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Prokineticins are neuroprotective in models of cerebral ischemia and ischemic tolerance *in vitro*

Elisa Landucci,[a] Roberta Lattanzi,[b] Elisabetta Gerace,[a] Tania Scartabelli,[a] Gianfranco Balboni,[c] Lucia Negri,[b] Domenico E. Pellegrini-Giampietro[a]

aDepartment of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Viale G. Pieraccini 6, 50139 Florence, Italy

bDepartment of Human Physiology and Pharmacology "Vittorio Erspamer", University of Rome "La Sapienza", Piazza A. Moro 5, 00185 Rome, Italy

cDepartment of Life and Environmental Sciences, Unit of Pharmaceutical, Pharmacological and Nutraceutical Sciences, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy

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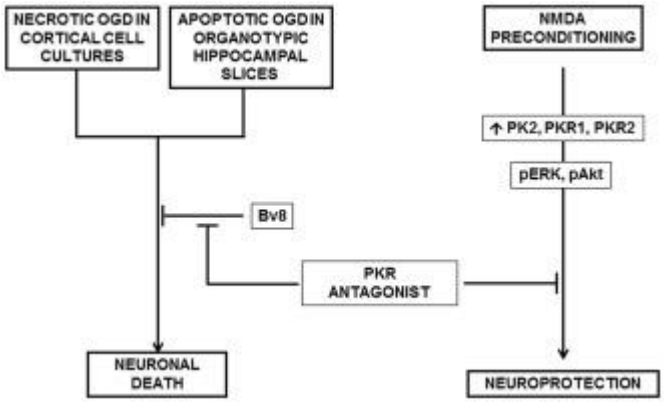
Highlights

- Bv8/PK2 are protective in apoptotic and necrotic models of cerebral ischemia.
- Prokineticins are involved in the development of ischemic tolerance *in vitro*.
- Prokineticin neuroprotection is mediated by activation of ERK1/2 and Akt signaling.

Abstract

Bv8/prokineticin 2 (PK2) is a member of a bioactive family of peptides that regulate multiple functions in the CNS including hyperalgesia, neurogenesis, neuronal survival and inflammation. Recent studies have associated PK2 and prokineticin receptors (PKR) with human diseases, but because their role in neuropathology is still debated we examined whether prokineticins exert a protective or deleterious role in models of cerebral ischemia and ischemic tolerance *in vitro*. In order to mimic cerebral ischemia, we exposed primary murine cortical cell cultures or rat organotypic hippocampal slices to appropriate periods of oxygen-glucose deprivation (OGD), which leads to neuronal damage 24 h later. Ischemic tolerance was induced by exposing hippocampal slices to a preconditioning subtoxic pharmacological stimulus (3 μM NMDA for 1 h) 24 h before the exposure to OGD. Bv8 (10–100 nM) attenuated OGD injury in cortical cultures and hippocampal slices, and the effect was prevented by the PKR antagonist PC7. The development of OGD tolerance was associated with an increase in the expression of PK2, PKR1 and PKR2 mRNA and proteins and was prevented by addition of the antagonist PC7 into the medium during preconditioning. Both Bv8 at protective concentrations and the NMDA preconditioning stimulus promoted the phosphorylation of ERK1/2 and Akt. These findings indicate that the prokineticin system can be up-regulated by a defensive preconditioning subtoxic NMDA stimulus and that PK2 may act as an endogenous neuroprotective factor through the activation of the ERK1/2 and Akt transduction pathways.

Graphical abstract



Keywords

Bv8, Prokineticins, Oxygen-glucose deprivation, Preconditioning, Ischemic tolerance Neuroprotection

Abbreviations

PK2 prokineticin 2
PKR PK receptor
PKR1 PK receptor 1
PKR2 PK receptor 2
PC7 (2-(5-(4-fluorobenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)-ethyl)-guanidine)
OGD oxygen-glucose deprivation
NMDA N-methyl-D-aspartate
PC preconditioning
PI propidium iodide
ERK extracellular-signal-regulated kinases
Akt protein kinasi B
GSk3β Glycogen synthase kinase 3 beta

1. Introduction

Stroke is the third leading cause of death in the Western world and the most common cause of disability. Unfortunately, clinical trials for stroke and related ischemic syndromes with neuroprotective drugs have been generally unsuccessful so far (Green and Shuaib, 2006, George and Steinberg, 2015). Along with the development of more appropriate experimental animal models, drugs with a better therapeutic index and aimed at alternative targets in the excitotoxic cascade are needed for the identification of new and effective neuroprotective therapies for cerebral ischemia. Ischemic tolerance, an evolutionary conserved cellular defense program in which exposure to a subtoxic preconditioning stimulus results in resistance to a subsequent otherwise lethal episode of ischemia, has been extensively investigated in the last few years as a powerful endogenous neuroprotective mechanism (Gidday, 2006, Dirnagl et al., 2009, Wang et al., 2015). Understanding the nature of the endogenous adaptive neuroprotective responses that are evoked by preconditioning stimuli has become an attractive experimental strategy to identify endogenous protective or regenerative mechanisms that can be therapeutically induced or supplemented. Along this line, in the past few years we have developed an *in vitro* model of pharmacological preconditioning and have studied a number of neuroprotective mechanisms that are evoked by low doses of glutamate receptor agonists in rat organotypic hippocampal slices exposed to oxygen-glucose deprivation (OGD) (Werner et al., 2007, Gerace et al., 2012b, Gerace et al., 2015).

Bv8 is a small peptide (8 kDa) extracted from the skin secretion of the yellow-bellied toad *Bombina variegata* (Wechselberger et al., 1999). The mammalian prokineticin family includes two small bioactive peptides (8–12 kDa) that stimulate guinea-pig ileum contraction termed prokineticin 1 (PK1) (also known as endocrine gland-vascular endothelial growth factor, EG-VEGF) and PK2 (the mammalian analog of Bv8). These two peptides are highly conserved across species (LeCouter et al., 2004, Giannini et al., 2009) and activate two highly homologous receptors, prokineticin receptor 1 (PKR1) and PKR2 (Negri and Lattanzi, 2011), which are coupled to a variety

