocampal cultures) or 14–15 d (striatal cultures). Cells were cultured at a density of 90,000 cells/cm^2 on poly-D-lysine-coated 6-well microplates (MW6) (for Western blot and real-time PCR experiments). Animals used in the preparation of cell cultures were handled according to National and Institutional guidelines. Experiments conducted at the Center for Neuroscience and Cell Biology were performed according to the European Union Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. These experiments did not require approval by an Institutional Animal Care and Use Committee.

Western blot

Hippocampal and striatal neuronal cultures were washed twice with ice-cold PBS and once more with PBS buffer supplemented with 1 mM DTT and a mixture of protease inhibitors: 0.1 mM PMSF and CLAP (1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml pepstatin; Sigma). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC, and 0.1% SDS at a final pH 7.5) supplemented with the mixture of protease inhibitors. After centrifugation at 16,100 × *g* for 10 min, protein in the supernatants was quantified using the BCA method, and the samples were denatured with 2× concentrated denaturing buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue) at 95°C for 5 min. Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels, transferred to PVDF membranes (Millipore), and immunoblotted, as previously described (Caldeira et al., 2007). Blots were incubated with primary antibodies (overnight at 4°C), washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20,000 dilution for anti-rabbit IgG and 1:10,000 dilution for mouse IgG; 1 h at room temperature). Alkaline phosphatase activity was visualized by enhanced chemifluorescence on the Storm 860 gel and blot imaging system, and quantified using the ImageQuant program (GE Healthcare). The following primary antibodies were used: anti-TrkB (1:750, Ab:156–322 aa; BD Biosciences), anti-RhoA (1:1000, Ab: C-terminal; Cell Signaling Technology), anti-spectrin (1:1000, Mab1622; Millipore Bioscience Research Reagents), anti-phospho-Trk (1:1000; Cell Signaling Technology), anti-phospho-Akt (Ser473, 1: 1000; Cell Signaling Technology), anti-Akt (1:1000; Cell Signaling Technology), and anti-PLCγ (1:1000; Cell Signaling Technology). The anti-β-tubulin I (1:500,000; Sigma) antibody was used as loading control.

mRNA semiquantification through real-time PCR

Total RNA was extracted from 7 DIV cultured hippocampal neurons using TRIzol Reagent (Invitrogen), as previously described (Santos and Duarte, 2008; Gomes et al., 2011). RNA quality and integrity was assessed using the Experion automated gel-electrophoresis system (Bio-Rad), as previously described (Santos and Duarte, 2008). Samples showing RNA degradation or contamination by DNA were discarded. RNA concentration was determined using the fluorescent dye RiboGreen (Invitrogen) or NanoDrop 1000 (Thermo Scientific). The samples were aliquoted and stored at −80°C until further use. cDNA synthesis was performed using 2 μg of total RNA and the AMV Reverse Transcriptase (10 U; Roche), as previously described (Santos and Duarte, 2008). Samples were stored at −80°C until further use.

Primers for TrkB isoforms in real-time PCR were as previously described (Silhol et al., 2007). GAPDH was used as the reference gene with the following primers: forward, 5′CTCCCATTCCTCCACCTTTG3′ and reverse, 5′TGTAGCCATATTCATTGTCATACC3′. The annealing temperature was 63.5°C.

For gene expression analysis, 2 μL of 1:100 diluted cDNA was added to 10 μL of 2× SYBR Green Master Mix (Bio-Rad), and the final concentration of each primer was 250 nM in 20 μL total volume. The thermocycling reaction was initiated with activation of the TaqDNA polymerase by heating at 95°C during 30 s, followed by 55 cycles of a 10 s denaturation step at 95°C and 30 s annealing and 30 s elongation steps at 72°C. The fluorescence was measured after the extension step, using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction, the melting step was performed with slow heating, starting at 55°C and with a rate of 0.5°C per 10 s, up to 95°C, with continuous measurement of fluorescence. Data analysis was performed using the Pfaffl method for efficiency correction (Pfaffl, 2001). The results were normalized with GAPDH as the internal reference gene, because it showed a stable expression in hippocampal neurons stimulated with glutamate.

DNA constructs/lentivirus production

The rat TrkB isoforms were kindly provided by V. Leßmann (Institut für Physiologie, Otto-von-Guericke-Universität Magdeburg, Magdeburg, Germany) (Hartmann et al., 2004). Briefly, the cDNA sequences from TrkB full-length (TrkB.FL) and TrkB truncated isoform 1 (TrkB.T1) were amplified from the plasmids provided. Fragments were then subcloned into pRRLSIN (Addgene plasmid 12252) using BamHI and SalI restriction sites in 5′ and 3′, respectively. The resulting plasmids were verified by automated sequencing (STABVIDA). Lentivirus were then generated as previously described (Zufferey et al., 1998). Briefly, HEK 293T cells were transfected by the calcium phosphate method and media collected 24 and 48 h after transfection. The virus were then concentrated by centrifugation at ∼80,000 × *g* for 2 h and the virus pellet resuspended in PBS supplemented with 1% BSA, aliquoted, and stored at −80°C until further use. Viral titers were determined by infection of HEK 293T cells.

Cell death assay

Hippocampal neurons were cultured for 7 d on poly-D-lysine-coated glass coverslips as previously described. After the experiments cells were fixed in 4% sucrose/4% paraformaldehyde (in PBS). The cells were washed twice with PBS and were then incubated with Hoechst 33342 (0.5 μg/ml) to stain nuclei. Analysis of the nuclear morphology was performed on a Zeiss Axiovert 200 florescence microscope, under a 40× objective.

Intrahippocampal injection of kainate

Injection. Intrahippocampal injection of kainate (Tocris Bioscience) was performed as previously described (Tomioka et al., 2002; Takano et al., 2005), with minor modifications. Briefly, adult male mice (C56BL6) were deeply anesthetized with avertin (2,2,2 tribromoethanol, 2-methyl-2-butanol), placed in a stereotaxic apparatus (Stoelting), and given a unilateral injection of 1 nmol of kainate (in 0.3 μl of PBS) into the hippocampal CA1 region, using a 10 μl motorized syringe (Hamilton), after drilling a small hole with a surgical drill. The coordinates of the injection were anterior–posterior −2.3 mm, mediolateral −1.5 mm, and dorsoventral −1.7 mm from the bregma. Two minutes after the needle insertion, kainate was injected at a constant flow rate of 0.05 μl/min. The needle remained in place for an additional 2 min to prevent reflux of fluid. The body temperature of mice was monitored and maintained at 37°C during surgery and 30 min after the injection, with a homeothermic heating blanket (Harvard Apparatus), with feedback regulation. Experiments were conducted according to the European Union Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

Western blot. 12 h after injection mice were killed and a 2 mm section around the hippocampus was taken with the help of a 1 mm coronal mouse matrix (Harvard Apparatus). The slices were immediately frozen with dry ice and the damaged ipsilateral and contralateral areas of the hippocampal slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for Western blot.

Middle cerebral artery occlusion

Focal cerebral ischemia was induced by the transient occlusion of the right middle cerebral artery (MCA), using the intraluminal filament placement technique as described previously (Nygren and Wieloch, 2005; Gomes et al., 2011). Briefly, adult male mice were anesthetized by inhalation of 2.5% isoflurane (IsobaVet; Schering-Plough) in O_2:N_2O (30:70). Anesthesia was subsequently reduced to 1.5–1.8% isoflurane and sustained throughout the occlusion period. Body temperature was kept at ∼37°C throughout the surgery period. To monitor regional cerebral blood flow (rCBF), an optical fiber probe (Probe 318-I; Perimed) was fixed to the skull at 2 mm posterior and 4 mm lateral to bregma and connected to a laser Doppler flow meter (Periflux System 5000; Perimed). A filament composed of 6–0 polydioxanone suture (PSD II; Ethicon) with a silicone tip (diameter of 225–275 μm) was inserted into the external carotid artery and advanced into the common carotid artery. The filament was retracted, moved into the internal carotid artery, and advanced until the origin of the MCA, given by the sudden drop in rCBF (∼70% of baseline). After 45 min, the filament was withdrawn and reperfusion observed. The animals were placed in a heating box at 37°C for the first 2 h postsurgery and thereafter transferred into a heating box at 35°C to avoid postsurgical hypothermia. Thirty minutes after the onset of reperfusion, 0.5 ml of 5% glucose was administered subcutaneously. Temperature and sensorimotor deficits were assessed at 1 and 2 h and in the morning after the surgery. The Ethics Committee for Animal Research at Lund University approved animal housing conditions, handling, and surgical procedures. C57BL/6J male mice, 9 to 11 weeks old (weight, 21.5–27.9 g; Taconic), were housed under diurnal conditions with free access to water and food before and after surgery.

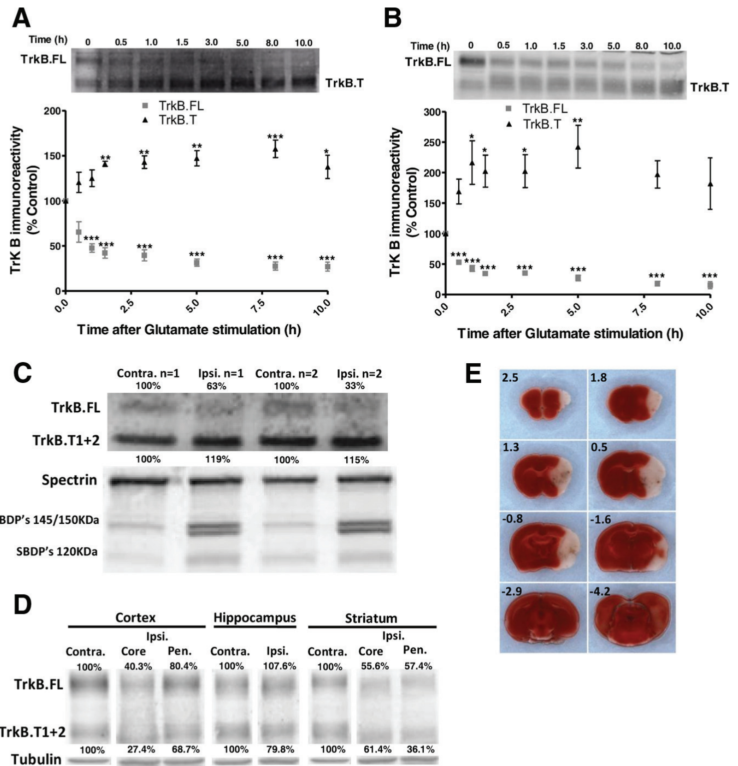

Figure 1. TrkB.FL cleavage and upregulation of a truncated TrkB isoform (TrkB.T) in cultured hippocampal and striatal neurons subjected to excitotoxic conditions. Cultured hippocampal (***A***) and striatal (***B***) neurons were subjected to excitotoxic stimulation with glutamate (125 μM glutamate, 20 min) and further incubated in culture-conditioned medium. TrkB full-length and truncated TrkB protein levels were determined by Western blot at the indicated time points after excitotoxic stimulation. The results for the hippocampal neurons are the average ± SEM of 3–7 independent experiments, performed in different preparations, whereas 4–15 independent experiments were performed with striatal neurons. Statistical analysis was performed using one-way ANOVA followed by Dunnett's comparison test performed for each condition as compared with the control, not exposed to excitotoxic conditions (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$). ***C***, Intrahippocampal injection of kainate changes full-length and truncated TrkB immunoreactivity in the ipsilateral (Ipsi) hippocampus of adult mice (C56Bl 6). TrkB and Spectrin breakdown product's (SBDP) protein levels in the injected and contralateral hippocampus were determined by Western blot 12 h after the lesion. The results are representative of two independent experiments, performed in different animals. ***D***, Changes in TrkB isoforms immunoreactivity in the ipsilateral (Ipsi) and contralateral (Contra) brain hemispheres of adult mice (C56Bl6) subjected to a transient 45 min occlusion of the right MCA. TrkB protein levels in the cerebral cortex, hippocampus, and striatum were determined by Western blot 6 h after the lesion. Core and penumbra (Pen) regions were dissected separately in the cerebral cortex and striatum. ***E***, TTC staining of 1-mm-thick sections of the mouse brain (with the bregma stereotaxic coordinates indicated), 24 h after the occlusion, providing an evaluation of the infarct volume and damaged areas in the brain after the 45 min occlusion of the right MCA.

Western blot. Mice were killed 6 h after middle cerebral artery occlusion (MCAO) and 1-mm-thick coronal brain sections were generated using a mouse brain matrix. The slices were immediately frozen with dry ice and the damaged ipsilateral and contralateral areas of the slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for Western blot as described above.

2,3,5-Triphenyltetrazolium chloride staining

After 24 h of recovery, animals were killed to evaluate the infarct volume. The brain was removed and the forebrain was sliced into 1-mm-thick sections using a mouse brain slicer on ice. The sections were rinsed once in ice-cold 0.9% NaCl for 10 min and subsequently immersed in 50 ml of 0.01% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.9% NaCl at 37°C for 15 min. Slices were fixed in 4% formalin and images were acquired

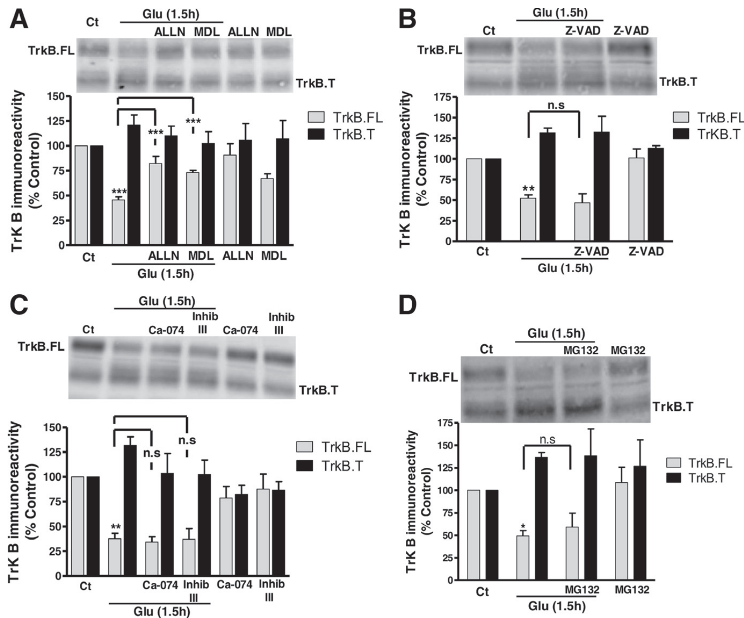

Figure 2. Calpain mediates TrkB.FL downregulation under excitotoxic conditions. ***A***, Cultured hippocampal neurons were preincubated (2 h) with the calpain inhibitors MDL 28170 (100 μM) or ALLN (50 μM), before and during glutamate stimulation (Glu; 125 μM, 20 min). The cells were further incubated in culture-conditioned medium for 1.5 h with calpain inhibitors and extracts were analyzed by Western blot with anti-TrkB antibody. ***B***, The effect of the pan-caspase inhibitor Z-VAD-FMK (50 μM) on glutamate-evoked TrkB.FL downregulation was tested using the experimental conditions described in (***A***). The effect of cathepsin B (Ca-074, 50 μM) and L (Inhibitor III, 10 μM) inhibitors is shown in (***C***). ***D***, Experiments where the effect of the proteasome inhibitor MG132 (1 μM) on TrkB.FL degradation were tested. The cells were preincubated for 30 min with the proteasome inhibitor before glutamate (Glu) stimulation. After excitotoxic stimulation the cells were incubated in culture-conditioned medium in the presence or in the absence of the tested inhibitor, for 1.5 h. Extracts were analyzed by Western blot with an antibody against TrkB. The results of (***A–D***) are the average ± SEM of at least 3 different experiments performed in independent preparations. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test. $***p < 0.001$; $**p < 0.01$; $*p < 0.05$; n.s., not significant as compared with the control protein levels for TrkB.FL or for the indicated comparisons.

with a MicroPublisher 3.3 RTV CCD camera (QImaging) using standard conditions.

