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TrkB-mediated activation of the phosphatidylinositol-3-kinase/Akt cascade reduces the damage inflicted by oxygen-glucose deprivation in area CA3 of the rat hippocampus

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Abstract

The selective vulnerability of hippocampal area CA1 to ischemia-induced injury is a well-known phenomenon. However, the cellular mechanisms that confer resistance to area CA3 against ischemic damage remain elusive. Here, we show that oxygen-glucose deprivation-reperfusion (OGD-RP), an in vitro model that mimics the pathological conditions of the ischemic stroke, increases the phosphorylation level of Tropomyosin-receptor-kinase B (TrkB) in area CA3. Slices preincubated with Brain-derived neurotrophic factor (BDNF) or 7,8-Dihydroxyflavone (7,8-DHF) exhibited reduced depression of the electrical activity triggered by OGD-RP. Consistently, blockade of TrkB suppressed the resistance of area CA3 to OGD-RP. The protective effect of TrkB activation was limited to area CA3, as OGD-RP caused permanent suppression of CA1 responses. At the cellular level, TrkB activation leads to phosphorylation of the accessory proteins SHC and Gab as well as the serine/threonine kinase Akt, members of the phosphoinositide 3-kinase/Akt (PI-3-K/Akt) pathway, a cascade involved in cell survival. Hence, acute slices pretreated with the Akt antagonist MK2206 in combination with BDNF lost the capability to resist the damage inflicted with OGD-RP. Consistently with these results, CA3 pyramidal cells exhibited reduced propidium iodide uptake and caspase-3 activity in slices pre-treated with BDNF and exposed to

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OGD-RP. We propose that PI-3-K/Akt downstream activation mediated by TrkB represents an endogenous mechanism responsible for the resistance of area CA3 to ischemic damage.

Graphical Abstract

This study demonstrates that oxygen-glucose deprivation-reperfusion (OGD-RP), an *in vitro* model that mimics the condition of ischemic stroke, increases the phosphorylation level of TrkB receptors in hippocampal area CA3. TrkB phosphorylation leads to activation of the PI-3-K/Akt pathway; blocks apoptosis, promotes membrane integrity and reduces the damage inflicted by OGD-RP. TrkB and PI-3-K/Akt represents an endogenous mechanism responsible for the resistance of area CA3 to ischemic damage.

Keywords

TrkB; BDNF; Mossy Fibers; CA3 pyramidal cells; Oxygen-glucose deprivation; Reperfusion; PI-3-K/Akt

Introduction

In response to momentary reductions in oxygen and glucose supply, the pyramidal cells (PCs) in the hippocampal formation react differently. Whereas CA1 PCs exhibit abrupt signs of cellular damage, PCs in area CA3 are resilient to the lack of oxygen and glucose. Interestingly, although a significant amount of knowledge regarding the mechanisms mediating the ischemic damage in area CA1 is regularly reported, little is known about the intrinsic mechanisms that provide resistance to CA3 PCs to ischemic episodes. Indeed, it is well known that CA3 PCs scarcely exhibit cellular alterations as compared to CA1 PCs in response to ischemia (Cherubini *et al.*, 1989; Leblond & Krnjevic, 1989; Nitatori *et al.*, 1995), and the synaptic plasticity in area CA3 persists during ischemic damage (Cherubini *et al.*, 1989; Kirino *et al.*, 1992). Furthermore, a brief exposure to oxygen-glucose deprivation followed by reperfusion (OGD-RP), an *in vitro* model that mimics the lack of oxygen and glucose that occurs during stroke (Noraberg *et al.*, 2005), barely causes synaptic depression of CA3-evoked excitatory potentials (Gee *et al.*, 2006; Dennis *et al.*, 2011).

What are the cellular mechanisms that mediate the inherent resilience of area CA3 during transitory anoxia/aglycemia? Here, we explored the hypothesis that activation of Tropomyosin-related kinase B receptor (TrkB), mediated by its endogenous ligand, brain-derived neurotrophic factor (BDNF), reduces the negative impact inflicted by OGD-RP. TrkB and BDNF are robustly expressed in the mossy fibers (MF) and area CA3 of the hippocampus (Kokaia *et al.*, 1996; Danzer & McNamara, 2004; Spencer-Segal *et al.*, 2011). In addition, TrkB activates a series of downstream signaling cascades including the Phosphatidylinositol-3 Kinase (PI-3-K)/serine threonine kinase (Akt), the Mitogen-Activated Protein Kinase MAPK/ERK 1/2 or the phospholipase C- γ . Although these cascades are critical for neuronal function and plasticity (Minichiello *et al.*, 2002; Danzer & McNamara, 2004; Nagappan & Lu, 2005), BDNF and TrkB activation also block apoptosis and promotes cell survival following ischemic damage (Hetman *et al.*, 1999; Han & Holtzman, 2000; Sun *et al.*, 2008).

Here, we demonstrate that blockade of TrkB with ANA-12, suppresses the resistance of MF excitatory postsynaptic potentials (MF EPSPs) and CA3 antidromic population spikes (CA3 PS) to OGD-RP. Conversely, preincubation of hippocampal slices with BDNF or its specific agonist, 7,8-dihydroxyflavone, reduces the depression triggered by OGD-RP. We also found that TrkB phosphorylation activates the PI-3-K/Akt, a signaling cascade involved in cell survival during ischemic damage (Han & Holtzman, 2000; Lu *et al.*, 2013). Furthermore, TrkB activation blocked expression of caspase-3 enzyme and reduced propidium iodide uptake, indicating reduced cellular damage (Laake *et al.*, 1999; Sun *et al.*, 2010). These results offer a mechanistic explanation of the endogenous resistance of area CA3 to the damage inflicted by oxygen-glucose deprivation associated with several forms of ischemic injury.

Material and methods

All of the experimental procedures performed throughout the length of the study were carried out in strict accordance with the Mexican Official Norm for utilization and care of laboratory animals “NOM-062-ZOO-1999” and by the local Ethics Committee of our Institution (Cinvestav-IPN, México. Authorization number 0090-14) that mandates minimization of suffering and reducing the number of experimental animals. Additionally, the experimental procedures comply with the ARRIVE guidelines. We performed 116 experiments in acute hippocampal slices from 45 Sprague-Dawley rats (35-45 days old). The animals were bred and housed with ad libitum access to food and water and continuous veterinary supervision at the local animal facility under a 12:12 h light/dark cycle. Animals were maintained in a climate-controlled room at $22 \pm 2^\circ\text{C}$ and ~50% relative humidity; with 18% fat feed (Harlan, Indianapolis, IN, USA). Sprague-Dawley male rats (35-45 days old) were deeply anaesthetized (Pentobarbital Sodium I.P., 60mg/Kg of body weight) decapitated, and their brains were quickly removed and placed in an ice-cold sucrose slicing solution containing (in mM): 210 Sucrose, 2.8 KCl, 2 MgSO₄, 1.25 Na₂HPO₄, 26 NaHCO₃, 1 MgCl₂, 1 CaCl₂ and 10 D-(+)-Glucose. Hippocampal tissue blocks were glued to the plate of a VT1000S microtome (Leica, Nussloch, Germany) and horizontal hippocampal slices (385 μm thick) were obtained. The slices were maintained for 30 min at $33 \pm 2^\circ\text{C}$ in an incubation solution with the following composition (in mM): 125 NaCl, 2.5 KCl, 1.25

Na₂HPO₄, 25 NaHCO₃, 4 MgCl₂, 1 CaCl₂ and 10 D-(+)-Glucose. After that, slices were incubated at room temperature for one hour before the recordings. Individual slices were transferred to a submersion recording chamber (total volume, 1.5 ml; exchange rate including the volume inside the tubing 2.5 ± 0.5 ml/min) for at least 20 min before the beginning of the experiments. The slices were maintained at a constant flow (4-4.5 ml/min) with a standard ACSF containing (in mM): 125 NaCl, 2.5 KCl, 1.25 Na₂HPO₄, 25 NaHCO₃, 2 MgCl₂, 2 CaCl₂ and 10 D-(+)-Glucose, heated to 33 ± 2 °C with the help of an in-line solution heater coupled to a temperature controller (TC-324C, Warner Instruments). Solutions were continuously bubbled with 95% O₂/5% CO₂.

Oxygen and Glucose Deprivation

The Oxygen-Glucose Deprivation (OGD) solution consisted of a modified ACSF in which the D-(+)-Glucose was substituted with an equimolar concentration of sucrose (10 mM) and continuously bubbled with a nitrogen-based mixture N₂ (95%) / CO₂ (5%). The OGD solution was bubbled during preparation, and for at least one hour before the beginning of the recordings. When OGD was perfused in the recording chamber, a strong bubbling was used to ensure N₂ saturation.

