Published in final edited form as:

J Neuroendocrinol. 2010 October ; 22(10): 1072–1081. doi:10.1111/j.1365-2826.2010.02054.x.

Vasopressin protects hippocampal neurones in culture against nutrient deprivation or glutamate-induced apoptosis

Jun Chen and Greti Aguilera

Developmental Endocrinology Branch, National Institute of Child Health and Human Development, NIH, Bethesda MD 20892, USA

Abstract

Vasopressin (VP) secreted within the brain modulates neuronal function by acting as a neurotransmitter. Recent reports show that VP prevents serum deprivation-induced apoptosis in the neuronal cell line, H32. To determine whether VP is antiapoptotic in hippocampal neurones, primary cultures of these neurones were used to examine the effect of VP on neuronal culture supplement (B27) deprivation-, or glutamate-induced apoptosis, and the signaling pathways mediating the effects. Removal of B27 supplement from the culture medium for 24 hours or addition of glutamate (3 to 10 μ M) decreased neuronal viability ($P < 0.05$) and increased Tdt-mediated dUTP nick-end labeling (TUNEL) staining and caspase-3 activity ($P < 0.05$), which is consistent with apoptotic cell death. VP (10nM) reduced B27 deprivation- or glutamate-induced cell death ($P < 0.05$). These antiapoptotic effects of VP were completely blocked by a V1 but not a V2 receptor antagonist, indicating that they are mediated via V1 VP receptors. The antiapoptotic effect of VP in neurones involves activation of MAPK/ERK and IP3/Akt signaling pathways. This was shown by the transient increases in phospho-ERK and phospho-Akt after incubation with VP revealed by western blot analyses, and the ability of specific inhibitors to reduce the inhibitory effect of VP on caspase-3 activity and TUNEL staining by 70% and 35%, respectively ($p < 0.05$). These studies demonstrate that VP has antiapoptotic actions in hippocampal neurones, an effect which is mediated by the MAPK/ERK and PI3/Akt signaling pathways. The ability of VP to reduce nutrient deprivation or glutamate overstimulation-induced neuronal death suggests that VP acts as a neuroprotective agent within the brain.

Keywords

Vasopressin; V1 receptor; apoptosis; MAPK/ERK; PI3/Akt; hippocampus

INTRODUCTION

Vasopressin (VP) produced in magnocellular neurones of the hypothalamus is an important neuropeptide involved in water conservation, blood pressure control and pituitary ACTH secretion (1–3). In addition, VP neurones in the medial amygdala and the bed nucleus of the stria terminalis project to the lateral septum and ventral hippocampal sites affecting memory and behavior (4–5). Previous studies showed that VP has trophic actions in a variety of cell lines, primary cultures of neurones, and in vivo in the brain (6–8). Such trophic actions of VP have been implicated in the mechanism by which VP facilitates learning and memory in the hippocampus (9). The actions of VP are mediated through G-protein coupled receptors

located in the plasma membrane of target cells (1). There are two major VP receptor subtypes; V1 receptors, which are coupled to phospholipase C (PLC), increase intracellular Ca^{2+} and PKC activity (10), and transactivating the MAPK/extracellular signal-regulated kinase (Erk) and PI3 kinase/Akt pathways (11–12). V1 receptors mediate the effects of VP in the liver, smooth muscle and brain (13–14). The MAPK/ERK and PI3/Akt pathway are known to be involved in neuronal development, memory formation, synaptic plasticity and neuronal survival (15–17). On the other hand, V2 VP receptors, which are responsible for the effects of VP on water homeostasis in the kidney, are coupled to adenylyl cyclase/cAMP/protein kinase A-dependent pathways (10).

We have recently reported that VP prevents serum deprivation-induced apoptotic cell death (18) in the neuronal cell line, H32, which expresses endogenous V1 receptors. Vasopressin exerts this effect through activation of protein kinase C alpha and beta, the Erk/ribosomal S6 kinase (RSK) MAP kinase pathway, and the PI3 kinase/Akt pathways (18–19). These findings in the neuronal cell line H32 strongly suggest that VP has neuroprotective actions in the brain. To test this hypothesis, we used primary cultures of rat hippocampal neurones to examine the effects of VP on apoptotic cell death induced by removal of the neuronal culture supplement (equivalent to serum deprivation) or glutamate exposure. The results show that activation of endogenous V1 receptors by VP protects hippocampal neurones against culture supplement deprivation and glutamate-induced neuronal cell death. As in the cell line H32, the effect of VP involves multiple signaling pathways including MAPK/Erk and Akt pathways.

Materials and Methods

Materials

The PI3 kinase inhibitors, LY294002 and wortmannin, and the MEK inhibitors SL327 and U0126 were purchased from Calbiochem (San Diego, CA), and used at concentrations of 10 μM , 100 nM, 1 and 1 μM , respectively. Glutamate was purchased from Sigma (St. Louis, MO). Antibodies against phospho-p44/42 MAP Kinase (Thr202/Tyr204), p44/42 MAP Kinase, phospho-Akt (Ser473) and Akt were purchased from Cell Signaling TechnologyTM (Beverly, MA). The non-selective peptide V1 VP receptor antagonist, (Phenylac¹, D-Tyr(Et)², Lys⁶, Arg⁸, des-Gly⁹)-Vasopressin, which has similar affinity for V1a and V1b receptors (20), and the selective peptide V2 receptor antagonist, [d(CH₂)₅, D-Phe², Ile⁴, Ala⁹-NH₂]-vasopressin (21), were purchased from Bachem (Torrance, CA). Both of these peptides bind to the specific receptor subtype but are unable to signal, thus antagonizing the effects of VP by competing with the active ligand, VP.

