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ORIGINAL ARTICLE

Homeostatic NMDA receptor down-regulation via brain derived neurotrophic factor and nitric oxide-dependent signalling in cortical but not in hippocampal neurons

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Abstract

Nitric oxide (NO) has been proposed to down-regulate NMDA receptors (NMDA-Rs) in a homeostatic manner. However, NMDA-R-dependent NO synthesis also can cause excitotoxic cell death. Using bicuculline-stimulated hippocampal and cortical cell cultures, we have addressed the role of the brain-derived neurotrophic factor-NO pathway in NMDA-R down-regulation. This pathway protected cortical cells from NMDA-induced death and led to NMDA-R inhibition. In contrast, no evidence was gained for the presence of this protective pathway in hippocampal neurons, in which NMDA-induced NO synthesis was confirmed to be toxic. Therefore, opposing effects of NO depended on the activation of different signalling pathways. The pathophysiological relevance of this observation was investigated in synaptosomes and post-synaptic densities isolated from rat hippocampi and cerebral cortices following kainic acid-induced *status epilepticus*. In cortical, but not in hippocampal synaptosomes, brain-derived neurotrophic factor induced NO synthesis and inhibited NMDA-R currents present in isolated post-synaptic densities. In conclusion, we identified a NO-dependent homeostatic response in the rat cerebral cortex induced by elevated activity. A low performance of this pathway in brain areas including the hippocampus may be related to their selective vulnerability in pathologies such as temporal lobe epilepsy.

Keywords: cerebral cortex, epilepsy, excitotoxicity, neuronal homeostasis.

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Homeostatic responses compensate for activity perturbations in the CNS both at a synaptic as well as cellular level (Turrigiano and Nelson 2004). Although important advances have been achieved in the unravelling of homeostatic mechanisms following activity blockade (Pozo and Goda 2010), processes involved in negative feedback following elevated activity are less well-known. Understanding of such mechanisms should help to identify potentially protective processes in diseases characterized by elevated neural

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Abbreviations used: APV, 2-amino-5-phosphonovalerate; BDNF, brain-derived neurotrophic factor; BIC, bicuculline; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DAF-fm, 3-amino,4-aminomethyl-2′,7′-difluorofluorescein; Fura-2-AM, Fura-2 acetoxymethylester; GC, guanylyl cyclase; L-NNA, *N*[Ω]-nitro-L-arginine; NMDA-R, NMDA receptor; NO, nitric oxide; NOS, NO synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3,-a] quinoxalin-1-one; PKG, protein kinase G; pro-NGF, precursor form of nerve growth factor; PSD, post-synaptic density; ROS, reactive oxygen species; SE, status epilepticus; SNAP, *S*-nitroso-*N*-acetylpenicillamine; VEH, vehicle.

activity, such as epilepsy. Chronic enhancement of neuronal activity leads to Ca^{2+}-dependent cell death by excessive activation of NMDA-type glutamate receptors (NMDA-Rs) in susceptible brain areas (Arundine and Tymianski 2004). This type of glutamate-mediated excitotoxicity is involved in hypoxic-ischemic brain injuries that may lead to temporal lobe epilepsy (Pitkanen and Sutula 2002). It is unknown why selective brain areas, such as the hippocampus, are more vulnerable to excitotoxicity (Wang *et al.* 2009): differential intrinsic neuronal properties in this brain area may favour imbalances in synaptic excitation and inhibition rather than homeostatic responses that could counteract the genesis of hyperexcitability.

NMDA-receptors are tetramers composed of two NR1 subunits in combination with two NR2 subunits. While NR1 subunits derive from a single gene, four different genes code for the NR2 subunits (NR2A-D) (Cull-Candy and Leszkiewicz 2004). In the adult telencephalon, NMDA-Rs contain mainly NR2A and/or NR2B subunits in addition to NR1. NMDA-R-mediated Ca^{2+} influx activates efficiently Ca^{2+}-calmodulin-dependent neuronal nitric oxide synthase (nNOS), which is physically linked to NMDA-Rs via the scaffolding protein PSD-95 (post-synaptic density-95) (Sattler *et al.* 1999; Aarts *et al.* 2002). Excessive nitric oxide (NO) has been shown to be neurotoxic (Calabrese *et al.* 2007), however, it can also act in a protective manner: cysteine thiol groups in NR1 and NR2A subunits can be *S*-nitrosylated leading to down-regulation of NMDA-R function, providing negative feedback to elevated activity (Choi and Lipton 2000).

Opposing biological functions of NO have been ascribed to high versus low levels (Calabrese *et al.* 2007). Although the measurement of absolute NO levels has technical limitations, low NO has in the past been estimated in the 0.2–20 nM range, consistent with the EC_{50} of its receptor, soluble guanylyl cyclase (GC). However, a highly sensitive method using the GC-coupled NO receptor as biosensor has recently revealed that physiological and in particular, synaptic NO, ranges from picomolar up to a maximal 5 nM concentration, and therefore higher concentrations may cause toxicity (Wang *et al.* 2006; Hall and Garthwaite 2009; Batchelor *et al.* 2010).

The notion that toxicity depends on high NO has recently been challenged by several studies performed in cortical neurons, indicating that NO is neuroprotective in this cell type (Mannick *et al.* 1999; Riccio *et al.* 2006; Nott *et al.* 2008; Sen *et al.* 2009). Even nitric peroxide, the product of the reaction between NO and superoxide ($O_2\bullet$) that is reportedly extremely toxic (Guix *et al.* 2005), exerts neuroprotective effects in primary cortical cultures (Garcia-Nogales *et al.* 2003; Delgado-Esteban *et al.* 2007). In neurons, NO synthesis is regulated by the neurotrophin brain-derived neurotrophic factor (BDNF) (Riccio *et al.* 2006; Nott *et al.* 2008). Because BDNF acts positively on cell survival, growth and plasticity (Huang and Reichardt 2003), we decided to study the contribution of BDNF-dependent NO synthesis to the negative regulation of NMDA-Rs in dissociated cell cultures and in rat brain synaptic protein fractions. To assess a potential homeostatic NO-dependent NMDA-R modulation, neuronal cultures were stimulated chronically with bicuculline and then subjected to a short excitotoxic challenge. In the brain, elevated activity was obtained by intraperitoneal kainic acid injections leading to status epilepticus (SE). We found that cortical neurons in culture were less vulnerable to excitotoxicity than hippocampal ones, and this depended critically on BDNF–induced NO production that was able to inhibit NMDA-R function. NMDA-R modulation depended on the specific BDNF receptor, TrkB, but not on the pan-neurotrophin receptor $p75^{NTR}$. This homeostatic response was induced by kainic acid-induced SE in rat synaptosomes and PSDs isolated from the rat cerebral cortex, but not from the hippocampus, underscoring the relevance of such a signalling pathway in the intact brain.

