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Research Report

# Neuroprotective effects of serotonin 5-HT$_{1A}$ receptor activation against ischemic cell damage in gerbil hippocampus: Involvement of NMDA receptor NR1 subunit and BDNF

*Pablo Salazar-Colocho*, Joaquín Del Río, Diana Frechilla*

*Division of Neuroscience, CIMA, University of Navarra, Av. Pío XII 55, 31008, Pamplona, Spain*

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ABSTRACT

It is known that the activation of 5-hydroxytryptamine receptor type 1A (5HT$_{1A}$ receptor) may protect against brain damage induced by transient global ischemia. The biochemical mechanisms that underlie this neuroprotective effect remain however to be fully elucidated. Given that serotonergic drugs may regulate *N*-methyl-D-aspartate (NMDA) receptor function, which is implicated in events leading to ischemia-induced neuronal cell death, and also stimulate the expression of brain-derived neurotrophic factor (BDNF), which is down-regulated in cerebral ischemia, we sought to determine the effects of the selective 5-HT1A receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), on the levels of NMDA receptor NR1 subunit and BDNF in gerbil hippocampus after transient global cerebral ischemia. Pretreatment with 8-OH-DPAT (1 mg/kg) prevented the neuronal loss in CA1 subfield 72 h after ischemia and also the dramatic decrease in BDNF immunoreactivity observed in this area at an earlier time. NMDA receptor NR1 levels in whole hippocampus were not affected 24 h after ischemia, but the levels of the subunit phosphorylated at the protein kinase A (PKA) site, pNR1(Ser897), were significantly increased, and this increase was prevented by the same 8-OH-DPAT dose, a probable consequence of the increased phosphatase 1 (PP1) enzyme activity found in ischemic gerbils pretreated with the 5-HT$_{1A}$ receptor agonist. The results indicate that both NR1 subunit phosphorylation and the neurotrophin BDNF account, at least in part, for the neuroprotective effect of 8-OH-DPAT on cell damage induced by global ischemia in the gerbil hippocampus and support the potential interest of 5-HT1A receptor activation in the search for neuroprotective strategies.



## 1. Introduction

Transient ischemia results in a number of neuropathological changes that lead to severe brain damage, including the delayed death of neurons in vulnerable areas, such as CA1 subfield of the hippocampus (Kirino, 1982; Pulsinelli et al., 1982; Uchino et al., 2002). The excessive release of excitatory amino acids, especially glutamate, during ischemia plays a major role in the mediation of ischemic neuronal damage (Rothman and Olney, 1986) by producing a sustained activation of postsynaptic ionotropic glutamate receptors with the consequent rise of intracellular free calcium ($Ca^{2+}$), initiating a cascade of metabolic events leading to cell death (Siesjo, 1988; Choi, 1992).

* *Corresponding author*. Fax: +34 948 194700/15.
E-mail addresses: pscolocho@alumni.unav.es, pabloesalazar@gmail.com (P. Salazar-Colocho).

*Abbreviations:* BrdU, 5′-bromodeoxyuridine; CNS, central nervous system; DG, dentate gyrus; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; PB, phosphate buffer; PBS, phosphate-buffered saline; SGZ, subgranular zone; SVZ, subventricular zone

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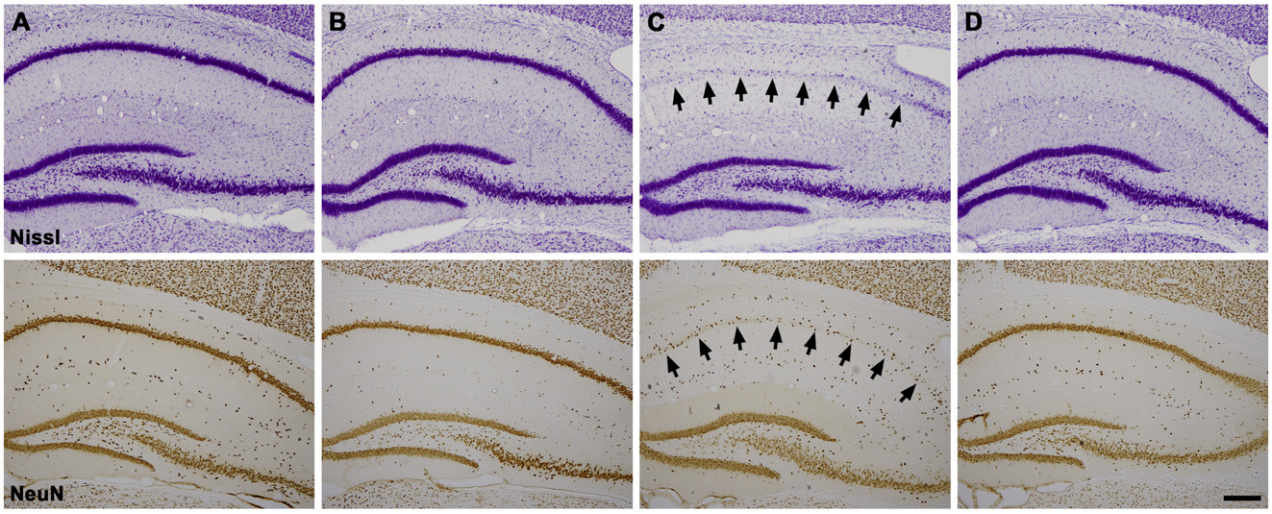


**Fig. 1 – Representative images of hippocampus; upper row: Nissl-stained sections; lower row: NeuN immunohistochemistry. A: sham-control animals, B: 24 h after ischemia, C: 72 h after ischemia and D: 72 h after ischemia +8-OH-DPAT. Note the extensive and localized neuronal loss in CA1 72 h after ischemia (indicated by arrows in C) compared with controls. No pyramidal neuronal death was observed 24 h after ischemic insult in this area (B). The selective 5-$HT_{1A}$ receptor agonist protected CA1 against delayed neuronal death (D). Scale bar=250 μm.**

