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Sigma Receptor Agonists Provide Neuroprotection *In Vitro* by Preserving *bcl-2*

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Abstract

Background—Sigma (σ)-receptor agonists attenuate brain injury after experimental focal cerebral ischemia in several species. We tested the hypothesis that the potent, prototypical σ_1 -receptor agonist, 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP), protects neurons by a mechanism involving the antiapoptotic protein *bcl-2*.

Methods—Primary cortical neuronal cultures were exposed to either 2 h of oxygen–glucose deprivation (OGD) or glutamate (100 μ M). PPBP treatment was initiated either 15 min prior to the insult or at 15 min postinsult then continued for 24 h. In another set of experiments, cultured neurons were preincubated for 2 h prior to PPBP treatment with σ_1 -receptor antagonist, rimcazole, in a dose-dependent manner. Alive and dead cells were detected with calcein-AM and propidium iodide respectively. *Bcl-2* and *bax* expression were determined by quantitative real time reverse transcription polymerase chain reaction and western blotting, and DNA damage was detected by TUNEL staining.

Results—PPBP pretreatment attenuated neuronal injury induced by OGD or glutamate (50 or 100 μ M). This protection was reversed with rimcazole (cell death: OGD 48 \pm 2%, OGD plus PPBP 31 \pm 3%, OGD plus PPBP with rimcazole 46 \pm 2%). PPBP treatment increased *bcl-2* but not *bax* mRNA levels. PPBP's ability to preserve *bcl-2* protein after OGD by PPBP was fully abolished by rimcazole. Lastly, PPBP reduced the number of TUNEL-positive cells after OGD, suggesting fewer cells with overt DNA damage.

Conclusions—These data demonstrate that PPBP reduces cell death *in vitro* by a mechanism involving receptor-dependent preservation of protective genes such as *bcl-2*.

Sigma (σ) receptor agonists are robustly neuroprotective in animal models of cerebral ischemia (1–5). We (1–5) have previously demonstrated that the potent and prototypic sigma 1 (σ_1) receptor agonist, 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP), attenuates stroke volume after focal cerebral ischemia in three animal species (cat, rat, and mouse). Sigma receptor ligands interact with numerous neurotransmitter systems in the brain (6) and may have potent antiexcitotoxic properties. While some studies suggest a presynaptic mechanism involving inhibition of ischemia-induced presynaptic glutamate release (7), others suggest receptor-mediated or postsynaptic mechanisms such as inhibition of dopaminergic neurotransmission (8), prevention of cortical spreading depression (9), modulation of neuronal responses to *N*-

methyl-D-aspartate (NMDA) receptor stimulation (10), attenuation of postsynaptic glutamate-evoked Ca^{2+} influx (11), attenuation of glutamate- and NMDA-induced nitric oxide (NO) synthase activation (10), and attenuation of ischemia-evoked NO production (5). Accordingly, we tested the hypothesis that PPBP protects neurons *in vitro* by a mechanism specific to the σ_1 receptor and to modulation of antiapoptotic *bcl-2* by determining neuronal viability after exposure of primary neuronal cultures to PPBP and the selective σ_1 -receptor antagonist, rimcazole as well as assessed DNA fragmentation, *bcl-2* and *bax* mRNA with quantitative polymerase chain reaction (PCR) and immunoblotting techniques.

Methods

Experimental protocols were approved by the Institutional Animal Care and Use Committee and conform to the National Institutes of Health guidelines for the care and use of animals in research.

Chemicals

PPBP was obtained from Tocris (Ellisville, USA). Glutamate, rimcazole dihydrochloride, propidium iodide (PI), and the antibody for β -actin were obtained from Sigma (St. Louis, MO), and calcein AM from Molecular Probes (Eugene, OR). The antibody against *bcl-2* was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Primary Neuronal Cell Cultures

Primary cortical neuronal cultures were established from E18 Sprague–Dawley rat pups (Charles River, MA), as described previously (12) with modifications. Dissociated cells were plated onto poly-L-ornithine coated plates (24 well plates, 2.5×10^5 cells/well or six well plates, 12×10^5 cells/well or 25-mm coverslip 3.5×10^5) in minimum essential medium supplemented with 10% horse serum, 2 mmol/L L-glutamine, 50 U/50 $\mu\text{g}/\text{mL}$ penicillin-streptomycin, and 25 mM glucose. Cells were kept for 30 min in minimum essential medium, which was subsequently replaced with cultivating medium (Neurobasal medium supplemented with 2% B27 and 2.0 mM Glutamax) and grown at 37°C in 100% humidity, 95% room air/5% CO_2 . All experiments were performed at 11–12 DIV and 37°C.