Materials and methods

Materials

Chemical reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated. Tocris Bioscience (Bristol, UK) provided us with NMDA, 2-amino-5-phosphonovalerate (APV), Ifenprodil and tetrodotoxin. Recombinant *Escherichia coli*-derived BDNF, precursor form of nerve growth factor (pro-NGF) and K252a were from Alomone Labs (Jerusalem, Israel). Nimodipine, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-amino,4-aminomethyl-2′,7′-difluorofluorescein (DAF-fm) were from Calbiochem (San Diego, CA, USA). Fura-2 acetoxymethylester (Fura-2-AM) was from Molecular Probes (Eugene, OR, USA). Neurobasal medium and B27 were from Gibco-Invitrogen (San Diego, CA, USA). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from RBI (Natick, MA, USA). TrkB receptor bodies (TrkB-Fc) were from R&D Systems (Minneapolis, MN, USA). IgG-Fc was from Jackson Laboratories (Sacramento, PA, USA). 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was from Cayman (Ann Arbor, MI, USA). Kainic acid was from NANOCS (New York, NY, USA). Antibodies were provided by the following companies: Millipore Corporation, Bedford, MA, USA (anti-calretinin and anti-GAD65); Pharmingen, San Diego, CA, USA (anti-NR1); BD Transduction Laboratories, San Jose, CA, USA (anti-TrkB, anti-synaptophysin and anti-nNOS).

Animals

Procedures involving animals and their care were performed in accordance with the Universidad de los Andes Bioethical Committee and the ARRIVE Guidelines. All efforts were made to minimise animal suffering. We used adult male Sprague–Dawley rats (250–400 g) for subcellular fractionation. Rats were injected i.p. with either saline or kainate (10 mg/kg body weight) dissolved in saline. The severity of seizures was estimated as previously described (Wyneken *et al.* 2001), and only rats suffering from grade 4–6

seizures were included. In total, 120 rats were rapidly decapitated 24 h following kainic acid or saline injection in order to obtain samples for subcellular fractionation (n = 10 per group; in total, six independent preparations were performed).

Neuronal cultures

Primary cultures of cortical and hippocampal neurons were obtained from day-18 embryos of Sprague–Dawley rats as described (Banker and Goslin 1988), see further details in Appendix S1. Sustained stimulation of cultures was achieved by incubations with the γ-aminobutyric acid ($GABA_A$) receptor antagonist bicuculline (10 μM, BIC) dissolved in dimethyl sulfoxide for 48 h. The corresponding controls were incubated with the vehicle (VEH), that is, dimethyl sulfoxide to a final concentration of 0.005%. The 10 μM BIC dose was chosen because its acute application to cell cultures induced indistinguishable calcium influxes in both culture types, but at the same time did not lead to significant cell death after 48-h incubations (n = 4, not shown). Subsequently, the excitotoxic challenge was performed as described by Sattler and colleagues (Sattler *et al.* 1999). Briefly, neuronal cultures were incubated with 30 μM NMDA for 1 h in the presence of 10 μM CNQX, 2 μM nimodipine, 1 μM tetrodotoxin (to block α-amino-3-hydroxy-5-methylisoxazole-4-propionate, Ca^{2+} and Na^{+} channels, respectively) and the indicated compounds: 2 μM *N*[Ω]-nitro-L-arginine (L-NNA), 0.2 μg/mL TrkB-Fc (a BDNF scavenger), 0.2 μg/mL BDNF, 5 μM KT5823, 100 μM ODQ. After stimulation, neurons were returned to fresh Neurobasal/B27 medium containing CNQX, nimodipine and 10 μM APV.

Cell survival

The percentage of surviving neurons was assessed 24 h after the NMDA challenge using the trypan blue exclusion test. Neurons were exposed to 0.05% (v/v) trypan blue in phosphate-buffered saline for 5 min. The cells were immediately examined under a phase-contrast microscope, and then images of ten random fields near the centre of the dish (containing 150–200 cells) were recorded. An observer (A.C.), blind to the experimental conditions, examined the images to quantify the numbers of living neurons (which exclude trypan blue) and dead (stained) neurons.

Fluorescence imaging

Fluorescence was measured using an Eclipse E400 epifluorescence microscope with a FluorX40 water immersion objective (Nikon Corporation, Melville, NY, USA) equipped with a Sutter Lambda 10-2 optical filter changer. Emitted fluorescence was registered with a cooled charge-coupled device video camera (Retiga 2000R Fast 1394, QImaging, Surrey, BC, Canada) and data obtained were processed using imaging software (IPLab 4.0, Scanalytics, Buckinghamshire, UK).

Measurement of intracellular nitric oxide

Neuronal cultures were loaded for 1 h at 37ºC with the membrane-permeant dye DAF-fm (10 μM, Calbiochem) plus 0.015% pluronic acid in recording solution (in mM: 116 NaCl, 5.4 KCl, 0.9 NaH_2PO_4, 1.8 $CaCl_2$, 0.9 $MgCl_2$, 20 HEPES, 10 glucose and 0.1 L-arginine, pH 7.4). Cultures were then washed five times and placed in recording solution. Light exposure was minimised throughout the experiment. Fluorescence (emission at 510 nm; excitation at 495 nm) from cells was acquired for 250 ms each 5-min to minimise photo-oxidation and photobleaching of DAF-fm (Balcerczyk *et al.* 2005; Kovacs *et al.* 2009). Signals were averaged over regions of interest of somas (excluding the nuclei) and 20 μm of dendrite; background was subtracted and relative intracellular NO levels were calculated from emission at 510 nm. There was a linear decay of the fluorescence baseline because of photobleaching. The baseline negative slope was determined for each experiment before addition of the stimulus (BDNF or NMDA) and the experimental slope was corrected for this (Figure S1a). Nevertheless, at the end of each experiment, application of an external NO donor (10 μM SNAP) resulted in an immediate increase in fluorescence, indicating that a considerable fraction of NO-sensitive dye was still available. Experiments in which SNAP did not increase fluorescence were discarded. Only cells of pyramidal morphology were selected for measurements (Figure S1b). For statistical analysis, the mean slopes ± SEM of five independent experiments were compared by two-tailed t-tests.

Measurement of intracellular calcium

Neuronal cultures were loaded with 10 μM of the cell permeant dye Fura-2-AM (Molecular Probes) plus 0.005% of pluronic acid in recording solution for 20 min at 37°C followed by 20 min at 20–22°C, washed five times and placed in recording solution with the corresponding pharmacological compounds. Fluorescence ratio at 510 nm was recorded (excitation at 340 nm and 380 nm) and acquired every 5 s. Signals were averaged over regions of interest for somata (without nucleus) and 20 μm of dendrites; background was subtracted. Intracellular calcium levels changes were estimated by normalization of ratio of fluorescence emission at 510 nm (340 nm/380 nm excitation) to the basal fluorescence emission ratio at t = 0.

Subcellular fractionation

Rat brain synaptosomes and PSDs were obtained as previously reported (Wyneken *et al.* 2001).

Homogenates of cell cultures were obtained in a buffer containing 5 mM Tris–Cl, 0.5% Triton X-100 and a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany). The detergent-insoluble fraction (i.e. enriched in PSDs) was obtained by centrifugation of homogenates at 100 000 g for 1 h.

Electrophysiology

We used the method described previously to measure NMDA-activated ion currents present in PSDs transplanted into the *Xenopus laevis* oocyte plasma membrane (Sandoval *et al.* 2007; Eusebi *et al.* 2009). PSDs (100–130 ng) suspended in 50 mM HEPES, pH 7.4 were injected into oocytes that were kept at 16°C in modified Barth's solution [in mM: 88 NaCl; 1 KCl; 0.33 $Ca(NO_3)_2$; 0.82 $MgSO_4$; 2.4 $NaHCO_3$; 10 HEPES; pH 7.4; supplemented with 100 IU/mL penicillin and 0.1 mg/mL streptomycin]. As controls, oocytes from the same batch were injected with 65 nL of buffer.