of G proteins and hence to intracellular Ca^{2+} mobilization and activation of the ERK1/2 and Akt pathways (Lin et al., 2002, Masuda et al., 2002, Soga et al., 2002). Prokineticins (especially PK2) and their receptors are largely expressed in CNS (Cheng et al., 2006) and regulate multiple biological functions in the CNS including hyperalgesia (Mollay et al., 1999), neurogenesis in the olfactory bulb (Ng et al., 2005), neuronal survival (Melchiorri et al., 2001) and inflammation (Martucci et al., 2006). Recent studies have implicated PK2 in human diseases such as, for example, Kallmann syndrome, which can be determined by loss-of-function mutations in the gene encoding for PK2 (Pitteloud et al., 2007) or CNS autoimmune demyelination, in which PK2 has been identified as a critical immune regulator (Abou-Hamdan et al., 2015).
Although many effects of prokineticins under physiological conditions have been recognized, their role in neuropathology has not been fully elucidated. Hypoxic conditions in human adrenal carcinoma (LeCouter et al., 2001) or experimental stroke (Cheng et al., 2012) are known to induce the transcription of prokineticins. However, the results of experimental studies are conflicting on whether these chemokines may exert a protective or deleterious role in neurodegeneration. Bv8 has been shown to protect against NMDA excitotoxicity in cerebellar granule cell cultures (Melchiorri et al., 2001), to protect cardiomyocytes against oxidative stress (Urayama et al., 2008), and to promote neurogenesis in the olfactory bulb (Ng et al., 2005). On the other hand, PK2 induces a proinflammatory phenotype in mouse macrophages (Martucci et al., 2006) and worsens the infarct volume, central inflammation and the behavioural outcome in models of stroke *in vivo* (Cheng et al., 2012).
In this study, we examined the neuroprotective effects of Bv8 in murine cortical cell cultures and in organotypic hippocampal slices exposed to OGD, two *in vitro* models of cerebral ischemia routinely in use in our laboratory that allow chronic treatment with drugs in a relatively isolated environment that closely reproduces what occurs *in vivo* (Conti et al., 2010, Gerace et al., 2012a). We also examined the role of prokineticins in the induction of OGD tolerance and the expression of prokineticins and prokineticin receptors following NMDA preconditioning in organotypic hippocampal slices. The results of our study indicate that prokineticins are neuroprotective in our models and may play a therapeutic role in stroke.

2. Materials and methods

Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The experimental protocols were approved by the Animal Care Committee of the Department of Pharmacology, University of Florence, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.1. Materials

N-methyl-D-aspartate and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Bv8 was isolated from skin secretions of the frog *Bombina variegata* as previously described (Giannini et al., 2009) and purified to 98% as assessed by high-performance liquid chromatography. PC7 (2-(5-(4-fluorobenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)-ethyl)-guanidine), a triazinic compound which displays approximately 75-fold higher affinity for PKR1 than for PKR2, was synthesized as previously described (Congiu et al., 2014). Tissue

culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA).

2.2. Oxygen-glucose deprivation (OGD) in cortical cell cultures

Cerebral cortices were dissected from ED 17–18 CD1 mice (Harlan, Italy), minced using medium stock (MS; composed of Eagle's minimal essential medium, with Earle's salts, glutamine-and $NaHCO_3$-free, supplemented with 38 mM $NaHCO_3$, 22 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin), and incubated for 10 min at 37 °C in MS with 0.25% trypsin and 0.05% DNase. Enzymatic digestion was terminated by a second incubation (10 min at 37 °C) in MS supplemented with 10% heat-inactivated horse serum and 10% fetal bovine serum. After brief centrifugation, cells were resuspended (approximately 4×10^5 cells/ml) and plated in 15-mm multiwell plates on a layer of confluent astrocytes using a plating medium of MS supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, and 2 mM glutamine for mixed cortical cultures. Cultures were kept in an incubator at 37 °C, with 100% humidity and 95% air/5% CO_2 environment. After 4–5 DIV, non-neuronal cell division was halted with 3 μM cytosine arabinoside for 24 h. Cultures were then shifted to a maintenance medium identical to the plating medium without fetal bovine serum.
Cultures of mixed murine cortical cells were exposed to oxygen-glucose deprivation (OGD) as previously described in detail (Pellegrini-Giampietro et al., 1999a, Pellegrini-Giampietro et al., 1999b). Briefly, culture medium was replaced by a glucose-free balanced salt solution saturated with 95% N2/5% CO2 and heated to 37 °C. Multiwells were then sealed into an airtight incubation chamber equipped with inlet and outlet valves and 95% N2/5% CO2 was blown through the chamber for 10 min to ensure maximal removal of oxygen. The chamber was then sealed and placed into the incubator at 37 °C for 60 min. OGD was terminated by removing the cultures from the chamber, replacing the exposure solution with oxygenated medium and returning the multiwells to the incubator under normoxic conditions. The extent of cell detah was quantitatively evaluated by measuring the amount of LDH released from injured cells into culture media 24 h following exposure to OGD, as previously described (Pellegrini-Giampietro et al., 1999a, Pellegrini-Giampietro et al., 1999b). The LDH level corresponding to complete neuronal death (with no glial death) was determined for each experiment by assaying sister cultures exposed to 1 mM glutamate for 24 h. Background LDH release was determined in control cultures not exposed to OGD and subtracted from all experimental values. The resulting value correlated linearly with the degree of cell loss estimated by observation of cultures under phase-contrast microscopy or under bright-field optics following 5 min incubation with 0.4% trypan blue, which stains debris and nonviable cells.

2.3. OGD and NMDA preconditioning in rat organotypic hippocampal cultures

Organotypic hippocampal slice cultures were prepared from 8-day-old rats, used at 14 DIV and exposed to OGD as previously reported in detail (Pellegrini-Giampietro et al., 1999a, Pellegrini-Giampietro et al., 1999b, Gerace et al., 2012a). Briefly: the slices were pre-incubated for 5 min in serum-free medium and then subjected to OGD by exposure to a serum-free medium devoid of glucose and previously saturated with 95% N2/5% CO2. Following 30 min incubation at 37 °C in the airtight anoxic chamber, the cultures were transferred to oxygenated serum-free medium containing 5 mg/ml glucose and returned to the incubator under normoxic conditions until neuronal injury was evaluated 24 h later. For pharmacological preconditioning experiments with NMDA, slices were pre-exposed to the ionotropic glutamate receptor agonist NMDA (3 μM) for 60 min and then, 24 h later, to 30 min OGD as previously reported in detail (Gerace et al., 2012b). Cell injury

was assessed in organotypic hippocampal cultures using PI, a polar dye which enters the cells only if the membrane is damaged and becomes fluorescent upon binding to DNA. PI was added to the medium at the end of the 24 h recovery period following OGD. Thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (4X) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1™; Intracellular Imaging Inc., Cincinnati, OH, USA) and subsequently analyzed using the Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, USA). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected. There was a linear correlation between CA1 PI fluorescence and the number of injured CA1 pyramidal cells as detected by morphological criteria (Pellegrini-Giampietro et al., 1999a).

