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Norepinephrine and nitric oxide promote cell survival signaling in hippocampal neurons

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ABSTRACT

Nitric oxide (NO), physical exercise and/or antidepressant drugs, through the increased release of norepinephrine and brain-derived neurotrophic factor (BDNF), have been shown to exert profound protective, pro-survival effects on neurons otherwise compromised by injury, disease, prolonged stress, and subsequent depression *in vivo*. We sought, therefore, to evaluate such survival and neuroprotection in hippocampal neurons in culture, which, in an analogous model of in vivo cellular stress, was deprived of several vital nutrients. We assessed pro-survival outcomes following the application of norepinephrine or the noradrenergic partial agonist, clonidine, a general nitric oxide synthase inhibitor and NO donor, using a cell survival assay and quantitative Western blotting of the survival signaling molecules, BDNF, P-CREB, P-Akt, and P-MAPK in hippocampal neuronal lysates. We demonstrate that norepinephrine, clonidine, the NO donor and various combinations of these drugs increased cell survival and the immunoreactivity of the four survival signaling molecules in the face of nutrient deprivation stress, whereas the NO synthase inhibitor, and each of several survival signaling pathway inhibitors all decreased cell survival even below that of controls without nutrient supplementation. These results demonstrate that conditions that make cells vulnerable to environmental/toxic insult can be offset by norepinephrine and its related drugs or by NO donors and exacerbated by drugs that specifically inhibit a key survival signaling pathway. These results indicate that pharmacological intervention can promote neuroprotection and survival signaling in the face of nutrient withdrawal, but that this may require that several pathways remain intact.

1. Introduction

In the hippocampus, NO mediates cellular transduction mechanisms, regulates neuronal plasticity (Palumbo et al., 2007) and suppresses neuronal apoptotic cell death (Contestabile and Ciani, 2004). Thus, NO may be neuroprotective or restorative after a stroke (Endres et al., 2003; Hashiguchi et al., 2004; Zhang et al., 2001), traumatic brain injury (Lu et al., 2003), during Alzheimer's (Puzzo et al., 2005), and depression (Hua et al., 2008).

Voluntary exercise and antidepressant treatment have been demonstrated to enhance hippocampal BDNF expression (Russo-Neustadt et al., 1999), as well as cellular survival signaling activation under stress (Chen and Russo-Neustadt, 2005), and these actions appear to depend on noradrenergic (Chen et al., 2007; Garcia et al., 2003; Ivy et al., 2003), as well as on increased endothelial nitric oxide synthase, activity (Endres et al., 2003), thereby promoting the plasticity of hippocampal neurons (Manji et al., 2004). We have demonstrated that noradrenergic system activation may serve as an important neuroprotector, enhancing cell survival and elevating BDNF synthesis (Chen et al., 2007).

Norepinephrine promotes cell survival via augmentation of PI-3K activity both *in vivo* (Chen and Russo-Neustadt, 2005) and in the primary hippocampal neuronal cell culture model (Chen and Russo-Neustadt, 2007). Earlier evidence indicates that NO signaling plays an important role in the mechanism of norepinephrine-induced neuroprotection in both the *in vivo* and *in vitro* models (Chen et al., 2006; Chen and Russo-Neustadt, 2007; Lonart et al., 1992). Furthermore, norepinephrine-mediated increases in BDNF and Akt levels is cGMP-dependent (Chen and Russo-Neustadt, 2007), indicating that NO and cGMP both play an important role in norepinephrine-mediated cell survival.

The PI-3K/Akt pathway is a major mediator of cell survival signaling, leading to the transcription of many pro-survival genes (Brunet et al., 2001; Kang et al., 2004), such as increased BDNF expression (Almeida et al., 2005; Chen et al., 2007). BDNF-mediated protection against apoptosis is completely abolished by the inhibition of PI-3K (Hetman et al., 1999). Furthermore, evidence from our primary hippocampal neuronal cell culture indicates that norepinephrine stimulation leads to increased PI-3K pathway activation, which depends on overall NO availability, as such activation, as well as that of CREB (Ciani et al., 2002b; Contestabile and Ciani, 2004) decreases in the presence of nitric oxide synthase (NOS) inhibition (Chen and Russo-Neustadt, 2007).

Cell death can be facilitated by the withdrawal of vital nutrients (Tong et al., 2004; Zheng and Quirion, 2009), where such deprivation

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is known to decrease PI-3K activity, leading to apoptosis (Hetman and Xia, 2000). It is possible, therefore, that depriving cells of these important components *in vitro* may serve as a model and perhaps even imitate cellular compromises that may occur *in vivo*.

In this study, we demonstrate that clonidine is neuroprotective in the face of nutrient deprivation stress, and that this neuroprotective mechanism is at least partly NO-dependent. To test this hypothesis, we have evaluated both cell survival and the level of the pro-survival signaling molecules, Akt, MAPK, CREB and BDNF after clonidine or norepinephrine application under conditions where cells are deprived of important nutritional supplements and/or NO.

2. Materials and methods

2.1. Drugs and chemicals

Clonidine, norepinephrine, sodium nitroprusside (SNP), N-nitro-methyl-arginine (l-NAME), and 1H-[1, 2, 4]oxodiazolo[4, 3-a] quinoxalin-1-one (ODQ) were all purchased from Sigma Chemical Co. (St. Louis, MO). 2-(4-Carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) was purchased from A.G. Scientific Inc. (San Diego, CA). Two-(2-amino-3-methoxyphenyl)-4H-1-Benzopyran-4-one (PD98059) and 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyrene-4-one (LY294002) were purchased from Assay Designs (Ann Arbor, MI). Dulbecco's modified eagle medium (DMEM) was purchased from ATCC (Manassas, VA). And N2 was purchased from Invitrogen (Eugene, OR).

2.2. Hippocampal dissection at embryonic day 18 (E18)

Female rats (Sprague–Dawley, Charles River Corp., Wilmington, MA) were sacrificed with an overdose of isofluorane on their 18th day of pregnancy. The embryos were removed by cesarean section and immediately placed on ice. The two hippocampi of each embryo were excised and cultured in a Ca–Mg-free medium according to the method of Banker and Cowan (1977). The dissected hippocampi were then rinsed twice with 5–10 ml/rinse of Ca–Mg-free medium in 15-ml conical tubes. Trypsin (0.125%) was added to the Ca–Mg-free medium (~4–5 ml) and then the conical tube was placed in a water bath at 37 °C for 5–10 min with gentle inversion every 2–3 min. Neurons were quenched in 10% fetal bovine serum/DMEM (warmed to 37 °C, 1–2 volume ~8 ml) to stop the reaction and centrifuged at 200 *g* for 5 min. The supernatant was then removed and the pellet resuspended in ~2 ml serum-free DMEM. Neurons were then triturated (pipetting up and down mechanically for separation) with a silicote-coated constricted glass pipette and filtered through a 40-μm cell strainer (Falcon) into a sterile 50-ml conical tube. The volume was then supplemented with serum-free 1% N2/DMEM to a total volume of 5 ml. Trypan blue (10 μl) was then added to 10 μl of cells, which were counted using a hemacytometer. Neurons, which probably contained some glial cells, were then plated in poly-l-lysine-coated plates at a density of 50,000 cells/cm^2. Treatment of such mixed hippocampal neuronal cultures was conducted in duplicate where each experiment was performed at least twice.