Primary hippocampal neuronal cultures

Primary cultures of rat hippocampal neurones were prepared from e18 fetal rat brains. Pregnant Spague Dawley rats were killed by decapitation after CO₂ sedation. Fetal rats were rapidly harvested and decapitated with sharp scissors. Heads were collected in ice cold PBS and transported to the laboratory for brain removal and isolation of the hippocampus using a dissecting microscope. All animal procedures were approved by NICHD Animal Care and Use Committee. After 1h incubation with collagenase II, 1mg/ml (Worthington Biochemical Corp, Lakewood, NJ) dissolved in 25 mM Hepes (Invitrogen, Carlsbad, CA), pH 7.4, containing 137 mM sodium chloride (Sigma), 5 mM potassium chloride (Sigma), 0.7 mM dibasic sodium phosphate (Sigma), gentamicin 100 $\mu\text{g}/\text{ml}$ (Invitrogen), 4 mg/ml BSA (Invitrogen), 1.0 mg/ml glucose (Sigma) and 0.2 mg/ml DNase (Sigma, activity 500 U/mg), hippocampal cells were mechanically dissociated by gentle pipetting. Following filtration through a nylon gauze (BD, Franklin Lakes, NJ), dispersed neurones were plated into 100 mm plates (3×10^6 cell per plate) for caspase-3 activity assay, or six-well plates (5×10^5 cell

per well) for cell viability assay, or on glass coverslips coated with poly-L-lysine (Sigma) for immunofluorescence analysis. Cultures were maintained in DMEM/F12 medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 24 hours before changing into Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen). B27 is a defined culture medium supplement used instead of serum, especially designed for neuronal cultures. The supplement contains insulin, triiodo-L-thyronine, corticosterone, progesterone, fatty acids, antioxidants, putrescine and other nutrients (22). Medium was replaced every 2 or 3 days for an additional 10 days of culture. Five μ M cytosine arabinoside (Sigma) was added to the medium at day-3 of culture to suppress glial cell proliferation. At day 10 the majority of cells showed a neuronal phenotype, as shown by neurone-specific microtubule-associated protein 2 (MAP2) staining (1:500, Millipore, Billerica, MA). These highly enriched neuronal cultures (~90%) were used for all experiments. Neuronal cell apoptosis was triggered by removal of B27 supplement from the medium (which is equivalent to serum deprivation) or by exposure to glutamate (1–10 μ M).

Evaluation of neuronal cell number and viability

Following the experimental manipulations (B27 supplement deprivation or glutamate exposure in the presence or absence of VP and signaling inhibitors), the number of neuronal cells was counted from 3 different fields in each of three coverslips under a Leica DMRX fluorescence microscope with 10x magnification. Neuronal cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) colorimetric assay following standard procedures (23). Briefly, hippocampal neurones were incubated for 4 h at 37°C with 0.5 mg/ml MTT. In living cells, MTT is converted into insoluble formazan, in direct correlation with the number of viable cells. Cells were washed and formazan extracted with 1 ml isopropanol/1 M HCl (24:1) (Sigma), before measurement of absorbance values using a FLUOStar OPTIMA microplate reader (BMG Labtechnologies Inc, Durham, NC) at 550 nm.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) Assay

Apoptotic hippocampal neurones were detected using *In Situ* Cell Death Detection Kit reagents (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, hippocampal neurones grown on glass coverslips were fixed with 3% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 (Invitrogen) in PBS for 5 min, and incubated with the TUNEL reaction mixture for 1 h at 37 °C. Neurones were counterstained with propidium iodide (PI) (1:5000, Invitrogen) or 4',6-diamidino-2-phenylindole (DAPI) (1:10,000, Invitrogen). Photomicrographs from 4 different fields in each of three coverslips were captured under Zeiss LSM 510 inverted confocal microscope. Images were digitized (magnification 60X). Typically, ~300 cells were analyzed for the number of TUNEL-positive (apoptotic) staining.

Caspase-3 activity measurement

Caspase-3 activity was measured using a Caspase-3/CPP32 fluorometric protease assay kit (BioSource International, Inc., Camarillo, CA) according to the manufacturer's protocol. Cells were washed with PBS, centrifuged for 5 min at 800 × g, the supernatant removed and the pellet resuspended in ice cold lysis buffer. After 20 min incubation at room temperature, samples were centrifuged at 16,000 × g for 10 min at 4 °C, and protein concentrations in the supernatants determined using BCATM protein Assay (PIERCE, Rockford, IL). Aliquots containing 100 μ g of protein were incubated with substrate DEVD (Asp-Glu-Val-Asp)-7-amino-4-trifluoromethyl coumarin (AFC) (BioSource International, Inc) for 90 min at 37 °C. Upon cleavage of the substrate by Caspase-3, free AFC, which emits a yellow-green

fluorescence, was measured by using a FLUOStar OPTIMA microplate reader (BMG Labtechnologies Inc, Durham, NC), with a 405 nm excitation and 505 nm emission filter.

Western blot analysis

Western blot analysis was performed essentially as described previously. Briefly, cells were lysed with T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with proteinase and phosphate inhibitor cocktail (Sigma). Protein concentrations were determined by BCATM Protein Assay (Pierce) and 20 µg of protein were loaded and separated in a 4–20% SDS-PAGE (Invitrogen,). Proteins were transferred from the gel to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ), incubated with 5% nonfat dried milk in Tri-buffered saline (TBS plus 0.1% Tween-20 [TBST]) (Invitrogen) for 1h and incubated with phospho-p44/42 MAP Kinase (Thr202/Tyr204) or phospho-Akt (Ser473) antibody (Cell Signaling TechnologyTM, Beverly, MA) at a 1:1,000 dilution overnight. After washing in TBST, membranes were incubated for 2h with peroxidase-linked anti-Rabbit IgG (Thermo Fisher Scientific, Rockford, IL) at a 1:10,000 dilution or anti-mouse IgG (Thermo Fisher Scientific, Rockford, IL) at a 1:5,000. In order to correct for protein loading, the same membranes were stripped for 5 min at room temperature with stripping buffer (Thermo Fisher Scientific, Rockford, IL), and then blotted with p44/42 MAP Kinase or Akt antibody (Cell Signaling TechnologyTM, Beverly, MA). Detection of the immunoreactive band was performed by using ECL PlusTM reagents (Amersham Pharmacia Biotech) and exposure to BioMax MR film (Kodak, Rochester, NY). Densitometric quantification of the immunoblots was performed using the public domain NIH Image program (ImageJ 1.36b developed at the US National Institutes of Health, and available on the Internet at: <http://rsb.info.nih.gov/ni-image>). Changes in phospho-Erk and phospho-Akt were expressed as percent of basal values after correction for total Erk or Akt.