2.4. Quantitative real time PCR

Total RNA was extracted from rat organotypic hippocampal slices using the RNeasy column kit (Qiagen, Milano, Italy). Purified RNA (1 μg) was used for cDNA synthesis using reverse transcriptase (Promega, Milan, Italy). cDNAs were amplified by real-time PCR (iCycler; Bio-Rad) using iQ SYBER Green Supermix (Bio-Rad). Specific sense and antisense primers were synthesized (Biogen, Rome, Italy) to PCR amplify the rat PK2, PKR1 and PKR2 cDNA, according to the following sequences previously described (Giannini et al., 2009):
PK2, 5′-CAAGGACTCTCAGTGTGGA-3′ and 5′-AAAATGGAACTTTCCGAGTC-3′; PKR1, 5′-CGCACCGTCTCCCTCTAC-3 and 5′-GTTTGACACTTCATCCGCG-3′; PKR2, 5′CTCCGTCAACTACCTTCGTA-3′ and 5′-GAGGCGGTCTGGTAATTCA-3′.

β-Actin was used as internal standard. Results are presented as the Ct (cycle threshold) values of the specific gene of interest or as input copy number of the gene of interest per ng of total RNA. cDNA standards for each analyzed gene were generated, and serial dilutions ranging from 10 to 10^9 input copies were used as a standard curve in each PCR run.

2.5. Western blotting

Cultured slices (4 slices/sample) and mixed cortical cells (3 wells/sample) were washed with cold 0.01 M phosphate-buffered saline, pH 7.4 and were gently transferred and dissolved in a tube containing 1% SDS. Total protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay. Lysates (20 μg/lane of protein) were resolved by electrophoresis on a 4–20% SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Blots were blocked for 1 h at room temperature in 20 mM Tris-buffered saline, pH 7.6–0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk, and then incubated overnight at 4 °C with polyclonal-goat antibodies against PK2, PKR1 and PKR2 (all from Santa Cruz Biotechnology, CA, U.S.A) diluited 1:500 in TBS-T containing 5% non-fat dry milk or polyclonal-rabbit antibody against phospho-ERK1/2 (Thr202/Thr204), phospho-Akt (Ser473) or phospho-GSK3-β (Ser9) (all from Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000 in TBS-T containing 5% bovine serum albumin. The loading control anti-β-actin antibody was monoclonal from Sigma (St Louis, MO, USA). Immunodetection was performed with secondary antibodies (1:2000 anti-mouse, anti-rabbit or anti-goat IgG from donkey, Amersham Biosciences, UK) conjugated to horseradish peroxidase in TBS-T containing 5% non-fat dry milk. Membranes were

washed with TBS-T and then reactive bands were detected using chemiluminescence (ECLplus; Euroclone, Padova, Italy). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analysis

Data are presented as means ± SEM of *n* experiments. Statistical significance of differences between PI fluorescence intensities or Western blot optical densities was analyzed using one-way ANOVA with a *post hoc* Tukey's *w*-test for multiple comparisons. Analysis of differences in mRNA levels as detected by PCR were analyzed using the Student's *t*-test or using one-way ANOVA with a *post hoc* Tukey's *w*-test for multiple comparisons. All statistical calculations were performed using Graph-Pad Prism v. 5 for Windows (GraphPad Software, San Diego, CA, USA). A probability value (*P*) of <0.05 was considered significant.

3. Results

3.1. Neuroprotective effects of Bv8 (PK2) in mixed murine cortical cultures and rat organotypic hippocampal slices exposed to oxygen and glucose deprivation (OGD)

Bv8 can be used as a suitable pharmacological tool to investigate the effects of PK2 *in vitro* and *in vivo* (Giannini et al., 2009, Severini et al., 2015). To investigate the possible neuroprotective effects of Bv8 we carried out experiments in cultured murine cortical cells exposed to OGD, an *in vitro* model of focal cerebral ischemia routinely used in our lab (Pellegrini-Giampietro et al., 1999a). We have demonstrated that OGD induces necrotic neuronal death in this system, as detected by the appearance of necrotic ultrastructural features 24 h later and the lack of caspase-3 activation (Moroni et al., 2001). As previously shown, exposure to OGD for 60 min produced an intermediate level of neuronal damage in this system: the release of LDH was approximately 75% of that observed by exposing the cultures to a high concentration of glutamate (1 mM) for 24 h, which elicited complete neuronal cell death. When Bv8 (1–1000 nM) was added to the incubation medium during OGD exposure and the subsequent 24 h recovery period, we observed a concentration-dependent reduction of OGD injury (Fig. 1A). This effect was completely reverted by the co-incubation with the PKR antagonist PC7 (10 μM) (Fig. 1B). None of the tested Bv8 concentrations produced any significant increase in the release of LDH when added alone to the incubation media (data not shown). Because it has been reported that Bv8 may support neuronal survival by activating the MAP-kinase/PI-3 kinase pathways (Melchiorri et al., 2001), we analyzed by Western blot the phophorylation of ERK 1/2, Akt and GSK3-β in cultures treated with Bv8 (10 nM). Bv8 produced significant increases in the phosphorylation of ERK 1/2 and Akt and a more robust increase in the phosphorylation of GSK3-β (Fig. 1C).

We then tested whether Bv8 could also be neuroprotective in an apoptotic model of cerebral ischemia in use in our laboratory, namely rat organotypic hippocampal slices exposed to 30 min OGD, which produces selective CA1 injury 24 h later and the appearance of morphological characteristics of apoptosis, caspase-3 activation and PARP-1 cleavage (Moroni et al., 2001, Gerace et al., 2012b). Fig. 2 shows that Bv8 (at the selected concentration of 10 nM) produced a significative protective effect when present in the incubation medium during OGD and the subsequent 24 h recovery period. This effect was prevented when the PKR antagonist PC7 (1–10 μM) was added to the incubation medium together with Bv8. Hippocampal slices exposed to Bv8 and PC7 alone for 24 h displayed no apparent signs of neurodegeneration (data not shown).