Twenty-four hours following plating, l-NAME and SNP each at a final concentration of 500 μM (Chen and Russo-Neustadt, 2007) or CPTIO at a final concentration of 200 μM (Griffiths et al., 2003) were added to the designated cells. Cells were then returned to the incubator at 37 °C and 5% CO_2. On the third day following plating, clonidine (100 μM, Chen et al., 2007) or norepinephrine (100 nM) was added to the appropriate treatment wells, and plates were returned to the incubator for 6 h. For the inhibition experiments, 3 days after plating, ODQ (50 μM), PD98059 (10 μM), or LY294002 (10 μM) were added to the appropriate wells 2 h before norepinephrine or clonidine were applied and were allowed to incubate for an additional 2 h. At the end of the 6 h, the media in all plates were aspirated off, washed twice with plain DMEM, and half the wells of each treatment condition were given fresh 1% N2/DMEM, and the other half given only fresh DMEM (−N2). The nutrients contained in the N2 supplement include insulin, insulin-like growth factors, transferrin, putrescine and selenium: 1% N2/DMEM was removed, cells were washed twice in DMEM, which was then added to them. Thereafter, the plates were returned to the incubator (37 °C, 5% CO_2) for 48 h.

2.3. Cell death assay

Neuronal cell death was evaluated using the Live/Dead Cytotoxicity Kit (Invitrogen, Eugene, OR). Live *vs.* dead cells were counted in 3–4 separate visual fields per tissue culture well in 24-well plates. Briefly, the live/dead cytotoxicity kit consists of 2 components: calcein AM, which marks live cells and appears green under fluorescence and ethidium bromide homodimer, which infiltrates dead cells and appears red under fluorescence. For a 24-well plate, to 240 μl CMF, 6 μl calcein AM and 14 μl ethidium Br homodimer are added. Ten μl of this mixture is then added to each well (containing 500 μl) and allowed to incubate at 37 °C for 10 min. Each well of the plate is then examined under fluorescence and the green (live) *vs.* red (dead) cells are counted. For example, for one such treatment, we counted 33, 36, and 42 live (green) cells in 3 separate visual fields in one well; in this same well and same visual fields, 12, 11, and 11 dead (red) cells were counted. Then, the % live was calculated, using the number of live cells divided by (the number of live + dead cells) × 100. Thus, $33/(33+12)\times 100 = 73\%$; $36/(36+11)\times 100 = 77\%$; and $42/(42+11)\times 100 = 70\%$. This represented one triplicate, whose mean is 76%.

2.4. Griess reagent system for measuring nitrite concentration

Because nitrite is the stable indicator of NO release, the Griess Reagent Kit (Promega, Madison, WI) was used for measuring nitrite levels released into the media from each set of treatments.

2.5. SDS-PAGE/Western blotting

2.5.1. Antibodies

Anti-BDNF was purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-phospho-CREB, anti-CREB, anti-phospho-T308-Akt, and anti-Akt were purchased from Cell Signaling Technology (Danvers, MA). And anti-phospho-MAPK, anti-MAPK, and anti-tubulin were purchased from Millipore (Temecula, CA). Secondary antibodies, anti-rabbit IgG and anti-mouse IgG, were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ).

Forty-eight hour following N2-deprivation, the media was aspirated off, 75 μl lysis buffer (10 mM tris, pH 7.4, 1% SDS) per well was added, the cells scraped from their wells, boiled for 5 min, tricturated through a 26-gauge needle, centrifuged at 14,000 *g* for 5 min, and an equal volume of gel-loading buffer (Laemmli, 1970) added to the supernatant. Samples were stored at −80 °C until ready for SDS-PAGE/Western blotting.

Equal amounts of cell lysates were applied to each lane of an SDS-PAG, electrophoresed, and the separated proteins subsequently electro-transferred to nitrocellulose (Amersham-Pharmacia Biotech, Piscataway, NJ). Western blotting on nitrocellulose membranes was performed according to the specifications of the respective manufacturers. Protein immunoreactivity was visualized by enhanced chemiluminescence (ECL), followed by apposition of blots to hyperfilm (Amersham-Pharmacia Biotech, Piscataway, NJ). To control for inadvertent differences in protein loading, all blots were then stripped in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM tris–HCl, pH 6.7, at 55 °C for 10 min, with agitation and then re-probed with the antibody against the respective total form (anti-Akt, anti-MAPK, or anti-CREB), followed by ECL and apposition to hyperfilm. Blots first probed with anti-BDNF were stripped and then re-probed with anti-tubulin. Blots first probed with the phospho-forms, followed by the total forms, were

then stripped once more and re-probed with anti-tubulin. All anti-tubulin reactions were then reacted once again with ECL and apposed to hyperfilm.

Optical densities of lightly exposed phospho-proteins, BDNF, total forms (e.g., total CREB), and tubulin bands corresponding to the relative mobility of each respective signaling protein were quantified using computer-assisted densitometry (MCID, Interfocus Imaging Ltd, London). Optical densities of the phospho-forms were then arithmetically divided by those of their respective total forms; this quotient was then arithmetically divided by the respective tubulin band. Optical density of BDNF bands was arithmetically divided by those of their respective tubulin bands. All exposed bands quantified within the linear range of a standard curve were used in gathering densitometry data.

2.6. Statistical analyses

With respect to values obtained with controls (vehicle-treated in the presence of N2), data were then statistically normalized to 1.00 for the Griess Nitrite Assay and for Western blotting or to 100.00 for the live/dead cytotoxicity assay. Statistical analyses were implemented using 2-way ANOVAs (drug × N2), followed by Fisher's post-hoc LSD test for each of the 5 outcome measures of cell survival, BDNF, P-CREB, P-Akt, and P-MAPK Westerns and a 1-way ANOVA, followed by Fisher's post-hoc LSD test for nitrite (NO) levels. To determine the extent of co-variation among cell survival signaling molecules, including cell survival itself under both N2+ and N2− conditions, we opted to analyze our data using Hedge's effect sizes ($g = (\text{mean}_1 - \text{mean}_2)/\text{pooled standard deviation}$ (Kline, 2005)), where mean_1 = the mean of optical density values of, say, BDNF immunoreactivity resulting from treatment with, say, clonidine. And mean_2 = the mean optical density of, say, BDNF immunoreactivity resulting from treatment with the vehicle (controls); mean_2 is always the vehicle. Thus, the comparisons were made of the optical densities of each of the 4 signaling molecules and that of cell survival between vehicle-treated cells (mean_2) and the cells of and each drug-treatment (mean_1) in the presence of N2 and absence of N2. Pearson correlations were then calculated between the effect sizes yielded by the vehicle-treated *vs.* other treatments for both media conditions (with or without N2) of BDNF, P-CREB, P-Akt, P-MAPK, and cell survival.