A major route for $Ca^{2+}$ entry into the postsynaptic neuron is through activation of the *N*-methyl-D-aspartate (NMDA) receptors which are directly implicated in events leading to ischemia-induced delayed neuronal death (Takagi et al., 1997). The NMDA receptor is formed by a combination of two major subunits, NR1 and NR2 (Parsons et al., 1998; Swope et al., 1999). The four types of NR2 subunit do not form functional channels but rather modulate the properties of heteromeric receptors consisting of NR1 plus one or more NR2 subunits (Hollmann and Heinemann, 1994). NMDA receptors are regulated through phosphorylation by the kinases PKC and PKA (Leonard and Hell, 1997; Tingley et al., 1997). In particular, NR1 subunits are phosphorylated by cAMP-dependent protein kinaseA (PKA) on Ser897 (Tingley et al., 1997). PKA activation may increase $Ca^{2+}$ influx through NMDA receptors (Raman et al., 1996), so the enhanced phosphorylation of NR1 at Ser897 may contribute to alterations in NMDA receptor function in the postischemic brain which may be related to pathogenic events leading to

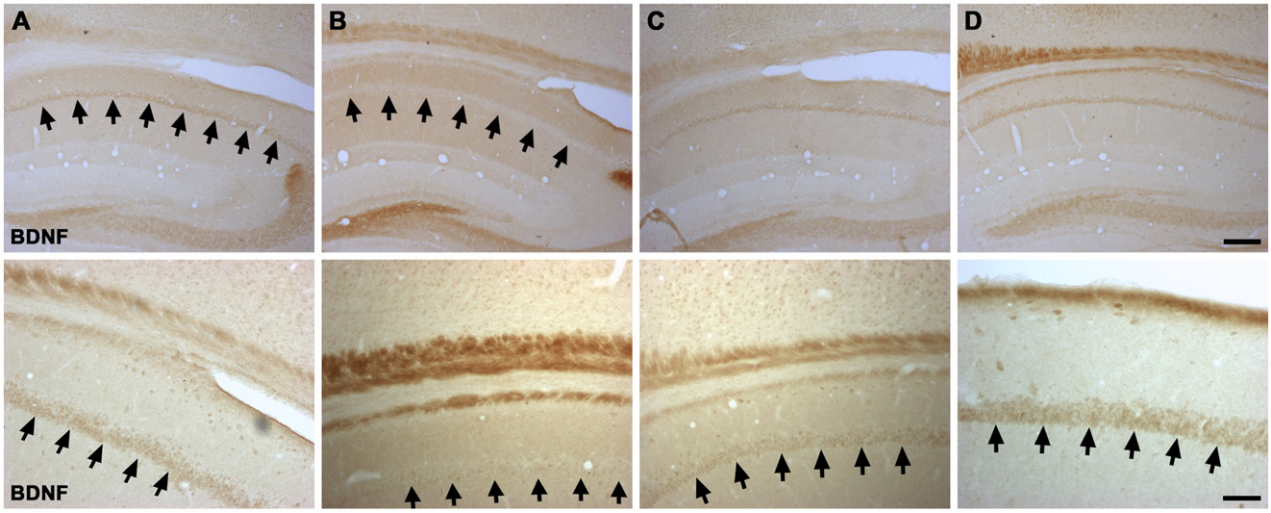


**Fig. 2 – Representative BDNF immunostained sections of hippocampus at different magnification, upper row: 4×; lower row: 20×. A: sham-control animals, B: 24 h after ischemia, C: 24 h after ischemia +8-OH-DPAT and D: non ischemic 8-OH-DPAT treated animals. CA1 pyramidal layer of hippocampus is indicated by arrows. Note the marked and localized loss of BDNF immunoreactivity in CA1 pyramidal neurons 24 h after ischemia (B) compared with control; these cells die 72 h after ischemic insult, but are Nissl and NeuN positive 24 h after ischemia (Fig. 1). Also note that 8-OH-DPAT prevents the BDNF loss at this time (C). No changes were observed in non ischemic 8-OH-DPAT treated animals (D) compared with controls. Scale bar upper row=250 μm. Scale bar lower row=50 μm.**

neuronal death (Cheung et al., 2001). Transient forebrain ischemia also induces the expression of serine/threonine protein phosphatase 1 (PP1) mRNA in the vulnerable regions of the gerbil brain 24 h after bilateral carotid artery occlusion, as a possible compensatory reaction against hyperphosphorylation (Horiguchi et al., 2002). The selective 5-$HT_{1A}$ receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) and also other 5-$HT_{1A}$ receptor agonists, such as buspirone and urapidil, inhibit NMDA receptor-mediated ionic currents (Yuen et al., 2005), attenuate excitotoxicity (Madhavan et al., 2003) and show neuroprotective properties in models of global and focal cerebral ischemia in mice, rats and gerbils (Bielenberg and Burkhardt, 1990; Prehn et al., 1991; Peruche et al., 1994; Piera et al., 1995; Torup et al., 2000). Moreover, activation of 5-$HT_{1A}$ receptors by 8-OH-DPAT reduces PKA and increases PP1 activity in the hippocampus (Moyano et al., 2004) while, conversely, the selective 5-$HT_{1A}$ receptor antagonist WAY-100635 increases PKA and reduces PP1 activity in this brain region (Schiapparelli et al., 2005).

The neurotrophin brain-derived neurotrophic factor (BDNF) is abundant in the rodent hippocampus (Wetmore et al., 1990; Dugich-Djordjevic et al., 1995) and it is generally accepted that this growth factor, like others, confers protection mainly by interfering with apoptotic pathways (Leker and Shohami, 2002). A marked reduction in BDNF expression in CA1 area of the hippocampus has been found 24 h after transient global cerebral ischemia (Ferrer et al., 1997; Ferrer et al., 1998). Different neurotrophic factors may prevent cell damage following transient brain ischemia or injury (Wu, 2005) and, in particular, much attention has been paid to the neuroprotective effects of BDNF in several brain ischemia models (Beck et al., 1994; Tsukahara et al., 1994; Schabitz et al., 1997; Yamashita et al., 1997; Schabitz et al., 2000). Both BDNF and serotonin (5-hydroxytryptamine, 5-HT) are known to regulate synaptic plasticity and neuronal survival in the adult brain. These two signals co-regulate one another so 5-HT stimulates the expression of BDNF, and BDNF enhances the growth and survival of 5-HT neurons (Mattson et al., 2004). In particular, both 5-HT and the selective 5$HT_{1A}$ receptor agonist 8-OH-DPAT are able to produce an increased expression of BDNF mRNA (Galter and Unsicker, 2000).