Expression of recombinant GST-Rhotekin-Rho-binding domain

Expression of the pGEX-Rhotekin-Rho-binding domain (amino acids 7–89) fusion protein, kindly provided by Michael Courtney (University of Kuopio, Finland) (Semenova et al., 2007), was induced by treating an *Escherichia coli* BL21 culture in the exponential phase of growth (37°C, $A_{600\ nm}$ = 0.7) with 0.3 mM isopropyl β-D-thiogalactoside, for 4 h at 25°C. The protein was extracted and purified from the bacterial pellet through affinity chromatography on glutathione Sepharose 4B (GE Healthcare), according to the manufacturer's recommendations.

Pull-down assay

Rho activity was measured as previously described (Semenova et al., 2007) with some modifications. Cultured striatal neurons with 15 DIV were treated with or without glutamate (125 μM, 5 or 20 min) and BDNF (100 ng/ml), for the periods of time indicated. Cells were rinsed with ice-cold PBS once and lysed in 900 μl lysis buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitors). Three wells, from the 6-well microplates (MW6), were used for each experimental condition. Homogenized lysates were frozen in liquid nitrogen to promote cell disruption and were then centrifuged at 16,000 × *g* for 10 min. The supernatant was collected and incubated with 30 μg of GST-Rhotekin and glutathione-Sepharose for 1 h at 4°C with constant and gentle rotation. Beads were spun out (5 min, 500 × *g*) and washed three times with 50 mM Tris, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitors. Finally, beads with GTP-RhoA were denatured with 5× concentrated denaturing buffer and boiled at 95°C for 5 min, followed by a centrifugation in a microtube provided with a filter to separate beads from the supernatant. Active RhoA was detected by immunoblotting with monoclonal anti-RhoA antibody.

Bio-Plex phosphoprotein testing

Bio-Plex phosphoprotein assays and Bio-Plex total target assays are bead-based multiplex assays (Luminex xMAP technology) that detect the phosphorylation of proteins in lysates derived from cell culture. The Bio-Plex total target assay was used to determine the total abundance of Erk in the extracts and the Bio-Plex phosphoprotein assay was used to determine Erk phosphorylation in separate wells. In the phosphoprotein assay antibodies directed against pErk are covalently coupled to internally dyed beads, and the coupled beads then react with the phosphorylated and dephosphorylated forms of Erk. After a series of washes to remove unbound protein, biotinylated detection antibodies for Erk (second antibodies) are added to the reaction forming a sandwich of antibodies around the target proteins (Erk). Streptavidin-PE is finally added to bind to the biotinylated detection antibodies on the bead surface. Data from the reaction are acquired using the Bio-Plex suspension array system (Bio-Rad). The contents of the well are draw up into the reader. The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent signal on the bead surface. This identifies each assay and reports the level of target proteins (pErk and total protein levels). In the preparation of extracts for the Bio-Plex assays, the cells were washed with cell wash buffer (Bio-Rad) and then lysed with cell lysis buffer (Bio-Plex cell lysis kit, Bio-Rad). After 30 min of agitation, the protein content was determined using the BCA method and the concentration was equalized with assay buffer (Bio-Plex phosphoprotein testing assay buffer). Finally, the samples were analyzed with the Bio-Plex suspension array system.

Statistical analysis

The immunoreactivity obtained in each experimental condition was calculated as a percentage of the control. Data are presented as mean ± SEM of at least three different experiments performed in independent preparations. Statistical analysis of the results was performed using one-way ANOVA analysis followed by either the Dunnett's or Bonferroni's post-test: $***p < 0.001$, $**\ p < 0.01$, and $*\ p < 0.05$.

Results

Excitotoxicity downregulates TrkB.FL *in vitro* and *in vivo*, and upregulates the truncated TrkB isoform

To understand how excitotoxic conditions influence the protein levels of the TrkB receptors, cultured neurons isolated from different brain regions were stimulated with glutamate (125 μM, for 20 min) and further incubated in culture-conditioned medium for different time periods. Stimulation of cultured hippocampal neurons under these conditions induces 40–50% cell death (Almeida et al., 2005), and Western blot experiments using an antibody against an extracellular epitope of TrkB showed a downregulation of full-length TrkB (145 kDa) and upregulation of a truncated form of the receptor with ~95 kDa (Fig. 1*A*). TrkB.FL protein levels dropped rapidly by

~50% within the first hour after the toxic insult, and a secondary slower decrease in the receptor protein levels was observed during the following 7 h. Concomitantly, truncated TrkB protein levels were upregulated with similar kinetics but with a delayed onset. This pattern of change in TrkB protein levels following excitotoxic stimulation was also observed in striatal neuronal cultures, but in this case the upregulation of the truncated TrkB receptors showed a greater magnitude being upregulated to >200% of the control (Fig. 1*B*).

The hippocampus and the striatum are two brain regions particularly sensitive to excitotoxic neuronal death after cerebral ischemia, and the latter region is highly enriched in TrkB receptors (Eide et al., 1996; Dos-Anjos et al., 2009). To address the changes of the TrkB isoform protein levels in an *in vivo* model of excitotoxicity we tested the effect of kainate injection into the CA1 region of the hippocampus, which leads to excitotoxic neuronal death (Tomioka et al., 2002; Higuchi et al., 2005; Takano et al., 2005). A downregulation of the TrkB.FL protein levels was observed together with an upregulation of the TrkB.T protein levels (Fig. 1*C*), similar to the results obtained in the neuronal cultures. As expected, kainate injection into the CA1 region of the hippocampus activated calpains, as determined by the formation of 145 kDa spectrin cleavage product; negligible amounts of the 120 kDa cleavage products were detected, indicating a minor contribution of caspase-3 to spectrin cleavage under the experimental conditions used (Wang, 2000).

The pattern of TrkB downregulation in cultured hippocampal and striatal neurons subjected to excitotoxic stimulation was also compared with that observed in the brain of mice subjected to the MCAO model of transient focal ischemia (Traystman, 2003). Adult mice (C56BI6) were subjected to transient 45 min occlusion of the right MCA, and extracts were prepared from the ipsilateral (Ipsi) and contralateral (Contra) brain hemispheres (cortex, striatum, and hippocampus) 6 h after injury. A downregulation of TrkB.FL protein levels was observed in the ipsilateral striatum and cerebral cortex 6 h after the ischemic insult, and this effect was more significant in the core than in the penumbra region (Fig. 1*D*). Truncated TrkB levels were also downregulated in the two brain regions, in contrast with the upregulation observed in the excitotoxicity studies performed *in vitro* and *in vivo* (see above). This may be due to (1) the presence of truncated receptors in astrocytes (Rose et al., 2003; Ohira et al., 2005a,b), which are vestigial in the neuronal cultures used in this work (Brewer et al., 1993), and (2) to a stronger injury induced by kainate in the *in vivo* experiments when compared with MCAO. MCAO did not change the expression of TrkB isoforms in the hippocampus, a region that is not primarily affected in this model of ischemia, as observed in 1-mm-thick sections of the mouse brain, 24 h after the occlusion, using TTC staining (Fig. 1*E*).

TrkB.FL is downregulated by a calpain-dependent mechanism

Chemical inhibitors of different classes of proteases were used to determine the mechanism(s) of downregulation of TrkB.FL under excitotoxic conditions. Both calpain inhibitors used, ALLN (50 μM) and MDL28170 (100 μM), prevented TrkB.FL loss as determined 1.5 h after glutamate stimulation (Fig. 2*A*). Z-VAD-FMK (50 μM), a general inhibitor of caspases, did not affect glutamate-induced downregulation of TrkB.FL (Fig. 2*B*), indicating that this class of proteases does not contribute to the observed response. Cathepsins, mainly the B and L isoforms, also contribute to protein degradation in ischemic cell death (Yamashima, 2000). The use of specific pharmacological inhibitors of cathepsin B and L (CA-074 Me and Inhibitor III, respectively,

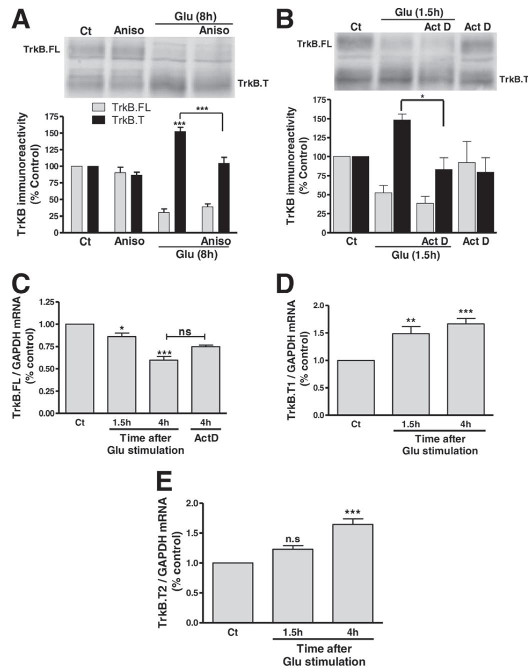

Figure 3. Truncated TrkB is upregulated under excitotoxic conditions by a transcription-dependent mechanism. ***A***, Cultured hippocampal neurons were preincubated (15 min) with the translation inhibitor anisomycin (5 μM) before and during glutamate stimulation (125 μM; 20 min). The cells were then incubated with the inhibitor in culture-conditioned medium for 8 h and extracts were analyzed by Western blot with an anti-TrkB antibody. The results are the average ± SEM of 5–9 different experiments performed in independent preparations. ***B***, Cultured hippocampal neurons were preincubated (30 min) with the transcription inhibitor actinomycin D (1.5 μM), before and during glutamate stimulation (Glu; 125 μM, 20 min). The cells were then incubated with the inhibitor in culture-conditioned medium for 1.5 h and extracts were analyzed by Western blot with anti-TrkB antibody. The results are the average ± SEM of 4 –7 different experiments performed in independent preparations. ***C–E***, Cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate (125 μM, 20 min) and further incubated in culture-conditioned medium for the indicated periods of time. Alternatively, cultures were incubated with the transcription inhibitor actinomycin D (1.5 μM) for 4 h. Total RNA was extracted and Trk.FL (***C***), TrkB.T1 (***D***), TrkB.T2 (***E***), and GAPDH (***C–E***) mRNA were semiquantified through real-time PCR. The results are the average ± SEM of 3– 8 different experiments performed in independent preparations. Statistical analysis from (***A–E***) was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n.s., not significant as compared with the control or for the indicated comparisons.

at 50 and 10 μM) showed that these proteases are also without effect on the cleavage of TrkB.FL under excitotoxic conditions (Fig. 2*C*). Similar results were obtained in the presence of the proteasome inhibitor MG132 (1 μM).

Truncated TrkB is upregulated by a transcription-dependent mechanism

The distinct onset of the TrkB.FL downregulation when compared with the delayed upregulation of a shorter form of the receptor under excitotoxic conditions, both in hippocampal and striatal neurons (Fig. 1*A*,*B*), suggests that the latter population of receptors corresponds to a TrkB.T isoform synthesized *de novo* rather than to a cleavage product. This hypothesis is corroborated by the results showing that inhibition of TrkB.FL downregulation by calpain inhibitors is not associated with a corresponding loss of the low molecular weight form of the receptor (Fig. 2*A*). In fact, the results rather suggest that the latter population of TrkB receptors corresponds to a TrkB.T isoform that is synthesized *de novo* upon excitotoxic stimulation. To test this hypothesis, cul-

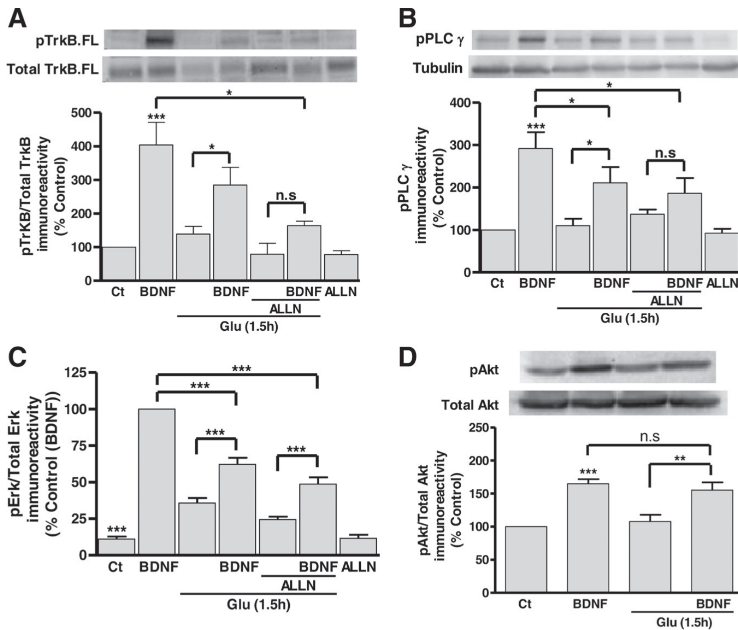

Figure 4. TrkB.FL-dependent signaling activity is downregulated under excitotoxic conditions. TrkB.FL signaling activity under excitotoxic conditions was assessed by analyzing TrkB.FL phosphorylation (***A***), PLCγ phosphorylation (***B***), ERK phosphorylation (***C***), and Akt phosphorylation (***D***), following stimulation of hippocampal neurons with BDNF (100 ng/ml) for 10 min, under control condition and after excitotoxic stimulation with glutamate (Glu; 125 μM, 20 min). In the latter experimental conditions the cells were incubated in culture-conditioned medium for 80 min after the toxic insult before stimulation with BDNF. When the effect of the calpain inhibitor ALLN (50 μM) was tested the cells were preincubated with the inhibitor for 2 h before glutamate stimulation, and the inhibitor was also present during all additional experimental manipulation. Erk phosphorylation was analyzed using the Bio-Plex System, and PLCγ, TrkB.FL, and Akt phosphorylation were measured by Western blot. The results are the average ± SEM of 3–9 different experiments performed in independent preparations. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test. $^{***}p < 0.001$; $^{*}p < 0.05$; n.s., not significant as compared with the control or for the indicated comparisons.

tured hippocampal neurons were stimulated with glutamate in the presence of the translation inhibitor anisomycin (5 μM), and the protein levels of TrkB.FL and the short form of the receptor were analyzed 8 h later (Fig. 3). Inhibition of protein synthesis abrogated the upregulation of the TrkB truncated form, showing that the effect is dependent on translation activity. Similar results were obtained in the presence of the transcription inhibitor actinomycin D, further indicating that the short form of the TrkB receptor corresponds to a TrkB.T isoform that is synthesized *de novo* following excitotoxic stimulation (Fig. 3*B*). Translation inhibition for 8 h, the maximum time period used in this set of experiments, had a minor impact in the total amount of TrkB.FL and TrkB.T isoforms under control conditions, showing their slow turnover rate (Fig. 3*A*).