For oxygen–glucose deprivation (OGD) experiments, media was replaced with buffer (phosphate buffered saline with 1 mM CaCl_2 , 0.8 mM MgCl_2 , previously saturated with 95% $\text{N}_2/5\%$ CO_2 for 10 min) and then placed in a hypoxic environment incubator for 2 h (Thermo Forma 3130, OH; 94% N_2 , 1% O_2 , and 5% CO_2). For reoxygenation, cells were again placed into the cultivating medium and returned to normoxic conditions. Control cells were washed with OGD buffer and maintained in media. In drug treatment studies, cells were pretreated with PPBP in OGD buffer for 15 min prior to OGD. For postinjury treatment, PPBP (10 μM) was added 15 min and 1 h after completion of OGD. To assess the role of the σ_1 -receptor, the selective receptor antagonist rimcazole (1 or 5 μM) was added to the culture medium (13). Rimcazole was applied for 2 h, followed by PPBP for 30 min, and then cells were exposed to 2 h OGD at 37°C and 24 h of recovery. To assess excitotoxicity, cultures were exposed to 24 h of glutamate (100 μM). Cells were either pretreated with PPBP (10 μM , administered 15 min prior to glutamate exposure) or posttreated (administered at 15 min of glutamate exposure).

Assessment of Neuronal Viability

Cell viability was assessed by PI and Calcein-AM uptake (14) using the Nikon inverted microscope (Nikon Eclipse TE200). Cultured neurons were incubated with 1 $\mu\text{g}/\text{mL}$ calcein-AM for 30 min; 10 $\mu\text{g}/\text{mL}$ PI was then added for 15 min in medium at 37°C. For cell counting, cells were visualized using a Nikon 40 \times objective lens (Nikon DXM1200). In a blinded fashion, three images per well (24 wells) were generated and three wells for each condition were

analyzed. Cell mortality is expressed as the ratio of PI-positive cells/calcein positive + PI positive cells.

DNA Fragmentation

Cells were fixed in 10% formalin and then washed in phosphate-buffered saline. The fluorescein-based terminal, deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique (Roche Molecular Biochemicals, IN) was used to label double-stranded DNA breaks as described previously (15). Cells were then counterstained with 4',6-dimidino-2-phenylindole (DAPI) to assess nuclear morphology. The number of TUNEL and DAPI stained cells were counted in OGD- and OGD with PPBP-treated cultures, then averaged over three separate experiments and are expressed as the ratio of TUNEL positive to DAPI stained cells.

Determination of *bcl-2* and *bax* mRNA with Quantitative PCR (qPCR)

Total RNA was isolated (Promega total RNA system, Madison, WI) using the manufacturer's protocol. cDNA was reverse transcribed from 2.5 μ g total RNA in 50 μ L RT reaction mix at 37°C for 2 h using random hexamers as primers (High-Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA); cDNA (50 ng) was used for qPCR. Primers and Taqman probes for rat *bcl-2* and *bax* were designed from known sequences for rat *bcl-2* mRNA (NM016993) and *bax* (AF235993) according to the recommended criteria using Primer Express (Version 2.0, Applied Biosystem). For *bcl-2*, the sequences were 5'-TGG GAT GCC TTT GTG GAA CTA T-3' (forward primer), 5'-AGA GAC AGC CAG GAG AAA TCA AAC-3' (reverse primer) and 5'-FAM-TGG CCC CAG CAT GCG ACC TC-TAMRA (Probe), and the amplicon spanned from 808 to 875 bp. For *bax*, the sequences were: 5'-GGG TGG TTG CCC TTT TCT ACT-3' (forward primer), 5'-CCC GGA GGA AGT CCA GTG TC-3' (reverse primer) and 5'-FAM-ACT GGT GCT CAA GGC CCT GTG CA-TAMRA (Probe), and the amplicon spanned from 780 to 890 bp. qPCR was performed in ABI Prism 7000 DNA Detection System with probe and primer concentrations of 250 and 300 nM, respectively, in the final PCR reaction mix. Cycling variables were: 2 min at 50°C, 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Controls were performed under the same conditions, including No RNA template control and reverse transcription-negative control *bax* PCR primers and probe were validated using RNA isolated from thymus as positive control. Data were normalized to 18S RNA for each sample (18S Genomic Endogenous Control Kit; Eurogentec, North America, San Diego, CA) and expressed as a percentage of control values.