Here, we sought to determine the effects of 8-OH-DPAT on the levels of NMDA receptor NR1 subunit and BDNF in the gerbil hippocampus after transient ischemia looking for a correlation with the neuroprotective effect of the 5$HT_{1A}$ receptor agonist.

## 2. Results

### 2.1. *Neuroprotective effect of 8-OH-DPAT in transient global cerebral ischemia*

By using Nissl staining, we initially characterized the pattern of neuronal death in the CA1 area of the hippocampus in this model of cerebral ischemia. No neuronal death was observed 24 h after the arterial occlusion, and neuronal loss was found 72 h after ischemia. Animals treated with 8-OH-DPAT (1 mg/kg) 30 min before arterial occlusion were protected against pyramidal neuronal loss in CA1 (Fig. 1).

### 2.2. *Prevention by 8-OH-DPAT of the reduction in BDNF levels after ischemia*

In order to ensure that CA1 pyramidal neurons of brain sections of gerbils subjected to transient global ischemia were still present 24 h after arterial occlusion, the expression of the phenotypic marker for neurons, NeuN, in the hippocampus of the four animals groups was initially studied, and no differences were observed (Figs. 1A and B).

Inspection of hippocampal sections revealed a marked reduction of BDNF immunoreactivity in the CA1 area 24 h after transient global ischemia (Fig. 2B). 8-OH-DPAT protected against the reduction in BDNF immunoreactivity in gerbils treated with this selective 5-$HT_{1A}$ receptor agonist 30 min before transient ischemia (Fig. 2C). There were no significant differences between the sham-operated and the 8-OH-DPAT-treated groups. BDNF-labelled neurons were present in areas

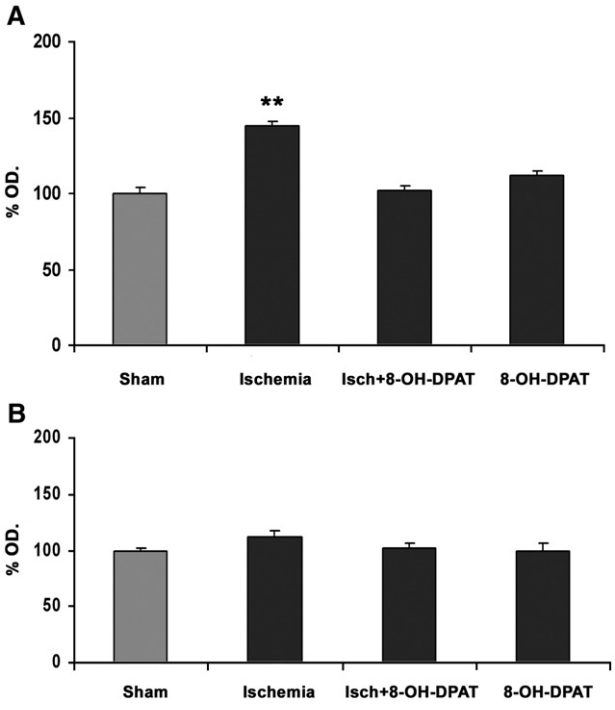


**Fig. 3 – A. Levels of pNR1 (Ser897) in P2 membrane preparations from the hippocampus of gerbils killed 24 h after carotid arterial occlusion or 8-OH-DPAT administration. The experiment was performed in duplicate. Values, means ± SEM of 7 animals or 5–6 animals (all other groups), are expressed as percentage of optical density (OD) values of sham animals. It is shown that 8-OH-DPAT prevents the ischemia-induced increase phosphorylation of NR1 subunit 24 h after the ischemic insult. ** $p < 0.01$ vs. sham controls, (one way ANOVA followed by Scheffé test). B. Total levels of NR1 in P2 membrane preparations from the hippocampus of gerbils killed 24 h after ischemia or 8-OH-DPAT administration. The experiment was performed in duplicate. Values, means ± SEM of 7 animals or 5–6 animals (all other groups), are expressed as percentage of optical density (OD) values of sham animals. No differences were observed (one way ANOVA).**

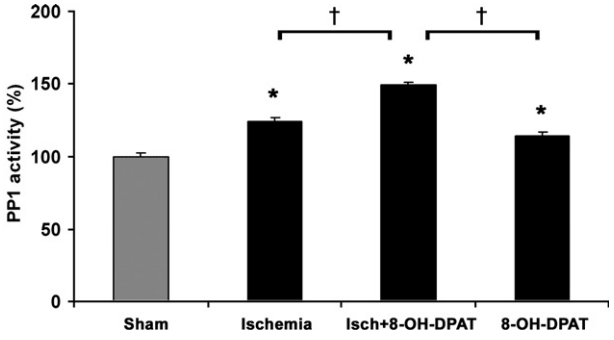


**Fig. 4 – Phosphatase 1 (PP1) activity in tissue lysates from the hippocampus of gerbils killed 24 h after carotid arterial occlusion or 8-OH-DPAT administration. Enzyme activities are expressed as percentage of sham PP1 activity (2.4 ± 0.06 pmol/min/μg). The experiment was performed in duplicate. Values are means ± SEM of 7 animals or 5 animals (all other groups). Ischemia and 8-OH-DPAT increased PP1 activity and this increase was more pronounced in ischemic animals treated with 8-OH-DPAT. *$p < 0.05$ vs. sham controls, †$p < 0.05$ vs. ischemia or 8-OH-DPAT treated groups (one way ANOVA followed by Scheffé test).**

not showing delayed neuronal death 72 h after ischemia, such as the dentate gyrus and cerebral cortex (Fig. 2).