Data analysis

Statistical significance of the differences between groups was calculated by one-way or two-way analysis of variance (ANOVA), or by Student's *t* test for paired data, as appropriate. Statistical significance was set at $p < 0.05$. Data are presented as means $\pm$ standard error of the mean (SEM) from the values in the number of observations indicated in results or legends to Figures.

RESULTS

VP attenuates nutrient deprivation-induced apoptosis in primary cultures of hippocampal neurones

In the culture conditions employed, about 97% of the primary hippocampal cultures were positive for the neuronal marker MAP-2. Only a minor proportion of cells (~ 3%) were positive for the glial marker, GFAP (not shown). Incubation of hippocampal neurones for 24h under nutrient deprivation conditions, achieved by replacing the B27 supplement with 0.1% BSA, decreased the number of neurones to 54% of the values in B27 supplemented (+B27) control cultures (Fig. 1A). Consistently, MTT absorption was 64% of the values in +B27 controls (Fig. 1B), and the number of TUNEL positive cells 5.8-fold higher than in controls (Fig. 1C and D). Co-incubation of B27 deprived neurones with 10nM VP partially reversed these effects, increasing the number of neurones (Fig 1A) and neuronal viability as reflected by MTT absorption (Fig. 1B), and decreasing the number of TUNEL-stained cells (Fig. 1C and D), compared with the values in B27-deprived cultures without VP ($p < 0.05$ for all 3 parameters). To further demonstrate that VP exerts antiapoptotic actions in hippocampal neurones, we examined caspase-3 activity levels in control and nutrient-deprived cultures in the presence and absence of VP. As shown in Fig. 1E, removal of the

B27 supplement induced a 5-fold increase in caspase-3 activity ($p < 0.01$). This effect was significantly reduced by co-incubation with VP (62% of nutrient-deprived values, $p < 0.05$).

VP attenuates glutamate-induced apoptosis in primary cultures of hippocampal neurones

Similar to the effects of B27 supplement deprivation, incubation of hippocampal neurones with glutamate, 1 to 10 μM for 24 h, caused a concentration-dependent decrease in MTT absorption, with an IC_{50} of 3.6 μM (Fig. 2A). Co-incubation of glutamate-treated neurones with 10nM VP significantly reduced the effects of glutamate, increasing MTT absorption by 65% and 40% of the values with 3 μM and 10 μM , respectively (Fig. 2B). Glutamate at concentrations of 3 and 10 μM increased caspase 3 activity by 3 and 5 fold, respectively. As with MTT absorption, co-incubation with 10 nM VP decreases caspase 3 activity by 70% and 45% of the values in the presence of 3 μM and 10 μM glutamate alone, respectively (Fig. 2C). As shown in Fig 2D and 2E, glutamate also caused a marked increase in TUNEL staining. Co-incubation of neuronal cultures with 10 nM VP reduced glutamate induced TUNEL staining by 75% and 50% for 3 μM and 10 μM glutamate, respectively (Fig. 2D and 2E).

The antiapoptotic effect of VP is mediated via V1 VP receptors

To determine the vasopressin receptor subtype mediating the antiapoptotic effect of VP, we examined the effect of V1 and V2 receptor antagonists on the effects of VP on MTT absorption and caspase 3 activity in hippocampal neuronal cultures with B27 deprivation or exposure to glutamate, with or without 10 nM VP. Consistent with the data above, removal of the B27 supplement from the culture medium (Fig 3A) or 5 μM glutamate (Fig 3B) for 24h significantly reduced MTT absorption ($p < 0.001$) compared with control cultures, and addition of VP prevented this effect. As shown in Fig 3A and B, the highly selective V1 VP antagonist, (Phenylac¹, D-Tyr(Et)², Lys⁶, Arg⁸, des-Gly⁹)-Vasopressin (800nM), completely blocked the protective action of VP, while the selective V2 antagonist, [d(CH₂)₅, D-Phe², Ile⁴, Ala⁹-NH₂]-vasopressin (1 μM), had no effect. A similar V1 receptor dependence was shown for the effects of VP reducing caspase 3 activity in B27 supplement deprived cultures. As shown in Fig 3C, VP reduced B27 deprivation-induced caspase 3 activity by about 60%, and this effect was completely prevented by the V1 antagonist.

Signaling transduction pathways mediating the antiapoptotic effect of VP

Since we have previously demonstrated the involvement of the MEK/Erk MAPK and the PI3 kinase/Akt pathways in mediating the antiapoptotic effect of VP in the neuronal cell line H32, we examined the effect of VP on these pathways as well as the effect of the MEK and PI3 kinase inhibitors on the protective actions of VP in hippocampal neurones. Western blot analysis revealed time-dependent changes in phospho-Erk levels, with a marked increase by 5 min exposure to 10nM VP, followed by a decline to about 50% of these values by 30 min and remaining at these levels up to 1 h (Fig. 4A and B). VP also increased phospho-Akt levels with a slightly delayed and transient time course. Phospho-Akt levels reached a maximum by 10 min and declined to near basal levels by 30 and 60 min (Fig 4C and D).

The participation of the Erk/MAP kinase and IP3 kinase pathways in mediating the antiapoptotic effect of VP in hippocampal neurones, was studied using the MEK inhibitors, UO126 and SL327, and the PI3 kinase inhibitors, LY294002 and wortmannin on TUNEL staining and caspase 3 activity. As shown by the representative images in Fig 5-A and B, VP markedly reduced B27 deprivation-induced TUNEL staining in hippocampal neurones. The MEK inhibitors, UO126 (Fig 5A and B) and SL327 (not shown), markedly increased TUNEL staining on their own, and totally prevented the protective action of VP. The PI3 kinase inhibitor, LY294002, had no effect on TUNEL staining in neurones cultured with B27, but it significantly reduced the protective effect of VP (Fig 5A and B).