Immunoblotting

Western blotting was performed as described previously (16), with modifications. Cell culture extracts were lysed in buffer (50 mM Tris-HCl (pH 7.5), 0.1% SDS, 1% NP-40, 150 mM NaCl, 0.04% deoxycholic acid sodium salt, 5 mM EDTA, 50 mM NaF with protease inhibitors). Protein concentration was determined with a BCA kit (Pierce, Rockford, IL), separating 30 μ g of protein on 12% SDS-PAGE then transferred to nitrocellulose membrane. Anti-*bcl-2* antibody was diluted 1:1000, anti- β -actin 1:3000 and incubated at room temperature for 2 h. Membranes were incubated with anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immunoresearch, Plymouth, PA) for 1 h. Protein bands were visualized with ECL (Amersham Biosciences, Buckinghamshire, England), and optical density was quantified (MCID Imaging Research).

Statistical Analysis

All results are presented as mean \pm SEM. Statistical significance was determined by one-way ANOVA (SigmaStat 3.0), with *post hoc* correction using the Tukey multiple comparison test. For western blot optical densitometry, a Student-Newman-Keuls test was used. Student's *t*-

test was used for variables in which only a single comparison was necessary. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of PPBP on Glutamate or OGD-Induced Cell Death

PPBP treatment did not result in detectable cell death at concentrations of 5, 10, or 20 μM when applied for 24 h of normoxic conditions. However, high concentrations of PPBP (100 μM) for 24 h resulted in significant cell death (78.5% versus untreated cells) (data not shown). Glutamate exposure (10–100 μM) resulted in dose-dependent cell death that was ameliorated by 10 μM PPBP (Fig. 1). PPBP pretreatment completely prevented glutamate-induced cell death, while PPBP applied 15 min into glutamate exposure was ineffective (glutamate: $40 \pm 1\%$, glutamate + PPBP: $37 \pm 1\%$; $n = 3$). Two hours of OGD resulted in significant cell death as assessed 24 h postinjury. Pre-OGD treatment with PPBP (5, 10, or 20 μM) significantly attenuated neuronal injury (Fig. 2A). PPBP 10 μM post-ODG treatment, whether early (at 15 min of reoxygenation) or delayed (1 h of reoxygenation), also attenuated OGD-induced cell death (Fig. 2B). Preincubation with the σ_1 -receptor antagonist, rimcazole (1 or 5 μM) blocked the protective effect of PPBP treatment (Fig. 3).

Modulation of Postinjury *bcl-2* Gene Expression and TUNEL Positive Cells by PPBP

Under controlled conditions, Western blot analysis did not demonstrate any effect of PPBP alone on *bcl-2* protein expression (Fig. 4). To further characterize the effects of PPBP after OGD and glutamate-induced neuronal cell death, we used qPCR analysis of *bcl-2* and *bax* mRNA. Cells were treated in two ways: (1) 2 h OGD then recovered for 3 h with or without pretreatment with 10 μM PPBP or (2) 100 μM glutamate for 3 h with or without PPBP. PPBP treatment enhanced *bcl-2* mRNA expression after glutamate (3 h) or OGD (3 h). Figures 5A and B depicts *bcl-2* mRNA levels after normalization to 18SRNA, then expressed as a percentage of control values. In contrast, there were no changes observed in *bax* gene expression with PPBP in either condition. In companion experiments, pretreatment with PPBP blunted loss of *bcl-2* protein in OGD (2 h followed by 6 h recovery) (Fig. 6). PPBP-induced *bcl-2* preservation was abolished by treatment with rimcazole (Fig. 7). Lastly, PPBP reduced TUNEL-positive cells (Fig. 8) after OGD, suggesting fewer cells with overt DNA damage, (TUNEL-positive/DAPI stained cells after 2 h OGD and 24 h recovery: OGD $26 \pm 2\%$, OGD with PPBP $11 \pm 2\%$ $P < 0.05$).