Extracellular recordings

The extracellular responses were recorded with pipettes pulled from borosilicate glass with resistances of 1-2 MΩ when filled with a NaCl solution (3M). The extracellular responses (See Figure 1A, 1B) were evoked via bipolar electrodes made of nichrome wire (38 μm bare diameter). Orthodromic mossy fibers synaptic responses were evoked from the dentate gyrus hilus (Henze *et al.*, 1997) whereas antidromic population spike responses were evoked by stimulating the CA3a stratum pyramidale. The stimulation electrodes were always positioned on either side of the recording pipette. Test stimuli (0.06Hz) consisted of paired monopolar pulses (15-30 μA intensity: 100-120 μs duration) that evoked 30-50% of the maximal response previously determined by an input-output curve. The sequential stimulation of mossy fibers (or Schaffer collaterals) and population spikes inputs converging onto the same recording site was delivered at 1000-ms inter-stimuli to minimize synaptic temporal summation. The pulses were delivered via a high voltage isolation unit (A365D; World Precision Instruments, Sarasota FL), controlled with a Master-8 pulse generator (AMPI, Jerusalem, Israel). Responses were amplified with a Dagan BVC-700A amplifier (Minneapolis, MN) coupled with an extracellular headstage (Dagan, model 8024) and high-pass filtered at 0.3 Hz. Additional electrical noise suppression was achieved with a Humbug noise eliminator (Quest Scientific Instruments; North Vancouver, BC). Evoked responses were displayed on a PC-based oscilloscope and digitalized for storage and off-line analysis with custom written software (Lab View system, National Instruments, Austin TX).

The following criteria were used to identify and accept evoked responses as mossy fiber field excitatory potentials (Claiborne *et al.*, 1993; Urban & Barrionuevo, 1996): 1) the negative-evoked sink was restricted to the *stratum lucidum*, and the duration was >4 ms; 2) the excitatory potential onset latency was <5 ms; 3) the paired-pulse exhibited strong facilitation; and 4) the evoked responses were depressed by the group II metabotropic

glutamate receptor agonist (2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine, DCG-IV (1 μ M).

Oxygen measurement

An oxygen microsensor (OX-25, Unisense, Denmark) equipped with three electrodes, was used to measure the oxygen partial pressure (pO_2) in the recording chamber (total volume 600 μ l). The measurements were stored via digital logger. (NI USB-6008, National Instruments Co. TX, USA). The sensor has a rapid response to oxygen pressure changes and the current signal changes linearly with oxygen partial pressure (Yin *et al.*, 2015), and was polarized at -800 mV (working & guard vs. reference) before use. Three-point calibration was performed with zero-oxygen solution (0.1 M NaOH + 0.1M sodium ascorbate), artificial cerebrospinal fluid (ACSF) saturated with carbogen mixture (5% CO_2 / 95% O_2) and OGD-based solution (see below) saturated with 95% N_2 / 5% CO_2 . An LC-4C amperometric detector (BASi, IN, USA) was used as the potentiostat. Acute slices or organotypic hippocampal slices were treated with control solution for 10 min, oxygen-glucose deprivation (OGD) for 10 min, and reperfusion (RP), 30-60 min. ACSF gassed with 95% O_2 / 5% CO_2 is used for the control solution and RP, and MACSF gassed with 95% N_2 / 5% CO_2 is used in OGD. The individual values obtained were averaged and shown in Figure 2.

Western Blotting

The antibodies used in this study are: TrkB (Abcam Cat# ab75173 RRID:AB_1281172. (Y816), 1:1000); pSHC (Cell Signaling Technology Cat# 2434P RRID:AB_10841301. (Y239/240), 1:1000); pGAB (Cell Signaling Technology Cat# 3231S RRID:AB_2305002. (Y627), 1:1000); pAkt (Cell Signaling Technology Cat# 4060 also 4060L, 4060S, 4060P RRID:AB_2315049. (S473), 1:2000). pERK 1/2 (Cell Signaling Technology Cat# 4370 also 4370L, 4370S, 4370P RRID:AB2315112 (T202/Y204); 1:2000). pCaMKII (Abcam Cat# ab2724 RRID:AB_303249 (T286); 1:1000) and Actin-D (Cell Signaling Technology Cat# 4370 also 4370L, 4370S, 4370P RRID:AB_2315112) with a dilution 1:1000. For the secondary antibodies, anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (dilution, 1:20,000). Cell lysates were prepared from hippocampal slices exposed to OGD (5 min or 10 min) or preincubated with 7,8-DHF (45 min) or 7,8-DHF + ANA-12 (45 min). Following the treatments, the micro slices containing DG-CA3 or area CA1 (see Figure 3) were mechanically disaggregated. Three sections of each field were immersed in Laemmli buffer 2X supplemented with 4 mM orthovanadate and 0.28 mM β -mercaptoethanol. Then, the samples were placed in a bath sonicator for 1 min to dissolve the tissue. Next, the proteins were separated in 8%, 10% or 12% SDS-PAGE gels. The sample was electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 4% dry milk in Tris-buffered saline containing 0.1% Tween 20. The resulting membranes were tested for the different primary antibodies and adequate secondary HRP-conjugated Ab before being visualized by chemiluminescence using Image Studio Software (LI-COR, Biosciences). The densitometric analysis was performed using ImageJ software.

Propidium Iodide uptake and Immunofluorescence analysis

The CA3 pyramidal cell viability was determined with Propidium Iodide (PI) uptake. PI is a non-diffusible agent that stains nucleic acids. Cell membrane integrity excludes PI from staining viable and apoptotic cells. Random slices selected from each experimental group were preincubated with PI (2 μ M) during 15 min with ACSF, followed by a cycle of five washes with bubbled ACSF.

For immunofluorescence analysis, three slices were used for each experimental condition. After PI staining, slices were post-fixed overnight in 4% PFA, then transferred into 30% sucrose solution. Slices were then re-sectioned into 60 μ m sections for the immunohistochemical experiments. The sections were incubated with BSA 5% in PBS 0.2% - Tween 20 in PBS for 1.5 hr at room temperature to prevent non-specific staining. Sections were then incubated with primary antibodies at room temperature overnight, followed by several PBS washes. Sections were then incubated with second antibodies at room temperature for two hr.

Antibodies

The rabbit anti-Caspase 3 (Cell Signaling Technology Cat# 9662P RRID:AB 10839261; 1:1000) and mouse anti-NeuN (Millipore Cat# MAB377 RRID:AB_2298772; 1:500) were used in our study. As a negative control, random-selected slices from the same tissue (60 μ m thickness) were simultaneously processed in the absence of the primary antibody. After rinsing three times, slices were incubated with secondary antibodies for two hours at room temperature as follows: goat anti-rabbit Cy5 and goat anti-mouse Alexa flour 488 (1:500, Molecular Probes, Eugene Or). Finally, slices were rinsed three times with PBS and mounted on slides using Vectashield with DAPI mounting medium (Vector). All slices were examined with an epifluorescent microscope 40x (Axio Scope, Carl Zeiss; Oberkochen, Germany). The stitching of sequential photos from the horizontal sections of the hippocampus and the measuring of the fluorescence intensity of the stained slices was performed with the open-source software Fiji (Schindelin *et al.*, 2012).

Stereological analysis

The stereological analyses were performed on hippocampal slices that were used for extracellular recordings. The image acquisition of NeuN, Propidium Iodide and Caspase-3 immunoreactive cells counterstained with DAPI (4',6-diamidino-2-phenylindole) was conducted with an Axio Observer Z1 Microscope (Carl Zeiss; Oberkochen, Germany) equipped with a motorized scanning stage (X, Y, Z); LSM 800 Airyscan camera, Plan-Apochromat 63 $\times$ /1.4 oil objective lens and Zen software (blue edition). The cell quantification was performed with the open source software Fiji (Schindelin *et al.*, 2012). The cell density of PCs located in the Stratum Pyramidale of CA3b region was estimated using an unbiased methodology (Zhu *et al.*, 2015). A counting frame of 173.15 μ m was set in both x and y-axis, resulting in an area of 29980.92 μ m² and a subsequent acquisition of seven-to-nine microphotographs of area CA3b (3 μ m distance between each image) was used for the estimates. The first focal plane was discarded and the cell computing was performed from cells that showed NeuN, PI and Caspase-3 positive signal. Otherwise, neurons were discarded. The measurements were performed using the cell counter plugging

(Fiji). Each count included three consecutive sections obtained from three slices obtained from different animals per treatment. The total cell density was determined by the average of the number of nuclei and slice thickness per condition. Cell density remained similar among the different treatments.