3.2. Prokineticins are involved in the induction of OGD tolerance in organotypic hippocampal slices

As previously reported (Gerace et al., 2012b), pre-exposure of hippocampal slices to a preconditioning subtoxic concentration of NMDA (3 μM for 60 min) was able to significantly reduce (by 53 ± 5%) the CA1 injury induced 24 h later by a lethal exposure to 30 min OGD (Fig. 3). Fig. 3B and C shows that the prokineticin receptor antagonist PC7 (10 μM) prevented the development of OGD tolerance induced by NMDA when present in the incubation medium during NMDA preconditioning and the subsequent 24 h period. Because activation of the prosurvival ERK1/2 and PI-3 kinase/Akt signaling pathways has been implicated in the neuroprotective mechanisms of OGD preconditioning (Gerace et al., 2012b), we used phospho-specific antibodies to measure the relative levels of the phosphorylated, active forms of ERK 1/2 and Akt after exposure to 3 μM NMDA for 60 min and evaluate whether the activation of these factors was dependent on prokineticin receptors. Fig. 3D shows that preconditioning with NMDA induced a significant and immediate increase in the phosphorylation of ERK 1/2 and a delayed increase of Akt 6 h later, that were both prevented by the prokineticin receptor antagonist PC7 (10 μM).

3.3. Expression of prokineticins and prokineticin receptors following NMDA preconditioning in organotypic hippocampal slices

In order to determine the levels of prokineticins and prokineticin receptor transcripts, we analyzed hippocampal slices using quantitative real time PCR at various time points after NMDA preconditioning. In preliminary experiments, we observed that whereas the PK1 transcript was not detectable, PK2 and PKR2 mRNAs were more abundant than those of PKR1: their levels were highest in freshly prepared organotypic hippocampal slices and decreased somewhat after slices were cultured for 14-days *in vitro* (Fig. 4A). NMDA preconditioning induced an increase in PK2 mRNA that reached a significant peak 3 h after incubation with NMDA was terminated (Fig. 4A). Western blot analysis confirmed and extended these results, showing that PK2, PKR1 and PKR2 proteins were all significantly increased 3–6 h after NMDA preconditioning (Fig. 4B). Notably, the increase in PK2 protein expression 6 h after NMDA preconditioning was prevented by the PKR antagonist PC7.

4. Discussion

The present study shows that the chemokine PK2 and its receptors are involved in the mechanisms that confer neuroprotection against cerebral ischemia. In particular, we have shown that Bv8 (PK2) attenuates both necrotic and apoptotic neuronal death in two different *in vitro* models of cerebral ischemia routinely used in our laboratory, namely mixed murine cortical cells and rat organotypic hippocampal slices exposed to appropriate periods of OGD (Pellicciari et al., 1999, Pellegrini-Giampietro et al., 1999b, Gerace et al., 2012a). The protective effects of Bv8 appear to be mediated by prokineticin receptors, in that they were blocked by the prokineticin receptor antagonist PC7. Moreover, during the development of a defensive mechanism such as *in vitro* OGD tolerance evoked by sublethal concentrations of NMDA (as described in (Gerace et al., 2012b), mRNA and protein levels of PK2, PKR1 and PKR2 were up-regulated. The prokineticin receptor

antagonist PC7 prevented the induction of tolerance in our slices, further indicating that the prokineticin family may play an endogenous neuroprotective role in the CNS.

These findings are in agreement with a number of studies suggesting that the prokineticin system may be involved in protective mechanisms in a variety of experimental models. For example, Bv8 has been shown to reduce the extent of NMDA excitotoxicity and apoptotic cell death induced by a low K^+ medium in cultured cerebellar granule cells (Melchiorri et al., 2001). In cardiac tissue, Bv8 was able to attenuate cardiomyocyte oxidative stress (Urayama et al., 2008) and, in a very recent report, a novel PKR1 agonist was demonstrated to prevent myocardial infarction in mice via an angiogenic mechanism (Gasser et al., 2015). However, reports also exist showing that Bv8/PK2 prokineticins induce a pro-inflammatory phenotype in mouse macrophages (Martucci et al., 2006), exacerbate the infarct volume in rats subjected to transient or permanent cerebral focal ischemia (Cheng et al., 2012), or reduce cell viability in primary rat cortical cultures (Severini et al., 2015). In these latter studies, prokineticin receptor blockers were able to protect against post-ischemic cell death (Cheng et al., 2012) or amyloid beta-induced neurotoxicity (Severini et al., 2015).

It is interesting to note that Bv8 and PK2 were used in the nanomolar range (10–100 nM) in our study as well as in all other studies in which they provided protective or pro-survival effects (Melchiorri et al., 2001, Ng et al., 2005, Urayama et al., 2008, Gasser et al., 2015). Conversely, the pro-apoptotic effects of these prokineticins were always obtained at much lower concentrations (10–100 pM), with bell-shaped dose-response curves and no neurotoxic effects at higher concentrations (Cheng et al., 2012, Gasser et al., 2015, Severini et al., 2015). Because the affinity of Bv8 for PKR1 and PKR2 is about 0.5–0.7 nM and that of PK2 is ten-fold lower (Negri et al., 2007), it is possible that only full (nanomolar) concentrations of Bv8 or PK2 may be able to activate a significant fraction of receptors and their pro-survival ERK1/2 and Akt transduction pathways (Negri et al., 2005). Along this line, the harmful effects of picomolar concentrations of PK2 have been suggested to be contributed by its unique effects on the SAP/JNK pathway under excitotoxic or OGD conditions (Cheng et al., 2012), whereas the pro-survival effects of prokineticins in the nanomolar range appear to require activation of the ERK1/2 and Akt signalling pathways (Melchiorri et al., 2001, Urayama et al., 2008, Gasser et al., 2015); this study). Other mechanisms are also possible: for example, the hyperalgesic effects of very low (femtomolar) concentrations of Bv8 are at least partly mediated by activation of the TRPV1 receptor (Negri et al., 2006), which has been shown to produce toxic effects and exacerbate OGD injury in our organotypic hippocampal slices (Landucci et al., 2011). Because of the absence of a vascular system and inflammatory cells in cortical cell or hippocampal slice cultures, other possible mechanisms known to be triggered by prokineticins, such as the pro-inflammatory (Martucci et al., 2006, Cheng et al., 2012) or angiogenic (LeCouter and Ferrara, 2003, Monnier and Samson, 2010) effects, can be ruled out in our experimental models.