Discussion

This study demonstrates two important and novel findings. First, the σ_1 -receptor agonist PPBP strongly attenuates neuronal injury *in vitro* after OGD or excitotoxic insult. Protection is specific to the σ_1 -receptor, as the selective antagonist rimcazole reverses PPBP's beneficial action. Second, PPBP enhances antiapoptotic *bcl-2* mRNA levels and preserves *bcl-2* protein load, an effect reversed by the inhibitor rimcazole. The effect was specific, in that no effect was observed in expression of proapoptotic *bax*. These data suggest that neuroprotection with PPBP is mediated by mechanism(s) involving *bcl-2* transcription and preservation of a favorable *bcl-2/bax* ratio in injured neurons.

First described almost three decades ago, σ -receptors are unique binding sites for selected endogenous and pharmacological ligands (17). The σ_1 -receptor has been cloned from human (18), mouse (19) and rat brain (20) has widespread labeling (21), and interacts with several excitatory neurotransmitter systems. We and others have repeatedly demonstrated ischemic neuroprotection *in vivo*, using well characterized rodent and nonrodent models of focal cerebral

ischemia (1–5). The present findings extend these *in vivo* observations to characterize PPBP's ability to block cell death after excitotoxic killing and energy failure *in vitro*.

Our previous work (1–3) strongly supports the potential for σ_1 -agonists as neuroprotective agents in ischemic stroke based on the observations that PPBP provides robust ischemic neuroprotection in three animal models (mouse, rat, and cat) of cerebral ischemia. Ischemic neuroprotection with NMDA receptor antagonists has long been the focus of human stroke clinical trials because of their robust neuroprotective effects in animal models. The limitation of these drugs has been their phencyclidine-like central nervous system effects, including hallucinations, agitation, confusion, and delirium. In our experimental work in the rat model of focal ischemia, although middle cerebral artery occlusion was associated with several alterations in behavior, treatment with PPBP had no effect on behavioral outcomes (1) over a 7-day observation period. In a previous study, histopathologic evaluation showed no evidence of neuronal dropout or morphologic changes between PPBP and vehicle-treated rats (22). Specifically, there were no morphologic changes observed in the cingulate gyrus and the retrosplenial cerebral cortex typically seen with NMDA toxicity (23).

In part, mechanisms that account for these neuroprotective properties may be linked to the concept that σ_1 -receptors modulate NMDA receptor activation (24). Previous studies indicate that σ -receptor stimulation inhibits ischemia-induced, presynaptic glutamate release (7). However, σ_1 /NMDA receptor interactions may be dependent on the physiologic response being studied, and on the brain region involved. Postsynaptic, receptor-mediated mechanism(s) have also been described, including inhibition of dopaminergic neurotransmission (8), attenuation of glutamate and NMDA induced (10), and ischemia-evoked NO production (5) and attenuation of postsynaptic glutamate-evoked Ca^{2+} influx (11). The present findings with rimcazole suggest that PPBP's protection in cortical neurons is transduced by the σ_1 receptor. Two known sub-types have been identified, σ_1 and σ_2 , based on regional distribution, enantiomeric selectivity, molecular weights, and downstream signaling partners (10). In the present study, we used PPBP as a prototypical σ_1 -receptor agonist with high subtype selectivity. The σ_1 -receptor agonist, (+) pentazocine, has likewise been shown to be effective in focal cerebral ischemia (25). However, the selectivity of many σ -receptor agonists are concentration-dependent. While both PPBP and (+) pentazocine are considered selective σ_1 -receptor agonists (21), (+) pentazocine, at high concentrations, inhibits the NMDA-receptor phencyclidine site (26). We have previously demonstrated that PPBP enhanced NO production *in vivo* is completely attenuated by the σ_1 -receptor antagonist 1-(cyclopropylmethyl)-4-(2'-oxoethyl) piperidine hydrobromide (DuP 734) indicating that the effects of PPBP are mediated via the σ_1 -receptor. The specific affinity of PPBP for σ_1 -receptor is not known. The IC_{50} and K_i for rimcazole for the binding sites for (+)-3-PPP [(3-hydroxyphenyl)-*N*-(1-propyl) piperidine]] is in the 1–2 μM range (13). Therefore, we sought in the present study to verify that PPBP attenuates neuronal injury in a dose-dependent fashion and that its actions are secondary to its direct interaction with the σ_1 -receptor.