### 2.3. *Reversion by 8-OH-DPAT of the increased phosphorylation of NMDA receptor NR1 subunit following ischemia*

Increased levels of the NR1 subunit phosphorylated at Ser897 were found in gerbil hippocampus 24 h after ischemia. 8-OH-DPAT prevented the ischemia-induced increase in NR1 subunit phosphorylation (Fig. 3A). Total levels of NR1 subunit remained unchanged in the four experimental groups, indicating that the increase in phosphorylated NR1 subunit found in ischemic animals was not a consequence of increased NR1 levels (Fig. 3B).

### 2.4. *Effects of ischemia and 8-OH-DPAT on PP1 enzyme activity*

The method for PP1 enzyme activity assays in tissue lysates was initially validated using the PP1 inhibitor okadaic acid. No differences were observed between sham-operated and control animals. Ischemia and 8-OH-DPAT administration increased PP1 activity; the enzyme activity was higher in ischemic animals pretreated with 8-OH-DPAT (Fig. 4).

## 3. Discussion

Activation of serotonin $5HT_{1A}$ receptors exerts a neuroprotective effect in different animal models of ischemia. Here we show that the neuroprotective effect of the $5HT_{1A}$ agonist, 8-OH-DPAT, on hippocampal cell damage in the gerbil induced by transient global ischemia is correlated with a reduced phosphorylation of the NMDA receptor NR1 subunit along with a prevention of the marked reduction of hippocampal BDNF levels elicited by global ischemia.

Serotonin 5-$HT_{1A}$ receptor activation by 8-OH-DPAT has been associated with neuroprotection against NMDA-induced apoptotic cell death in cell cultures (Madhavan et al., 2003). Other studies have shown that 5-$HT_{1A}$ receptor stimulation protects against ischemic neuronal cell death (Nakata et al., 1997; Harkany et al., 2001). Although the cellular mechanisms underlying the neuroprotective effect of 5-$HT_{1A}$ receptor activation remain unclear, a proposed mechanisms is the hyperpolarization of pyramidal neurons that inhibits the excitotoxicity by glutamate after cerebral ischemia (Torup et al., 2000). Previous studies have shown that 5-$HT_{1A}$ receptor stimulation can modulate NMDA receptor-induced $Ca^{2+}$ influx (Strosznajder et al., 1996; Matsuyama et al., 1997), and other reports have provided evidence for functional interactions between NMDA channels and 5-$HT_{1A}$ receptors (Ross et al., 1992; Becquet et al., 1993), suggesting that 5-$HT_{1A}$ receptors may serve as mediators of autoprotective mechanisms of the brain against the effects of excitatory amino acid overstimulation (Oosterink et al., 1998).

In the present study, we found an increased phosphorylation of the NMDA receptor NR1 subunit at the PKA phosphorylation site (Ser897) in P2 membrane preparations from the hippocampus of gerbils killed 24 h after ischemia, a result in keeping with a previous finding in a rat model of transient global ischemia (Cheung et al., 2001). Increased phosphorylation of NR1 by PKA may be caused by ischemia-induced increases in intracellular $Ca^{2+}$ levels which may activate $Ca^{2+}$-dependent enzymes, among others PKA. A possible activation of this enzyme by an increase in cAMP levels associated to ischemia cannot be ruled out (Blomqvist et al., 1985). Given that enhanced NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) by activation of PKA can increase charge transfer and $Ca^{2+}$ influx through NMDA receptors (Raman et al., 1996), and that intracellular $Ca^{2+}$ overload that results from glutamatergic NMDA receptor-mediated $Ca^{2+}$ influx after cerebral ischemia leads to cellular death (Choi, 1995; Leker and Shohami, 2002), it is possible that the ischemia-induced enhancement in NR1 phosphorylation play an important role in neuronal death after global cerebral ischemia. The 5-$HT_{1A}$ receptors are coupled to Gi/Go proteins to mediate a range of biological effects, especially a negative modulation of adenylate cyclase and cAMP formation, which subsequently reduces the phosphorylation of ion channels and thereby reduces neuronal excitation (Raymond et al., 2001; Carr et al., 2002). It should be noted that 8-OH-DPAT prevented the ischemia-induced increase in NR1 subunit phosphorylation, but total levels of the NR1 subunit remained unchanged, indicating that the increase in phosphorylated NR1 subunit was not a consequence of an increase in total NR1 levels.

On the other hand, transient forebrain ischemia enhanced PP1 mRNA expression in the vulnerable regions of gerbil brain (CA1) 24 h after bilateral carotid artery occlusion as a possible reaction against the hyperphosphorylated state, suggesting that PP1 may play a key role in delayed neuronal death (Horiguchi et al., 2002). In general, a crucial role in cell survival has been associated to protein phosphatases (Nuydens et al., 1998). In the present study, we found, in line with our previous data (Moyano et al., 2004), that 8-OH-DPAT increased PP1

activity in the hippocampus of ischemic animals and such an increase in phosphatase activity may account for the prevention of the ischemia-induced phosphorylation of the NR1 subunit.

In keeping with previous findings (Ferrer et al., 1997; Ferrer et al., 1998), we found that there was a marked reduction in BDNF protein expression in the CA1 subfield of hippocampus 24 h after 5 min of transient global cerebral ischemia. As demonstrated by NeuN-labelling of the same tissue sections, pyramidal neurons were still present in this area 24 h after the ischemic insult, indicating that the decrease in BDNF expression was not a result of cellular death, which was not apparent until 72 h after ischemia. Endogenous BDNF plays an important role in preventing neuronal cell death during development, and promotes cell survival during stressful conditions such as ischemia and trauma in the adult brain (Larsson et al., 1999). It seems consequently that reduction of this neurotrophic factor leads to the appearance of delayed cell death in this area. The prevention in the decrease of the neurotrophin BDNF observed after administration of the selective $5HT_{1A}$ receptor agonist, 8-OH-DPAT, might play a crucial role in the protection against ischemia-induced delayed pyramidal neuronal death in the CA1 subfield of hippocampus showed by this agonist.