3. Results

3.1. Cell survival

There was a statistically significant drug × N2 interaction in cell survival [$F(17, 162) = 22.74$, $P<0.0001$]. Thus, cell survival was significantly higher in vehicle-treated neurons in the presence than in the absence of N2 supplements [$F(1, 162) = 2316$, $P<0.0001$; Fig. 1]. In the presence of N2 supplements, clonidine ($P<0.0001$), norepinephrine ($P<0.0001$), SNP ($P<0.0001$), or the combinations of SNP + clonidine ($P<0.0001$) and SNP + norepinephrine ($P<0.0001$) significantly increased cell survival above that of vehicle-treated controls by about 30% /INS; [$F(17, 162) = 91.44$, $P<0.0001$; Fig. 1A], indicating a significant drug effect. Conversely, the inhibitors, l-NAME ($P<0.0001$), PD98059 ($P<0.0001$), ODQ ($P<0.0001$) and LY294002 ($P<0.0001$) decreased cell survival by ~10–15% (Fig. 1B). When combined with ODQ, PD98059, or LY294002, clonidine did not increase cell survival above that of vehicle-treated controls (Fig. 1B); however, clonidine or norepinephrine was still able to increase cell survival ~15% above that of vehicle-treated cells when combined with both l-NAME and CPTIO to about 20% (Fig. 1C). Without N2, clonidine ($P<0.0001$), norepinephrine ($P<0.0001$), SNP ($P<0.0001$), but neither the SNP + clonidine nor the SNP + norepinephrine combination significantly increased cell survival above that of vehicle-treated controls (Fig. 1A, C). And norepinephrine increased cell survival even above that of N2-supplemented controls to levels ($P<0.0001$) comparable to that of N2-supplemented levels of

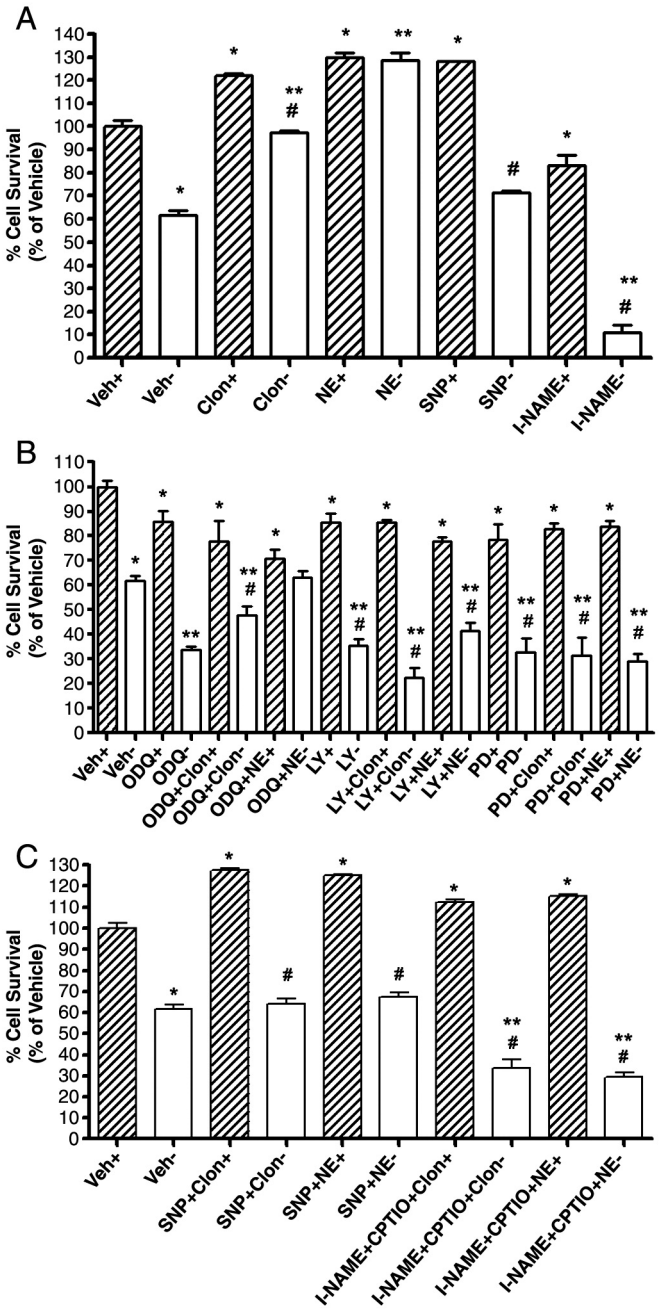

Fig. 1. Noradrenergic activation and exogenous NO donation both augment neuronal survival, which can be reversed by the inhibition of any one of several key survival-promoting pathways. Clonidine, norepinephrine, SNP, and the combination treatment, SNP + clonidine and SNP + norepinephrine each increase neuronal survival above that of controls (A), whereas NOS inhibition (by l-NAME), guanylate cyclase, PI-3K, and MAPK inhibition (by ODQ, LY294002, and PD98059, respectively) all decrease cell survival below that of controls (B). In the face of nutrient deprivation, both clonidine and norepinephrine, but not SNP, rescued cell survival above that of controls deprived of N2 (each $P<0.05$). The four inhibitors, l-NAME, ODQ, LY294002, and PD98059, all suppressed cell survival far below that of controls deprived of N2 (B; each $P<0.05$). Without N2, inclusion of CPTIO with l-NAME + clonidine or l-NAME + norepinephrine resulted in significantly decreased cell survival (C). Bars represent the mean (±SEM) of at least 2 experiments with each sample conducted in triplicate or quadruplicate. Striped bars represent the plus (+) N2 condition; white bars represent the minus (−) N2 condition. For convenience, the Vehicle+ and Vehicle− conditions are reproduced in all 3 panels (A, B, C). *, statistically and significantly different from the Veh+ condition at $P<0.05$. #, statistically and significantly different from the corresponding N2+ condition at $P<0.05$. **, statistically and significantly different from the Veh− condition at $P<0.05$. Veh, vehicle; Clon, clonidine; NE, norepinephrine; PD, PD98059; and LY, LY294002.

norepinephrine treatment (Fig. 1A). Conversely, ODQ ($P<0.0001$), LY294002 ($P<0.0001$), and PD98059 ($P<0.0001$), whether clonidine or norepinephrine was present or not, managed to keep cell survival significantly lower than that of vehicle-treated controls (Fig. 1B). However, when paired with norepinephrine, ODQ was no longer able to suppress cell survival, compared to that of vehicle-treated controls (−N2) (Fig. 1B).