Consistent with the data in Fig 1, removal of the B27 supplement for 24h increased caspase 3 activity by 3.8-fold the control values (with B27 supplement). VP significantly reduced this effect to 1.8-fold the values in control cultures. As observed for TUNEL staining, addition of the MEK inhibitor, U0126, to cultures with B27 supplement increased caspase 3 activity by 3-fold on its own, and abolished the inhibitory effect of VP on B27 deprivation-induced caspase 3 activity (Fig 5C). Similar to the effects on TUNEL staining, the PI3 kinase inhibitor, LY294002 (10 μ M) had no effect on nutrient-deprivation induced caspase 3 activation on its own, but it reduced the protective effect of VP by 35%, $P < 0.05$. The PI3K inhibitor, wortmannin (100 nM), had the same effect, reducing the inhibitory effect of VP on caspase 3 activity by 38%, $p < 0.05$ (Data not shown). As shown in Fig 5-D, a similar attenuating effect of MEK and PI3 kinase inhibitors was observed on the protective action of VP against glutamate-induced caspase 3 activation. Glutamate 3 μ M caused a 4.2-fold increase in caspase 3 activity compared with control values in the absence of glutamate. Co-incubation with VP (10 nM) reduced this effect to 2.5-fold the control values. This protective effect of VP was attenuated by 45% when cultures were pre-incubated with the MEK inhibitor SL327 (1 μ M). Similarly, the PI3 kinase inhibitors, LY294002 (10 μ M) and wortmannin (100 nM) reduced the protective effect of VP by 35%.

DISCUSSION

The recent finding that activation of endogenous V1 receptors by VP prevents serum deprivation-induced apoptotic cell death in the neuronal cell line, H32, (18), prompted us to test the hypothesis that VP has antiapoptotic actions in the brain. Since stress induces VP secretion within the brain, and V1 VP receptors are expressed in neurones (5,24–27), it is likely that the peptide acts locally in neurones and exerts neuroprotective effects. An important brain region displaying both V1 VP receptors (25,28) and VP release is the hippocampus (24,29). A number of autoradiographic studies in the brain have mapped V1 VP receptors in CA1, CA2, CA3 pyramidal cell layer and granule cells in the dentate gyrus (30–31). The hippocampus is a critical site for learning, memory and glucocorticoid feedback, and its neurones are especially vulnerable to hypoxia-ischemia, seizure and prolonged stress (32). For these reasons, we sought to use primary cultures of hippocampal neurones as an in vitro model to investigate the potential role of VP as a neuroprotective agent. As shown by the present experiments, these neuronal cultures express functional VP receptors, as shown by the ability of VP to induce Erk and Akt phosphorylation. These neuronal cultures undergo apoptotic cell death following removal of the culture supplement, B27, or exposure to micromolar concentrations of glutamate, thus, providing a good in vitro system to study the effects of VP on apoptotic neuronal death. Using this system we were able to demonstrate that nanomolar concentrations of VP activation have protective effects against nutrient deprivation- or glutamate-induced apoptosis in hippocampal neurones, and that activation of the MAPK/Erk and PI3/Akt signaling pathways contribute to this effect of VP. The concentration of VP used in this study (10nM) was previously shown to exert maximal antiapoptotic effects in dose-response studies in the cell line H32, and it is likely to be achieved during synaptic release in the hippocampus (33). As shown by binding studies, this concentration of VP causes maximal receptor occupancy (10).

The fact that VP has antiapoptotic actions in highly enriched neuronal cultures containing less than 5% glial cells (after cytosine arabinoside treatment) strongly suggests that VP exerts its antiapoptotic actions directly by activating receptors in the neurone and not via glial cells or interactions with other neurotransmitters. However, previous studies in cultured astrocytes have shown that VP inhibits the expression of pro-inflammatory cytokines (34). Such an indirect effect on glial cells is also likely to contribute to neuroprotective effects of VP in the brain. It should be noted that in contrast to H32 cells in which VP totally prevented serum deprivation-induced apoptosis, VP was only partially protective in primary

cultures of hippocampal neurones. The lower effectiveness of VP probably reflects the lower proportion of hippocampal neurones containing V1 VP receptors, compared with a homogeneous cell line. Although it is not possible to exclude the possibility that VP could more effectively prevent neuronal death by partial B27 deprivation, a full protective effect is unlikely, since VP was not completely effective against a submaximal concentration of glutamate (Fig 2).

Glutamate, the most abundant excitatory neurotransmitter in the nervous system, is released from presynaptic nerve endings, and activates postsynaptic glutamate receptors, such as the NMDA, AMPA or mGluR1 receptors. Although glutamatergic transmission is essential for synaptic plasticity, cognition, learning and memory in the hippocampus and cortex (35–37), pathologically high levels of the neurotransmitter can lead to neuronal damage in the brain (36,38–39). Thus, overactivation of NMDA, AMPA or mGluR1 receptors may contribute to brain damage occurring acutely after epileptic crisis, cerebral ischemia or traumatic brain injury (39–40). It has been postulated that glutamate excess contributes to chronic neurodegeneration in disorders such as amyotrophic lateral sclerosis and Huntington's chorea (41–42). The concentration of glutamate used to induce neuronal death, TUNEL staining, and caspase 3 activation (3 μ M), was consistent with the reported concentrations of the neurotransmitter in the brain in microdialysis studies in rats (43). This glutamate concentration is in the range of the affinity of the NMDA receptor for glutamate (ED50 about 2 μ M) (44).

Surface expression of VP receptors was not measured in the primary neuronal cultures in these experiments. However, previous binding autoradiography, in situ hybridization and RT-PCR studies have shown expression of V1a and V1b but not V2 receptors in the hippocampus (9,25,28,31,45). The finding that the V1 but not a V2 receptor antagonist prevented the antiapoptotic effect of VP in hippocampal neurones indicates that the effect of VP is mediated by V1 receptors. Since no receptor subtype specific antagonists were used in the present study, the relative contribution of V1a or V1b receptors in mediating the antiapoptotic effect of VP in hippocampal neurones could not be determined. In the neuronal cell line, H32, which expresses mostly V1a and a small proportion of V1b receptors, pharmacological antagonism of both receptor subtypes is required to completely block the antiapoptotic effects of VP. In situ hybridization and electrophysiological studies have shown expression of both V1a and V1b receptors in hippocampal neurones (31,46). Thus, it is likely that both receptor subtypes contribute to the antiapoptotic effects of VP in hippocampal neurones described in this study.