It is known that BDNF and other growth factors confer protection against cerebral ischemia mainly by both suppression of apoptotic pathways and activation of anti-apoptotic pathways (Kaplan and Miller, 2000; Leker and Shohami, 2002). Global ischemia induces expression of activated caspase-3 (Chen et al., 1998; Namura et al., 1998), which activates a host of downstream events leading to fragmentation of genomic DNA, a hallmark of apoptotic cell death (Cohen, 1997; Nicholson and Thornberry, 1997). It was found (Madhavan et al., 2003) that NMDA-induced caspase-3 activity and DNA fragmentation were almost completely blocked by 8-OH-DPAT in cell cultures, and these protective effects of 8-OH-DPAT were reversed by pretreatment with the 5-$HT_{1A}$ antagonist *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide (WAY 100635). Serotonin 5-$HT_{1A}$ receptor stimulation could then represent a useful therapeutic approach for modulating apoptotic processes in neurodegenerative processes.

The anti-apoptotic properties of 5-$HT_{1A}$ receptor stimulation against excitotoxic insult as well as the protective effect of 8-OH-DPAT on ischemia-induced hippocampal cell damage found in our study might to be related, among others, to a facilitation of the anti-apoptotic effect of neurotrophin BDNF. In addition to the potential effect on cell survival, BDNF may also have a role in tissue regeneration and it seems that surviving BDNF containing neurons participate in synaptic remodelling of the damaged hippocampus following transient forebrain ischemia in the gerbil (Ferrer et al., 1997). It is known that 5-HT and BDNF often function in a cooperative manner to regulate neuronal plasticity and survival. Activation of 5-HT receptors coupled to cAMP production and CREB activation can induce transcription of the BDNF gene but also, interestingly, it was found that serotonin acting on 5-$HT_{1A}$ autoreceptors increases BDNF expression (Galter and Unsicker, 2000). Conversely, BDNF can stimulate the growth and sprouting of 5-HT neuron axons innervating the cerebral cortex, presumably increasing the number of 5-HT synapses in this brain region (Mattson et al., 2004). Recent research has identified BDNF and serotonin as two prominent signals that act in concert to regulate aspects of neural plasticity in multiple brain regions.

Overall, the present results suggest that the NMDA receptor NR1 subunit and BDNF are implicated in the neuroprotective effects of the selective 5-$HT_{1A}$ receptor agonist, 8-OH-DPAT, against CA1 neurons apoptotic death after transient global cerebral ischemia. 5-$HT_{1A}$ receptors seem to be a promising target for the development of neuroprotective agents designed to interfere with excitotoxic and apoptotic cell death processes in the postischemic brain.

## 4. Experimental procedure

### 4.1. *Animals and experimental design*

Adult male Mongolian gerbils (11–12 weeks of age; Harlan, Barcelona, Spain) weighing 60–65 g were used for these studies and divided into five groups: controls ($n=15$), animals subjected to 5 min of global cerebral ischemia ($n=20$; see below), sham controls ($n=20$), animals subjected to ischemia and treated with 8-OH-DPAT (1 mg/kg sc) 30 min before ischemia ($n=20$) and animals treated only with 8-OH-DPAT ($n=20$). The gerbils were sacrificed 24–72 h later. One third of the animals was used to measure protein levels by Western blotting, another third for enzyme assay activity, and the rest for immunohistochemistry. Procedures involving animals were in accordance with the guidelines established by the normative of the European Community of November 24, 1986 (86/609/EEC).

### 4.2. *Transient global cerebral ischemia*

Gerbils were anesthetized with Equithesin (chloral hydrate 4.26%, sodium pentobarbital 0.96% and magnesium sulfate 2.12%, in aqueous solution with 9.5% ethanol), 3 ml/kg ip (Gaese and Ostwald, 1995, 2001). After a medial neck incision, both common carotid arteries (CCAs) were exposed and occluded with aneurysm clips for 5 min. The clips were then removed to restore cerebral blood flow. The rectal temperature was controlled with a heating blanket to prevent hypothermia. Only animals showing a restless behaviour with continuous circling for about 9 h after ischemia were considered in this study. Sham-operated animals were treated identically, except that the CCAs were not occluded after the neck incision and carotid exposition.

### 4.3. *Fixation and tissue processing*

For immunohistochemistry, gerbils were anaesthetized as above and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed and cryoprotected in 30% sucrose solution in phosphate buffer (PB) overnight. Microtome sections (30-μm-thick) were cut coronally through the entire hippocampus, collected free-floating and stored in 30% ethylene glycol, 30% glycerol, and 0.1 M PB at −20°C until processed for immunostaining. Some sections were processed

for staining with thionin (Nissl staining) for histological assessment of damage. For biochemical studies animals were killed by decapitation, the brains were removed and immediately frozen in isopentane/dry ice.

### 4.4. *Immunohistochemistry*

For BDNF immunostaining free-floating sections (4 per animal) were first washed in three changes of PBS 0.1 M (pH 7.4) and treated with 0.3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. Sections where then washed in 0.05 M Tris-buffered saline (TBS) containing 0.3% Triton X-100 at pH 8.0 (TBS-Tx) and preincubated for 1 h in 5% normal goat serum in TBS-Tx at room temperature. Afterwards, the sections were incubated for 60 h at 4 °C with the primary antibody, rabbit polyclonal anti-Brain Derived Neurotrophic Factor (1:1500; Chemicon), in the same preincubation solution and then washed three times for 10 min each in TBS-Tx and incubated with the secondary antibody, biotinylated goat-anti-rabbit (1:300; Vector Lab.), for 2 h. Sections were again rinsed in TBS-Tx three times and the subsequent visualization procedure was performed by the avidin-biotin complex (ABC) method (Vectastain ABC Kit; Vector Lab.) with diaminobenzidine (DAB) as chromogen (peroxidase substrate kit, Vector Lab.). Sections were mounted on super frost plus slides, air dried for 24 h, dehydrated, cleared with xylene and cover-slipped with DPX mounting medium.