The inhibition of glutamate-evoked upregulation of TrkB.T upon transcription inhibition suggests that excitotoxicity shifts the splicing of the TrkB pre-mRNA to increase the amount of mature mRNA coding or the truncated form(s) of the receptor. This may not have an impact in the amount of TrkB.FL detected under the experimental conditions used given the low turnover rate of the receptors (see above). This hypothesis was addressed through analysis of the excitotoxicity-induced changes in the mRNA for TrkB.FL and the truncated isoforms, using real-time PCR. TrkB.FL mRNA was downregulated 1.5 and 4 h after glutamate stimulation (125 μM, 20 min) (Fig. 3*C*). This downregulation was likely due to active degradation of TrkB.FL transcripts since inhibition of transcription with actinomycin D, for the same period of time (4 h), did not significantly change TrkB.FL mRNA (Fig. 3*C*). In contrast, TrkB.T1 and TrkB.T2 mRNAs were upregulated after the excitotoxic injury, in agreement with the results obtained when TrkB.T protein levels were analyzed (Fig. 3*D*,*E*).

BDNF-dependent signaling activity is downregulated under excitotoxic conditions

The TrkB.FL degradation after excitotoxic injury and the upregulation of TrkB.T under the same conditions is likely to have a significant impact in the signaling activity of the full-length receptors. This was addressed at three different levels: (1) TrkB phosphorylation, the first step following binding of BDNF to the receptors and their dimerization; (2) PLCγ phosphorylation, which follows the interaction of the enzyme with the active receptor; and (3) phosphorylation (presumably activation) of ERK and Akt, downstream mediators of two signaling cascades activated by TrkB receptors (Middlemas et al., 1994; Huang and Reichardt, 2003; Manadas et al., 2007). Activation of TrkB.FL by BDNF was evaluated by Western blot, using an antibody against the phosphorylated form of the receptor, and the results were calculated as a ratio to the total amount of TrkB.FL protein levels, under control conditions and in cells subjected to excitotoxic stimulation. Stimulation of hippocampal neurons with BDNF (100 ng/ml) was performed 1.5 h after the excitotoxic insult, a time point when there is a significant degradation of TrkB.FL protein levels and TrkB.T receptors are already upregulated (Fig. 1*A*). The cells were stimulated with the neurotrophin for 10 min since this time period allows inducing maximal signaling activity (Almeida et al., 2005). Excitotoxic stimulation of hippocampal neurons decreased the total amount of pTrkB detected following stimulation with BDNF (data not shown), due to degradation of a subpopulation of receptors (50%) (Fig. 1*A*). However, the remaining receptors are still partially responsive to BDNF stimulation, as determined by the increase in the ratio pTrkB /total TrkB.FL (Fig. 4*A*). When the experiments were performed in the presence of the calpain inhibitor ALLN (50 μM), which prevents the degradation of TrkB.FL (but had no effect on the upregulation of TrkB.T), the ratio pTrkB/total TrkB was still much lower than that obtained under control conditions (Fig. 4*A*). After excitotoxic injury there was also a decrease in the BDNF-induced PLCγ phosphorylation (Fig. 4*B*) and ERK activation (Fig. 4*C*), and the activity of both signaling pathways was still downregulated when the experiments were conducted in the presence of the calpain inhibitor ALLN. In contrast with the results obtained for TrkB, PLCγ, and ERK phosphorylation, excitotoxic stimulation of hippocampal neurons did not significantly change the BDNF-induced accumulation of

phosphorylated Akt (Fig. 4*D*). This may be due to differences in the activity of the Akt phosphatase(s), when compared with the phosphatases responsible for the regulation of TrkB, ERK, and PLCγ phosphorylation state. Together the results clearly show that TrkB.FL signaling activity is downregulated in excitotoxic conditions, but the effect cannot be explained solely based on the degradation of TrkB.FL.

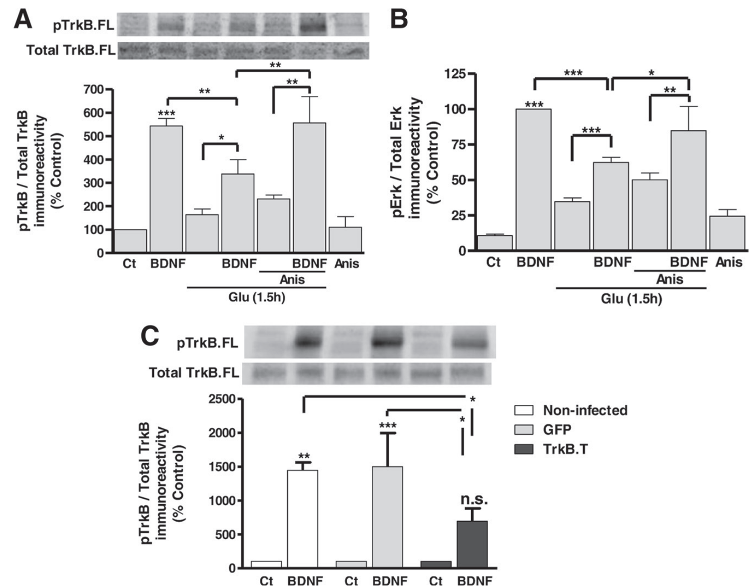

Figure 5. Truncated TrkB acts as dominant-negative inhibiting signaling by TrkB.FL. TrkB.FL signaling activity under excitotoxic conditions was measured after blocking the upregulation of truncated TrkB with the translation inhibitor anisomycin (5 μM). Cultured hippocampal neurons were preincubated (15 min) with anisomycin before and during glutamate stimulation (125 μM, 20 min). TrkB.FL phosphorylation (***A***) and ERK phosphorylation (***B***) were then measured upon stimulation of hippocampal neurons with BDNF (100 ng/ml) for 10 min under control condition or after excitotoxic stimulation with glutamate (125 μM, 20 min). In the latter experimental conditions, after the excitotoxic insult the cells were incubated in culture-conditioned medium for 80 min and then stimulated with BDNF. When the effect of the translation inhibitor anisomycin (5 μM) was tested the cells were preincubated with the inhibitor for 15 min before glutamate stimulation, and the inhibitor was also present during all additional experimental manipulations. ***C***, TrkB.FL signaling activity under control conditions was also measured in cultured hippocampal neurons infected with lentiviral constructs of GFP or TrkB.T. TrkB.FL phosphorylation was measured upon stimulation of hippocampal neurons with BDNF (100 ng/ml) for 10 min. Erk phosphorylation was analyzed using the Bio-Plex System, and TrkB.FL phosphorylation was measured by Western blot. The results are the average ± SEM of 3– 6 different experiments performed in independent preparations. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, as compared with the control or for the indicated comparisons.

Truncated TrkB may act as dominant-negative preventing the activation of TrkB.FL receptors

Truncated TrkB receptors have been described to exert a dominant-negative effect over TrkB.FL, preventing the association of two full-length receptors which is required for their transphosphorylation and activation of signaling pathways (Manadas et al., 2007). The results showing preservation of TrkB.FL receptors but not their signaling activity in hippocampal neurons subjected to excitotoxic stimulation in the presence of ALLN may be partially explained considering that TrkB.T receptors, which are upregulated under the same conditions, may exert such a dominant-negative effect. This may arise from the dimerization of Trk.FL and TrkB.T receptors, which will prevent tyrosine phosphorylation of TrkB.FL and consequently, no signaling activity should be generated (Eide et al., 1996; Li et al., 1998; Haapasalo et al., 2001). This hypothesis was tested by inhibiting translation with anisomycin (5 μM), thereby preventing the upregulation of TrkB.T under excitotoxic conditions (Fig. 3*A*). Figure 5 shows that translation inhibition prevents the excitotoxicity-induced downregulation of TrkB.FL signaling activity, both at the level of the receptor (pTrkB/TrkB Total; Fig. 5*A*) and activation of the ERK signaling pathway (Fig. 5*B*). Together the results strongly suggest that TrkB.FL signaling activity is downregulated under excitotoxic conditions due to the dominant-negative effect exerted by TrkB.T isoforms that are upregulated under the same conditions. The dominant-negative effect of TrkB.T isoforms was further investigated in experiments with cultured hippocampal neurons transduced with lentivirus expressing GFP or TrkB.T1, and the effect of BDNF on TrkB-FL phosphorylation was determined by Western blot. Upregulation of the expression of TrkB.T1 significantly decreased the BDNF-induced TrkB.FL phosphorylation (Fig. 5*C*) in agreement with the dominant-negative hypothesis.

The dominant-negative effect of TrkB.T receptors may not fully account for the loss of TrkB.FL activity

To evaluate whether the upregulation of TrkB.T receptors may fully account for the loss of TrkB.FL receptor activity in hippocampal neurons subjected to excitotoxic stimulation, a theoretical analysis was performed to compare the observed changes in TrkB.FL phosphorylation with the results predicted based on the changes in the relative abundance of the TrkB.FL and TrkB.T forms.

Addition of BDNF to a system containing TrkB.FL and TrkB.T receptors leads to the formation of TrkB.FL–TrkB.FL, TrkB.T–TrkB.FL, and TrkB.T–TrkB.T dimers. The total number of dimers formed is as follows:

$$N_{dimers} = \binom{N_R}{2},\ N_{FL\text{-}FL} \sim \binom{N_{FL}}{2},\ N_{T-T} \sim \binom{N_T}{2},\ N_{FL-T} \sim \frac{N_{FL}N_T}{2}, \tag{1}$$

where N_R is the total number of TrkB receptors, and N_{FL} and N_T the total number of TrkB.FL and TrkB.T receptors, respectively. At equilibrium, the total number of receptor homodimers and heterodimers is given by the following equations:

$$N_{FL\text{-}FL} \propto N_{FL}^2,$$

$$N_{T\text{-}T} \propto N_T^2,$$

$$N_{FL\text{-}T} \propto N_{FL}N_T, \tag{2}$$

$$N_{FL-T} + N_{FL-FL} + N_{T-T} = \frac{N_R}{2}.$$

The first two terms in Equation 2 state that, from Equation 1, the total number of Trk.FL–TrkB.FL or TrkB.T–TrkB.T homodimers is approximated by proportionality to the square of the total number of receptors belonging to the corresponding class if their expression is large enough.

The last term in Equation 2 states that in the presence of a saturating concentration of BDNF all receptors are in a dimerized form at equilibrium. The model assumes that the formation of the different binary complexes is equally probable and, therefore, the formation of TrkB.FL–TrkB.FL or TrkB.T–TrkB.T homodimers is not preferred to the formation of TrkB.T–TrkB.FL heterodimers.

Under resting conditions (Eqs. 3 and 4) and following stimulation with saturating concentrations of BDNF (Eqs. 5 and 6), the phosphorylation of TrkB.FL receptors under control conditions (Eqs. 3 and 5) and after excitotoxic stimulation (Eqs. 4 and 6) can be described by the following equations:

$$N_{FL-T}^{control}\, k_{rest-e} + N_{FL-FL}^{control}\, k_{rest-h} = N_{resting}^{control}, \tag{3}$$

$$N_{FL-T}^{toxic}\, k_{rest-e} + N_{FL-FL}^{toxic}\, k_{rest-h} = N_{resting}^{toxic}, \tag{4}$$

$$N_{FL-FL}^{control}\, k_{act} = N_{BDNF}^{control} - N_{resting}^{control}, \tag{5}$$

$$N_{FL-FL}^{toxic}\, k_{act} = N_{BDNF}^{toxic} - N_{resting}^{toxic}. \tag{6}$$

In these equations $k_{\text{rest-e}}$ = rate of oligomerization of TrkB.FL–TrkB.T heterodimers, $k_{\text{rest-h}}$ = rate of oligomerization of TrkB.FL–TrkB.FL homodimers, and k_{act} = rate of activation of TrkB.FL receptors following stimulation with BDNF.

To identify the correct set of parameters we used the constrained least-squares as follows:

$$k = \begin{bmatrix} k_{rest-h} \\ k_{rest-e} \\ k_{act} \end{bmatrix},$$

and to attain the minimum of the following quantity

$$||M_{\text{dimers}} \cdot k - N_{\text{data}}||^2, k \geq 0, \tag{7}$$

where the terms in Equation 7 are defined as:

$$M_{dimers} = \begin{bmatrix} N_{FL-FL}^{control} & N_{FL-T}^{control} & 0 \\ N_{FL-FL}^{toxic} & N_{FL-T}^{toxic} & 0 \\ 0 & 0 & N_{FL-FL}^{control} \\ 0 & 0 & N_{FL-FL}^{toxic} \end{bmatrix},$$

and

$$N_{data} = \begin{bmatrix} N_{resting}^{control} \\ N_{resting}^{toxic} \\ N_{BDNF}^{control} - N_{resting}^{control} \\ N_{BDNF}^{toxic} - N_{resting}^{toxic} \end{bmatrix}.$$

In M_{dimers} we consider that BDNF-induced TrkB.FL–TrkB.FL homodimerization leads to receptor transphosphorylation with a probability of 1 as follows:

$$\text{Number of phosphorylation events} = k_{act} N_{FL-FL},$$

where

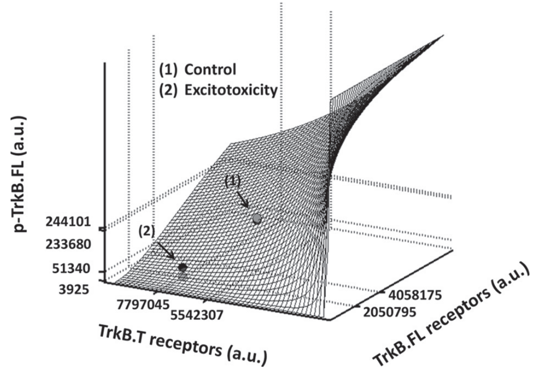

Figure 6. Prediction of the dominant-negative effect of the TrkB.T receptors over TrkB.FL. The response—measured by TrkB.FL phosphorylation induced by BDNF—is predicted as a function of the number of TrkB.FL and TrkB.T receptors. The surface interpolates the experimental values (gray bullets) in a least-squares sense and predicts the cellular response upon varying the concentration of TrkB.FL and TrkB.T. The gray bullets represent the observed experimental values. The BDNF-induced changes in TrkB.FL phosphorylation 90 min after the excitotoxic insult (3925 a.u. as measured in Western blot experiments; data taken from Fig. 5*A*) were determined under conditions when the abundance of TrkB.FL = 2050795 (a.u.) and TrkB.T = 7797045 (a.u.), as determined by Western blot (data taken from Fig. 1*A*). TrkB.FL phosphorylation predicted by the model (51340 a.u.), considering the effects depending on the numbers of TrkB.FL and TrkB.T and their dimerization only, is represented by the black dot (on the surface). A similar analysis was performed for BDNF-induced TrkB.FL phosphorylation under control conditions: 244101 a.u. as measured in Western blot experiments (data taken from Figure 5*A*) (shown by the gray bullet above the surface). Under these conditions the abundance of TrkB.FL = 4058175 (a.u.) and TrkB.T = 5542307 (a.u.), as determined by Western blot (data taken from Fig. 1*A*). The predicted increase of TrkB.FL phosphorylation is 233680 (a.u.) (black dot on the surface).

$$N_{FL-FL} = \frac{N_{FL}^2}{N_{FL}^2 + N_{FL}N_T + N_T^2}, \tag{8}$$

and analogously

$$N_{FL-T} = \frac{N_{FL}N_T}{N_{FL}^2 + N_{FL}N_T + N_T^2}. \tag{9}$$

In the above expressions (Eqs. 8 and 9) we consider that the total number of homodimers and heterodimers is computed as the likelihood of forming a homodimer or heterodimer, respectively (Eq. 2), over the total number of dimers that can be formed. Figure 6 shows the predicted phosphorylation of TrkB.FL receptors considering different expression levels of TrkB.FL and TrkB.T receptors. As expected, maximal TrkB.FL phosphorylation is predicted in the absence of TrkB.T receptors. Based on the quantification of the results of Figure 1*A* showing the relative expression of TrkB.FL and TrkB.T receptors in cultured hippocampal neurons under control conditions, the phosphorylation levels of TrkB.FL predicted by the model (black circle) were slightly below the immunoreactivity obtained in the Western blot experiments with the *p*-TrkB.FL receptors (Fig. 5A; Fig. 6, gray bullet). The change in the relative expression of TrkB.FL and TrkB.T receptors following excitotoxic stimulation decreased the predicted *p*-TrkB.FL response to BDNF stimulation (black circle), in agreement with the dominant-negative hypothesis. In this case, the response to BDNF stimulation was even lower than the predicted results, suggesting that additional factors may contribute to the downregulation of TrkB.FL receptor activity under excitotoxic conditions.