Our results showing that hippocampal slices freshly prepared from neonate rat brain display negligible amounts of PK1 mRNA and a more abundant expression of PK2, PKR1 and PKR2 than what observed in slices cultured for 14 days *in vitro* corroborate the findings of previous studies showing that prokineticins and their receptors are developmentally regulated (Negri et al., 2007) and that in adult brain, whereas PK1 expression is confined to the olfactory bulb and discrete brainstem areas, Bv8/PK2 is present at high levels in all other brain regions (Melchiorri et al., 2001, Cheng et al., 2012, Negri et al., 2007). Our results also confirm previous reports showing that both PKR1 and PKR2 are present in neonate and adult brain, with PKR2 prevalently expressed in neurons and PKR1 in astrocytes and microglia (Koyama et al., 2006, Cheng et al., 2012). Notably, the expression of prokineticins can be induced or up-regulated in response to various pathological stressors: PK2 mRNA is increased in primary cortical cultures exposed to hypoxia, H_2O_2, and excitotoxins or in the cortex and hippocampus of rats subjected to transient focal ischemia (Cheng et al., 2012), and so are the levels of PK2, PKR1 and PKR2 mRNA and proteins in response to amyloid beta-induced neurotoxicity *in vitro* and *in vivo* (Severini et al., 2015). Our study shows for the first time that exposure to a defensive preconditioning subtoxic NMDA stimulus is similarly able to produce 3 h later an up-regulation in the expression of PK2 mRNA, which is followed by a peak (after 6 h) in the induction of PK2, PKR1 and PKR2 proteins. It is interesting to note that in the study by Cheng et al. (2012) glutamate was able to induce a significant increase in the expression of PK2 mRNA even at quite low, presumably non toxic, concentrations (i.e. 20 μM), and that the effect of glutamate was dependent on the activation of NMDA receptors. Similarly to what reported by Lattanzi et al. (Lattanzi et al., 2015), the increase in PK2 protein levels under our experimental conditions was dependent on the activation of PK receptors, again suggesting a possible paracrine mechanism for the increase in prokineticin expression.

The neuronal localization of PKR2, together with data demonstrating that the hippocampal CA1 region is particularly enriched with PKR2, as compared to CA3 and the dentate gyrus that express both subtypes of prokineticin receptors (Cheng et al., 2012), strongly suggest that the protection of vulnerable CA1 pyramidal cells produced by Bv8 in our hippocampal slices is most likely mediated by PKR2. The antagonist that we used (PC7) displays a relatively higher affinity for PKR1, but our working concentration (10 μM) is most likely able to block PKR2 as well, being its IC_{50} for this receptor subtype around 4–5 μM (Congiu et al., 2014). In cardiac tissue, PKR1 and PKR2 have been proposed to play opposite roles: whereas PK2/PKR1 signalling in endothelial cells promotes proliferation, migration and angiogenesis and has an important role in attenuating post-ischemic injury (Boulberdaa et al., 2011, Gasser et al., 2015), PKR2 appears to mediate mostly detrimental effects inducing cardiac hyperthrophy and vascular leakage (Urayama et al., 2008). In the brain, the relative contribution of PKR1 and PKR2 to the mechanisms leading to neurodegeneration or neuroprotection has not been yet elucidated, mainly because of the lack of specific receptor subtype

antagonists (Cheng et al., 2012, Severini et al., 2015). However, it has been clearly shown that prokineticins need to activate the pro-survival MAPK/ERK1-2 and PI-3 kinase/Akt transduction pathways for the induction of a defensive or protective response (Melchiorri et al., 2001, Urayama et al., 2008, Gasser et al., 2015), whereas the deteriorating effects of PK2 under excitotoxic or OGD conditions appear to be mediated by other mechanisms, such as the SAP/JNK pathway (Cheng et al., 2012). This view is further supported by our data showing that both Bv8 at protective concentrations and the NMDA preconditioning stimulus promote the activation of components of both the MAPK/ERK1-2 and PI-3 kinase/Akt transduction pathways, and that the increase in the phosphorylation of ERK1/2 and Akt is mediated by prokineticin receptors in a time-dependent fashion.

In conclusion, our study supports the idea that prokineticins may play a dual role in the mechanisms leading to neurodegeneration or neuroprotection. Whereas both pathological and defensive mechanisms can induce an up-regulation of PK2 and its receptors, the relative contribution of PKR1 and PKR2 in these mechanisms still awaits the development of appropriate experimental tools. The stimulation of a significant fraction of receptors with full (nanomolar) concentrations of agonists and the activation of the pro-survival MAPK/ERK1-2 and PI-3 kinase/Akt transduction pathways appear to be required for the induction of a protective phenotype in response to prokineticins.