Drugs

(2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine (DCG-IV), and KN-93 phosphate were purchased from TOCRIS (Ellisville, MO) and dissolved in double distilled water and DMSO, respectively. Brain-derived neurotrophic factor human (BDNF), 7,8-Dihydroxyflavone (7,8-DHF), N-[2-[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide (ANA-12), UO126 monoethanolate and 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (Propidium Iodide) were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO). MK-2206 2HCl was purchased from Selleck Chemicals (Houston, TX). 7,8-DHF, ANA-12, UO126, PI and MK-2206 2HCl were dissolved in DMSO. The concentration of DMSO in the final bath solution was 0.1%.

Statistics

Group measures are expressed as means $\pm$ SEM. The statistical significance of the changes in the Mossy Fiber field excitatory potentials and Population Spike amplitude were determined by comparing the average value of the EPSP in baseline condition with the mean at 10 min of OGD and finally at 120 min RP. The Normality distribution of the populations was validated using a Kolmogorov-Smirnov test ($p < 0.05$) and the comparison among experimental groups was determined by unpaired t-test ($p < 0.05$). For the Western blots analyses, the Student t-test was used to determine the statistic difference. For all the experiments included in this study, the statistical difference was considered significant if $p < 0.05$.

Results

Effect of transient exposure to OGD in the acute hippocampal slice

MF EPSPs and antidromic CA3 PS or CA1 EPSPs and CA1 PS were used to contrast the effect of TrkB activation during OGD-RP (see methods for acquisition details). Figure 1A shows the stimulation and recording sites and Figure 1B, the typical electrophysiological responses obtained from each hippocampal subregion. The MF EPSPs exhibited strong paired-pulse facilitation (2.19 ± 0.49 ; $n = 70$ slices), 1 Hz-stimulation facilitation ($326 \pm 71\%$; $n = 22$ slices) and suppression $\approx 90\%$ when the metabotropic glutamate receptor agonist, DCG-IV (1 μ M), was bath perfused to the recording chamber (Figures 1B-1E). The CA3 PS exhibited a less pronounced PPF (1.27 ± 0.25 ; $n = 70$ slices) and 1 Hz-stimulation facilitation (156.9 ± 6.84 ; $n = 22$ slices) but were insensitive to bath perfusion of DCG-IV (Figures 1C-1E, gray bars). The CA1 EPSPs and CA1 PS exhibited reduced facilitation when compared to CA3 responses (CA1 EPSPs PPF = 1.42 ± 0.35 ; CA1 PS PPF = 1.14 ± 0.3 ; $n = 29$ slices). Table 1 summarizes the kinetic properties of CA3 and CA1 evoked responses recorded in this study.

First, we corroborated the selective vulnerability of the electrophysiological responses of the hippocampus to OGD-RP. A stable 25 min baseline of MF EPSPs and CA3 PS was recorded followed by a transient OGD exposure (10 min) and ACSF-RP continued up to 120 min. In agreement with previous reports (Gee *et al.*, 2006; Dennis *et al.*, 2011) we found that OGD-RP caused depression of both MF EPSPs and CA3 PS (MF EPSPs at 120 min RP = $29.4 \pm 4.2\%$ of baseline; $p < 0.001$ paired t-test; CA3 PS at 90 min OGD-RP = $26.95 \pm 3.8\%$ of baseline; $p < 0.001$; paired t-test; $n = 28$ slices; Figure 2A). Contrary to this observation, the same recording protocol (10 min OGD and 120 min RP) caused a permanent suppression of the EPSP and PS of area CA1 (CA1 EPSPs at 120 min RP = $0.91 \pm 0.15\%$ of baseline; $p < 0.001$. CA1 PS at 120 min RP = $1.97 \pm 0.55\%$ of baseline; $p < 0.001$; paired t-test. $n = 14$ slices; Figure 2B). The individual responses from each experiment conducted in area CA3 and area CA1 are plotted in Figures 2C and 2D and collectively shows that the response to OGD-RP is more variable in CA3 than CA1.

We also measured the changes in the interstitial partial oxygen pressure (pO_2) to monitor the gas exchange ($O_2/CO_2 \rightarrow N_2/CO_2$) (Yin *et al.*, 2015) during OGD-RP. The pO_2 measure was performed at $150 \pm 25 \mu m$ below the hippocampal slice surface, the depth at which we regularly placed the recording pipette for the extracellular recordings. During baseline, the average pO_2 concentration was 338.8 ± 0.2 mm/Hg (Blue traces in the top panel of Figure 2A and 2B). The OGD perfusate (indicated by a gray bar in the time course graphs) caused a pO_2 drop $\approx 85\%$ of the baseline measurement (to 45 ± 0.5 mm/Hg) that concurs with the decrease of the electrical responses. Remarkably, within 6.3 ± 1 min of RP the pO_2 level returned to baseline level (332.4 ± 0.6 mm/Hg), and continued stable during the rest of the recordings (pO_2 level at 90 min RP = 340 ± 0.12 mm/Hg; $n = 6$ slices for each hippocampal region). The pO_2 levels observed during normoxia, OGD and RP are in the same range to pO_2 values previously reported using acute slices in submerged chambers (McBean *et al.*, 1995; Pugliese *et al.*, 2003; Huchzermeyer *et al.*, 2008). Collectively, these data show that suppression of the hippocampal responses concurs with OGD and confirms previous observations regarding the differential susceptibility of the hippocampal formation to ischemic damage.

OGD activates TrkB phosphorylation in the hippocampus

We also noticed that bath perfusion of ANA-12 (100 μM), a potent ligand that prevents activation of TrkB, accelerates the decay of CA3 responses to a comparable extent to that observed in CA1 (MF EPSPs time decay during OGD = 5.68 ± 0.07 min; in the presence of ANA-12 = 3.48 ± 0.06 min; CA1 EPSPs decay during OGD = 2.56 ± 0.07 min. Figure 3A, left panel; CA3 PS decay = 5.25 ± 0.11 min; in the presence of ANA-12 = 3.31 ± 0.08 min; CA1 PS decay during OGD = 3.61 ± 0.09 min; Figure 3A, right panel). Because the leftward shift of the decay curves, we assumed that the endogenous resilience of area CA3 to OGD-RP involves TrkB activation. To further investigate this idea, western blots from lysates containing area CA3 or area CA1 were processed, and phosphorylation of TrkB (pTrkB) was used as a surrogate measure of TrkB activation. We found that basal activity of TrkB was three-fold higher in area CA3 than CA1 ($p < 0.001$; unpaired t-test; $n = 6$). Moreover, OGD exposure (10 min), markedly increased TrkB phosphorylation in CA3 compared to CA1 (CA3 = 3.27 ± 0.26 fold; $p < 0.001$; CA1 = 2.33 ± 0.18 fold; $p = 0.012$; unpaired t-test; $n =$

6). When OGD was applied in the presence of ANA-12, TrkB phosphorylation was prevented (Figures 3B1-3B2). Together, these results confirm that TrkB activity is stronger in area CA3 than area CA1, and demonstrate that phosphorylation of TrkB selectively occurs in the hippocampus after a transient exposure to OGD.

TrkB activation increases the CA3 recovery during OGD-RP

Next, we explored whether TrkB activation is capable of reverting the depression of the evoked responses caused by OGD-RP. Two experiments were designed to test this hypothesis. First, the hippocampal slices were preincubated with BDNF (15 ng/ml; 45 min). The preincubation did not alter the kinetics of the CA3 responses (Table 1) nor caused exacerbation of the synaptic activity (Scharfman, 1997, 2005). After a stable baseline, OGD-RP (10 and 120 min, respectively) was recorded. Preincubation with BDNF reduced the negative effect of OGD-RP, as CA3 responses (MF EPSPs and CA3 PS) exhibited an increase in the recovery at 120 min RP compared with naïve, OGD-exposed slices (percentage of MF EPSP recovery in the BDNF-treated, vs. the non-treated slices = 52.48%; $p = 0.0414$; unpaired t-test; percentage of recovery of CA3 PS = 59.6%; $p = 0.0101$; unpaired t-test; $n = 15$ slices, Figure 4A, 4B and 4C). We also noticed that the recovery observed with BDNF preincubation was accompanied with a reduction in the paired-pulse facilitation of both CA3 responses (MF PPF = 2.63 ± 0.08 ; at 120 min RP = 1.24 ± 0.7 ; $p = 0.0162$. CA3 PS PPF = 1.23 ± 0.03 ; at 120 min RP = 0.96 ± 0.1 ; $p = 0.01$; paired t-test; $n = 15$ slices; data not shown). These results support the notion that glutamatergic transmission exhibits a reduction following anoxia/aglycemia, possibly via a presynaptic mechanism (Howard *et al.*, 1998; Pang *et al.*, 2002). The mossy fiber origin of the recovered responses was confirmed by bath perfusion of DCG-IV (1 μ M) (MF EPSP in the presence of DCG-IV = 4.78 ± 6.4 ; $p < 0.001$; paired t-test; CA3 PS in the presence of DCG-IV = 102.5 ± 11 ; ns; paired t-test; $n = 8$; Figure 4A and 4C). To confirm the participation of TrkB in the resistance to OGD-RP of area CA3, another group of slices was preincubated with BDNF (15 ng/ml) in combination with ANA-12 (100 μ M). Notably, the pharmacological blockade of TrkB with ANA-12, abolished the recovery of the CA3 responses (MF EPSPs in the presence of ANA-12 at 120 min RP = $0.2 \pm 0.5\%$ of baseline; $p < 0.001$; paired t-test; CA3 PS at 120 min RP = $3 \pm 0.5\%$ of baseline; $p < 0.001$; paired t-test; Figure 4A and 4B, $n = 12$ slices).