3.2. Nitrite levels

Neither clonidine nor norepinephrine alone resulted in nitrite levels (and therefore, NO levels) that were different from vehicle-treated control cells (Fig. 2). Expectedly, the NO donor, SNP, revealed a ~2.5-fold increase in nitrite levels, while l-NAME, as a NOS inhibitor, decreased nitrite levels, compared to those of controls [$F(10, 66) = 257$, $P<0.0001$, Fig. 2]. Further, when combined with SNP, norepinephrine substantively increased nitrite levels above those of not only vehicle-treated ($P<0.0001$), but also, SNP-treated ($P<0.001$) cells, whereas clonidine in combination with SNP had no effect on SNP-induced nitrite levels (Fig. 2). And when combined with l-NAME, although both clonidine ($P<0.0001$) and norepinephrine ($P<0.0001$) resulted in small, but significant, increases in nitrite production (Fig. 2), the inclusion of the NO scavenger, CPTIO, significantly decreased nitrite levels well below those of controls.

3.3. Neuron Survival Signaling — Immunoblotting

3.3.1. BDNF

There was a statistically significant drug × N2 interaction in BDNF immunoreactivity [$F(18, 135) = 2.30$, $P = 0.005$]. BDNF immunoreactivity was significantly higher in vehicle-treated neurons in the presence than in the absence of N2 supplements [$F(1, 135) = 24.1$, $P<0.0001$; Fig. 3A]. In the presence of N2 supplements, clonidine ($P<0.0001$), norepinephrine ($P<0.0001$), and SNP ($P<0.0001$) all up-regulated, whereas all inhibitors (l-NAME ($P<0.0001$), CPTIO ($P<0.0001$), ODQ ($P<0.0001$), PD98059 ($P<0.0001$), and LY294002 ($P<0.0001$) substantively down-regulated BDNF immunoreactivity with respect to vehicle-treated controls [$F(18, 135) = 6.05$, $P<0.0001$; Fig. 3A, B]. Pairing of clonidine or norepinephrine with these inhibitors resulted in significant restoration of BDNF immunoreactivity to levels comparable to (clonidine + PD98059, clonidine + LY294002, norepinephrine + PD98059), or higher than (clonidine + ODQ, norepinephrine + ODQ) that of the vehicle alone ($P<0.0001$) (Fig. 3A, B). Combining clonidine ($P<0.0001$) or norepinephrine ($P<0.0001$) with both kinds of NO inhibitors (l-NAME + CPTIO) significantly decreased BDNF immunoreactivity below that of vehicle-treated controls (Fig. 3C). Furthermore, exogenously added NO (SNP) + clonidine or norepinephrine significantly increased, but complete NO deprivation (l-NAME + CPTIO) + clonidine or norepinephrine decreased BDNF immunoreactivity, compared to those of vehicle-treated (+N2) cells (Fig. 3C).

Without N2, clonidine ($P<0.0001$), norepinephrine ($P<0.0001$), and SNP all restored BDNF immunoreactivity above these N2-deprived vehicle-treated cells, but still less than cells treated with one of these respective drugs in the presence of the supplements (Fig. 3A). None of these three compounds, however, were able to restore BDNF immunoreactivity in the face of both N2 deprivation and any of the inhibitors used (Fig. 3B, C). However, even without N2, norepinephrine in the presence of l-NAME + CPTIO was still able to increase BDNF immunoreactivity significantly above that of vehicle-treated controls (−N2, ($P<0.0001$)), whereas this result was not observed with clonidine (Fig. 3C).

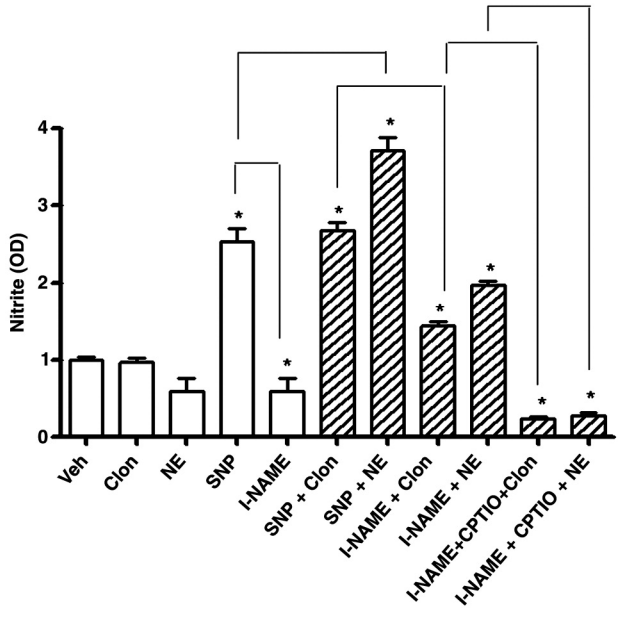

Fig. 2. Noradrenergic activation stimulates NO production in the presence of a NO donor. SNP increased, whereas l-NAME decreased nitrite (an indicator of NO) levels with respect to control levels. When applied alone, neither clonidine nor norepinephrine increased nitrite (and therefore NO) levels with respect to that of controls; but when combined with SNP, norepinephrine, increased NO levels above those of controls. Brackets indicate statistically significant differences ($P<0.05$) between the indicated treatments. Veh, vehicle; Clon, clonidine; and NE, norepinephrine.

3.3.2. P-CREB

There was a statistically significant drug × N2 interaction in P-CREB immunoreactivity [$F(18, 94) = 2.53$, $P<0.0001$]. P-CREB immunoreactivity was significantly higher in vehicle-treated neurons in the presence than in the absence of N2 supplements [$F(1, 94) = 75.91$, $P<0.0001$; Fig. 4A]. In the presence of N2 supplements, clonidine ($P<0.0001$), norepinephrine ($P<0.0001$), and SNP ($P<0.0001$) all significantly up-regulated, whereas none of the inhibitors evaluated (l-NAME, CPTIO, ODQ, PD98059, and LY294002) had any effect on P-CREB immunoreactivity, compared to levels of vehicle-treated controls [$F(18, 94) = 11.59$, $P<0.002$; Fig. 4A, B]. Pairing of clonidine, norepinephrine, or SNP with these inhibitors increased P-CREB immunoreactivity to levels comparable to or higher than those of vehicle alone (Fig. 4B, C). Pairing of clonidine ($P<0.0001$) or norepinephrine ($P<0.0001$) with l-NAME and the NO scavenger, CPTIO, resulted in a 4–5-fold increase in P-CREB immunoreactivity (Fig. 4C).