V1 receptors are coupled to G_{q/11} (10,47–48), and upon activation leads to PLC-mediated phosphatidylinositol-4, 5-bisphosphate (PIP2) hydrolysis to IP3 and DAG. The increase in IP3 releases Ca²⁺ from the endoplasmic reticulum and activates CaMK, and DAG stimulates PKC activity. In addition, as shown by the present experiments and consistent with findings in other tissues and cell lines, VP also transactivates the EGFR in neurones leading to stimulation of the MAPK/Erk pathway (18,49). The ability of the MEK inhibitors, UO126 and SL327, to block the protective effect of VP on cell viability, TUNEL staining and caspase 3 activity, indicates that the antiapoptotic effects of VP involve the Erk/MAP kinase pathway. This is in agreement with previous observations in the neuronal cell line H32, in which activation of this pathway by VP partially prevented apoptotic cell death through phosphorylation of RSK and phosphorylation-inactivation of the pro-apoptotic protein Bad (18). It should be noted that two different MEK inhibitors increased TUNEL staining and caspase 3 activity on their own in the absence of glutamate or nutrient deprivation. This suggests that basal levels of Erk/MAP kinase activation are required for neuronal viability in the absence of a challenge. Since these inhibitors are not fully specific, a contribution of other kinases, such as the neuroprotective kinase, Erk 5 cannot be ruled out

(50). However, the fact that two structurally different inhibitors have the same effect renders this unlikely. In addition, a similar requirement for the Erk/MAP kinase pathway has been described in the dopaminergic cell line Mn9D, in which 24h exposure to UO126 or transfection with a MEK dominant negative decreases basal cell viability (51).

An additional V1 VP receptor-activated pathway involved in the antiapoptotic effect of VP in neuronal line, H32, is the PI3 kinase/Akt pathway (18,52), which has been shown to inactivate several pro-apoptotic molecules (53). Consistent with the effects in other systems (19,54), VP induces a rapid but transient phosphorylation of Akt in hippocampal neurones. The present demonstration that the PI3 kinase inhibitor, LY 294002, at a concentration about 7-fold the IC50, attenuates the protective effects of VP on caspase-3 activity and TUNEL staining, strongly suggest that activation of the PI3/Akt pathway is involved in the antiapoptotic effects of VP in hippocampal neurones. It has been reported that at this concentration the compound is specific for PI3 kinase and does not affect the activity of PI4 kinase, PKC, MAP kinase or c-Src (55), thus a non-specific effect is possible but unlikely. In addition, similar effects were observed by using another chemically unrelated PI3 kinase inhibitor, wotmannin.

A major signaling pathway triggered by V1 receptors is activation of PLC and PKC (10). Although the role of PKC was not examined in the present study, it is likely that PKC is also involved in the antiapoptotic actions of VP in hippocampal neurones. PKC could exert antiapoptotic effects indirectly through activation of the MAP kinase pathway, and by directly inhibiting pro-apoptotic pathways (56–57). Studies in the hypothalamic neuronal line H32 have shown subtype specific effects of PKC on neuronal survival, with PKC alpha and beta having antiapoptotic effects, and PKC delta being pro-apoptotic (19). In H32 cells, VP has no effect on PKC delta activity but stimulates PKC alpha and beta activity. The latter effect was shown to contribute to the stimulation of BAD phosphorylation and antiapoptotic actions of VP (18–19). Similar protective effects of VP have been shown in other systems (58–59).

While the present study provides clear evidence that VP has direct antiapoptotic actions in hippocampal neurones in culture, the actions of VP in the brain in vivo are more complex because of its vasoactive properties and effects on water transport (60–61). One report claims that VP given peripherally (i.p.) acts as a neuroprotective agent against magnesium chloride induced cerebral ischemia (62). Since magnesium chloride causes hypotension, it is likely that the effects of VP described in the latter report are mediated by the vasopressor actions of the peptide. It is also established that VP acting through V1 receptors, upregulates aquaporin 4 in glial cells contributing to water retention in the brain (63). In fact, V1a receptors have been implicated in the pathogenesis of brain edema and the subsequent development of brain damage following traumatic brain injury (56). Thus, it has been reported that V1a receptor antagonists are beneficial for the treatment of these conditions (64–65). Although elucidation of the relative importance of VP on brain function in different physiological and pathological situations will require further studies, it is clear that within its multiplicity of actions, VP can exert direct antiapoptotic effects in hippocampal neurones.

In summary, the data shows that in addition to its recognized actions on neuronal plasticity, VP has direct antiapoptotic actions in hippocampal neurones through activation of V1 receptors. This protective effect of VP in preventing apoptotic cell death is mediated by multiple signaling pathways, including phosphorylation-activation of MAPK/Erk and Akt. Since VP is released in the brain during stress conditions and VP V1 receptors are present in neurones at sites controlling behavior and learning, it is likely that the peptide plays a role as a neuronal protective agent. This novel function expands the potential range of actions of VP in the brain and supports a role of the peptide as a neuroprotective agent.

Acknowledgments

This work was supported by the Intramural Research Program of Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH.