We observed early in our study that PPBP attenuated the appearance of TUNEL-positive cells after OGD, and hypothesized that the agonist could be altering delayed cell death by elaborating pro-survival *bcl-2* or by suppressing *bax*. Although our data do not address transcriptional mechanisms by which postinjury *bcl-2* expression is enhanced by PPBP, the study is the first to demonstrate an interaction of σ_1 -receptor agonist with antiapoptotic gene expression. It is important to note that *bax* mRNA was completely unaltered by PPBP, while changes in *bcl-2* were readily evident. Therefore, it seems unlikely that changes in *bcl-2* transcripts or protein load are simply a function of enhanced numbers of live cells surviving due to PPBP. The present study cannot address no change in *Bax* RNA and protein concentration from our data. Unlike the *in vivo* literature, documented changes in *bax* as assessed in primary neuronal culture after “ischemia relevant” challenges are far from clear. In general, *bcl-2* and *bax* have

been less well studied in culture than *in vivo*. Furthermore, few investigations are available that evaluate *bax* mRNA or protein after OGD. More typical is the evaluation of serum deprivation, exposure to toxic apoptotic stimuli or exposure to oxidants or 3-NP. Others have reported no change in *bax* after these latter stimuli, e.g., after 3-nitropropionic acid challenge to rat cortical neurons (27), and to serum deprivation in mouse neurons (28).

Our data from primary cortical neurons agree with these studies in that postinsult *bax* mRNA levels as assessed by real-time PCR were not altered by PPBP. In contrast, studies that have used culture paradigms to follow *bax* translocation to the mitochondria during apoptosis have shown positive results, i.e., increases in *bax* in mitochondrial fraction. It may be that PPBP does alter translocation of *bax*, but our measurements do not allow us to comment on this possibility. Lastly, it is also possible that astrocytes provide a large amount of the *bax* signal when measurements are done *in vivo*. Others have shown that *in vitro* injury and ischemia alter *bax* levels in astrocytes, in a time-dependent manner (29).

In conclusion, these data obtained from *in vitro* cell injury models suggest novel downstream effects of PPBP application that are important to its neuroprotective properties. σ -receptor agonists such as PPBP have consistently shown efficacy across species and in varying animal models, perhaps due to multiple mechanisms involving presynaptic glutamate release and postsynaptic events, including expression of protective genes such as *bcl-2*.

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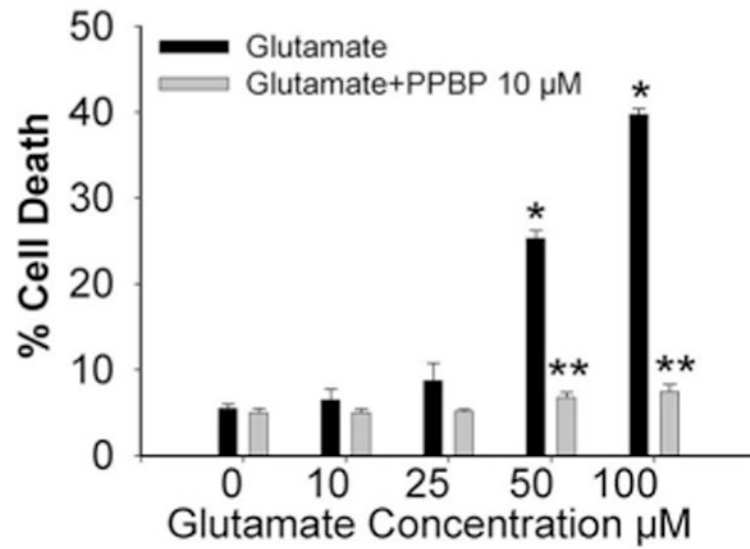


Figure 1.