Without N2, clonidine ($P<0.0001$), but neither norepinephrine nor SNP restored P-CREB immunoreactivity above these N2-deprived vehicle-treated cells by up to 2.5-fold (Fig. 4A), but still significantly less than cells treated with clonidine in the presence of N2. None of these three compounds, however, were able to restore P-CREB immunoreactivity in the face of both N2 deprivation and any of the inhibitors used (l-NAME, ODQ, PD98059, LY294002; Fig. 4B, C). It is noteworthy that under N2(−) conditions, clonidine alone significantly increased P-CREB immunoreactivity to levels higher than those of the SNP + clonidine combination (Fig. 4A, C).

3.3.3. P-Akt

P-Akt immunoreactivity was significantly higher in vehicle-treated neurons in the presence than in the absence of N2 supplements [$F(1, 94) = 0.524$, $P = 0.471$; Fig. 5A]. In the presence of N2 supplements, clonidine ($P<0.0001$), norepinephrine ($P<0.0001$), and SNP ($P<0.0001$) all significantly up-regulated, whereas all inhibitors (l-NAME ($P<0.0001$), ODQ ($P<0.0001$), PD98059 ($P<0.0001$), and LY294002 ($P<0.0001$) significantly down-regulated P-Akt immunoreactivity with respect to vehicle-treated controls [$F(18, 94) = 8.11$, $P<0.0001$; Fig. 5A, B]. In most cases, pairing of clonidine, norepinephrine, or SNP with each of the inhibitors still resulted in less P-Akt activation than that of vehicle-treated cells (Fig. 5B); only the clonidine + PD98059 combination

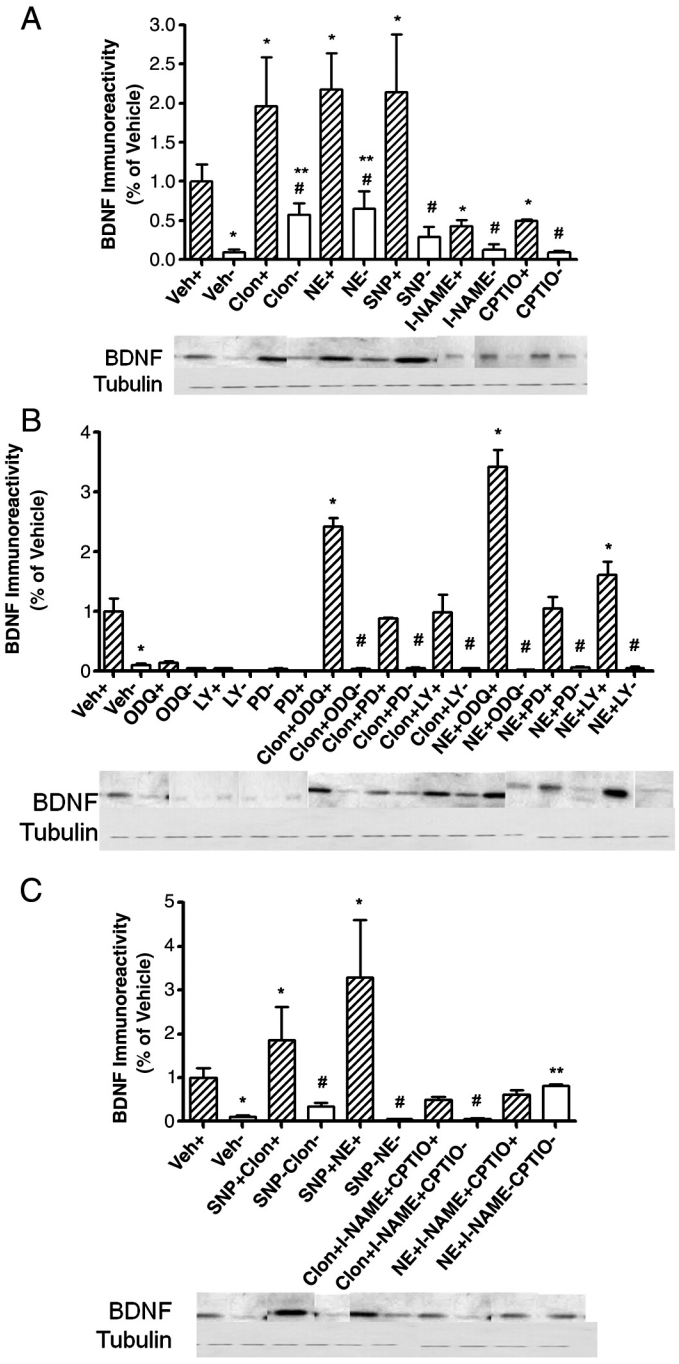

Fig. 3. Clonidine, norepinephrine and exogenous NO increase BDNF immunoreactivity and are neuroprotective in the face of nutrient deprivation. In the presence of N2 supplements, clonidine, norepinephrine, and SNP all up-regulated (A), whereas all inhibitors evaluated (l-NAME, CPTIO, ODQ, PD98059, and LY294002) tended to down-regulate BDNF immunoreactivity with respect to vehicle-treated controls (A, B). Pairing of clonidine, norepinephrine, or SNP with these inhibitors resulted in significant restoration of BDNF immunoreactivity to levels comparable (clonidine + PD98059, clonidine + LY294002, norepinephrine + PD98059, l-NAME + clonidine + CPTIO) or higher [(clonidine + ODQ, $P<0.0001$; norepinephrine + ODQ, $P<0.0001$; (C)] than that of vehicle alone. Bars represent the mean ± SEM) of at least 3 experiments with each sample conducted in duplicate or triplicate. Striped bars represent the plus (+) N2 condition; white bars represent the minus (−) N2 condition. For convenience, the Vehicle+ and Vehicle− conditions are reproduced in all 3 panels (A, B, C). *, statistically and significantly different from the Veh+ condition at $P<0.05$. #, statistically and significantly different from the corresponding N2+ condition at $P<0.05$. **, statistically and significantly different from the Veh− condition at $P<0.05$. Veh, vehicle; Clon, clonidine; NE, norepinephrine; PD, PD98059; and LY, LY294002.