References

1. Peter J, Burbach H, Adan RA, Lolait SJ, van Leeuwen FW, Mezey E, Palkovits M, Barberis C. Molecular neurobiology and pharmacology of the vasopressin/oxytocin receptor family. *Cell Mol Neurobiol.* 1995; 15:573–95. [PubMed: 8719042]
2. Aguilera G, Subburaju S, Young S, Chen J. The parvocellular vasopressinergic system and responsiveness of the hypothalamic pituitary adrenal axis during chronic stress. *Prog Brain Res.* 2008;17029–39.
3. Antunes-Rodrigues J, de Castro M, Elias LL, Valença MM, McCann SM. Neuroendocrine control of body fluid metabolism. *Physiol Rev.* 2004; 84(1):169–208. [PubMed: 14715914]
4. Alescio-Lautier B, Paban V, Soumireu-Moura B. Neuromodulation of memory in the hippocampus by vasopressin. *Eur J Pharmacol.* 2000; 405:63–72. [PubMed: 11033315]
5. Caffé AR, van Leeuwen FW, Luiten PG. Vasopressin cells in the medial amygdala of the rat project to the lateral septum and ventral hippocampus. *J Comp Neurol.* 1987; 261(2):237–52. [PubMed: 3305600]
6. Subburaju S, Aguilera G. Vasopressin mediates mitogenic responses to adrenalectomy in the rat anterior pituitary. *Endocrinology.* 2007; 148(7):3102–10. [PubMed: 17412807]
7. Hiroyama M, Wang S, Aoyagi T, Oikawa R, Sanbe A, Takeo S, Tanoue A. Vasopressin promotes cardiomyocyte hypertrophy via the vasopressin V1A receptor in neonatal mice. *Eur J Pharmacol.* 2007; 559(2–3):89–97. [PubMed: 17275806]
8. Thibonnier M, Conarty DM, Plesnicher CL. Mediators of the mitogenic action of human V(1) vascular vasopressin receptors. *Am J Physiol Heart Circ Physiol.* 2000; 279(5):H2529–39. [PubMed: 11045991]
9. Brinton RD, Monreal AW, Fernandez JG. Vasopressin-induced neurotrophism in cultured hippocampal neurons via V1 receptor activation. *J Neurobiol.* 1994; 25(4):380–94. [PubMed: 8077964]
10. Birnbaumer M. Vasopressin receptors. *Trends Endocrinol Metab.* 2000; 11(10):406–10. [PubMed: 11091117]
11. Volpi S, Liu Y, Aguilera G. Vasopressin increases GAGA binding activity to the V1b receptor promoter through transactivation of the MAP kinase pathway. *J Mol Endocrinol.* 2006; 36:581–90. [PubMed: 16720725]
12. Ghosh PM, Mikhailova M, Bedolla R, Kreisberg JJ. Arginine vasopressin stimulates mesangial cell proliferation by activating the epidermal growth factor receptor. *Am J Physiol Renal Physiol.* 2001; 280(6):F972–9. [PubMed: 11352836]
13. Winslow JT, Insel TR. Neuroendocrine basis of social recognition. *Curr Opin Neurobiol.* 2004; 14(2):248–53. [PubMed: 15082332]
14. Ostrowski NL, Lolait SJ, Bradley DJ, O'Carroll AM, Brownstein MJ, Young WS. Distribution of V1a and V2 vasopressin receptor messenger ribonucleic acids in rat liver, kidney, pituitary and brain. *Endocrinology.* 1992; 131(1):533–5. [PubMed: 1535312]
15. Zhao H, Shimohata T, Wang JQ, Sun G, Schaal DW, Sapolsky RM, Steinberg GK. Akt contributes to neuroprotection by hypothermia against cerebral ischemia in rats. *J Neurosci.* 2005; 25(42):9794–806. [PubMed: 16237183]
16. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem.* 2000; 268(20):14553–6. [PubMed: 8325833]
17. Sweatt JD. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem.* 2001; 76(1):1–10. [PubMed: 11145972]
18. Chen J, Volpi S, Aguilera G. Anti-apoptotic actions of vasopressin in H32 neurons involve MAP kinase transactivation and Bad phosphorylation. *Exp Neurol.* 2008; 211(2):529–38. [PubMed: 18402937]

19. Chen J, Liu Y, Soh JW, Aguilera G. Antiapoptotic effects of vasopressin in the neuronal cell line H32 involve protein kinase Calpha and beta. *J Neurochem.* 2009; 110(4):1310–20. [PubMed: 19519660]
20. Manning M, Stoev S, Kolodziejczyk A, Klis WA, Kruszynski M, Misicka A, Olma A, Wo NC, Sawyer WH. Design of potent and selective linear antagonists of vasopressor (V1-receptor) responses to vasopressin. *J Med Chem.* 1990; 33(11):3079–86. [PubMed: 2231609]
21. Manning M, Stoev S, Chini B, Durroux T, Mouillac B, Guillon G. Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents. *Prog Brain Res.* 2008; 170:473–512. [PubMed: 18655903]
22. Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res.* 1993; 35(5): 567–76. [PubMed: 8377226]
23. Hussain RF, Nouri AM, Oliver RT. A new approach for measurement of cytotoxicity using colorimetric assay. *J Immunol Methods.* 1993; 160:89–96. [PubMed: 8450240]
24. Orlowska-Majdak M, Traczyk WZ, Szymański D. Hippocampal vasopressin release evoked by N-methyl D-aspartate (NMDA) microdialysis. *Physiol Res.* 2003; 52 (3):373–82. [PubMed: 12790771]
25. Ostrowski NL, Lolait SJ, Young WS. Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. *Endocrinology.* 1994; 135(4):1511–28. [PubMed: 7925112]
26. Wotjak CT, Kubota M, Liebsch G, Montkowski A, Holsboer F, Neumann I, Landgraf R. Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotrophic hormone secretion? *J Neurosci.* 1996; 16(23):7725–32. [PubMed: 8922428]
27. Landgraf R, Wotjak CT, Neumann ID, Engelmann M. Release of vasopressin within the brain contributes to neuroendocrine and behavioral regulation. *Prog Brain Res.* 1998; 119:201–20. [PubMed: 10074790]
28. Szot P, Bale TL, Dorsa DM. Distribution of messenger RNA for the vasopressin V1a receptor in the CNS of male and female rats. *Mol Brain Res.* 1994; 24(1–4):1–10. [PubMed: 7968346]
29. Keck ME, Sillaber I, Ebner K, Welt T, Toschi N, Kaehler ST, Singewald N, Philippu A, Elbel GK, Wotjak CT, Holsboer F, Landgraf R, Engelmann M. Acute transcranial magnetic stimulation of frontal brain regions selectively modulates the release of vasopressin, biogenic amines and amino acids in the rat brain. *Eur J Neurosci.* 2000; 12(10):3713–20. [PubMed: 11029641]
30. Brinton RE, McEwen BS. Vasopressin neuromodulation in the hippocampus. *J Neurosci.* 1989; 9(3):752–9. [PubMed: 2564430]
31. Young WS, Li J, Wersinger SR, Palkovits M. The vasopressin 1b receptor is prominent in the hippocampal area CA2 where it is unaffected by restraint stress or adrenalectomy. *Neuroscience.* 2006; 143(4):1031–9. [PubMed: 17027167]
32. Bachevalier J, Vargha-Khadem F. The primate hippocampus: ontogeny, early insult and memory. *Curr Opin Neurobiol.* 2005; 15(2):168–74. [PubMed: 15831398]
33. Landgraf R, Malkinson TJ, Veale WL, Lederis K, Pittman QJ. Vasopressin and oxytocin in rat brain in response to prostaglandin fever. *Am J Physiol.* 1990; 259(5 Pt 2):R1056–62. [PubMed: 2240266]
34. Zhao L, Brinton RD. Suppression of proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha in astrocytes by a V1 vasopressin receptor agonist: a cAMP response element-binding protein-dependent mechanism. *J Neurosci.* 2004; 24(9):2226–35. [PubMed: 14999073]
35. Nishi N, Odagaki Y, Koyama T. Pharmacological characterization of metabotropic glutamate receptor-mediated high-affinity GTPase activity in rat cerebral cortical membranes. *Br J Pharmacol.* 2000; 130:1664–70. [PubMed: 10928972]
36. Meldrum BS. Glutamate as a Neurotransmitter in the Brain: Review of Physiology and Pathology. *Journal of Nutrition.* 2000; 130:1007S–15S. [PubMed: 10736372]