Protective effect of 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) against glutamate-induced neurotoxicity in rat cortical cell cultures. Administration of PPBP (10 μM) 15 min prior to 24 h of glutamate exposure resulted in prevention of cell death at glutamate concentrations of 50–100 μM ($n = 5$). * $P < 0.05$ glutamate versus control, ** $P < 0.05$ glutamate versus PPBP.

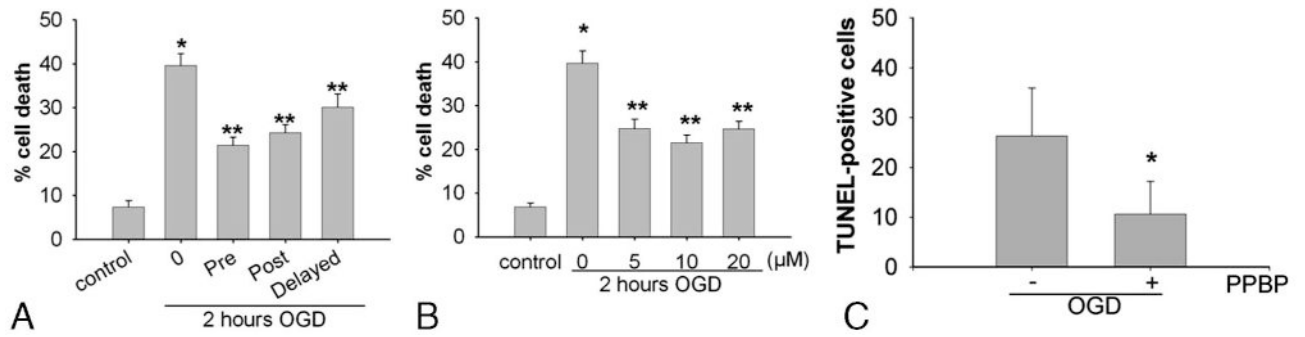


Figure 2.

A. Protective effects of 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) against oxygen-glucose deprivation (OGD)-induced neuronal injury. After 2 h of OGD, treatment with 5, 10, and 20 μM PPBP treatment initiated 15 min before OGD and continued for 24 h postreoxygenation reduced neuronal death. $*P < 0.05$ versus control and $**P < 0.05$ vs. 2 h OGD ($n = 5$). B. Protection conferred by pretreatment and posttreatment after 2 h of oxygen-glucose deprivation (OGD) followed by reoxygenation. Neuronal death was decreased by PPBP pretreatment (10 μM , initiated 15 min before OGD), early posttreatment (10 μM , initiated 15 min after reoxygenation) or delayed posttreatment (10 μM , initiated 1 h after reoxygenation). Cell death was assessed 24 h after OGD ($n = 5$). $*P < 0.05$ versus control and $**P < 0.05$ versus 2 h OGD. C. Effect of PPBP treatment with OGD on TUNEL-positive cells. $*P < 0.05$ versus without OGD treatment.