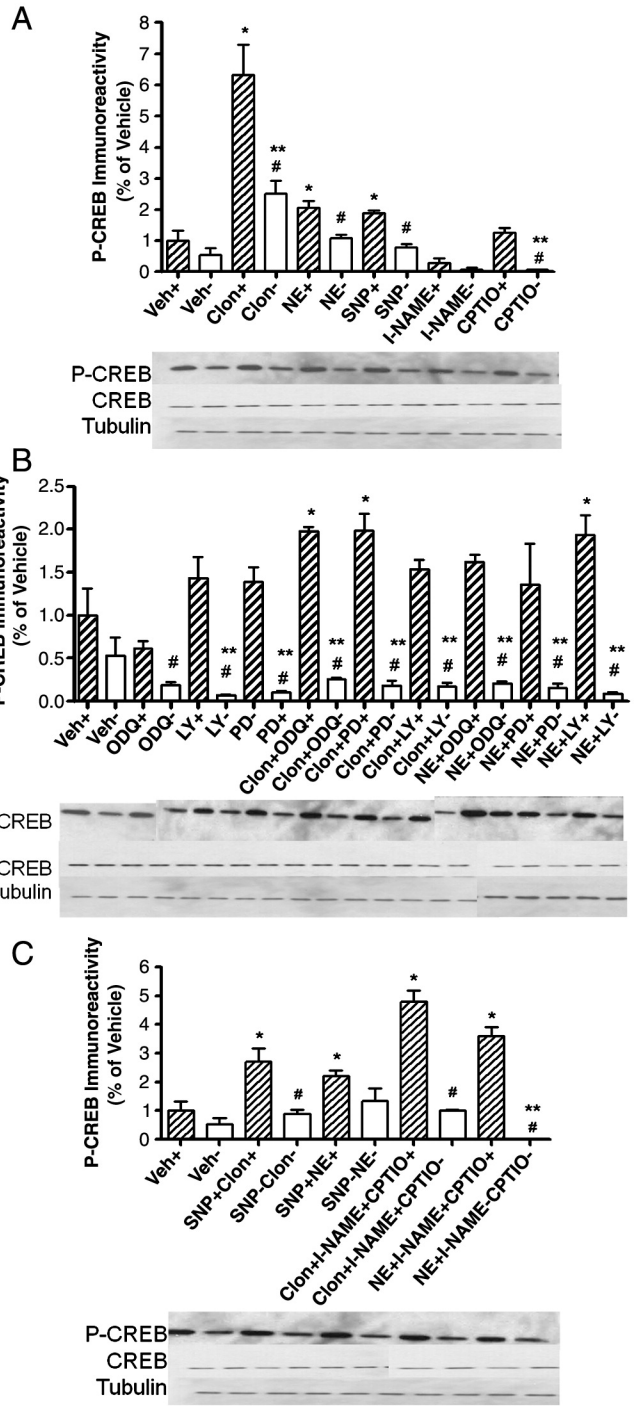

Fig. 4. Clonidine, norepinephrine, and exogenous NO increase CREB phosphorylation. In the presence of N2 supplements, clonidine, norepinephrine, and SNP all up-regulated (A), whereas all inhibitors evaluated (l-NAME, CPTIO, ODQ, PD98059, and LY294002) did not allow P-CREB immunoreactivity to increase with respect to vehicle-treated controls (A, B). Pairing SNP with clonidine or norepinephrine resulted in increases in P-CREB immunoreactivity levels comparable to each of these drugs applied alone (C). Without N2 supplements, vehicle-treated cells expressed substantively less P-CREB than that of N2-supplemented controls (A); only clonidine, restored P-CREB immunoreactivity above those of N2-deprived vehicle-treated cells. Bars represent the mean (± SEM) of at least 3 experiments with each sample conducted in duplicate or triplicate. Striped bars represent the plus (+) N2 condition; white bars represent the minus (−) N2 condition. For convenience, the Vehicle+ and Vehicle− conditions are reproduced in all 3 panels (A, B, C). *, statistically and significantly different from the Veh+ condition at $P<0.05$. #, statistically and significantly different from the corresponding N2+ condition at $P<0.05$. **, statistically and significantly different from the Veh− condition at $P<0.05$. Veh, vehicle; Clon, clonidine; NE, norepinephrine; PD, PD98059; and LY, LY294002.