In the next set of experiments, the slice preincubation was performed with the TrkB specific agonist, 7,8-DHF (25 μ M) in lieu of BDNF. In this group of slices, the CA3 responses also exhibited marked recovery compared to naïve OGD-RP-exposed slices (percentage of MF EPSPs recovery in the 7,8-DHF-treated slices vs. the non-treated slices = 74.4%; $p = 0.01$; unpaired t-test; percentage of recovery of CA3 PS = 74.5%; $p = 0.0117$; unpaired t-test; $n = 15$ slices, Figure 4D). No statistical difference was found in the percentage of recovery observed with BDNF vs. 7,8-DHF of MF EPSPs ($p = 0.589$; unpaired t-test) or CA3 PS ($p = 0.633$; unpaired t-test) as summarized in the box plots of Figure 4D.

Lastly, we replicate these experiments in area CA1 using the same preincubation and TrkB agonists concentration. However, neither preincubation with BDNF nor 7,8-DHF were capable of reverting the suppressive effects of OGD ($n = 8$ slices for BDNF and 8 slices for

7,8-DHF; Figure 4E). Collectively, these experiments show that activation of TrkB receptor mediates a mechanism of neuroprotection against OGD-RP restricted to area CA3. More importantly, the OGD-mediated suppression of CA3 responses with ANA-12, demonstrates the central role of activation of TrkB on the selective vulnerability of area CA3 to the damage inflicted during OGD-RP.

Identification of the signaling cascades activated by TrkB in area CA3 of the hippocampus

Because our data demonstrate the regional significance of TrkB in the neuroprotection against OGD-RP, in the next experiments we utterly focused on determining the signaling cascades activated by TrkB receptors in area CA3. To discard the possibility that other members of the Trk family receptors may activate signaling cascades in response to BDNF, the slices were preincubated with 7,8-DHF. The western blots showed phosphorylation of the docking proteins pSHC and pGab as well as pAkt, indicating downstream activation of the IP-3-K/Akt signaling cascade. Also, CaMKII showed increased phosphorylation (Figures 5A and 5B; $n = 4$). Notably, stimulation with 7,8-DHF reduced the phosphorylation levels of pERK 1/2. However, this is not surprising, as PI-3-K/Akt and MAPK/ERK 1/2 have been shown to act antagonistically (Van der Heide *et al.*, 2003; Hui *et al.*, 2005; Sun *et al.*, 2010). Together, these results strongly indicate that the signaling cascades activated in area CA3 in response to TrkB phosphorylation are the PI-3-K/Akt and CaMKII but not MAPK/ERK 1/2.

The PI-3-K/Akt signaling cascade mediates the TrkB neuroprotection to OGD-RP in area CA3

Our finding that in response to TrkB activation, the PI-3-K/Akt and CaMKII are activated in CA3 raises the question about the role that those cascades play in the resistance to OGD-RP. To address this issue, we explored whether the selective blockade of Akt, CaMKII or MAPK/ERK 1/2 interfere with the recovery observed during RP. Thus, slices were preincubated with BDNF (45 min) in combination with the selective blockers of Akt, CaMKII or ERK 1/2 (MK-2206, 10 nM, KN-97, 10 μ M or U0126, 100 nM, respectively; See diagram in the bottom panel of Figure 6C). We found that blockade of Akt with MK-2206 suppressed the recovery of the evoked responses in a similar extent that the observed in slices preincubated with BDNF+ANA-12 (MF EPSPs at 120 min RP = $3.4 \pm 6\%$ of baseline; $p < 0.001$. CA3 PS at 120 min RP = $2.0 \pm 0.55\%$ of baseline; $p < 0.001$; paired t-test; triangles in Figure 6A and 6B; $n = 9$ slices). Contrary to this observation, slices preincubated with BDNF in combination with the CaMKII blocker KN-93 or the MAPK/ERK 1/2 selective inhibitor U0126, recovered in the same extent to the observed with BDNF alone. These experiments indicate that neither PLC-IP3-CamKII ($n = 7$ slices) nor the MAPK/ERK 1/2 ($n = 8$ slices) signaling pathways play a significant role in the resistance of area CA3 to OGD-RP. Moreover, the recovery observed in these experimental groups was indistinguishable from the recovery observed with BDNF alone ($p = 0.805$ for UO-126 and $p = 0.525$ for KN-93). The scatter plots in Figure 6B summarize the effects of the blockade of the kinases in response to OGD-RP and the MF EPSPs in Figure 6C illustrate the recovery of the synaptic responses when the respective kinase blockers were present. Taken together, these experiments demonstrate that activation of PI-3-K/Akt is a

fundamental step underlying the neuroprotection exerted by TrkB in area CA3 of the hippocampus.

OGD-RP induces cell damage and caspase-3 activation in CA3 pyramidal cells

It is well known that exposure to OGD-RP compromise the cellular integrity and induces activation of apoptotic cascades (Chen *et al.*, 1998; Namura *et al.*, 1998; Han *et al.*, 2000; Le *et al.*, 2002). Thus, we investigated the effect of TrkB activation in CA3 PCs exposed to OGD-RP. We assumed that, if TrkB activation exerts neuroprotection, the membrane integrity would be preserved and activation of apoptotic proteins would be reduced in the BDNF-incubated slices exposed to OGD-RP. Thus, we performed a fluorescence imaging analysis of propidium iodide (PI) uptake as an indicator of membrane integrity combined with the immunohistochemical expression of caspase-3 in neuronal nuclear antigen (NeuN)-positive CA3 PCs. In control slices, the NeuN-positive CA3 PCs did not exhibit PI uptake ($3.7 \pm 0.3\%$; $p < 0.001$) or expressed Caspase-3 ($0.4 \pm 0.2\%$; $p < 0.001$; Figure 7A1). On the other hand, slices exposed to OGD-RP showed an increase in PI uptake and nuclear expression of Caspase-3 (PI uptake after OGD-RP = $79 \pm 6\%$; $p < 0.001$. Caspase-3 expression after OGD-RP = $50.4 \pm 3.4\%$; $p < 0.001$; Figure 7A2). In contrast, when slices were preincubated with BDNF (15 ng/ml for 45 min), a reduction in both PI uptake and Caspase-3 expression was observed (PI uptake in BDNF preincubated slices = $43.7 \pm 7\%$; $p = 0.0031$. Caspase-3 expression = $21.4 \pm 4.5\%$; $p < 0.001$; Figure 7A3). Lastly, slices in which ANA-12 was included during BDNF preincubation, exhibited increased PI and Caspase 3 expression (PI uptake = $79.6 \pm 4\%$; $p < 0.001$; Caspase-3 expression = 49 ± 4 ; $p < 0.001$. Figure 7A4). The bar graphs in Figure 7B1-B3 summarizes the quantification of the PI and caspase-3 expression regarding the pharmacological treatments ($n = 3$). Together, these data demonstrate that in area CA3, BDNF-mediated activation of TrkB receptor reduces pyramidal cell damage and inhibits the activation of apoptotic mechanisms following OGD-RP.

Discussion

We have demonstrated that activation of TrkB plays a significant role in the resistance of hippocampal area CA3 to the damage inflicted by OGD-RP in acute hippocampal slices. Our western blots showed that OGD-RP induces rapid phosphorylation of TrkB and Akt in area CA3. In turn, TrkB activates the PI-3-K/Akt cascade that reduces the OGD-RP-mediated depression of electrical activity, contributed to the preservation of CA3 PCs membrane integrity and reduced the expression of caspase-3 enzyme. Moreover, our data consistently showed that pharmacological blockade of TrkB or the Akt signaling pathway suppressed the resistance of CA3 to OGD-RP. Taken together, our studies have uncovered an intrinsic mechanism to cope with the ischemic damage in area CA3.