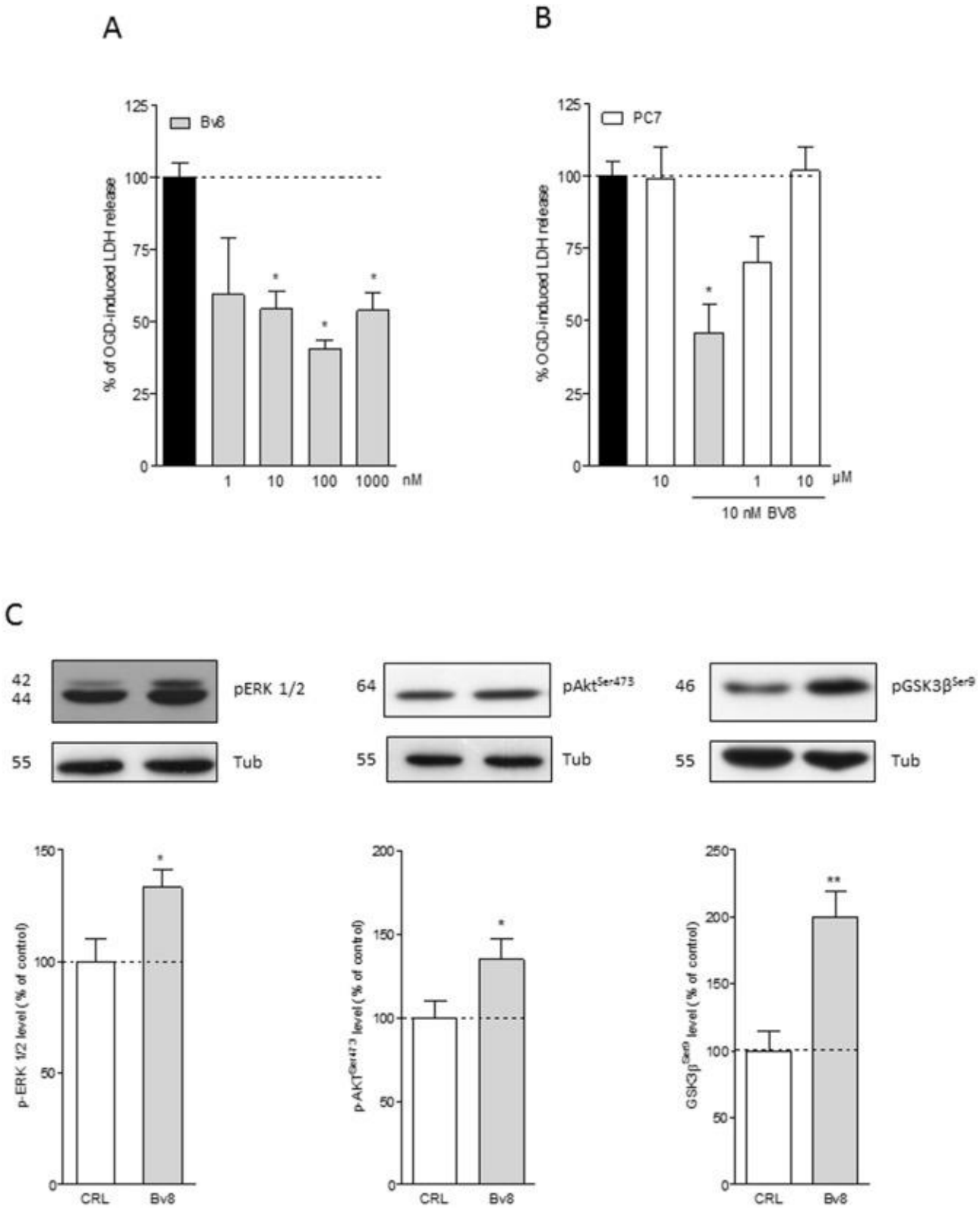


Fig. 1. **A**-**B**) Neuroprotective effects of Bv8 in mixed murine cortical cultures exposed to OGD. OGD was applied for 60 min and 24 h later neuronal death was assessed by measuring the release of LDH in the medium. Neuronal death was significantly reduced in a dose-dependent manner when Bv8 (1–1000 nM) was added to the incubation medium during OGD exposure and the subsequent 24 h recovery period; the effect was completely reverted by co-incubation with the prokineticin receptor antagonist PC7 (1–10 μM). Data are expressed as percentage of OGD-induced LDH release. Values represent the mean ± SEM of at least three experiments

performed in quadruplicate. *P < 0.05 vs. 60 min OGD alone (ANOVA + Tukey's w-test). **C**) Effects of Bv8 on ERK1/2, Akt and GSK3β phosphorylation. Cortical cultures were incubated with 10 nM BV8 for 60 min and then lysed and processed for Western blotting. *Top panels*: Representative Western blots using antibodies directed against phospho-ERK1/2 (Thr202/Thr204), phospho-Akt (Ser473) and GSK3-β (Ser9). The numbers on the left indicate the position of the estimated molecular mass markers (kDa). Tubulin was used as loading control. *Bottom*: Quantitative analysis of immunoreactive bands, showing a significant increase in phospho-ERK1/2, phospho-Akt and phospho-GSK3β expression levels induced by Bv8 exposure. Data are expressed as percentage of phosphorylation in control untreated cultures. Bars represent the mean ± SEM of at least 6 experiments **p < 0.01 vs. control (Student's *t*-test).

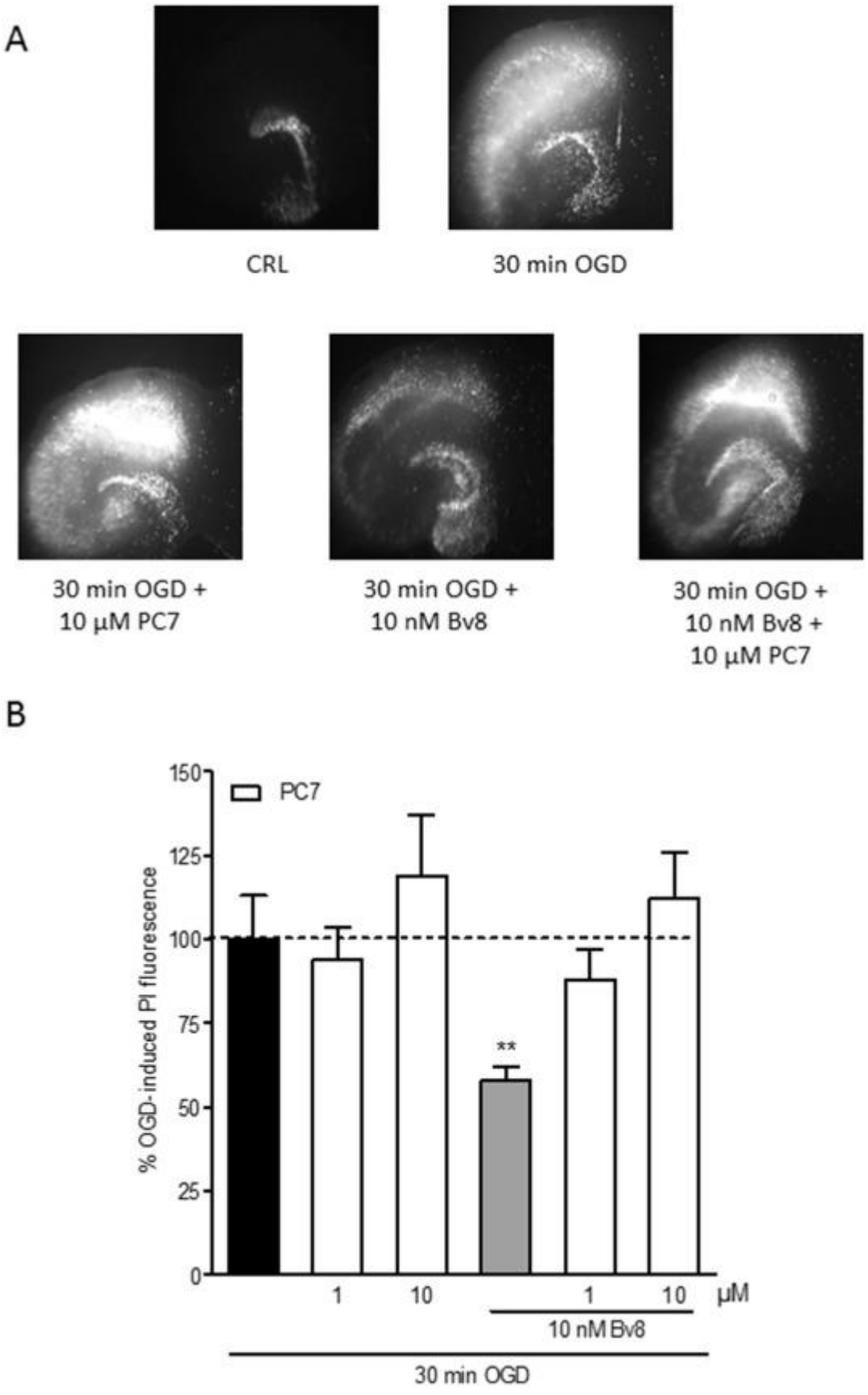


Fig. 2. Neuroprotective effects of Bv8 in organotypic hippocampal slices exposed to OGD. **A**) Hippocampal slices, photographed under fluorescence optics, displaying background levels of fluorescence under control conditions, an intense PI labeling in the CA1 sub-