For NeuN immunostaining, free-floating sections (2 per animal) were rinsed in several changes of PBS 0.1 M (pH 7.4), treated with 1% hydrogen peroxide in PBS during 15 min, preincubated for 2 h in blocking solution (PBS 0.1 M, pH 7.4, containing 0.3% Triton X-100, 2% normal goat serum and 0.1% BSA) and then incubated overnight at 4 °C with the primary antibody, mouse monoclonal anti-NeuN (1:200; Chemicon) in blocking solution. The sections were then washed in PBS three times and incubated with the secondary antibody, biotinylated goat-anti-mouse (1:200; Vector Lab.), for 2 h at room temperature. The subsequent visualization and mounting procedures were performed as above.

To ensure comparable immunostaining, sections were processed together at the same time under the same conditions. For the assessment of non-specific immunostaining, sections from each experimental group were incubated without the primary antibodies. In this case no immunostaining was observed.

### 4.5. *Production of protein extracts*

To obtain P2 membrane proteins, the hippocampus was homogenized in ice-cold 10 mM Tris–HCl (pH 7.4)/5 mM EDTA buffer containing 320 mM sucrose, protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 5 μg/mL aprotinin, 5 μg/mL leupeptin) and phosphatase inhibitors (0.1 mM $Na_3$ $VO_4$, 1 mM NaF) and centrifuged at 700 g for 10 min. The supernatant was centrifuged again at 37000 g for 40 min at 4 °C and the pellet (P2) was resuspended in 10 mM Tris–HCl (pH 7.4) in the presence of the indicated enzyme inhibitors (Dunah et al., 2000). The protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard and the aliquots were frozen at −80 °C. For Western blot analysis, aliquots of the P2 membrane preparation were solubilized in denaturing conditions by adding 0.1 volumes of 20% sodium dodecyl sulphate (SDS) containing 50% β-mercaptoethanol and boiled for 5 min. The denatured preparations were diluted 20-fold in 50 mM Tris–HCl (pH 7.4)/0.1% Triton X-100 buffer in the presence of protease and phosphatase inhibitors and centrifuged at 37000 g for 10 min at 4 °C. The supernatant was used for immunoblotting.

### 4.6. *Western blotting*

Solubilized membrane enriched proteins were separated onto 10% SDS-polyacrylamide gels. Samples were diluted in an equal volume of electrophoresis buffer and boiled for 5 min. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using a trans-Blot® SD semidry (Bio-Rad) system for 30 min at 12 V. The membranes were blocked with 5% milk, 0.05% Tween-20 in PBS followed by overnight incubation with the primary antibodies, rabbit anti-pNR1 (Ser897) and rabbit anti-NR1 (both for Upstate Biotechnology, dilution 1:500), in the corresponding buffer. Membranes were washed three times in PBS/Tween-20 at room temperature, and horseradish peroxidase-conjugated anti-rabbit antibody (Dako, dilution 1:1500) was added and incubated for 60 min. Following two washes in PBS/Tween-20 and one in PBS alone, immunolabelled protein bands were detected using an enhanced chemiluminiscence system (ECL Amersham Biosciences), following an autoradiographic exposure to Hyper-film™ ECL (Amersham Biosciences). The quantification of signals was determined by densitometry using the program ImageMaster I-D (Pharmacia).

### 4.7. *Phosphatase 1 assay*

PP1 activity was measured in tissue lysates using the 'Protein Serine/Threonine Phosphatase (PSP) Assay Kit' (BioLabs), which quantifies the release of inorganic phosphate from a labelled protein. The substrate used was myelin basic protein, labelled with [$\gamma$-$^{32}$P]ATP following the kit recommendations. Ten μg of tissue lysate, prepared without phosphatase inhibitors, were added to 30 μl of assay buffer containing 2 nm okadaic acid and 50 μm FK-506 as PP2A and calcineurin inhibitors, respectively. After a preincubation for 5 min at 30 °C, the reaction was started by adding 10 μl of substrate and incubated for 10 min at 30 °C. The reaction was terminated by adding 200 μl of cold 20% trichloroacetic acid and, after centrifugation at 12000 g, radioactivity was counted in the supernatant using the BCS Scintillation Cocktail (Amersham Biosciences). Activity of PP1 was expressed as pmol of phosphate released from labelled myelin basic protein/min/μg protein (Schiapparelli et al., 2005).

### 4.8. *Statistical analysis*

Results, reported as means ± SEM, were analyzed using one way ANOVA, and post hoc comparisons were made using the Scheffé test.

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## REFERENCES

Beck, T., Lindholm, D., Castren, E., Wree, A., 1994. Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus. J. Cereb. Blood Flow Metab. 14, 689–692.

Becquet, D., Hery, M., Deprez, P., Faudon, M., Fache, M.P., Giraud, P., Hery, F., 1993. N-methyl-D-aspartic acid/glycine interactions on the control of 5-hydroxytryptamine release in raphe primary cultures. J. Neurochem. 61, 1692–1697.

Bielenberg, G.W., Burkhardt, M., 1990. 5-hydroxytryptamine1A agonists. A new therapeutic principle for stroke treatment. Stroke 21, IV161–IV163.

Blomqvist, P., Lindvall, O., Stenevi, U., Wieloch, T., 1985. Cyclic AMP concentrations in rat neocortex and hippocampus during and following incomplete ischemia: effects of central noradrenergic neurons, prostaglandins, and adenosine. J. Neurochem. 44, 1345–1353.

Carr, D.B., Cooper, D.C., Ulrich, S.L., Spruston, N., Surmeier, D.J., 2002. Serotonin receptor activation inhibits sodium current and dendritic excitability in prefrontal cortex via a protein kinase C-dependent mechanism. J. Neurosci. 22, 6846–6855.