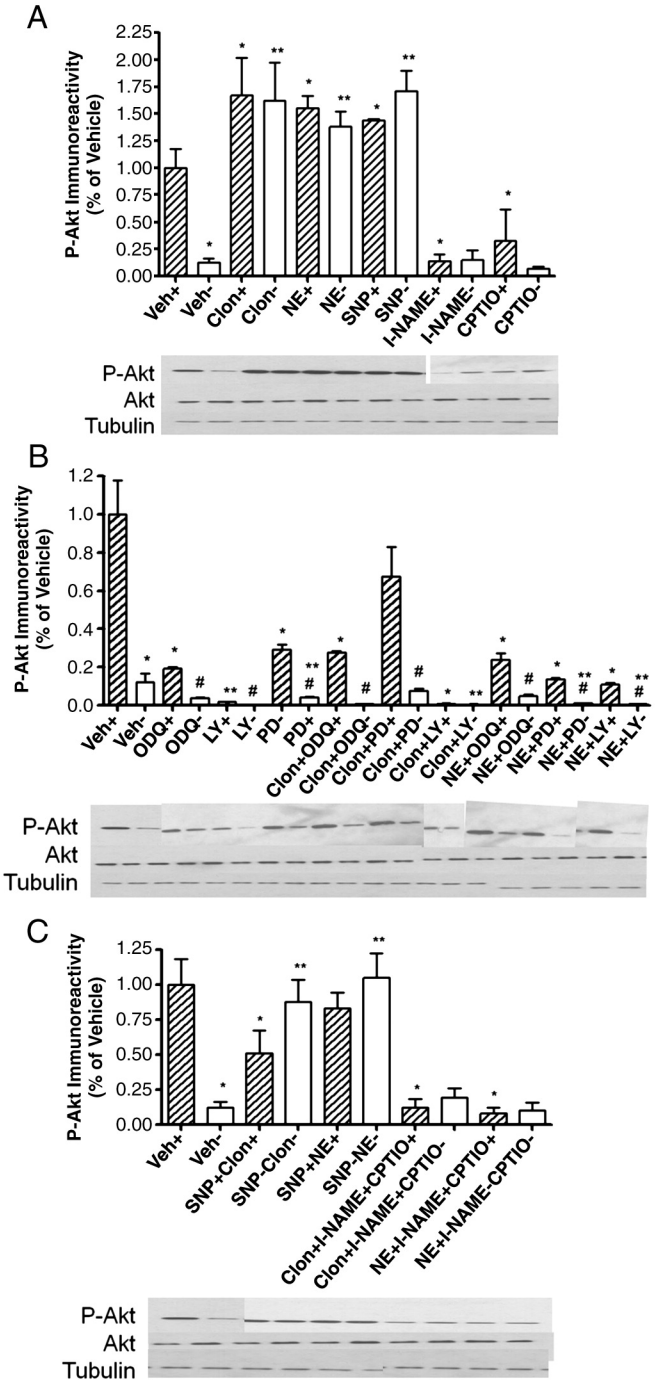

Fig. 5. In the presence of N2 supplements, clonidine, norepinephrine, and SNP all up-regulated (A), whereas all inhibitors (l-NAME, CPTIO, ODQ, PD98059, and LY294002) significantly down-regulated P-Akt immunoreactivity with respect to vehicle-treated controls (A, B). Without N2 supplements, vehicle-treated cells expressed less P-Akt than that of the N2-supplemented controls (+N2); clonidine, norepinephrine, and SNP, however, all restored P-Akt immunoreactivity to values comparable to those of N2-supplemented (+N2) cells, surpassing that of even vehicle-treated controls. Bars represent the mean (±SEM) of at least 3 experiments with each sample conducted in duplicate or triplicate. Striped bars represent the plus (+) N2 condition; white bars represent the minus (−) N2 condition. For convenience, the Vehicle+ and Vehicle− conditions are reproduced in all 3 panels (A, B, C). *, statistically and significantly different from the Veh+ condition at $P<0.05$. #, statistically and significantly different from the corresponding N2+ condition at $P<0.05$. **, statistically and significantly different from the Veh− condition at $P<0.05$. Veh, vehicle; Clon, clonidine; NE, norepinephrine; PD, PD98059; and LY, LY294002.

allowed P-Akt immunoreactivity to reach levels comparable to that of vehicle-treated controls (Fig. 5B).

Without N2, clonidine ($P<0.001$), norepinephrine ($P<0.001$), and SNP ($P<0.001$) all surpassed that of vehicle-treated controls (−N2) in P-Akt immunoreactivity (Fig. 5A). None of these three drugs, however, were able to restore P-Akt immunoreactivity in the face of both N2 deprivation and any of the inhibitors used (Fig. 5B). However, both paired treatments, SNP + clonidine and SNP + norepinephrine, restored P-Akt immunoreactivity levels to those comparable in the presence of N2 (Fig. 5C). And complete abolishment of NO (l-NAME + CPTIO) paired with either clonidine or norepinephrine resulted in significantly less P-Akt immunoreactivity whether N2 was present or not (Fig. 5C).

3.3.4. P-MAPK

P-MAPK immunoreactivity was significantly higher in vehicle-treated neurons in the presence than in the absence ($P<0.05$) of N2 supplements [$F(1, 94) = 0.204$, $P = 0.653$; Fig. 6). In the presence of N2 supplements, clonidine ($P<0.001$), norepinephrine ($P<0.001$), and SNP ($P<0.001$) all significantly up-regulated, whereas only l-NAME ($P<0.001$), CPTIO ($P<0.001$), and PD98059 ($P<0.001$) significantly decreased P-MAPK immunoreactivity with respect to vehicle-treated controls [$F(18, 94) = 2.86$, $P = 0.001$; Fig. 6A, B]. Pairing of clonidine or norepinephrine with ODQ or LY294002 did not affect P-MAPK activation with respect to vehicle-treated cells (Fig. 6B); whereas pairing with PD98059 significantly decreased P-MAPK immunoreactivity (Fig. 6B).

Without N2 supplements, clonidine ($P<0.001$), norepinephrine ($P<0.001$), and SNP ($P<0.001$) each surpassed that of even vehicle-treated controls (+N2) to values comparable to those of cells treated, in the presence of N2, with clonidine, norepinephrine, or SNP, respectively (Fig. 6C). None of these three drugs, however, were able to restore P-MAPK immunoreactivity in the face of both N2 deprivation and any of the inhibitors used (Fig. 6B). However, when clonidine or norepinephrine were combined with either exogenous NO (SNP) or with complete NO deprivation (l-NAME + CPTIO), P-MAPK immunoreactivity was comparable to or significantly higher than, respectively, vehicle-treated controls ((−N2, $P<0.001$), Fig. 6C).

3.4. Nutrient withdrawal reveals more survival signaling

When effect sizes comparing vehicle treatment *vs.* each of the various other treatments in the presence of N2, 4 significant positive correlations were found for BDNF:P-CREB, BDNF:P-MAPK, P-Akt:P-MAPK, and P-Akt:survival (Table 1A). Without N2, however, all correlations of effect sizes between vehicle *vs.* other treatments were statistically significant, except for that of BDNF:P-MAPK (Table 1B), which was significant in the presence of N2 (Table 1A).

4. Discussion

Our results support the hypothesis that a norepinephrine-mediated cell survival signaling mechanism protects hippocampal neurons under nutrient deprivation stress, and that this mechanism is NO-dependent (Stout and Woodward, 1995). All five outcome measures evaluated were increased as a result of exogenously applied NO in the form of SNP and decreased as a result of the inhibition of endogenously produced NO via the NOS inhibitor, l-NAME and the NO scavenger, CPTIO. Recently, Hua et al. (2008) showed that a NO donor conferred antidepressant-like effects and increased hippocampal neurogenesis. As noted above, others have provided evidence that NO is neuroprotective (Contestabile et al., 2003; Mohanakumar et al., 2002; Puzzo et al., 2005; Rauhala et al., 2005) through the PI-3K/Akt (Culmsee et al., 2005; Hashiguchi et al., 2004) and MAPK (Culmsee et al., 2005; Kanterewicz et al., 1998;) pathways. And Riccio et al. (2006) demonstrated that cortical neurons treated with SNP increased the binding of CREB to its target gene promoters even without BDNF stimulation.