Activation of TrkB.T receptors by BDNF blocks the activation of RhoA GTPase under excitotoxic conditions

TrkB.T1 receptors are coupled to the inhibition of RhoA (Ohira et al., 2006), a small GTPase that mediates the Ca^{2+}-dependent activation of p38 MAPK followed by neuronal death under excitotoxic conditions (Semenova et al., 2007). Therefore, we investigated whether the TrkB.T receptors upregulated under excitotoxic conditions could express their own signaling activity. RhoA activity was assessed by a pull-down assay using a GST fusion protein with the Rhotekin amino acid sequence responsible for binding to the active form of the GTPase. Striatal neurons were subjected to excitotoxic stimulation (125 μM glutamate, 20 min) to induce an upregulation of TrkB.T expression, and 1.5 h later a short stimulation (5 min) with glutamate was performed in the absence or in the presence of BDNF. Alternatively, the effect of BDNF was tested in cells exposed to glutamate for 5 min without previous excitotoxic stimulation. The former (but not the latter) experimental conditions allow the upregulation of TrkB.T before testing for the activity of TrkB.T receptors in response to stimulation with BDNF. In striatal neurons previously exposed to excitotoxic conditions the activation of RhoA induced by 5 min incubation with glutamate was significantly inhibited by BDNF (Fig. 7*A*). This inhibitory effect was not observed in cells treated with glutamate and BDNF without previous excitotoxic stimulation (Fig. 7*A*). Although the upregulation of TrkB.T in hippocampal neurons following excitotoxic stimulation is not as robust as in striatal neurons (Fig. 1*A*,*B*), the results of Figure 7*B* show that hippocampal cultures behave similarly to striatal neurons. Interestingly, inhibition of RhoA activity by BDNF in cells previously subjected to excitotoxic stimulation was not observed when the experiments were conducted in the presence of anisomycin to inhibit translation. Since under these conditions there is no upregulation of TrkB.T following the excitotoxic insult (Fig. 3*A*), the results suggest that the inhibitory effects of BDNF on RhoA GTPase signaling are mediated by activation of the truncated receptors. Accordingly, control experiments showed that the inhibition of RhoA by BDNF is insensitive to the TrkB.FL receptor inhibitor K252A (Fig. 7*B*). Together these results indicate that the upregulation of TrkB.T following an excitotoxic insult significantly interferes with the activation of RhoA signaling pathways which are known to be coupled to excitotoxic cell death (Semenova et al., 2007).

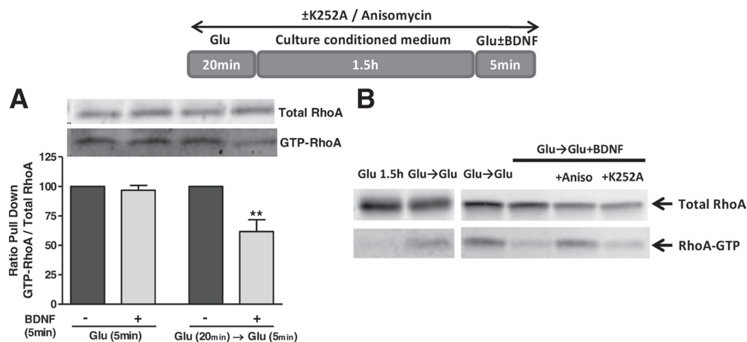

Figure 7. Activation of TrkB.T receptors by BDNF inhibits the stimulation of Rho A under excitotoxic conditions. Activated GTP-bound RhoA was measured by pull-down from cell lysates with immobilized recombinant Rhotekin-RBD and detected by immunoblotting with an anti-RhoA antibody. ***A***, RhoA activity was measured in striatal neurons following 5 min stimulation with glutamate, in the presence or in the absence of BDNF (100 ng/ml). Where indicated the cells were subjected to excitotoxic stimulation with glutamate (125 μM, 20 min) before the short incubation with the amino acid. Active RhoA was measured by pull-down and measured as a ratio to the total amount of RhoA. The results are the average ± SEM of 3–5 different experiments performed in independent preparations. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test. $^{**}p < 0.01$, as compared with the respective control, i.e., cells exposed to excitotoxic stimulation and later exposed to glutamate for 5 min. ***B***, The same experimental procedure was followed in cultured hippocampal neurons. Truncated TrkB upregulation was blocked with anisomycin (5 μM) added to the medium 30 min before glutamate stimulation and present throughout the experiment. When the effect of K252A (200 nM) was tested the cells were preincubated with the inhibitor (30 min) before glutamate stimulation and the inhibitor was present throughout the experiment. The results are representative of two independent experiments performed in distinct preparations.

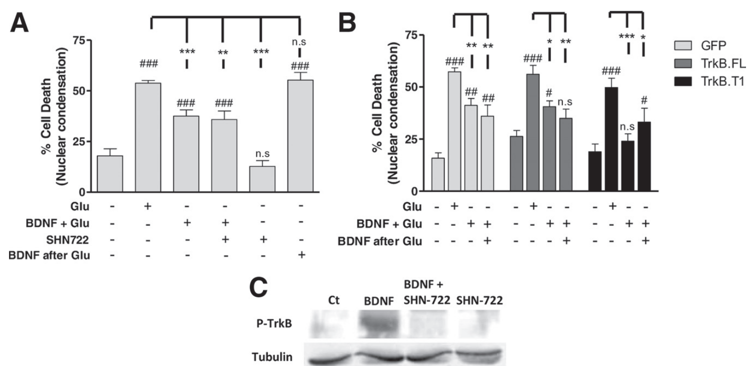

Figure 8. BDNF protects neurons through TrkB.T receptors. ***A***, Cultured hippocampal neurons (DIV 7) were challenged with glutamate (125 μM, 20 min), and BDNF (100 ng/ml) was added during glutamate stimulation or immediately after. Where indicated, 1 μM SHN722 was present during the period of glutamate stimulation, in the presence or in the absence of BDNF, and the cells were preincubated with the inhibitor for 30 min. Cell death was assessed 14 h later by fluorescence microscopy, using the fluorescent dye Hoechst 33342. The results are the average ± SEM of 5–8 different experiments performed in independent preparations. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test. ***B***, Cultured hippocampal neurons (DIV 7) were transduced with lentiviral constructs of GFP, TrkB.FL, or TrkB.T1 and 48–72 h after infection cultures were challenged with glutamate (125 μM, 20 min) in the presence or absence of BDNF (100 ng/ml), and where indicated the neurotrophin was also present in the postincubation period. Fourteen hours after this stimulation cells were fixed and cell death was assessed as indicated in (***A***). The results are the average of 3–10 experiments performed in independent preparations. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test. $^{***}p < 0.001$; $^{**}p < 0.01$; and n.s., not significant as compared with glutamate stimulation. $^{\#\#\#}p < 0.001$; $^{\#\#}p < 0.01$; $^{\#}p < 0.05$; and n.s., not significant as compared with the control. ***C***, Cultured hippocampal neurons were stimulated or not with BDNF (100 ng/ml) for 10 min, in the presence or in the absence of the inhibitor SHN-711, and TrkB receptor phosphorylation was analyzed by Western blot.

BDNF protects neurons through TrkB full-length but also through truncated TrkB

To determine how the changes in TrkB protein levels affect the neurotrophic effects of BDNF in hippocampal neurons, we compared the protective activity of the neurotrophin added to hippocampal neurons during the period of glutamate stimulation or

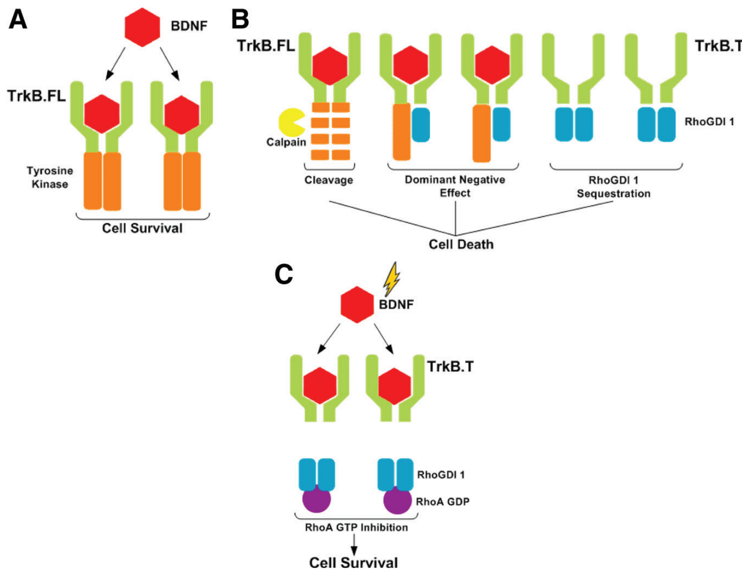

Figure 9. Role of TrkB isoforms in cell survival/cell death upon excitotoxic stimulation. Proposed model for effects of TrkB isoforms under excitotoxic conditions, in the absence (***B***) and in the presence (***A***,***C***) of BDNF. ***A***, In normal physiological conditions the signaling activity of TrkB.FL receptors is induced by BDNF contributing to cell survival (trophic support). ***B***, Under excitotoxic conditions TrkB.FL is degraded by a calpain-dependent mechanism, resulting in a loss of the trophic support. At the same time there is an upregulation of TrkB.T which (1) has a dominant-negative effect over the TrkB.FL receptors and (ii) sequesters RhoGDI 1, allowing the activation of RhoA signaling that ultimately may activate p38MAPK and stress signaling pathways, contributing to cell death. ***C***, Under excitotoxic stimulation in the presence of BDNF neuroprotection may result from the activity of the remaining functional TrkB.FL receptors, in addition to the effect of TrkB.T signaling. Binding of BDNF to the latter receptors releases RhoGDI 1 from an intracellular binding domain, allowing the stabilization of GDP-RhoA. This inactivates the RhoA signaling pathway promoting cell survival.

after the toxic insult. BDNF protected hippocampal neurons only when incubated during the period of excitotoxic stimulation, and no effect was observed when the neurotrophin was added after the insult (Fig. 8*A*). The protective effect of BDNF when added during excitotoxic stimulation was insensitive to the TrkB.FL chemical inhibitor SHN722 (1 μM) (Martin et al., 2011), suggesting that it may be mediated by the truncated TrkB receptors. Control experiments showed that 1 μM SHN722 inhibits BDNF-induced TrkB.FL phosphorylation (Fig. 8*C*). The lack of effect of BDNF when applied after the toxic insult indicates that activation of the truncated receptors expressed after the toxic insult does not provide neuroprotection, possibly because the excitotoxic machinery had been already activated during the period of glutamate stimulation.

To further investigate putative trophic effects of the TrkB receptor isoforms, cultured hippocampal neurons were transduced with lentivirus expressing GFP, TrkB.FL, or TrkB.T1, and the toxic effects of glutamate were investigated in the presence and in the absence of BDNF. In these experiments the cells were incubated with the neurotrophin only during the period of glutamate stimulation or both during the excitotoxic insult and the postincubation in culture-conditioned medium. Incubation with BDNF during the period of excitotoxic stimulation partially protected hippocampal neurons (Fig. 8*B*), similarly to the results obtained in nontransduced cells (Fig. 8*A*). Remarkably, BDNF fully protected hippocampal neurons transduced with TrkB.T when the neurotrophin was added during the period of stimulation with glutamate, and similar results were observed when BDNF was also present during the postincubation period (Fig. 8*B*). In contrast, overexpression of TrkB.FL did not further increase the neuroprotective effects of BDNF when tested during the period of glutamate stimulation, since the effects were similar to those obtained in hippocampal neurons transduced with GFP (Fig. 8*B*). Furthermore, when BDNF was also present during the postincubation period no additional neuroprotection was observed.

Discussion

In this work we show that TrkB.FL receptors are degraded under excitotoxic conditions (*in vitro* and *in vivo*) and in transient focal cerebral ischemia, and excitotoxicity also upregulated a truncated form of the TrkB receptor. Evidence indicates that the truncated TrkB receptor is not a cleavage product of TrkB.FL: (1) inhibition of calpains prevented glutamate-evoked degradation of the TrkB.FL but had no effect on the upregulation of TrkB.T; (2) transcription and translation inhibitors prevented the upregulation of TrkB.T induced by excitotoxic stimulation; and (3) glutamate stimulation upregulated TrkB.T1 and TrkB.T2 mRNA in cultured hippocampal neurons, with a concomitant decrease in the number of transcripts for TrkB.FL. The latter results suggest that the splicing mechanisms of the TrkB pre-mRNA are changed under excitotoxic conditions, leading to a sustained synthesis of more mature mRNAs coding for TrkB.T rather than TrkB.FL (Barbacid, 1994; Stoilov et al., 2002; Luberg et al., 2010). However, this may not have an impact in the amount of TrkB.FL protein levels detected under the experimental conditions used due to the low turnover rate of the receptors.

Calpains have been shown to play an important role in ischemic neurodegeneration (Bevers and Neumar, 2008), in agreement with the results obtained here showing a role for these proteases in the degradation of TrkB.FL under excitotoxic conditions. Other proteases also involved in excitotoxicity and ischemic cell death do not seem to participate in the downregulation of the receptor as their chemical inhibitors did not affect glutamate-induced TrkB.FL cleavage (Yamashima, 2000). It is not clear at this point how calpains contribute to the degradation of the receptors, since these proteases usually give rise to truncated cleavage products that were not detected in the present work (Haacke et al., 2007; Meary et al., 2007; Xu et al., 2007; Grumelli et al., 2008; Gomes et al., 2011).