About 12–16 h after injection, membrane currents were recorded from two-microelectrodes voltage-clamped oocytes placed in a recording chamber containing 0.5 mL oocyte Ringer's solution (in mM: 115 NaCl, 2 KCl, 1.8 $BaCl_2$, 5 HEPES, pH 7.4) at room temperature (20–22°C). The oocyte membrane potential was held at −60 mV and NMDA was applied continuously for 2 min. Between

agonist applications, oocytes were superfused with Ringer's solution for 10 min (5 mL at 0.5 mL/min). About 60% of oocytes expressed NMDA-R currents. The current integral (I, total current) during agonist application was calculated. Neurotrophins and drugs were added at the indicated final concentrations to the Ringer's solution 1 min before NMDA application (plastic material used to handle BDNF was blocked with 0.1 g/L bovine serum albumin prior to use). To estimate the apparent inhibition constant for BDNF, data were fitted using least-square routines to the following equation: $I = I_0 + (1 - I_0) * (K_i/([\text{BDNF}] + K_i))$, where I is the normalised current and $1 - I_0$ expresses maximal inhibition. The WCP program, provided by John Dempster (University of Strathclyde, UK), was used for data acquisition and analysis.

Data analysis

Average values are expressed as means ± SE. Statistical significance of results was assessed using two-tailed Student's *t*-test or one-way ANOVA followed by Bonferroni post-tests, as indicated.

Results

Differential modulation of cell viability by the BDNF–NO pathway in cortical and hippocampal cultures

Hippocampal and cortical primary cultures were established under similar conditions and the proportions of cells expressing the neuronal marker protein microtubule associated protein-2 (MAP-2) and the astroglial marker protein glial fibrillary acidic protein were assessed in both cultures. Cell nuclei were stained with 4′,6′-diamidino-2-phenylindole. At 14 DIV, 71 ± 3.2% of the cells were immunopositive for MAP-2, consistent with 23.2 ± 2.3% of cells expressing glial fibrillary acidic protein. Both culture types contained a similar number of GABAergic cells: on immunoblots for calretinin, a calcium-binding protein present in a subpopulation of GABAergic cells, no difference was found in cell homogenates among cultures (not shown). Accordingly, the fraction of GAD65-positive cells was 4.4 ± 0.3% in hippocampal cultures and 6.4 ± 1% in cortical cultures. To test whether chronic overstimulation was able to induce an adaptive response, 10- to 12-day-old sister cultures (10–12 DIV) were incubated for 2 days with either 10 μM BIC or VEH (until 12–14 DIV). Then, they were exposed acutely for 1 h to 30 μM NMDA and cell viability was measured. To isolate NMDA-R-dependent Ca^{2+} influx and cell death from other pathways, α-amino-3-hydroxy-5-methylisoxazole-4-propionate and kainate receptors, and Ca^{2+} and Na^{+} voltage-sensitive channels were blocked. Similar to previous reports, 30 μM NMDA evoked reproducible Ca^{2+} transients that could be blocked by 10 μM APV or 10 μM MK801 (data not shown; Sattler *et al.* 1999). In addition, this NMDA concentration induced near-maximal cell death in hippocampal cultures, with no effect on cortical cultures (Kambe *et al.* 2008) and therefore represented a useful concentration to study potentially neuroprotective signalling pathways in cortical cells. At 12–14 DIV, both types of cell cultures had achieved similar maturity and expressed high levels of the synaptic markers synaptophysin and PSD-95 when compared to 1 DIV (not shown).

Using the trypan-blue exclusion test, we found that NMDA reduced cell viability in VEH cortical and hippocampal cultures to 72.9 ± 4.7% and 33 ± 3.6% of control values (i.e. without NMDA), respectively ($p < 0.05$ and $p < 0.01$) (Fig. 1a). Following BIC treatment, NMDA application reduced cortical and hippocampal cell viability to 71.2 ± 2.8% and 24.6 ± 2.2% of control, respectively ($p < 0.05$ and $p < 0.001$). Thus, elevated network activity induced by BIC during 2 days decreased the viability of hippocampal, but not cortical, cells ($p < 0.05$ for BIC vs. VEH). To test whether BDNF was involved in this response, cultures were incubated during the 1-h excitotoxic challenge either with BDNF or with the BDNF scavenger TrkB-Fc. The addition of BDNF increased cortical cell viability to 95.8 ± 12.8% and 95.6 ± 5.3% in VEH and BIC cultures, respectively, values close to that observed in absence of NMDA (100%). Consistently, cortical viability decreased in the absence of endogenous BDNF (i.e. plus TrkB-Fc) in BIC

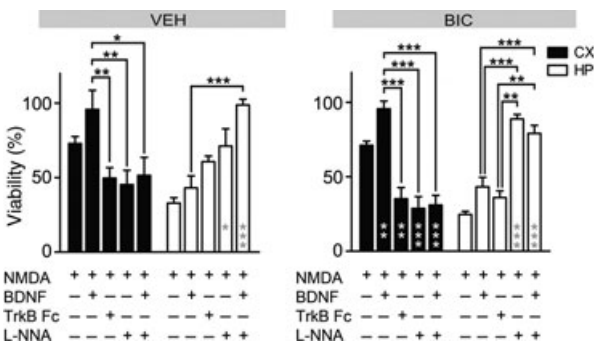

Fig. 1 BDNF and NO are necessary for cell survival in cortical but not hippocampal primary cultures. These opposing effects are augmented following treatment with bicuculline (BIC). Cortical and hippocampal neuronal cultures were incubated for 2 days with vehicle (VEH, left panel) or 10 μM BIC (right panel). Then, 30 μM NMDA in presence of 10 μM CNQX, 2 μM nimodipine, 1 μM tetrodotoxin was added for 1 h. The modulation of excitotoxicity by NOS or BDNF was evaluated by addition of 2 μM *N*[2]-nitro-L-arginine (L-NNA), 0.2 μg/mL receptor bodies (TrkB-Fc, a BDNF scavenger) or 200 ng/mL BDNF. Cell death was determined 24 h later by counting the number of stained cells using the trypan blue exclusion test. Cell viability in the absence of NMDA was quantified in each experimental series and considered as 100%. For each condition, $n = 5–7$ culture dishes obtained at different dates were analyzed. Statistical significance was assessed in a one-way ANOVA followed by Bonferroni *post-hoc* tests. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Symbols within bars indicate significance compared to viability following the NMDA challenge (first bar, with exception to TrkB-Fc, that was compared to NMDA plus IgG-Fc). Symbols on brackets indicate significance between indicated experimental conditions. Black bars: CX, cortical cultures; white bars: HP, hippocampal cultures.

cultures compared to its control with IgG-Fc (35.3 ± 7.4% vs. 70.8 ± 3.2%; $p < 0.001$) but not in VEH cultures (49.7 ± 7% vs. 73.6 ± 2.6%, n.s.). In addition, the viability of BIC-treated cortical cultures decreased in the presence of the NOS inhibitor L-NNA. The requirement of NOS activity for cell survival was not eliminated by the addition of exogenous BDNF, suggesting that BDNF acted upstream of NOS. Contrasting results were observed in hippocampal cell cultures, in which neither the presence of exogenous BDNF nor its absence had any effect on cell survival, while the inhibition of NO production was neuroprotective. These marked differences in cell viability under the different experimental conditions were evident in the presence of NMDA, but not in the corresponding control situations, i.e., without the excitotoxic NMDA challenge (data not shown). These results suggested that NO, when synthesised in response to BDNF, was able to counteract an excitotoxic challenge providing neuroprotection.