Cohen, G.M., 1997. Caspases: the executioners of apoptosis. Biochem. J. 326 (Pt 1), 1–16.

Chen, J., Nagayama, T., Jin, K., Stetler, R.A., Zhu, R.L., Graham, S.H., Simon, R.P., 1998. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J. Neurosci. 18, 4914–4928.

Cheung, H.H., Teves, L., Wallace, M.C., Gurd, J.W., 2001. Increased phosphorylation of the NR1 subunit of the NMDA receptor following cerebral ischemia. J. Neurochem. 78, 1179–1182.

Choi, D.W., 1995. Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci. 18, 58–60.

Choi, D.W., 1992. Excitotoxic cell death. J. Neurobiol. 23, 1261–1276.

Dugich-Djordjevic, M.M., Peterson, C., Isono, F., Ohsawa, F., Widmer, H.R., Denton, T.L., Bennett, G.L., Hefti, F., 1995. Immunohistochemical visualization of brain-derived neurotrophic factor in the rat brain. Eur. J. Neurosci. 7, 1831–1839.

Dunah, A.W., Wang, Y., Yasuda, R.P., Kameyama, K., Huganir, R.L., Wolfe, B.B., Standaert, D.G., 2000. Alterations in subunit expression, composition, and phosphorylation of striatal N-methyl-D-aspartate glutamate receptors in a rat 6-hydroxydopamine model of Parkinson's disease. Mol. Pharmacol. 57, 342–352.

Ferrer, I., Lopez, E., Pozas, E., Ballabriga, J., Marti, E., 1998. Multiple neurotrophic signals converge in surviving CA1 neurons of the gerbil hippocampus following transient forebrain ischemia. J. Comp. Neurol. 394, 416–430.

Ferrer, I., Ballabriga, J., Marti, E., Pozas, E., Planas, A.M., Blasi, J., 1997. BDNF and TrkB co-localize in CA1 neurons resistant to transient forebrain ischemia in the adult gerbil. J. Neuropathol. Exp. Neurol. 56, 790–797.

Gaese, B.H., Ostwald, J., 1995. Temporal coding of amplitude and frequency modulation in the rat auditory cortex. Eur. J. Neurosci. 7, 438–450.

Gaese, B.H., Ostwald, J., 2001. Anesthesia changes frequency tuning of neurons in the rat primary auditory cortex. J. Neurophysiol. 86, 1062–1066.

Galter, D., Unsicker, K., 2000. Sequential activation of the 5-HT1(A) serotonin receptor and TrkB induces the serotonergic neuronal phenotype. Mol. Cell Neurosci. 15, 446–455.

Harkany, T., Mulder, J., Horvath, K.M., Keijser, J., van der Meeberg, E.K., Nyakas, C., Luiten, P.G., 2001. Oral post-lesion administration of 5-HT(1A) receptor agonist repinotan hydrochloride (BAY×3702) attenuates NMDA-induced delayed neuronal death in rat magnocellular nucleus basalis. Neuroscience 108, 629–642.

Hollmann, M., Heinemann, S., 1994. Cloned glutamate receptors. Annu. Rev. Neurosci. 17, 31–108.

Horiguchi, T., Shima, H., Suga, S., Ogino, M., Shimizu, K., Toya, S., Nagao, M., Kawase, T., 2002. Transient forebrain ischemia induces expression of serine/threonine protein phosphatase 1 mRNA in the vulnerable regions of gerbil brain. Neurosci. Lett. 325, 115–118.

Kaplan, D.R., Miller, F.D., 2000. Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. 10, 381–391.

Kirino, T., 1982. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. 239, 57–69.

Larsson, E., Nanobashvili, A., Kokaia, Z., Lindvall, O., 1999. Evidence for neuroprotective effects of endogenous brain-derived neurotrophic factor after global forebrain ischemia in rats. J. Cereb. Blood Flow Metab. 19, 1220–1228.

Leker, R.R., Shohami, E., 2002. Cerebral ischemia and trauma-different etiologies yet similar mechanisms: neuroprotective opportunities. Brain Res. Brain Res. Rev. 39, 55–73.

Leonard, A.S., Hell, J.W., 1997. Cyclic AMP-dependent protein kinase and protein kinase C phosphorylate N-methyl-D-aspartate receptors at different sites. J. Biol. Chem. 272, 12107–12115.

Madhavan, L., Freed, W.J., Anantharam, V., Kanthasamy, A.G., 2003. 5-hydroxytryptamine 1A receptor activation protects against N-methyl-D-aspartate-induced apoptotic cell death in striatal and mesencephalic cultures. J. Pharmacol. Exp. Ther. 304, 913–923.

Matsuyama, S., Nei, K., Tanaka, C., 1997. Regulation of GABA release via NMDA and 5-HT1A receptors in guinea pig dentate gyrus. Brain Res. 761, 105–112.

Mattson, M.P., Maudsley, S., Martin, B., 2004. BDNF and 5-HT: a dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. Trends Neurosci. 27, 589–594.

Moyano, S., Del Rio, J., Frechilla, D., 2004. Role of hippocampal CaMKII in serotonin 5-HT(1A) receptor-mediated learning deficit in rats. Neuropsychopharmacology 29, 2216–2224.

Nakata, N., Suda, H., Izumi, J., Tanaka, Y., Ikeda, Y., Kato, H., Itoyama, Y., Kogure, K., 1997. Role of hippocampal serotonergic neurons in ischemic neuronal death. Behav. Brain Res. 83, 217–220.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K.J., Yuan, J., Moskowitz, M.A., 1998. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J. Neurosci. 18, 3659–3668.

Nicholson, D.W., Thornberry, N.A., 1997. Caspases: killer proteases. Trends Biochem. Sci. 22, 299–306.

Nuydens, R., de Jong, M., Van Den Kieboom, G., Heers, C., Dispersyn, G., Cornelissen, F., Nuyens, R., Borgers, M., Geerts, H., 1998. Okadaic acid-induced apoptosis in neuronal cells: evidence for an abortive mitotic attempt. J. Neurochem. 70, 1124–1133.