In a previous study we showed that TrkB.FL receptors mediate the neuroprotective effects of BDNF when preincubated before excitotoxic stimulation with glutamate, through a mechanism dependent of protein synthesis (Almeida et al., 2005). Activation of the TrkB.FL receptors is followed by receptor dimerization and transphosphorylation on tyrosine residues (Huang and Reichardt, 2003; Manadas et al., 2007). The tyrosine phosphorylated receptors activate the ERK and PI3-K signaling pathways, which account for the neurotrophic effects of BDNF and provide neuroprotection under excitotoxic conditions (Almeida et al., 2005;

Manadas et al., 2007). Both signaling pathways were also shown to contribute to the neurotrophic effects of BDNF, promoting neuronal survival (Dudek et al., 1997; Bonni et al., 1999). In the present work we show that BDNF present during the period of excitotoxic stimulation partially protects hippocampal neurons by a mechanism independent of TrkB.FL receptors. Furthermore, hippocampal neurons transduced with TrkB.T receptors were fully protected from excitotoxic stimulation when BDNF was present during the period of incubation with glutamate. This effect was more significant than that observed in neurons transduced with TrkB.FL or with GFP, which gave similar results. Although TrkB.T receptors were upregulated after excitotoxic stimulation, incubation of hippocampal neurons with BDNF after the insult did not prevent cell death, suggesting that the neuroprotective activity of truncated receptors should occur at an initial point, when the excitotoxic signaling cascade is activated.

Activation of TrkB.FL and TrkB.T receptors by BDNF induces independent signaling activity and, therefore, activation of the latter receptors may have distinct modulatory effects on excitotoxic neuronal damage. Activation of TrkB.T1 by BDNF leads to dissociation of Rho guanine nucleotide dissociation inhibitor 1 (Rho GDI 1), which interacts with the C-terminal region of the receptor. Once released from TrkB.T1, Rho GDI 1 acts as a negative regulator of Rho GTPases (Takai et al., 2001), including RhoA. Accordingly, we observed that the upregulation of TrkB.T under excitotoxic conditions was correlated with the induction of a BDNF-induced inhibition of RhoA response in cultured hippocampal and striatal neurons. This response was insensitive to inhibition of TrkB.FL but was blocked by anisomycin, which also prevented the upregulation of the truncated receptor, suggesting that the latter isoform is responsible for effect observed on RhoA signaling. RhoA mediates the Ca^{2+}-dependent activation of p38 MAPK, which is coupled to neuronal death under excitotoxic conditions (Semenova et al., 2007). The GTPase is also coupled to the activation of the stress kinase JNK (Marinissen et al., 2004) and there is evidence that the JNK/c-Jun signaling pathway is important for neuronal death induced by excitotoxicity (Yang et al., 1997). Therefore, the inhibition of RhoA by TrkB.T may account for neuroprotection by BDNF when present during the period of excitotoxic stimulation. This contrasts with the deleterious effects of TrkB.T receptors that have been proposed, resulting from the inhibition of the activity of TrkB.FL receptors through a dominant-negative mechanism (Haapasalo et al., 2001; De Wit et al., 2006).

In contrast with the role of TrkB.T receptors in neuroprotection during excitotoxic stimulation, the TrkB.FL receptors were without effect. Accordingly, neuroprotection by BDNF added during the period of excitotoxic stimulation was insensitive to the TrkB.FL receptor inhibitor SHN722, and no additional protection was observed in hippocampal neurons transduced with the tyrosine kinase receptors. Furthermore, the downregulation of TrkB.FL receptors observed following excitotoxic stimulation and in brain ischemia is expected to decrease their neurotrophic and neuroprotective signaling activity, and this may account, at least in part, for the lack of neuroprotective activity of BDNF when added after excitotoxic stimulation of cultured hippocampal neurons. *In vivo*, the loss of trophic support provided by TrkB.FL receptors may also contribute to neurodegeneration after the ischemic insult.

The effect of excitotoxicity on the signaling activity of TrkB.FL was investigated by measuring the phosphorylation of the receptor, PLCγ phosphorylation on tyrosine, and activation of the ERK and Akt protein kinases. Analysis of the ratio pTrkB/total TrkB in hippocampal neurons stimulated with BDNF showed a large reduction following excitotoxic stimulation, and similar results were obtained for the activation of PLCγ and ERK (Fig. 4). The reduction in TrkB.FL signaling activity was also observed in experiments conducted in the presence of calpain inhibitors, which prevent the degradation of TrkB.FL receptors, further indicating that under excitotoxic conditions the mechanisms that couple the receptor to the signaling pathways are affected to some extent. This may also partially account for the decrease in the activation of PLCγ and ERK. Since excitotoxic stimulation upregulates TrkB.T, these receptors may have a dominant-negative effect by preventing the dimerization of TrkB.FL thereby modulating the downstream signaling activity (Fig. 9). This hypothesis is supported by (1) the decrease in BDNF-induced TrkB.FL phosphorylation in hippocampal neurons transduced with lentivirus expressing TrkB.T1 and (2) the results showing no difference between the ratio pTrkB/total TrkB in hippocampal neurons stimulated with BDNF under control conditions and after excitotoxic stimulation in the presence of a translation inhibitor (Fig. 5). Similar results were obtained for the activation of the ERK pathway, strongly suggesting that the upregulation of TrkB.T under excitotoxic conditions plays a major role in the inhibition of the TrkB.FL signaling activity. Interestingly, the results showing that inhibition of protein synthesis almost fully restored BDNF-induced ERK activation under excitotoxic conditions suggest that the downregulation of TrkB.FL receptors is a minor player in the deregulation of the receptor signaling activity. The dominant-negative effect of TrkB.T receptors is also supported by the correlation observed between the experimental results and the effects predicted by mathematical modeling of the changes in TrkB.FL and TrkB.T receptors following excitotoxic stimulation of hippocampal neurons. This negative feedback of TrkB.T over TrkB.FL signaling activity in pathological situations has also been described in other studies (Saarelainen et al., 2000; Luikart et al., 2003; Dorsey et al., 2006). However, additional factors may also contribute to the downregulation of BDNF-induced ERK activity since activation of the kinase requires multiple intermediate components (Huang and Reichardt, 2003; Manadas et al., 2007) that may be lost and/or redistributed within the cell upon excitotoxic stimulation.

In the absence of BDNF, TrkB.T may also contribute to excitotoxic cell death due to their effect on the sequestration of Rho GDI 1 which interacts with the C-terminal region of the receptor. This sequestration allows the activation of GTP-RhoA, stimulating the stress signaling pathways (Fig. 9*B*) as shown for pancreatic cancer cells (Li et al., 2009).

Together, the results indicate that calpain mediates TrkB.FL cleavage under excitotoxic conditions, and this is accompanied by an upregulation of the truncated TrkB isoform. Although TrkB.T may have a dominant-negative effect on the signaling activity of the full-length receptors, this may be compensated at an early stage by the neuroprotective signaling activity of the truncated receptors (Fig. 9*C*). Thus, activation of TrkB.T receptors may constitute an endogenous neuroprotective strategy under conditions characterized by excitotoxic cell death, including brain ischemia, cerebral trauma, epileptic seizures, and in chronic neurodegenerative disorders.

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Chapter 3

Characterization of Gephyrin Cleavage under Excitotoxic Conditions

Abstract

GABA is the major inhibitory neurotransmitter in the CNS and changes in GABAergic neurotransmission affect the overall activity of neuronal networks. Gephyrin is a scaffold protein responsible for the traffic and anchoring of glycine and $GABA_A$ receptors, and changes in gephyrin expression and oligomerization may affectthe activity of GABAergic synapses. In this work we investigated the changes in gephyrin protein levels during ischemia and in excitotoxic conditions, which may affect the organization of clusters. We found that gephyrin is cleaved by calpains following excitotoxic stimulation of hippocampal or striatal neurons with glutamate, giving rise to a stable cleavage product. Similar results were obtained in cultured hippocampal neurons subjected to transient oxygen and glucose deprivation. Gephyrin cleavage was also observed after transient middle cerebral artery occlusion (MCAO) in mice, a model of focal brain ischemia, and following intrahippocampal injection of kainate. The latter effect was prevented in transgenic mice overexpressing calpastatin, an endogenous inhibitor of calpain. Collybistin is another important partner of gephyrin and $GABA_A$Rs clusters at synapses, and we showed that this protein is also cleaved under excitotoxic conditions by calpains. Our results show that excitotoxicity downregulates full-length gephyrin and collybistin, with a concomitant generation of truncated products, which are likely to affect $GABA_A$ receptor clustering at the synapse.

Introduction

Glutamate is the major excitatory neurotransmitter in the CNS and together with GABA, the major inhibitory neurotransmitter, controls the activity of neuronal networks. Excitotoxicity mediated by overactivation of glutamate receptors plays a key role in neuronal death characteristic of different disorders of the nervous system, including ischemia, epilepsy and neurodegenerative diseases (Szydlowska et al., 2010). The research in cerebral ischemia and excitotoxic neuronal damage has been mainly focused on the excitatory mediators and much less is known regarding the changes in GABAergic activity (Schwartz-Bloom et al., 2001). The release of GABA in the ischemic

brain and the consequent activation of $GABA_A$ receptors may be neuroprotective through reduction of membrane depolarization. However, Cl^- entry through $GABA_A$ receptors in association with overactivation of glutamate receptors may further increase the influx of water and cell swelling. The former mechanisms may be dominant since neuroprotective strategies to increase GABAergic neurotransmission, targeting both sides of the synapse, have been tested, and some of them were found to be quite efficacious in animal models of ischemia (Schwartz-Bloom *et al*., 2001). The impairment of GABAergic synaptic transmission in brain ischemia is partly due to a down-regulation of synaptic $GABA_A$ receptors, which may contribute to the ongoing neuronal excitability and possibly to neuronal death (Schwartz-Bloom *et al.*, 2001).

Gephyrin is a scaffold protein of $GABA_A$ and Glycine receptors and is the major protein determinant for the clustering of inhibitory neurotransmitter receptors (Bedet *et al.,* 2006; Yu *et al.,* 2007). Gephyrin is a cytosolic protein, which is deliveredto the membrane by a mechanism dependent on the interaction with other proteins, such as collybistin and dynein (Fuhrmann *et al.*, 2002), forming a hexagonal submembranous structure. Several studies suggested that gephyrin is essential for GABAergic clustering, since the protein forms intracellular aggregates, but not postsynaptic clusters, in neurons lacking certain $GABA_A$ receptor subunits *in vivo* (Kralic *et al.,* 2006; Studer *et al.,* 2006). Recent studies showed that this scaffold protein can bind directlyto α2 subunit of $GABA_A$ receptors, anchoring these receptors at GABAergic synapses (Maric *et al.,* 2011). Thus, putative changes in the levels of gephyrin and its oligomerization in the ischemic brain may influence the clustering of GABAergic receptors, its presence at synapse and therefore the balance between excitatory and inhibitory activity.

Calpains are cysteine proteases involved in ischemic/excitotoxic cell death and in several chronic neurodegenerative conditions (Bevers *et al.,* 2008; Vosler *et al.,* 2008). Under normal physiological conditions the activation of calpains is tightly regulated and plays a key role in the cleavage of selected protein targets (Liu *et al.*, 2008). Activation of these proteases due to a $[Ca^{2+}]_i$ overload under excitotoxic conditions leads to the cleavage ofkey synaptic proteins in glutamatergic synapses, including ionotropic (AMPA and NMDA receptor subunits) and metabotropic glutamate receptors (Xu *et al.,* 2007; Yu en *et al.,* 2007; Gascon *et al.,* 2008), and post-synaptic scaffold proteins (Jourdi *et al.*, 2005), but little information is available regarding the

effect of calpains on GABAergic synapses.A recent study showed that vesicular GABA transporter (VGAT) is cleaved by calpains following excitotoxic stimulation of hippocampal neurons with glutamate and following transient focal ischemia, giving rise to a stable product (tVGAT) (Gomes *et al.*, 2011).

The molecular mechanisms contributing to the downregulation of GABAergic neurotransmission at the postsynaptic level in brain ischemia is still not understood. A recent study showed a depletion of synaptic $GABA_A$ receptors containing β3 subunits in hippocampal neurons subjected to oxygen and glucose deprivation, by a mechanism dependent on a motif containing three arginine residues (405RRR407), present in the intracellular domain of this subunit, which is responsible for the interaction with AP2 adaptor for clathrin-mediated endocytosis (Smith *et al.,* 2012). In the present study we characterized the gephyrin cleavage under excitotoxic conditions as well as in in vivo and in vitro models of brain ischemia. Furthermore, we analysed the changes in the protein levels of collybistin under excitotoxic conditions due to the important role played by this protein in the traffic and clustering of gephyrin. The results show that gephyrin and collybistin are cleaved by calpains into new truncated forms, which may affect gephyrin and $GABA_A$ receptor clustering and function.

Material and Methods

Hippocampal and striatal cultures

Cultures of rat hippocampal and striatal neurons were prepared from E18-E19 Wistar rat embryos as previously described (Almeida *et al.,* 2005; Caldeira *et al.,* 2007). Briefly, after dissection, the hippocampi were treated with trypsin (0.06%,15 min, 37°C; GIBCO Invitrogen) in Ca^{2+}-and Mg^{2+}- free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_20$, 5mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were washed with HBSS containing 10% fetal bovine serum, to stop trypsin activity, and then washed once in HBSS to remove serum and avoid glia growth. Finally, the hippocampi were transferred to Neurobasal medium (GIBCO Invitrogen), supplemented with B27 (1:50 dilution; GIBCO Invitrogen),

glutamate (25 µM), glutamine (0.5 mM) and gentamicin (0.12 mg/mL), the cells were dissociated mechanically, and platted on poly-D-lysine coated 6 microwell plates (MW6) at a density of $9x10^4$ cells/cm^2. The cells were kept at 37°C in a humidified incubator with 5% CO_2 / 95% air, for 7or 14 days in vitro (DIV).

Excitotoxic stimulation with glutamate

Hippocampal neurons were exposed to 125 µM glutamate for 20 min in Neurobasal medium and further incubated in culture conditioned medium for the indicated periods of time. Pre-incubations of 2h were used when cells were treated with the calpain inhibitors MDL2817 (Calbiochem) and ALLN (Calbiochem) (50 µM). Under control conditions hippocampal neurons were not exposed to glutamate.

Oxygen-glucose deprivation(OGD)

Hippocampal neurons (14 DIV) were incubated in a glucose-free saline buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 26mM $NaHCO_3$), under an anaerobic atmosphere (10% H_2, 85% N_2, 5% CO_2) (Forma Anaerobic System, Thermo Scientific) at 37°C for the indicated time periods. After the OGD challenge, cultures were incubated in culture conditioned medium and returned to the humidified 95% air 5% CO_2 incubator for 8h. Control neurons were washed and incubated in the saline buffer described above, supplemented with 10 mM glucose, and kept in the humidified 95% air / 5% CO_2 incubator at 37°C for the indicated time periods. When appropriate the calpain inhibitor MDL28170 (50 µM) was added 2h before and during OGD.