The primary downstream cellular actions of NO are either activation of soluble GC and protein kinase G (PKG) (Garthwaite 2008) or protein modification by cysteine *S*-nitrosylation (Lipton *et al.* 2002). To evaluate the involvement of the GC-PKG pathway in cell survival, cultures were incubated in the presence of ODQ (a GC inhibitor) or KT5823 (a PKG inhibitor) (Fig. 2). Both inhibitors were able to prevent cell death in hippocampal cells but not in cortical cells that display a reduced mortality to start with. This reveals an additional difference between the two primary cultures.

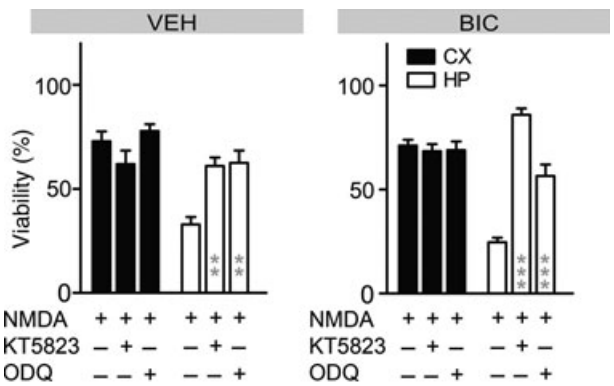

Fig. 2 The guanylyl cyclase (GC)/PKG pathway regulates excitotoxic cell death in hippocampal but not in cortical neurons. Viability following the NMDA challenge was assessed in cortical and hippocampal cells in the presence of ODQ, a GC inhibitor or KT5823, a PKG inhibitor. In cortical cells, the inhibitors did not modify viability whereas in hippocampal cells, the inhibitors were able to revert cell death. Statistical significance as in Fig. 1. $^{**}p < 0.01$; $^{***}p < 0.001$, $n = 5$–7 for each experimental condition. Symbols within bars indicate significance compared to the first bar (NMDA challenge without any other drug). Black bars: CX, cortical cultures; white bars: HP, hippocampal cultures.

NO measurements in cortical and hippocampal cultures

To directly evaluate whether NO can be produced independently in response to BDNF or NMDA, cells were loaded with the NO-sensitive dye DAF-fm (Fig. 3). Following 18 min of basal fluorescence measurement, 200 ng/mL BDNF was added to the culture dish (Fig. 3a). It has been reported that homeostatic regulation can occur through local mechanisms in neuronal subcompartments, for example, in

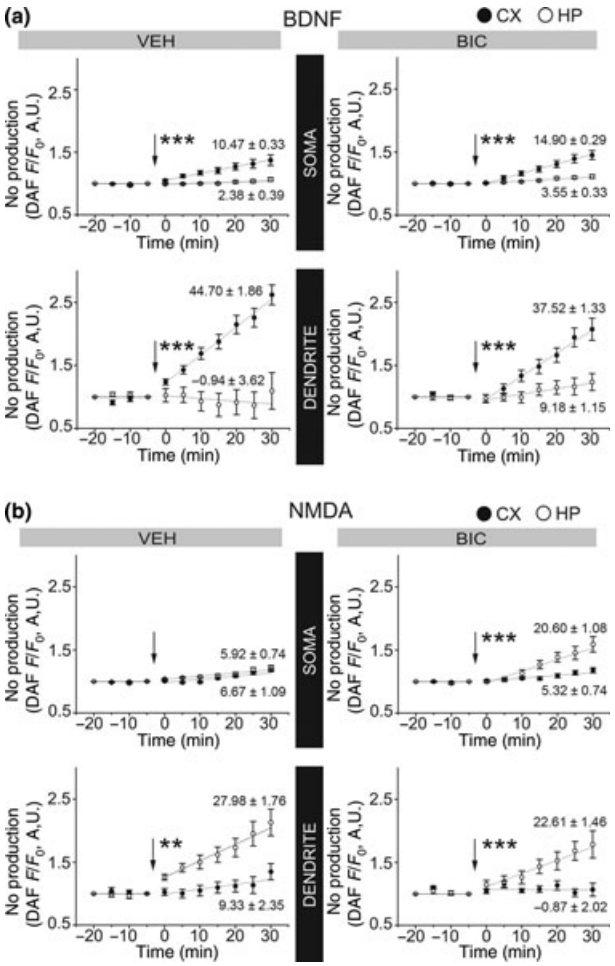

Fig. 3 NO production in cortical and hippocampal cultures in response to BDNF and NMDA treatment. (a) BDNF enhances NO production in cortical but not hippocampal neurons. NO production was measured following addition of 200 ng/mL BDNF (indicated by the arrow) in soma (upper panels) and a 20-μm stretch of dendrite (lower panels) in VEH- (left) or BIC-treated (right) cells. (b) NMDA enhances NO production mainly in hippocampal neurons. NO production was measured following addition of 30 μM NMDA (arrow) in soma (upper panels) and a 20-μm stretch of dendrite (lower panels) in VEH- (left) or BIC-treated (right) cells. Slopes of linear regressions following correction for the baseline negative slope are indicated. Black symbols: cortical cultures; white symbols: hippocampal cultures. At least five neurons were analysed from $n = 5$ to 7 different culture dishes. Slopes ± SEM of hippocampal vs. cortical cultures were compared by two-tailed *t*-tests and significance indicated in each panel. $^{**}p < 0.01$; $^{***}p < 0.001$.

dendrites (Yu and Goda 2009). Therefore, F/F_0 was measured in both soma (upper panel) as well as in dendrites (lower panel). BDNF induced NO synthesis both in the soma and dendrites of cortical cells that was always higher in cortical than in hippocampal cells. BDNF-dependent NO synthesis was completely abolished in the presence of the Trk receptor inhibitor K252a (Figure S2). In summary, cortical cells were able to synthesise NO in response to BDNF-TrkB, whereas hippocampal cells produced only low levels with the exception of VEH dendrites, in which no NO increases could be detected.

Because coupling of NMDA-Rs to NOS stimulation is one of the best-characterised pathways leading to NO synthesis (Aarts *et al.* 2002), we also measured NO increases following stimulation with NMDA in both cultures (Fig. 3b). NO was synthesised with significantly higher rates in hippocampal cells with the sole exception of the soma in VEH-treated cells, in which it was similar among culture types. In addition, no NO was produced in cortical dendrites after BIC, suggesting that homeostatic mechanisms induced by BIC are able to reduce harmful NMDA-induced NO production.

Assessment of NMDA-R function

The results suggested that in cortical cultures, protein *S*-nitrosylation could be involved in neuroprotection and that a target for such a modification could be the NMDA-R itself. To test this hypothesis, NMDA-induced calcium responses were recorded in BIC cultures by loading cells with the Ca^{2+}-sensitive dye Fura-2-AM (Fig. 4). NMDA induced weaker changes in $[Ca^{2+}]_i$ levels in the soma and dendrites of cortical cells ($p < 0.01$ and $p < 0.05$, respectively). As expected, NOS inhibition by L-NNA and the absence of BDNF-induced significant increases in $[Ca^{2+}]_i$ in cortical cells, which were suppressed by the addition of the NO donor, SNAP. In contrast, in hippocampal neurons, no differences in NMDA-R function were observed following L-NNA treatment, whereas the absence of BDNF reduced NMDA-R activity in the somatic region. Taken together, these results indicated that in hippocampal primary neurons, NO does not contribute to NMDA-R down-regulation.