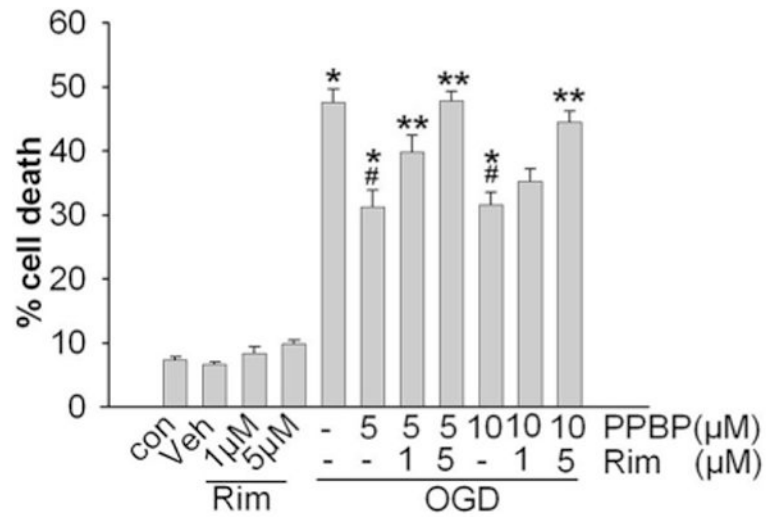


Figure 3.

Treatment with σ_1 receptor antagonist, rimcazole, reversed 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP)'s ability to reduce neuronal death after 2 h oxygen-glucose deprivation (OGD) and 24 h recovery ($n = 4$). Cells were treated with rimcazole for 2 h, then with PPBP 30 min prior to OGD. * $P < 0.05$ versus control, *# $P < 0.05$ versus OGD alone, ** $P < 0.05$ versus OGD + PPBP. Abbreviations: Con: Control; Veh: Vehicle treated; Rim: Rimcazole-treated.

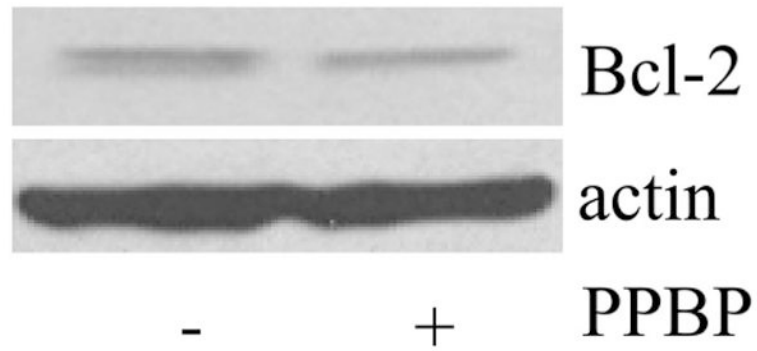


Figure 4. Western blot analysis did not demonstrate any effect of 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) alone on *bcl-2* protein expression.

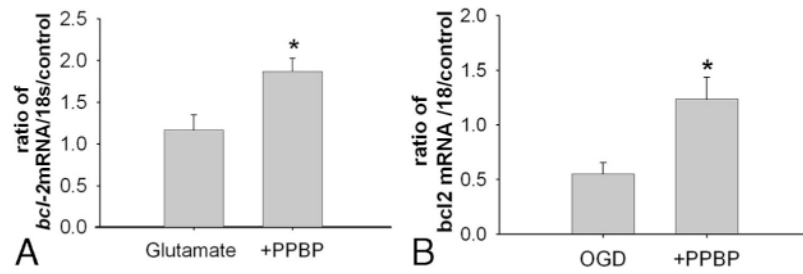


Figure 5.

A. Increased *bcl-2 mRNA* as measured by qPCR in cortical neurons pretreated with PPBP 10 μ M then exposed to glutamate. *bcl-2 mRNA* was normalized to 18S RNA and expressed as the percentage of control ($n = 6$). * $P < 0.05$ versus glutamate exposure. B. Increased *bcl-2 mRNA* as measured by qPCR in cortical neurons pretreated with 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) 10 μ M then exposed to oxygen–glucose deprivation (OGD) (2 h) with 3 h recovery. *bcl-2 mRNA* was normalized to 18S RNA and expressed as the percentage of control ($n = 5$). * $P < 0.05$ versus OGD.