One of the most noticeable observations of this study is the strong depression of CA3 responses triggered by OGD-RP. However, since area CA3 contains the higher levels of BDNF in the CNS (Conner *et al.*, 1997; Danzer & McNamara, 2004; Scharfman, 2005), why is that endogenous BDNF was ineffective on itself to protect the CA3-evoked responses against OGD-RP? This phenomenon could be explained by several ways. First, the physical

damage following brain slicing alters the ionic distribution and increases the release of metabolites and growth factors, including BDNF (Zhou *et al.*, 1995; Siklós *et al.*, 1997; Taubenfeld *et al.*, 2002). These events have been proposed to alter the integrity of the acute slice, and fail to replicate the intact brain condition (Taubenfeld *et al.*, 2002). If this is the case, the exogenous BDNF treatment would compensate for the lack of neuroprotective effect of the endogenous BDNF in response to OGD-RP in the acute slice. Along the same line, a significant increase in the induction of BDNF mRNA occurs in the hippocampus after the slicing procedure (Taubenfeld *et al.*, 2002; Danzer *et al.*, 2004). However, the BDNF mRNA increase is uncoupled from the synthesis of new BDNF protein, a phenomenon commonly associated to ischemic damage in hippocampal slices (Vornov & Coyle, 1991; Raley-Susman & Murata, 1995). Therefore, we cannot discard the possibility that the brain slicing procedure, in addition to OGD-RP, played a major role in the marked reduction of the CA3-evoked responses observed in our study. This scenario also explains the requirement of exogenous BDNF to unmask the neuroprotective role of BDNF acting via TrkB in response to OGD-RP. On the other hand, our data showed that blockade of TrkB receptor with ANA-12 not only accelerated the suppression of CA3 responses in response to OGD-RP, but abolished the recovery of the electrical responses, increased the cellular damage, and promoted expression of Caspase-3 enzyme. However, despite the experimental limitations of the in vitro preparation, one can speculate that in the intact brain, TrkB activation via BDNF plays a central role to reduce the damage associated with the ischemic stroke. Consistently, our results uncovered the significant role of TrkB in area CA3 in coping with the transient reduction of oxygen and glucose supply.

Although previous work has shown that responses from CA3 are insensitive or only exhibit partial sensitivity to OGD-RP, it should be noted that a larger part of these studies were performed in immature brains or organotypic slices. Since previous demonstrations that CA3 susceptibility to anoxia/aglycemia depends on the developmental stage (Nabetani *et al.*, 1995; Cheng *et al.*, 1997; Arabadzisz & Freund, 1999), our study was performed in acute slices from 35 - 45 days old animals. Given the critical role that suppression of oxygen plays for ischemic damage, it was critical to monitor the partial oxygen pressure during OGD and RP. For instance, a previous report showed that anoxic depolarization and ischemia in the in vivo hippocampus requires a reduction of $\approx 90\%$ of the cerebral blood level flow (Yonekura *et al.*, 2004). Our in vitro measurements showed that pO_2 is reduced $\approx 85\%$ during OGD, indicating that our experimental conditions mimicked the noxious environment that the hippocampal formation experiences during stroke.

BDNF/TrkB neuroprotection of CA3 pyramidal cells

Although the neuroprotective effects of BDNF are well documented, it is also well established that elevated concentrations of this neurotrophin may carry adverse effects, including hyperexcitability, spontaneous epileptiform discharges and exacerbated neuronal damage (Scharfman, 1997, 2005). This potential problem was diminished in our study by using a low BDNF concentration (15 ng/ml). Thus, in the present experimental conditions, we did not observe collateral effects (as summarized in Table 1) by BDNF preincubation. In response to an ischemic insult, TrkB-BDNF increases its expression level as a neuroprotective response or promoter of plasticity in the hippocampus (Lindvall *et al.*, 1992;

Kokaia *et al.*, 1993). Furthermore, the increased levels of BDNF have been hypothesized to protect the BDNF-synthesizing neuron itself (Lindvall *et al.*, 1992; Kokaia *et al.*, 1993). Thus, the elevated levels of BDNF/pTrkB of area CA3 are likely to function as an intrinsic mechanism of neuroprotection that also triggers signaling cascades associated with cell survival. In this sense, BDNF is recognized as a potent survival-promoting factor for neurons (Ferrer *et al.*, 2001; Sun *et al.*, 2008).

TrkB leads to the activation of at least two cascades involved in cell survival: the PI-3-K/Akt and the MAPK/ERK 1/2. Our results demonstrated activation of the PI-3-K/Akt in area CA3. Such activity plays a central role in the resistance to OGD-RP, a liable mechanism since different biochemical pathways can activate in response to specific cellular insults (Hetman *et al.*, 1999; Barnabé-Heider & Miller, 2003). In this sense, previous reports have shown the survival role of PI-3-K/Akt during ischemic insult (Sun *et al.*, 2008, 2010). See also the proposed model in Figure 8.

The Western blots also revealed that increased levels of pAkt were accompanied by reduced levels of pERK 1/2, a phenomenon that may be explained by an activation of an ERK 1/2 phosphatase or inhibition of an ERK 1/2 kinase (Van der Heide *et al.*, 2003); however, this is an issue that requires further investigation. Interestingly, the PI-3-K/Akt and MAPK/ERK 1/2 signaling cascades have been shown to act antagonistically (Van der Heide *et al.*, 2003; Sun *et al.*, 2010). In agreement with this observation, MAPK/ERK 1/2 plays a critical role in the induction of synaptic plasticity of area CA1 but is not required for CA3 LTP (Kanterewicz *et al.*, 2000), a demonstration that also indicates a divergence in the signaling cascades of these hippocampal subregions.

The role of one specific signaling cascade, the PI-3-K/Akt, associated with one specific type of neuron, the CA3 pyramidal cell, corroborates the idea that hippocampal neurons have unique physiological properties and unique molecular mechanisms to cope with different forms of neuronal damage. Nevertheless, we cannot discard additional cellular mechanisms involving TrkB activity that may act conjunctly with those reported here. For instance, autoreceptor phosphorylation of TrkB can occur in the absence of BDNF in hippocampal neurons. This mechanism is dependent on adenosine and A2 receptors (Lee & Chao, 2001) and involves activation of the PI-3-K/Akt cascade. Notably, the adenosine A2 receptors are highly expressed in the dentate MFs, and exert tonic inhibition of the glutamatergic transmission (Moore *et al.*, 2003). Hence, it can be speculated that upon activation of TrkB receptors, the OGD-sustained depolarization increases neurotransmitter release, including adenosine (Chu *et al.*, 2013), which in turn would activate the TrkB signaling cascades necessary to promote cell survival.

Lastly, although our experimental evidence supports the notion of a postsynaptic mechanism of neuroprotection, we cannot discard the possibility that CA3 resilience to OGD-RP includes a presynaptic component. Indeed, neurons from DG are well known to resist ischemic damage (Schmidt-Kastner & Freund, 1991). For example, a previous report demonstrated that ischemia also reduces the DG spontaneous firing rate and increases the stimulus intensity required for the generation of EPSPs (Howard *et al.*, 1998). Consistent with this idea, we found that OGD-RP reduces the MF PPF, suggesting that the depression

of the synaptic transmission may act as an additional protective mechanism against OGD-RP. Also, DG granular cells express TrkB receptors (Spencer-Segal et al., 2011), and its activation promotes cell survival and cellular homeostasis following ischemic damage (Horn et al., 2005). In both cases, the IP-3-K/Akt pathway is involved (Horn et al., 2005; Sun et al., 2010). Thus, it can be expected that the pharmacological manipulations performed in this study, also affected DG granular cells. Nevertheless, the contribution of BDNF/TrkB in this area of the hippocampus requires further investigation.

In conclusion, we have demonstrated that activation of the TrkB receptors is critical to counteract the effects of OGD-RP in area CA3 of the hippocampus. Blockade of TrkB abolishes the resistance of area CA3 to ischemic damage. Mechanistically, the PI-3-K/Akt signaling cascade mediates the neuroprotective effect.