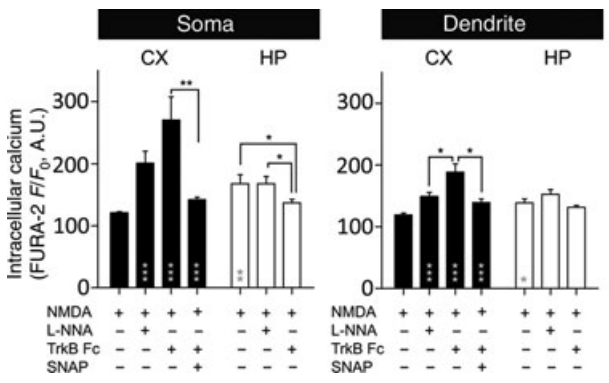

Fig. 4 The absence of BNDF or suppression of NO production augments NMDA-dependent $[Ca^{2+}]_i$ increases in cortical cells. Intracellular Ca^{2+} concentrations were measured under different experimental conditions in soma (left panel) and dendrites (right panel) of BIC-treated cultures using Fura-2. The fluorescence ratio (F340/F380) was determined before and after the application of NMDA, and the Ca^{2+} signal was integrated over a 20-min measurement period with 5-s intervals. Values of columns represent the mean ± SEM obtained from $n = 5$ different culture dishes (at least 5 neurons/dish). Statistical significance indicated within bars resulted from comparison with Ca^{2+} increase obtained following addition of NMDA to cortical cells (first bar). Black bars: CX, cortical cultures; white bars: HP, hippocampal cultures. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by two-tailed *t*-tests.

To check whether these opposing effects are because of differential levels of the implicated proteins, BDNF levels were measured in the culture media (Appendix S1 and Figure S3a). Interestingly, BDNF concentrations were always higher in hippocampal culture media. However, when the TrkB content in cell homogenates was detected by western blots, it was found to be 4.3 ± 0.8 (VEH) and 3.5 ± 0.02 (BIC) times higher in cortical cells when compared to that in hippocampal cells. (Appendix S1 and Figure S3b). Expression level of other involved proteins, such as nNOS and NR1, was similar in both culture types. Rat cerebrocortical homogenates also contained 1.33 ± 0.02 times higher TrkB levels when compared to hippocampal homogenates (not shown). The increased TrkB content in cortical neurons was confirmed in PSD-enriched fractions obtained from the cell cultures (4.6 ± 1-fold and 3.2 ± 0.6-fold higher in VEH and BIC cortical cultures, respectively). Therefore, the requirement of BDNF addition to cortical cultures to increase cell survival during the NMDA challenge to near control values might rely on lower BDNF release that is not able to maximally activate TrkB, that is highly expressed in these cells.

As NR2B subunits are involved in cell death, we wanted to test whether there is a differential involvement of these subunits in both culture types (Hardingham *et al.* 2002). To this end, 10 μM ifenprodil, a NR2B subunit antagonist, was added during the excitotoxic challenge to measure viability (Figure S4a). Under these conditions, viability returned to near control levels in hippocampal as well as cortical cultures (92.3 ± 1.9% and 95.1 ± 6.3%, respectively). This result is consistent with the idea that NR2B subunits are involved in cell death in both culture types. Ifenprodil was also able to reduce intracellular NMDA-induced Ca^{2+} increases (in the case of hippocampal cells) or NMDA plus L-NNA-induced Ca^{2+} increases (in the case of cortical cells). In this set of experiments (Figure S4b), the differential effect of NO on viability and intracellular Ca^{2+} was confirmed.

NO production and NMDA-R function in cortical and hippocampal subcellular fractions following SE

Taken together, the above results show that in cortical cells, a neuroprotective BDNF-TrkB-NO pathway is able to inhibit

NMDA-R-dependent Ca^{2+} influx. To test whether such a mechanism is present in *ex vivo* brain synapses, synaptosomes and PSDs were isolated by subcellular fractionation from control rats and from rats in which SE had been induced by kainic acid treatment. First, NO production was measured in cortical and hippocampal synaptosomes stimulated either with BDNF or NMDA (Appendix S1 and Figure S5). In cortical synaptosomes, 200 ng/mL BDNF led to NO production after SE but not in control synaptosomes, suggesting that SE induced a reorganisation of the synaptic compartment to establish BDNF-dependent NO signalling. On the other hand, NMDA led to NO production in control cortical synaptosomes, but not after SE. In contrast, BDNF failed to induce NO production in hippocampal synaptosomes. As expected, NMDA induced NO production in control hippocampal synaptosomes.

To test the functional effect of BDNF-dependent NO production on NMDA-R currents in isolated PSDs, we incorporated them into the cell membrane of *Xenopus laevis* oocytes (Sandoval *et al.* 2007; Eusebi *et al.* 2009). This procedure allows the characterisation of ion channels in their physiological cellular context. PSDs were isolated separately from hippocampi and from the cerebral cortices and injected into oocytes. Sequential experiments were performed (horizontal lines, Fig. 5a) in which inward currents were recorded during a 2-min application of 100 μM NMDA under different experimental conditions. To evaluate total current transfer during agonist application, the current integral (*I*) was calculated. When measuring the effect of BDNF on control PSDs, NMDA-R currents were either potentiated (hippocampus, 1.9 ± 0.07-fold over control) or not affected (cortex, 1.1 ± 0.1-fold over control) (Fig. 5a, upper panels).

As shown previously, the net effect of BDNF in forebrain PSDs resulted from interaction with different receptors, leading to TrkB-dependent potentiation and/or $p75^{NTR}$-dependent inhibition (Sandoval *et al.* 2007). The net potentiation in hippocampal PSDs suggested that the TrkB-dependent effect dominated over $p75^{NTR}$-dependent inhibition. Potentiation is probably mediated by phosphorylation of the NMDA-R (Kovalchuk *et al.* 2004), whereas the downstream mechanism involved in $p75^{NTR}$-dependent inhibition is currently unknown. Interestingly, following SE the application of BDNF to cortical PSDs elicited inhibition in 100% of the oocytes (0.64 ± 0.08 of control, Fig. 5a, lower panel). This finding contrasted with hippocampal PSDs, in which potentiation was indistinguishable from the control situation (1.6 ± 0.07-fold).

To test whether NMDA-Rs were functionally inhibited by NO, recordings of cortical PSDs isolated from SE brains were performed in the presence or absence of 1 μM L-NNA (Fig. 5b). In the presence of this inhibitor, BDNF was unable to inhibit the NMDA-R and the current integral values returned to baseline (1.04 ± 0.22 compared to control). This effect was dose-dependent with an apparent inhibition constant of 5.4 nM and a maximal inhibition of ≥ 79% (Fig. 5c). Given that NMDA-R inhibition could depend on the $p75^{NTR}$, we measured both neurotrophin receptor levels in cortical PSDs by immunoblot analysis (Figure S6). Consistent with previous results (Wyneken *et al.* 2001), TrkB increased by 5.8 ± 0.8 and 3.8 ± 0.6-fold in cortical PSDs, when measured 24 and 72 h following SE. In contrast, the $p75^{NTR}$ receptor levels decreased to 0.7 ± 0.08 of control and to almost non-detectable amounts at 72 h.