Preparation of hippocampal culture extracts

Hippocampal cultures were washed twice with ice-cold PBS, and once more with PBS buffer supplemented with 1mM dithiothreitol (DTT) and a cocktail of proteases inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), CLAP (1µg/ml chymostatin, 1µg/ml leupeptin, 1µg/ml antipain, 1µg/ml pepstatin;SIGMA). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5%DOC and 0.1% SDS at a final pH 7.5) supplemented with 50 mM sodium fluoride

(NaF), 1.5 mM sodium ortovanadate (Na_3VO_4) and the cocktail of protease inhibitors. After centrifugation at 16,100xg for 10 min at 4°C, protein in the supernatants was quantified using the Bicinchoninic acid (BCA) assay kit (Pierce), and the samples were denatured with 2x concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4 and 0.01% bromophenol blue).

Western blot

Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Milipore, MA), and immunoblotted, as previously described (Caldeira et al., 2007). Blots were incubated with primary antibodies overnight at 4°C, washed, and exposed to alkaline phosphatase-conjugated secondary antibodies overnight (1:20000 dilution for anti-rabitt IgG and 1:10000 diluition for mouse IgG) for 1h at room temperature. Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Storm 860 gel and blotimaging system, and quantified using the Image Quant program (GE Healthcare). The following primary antibodies were used: anti-gephyrin (1:1000, Synaptic Systems), anti-spectrin (1:1000, Chemicon) and Collybistin (1:1000,Sigma). The anti-synaptophysin (1:10000, Synaptic Systems) antibody was used as loading control.

Specific activation of synaptic and extrasynaptic NMDA receptors and stimulation of synaptic glutamate release

To stimulate synaptic glutamate release on hippocampal cultured neurons (14 DIV), neurons were treated with 50 µM bicuculline (Tocris), 2.5 mM 4-AP (Tocris), and 10 µM glycine for 20 min, and were then allowed to recover for 4 h in the original culture medium. To activate the extrasynaptic pool of NMDA receptors, hippocampal cultured neurons (14 DIV) were treated with 50 µM bicuculline, 2.5 mM 4-AP, 10 µM MK 801 and 10 µM glycine for 5 min and then washed with a similar solution but in the absence of MK 801; thereafter neurons were incubated with 100 µM NMDA for 20 min and recovered 4 h in the original medium. All experiments were performed in basal saline solutions (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM

glucose, and 10 mM HEPES). Cell extracts were prepared and described abovefor western blot experiments.

Intra-hippocampal injection of kainate

Intra-hippocampal injection of kainate was performed as previously described (Tomioka *et al.,* 2002, Takano *et al.,* 2005), with minor modifications. Briefly, wild type adult male mice (C56BL6) were deeply anesthetized with avertin (2,2,2 Tribromoethanol, 2-methyl-2-butanol), placed in a stereotaxic apparatus, and given a unilateral injection of 1nmol of kainate (in 0.3 µl of PBS) into the hippocampal CA1 region using a 10 µl motorized syringe (Hamilton), after drilling a small hole with a surgical drill. The coordinates of the injection were anterior-posterior: 2.3 mm, medial lateral: 1.5 mm, and dorsal-ventral: 1.7 mm from the bregma.Two min after the needle insertion, kainate was injected at a constant flow rate of 0.05 µl/min. The needle remained in place for an additional 2 min to prevent reflux of fluid. The body temperature of mice was monitoredand maintained at 37°C during surgery and up to 30 min after the injection, using an homeothermic heating blanket (Harvard Apparatus) with feedback regulation. The death rate in this experiment was less than 5%. 4 or 8 h after injection mice were sacrificed and a 2 mm section around the hippocampus was taken with the help of a 1 mm coronal mouse matrice. The slices were immediately frozen with dry ice and the contralateral and the damaged ipsilateral areas of the hippocampal slices were taken using a Harris Unicore 2mm tip (Pelco International). Samples were then homogenized in RIPA buffer supplemented with 50 mM NaF, 1.5 mM Na_3VO_4 and the cocktail of protease inhibitors (PMSF, DTT, CLAP), and processed for western blot.

Middle cerebral artery occlusion (MCAO)

Focal cerebral ischemia was induced by the transient occlusion of the right middle cerebral artery (MCA), using the intraluminal filament placement technique as described previously (Nygren and Wieloch, 2005). Briefly, adult male mice were anesthetized by inhalation of 2.5% isoflurane (IsobaVet, Schering-Plough Animal Health, England) in O_2:N_2O(30:70). Anesthesia was subsequently reduced to 1.5 - 1.8% isoflurane and sustained throughout the occlusion period. Body temperature was kept

at approximately 37°C throughout the surgery period. In order to monitor regional cerebral blood flow (rCBF), an optical fiber probe (Probe 318-I, Perimed,Sweden) was fixed to the skull at 2mm posterior and 4 mm lateral to the bregma and connected to a laser Doppler flow meter (Periflux System 5000, Perimed, Sweden). A filament composed of 6-0 polydioxanone suture (PSDII, Ethicon) with a silicone tip (diameter of 225 to 275 µm) was inserted into the External Carotid Artery and advanced into the Common Carotid Artery. The filament was retracted, moved into the Internal Carotid Artery and advanced until the origin of the MCA, given by the sudden drop in rCBF (approximately 70% of baseline). After 45 min, the filament was withdrawn and reperfusion observed. The animals were placed in a heating box at 37°C for the first 2h post-surgery and thereafter transferred into a heating box at 35°C, to avoid post-surgical hypothermia. Thirty minutes after the onset of reperfusion, 0.5 mlof 5% glucose were administered subcutaneously. Temperature and sensorimotor deficits were assessed at one and two hours, and in the morning after the surgery. The Ethics Committee for Animal Research at Lund University approved animal housing conditions, handling and surgical procedures. Nine to 11 weeks old C57BL/6J male mice (weight 21.5 g to 27.9 g; Taconic, Denmark) were housed under diurnal conditions with free access to water and food before and after surgery.

Mice were sacrificed 24 h after the occlusion and 1mm sections were done with the help of a 1 mm coronal mouse matrice. The slices were immediately frozen with dry ice and the damaged ipsilateral and contralateral areas of the slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for western blot.

Statistical analysis

The immunoreactivity obtained in each experimental condition was calculated as a percentage of the control. Data are presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis of the results was performed using One Way ANOVA analysis followed by either Dunnet`s or Bonferroni’s multiple comparison test: n.s. non significant, *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

Results

Gephyrin is cleaved after excitotoxic stimulation with glutamate

To investigate the effect of excitotoxic stimulation on the protein levels of gephyrin, cultured hippocampal and striatal neurons (7 DIV) were stimulated with 125 μM of glutamate for 20 min, and were further incubated in culture conditioned medium for different periods of time. Stimulation of cultured hippocampal neurons under these conditions causes 40-50% of cell death (Almeida et al., 2005), and western blot experiments, using an antibody against C-terminal region or G domain, showed a cleavage of gephyrin, giving rise to a product of about 47 kDa (Geph.T). The downregulation of the full-length gephyrin was associated with an upregulation of a 47 kDa imunoreactive band, suggesting that this is a cleavage product of the gephyrin. As the total amount of gephyrin (full length + truncated gephyrin) did not change significantly, the results in figure 3.1A were expressed asa percentage of cleaved gephyrin. Excitotoxic stimulation of hippocampal neurons induced a cleavage of about 30% of total gephyrin when determined 1h after the insult (Figure 3.1 A).

Since the striatum is enriched in GABAergic neurons and vulnerable to excitotoxic injury, we tested the effect of glutamate stimulation on gephyrin protein levels in cultured rat striatal neurons. The cells were stimulated with 125 μM glutamate for 20min and further incubated during 1.5h or 8h in culture conditioned medium. The results showed an increase of cleaved gephyrin by about 20% (Figure 3.1 B).

To determinewhether the downregulation ofgephyrin protein levels in hippocampal neurons subjected to excitotoxic stimulation could be attributed, at least in part, to a decrease in gene expression, hippocampal neurons (7 DIV) were subjected to glutamate stimulation for 20min and RNA extraction was performed 4h after excitotoxic injury. The results show that gephyrin mRNA levels were not affected under these experimental conditions (Figure 3.1 C).

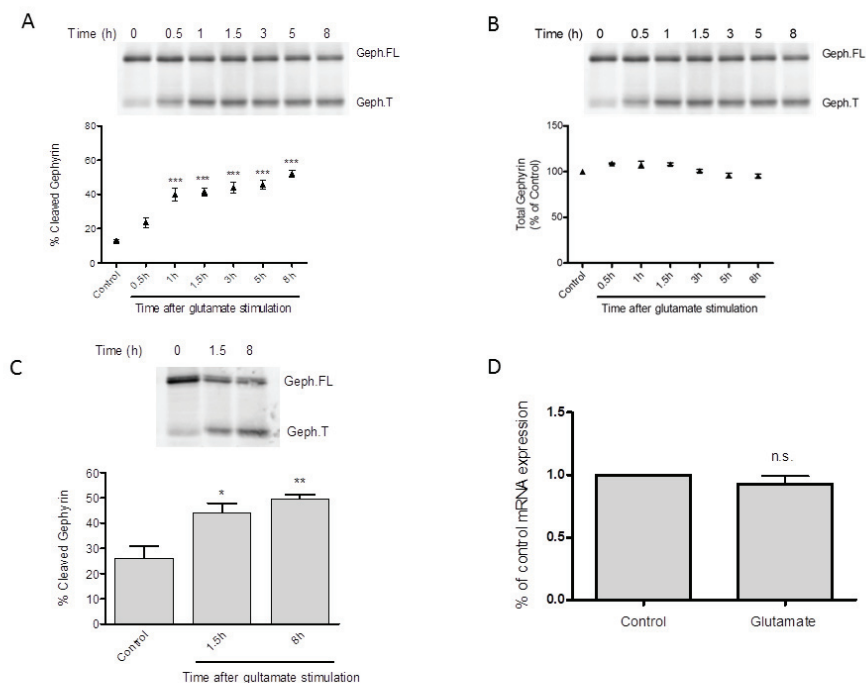

Figure 3.1. Glutamate-induced cleavage of gephyrin in cultured hippocampal and striatal neurons. The hippocampal cells (7DIV) (A) or striatal cells (C) were subjected to excitotoxic stimulation with glutamate (125 µM for 20 min) and the extracts were prepared after incubation in culture conditioned medium for the indicated periods of time. Samples were analysed by western blot with an antibody against the G domain of gephyrin and the results are expressed as a percentage of total gephyrin (FL and truncated gephyrin) (A-C). (B) Total gephyrin protein levels (full length + truncated gephyrin) in hippocampal neurons under control conditions and after excitotoxic stimulation with glutamate. (D) Effect of excitotoxic stimulation with glutamate on gephyrin mRNA. Cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate (125 µM, 20min) and further incubated in culture conditioned medium for 4h. Total RNA was extracted and gephyrin mRNA was semi-quantified through Real-Time PCR. The results are the average ± SEM of 5 to 7 independent experiments performed in distinct preparations. Statistical analysis was performed using One-Way ANOVA, followed by the Dunnet's multiple comparison test performed for each condition as compared to the control condition (n.s. non significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$).

Gephyrin is cleaved under excitotoxic conditions *in vivo*

To determine if gephyrin cleavage also occurs under excitotoxic conditions *in vivo,* adult mice were subjected to middle cerebral artery occlusion (MCAO), a model of transient focal ischemia (Traystman, 2003). Adult mice were subjected to a 45 min occlusion of the left middle cerebral artery, and extracts were prepared from the ipsilateral and contralateral brain hemispheres (cerebral cortex, hippocampus and striatum) 24h after injury. Gephyrin cleavage was observed in the striatum and

cerebral cortex,which are the brain areas preferentiallyaffected inthis model, but not in the hippocampus, as assessed by western blot using the antibody against gephyrin C-terminal region. Synaptophysin protein levels were used as loading control (Figure 3.2).

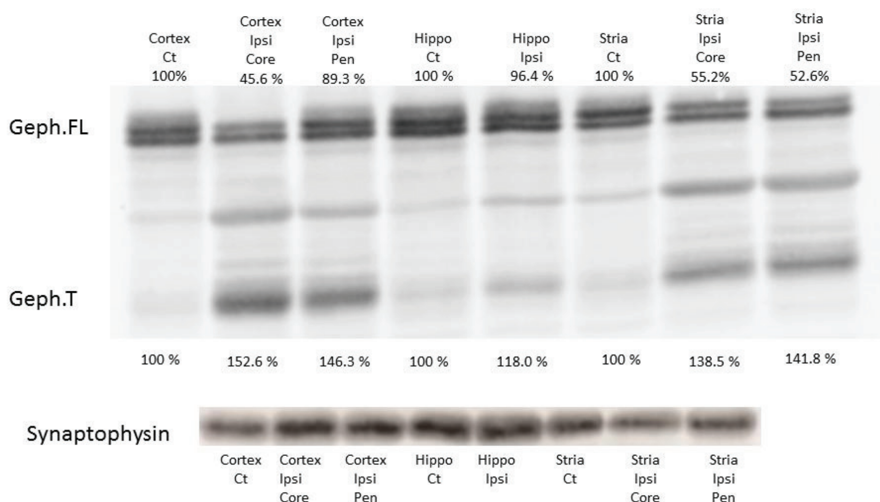

Figure 3.2. Gephyrin is cleaved following transient focalischemic induced bymiddle cerebral artery occlusion. Adult mice (C56Bl6) were subjected to a transient 45 min occlusion of the left middle cerebral artery (MCAO). Gephyrin and synaptophysin levels were determined by western blot in the cerebral cortex, hippocampus (Hippo) and striatum (Stria) of the Ipsilateral (Ipsi) and Contralateral (Ct) brain hemispheres, 24h after the lesion. The results are expressed as a percentage of total gephyrin (FL and truncated gephyrin). The results represent one of two independent experiments performed with extracts obtained from different animals.

Calpain cleaves gephyrin under *in vitro* and *in vivo*excitotoxic conditions

Since calpains play a key role in neuronal death following excitotoxicity and ischemia (Bevers and Neumar, 2008), we investigated whetherthese proteases are involved in gephyrin cleavage following anexcitotoxic insult with glutamate. Cultured hippocampal neurons (7 DIV) were pre-incubated with the chemical calpaininhibitors ALLN or MDL28170 for 2h, before glutamate stimulation (125 µM for 20 min) in the presence of the inhibitors. Gephyrin protein levels were analyzed by western blot using the antibody against gephyrin, 8 h after glutamate stimulation. Both calpain inhibitors used, ALLN and MDL28170, prevented gephyrin cleavage induced by glutamate (Figure 3.3 A).

Transient incubation of cultured neurons under oxygen and glucose deprivation (OGD) is often used as an in vitro model of transient global ischemia (Hertz, 2008). To test the effect of OGD on gephyrin protein levels, cultured hippocampal neurons (14 DIV) were pre-incubated with MDL 28170 for 2h, and then subjected to OGD for 2h in the presence of inhibitor. Gephyrin levels were assessed by western blot 2h after OGD using the antibody raised against C-terminal of gephyrin. The results show that incubation with MDL28170 prevented gephyrin cleavage caused by OGD (Figure 3.4 A). Taken together, the results indicate that gephyrin is cleaved by calpains after excitotoxic or ischemic *in vitro* insults.