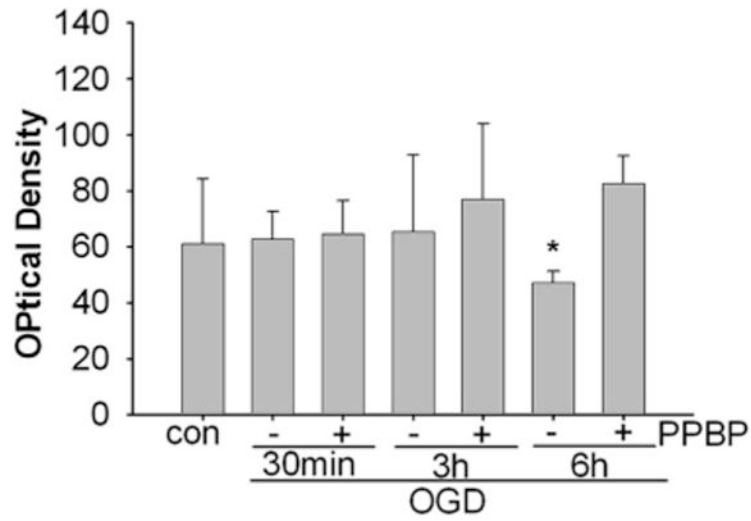


Figure 6. Increased *bcl-2* protein levels in 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP)- treated neurons exposed to 2 h oxygen–glucose deprivation (OGD) and 30 min, 3 or 6 h of recovery. Data are compiled from three separate experiments for each exposure and treatment condition. Protein levels are normalized to β -actin. * $P < 0.05$ from PPBP treatment at 6 h.

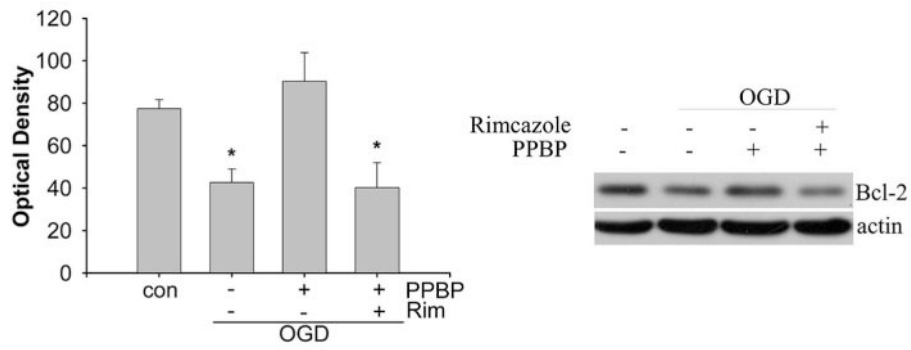


Figure 7. Right: Representative western blot showing preservation of *bcl-2* protein by 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) and reversal with rimcazole; neurons are exposed to 2 h oxygen–glucose deprivation (OGD) and 6 h of recovery. Left: Plot shows data compiled from three separate experiments for each treatment condition. Protein levels are normalized to β -actin. * $P < 0.05$ different from OGD with PPBP treatment group.

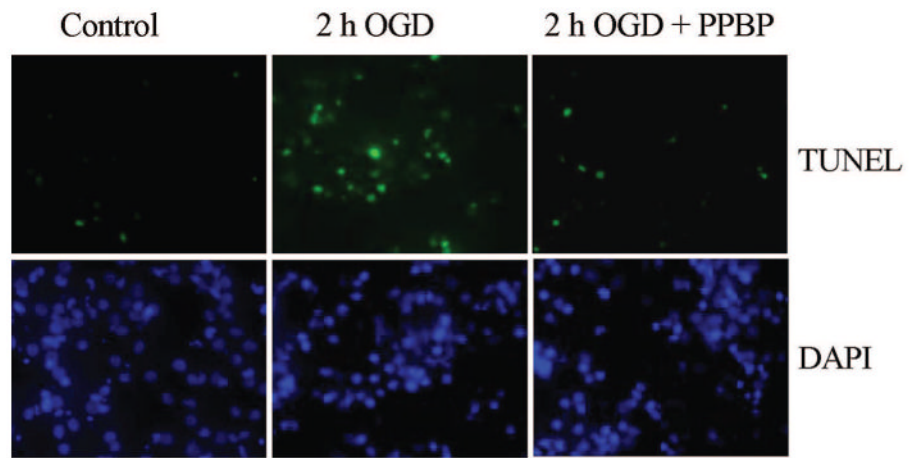


Figure 8. Representative photomicrograph of TUNEL staining with 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) treatment.