To further clarify the participation of $p75^{NTR}$ in inhibition, pharmacological strategies were used (Sandoval *et al.* 2007) (Fig. 5d). First, current integral measurements revealed that the pro-NGF, an agonist that interacts with $p75^{NTR}$ but not with TrkB (Nykjaer *et al.* 2005), was not able to inhibit NMDA-induced currents in PSDs obtained 24 h following SE. Similarly, the incubation of PSDs with the anti-$p75^{NTR}$ antibody (REX, kindly donated by Dr. Louis Reichardt) did not reverse BDNF-induced inhibition. In contrast, incubation with a TrkB antibody generated against its extracellular domain (Transduction Biosciences) prevented the inhibitory action of BDNF, suggesting that TrkB, but not $p75^{NTR}$, was involved in NMDA-R inhibition.

To further rule out the participation of $p75^{NTR}$ in total reactive oxygen species (ROS) production (this is, including NO), we measured BDNF and pro-NGF-induced increase in luminescence in cortical synaptosomes. The % of $p75^{NTR}$-dependent (i.e., sensitive the $p75^{NTR}$ block-of-function antibody REX) ROS production was plotted (Appendix S1 and Figure S7). Both BDNF and pro-NGF were able to induce $p75^{NTR}$-dependent ROS production in control synaptosomes. However, no significant amount of ROS was produced under these experimental conditions after SE, ruling out the possibility that NO production following SE was $p75^{NTR}$-dependent. The participation of NO in downstream $p75^{NTR}$ signalling in control synaptosomes and PSDs was not further evaluated. These results reveal that TrkB was involved in NO production following SE, but that this phenomenon is not significant in control synaptosomes, confirming that SE leads to an extensive reorganisation of the post-synaptic compartment (Wyneken *et al.* 2001). In addition to significant changes in the composition of PSDs, we now demonstrated the incorporation of a new TrkB signalling pathway into PSDs following exposure to elevated activity that provided a negative feedback mechanism to control neuronal activity.

Discussion

The main finding of the present study was that a neuroprotective BDNF-TrkB-NO signalling pathway leading to NMDA-R down-regulation was present in cerebrocortical, but not hippocampal cells both in dissociated cultures as

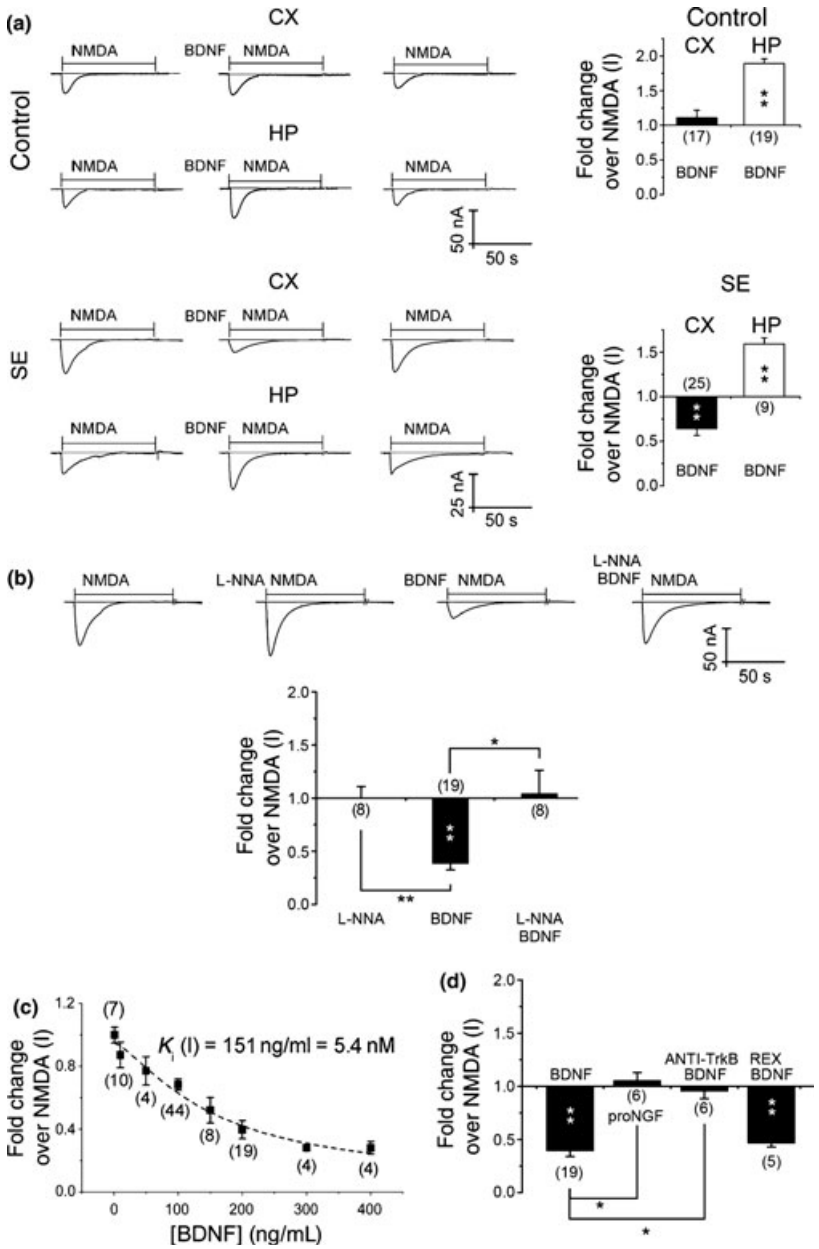

Fig. 5 BDNF-dependent inhibition of NMDA-R currents in cortical, but not hippocampal, post-synaptic densities (PSDs) following status epilepticus (SE) depends on TrkB and NO. Representative recordings of consecutive responses of PSD-injected oocytes to 100 μM NMDA + 5 μM glycine are shown (horizontal lines). To study the modulation of the NMDA-R by BDNF, the neurotrophin was added 1 min prior to the NMDA application, as indicated. Then, BDNF was washed out to test the reversibility of the modulatory effect. The column graphs show the mean change of the current integral ± SEM as compared to the control recording. (a) In control PSDs, BDNF induced no net effect on cortical NMDA-Rs, but potentiated hippocampal NMDA-R currents (upper panel). Following SE, BDNF continued to potentiate hippocampal NMDA-Rs but inhibited cortical NMDA-Rs (lower panel). (b) The NOS inhibitor L-NNA prevented BDNF-dependent NMDA-R inhibition in cortical PSDs obtained following SE. Representative recordings and quantification of results are shown. (c) Dose dependence of NMDA-R inhibition by BDNF in cortical PSDs. The mean fractional NMDA-R current in the presence of increasing concentrations of BDNF was fitted with the following equation: $I = I_0 + (1 - I_0) * (K_i/([BDNF] + K_i))$, where I = normalised current and $1 - I_0$ = maximal fractional inhibition. The maximal % of inhibition was ≥ 79% and the apparent inhibition constant was 5.4 nM. (d) The $p75^{NTR}$ is not involved in NMDA-R inhibition. Quantification of results obtained in electrophysiological recordings after application of the $p75^{NTR}$ agonist pro-NGF (150 ng/mL), an anti-TrkB antibody or the $p75^{NTR}$ antibody REX. Numbers of recorded oocytes are indicated. Data obtained in the same oocyte were compared using paired Student's *t*-tests. Symbols within bars denote statistical significance compared to the control current integral (i.e. NMDA application without any additional drug). $*p < 0.05$; $**p < 0.01$ in two-tailed *t*-tests.