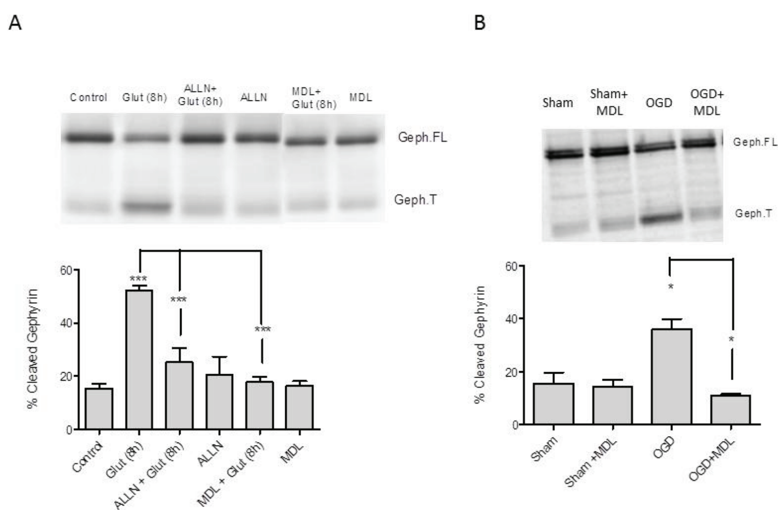

Figure 3.3. Calpains cleave gephyrin in hippocampal neurons subjected to transient OGD.(A) Cultured hippocampal neurons were preincubated (2 h) with the calpain inhibitors MDL 28170 (100 µM) or ALLN (50 µM), before and during glutamate stimulation (Glu; 125 µM, 20 min). The cells were further incubated in culture-conditioned medium for 1.5 h with calpain inhibitors and extracts were analyzed by Western blot with an anti-gephyrin antibody. (B) Cultured hippocampal neurons were subjected to OGD (2h) in the presence or in the absence ofthe calpain inhibitors MDL 28170 (100 µM) or ALLN (50 µM). The results are expressed as a percentage of total gephyrin protein levels (FL and truncated gephyrin), and are the average ± SEM of 4 to 7 independent experiments performed in different preparations.Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's multiple comparison test; ***$p<0.001$, *$p<0.05$.

To confirm the role of calpains in gephyrin cleavage under excitotoxic conditions *in vivo*, the effect of intra-hippocampal injection of kainate was compared in mice overexpressing calpastatin (Tomioka et al., 2002, Takano et al., 2005) and in wild-type mice. Gephyrin protein levels weredetermined at 4h and 8h after the insult by western blot using an antibody against the C-terminal region. Kainate injection in the

hippocampal CA1 regioninduced gephyrin cleavage in the ipsilateral hemisphere (as compared to the contralateral hemisphere) at 4h and 8h after the insult in the wild type mice. Interestingly, the effect of kainate was significantly decreased intransgenic mice overexpressing calpastatin (Figure 3.4 B), by about 71 % or 50 % when determined 4h and 8h after the excitotoxic lesion, respectively.

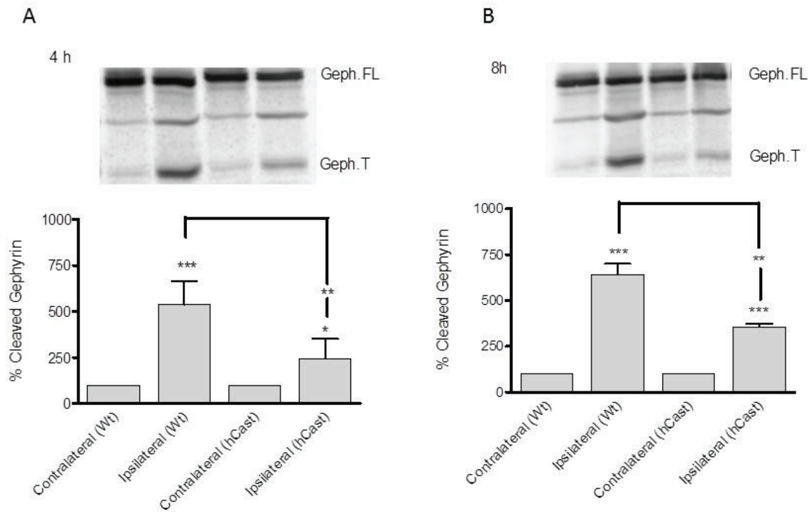

Figure 3.4. Gephyrin is cleaved by calpains under excitotoxic conditions *in vivo.*Adult wild type mice (Wt) (C56BL6) or calpastatin-transgenic mice (hCast) were injected with kainate (1 nmol in 0.3 µl phosphate buffer) in the right hippocampus and 4h (A) or 8h (B) later the ipsilateral and the contralateral hippocampi were collected from the coronal sliced brain. Hippocampal extracts were analysed by western blot using an antibody against gephyrin, and gephyrin cleavage was expressed as a percentage of total gephyrin (FL and truncated). The results are the average of ± SEM of 3-5 independent experiments performed in different preparations. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's multiple comparison test.*p<0.05,**p<0.01; ***p<0.001.

The role of NMDA receptors in inducing the gephyrin cleavage

The excessive activation of NMDA receptors is thought to play a key role in glutamate-induced neuronal death by inducing an $[Ca^{2+}]_I$ overload. It has been hypothesized that the extrasynaptic NMDA receptors are preferentially coupled to the activation of the excitotoxic machinery (Zhang et al., 2011). To better understand how calpains could mediate gephyrin cleavage and the role of NMDA receptors in this process, we stimulated cultured hippocampal neurons under conditions that allow activating synaptic or extrasynaptic NMDARs (Zhang et al., 2011). Gephyrin levels were analysed by western blot using an antibody against the C-terminal region of the protein and the

results show the cleavage of gephyrin only in response tostimulation of the extrasynaptic population of NMDARs (Figure 3.5 A).

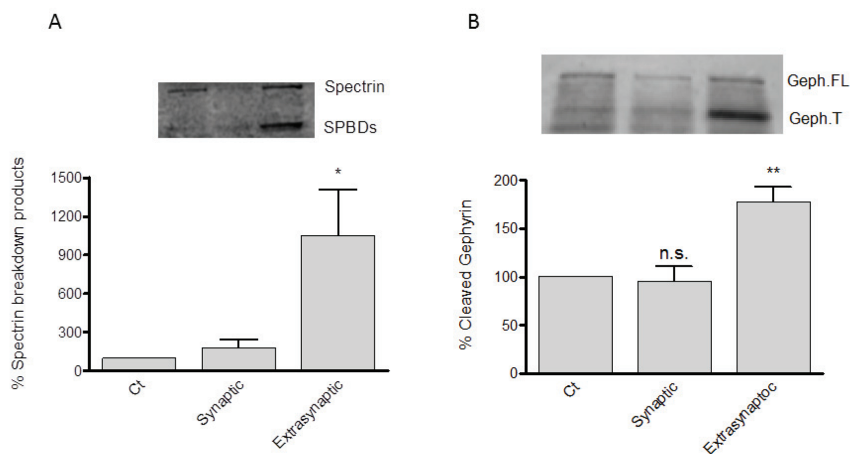

Figure 3.5. Role of extrasynaptic NMDA receptors in inducing gephyrin cleavage. Cultured hippocampal neurons (14 DIV) were incubated under conditions that allow the specific activation of synaptic or extrasynaptic NMDA receptors, as described in Material and Methods. Gephyrin (A), spectrin and spectrin breakdown products (B) protein levels were determined by western blot. The results are expressed as a percentage of the total amount of gephyrin (A) or spectrin (B). The results are the average ± SEM of 5 to 7 independent experiments performed in distinct preparations. Statistical analysis was performed using One Way ANOVA, followed by the Dunnet's multiple comparison test. n.s.-non significant,*p<0.05**p<0.01 when compared with the control.

To determine whether activation of extrasynaptic NMDA receptors is preferentially coupled to the activation of calpains, we compared the cleavage of spectrin, a calpain substrate, under the experimental conditionsused to specifically activate synaptic and extrasynaptic NMDA receptors. Spectrin cleavage was determined by western blot using anantibody which interacts with spectrin and its cleavage products. The results show that gephyrin cleavage is selectively induced by extrasynaptic NMDA receptors, similarly to the results obtained for gephyrin cleavage (Figure 3.5B). However, since the experimental conditions used did not allow separating the 145 kDa and 150 kDa cleavage products, resulting from the activity of calpains or calpains + caspase-3**)**, it is not possible to conclude whether stimulation of extrasynaptic NMDA receptors is selectively coupled to calpain activation. The product with 150 KDa is given by the

calpain or caspase 3 activity and 145 KDa spectrin breakdown product is just produced by calpain activation (Wang, 2000).

Collybistin is downregulated under excitotoxic conditions *in vitro*

As described in the introduction (section 1.3.1) collybistin is one of the most important partners of gephyrin, playing akey role in the traffic and clustering of gephyrin and $GABA_A$ receptors at GABAergic synapses. We performed western blot experiments to determine whether excitotoxic stimulation (125 µM glutamate; 20 min) of hippocampal neurons (7 DIV) also affects collybistinprotein levels. After glutamate stimulation the cells were further incubated in culture conditioned medium for different periods of time, and collybistin protein levels were assessed by western blot using a specific antibody. The results show a time-dependent downregulation of collybistin protein levels, which was correlated with the formation of a cleavage product. Thus, collybistin protein levels were decreased by about 50 % 8h after the insult, while a truncated collybistin form increased by about 70 %, showing that collybistin is also cleaved under excitotoxic conditions *in vitro*.

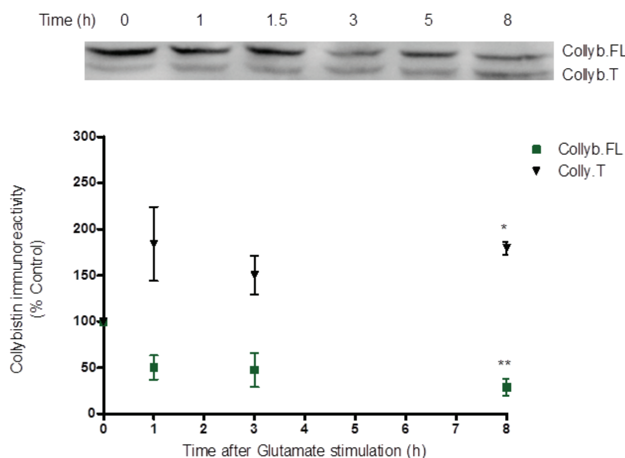

Figure 3.6. Collybistin is cleavedupon excitotoxicstimulation of cultured hippocampal neurons. The cells (7 DIV) were subjected to excitotoxic stimulation with glutamate (125 µM, 20 min) and the extracts were prepared after incubation in culture conditioned medium for the indicated periods of time. Samples were analysed by western blot with an antibody against collybistin. The results are the average ± SEM of 3 to 5 independent experiments performed in distinct preparations. Control (time 0) collybistin protein levels (FL or truncated) were determined in cells not exposed to glutamate, and were set independently to 100 %. Statistical analysis was performed using One-Way Anova, followed by the Dunnett`s multiple comparison test performed for each condition as compared to the control condition. Statistically significant differences are indicated in the graph: *$p<0.05$, **$p<0.01$.

Collybistin is Cleaved by Calpains under in vitro Excitotoxic Conditions

To identify the protease(s) responsible for the cleavage of collybistin under excitotoxic conditions, experiments were performed using calpain (ALLN) and proteosome (MG132) inhibitors. The cells were pre-incubated with the chemical inhibitors for 2h and 30 min, respectively, and subjected to glutamate stimulation (125 µM; 20 min) in the presence of inhibitors. Collybistin protein levels were analysed by western blot using the antibody against collybistin, 8h after stimulation. Both calpain and proteasome inhibitors used, ALLN and MG132, prevented collybistin cleavage induced by glutamate (Figure 3.7).

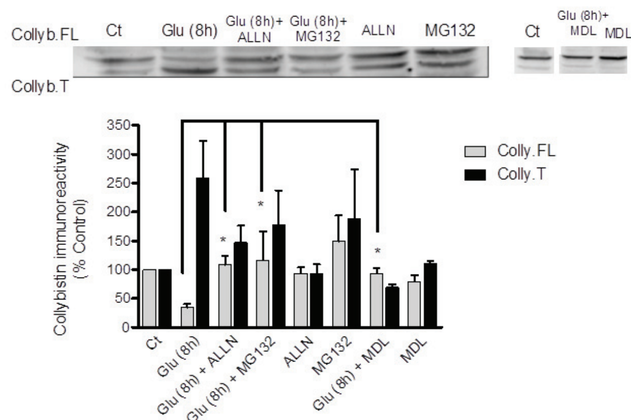

Figure 3.7. Collybistin is cleaved under excitotoxic conditions by calpains. The cells (7 DIV) were stimulated with 125 µM glutamate for 20 min, and further incubated in culture conditioned medium for 8h. Where indicated the cells were pre-incubated with MDL28170 (100 µM), ALLN (50 µM) or MG132 (1 µM), and the inhibitors were also present throughout the experiment. Collybistin protein levels were determined by western blot using aspecific antibody. The results are the average ± SEM of 5-8 experiments performed in independent preparations. Statistical analysis was performed using One Way ANOVA followed by Bonferroni's Multiple Comparison test.*$p<0.05$, as compared with the control protein levels for collybistin FL or truncated form or for indicated comparisons. The representative images shown for the experiments performed using the calpain inhibitor MDL28170 are from the same blot.

Discussion

In this work we show that gephyrin is cleaved by calpain under excitotoxic conditions in vitro and in vivo, giving rise to a stable truncated product, whichmay disorganizethegephyrin hexagonal lattice. These findings are in agreement with the key role played by calpains in neuronal damage, in excitotoxic damage and in brain ischemia (Bevers et al., 2008).

Gephyrin was found to be cleaved under the following excitotoxic conditions: i) upon stimulation of cultured hippocampal and striatal neurons with glutamate, ii) injection of kainate in the hippocampus in vivo, iii) following exposure of hippocampal neurons to oxygen and glucose deprivation, an in vitro model of global ischemia, and iv) after

transient cerebral occlusion artery in mice, which inducedgephyrin cleavage in the striatum and cerebral cortex, the brain areas affected inthis model. The role of calpains in gephyrin cleavage under excitotoxic conditions is supported by the following observations: i) the chemical inhibitors of calpains prevented the formation of a gephyrin cleavage product in cultured neurons subjected to excitotoxic stimulation with glutamate or subjected to oxygen and glucose deprivation; ii) transgenic mice overexpressing calpastatin, the endogenous calpain inhibitor, showed a decrease in kainate-evoked gephyrin cleavage. Calpains are also activated in the striatum and cerebral cortex after MCAO, contributing to neuronal death (Takagaki et al., 1997; Bevers et al., 2008), and may mediate the observed cleavage of gephyrin in mice exposed to this model of transient focal brain ischemia.In agreement with the present findings, incubation of hippocampal membranes with recombinant calpain was show to induce the cleavage of gephyrin, giving rise to a stable product of 48 - 50 kDa (Kawasaki et al.,1997).