Oosterink, B.J., Korte, S.M., Nyakas, C., Korf, J., Luiten, P.G., 1998. Neuroprotection against N-methyl-D-aspartate-induced excitotoxicity in rat magnocellular nucleus basalis by the 5-HT1A receptor agonist 8-OH-DPAT. Eur. J. Pharmacol. 358, 147–152.

Parsons, C.G., Danysz, W., Quack, G., 1998. Glutamate in CNS disorders as a target for drug development: an update. Drug News Perspect. 11, 523–569.

Peruche, B., Backhauss, C., Prehn, J.H., Krieglstein, J., 1994. Protective effects of 5-HT1A receptor agonists against neuronal damage demonstrated in vivo and in vitro. J. Neural. Transm. Park Dis. Dement. Sect. 8, 73–83.

Piera, M.J., Beaughard, M., Michelin, M.T., Massingham, R., 1995. Effects of the 5-hydroxytryptamine1A receptor agonists, 8-OH-DPAT, buspirone and flesinoxan, upon brain damage

induced by transient global cerebral ischaemia in gerbils. Arch. Int. Pharmacodyn. Ther. 329, 347–359.
Prehn, J.H., Backhauss, C., Karkoutly, C., Nuglisch, J., Peruche, B., Rossberg, C., Krieglstein, J., 1991. Neuroprotective properties of 5-HT1A receptor agonists in rodent models of focal and global cerebral ischemia. Eur. J. Pharmacol. 203, 213–222.
Pulsinelli, W.A., Brierley, J.B., Plum, F., 1982. Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann. Neurol. 11, 491–498.
Raman, I.M., Tong, G., Jahr, C.E., 1996. Beta-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase. Neuron. 16, 415–421.
Raymond, J.R., Mukhin, Y.V., Gelasco, A., Turner, J., Collinsworth, G., Gettys, T.W., Grewal, J.S., Garnovskaya, M.N., 2001. Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol. Ther. 92, 179–212.
Ross, S.B., Renyi, L., Kelder, D., 1992. N-methyl-D-aspartate receptor antagonists counteract the long lasting 5-HT1A receptor-induced attenuation of postsynaptic responses in the rat in vivo. Naunyn Schmiedebergs Arch. Pharmacol. 346, 138–143.
Rothman, S.M., Olney, J.W., 1986. Glutamate and the pathophysiology of hypoxic–ischemic brain damage. Ann. Neurol. 19, 105–111.
Schabitz, W.R., Sommer, C., Zoder, W., Kiessling, M., Schwaninger, M., Schwab, S., 2000. Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. Stroke 31, 2212–2217.
Schabitz, W.R., Schwab, S., Spranger, M., Hacke, W., 1997. Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. J. Cereb. Blood Flow Metab. 17, 500–506.
Schiapparelli, L., Del Rio, J., Frechilla, D., 2005. Serotonin 5-HT receptor blockade enhances Ca(2+)/calmodulin-dependent protein kinase II function and membrane expression of AMPA receptor subunits in the rat hippocampus: implications for memory formation. J. Neurochem. 94, 884–895.
Siesjo, B.K., 1988. Historical overview. Calcium, ischemia, and death of brain cells. Ann. N. Y. Acad. Sci. 522, 638–661.
Strosznajder, J., Chalimoniuk, M., Samochocki, M., 1996. Activation of serotonergic 5-HT1A receptor reduces Ca(2+)- and glutamatergic receptor-evoked arachidonic acid and No/cGMP release in adult hippocampus. Neurochem. Int. 28, 439–444.
Swope, S.L., Moss, S.J., Raymond, L.A., Huganir, R.L., 1999. Regulation of ligand-gated ion channels by protein phosphorylation. Adv. Second Messenger Phosphoprotein Res. 33, 49–78.
Takagi, N., Shinno, K., Teves, L., Bissoon, N., Wallace, M.C., Gurd, J.W., 1997. Transient ischemia differentially increases tyrosine phosphorylation of NMDA receptor subunits 2A and 2B. J. Neurochem. 69, 1060–1065.
Tingley, W.G., Ehlers, M.D., Kameyama, K., Doherty, C., Ptak, J.B., Riley, C.T., Huganir, R.L., 1997. Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. J. Biol. Chem. 272, 5157–5166.
Torup, L., Moller, A., Sager, T.N., Diemer, N.H., 2000. Neuroprotective effect of 8-OH-DPAT in global cerebral ischemia assessed by stereological cell counting. Eur. J. Pharmacol. 395, 137–141.
Tsukahara, T., Yonekawa, Y., Tanaka, K., Ohara, O., Wantanabe, S., Kimura, T., Nishijima, T., Taniguchi, T., 1994. The role of brain-derived neurotrophic factor in transient forebrain ischemia in the rat brain. Neurosurgery 34, 323–331 discussion 331.
Uchino, H., Minamikawa-Tachino, R., Kristian, T., Perkins, G., Narazaki, M., Siesjo, B.K., Shibasaki, F., 2002. Differential neuroprotection by cyclosporin A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. Neurobiol. Dis. 10, 219–233.
Wetmore, C., Ernfors, P., Persson, H., Olson, L., 1990. Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. Exp. Neurol. 109, 141–152.
Wu, D., 2005. Neuroprotection in experimental stroke with targeted neurotrophins. NeuroRx 2, 120–128.
Yamashita, K., Wiessner, C., Lindholm, D., Thoenen, H., Hossmann, K.A., 1997. Post-occlusion treatment with BDNF reduces infarct size in a model of permanent occlusion of the middle cerebral artery in rat. Metab. Brain Dis. 12, 271–280.
Yuen, E.Y., Jiang, Q., Chen, P., Gu, Z., Feng, J., Yan, Z., 2005. Serotonin 5-HT1A receptors regulate NMDA receptor channels through a microtubule-dependent mechanism. J. Neurosci. 25, 5488–5501.