region 24 h after exposure to 30 min OGD and when incubated with the prokineticin receptor antagonist PC7, and a reduction of CA1 PI fluorescence that was prevented by the PKR antagonist PC7 when incubated with the prokineticin receptor agonist Bv8 during OGD and the subsequent 24 h period. **B**) Quantitative analysis of OGD-induced CA1 PI fluorescence expressed as percentage of OGD toxicity. Incubation with Bv8 (10 nM) significantly attenuated OGD injury; the effect was completely reverted by the co-incubation with PC7 (1–10 μM). Values represent the mean ± SEM of at least three experiments performed in quadruplicate. **P < 0.01 vs. 30 min OGD alone (ANOVA + Tukey's w-test).

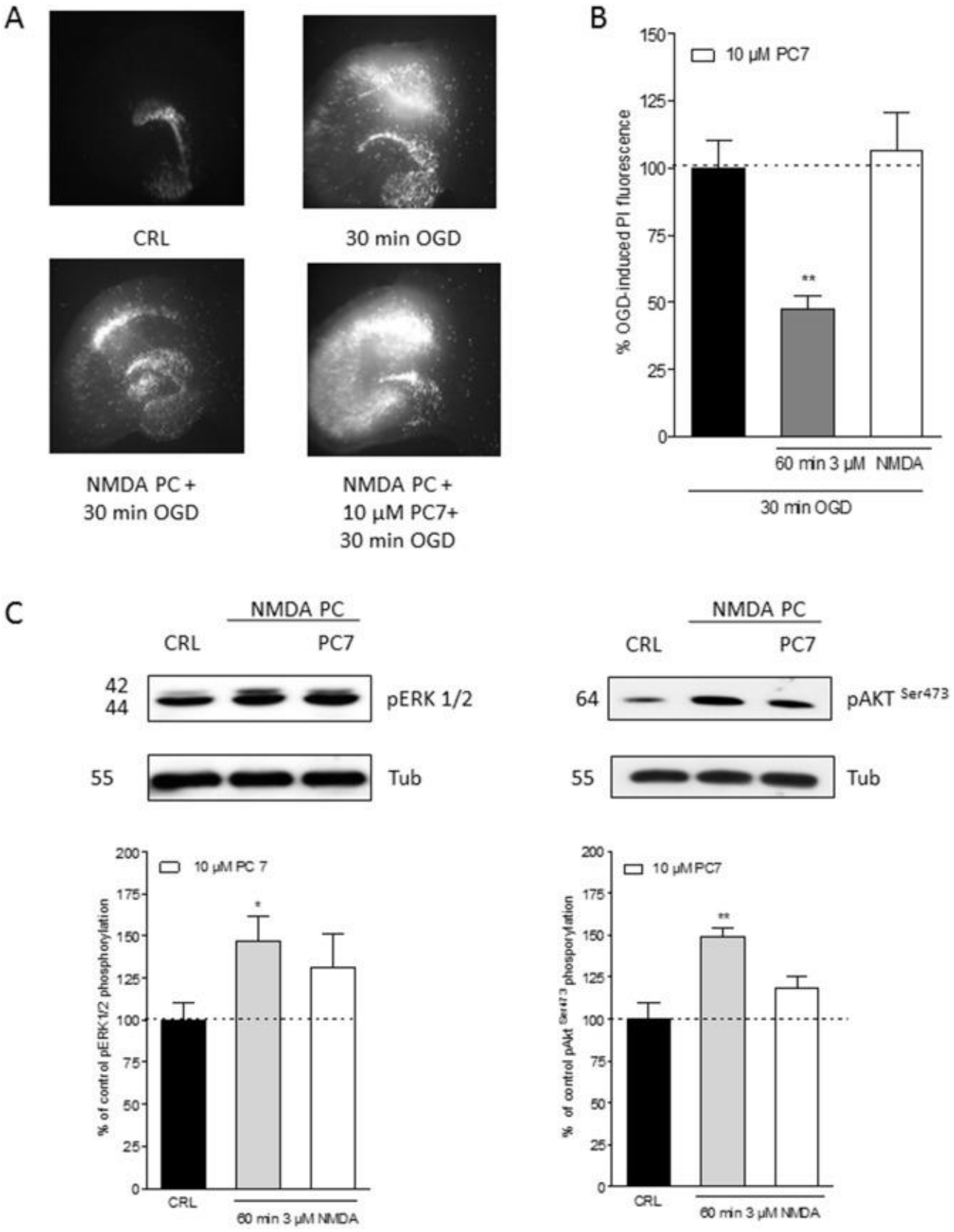


Fig. 3. The prokineticin receptor antagonist PC7 prevents the induction of ischemic tolerance in organotypic hippocampal slices. **A**) *Control* (CRL): slice exposed to a subtoxic preconditioning dose of NMDA (3 µM for 60 min) and 48 h later incubated with PI, displaying background levels of fluorescence. *30* min *OGD*: Slice exposed to 30 min OGD, displaying intense PI labeling in the CA1 subregion 24 h later. *NMDA PC + 30* min *OGD*: hippocampal slice exposed to NMDA PC

24 h prior to OGD, displaying reduced CA1 PI labeling as compared to OGD alone. *NMDA PC + 10 μM PC7 + 30* min *OGD*: slice exposed to the prokineticin receptor antagonist PC7 during NMDA PC and the subsequent 24 h period, displaying a prevention of the induction of tolerance to OGD. **B**) Quantitative analysis of CA1 PI fluorescence expressed as percentage of OGD toxicity. The prokineticin receptor antagonist PC7 prevented the development of OGD tolerance induced by NMDA PC. Bars represent the mean ± SEM of 3 experiments. **P < 0.01 vs. OGD alone (ANOVA + Tukey's w-test). **C**) Effects of PC7 on the increase in ERK1/2 and Akt phosphorylation induced by NMDA PC. Slices were exposed to the NMDA (3 μM for 60 min) preconditioning protocol and then processed for Western blotting immediately after (*left*) or 6 h later (*right*). PC7 (10 μM) was added to the incubation medium 15 min before and during the preconditioning exposure. *Top*: Representative Western blots using polyclonal rabbit antibodies directed against phospho-ERK1/2 (Thr202/Thr204) or phospho-Akt (Ser473). The numbers on the left indicate the estimated molecular mass (kDa). Tubulin was used as loading control. *Bottom*: Quantitative analysis of immunoreactive bands, showing that the prokineticin receptor antagonist PC7 prevented both the immediate increase in ERK1/2 and the delayed Akt phosphorylation induced by NMDA preconditioning. Data are expressed as percentage of phosphorylation in control untreated slices. Bars represent the mean ± SEM of three experiments. **P < 0.01 and *P < 0.05 vs. control (ANOVA + Tukey's w-test).