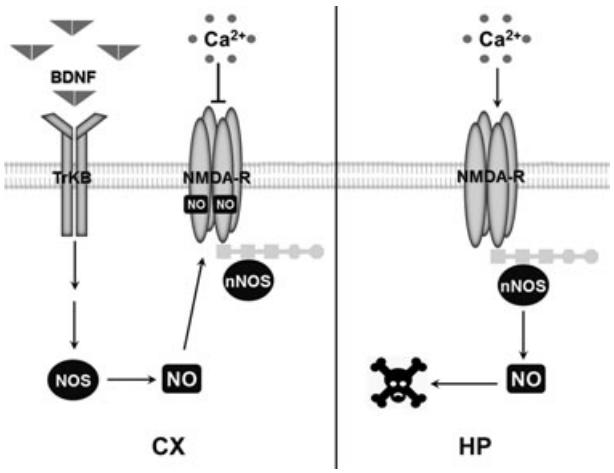

Fig. 6 Schematic representation of the BDNF-TrkB-NO pathway that leads to NMDA-R down-regulation in cortical cells in culture and in cortical synaptic fractions obtained after status epilepticus. Left: BDNF interacts with its receptor TrkB leading efficiently to NO synthesis that in turn is able to down-regulate NMDA-Rs. Glutamate, although present when neuronal activity is high, will not be able to induce Ca^{2+} influx, thus preventing NMDA-R-dependent cell death. In the brain, this homeostatic pathway was induced following SE, a brain insult leading to temporal lobe epilepsy. The NOS isoform leading to BDNF-dependent NO increases is still unknown. Right: no evidence was gained for such a protective pathway in the hippocampus, in which NMDA-dependent NO synthesis was not able to down-regulate NMDA-Rs and in contrast, leads to cell death as already documented in the literature.

well as in brain tissue. In contrast, in hippocampal cells the source of NO was primarily dependent on NMDA-R activation (Fig. 6). Whereas the BDNF-TrkB-NO pathway was detected in dissociated cortical VEH and BIC cells, although augmented in the latter condition, elevated activity was necessary to induce this signalling pathway in cerebrocortical synaptic fractions. This may be because of the fact that dissociated neurons are already subjected to oxidative stress, so they may have used homeostatic adaptations to survive to this condition (Erecinska and Silver 2001). The fact that the expression of the BDNF-TrkB-NO pathway was observed both in dissociated cultures that do not retain network architecture, and in brain subcellular fractions, indicates that it depends on inherent properties of each cell type and not on a differential network connectivity (Pozo and Goda 2010). This pathway contributes significantly to the selective resistance of dissociated cerebrocortical neurons to an excitotoxic challenge. It is plausible that the low physiological O_2 concentration in the brain, favouring free thiol groups and therefore their nitrosylation (Takahashi *et al.* 2007), allows an efficient negative control following over-excitation. However, whether the BDNF-TrkB pathway leads to such modification in cerebrocortical regions needs to be addressed in animal models in which glutamatergic activity is exacerbated, such as epilepsy or ischemia.

Cell-type specific homeostatic mechanisms

The higher vulnerability of hippocampal vs. cortical neurons in culture is consistent with a previous report (Kambe *et al.* 2008), although the involvement of BDNF-dependent NO production was not tested there. Similar to this study, our results reflect a dominant response of each culture type. We controlled the proportion of astroglia and of GABAergic cells in both culture types. Although our cortical cultures contain a mixture of neurons, some of them originating from cerebrocortical regions that are sensitive to excitotoxic neurodegeneration, such as the entorhinal cortex or certain frontal regions (Wang *et al.* 2009), the contribution of such cells to our measurements are minor. Subregional differences in vulnerability have also been observed in the hippocampus. For example, the hippocampal CA1 neurons are very sensitive to ischemic-hypoxic injuries (Mattson and Kater 1989; Ampuero *et al.* 2007). It is therefore very obvious that independent of their subregion origin, the majority of cells in the two culture systems differentially activated the BDNF-TrkB-NO pathway and this was exactly paralleled in synaptic fractions derived from adult brain hippocampi or cerebral cortices. Therefore, the relevant conclusion is that a specific pathway to generate NO was able to provide homeostatic NMDA-R down-regulation.

NO in neuroprotection vs. neurotoxicity

Nitric oxide induces neuronal death by necrosis, as a consequence of inhibition of mitochondrial respiration by several mechanisms, energy depletion and calcium overload, and apoptosis, as a consequence of caspase activation or cytochrome *c* release from mitochondria. In the presence of oxygen, NO gives rise to a mixture of ROS and nitrogen species (NOS), that cause oxidation of protein cysteine residues, protein nitration and nitrosation, lipid oxidation and DNA damage. One of the best characterized pathways leading to neuronal death is DNA fragmentation by peroxynitrite, thus activation of the DNA repair enzyme poly (ADP-ribose) polymerase-1, depletion of nicotinamide adenine dinucleotide and production of the toxic poly (ADP-ribose) polymer that kills cells through apoptosis inducing factor activation (Vosler *et al.* 2009; Brown 2010). Moreover, *S*-nitrosylation of a growing list of proteins, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), protein-disulphide isomerase, matrix metalloprotease-9 (MMP-9), Parkin, XIAP and cyclooxygenase-2, *N*-ethylmaleimide sensitive factor have been linked to neuronal death (Sen *et al.* 2008; Wang *et al.* 2010; Nakamura and Lipton 2011). In addition, several positive regulators of NMDA-Rs, for example, nitration (Mishra *et al.* 2002; Zanelli *et al.* 2002) and src kinase-mediated tyrosine phosphorylation (Chen *et al.* 2003) promote

pro-death pathways such as p38 MAPK activation (Soriano *et al.* 2008).

Opposing roles of NO have been discussed controversially and cell death-promoting effects have been ascribed to high NO levels (Calabrese *et al.* 2009). In addition to alternative signalling pathways that may control NO synthesis, species and developmental variations or > 20-fold differences in NMDA concentrations (Dawson *et al.* 1996; Blackshaw *et al.* 2003) or even culture conditions (Samdani *et al.* 1997) may account for differences. The excitotoxic condition that was selected here does not rule out that higher NMDA concentrations, or longer incubation periods, will ultimately lead to extensive neuronal death because of the activation of a competing neurotoxic NMDA-nNOS pathway also in cortical cells, thereby disturbing the delicate balance between opposing signalling pathways.

It is conceivable that in addition to NMDA-Rs, NO targets other proteins in cortical cells, such as GAPDH's competitor of Siah Protein Enhances Life (GOSPEL) (Sen *et al.* 2009), histone deacetylase 2 (Riccio *et al.* 2006; Nott *et al.* 2008) and/or caspases (Zhou *et al.* 2005), thus contributing to neuroprotection by *S*-nitrosylation in cortical cells. We propose that BDNF-NO, through this modification, is able to orchestrate a complex 'survival program' that accounts for the high resistance of cortical neurons to elevated activity. To test this hypothesis, the proteome of *S*-nitrosylated proteins following elevated activity should be identified.