37. McEntee W, Crook T. Glutamate: its role in learning, memory, and the aging brain. *Psychopharmacology*. 1993; 111(4):391–401. [PubMed: 7870979]
38. Novelli A, Reilly JA, Lysko PG, Henneberry RC. Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. *Brain Res*. 1988; 451(1–2):205–12. [PubMed: 2472189]
39. Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci*. 1990; 13:171–82. [PubMed: 1970230]
40. Xu W, Wong TP, Chery N, Gaertner T, Wang YT, Baudry M. Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. *Neuron*. 2007; 53(3):399–412. [PubMed: 17270736]
41. Olney JW, de Gubareff T. Glutamate neurotoxicity and Huntington's chorea. *Nature*. 1978; 271(5645):557–9. [PubMed: 146165]
42. Shaw PJ, Ince PG. Glutamate, excitotoxicity and amyotrophic lateral sclerosis. *Neurol*. 1997; 244(Suppl 2):S3–14.
43. Lerma J, Herranz AS, Herreras O, Abaira V, Martin Del Rio R. In vivo determination of extracellular concentration of amino acids in the rat hippocampus. *Brain Res*. 1986; 384:145–155. [PubMed: 3790989]
44. Patneau DK, Mayer ML. Structure–activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J Neurosci*. 1990; 10:2385–99. [PubMed: 2165523]
45. Alfonso J, Pollevick GD, Castensson A, Jazin E, Frasch AC. Analysis of gene expression in the rat hippocampus using Real Time PCR reveals high inter-individual variation in mRNA expression levels. *J Neurosci Res*. 2002; 67(2):225–34. [PubMed: 11782966]
46. Raggenbass M. Overview of cellular electrophysiological actions of vasopressin 2008 583: 243–254. *Eur J Pharmacol*. 2008; 583:243–54. [PubMed: 18280467]
47. Thibonnier M. Signal transduction of V1-vascular vasopressin receptors. *Regul Pept*. 1992; 38(1): 1–11. [PubMed: 1533467]
48. Peter J, Burbach H, Adan RA, Lolait SJ, van Leeuwen FW, Mezey E, Palkovits M, Barberis C. Molecular neurobiology and pharmacology of the vasopressin/oxytocin receptor family. *Cell Mol Neurobiol*. 1995; 15:573–95. [PubMed: 8719042]
49. Ghosh PM, Bedolla R, Thomas CA, Kreisberg JJ. Role of protein kinase C in arginine vasopressin-stimulated ERK and p70S6 kinase phosphorylation. *J Cell Biochem*. 2004; 91(4):1109–29. [PubMed: 15048868]
50. Mody N, Leitch J, Armstrong C, Dixon J, Cohen P. Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. *FEBS Lett*. 2001; 502:21–4. [PubMed: 11478941]
51. Cavanaugh JE, Jaumotte JD, Lakoski JM, Zigmond MJ. Neuroprotective role of ERK1/2 and ERK5 in a dopaminergic cell line under basal conditions and in response to oxidative stress. *J Neurosci Res*. 2006; 84(6):1367–75. [PubMed: 16941494]
52. Vanhaesebroeck B, Alessi DR. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J*. 2000; 346(3):561–76. [PubMed: 10698680]
53. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*. 2005; 9(1):59–71. [PubMed: 15784165]
54. Pisitkun T, Jacob V, Schleicher SM, Chou CL, Yu MJ, Knepper MA. Akt and ERK1/2 pathways are components of the vasopressin signaling network in rat native IMCD. *Am J Physiol Renal Physiol*. 2008; 295(4):F1030–43. [PubMed: 18667481]
55. Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*. 1994; 269(7): 5241–8. [PubMed: 8106507]
56. Zirngiebel U, Ohga Y, Carter B, Berninger B, Inagaki N, Thoenen H, Lindholm D. Characterization of TrkB receptor-mediated signaling pathways in rat cerebellar granule neurons: involvement of protein kinase C in neuronal survival. *J Neurochem*. 1995; 65:2241–50. [PubMed: 7595513]
57. Tan Y, Ruan H, Demeter MR, MJC. p90(RSK) blocks bad-mediated cell death via a protein kinase C-dependent pathway. *J Biol Chem*. 1999; 274:34859–67. [PubMed: 10574959]