Calpains cleave at preferred sequences in association with preferred tertiary structures of substrates, but without known absolute rules (Tompa et al., 2004).According to the PEST hypothesis, calpains cleave target proteins preferentially near PEST sequences (Rogers et al., 1986, Rechsteiner et al., 1996). Analysis of the amino acid sequence of gephyrin shows two potential PEST sequences with high score (Figure 3.8), located in the linker region between aminoacid 180 and 272.Considering the apparent molecular weight of gephyrin (93 kDa) and its cleavage product (47 KDa), and the specificity of the antibody used which binds to the E domain of the protein, the calpain the cleavage site is likely to be located in the begining of the E domain. However, additional studies are required to determine where gephyrin is cleaved by calpain.

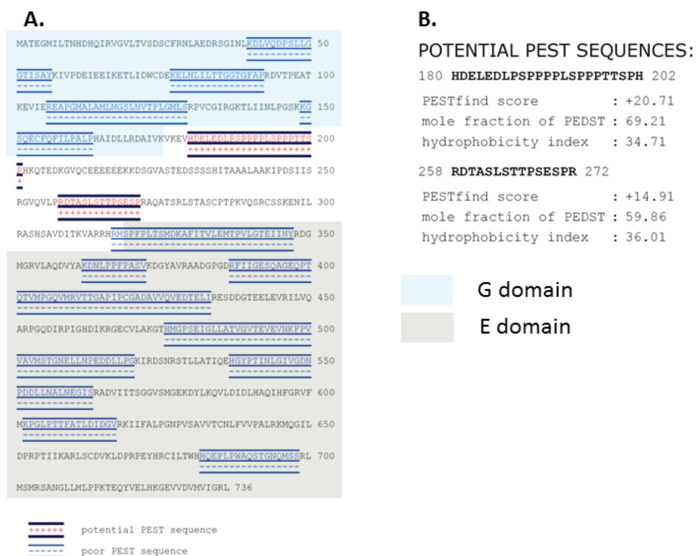

Figure 3.8. Amino acid sequence of rat gephyrin and analysis of predicted PEST sequences. The potential PEST sequences are highligted in red in panel A, and the PEST-find score is shown in B. The analysis was performed using the algorithm available in https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis webtool.htm.

In addition to the role in gephyrin cleavage observed in this work, calpain activation following kainate injection in the hippocapus in vivo was shown to play a key role in neuronal death (Takano et al., 2005). The latter study showed that calpain activity resulted in the proteolytic activation of a proapoptotic Bcl-2 subfamily member (Bid), in addition to nuclear translocation of apoptosis-inducing factor (AIF) and endonuclease G, DNA fragmentation, and chromatin condensation in pyramidal neurons. These apoptotic responses were significantly upregulatedin micedefficient in the endogenous calpain inhibitor calpastatin, and suppressed in transgenic mice overexpressing calpastatin. These results demonstrated that calpain mediates excitotoxic signals through mobilization of proapoptotic factors in a caspase-independent manner (Takano et al., 2005).

Using a pharmacological approach that allows stimulating independently the synaptic and extrasynaptic NMDA receptors we found that the two populations of receptors are differentially coupled to gephyrin cleavage and possibly to calpain activation. The selective coupling of extrasynaptic NMDA receptors to calpain activation and gephyrin cleavage may be explained based on their distinct cellular localization which may be

more favorable to activate calpains. In fact, the extrasynaptic NMDA receptors were also proposed to play an important role in excitotoxic cell death, in contrast with the synaptic population of NMDA receptors which is thought to contribute to neuronal survival and plasticity (Hardingham et al., 2002).

The total gephyrin protein levels (full length + cleaved product) in hippocampal and striatal neurons subjected to excitotoxic stimulation was not changed 8 h after the insult, suggesting that the cleavage of the protein represents a post-translational modification rather than a destructive process. Furthermore, no changes were observed in mRNA gephryrin levels following glutamate stimulation, further indicating that the loss in full length protein is caused by cleavage of gephyrin by calpain.The role of calpains in post-translational modification of specific protein targets in brain ischemia and in excitotoxic conditions is not limited to gephyrin, since the protease has been reported to cleave other proteins, giving rise to stable cleavage products (Chan et al., 1999; Hou et al., 2006; Cao et al., 2007; Xu et al., 2007; Bevers et al., 2008, Gascon et al., 2008; Gomes et al.,2011).

The isolated gephyrin G domain forms stable trimers, while isolated E domains can form dimers in solution (Fritschy et al., 2008).The current model of gephyrin aggregation, derived from the structure of the isolated G and E domains, postulates the formation of a regular hexagonal lattice by G domain trimerization and E domain dimerization (Sayed et al., 2007). The results shown in this section suggest that under excitotoxic conditions calpain cleaves gephyrin in the begining of the E domain, near to linker region. This cleavage may lead to disassembly of the gephyrin hexagonal structure and formation of isolated trimers (G domain) and dimers (E domain) (Figure 3.9). The disassembly of the gephyrin lattice is expected to have a significant impact at inhibitory synapses due to an increase in the mobility of $GABA_A$ and glycine receptors (Kirsch et al., 1998; Kralic et al.,2006; Maric et al.,2011).

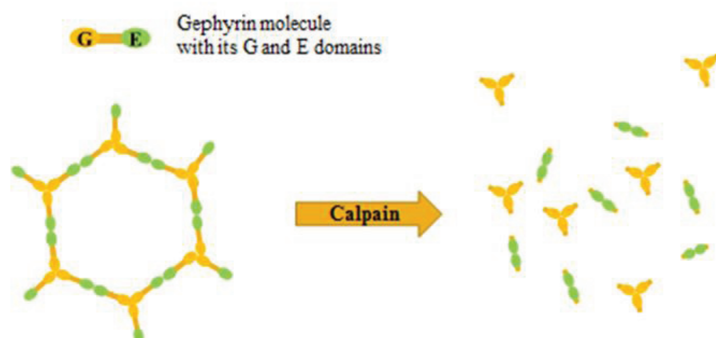

Figure 3.9.Model for disassembly of gephyrin clusters under excitotoxic conditions.

Accordingly, knocking down of gephyrin expression with shRNA in cultured hippocampal pyramidal cells decreased both the number of gephyrin and $GABA_A$receptor clusters (Yu et al., 2007). This study also showed that the disruption ofpostsynaptic gephyrin clusters had transsynaptic effects leading to a significant reduction of GABAergic presynaptic boutons. The cleavage of gephyrin under ischemic conditions may havesimilar effects.

Calpains may also be involved in gephyrin cleavage in the hippocampus of rats following systemic injection of kainate (Araujo *et al.,* 2008), a model of temporal lobe epilepsy. In this model, calpain activity was required for neuronal death in the CA1 region of the hippocampus. Calpain activity in this model may also contribute to the disruption of the gephyrin submembrane lattice responsible for anchoring $GABA_A$ receptors at the synapse, increasing $GABA_A$ receptor mobility at synaptic sites. The resulting decrease in $GABA_A$ inhibitory currents may compromise the balance with the excitatory machinery and may therefore contribute to neuronal death that accompanies seizures.

All gephyrin variants bind the GlyR β subunit cytoplasmic loop with high affinity regardless of their cassette composition. Coexpression experiments in COS-7 cells demonstrated that GlyR bound to gephyrin harboring cassette 5 cannot be stabilized at the cell surface. This gephyrin variant was found to deplete synapses from both GlyR and gephyrin in transfected neurons. These data suggest that the relative expression level of cellular variants influencethe overall oligomerization pattern of gephyrin and thus the turnover of synaptic GlyR (Bedet *et al.,* 2006). The same may apply to the

cleaved form of gephyrin generated under excitotoxic conditions, which may also impair the synaptic clustering of GlyR.

Collybistin is an important partner of gephyrin to regulate the traffic and clustering of $GABA_A$ receptors (Harvey *et al.,* 2004).We found that collybistin is also cleaved upon glutamate stimulation by a mechanism sensitive to the calpain inhibitor MDL28170 and ALLN. MG132also reduced the cleavage of collybistin under excitotoxic conditions, but this effect may be explained by the lack of specificity of this inhibitor which can also inhibit calpains besides the proteasome (Mailhes et al., 2002). Therefore, additional experiments with a specific inhibitor of the proteasome, like β-Lactone,are necessary to determine the role of the proteasome in collybistin downregulation under excitotoxic conditions.

Collybistin interacts with the M3-M4 loop of the $GABA_A$ receptor α2 subunit through the SH3 domain of collybistin, while the gephyrin E domain is responsible for the interaction with the M3-M4 loop of $GABA_AR$ α2 subunit (Saiepour *et al.,* 2010). It was proposed that the collybistin-gehyrin complex has an intimate role in the clustering of $GABA_ARs$ containing the α2 subunit. The clustering of collybistin under excitotoxic conditions may therefore have an impact in the surface distribution of $GABA_ARs$.

Future perspectives

In this work we characterized the calpain-mediated gephyrin cleavage in *in vitro* and *in vivo*models of excitotoxicity. However, the functional implications of these results still remain to be elucidated. The effect of gephyrin cleavage on oligomerization will be further studied *in vitro,* by crosslinking different ratios of the full length gephyrin and the calpain cleavage products with glutaraldehyde. As described in Chapter 1 gephyrin forms an hexagonal submembrane lattice onto which $GABA_A$receptors are anchored. Since the cleavage of gephyrin by calpain is likely to occur in the linker region, separating the G and E domains, this may disassemble the postsynaptic clusters of gephyrin (Figure 3.9). This hypothesis will be tested using immunocytochemistry, and the gephyrin distribution in dendrites of cultured hippocampal neurons will be tested under control conditions and after transfection with truncated gephyrin domains.

Whether the loss of gephyrin clusters is related with a decrease in synaptic $GABA_A$Rs will be determined by immunocytochemistry, using antibodies against subunits characteristic of synaptic receptors, such as α2 subunits, which interact with gephyrin and collybistin as described above. These experiments will allow investigating the impact of the loss of gephyrin clusters on $GABA_A$Rs anchoring, which is expected to affect GABAergic transmission under ischemic conditions.

Chapter 4

Conclusions

Conclusions

Stroke can produce a significant neurological impairment and is a leading cause of death in developed countries, including Portugal. Excitotoxicity is one of the first events in the ischemic stroke and is an important trigger and executioner of tissue damage in focal cerebral ischemia. Although it is still considered a prime target for stroke therapy, the extensive work in the field has not yet provided a drug for treating patients, being the only exception the thrombolytic drug tPA (tissue plasminogen activator) that disolves the clot and allows reperfusion of the injured area of the brain. The study of the molecular changes in neurons exposed to excitotoxic conditions may contribute to the development of new therapeutic strategies. In this work, we investigated the changes in the TrkB receptor for BDNF, a neurotrophic factor for different types of neurons (Almeida *et al.,* 2005, Jourdi *et al.,* 2009) and in gephyrin, a protein crucial for the traffic and to anchor inhibitory receptors (GlyRs and $GABA_A$Rs) at the synapse (Bedet *et al.,* 2006; Yu *et al.,* 2007).

In the first study we showed that:

- TrkB.FL receptors are degraded under excitotoxic conditions and in transient focal cerebral ischemia. Furthermore, glutamate stimulation upregulated a truncated form of TrkB receptors in cultured hippocampal and striatal neurons. These truncated receptors are isoform(s) of the *trkB* gene, and not a cleavage product of TrkB.FL.
- Excitotoxicity upregulates TrkB.T1 and TrkB.T2 mRNA in cultured hippocampal neurons, with a concominant decrease in the number of transcripts for TrkB.FL. This result suggests that the splicing mechanism of the TrkB pre-mRNA is changed under excitotoxic conditions, leading to a sustained synthesis of more mature mRNAs coding for TrkB.T rather than TrkB.FL (Barbacid, 1994; Stoilov *et al.,* 2002; Luberg *et al.,* 2010).
- The effect of excitotoxic conditions on the signaling activity of TrkB.FL was investigated by measuring the phosphorylation of the receptor, PLCγ phosphorylation on tyrosine, and activation of Erk.Analysis of the ratio pTrkB/total TrkB in hippocampal neurons stimulated with BDNF showed a large reduction in phosphorylation following excitotoxic stimulation and, as expected, the activation of PLCγ and ERK was also downregulated. However, the loss of TrkB.FL signaling activity

is not only due to receptor degradation and the studies performed strongly suggest that TrkB.T receptors upregulated under excitotoxic conditions can exert a dominant negative type of effect that prevents the signaling by the full-length receptors.

- In addition to the activation of TrkB.FL, BDNF also stimulates TrkB.T, which possesses an independent signaling activity that may have additional modulatory effects on excitotoxic neuronal damage. Activation of TrkB.T1 by BDNF acts as a negative regulator of Rho GTPases (Takai *et al.,* 2001), including RhoA. Accordingly, the upregulation of TrkB.T iscorrelated with the induction of a BDNF-induced inhibition of RhoA response in cultured hippocampal and striatal neurons subjected to excitotoxic stimulation. The inhibition of RhoA by TrkB.T may provide additional neuroprotection under excitotoxic conditions by inhibiting p38 MAPK and JNK/c-Jun (Marinissen *et al.,* 2004; Semenova *et al.,* 2007).

- The results obtained indicate that although TrkB.T may have a dominant-negative effect on the signaling activity of the full-length receptors, this may be compensated at an early stage by the neuroprotective signaling activity of the truncated receptors. Thus, activation of TrkB.T receptors may constitute an endogenous neuroprotective strategy underconditions characterized by excitotoxic cell death, including brain ischemia, cerebral trauma, epileptic seizures, and chronic neurodegenerative disorders.

In the second part of this work we showed that:

- The scaffold protein of Glycine and $GABA_A$ receptors, gephyrin, is cleaved under excitotoxic conditions *in vitro* and *in* vivo, and in transient focal ischemia, giving rise to a truncated formwhich may disorganize the submembranous lattice which anchors these inhibitory receptors. The cleavage of full length gephyrin upon intra-hipocampal injections of kainate was significant in the first 4h and 8h after the excitotoxic injury, suggesting that the functional consequences of gephyrin cleavage may be more relevant in the first hours after ischemia, when excitotoxicity takes place, and alterations in GABAergic synaptic transmission may interfere in the balance excitation/inhibition. Gephyrin was cleaved under excitotoxic conditions by calpains as shown by the results obtained with chemical inhibitors and in transgenic mice overexpressing calpastatin, the endogenous calpain inhibitor.

- Collybistin was also cleaved under excitotoxic conditions and the results obtained suggest that calpains may be involved in its degradation. Collybistin and gephyrin participate in multimeric complexes with $GABA_A$Rs, and the collybistin-gephyrin complex was proposed to play a key role in $GABA_A$Rs containing α2 subunits (Saiepour*et al.,* 2010). The clevage of gephyrin and collybistin under excitotoxic conditions is likely to have a great impact in the clustering of $GABA_A$Rs with other subunit compositions.

Together, the results presented in this thesis show the important role of calpains under ischemic conditions, contributing to a downregulation of the trophic effects mediated by TrkB.FL receptors and to the cleavage of gephyrin and collybistin which is likely to impair inhibitory synapses.

Chapter 5

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