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References

- Arabadzisz D, Freund TF. Changes in excitatory and inhibitory circuits of the rat hippocampus 12-14 months after complete forebrain ischemia. *Neuroscience*. 1999; 92:27–45. [PubMed: 10392828]
- Barnabé-Heider F, Miller FD. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. *J Neurosci*. 2003; 23:5149–5160. [PubMed: 12832539]
- Chen J, Nagayama T, Jin K, Stetler Ra, Zhu RL, Graham SH, Simon RP. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci*. 1998; 18:4914–4928. [PubMed: 9634557]
- Cheng Y, Gidday JM, Yan Q, Shah AR, Holtzman DM. Marked age-dependent neuroprotection by brain-derived neurotrophic factor against neonatal hypoxic-ischemic brain injury. *Ann Neurol*. 1997; 41:521–529. [PubMed: 9124810]
- Cherubini E, Ben-Ari Y, Krnjevi K. Anoxia produces smaller changes in synaptic transmission, membrane potential, and input resistance in immature rat hippocampus. *J Neurophysiol*. 1989; 62:882–895. [PubMed: 2809708]
- Chu S, Xiong W, Zhang DL, Soylu H, Sun C, Albeni BC, Parkinson FE. Regulation of adenosine levels during cerebral ischemia. *Acta Pharmacol Sin*. 2013; 34:60–66. [PubMed: 23064722]
- Claiborne BJ, Xiang Z, Brown TH. Hippocampal circuitry complicates analysis of long-term potentiation in mossy fiber synapses. *Hippocampus*. 1993; 3:115–121. [PubMed: 8353597]
- Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S. Distribution of Brain-Derived Neurotrophic Factor (BDNF) Protein and mRNA in the Normal Adult Rat CNS: Evidence for Anterograde Axonal Transport. *J Neurosci*. 1997; 17:2295–2313. [PubMed: 9065491]
- Danzer SC, McNamara JO. Localization of brain-derived neurotrophic factor to distinct terminals of mossy fiber axons implies regulation of both excitation and feedforward inhibition of CA3 pyramidal cells. *J Neurosci*. 2004; 24:11346–11355. [PubMed: 15601941]
- Danzer SC, Pan E, Nef S, Parada LF, McNamara JO. Altered regulation of brain-derived neurotrophic factor protein in hippocampus following slice preparation. *Neuroscience*. 2004; 126:859–869. [PubMed: 15207321]

- Dennis SH, Jaafari N, Cimarosti H, Hanley JG, Henley JM, Mellor JR. Oxygen/Glucose Deprivation Induces a Reduction in Synaptic AMPA Receptors on Hippocampal CA3 Neurons Mediated by mGluR1 and Adenosine A3 Receptors. *J Neurosci*. 2011; 31:11941–11952. [PubMed: 21849555]
- Ferrer I, Krupinski J, Goutan E, Martí E, Ambrosio S, Arenas E. Brain-derived neurotrophic factor reduces cortical cell death by ischemia after middle cerebral artery occlusion in the rat. *Acta Neuropathol*. 2001; 101:229–238. [PubMed: 11307622]
- Gee CE, Benquet P, Raineteau O, Rietschin L, Kirbach SW, Gerber U. NMDA receptors and the differential ischemic vulnerability of hippocampal neurons. *Eur J Neurosci*. 2006; 23:2595–2603. [PubMed: 16817862]
- Han BH, D'Costa A, Back SA, Parsadanian M, Patel S, Shah AR, Gidday JM, Srinivasan A, Deshmukh M, Holtzman DM. BDNF blocks caspase-3 activation in neonatal hypoxia-ischemia. *Neurobiol Dis*. 2000; 7:38–53. [PubMed: 10671321]
- Han BH, Holtzman DM. BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. *J Neurosci*. 2000; 20:5775–5781. [PubMed: 10908618]
- Henze DA, Urban NN, Barrionuevo G. Origin of the apparent asynchronous activity of hippocampal mossy fibers. *J Neurophysiol*. 1997; 78:24–30. [PubMed: 9242257]
- Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem*. 1999; 274:22569–22580. [PubMed: 10428835]
- Horn AP, Gerhardt D, Geyer AB, Valentim L, Cimarosti H, Tavares A, Horn F, Lenz G, Salbego C. Cellular death in hippocampus in response to PI3K pathway inhibition and oxygen and glucose deprivation. *Neurochem Res*. 2005; 30:355–361. [PubMed: 16018579]
- Howard EM, Gao TM, Pulsinelli Wa, Xu ZC. Electrophysiological changes of CA3 neurons and dentate granule cells following transient forebrain ischemia. *Brain Res*. 1998; 798:109–118. [PubMed: 9666096]
- Huchzermeyer C, Albus K, Gabriel HJ, Otahal J, Taubenberger N, Heinemann U, Kovacs R, Kann O. Gamma Oscillations and Spontaneous Network Activity in the Hippocampus Are Highly Sensitive to Decreases in pO₂ and Concomitant Changes in Mitochondrial Redox State. *J Neurosci*. 2008; 28:1153–1162. [PubMed: 18234893]
- Hui L, Pei DS, Zhang QG, Guan QH, Zhang GY. The neuroprotection of insulin on ischemic brain injury in rat hippocampus through negative regulation of JNK signaling pathway by PI3K/Akt activation. *Brain Res*. 2005; 1052:1–9. [PubMed: 16018989]
- Kanterewicz BI, Urban NN, McMahon DB, Norman ED, Giffen LJ, Favata MF, Scherle PA, Trzskos JM, Barrionuevo G, Klann E. The extracellular signal-regulated kinase cascade is required for NMDA receptor-independent LTP in area CA1 but not area CA3 of the hippocampus. *J Neurosci*. 2000; 20:3057–3066. [PubMed: 1077769]
- Kirino T, Robinson HP, Miwa A, Tamura A, Kawai N. Disturbance of membrane function preceding ischemic delayed neuronal death in the gerbil hippocampus. *J Cereb Blood Flow Metab*. 1992; 12:408–417. [PubMed: 1569136]
- Kokaia Z, Gidö G, Ringstedt T, Bengzon J, Kokaia M, Siesjö BK, Persson H, Lindvall O. Rapid increase of BDNF mRNA levels in cortical neurons following spreading depression: regulation by glutamatergic mechanisms independent of seizure activity. *Brain Res Mol Brain Res*. 1993; 19:277–286. [PubMed: 8231731]
- Kokaia Z, Nawa H, Uchino H, Elmer E, Kokaia M, Carnahan J, Smith ML, Siesjö BK, Lindvall O. Regional brain-derived neurotrophic factor mRNA and protein levels following transient forebrain ischemia in the rat. *Brain Res Mol Brain Res*. 1996; 38:139–144. [PubMed: 8737677]
- Laake JH, Haug FM, Wieloch T, Ottersen OP. A simple in vitro model of ischemia based on hippocampal slice cultures and propidium iodide fluorescence. *Brain Res Protoc*. 1999; 4:173–184.
- Le DA, Wu Y, Huang Z, Matsushita K, Plesnila N, Augustinack JC, Hyman BT, Yuan J, Kuida K, Flavell RA, Moskowitz MA. Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. *Proc Natl Acad Sci U S A*. 2002; 99:15188–15193. [PubMed: 12415117]

- Leblond J, Krnjevic K. Hypoxic changes in hippocampal neurons. *J Neurophysiol.* 1989; 62:1–14. [PubMed: 2547034]
- Lee FS, Chao MV. Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc Natl Acad Sci U S A.* 2001; 98:3555–3560. [PubMed: 11248116]
- Lindvall O, Ernfors P, Bengzon J, Kokaia Z, Smith ML, Siesjö BK, Persson H. Differential regulation of mRNAs for nerve growth factor, brain derived neurotrophic factor, and neurotrophin 3 in the adult rat brain following cerebral ischemia and hypoglycemic coma. *PNAS.* 1992; 89:648–652. [PubMed: 1731336]
- Lu B, Nagappan G, Guan X, Nathan PJ, Wren P. BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases. *Nat Rev Neurosci.* 2013; 14:401–416. [PubMed: 23674053]
- McBean DE, Winters V, Wilson AD, Oswald CB, Alps BJ, Armstrong JM. Neuroprotective efficacy of lifarizine (RS-87476) in a simplified rat survival model of 2 vessel occlusion. *Br J Pharmacol.* 1995; 116:3093–3098. [PubMed: 8719782]
- Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M. Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron.* 2002; 36:121–137. [PubMed: 12367511]
- Moore KA, Nicoll RA, Schmitz D. Adenosine gates synaptic plasticity at hippocampal mossy fiber synapses. *Proc Natl Acad Sci.* 2003; 100:14397–14402. [PubMed: 14608033]
- Nabetani M, Okada Y, Kawai S, Nakamura H. Neural activity and the levels of high energy phosphates during deprivation of oxygen and/or glucose in hippocampal slices of immature and adult rats. *Int J Dev Neurosci.* 1995; 13:3–12. [PubMed: 7793309]
- Nagappan G, Lu B. Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications. *Trends Neurosci.* 2005; 28:464–471. [PubMed: 16040136]
- Namura S, Zhu J, Fink K, Endres M, Srinivasan a, Tomaselli KJ, Yuan J, Moskowitz Ma. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci.* 1998; 18:3659–3668. [PubMed: 9570797]
- Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibana K, Kominami E, Uchiyama Y. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci.* 1995; 15:1001–1011. [PubMed: 7869078]
- Norberg J, Poulsen FR, Blaabjerg M, Kristensen BW, Bonde C, Montero M, Meyer M, Gramsbergen JB, Zimmer J. Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. *Curr Drug Targets CNS Neurol Disord.* 2005; 4:435–452. [PubMed: 16101559]
- Pang ZP, Deng P, Ruan YW, Xu ZC. Depression of fast excitatory synaptic transmission in large aspiny neurons of the neostriatum after transient forebrain ischemia. *J Neurosci.* 2002; 22:10948–10957. [PubMed: 12486190]
- Pugliese AM, Latini S, Corradetti R, Pedata F. Brief, repeated, oxygen-glucose deprivation episodes protect neurotransmission from a longer ischemic episode in the *in vitro* hippocampus: role of adenosine receptors. *Br J Pharmacol.* 2003; 140:305–314. [PubMed: 12970092]
- Raley-Susman KM, Murata J. Time course of protein changes following in vitro ischemia in the rat hippocampal slice. *BRAIN Res ELSEVIER Brain Res.* 1995; 694:94–102.
- Scharfman HE. Hyperexcitability in combined entorhinal/hippocampal slices of adult rat after exposure to brain-derived neurotrophic factor. *J Neurophysiol.* 1997; 78:1082–1095. [PubMed: 9307136]
- Scharfman HE. Brain-derived neurotrophic factor (BDNF) and the dentate gyrus mossy fibers: Implications for epilepsy. *Synaptic Plast Transsynaptic Signal.* 2005:201–220.
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012; 9:676–682. [PubMed: 22743772]
- Schmidt-Kastner R, Freund TF. Selective vulnerability of the hippocampus in brain ischemia. *Neuroscience.* 1991; 40:599–636. [PubMed: 1676492]