58. Nakajima T. Signaling cascades in radiation-induced apoptosis: roles of protein kinase C in the apoptosis regulation. *Med Sci Monit.* 2006; 12(10):RA220–4. [PubMed: 17006414]
59. Heasley LE, Johnson GL. Regulation of protein kinase C by nerve growth factor, epidermal growth factor, and phorbol esters in PC12 pheochromocytoma cells. *J Biol Chem.* 1989; 264:8646–52. [PubMed: 2785993]
60. Liu X, Nakayama S, Amiry-Moghaddam M, Ottersen OP, Bhardwaj A. Arginine-vasopressin V1 but not V2 receptor antagonism modulates infarct volume, brain water content, and aquaporin-4 expression following experimental stroke. *Neurocrit Care.* 2010; 12:124–31. [PubMed: 19806476]
61. Maturi MF, Martin SE, Markle D, Maxwell M, Burruss CR, Speir E, Greene R, Ro YM, Vitale D, Green MV. Coronary vasoconstriction induced by vasopressin. Production of myocardial ischemia in dogs by constriction of nondiseased small vessels. *Circulation.* 1991; 83(6):2111–21. [PubMed: 1904014]
62. Tyagi MG, Parthiban KV. Vasopressin mediates neuroprotection in mice by stimulation of V1 vasopressin receptors: influence of PI-3 kinase and gap junction inhibitors. *Indian J Exp Biol.* 2003; 41(6):574–80. [PubMed: 15266902]
63. Niermann H, Amiry-Moghaddam M, Holthoff K, Witte OW, Ottersen OP. A novel role of vasopressin in the brain: modulation of activity-dependent water flux in the neocortex. *J Neurosci.* 2001; 21(9):3045–51. [PubMed: 11312289]
64. Kleindienst A, Fazzina G, Dunbar JG, Glisson R, Marmarou A. Protective effect of the V1a receptor antagonist SR49059 on brain edema formation following middle cerebral artery occlusion in the rat. *Acta Neurochir Suppl.* 2006; 96:303–6. [PubMed: 16671476]
65. Trabold R, Krieg S, Schöller K, Plesnila N. Role of vasopressin V(1a) and V2 receptors for the development of secondary brain damage after traumatic brain injury in mice. *J Neurotrauma.* 2008; 25(12):1459–65. [PubMed: 19118456]

Fig. 1. Vasopressin (VP) protects hippocampal neuronal cultures against nutrient deprivation-induced apoptosis

Neuronal cell number (A) and cell viability (B) were measured following 24h incubation in neurobasal medium with B27 supplement (+B27), or without B27 supplement (-B27), in absence or in the presence of 10 nM VP (-B27+VP). Data are presented as percent of the controls (+B27). (C) and (D) TUNEL staining in primary cultures of hippocampal neurones following 24h incubation in neurobasal medium +B27, -B27 or -B27+VP. Neurones were counterstained with Propidium Iodide (PI). Bars represent the percentage of TUNEL positive stained (apoptotic) cells compared with +B27 controls (E) Caspase 3 activity in primary cultures of hippocampal neurones following 24h incubation in neurobasal medium +B27, -B27 or -B27+VP. All data are presented as the average $\pm$ S.E.M of the values obtained in 3 independent experiments conducted in duplicate. * $p < 0.05$, ** $p < 0.01$ compared to control (+B27) group; # $p < 0.05$ compared to -B27 group.

Fig. 2. Vasopressin (VP) protects hippocampal neuronal cultures against glutamate-induced apoptosis

(A) Glutamate caused a dose-dependent decrease in MTT absorption indicating decreased viability of hippocampal neuronal cultures. Co-incubation with 10nM VP reduced glutamate-induced decrease of neuronal cell viability (B) increases in caspase-3 activity (C) and TUNEL staining (D and E). Bars represent the average $\pm$ S.E.M of 3 independent experiments conducted in duplicate. * $p < 0.05$, compared to control group; # $p < 0.05$ compared to the corresponding glutamate group.

Fig. 3. The antiapoptotic effect of VP is mediated by V1 VP receptors

Hippocampal neurones cultured without B27 supplement (–B27), or in the presence of 5 uM glutamate for 24h were incubated with or without VP (10 nM). A selective V1 VP receptor antagonist (800 nM), or a selective V2 VP receptor antagonist (1uM), was added 30 min before VP or vehicle treatment. Cell viability measured as MTT absorption (A and B) and caspase 3 activity (C) were measured as described in “Materials and Methods”. Bars represent the mean $\pm$ S.E.M of the values obtained in three experiments conducted in duplicate. * $p < 0.05$, compared to –B27 (A and C) or glutamate (B) control group; # $p < 0.05$ compared to the corresponding –B27+VP (A and C) or glutamate +VP (B) control group.

Fig. 4.

Vasopressin (VP) caused a time-dependent increase in Erk phosphorylation (A and B) and Akt phosphorylation (C and D). Hippocampal neuronal cultures were incubated up to 60 min with 10 nM VP before protein preparation and western blot analysis of phospho-Erk (p-Erk) and phospho-Akt (p-Akt). Data points are the mean $\pm$ S.E.M of the values obtained in three experiments.

Fig. 5.

MAP kinase and PI3 kinase inhibitors reduce the antiapoptotic actions of VP in hippocampal neuronal cultures. The MEK inhibitor, UO126 (UO, 1 μ M), SL327 (SL, 1 μ M) or the PI3K inhibitor, LY294002 (LY, 10 μ M), Wortmannin (W, 100 nM) were added 15min before 24h culture of primary hippocampal neuronal cultures in neurobasal medium with (+B27) or without (-B27) B27 supplement, or glutamate exposure (Glu, 3 μ M), in absence or in the presence of 10 nM VP. TUNEL staining (A and B) and caspase 3 activity (C and D) were measured as described in "Materials and Methods". Bars represent the mean $\pm$ S.E.M of the values obtained in three experiments conducted in duplicate. In B and C, * $p < 0.05$, compared to +B27 vehicle group; # $p < 0.05$ compared to the corresponding -B27 group. & $P < 0.05$ compared to the -B27+VP vehicle group. In D, * $p < 0.05$, compared to control group; # $p < 0.05$ compared to Glu group. & $P < 0.05$ compared to the Glu+VP group.