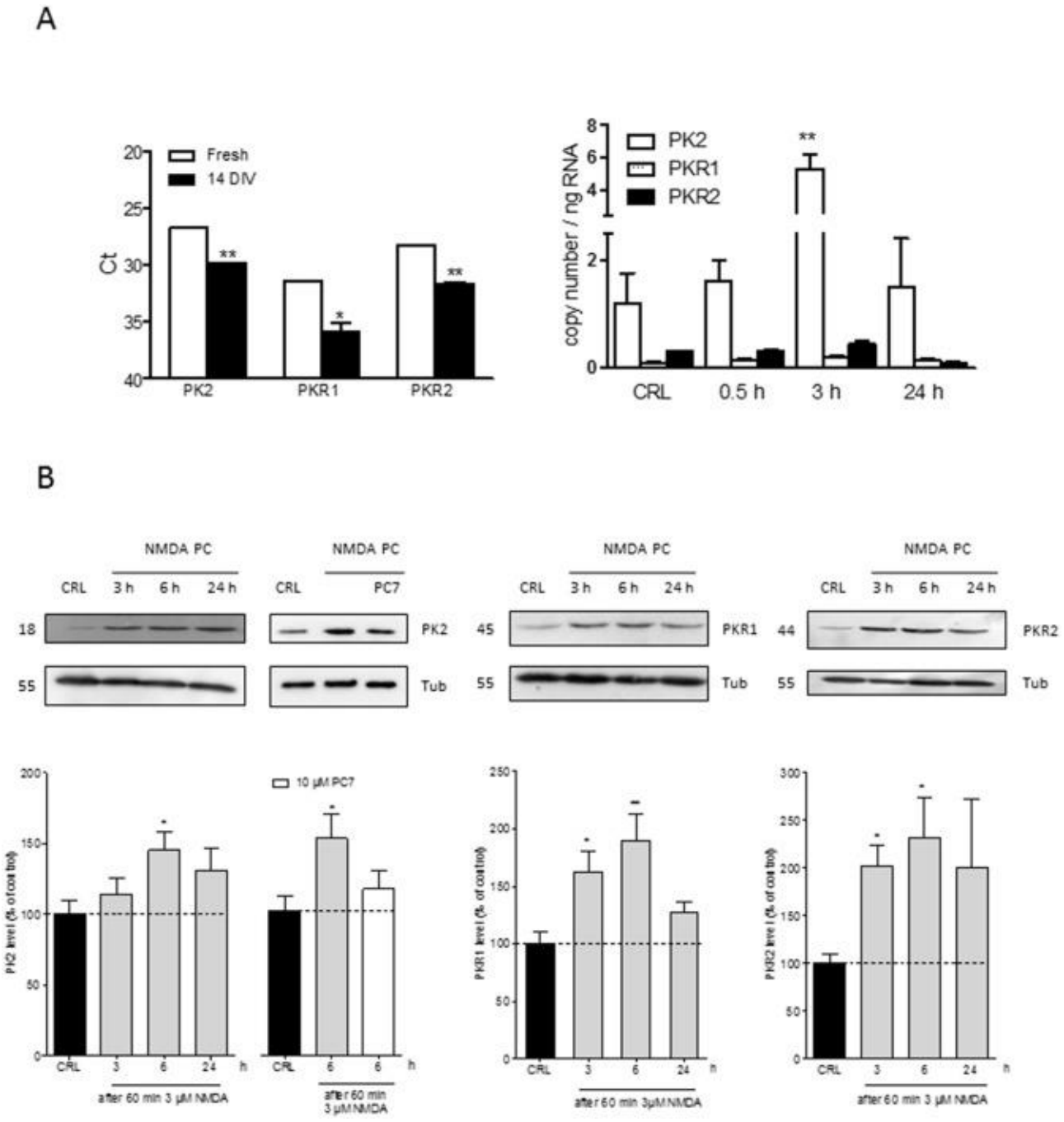


Fig. 4. A) Time course of the expression of PK2 and its receptors following NMDA preconditioning in organotypic hippocampal slices. Left: Expression of PK2, PKR1 and PKR2 during organotypic hippocampal slice maturation in vitro. Slices were prepared and processed for PCR immediately (fresh) or after 14 days in vitro (14 DIV). PK2, PKR1 and PKR2 mRNAs were detected at significant levels in fresh organotypic hippocampal slices and decreased with maturation. Data are expressed as Ct (cycle threshold) values of the specific gene of interest. Values represent the mean ± SEM of at least three experiments. *P < 0.05, **P < 0.01 14 DIV vs fresh organotypic hippocampal slices (Student's t-test). Right: Hippocampal slices were exposed to NMDA preconditioning (PC) and then processed for real time PCR analysis at different time points, as indicated in the diagram, to determine relative mRNA expression of PK2 and its receptors. NMDA preconditioning induced a significant increase in PK2 mRNA 3 h later. Results are presented as input copy number of the gene of interest per ng of total RNA. Bars represent the mean ± SEM of at least 5 experiments **p < 0.01 vs. control (ANOVA

+ Tukey's w-test). B) Top: Representative Western blot using polyclonal anti-PK2, anti-PKR1 or anti-PKR2 antibodies to detect PK2, PKR1 and PKR2 protein levels in organotypic hippocampal slices. The numbers on the left indicate the estimated molecular mass (kDa). Tubulin was used as loading control. Bottom: Quantitative analysis of immunoreactive bands, showing a significant increase in PK2 and its receptor protein levels 3 and 6 h after NMDA preconditioning. The increase in PK2 was prevented by the PKR antagonist PC7. Data are expressed as percent control levels of the corresponding protein. Bars represent the mean ± SEM of 4 experiments.**P < 0.01 and *P < 0.05 vs. control (ANOVA + Tukey's w test).

Conflicts of interest

The authors declare that they have no conflict of interest.

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