- Siklós L, Kuhnt U, Párducz Á, Szerdahelyi P. Intracellular calcium redistribution accompanies changes in total tissue Na⁺, K⁺ and water during the first two hours of in vitro incubation of hippocampal slices. *Neuroscience*. 1997; 79:1013–1022. [PubMed: 9219964]
- Spencer-Segal JL, Waters EM, Bath KG, Chao MV, McEwen BS, Milner TA. Distribution of Phosphorylated TrkB Receptor in the Mouse Hippocampal Formation Depends on Sex and Estrous Cycle Stage. *J Neurosci*. 2011; 31:6780–6790. [PubMed: 21543608]
- Sun X, Yao H, Douglas RM, Gu XQ, Wang J, Haddad GG. Insulin/PI3K signaling protects dentate neurons from oxygen-glucose deprivation in organotypic slice cultures. *J Neurochem*. 2010; 112:377–388. [PubMed: 19860861]
- Sun X, Zhou H, Luo X, Li S, Yu D, Hua J, Mu D, Mao M. Neuroprotection of brain-derived neurotrophic factor against hypoxic injury in vitro requires activation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *Int J Dev Neurosci*. 2008; 26:363–370. [PubMed: 18243629]
- Taubenfeld SM, Stevens KA, Pollonini G, Ruggiero J, Alberini CM. Profound molecular changes following hippocampal slice preparation: Loss of AMPA receptor subunits and uncoupled mRNA/protein expression. *J Neurochem*. 2002; 81:1348–1360. [PubMed: 12068082]
- Urban NN, Barrionuevo G. Induction of hebbian and non-hebbian mossy fiber long-term potentiation by distinct patterns of high-frequency stimulation. *J Neurosci*. 1996; 16:4293–4299. [PubMed: 8753890]
- Van der Heide LP, Hoekman MFM, Biessels GJ, Gispen WH. Insulin inhibits extracellular regulated kinase 1/2 phosphorylation in a phosphatidylinositol 3-kinase (PI3) kinase-dependent manner in neuro2a cells. *J Neurochem*. 2003; 86:86–91. [PubMed: 12807428]
- Vornov JJ, Coyle JT. Glutamate Neurotoxicity and the Inhibition of Protein Synthesis in the Hippocampal Slice. *J Neurochem*. 1991; 56:996–1006. [PubMed: 1671589]
- Yin B, Barrionuevo G, Weber SG. Optimized Real-Time Monitoring of Glutathione Redox Status in Single Pyramidal Neurons in Organotypic Hippocampal Slices during Oxygen-Glucose Deprivation and Reperfusion. *ACS Chem Neurosci*. 2015; 6:1838–1848. [PubMed: 26291433]
- Yonekura I, Kawahara N, Nakatomi H, Furuya K, Kirino T. A Model of Global Cerebral Ischemia in C57 BL/6 Mice. *J Cereb Blood Flow Metab*. 2004; 24:151–158. [PubMed: 14747741]
- Zhou Q, Abe H, Nowak TS. Immunocytochemical and in situ hybridization approaches to the optimization of brain slice preparations. *J Neurosci Methods*. 1995; 59:85–92. [PubMed: 7475255]
- Zhu Y, Liu F, Zou X, Torbey M. Comparison of unbiased estimation of neuronal number in the rat hippocampus with different staining methods. *J Neurosci Methods*. 2015; 254:73–79. [PubMed: 26238727]

Abbreviations

7,8-DHF	7,8-Dihydroxyflavone
ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANA-12	N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide
ATP	Adenosine triphosphate
BDNF	Brain-derived Neurotrophic Factor
CA3	Cornu Ammon 3
CA3 PS	Antidromic CA3 population spikes

CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
DCG-IV	(2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine
DG	Dentate Gyrus
MAPK	Mitogen-activated protein kinase
MF	Mossy Fibers
MF EPSP	Mossy Fiber-evoked field excitatory potentials
mTOR	Mechanistic target of the rapamycin
NeuN	Neuronal nuclear antigen
OGD-RP	Oxygen-Glucose Deprivation-reperfusion
pAkt	Phosphorylate protein kinase BPCs, Pyramidal cells
pERK ^{1/2}	Phosphorylate extracellular signal-regulated kinases ^{1/2}
PI	Propidium Iodide
PI-3-K/Akt	Phosphoinositide 3-kinase/Akt pathway
PLC	Phospholipase C
pO₂	Partial oxygen pressure
PPF	Paired pulse facilitation
pTrkB	Phosphorylated Tropomyosin-related kinase B receptor
RP	Reperfusion
TrkB	Tropomyosin-related kinase B receptor

Figure 1. Properties of the extracellular recordings

A) Schematic diagram of the hippocampal slice preparation showing the position of the stimulation and recording electrodes for CA3 and CA1. MF EPSP and CA3 antidromic Population Spikes were recorded in the CA3b section of the stratum lucidum; CA1 responses were recorded in the middle section of the CA1 Stratum Radiatum. **B)** Representative traces of MF EPSP and CA3 PS responses (upper panel). The gray trace in the first Mf EPSP indicated with an arrowhead shows the sensitivity to DCG-IV. Bottom panel shows the typical synaptic response and antidromic population spike evoked in area CA1. To accept MF EPSP responses, the following three criteria were rigorously met: **C)** Strong paired-pulse facilitation ($S2/S1 > 2$), **D)** Marked frequency facilitation during 1 Hz stimulation (300-400% increase in the extracellular response); and **E)** Suppression above 90% when DCG-IV was bath applied. CA3 experiments that did not accomplish these criteria were discarded from the study. Each symbol represents the mean value obtained from individual experiments and the bar represents the average response; error bars indicate SEM. *** $p < 0.001$ or higher statistical significance.

Figure 2. Effect of OGD-RP on hippocampal responses

A) Time-course graphs averaged from 28 experiments showing the effects of OGD-RP (OGD perfusion is indicated by the gray bar; RP continued for 120 min) on MF EPSP and CA3 PS (upper and bottom panels, respectively) or **B)** CA1 responses, averaged from 14 experiments. Insets show representative traces at the indicated conditions. In six slices, the partial Oxygen pressure (pO₂) was measured at 150 μm below the slice surface using an O₂ electrode (Blue lines; n = 6 slices for CA3 and n = 6 for CA1). Within 6 min of ACSF reperfusion, O₂ levels returned to baseline values. **C** and **D)** Scatter plots showing the individual responses for each experiment conducted in CA3 or in CA1. Each symbol represents the mean value obtained from an individual experiment and the superimposed red

trace indicates the mean response $\pm$ SEM for each experimental condition. *** $p < 0.001$ or higher statistical significance.