The role of NO in epilepsy has also been controversially disputed, most probably because of the use of different animal models and/or brain regions in these studies (Blackshaw *et al.* 2003). Consistent with our proposal, NO promoted the initiation of seizure-like events in hippocampal and entorhinal cortical slices (Kovacs *et al.* 2009) and, following SE, NO contributed to cell death in the CA3 subfield (Chuang *et al.* 2007, 2009). However, consistent with our results, in the rat visual cortex NO negatively modulated cortical synapses, thereby limiting possible excitotoxic cascades (Kara and Friedlander 1998) and serving a potentially neuroprotective function.

Regional differences have been reported concerning the activation and localization of NOS isoforms, however, the complex molecular mechanisms that underlie this differential regulation are still not fully understood. In the cerebral cortex, nNOS is expressed almost exclusively in a subpopulation of GABAergic interneurons that also contain somatostatin or neuropeptide-Y (Chung *et al.* 2004) and in some GABAergic projection neurons (Higo *et al.* 2007). nNOS is present within post-synaptic densities in the cerebral cortex (Valtschanoff and Weinberg 2001) and in pre-synaptic GABAergic terminals that contact dendritic spines receiving also asymmetric (i.e. glutamatergic) inputs, thereby modulating excitatory neurotransmission (Sancesario *et al.* 2000). In the hippocampus, nNOS is present in pyramidal cells throughout all regions of Ammon's horn (Chung *et al.* 2004; Kovacs *et al.* 2009). However, these pyramidal neurons seem to be GABAergic, while the CA1 region is innervated by glutamatergic nNOS-positive neurons localized in the subiculum (Seress *et al.* 2002). In the hippocampus, the co-localization of nNOS with somatostatin is low (Jinno and Kosaka 2004), indicating a differential nature of nNOS-containing neurons in both brain areas.

In addition, nNOS activity can be modulated by diverse interacting proteins, such as CAPON, nitric oxide synthase-interacting protein (NOSIP) or protein inhibitor of nitric oxide synthase (PIN) (Jaffrey and Snyder 1996; Jaffrey *et al.* 2002; Dreyer *et al.* 2004), or by post-translational modifications (Komeima *et al.* 2000). Cell-type specific actions have not yet been addressed.

If we hypothesize that NO synthesized following TrkB, but not NMDA receptor activation, targets NMDA-Rs, several controversies regarding NMDA-R down-regulation can be explained: NMDA-dependent NO synthesis did not down-regulate hippocampal NMDA-Rs (Hopper *et al.* 2004; Keynes *et al.* 2004), however, NO inhibited this receptor in cortical (Takahashi *et al.* 2007) and striatal neurons (Manzoni and Bockaert 1993). The participation of BDNF-TrkB in NO generation was not investigated in these studies.

Relevant cysteine thiol groups that lead to NMDA-R down-regulation are present on NR1 and NR2A subunits, whereby the cysteine residue at position 399 of the NR2A subunit accounts for the predominant effect of *S*-nitrosylation (Lipton *et al.* 2002). NR2A subunits are localized primarily at synapses and promote cell survival whereas NR2B subunits are contained predominantly in extra-synaptic NMDA-Rs and promote cell death (Hardingham *et al.* 2002). Consistent with the concept that NR2B is involved in cell death, we found that the selective pharmacological blockade of NR2B with ifenprodil prevented cell death in both hippocampal and cortical cultures. Different though not mutually exclusive hypotheses may explain how inhibition of NR2A-containing receptors decreased NR2B-dependent cell death. First, the down-regulation of heterotrimeric NMDA-Rs that contain both NR2A and NR2B subunits [about 30% of the population in hippocampal CA1 synapses (Al-Hallaq *et al.* 2007)] may be sufficient to counteract excitotoxicity. Second, *S*-nitrosylation-mediated inhibition of both dimeric and heterotrimeric NR2A-containing receptors may decrease post-synaptic depolarisation to such an extent that NMDA is not able to relieve Mg^{2+} blockade of the receptors, thus preventing activation of solely NR2B-containing receptors. The later scenario is favoured by our experimental setup where depolarisation by other ionic pathways was pharmacologically blocked. Similarly, removal of the final 400 amino acids of the NR2A receptor was able to reduce cell death (Anegawa *et al.* 2000).

It remains unknown why NMDA-dependent NO production is not able to *S*-nitrosylate NR2A subunits under physiologically relevant conditions (Hopper *et al.* 2004; Keynes *et al.*

2004). However, in this study we found that in cortical cells an alternative pathway was able to perform this function.

BDNF/TrkB-dependent NO synthesis

Although TrkB has been shown to be localized to synaptic spines and PSDs by immunoelectron microscopy (Aoki *et al.* 2000), the molecular mechanisms involved in its synaptic targeting remain unknown. We reported a massive association of TrkB to PSDs following SE (Wyneken *et al.* 2001), however, the functional relevance of this finding was not tested. The substantial enrichment of TrkB in cortical versus hippocampal neuronal cultures is consistent with the presence of additional TrkB-dependent mechanisms in this cell type, although the mechanistic coupling of TrkB-dependent NO synthesis to synaptic NMDA-R down-regulation deserves further investigation.

BDNF in epileptogenesis and in homeostatic signalling

Although BDNF is a key factor promoting cell growth, survival and synaptic plasticity (Huang and Reichardt 2003), it is also known to enhance excitability in the hippocampus under pro-epileptogenic conditions, thereby promoting temporal lobe epileptogenesis (Simonato *et al.* 2006). However, in cortical cells, BDNF exerted a protective effect following elevated activity, including homeostatic up-regulation of inhibitory neurotransmission (Kume *et al.* 1997; Peng *et al.* 2010), revealing that BDNF may induce a variety of homeostatic mechanisms following elevated activity.

Using kainate administration to induce activity elevation *in vivo*, we replicated the most relevant findings observed in dissociated cortical cells, i.e. BDNF-dependent NO synthesis and NMDA-R down-regulation, underscoring the relevance of our findings to the *in vivo* context. The possibility of potentiating such endogenous homeostatic mechanisms should be considered in the treatment of diseases in which excitotoxicity is relevant, such as temporal lobe epilepsies, where 30% of the patients are resistant to available pharmacological therapies.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Supplementary Methods.

Figure S1. (a) Instant increase in intracellular DAF-fm fluorescence in neuronal soma after application of the nitric oxide donor SNAP (10 μM). (b) Only neurons with pyramidal morphology were recorded.

Figure S2. NO production in cortical cells following 200 ng/mL BDNF depended on TrkB.

Figure S3. BDNF measurements in culture media and western blots of relevant proteins in homogenates and PSD-enriched fractions derived from cell cultures.

Figure S4. (a) Blockade of NR2B subunits of the NMDA-R prevented cell death in both culture types. (b) Ifenprodil reduced intracellular Ca^{2+} increases in both culture types.

Figure S5. NO is produced in response to BDNF in rat cortical synaptosomes obtained from animals with kainic acid-induced *status epilepticus* (SE).

Figure S6. TrkB levels increase while levels of the pan-neurotrophin receptor $p75^{NTR}$ decrease in cortical PSDs following SE.

Figure S7. BDNF-induced ROS production in control cortical synaptosomes, but not following SE, depends on $p75^{NTR}$.

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