Figure 3. OGD induces activity of TrkB receptors in the hippocampus

A1-A2) Sigmoid fitting of EPSPs or antidromic population spikes decay in response to OGD in the acute slice. Perfusion of ANA-12, a TrkB receptor antagonist, accelerates the decay of CA3 responses resembling the decay observed in CA1. **B)** Western blots obtained from area CA3 or CA1 showing pTrkB activity. pTrkB activity was higher in CA3 compared to CA1; OGD exposure (10 min) increased pTrkB in both hippocampal regions. The pTrkB activity was inhibited when slices were preincubated with ANA-12. **C)** Bar graphs summarizing pTrkB activity in response to OGD or OGD + ANA-12 in both hippocampal regions. Symbols show the values obtained from individual experiments; bars the average response; error bars indicate SEM. ** $p < 0.01$; *** $p < 0.001$ or higher statistical significance.

Figure 4. Preincubation with BDNF reduces the effect of OGD-RP in CA3 but not in CA1 responses

A) Time-course graphs averaged from 15 experiments. OGD-RP (10 and 120 min, respectively) was followed by DCG-IV application ($n = 8$ slices). In the ANA-12 + BDNF treated slices ($n = 12$ slices) OGD caused a permanent suppression of the evoked responses (triangles in both graphs). **B)** Scatter plots showing the individual responses for each CA3 experiment in BDNF preincubated slices or BDNF+ANA12. The superimposed red traces indicate the mean response $\pm$ SEM for each experimental condition. **C)** Representative MF EPSPs and CA3 PS from slices preincubated with BDNF and exposed to OGD-RP. The superimposed gray traces show the sensitivity to DCG-IV of the MF EPSP but not the CA3 PS. **D)** Box plots summarizing the effects of preincubation with BDNF or 7,8-DHF on MF

EPSPs and CA3 PS at 120 min of OGD-RP. E) Box plots summarizing the lack of neuroprotective effects of preincubation with BDNF (n = 8 slices) or 7,8-DHF (n = 8 slices) in CA1 EPSPs and CA1 PS. Error bars indicate SEM. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ or higher statistical significance.

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Figure 5. TrkB signaling profile activity in CA3 following 7,8-DHF incubation

A) Representative Western blots of CA3 lysates preincubated with 7,8-DHF showing the phosphorylation profile of the proteins SHC, Gab, Akt, CaMKII, and ERK 1/2. **B)** Bar graphs summarizing the quantification analysis by densitometry of protein levels of the different signaling pathways ($n = 4$ experiments). Symbols show values obtained from individual experiments; error bars indicate SEM. * $p < 0.05$; ** $p < 0.01$ or higher statistical significance.

Figure 6. The PI-3-K/Akt signaling cascade is critical for recovery following OGD

A) Time-course graphs of MF EPSP and CA3 PS (upper and bottom panel) obtained from slices preincubated with BDNF alone or in combination with the Akt blocker MK2206 (15 ng/ml and 10 nM; $n = 11$). Insets: representative MF EPSP and CA3 PS responses obtained at the indicated condition. **B)** Scatter plots showing individual experiments of slices preincubated with BDNF + MK2206; the ERK kinase inhibitor UO126 or the CaMKII blocker, KN-97. The superimposed red traces indicate the mean response $\pm$ SEM for each experimental condition. **C)** Representative MF EPSP obtained in the presence of the indicated drugs. Bottom panel schematic diagram showing the signaling cascades activated by TrkB and their corresponding pharmacological antagonists.

Figure 7. TrkB activation reverts the OGD-induced cell damage and caspase-3 activation in CA3 pyramidal cells

A1-A4) Higher magnifications of confocal microphotographs contrasting PI uptake and Caspase-3 expression with DAPI counterstained nucleus (blue) in acute hippocampal slices. OGD-RP (10 and 120 min, respectively) increased the PI uptake and Caspase-3 nuclear expression. The negative effect of OGD was reverted when slices were preincubated with BDNF (15 ng/ml; 45 min). A relapse in the BDNF-mediated neuroprotection was observed when slices were preincubated with BDNF+ANA-12. **B1)** Quantitative analysis of the cell density in area CA3. Neither the OGD nor the pharmacological manipulations altered the total number of CA3 cells. **B2)** Quantitative analysis of the total number of CA3 neurons positive to NeuN + PI on the different experimental conditions. **B3)** Quantitative analysis of

CA3 neurons positive to NeuN + PI + Caspase-3. In control slices, neither PI uptake nor caspase-3 nuclear expression is detected. 10 min OGD caused a significant increase in the PI uptake and expression of caspase-3 that was reverted when slices were preincubated with BDNF. PI uptake and caspase-3 expression were increased when slices were preincubated with BDNF+ANA-12. Symbols show values obtained from individual experiments; Error bars indicate SEM. ** $p < 0.01$ or higher statistical significance.

Figure 8. Schematic representation of the intrinsic mechanism of resilience to OGD-RP in hippocampal CA3 pyramidal cells

A) An episode of OGD-RP causes strong depolarization of CA3 PCs and neurotransmitter release, including glutamate and BDNF. BDNF can be released from the mossy boutons, filopodial extensions (Danzer & McNamara, 2004), dendritic trees of CA3 PCs (Kuczewski *et al.*, 2008) but also from astrocytes (Danzer & McNamara, 2004). In response to the OGD-mediated depolarization, fast-spiking interneurons in Stratum Lucidum also exhibit increased glutamatergic activity modulated by TrkB receptors (Herrera-López, Barrionuevo and Galván, unpublished observations). **B)** The proposed mechanism by which BDNF/TrkB counteract the deleterious effects of OGD-RP in CA3 PCs requires downstream activation of the PI-3-K/Akt, and possibly activation of the mechanistic target of the rapamycin (mTOR), to promote cell survival. Activation of this signaling cascade maintain the membrane integrity, and reduces the expression of Caspase-3 enzyme in OGD-RP treated slices. Simultaneously, the BDNF-mediated activation of TrkB receptor causes a downregulation of the MAPK-ERK 1/2 pathway and upregulation of the CaMKII pathway.

Table 1

Kinetic properties of mossy fiber EPSPs and antidromically-elicited CA3 population spikes or CA1 EPSPs and CA1 PS (See schematic diagram in Figure 1A) under the different pharmacological treatments. Neither the 45 min preincubation with BDNF (15 ng/ml) nor 7,8-DHF (25 μ M) altered the kinetic properties of the extracellular responses.

	Latency (ms)	Tau decay(ms)	Width (mv/ms)	PPF (S2/S1)	1 Hz Frequency Stim (%)	DCG-IV Sensitivity (%)
MF EPSP	3.2 $\pm$ 0.3	54 $\pm$ 4	4.3 $\pm$ 0.3	2.2 $\pm$ 0.05	326 $\pm$ 15	95.3 $\pm$ 6
MF EPSP (+BDNF)	3.13 $\pm$ 0.2	54 $\pm$ 0.2	3.9 $\pm$ 0.2	1.96 $\pm$ 0.1	361 $\pm$ 17	96.7 $\pm$ 6
MF EPSP (+7,8-DHF)	3.1 $\pm$ 0.15	53 $\pm$ 0.5	4.25 $\pm$ 0.3	2.04 $\pm$ 0.6	-----	93.3 $\pm$ 4
CA3 PS	0.2 $\pm$ 0.3	36 $\pm$ 11	1.3 $\pm$ 0.2	1.3 $\pm$ 0.02	171.4 $\pm$ 15	0.4 $\pm$ 1.7
CA3 PS (+BDNF)	0.19 $\pm$ 0.2	36 $\pm$ 0.4	1.25 $\pm$ 0.2	1.18 $\pm$ 0.1	161 $\pm$ 14	2.6 $\pm$ 1.3
CA3 PS (+7,8-DHF)	0.19 $\pm$ 0.2	35.4 $\pm$ 2.0	1.3 $\pm$ 0.4	1.17 $\pm$ 0.1	-----	1.12 $\pm$ 0.05
CA1 EPSP	4.1 $\pm$ 1	71.6 $\pm$ 2	12 $\pm$ 0.4	1.4 $\pm$ 0.06	-----	-----
(+BDNF)	4 $\pm$ 2.5	69.4 $\pm$ 3	13.3 $\pm$ 1.2	1.38 $\pm$ 0.8	-----	-----
(+7,8-DHF)	3.92 $\pm$ 1	70.2 $\pm$ 2.3	11.9 $\pm$ 3.3	1.45 $\pm$ 0.2	-----	-----
CA1 PS	0.2 $\pm$ 0.1	38 $\pm$ 9	1.2 $\pm$ 0.1	1.14 $\pm$ 0.05	-----	-----
(+BDNF)	0.19 $\pm$ 0.5	37.2 $\pm$ 0.4	1.19 $\pm$ 0.02	1.21 $\pm$ 0.3	-----	-----
(+7,8-DHF)	0.2 $\pm$ 0.01	41.1 $\pm$ 0.6	1.06 $\pm$.2	1.19 $\pm$ 0.05	-----	-----