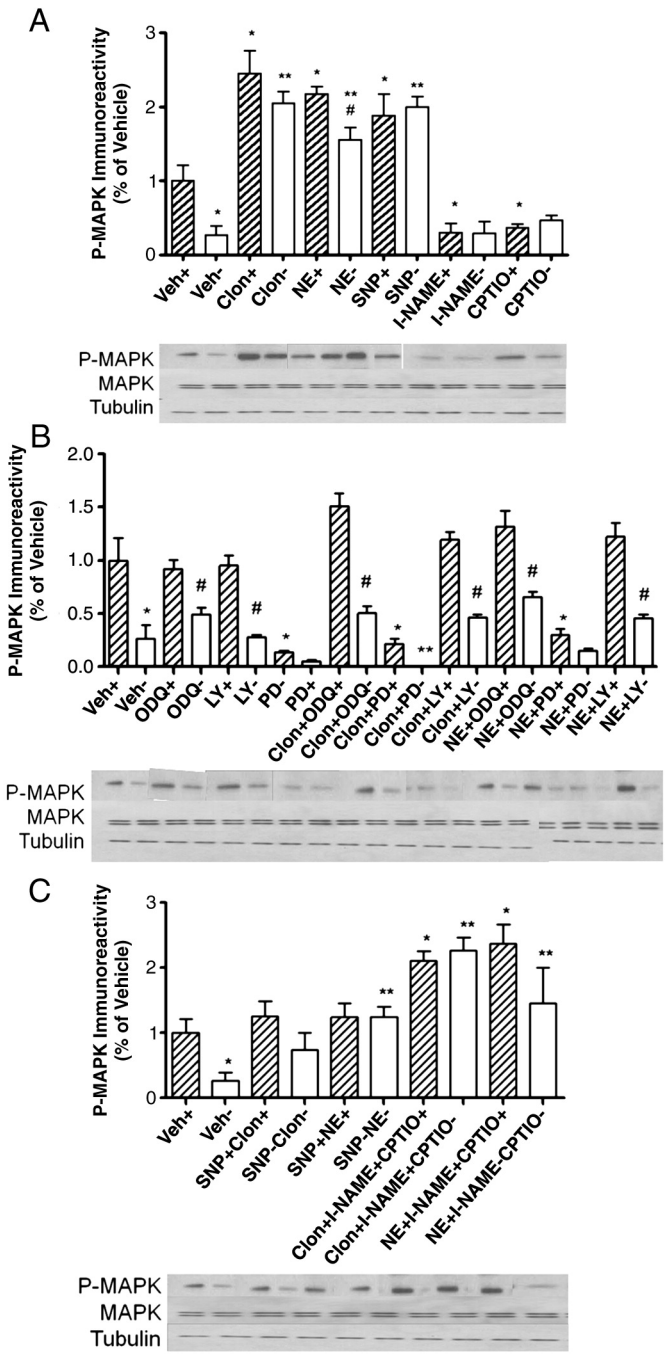

Fig. 6. In the presence of N2 supplements, clonidine, norepinephrine, and SNP all up-regulated (A), whereas the inhibitors, l-NAME, CPTIO, ODQ, and LY294002, prevented P-MAPK immunoreactivity from increasing with respect to vehicle-treated controls (A, B). Without N2 supplements, vehicle-treated cells expressed less P-MAPK than that of the N2-supplemented (+) controls, with clonidine, norepinephrine, SNP (A, B), and the combination treatment, SNP + norepinephrine (C), however, all restoring P-MAPK immunoreactivity to values comparable to those of N2-supplemented cells (+N2). Bars represent the mean (±SEM) of at least 3 experiments with each sample conducted in duplicate or triplicate. Striped bars represent the plus (+) N2 condition; white bars represent the minus (−) N2 condition. For convenience, the Vehicle + and Vehicle− conditions are reproduced in all 3 panels (A, B, C). *, statistically and significantly different from the Veh+ condition at $P<0.05$. #, statistically and significantly different from the corresponding N2+ condition at $P<0.05$. **, statistically and significantly different from the Veh− condition at $P<0.05$. Veh, vehicle; Clon, clonidine; NE, norepinephrine; PD, PD98059; and LY, LY294002.

Consistently, we show herein that SNP, in combination with clonidine or norepinephrine, increases cell survival robustly (Fig. 1), suggesting that norepinephrine and NO-stimulated mechanisms may

Table 1
Pearson correlations (2-tailed) of the effect size values between all possible pairs of the five survival signaling measures evaluated in the presence (A) or absence (B) of N2, respectively. Using $P<0.05$, four of the measures are significantly correlated with each other in the presence of N2 (A), whereas nine of the measures are significantly correlated with each other without N2 (B). *r*, Pearson correlation coefficient; *P*, significance level; and *n*, sample size.

		BDNF	P-CREB	P-Akt	P-MAPK	Survival
A						
BDNF	*r*	1				
	P					
	n	17				
P-CREB	*r*	0.645	1			
	P	0.005				
	n	17	17			
P-Akt	*r*	0.446	0.445	1		
	P	0.073	0.073			
	n	17	17	17		
P-MAPK	*r*	0.787	0.454	0.506	1	
	P	<0.0001	0.067	0.038		
	n	17	17	17	17	
Survival	*r*	0.348	0.381	0.735	0.446	1
	P	0.204	0.161	0.002	0.096	
	n	15	15	15	15	15
B						
BDNF	*r*	1				
	P					
	n	17				
P-CREB	*r*	0.72	1			
	P	0.001				
	n	17	17			
P-Akt	*r*	0.794	0.835	1		
	P	<0.0001	<0.0001			
	n	17	17	17		
P-MAPK	*r*	0.426	0.654	0.552	1	
	P	0.088	0.004	0.021		
	n	17	17	17	17	
Survival	*r*	0.519	0.79	0.778	0.64	1
	P	0.047	<0.0001	0.001	0.010	
	n	15	15	15	15	15

enhance one another (Stout and Woodward, 1995), which may be in part due to the stimulation of the synaptic release of norepinephrine from intracellular stores (Lonart et al., 1992).

We previously demonstrated that the norepinephrine-mediated enhancement of BDNF expression depends on NO signaling (Chen and Russo-Neustadt, 2007); and our current results suggest that a norepinephrine–NO mechanism is also neuroprotective in a situation where neurons are deprived of important nutritional supplements. Further, when any one of four key survival-promoting pathways is inhibited, neuronal survival decreases by approximately 10–20% of levels elicited by controls; and in the face of N2 deprivation, neuronal survival further decreases to levels approximately one half of that produced by controls with N2.

This nutrient deprivation model of stress resulted in ~40% neuronal death (Fig. 1) to ~90% decrease in BDNF immunoreactivity (Fig. 3). This dramatic decline was offset by clonidine, norepinephrine, SNP, and/or a combination of two of these drugs, indicating a possible neuroprotective role conferred by these compounds. When compared to the vehicle-without-N2 condition, however, clonidine, SNP, and norepinephrine, but not the clonidine/SNP, and norepinephrine/SNP combination all increased the immunoreactivity of signaling molecules to varying degrees, as well as cell survival. Nutrient deprivation resulting in decreased survival has previously been reported in cortical (Tong et al., 2004) and hippocampal (Zheng and Quirion, 2009) neurons. Others have also reported the survival-promoting effects of exogenously applied NO: when chick retinal neuronal survival was compromised by ~40% as a result of refreshing the media, an exogenously applied NO donor significantly increased cell survival to nearly control levels (Mejia-Garcia and Paes-de-Carvalho, 2007); this

compromise in cell survival was further exacerbated by incubation with several survival-promoting pathway (PI-3K, MAPK, and CAMK) inhibitors (Mejia-Garcia and Paes-de-Carvalho, 2007).

We expected that when guanylate cyclase, PI-3K, or the MAPK pathway is inhibited by ODQ, LY294002, and PD98059, respectively, cell survival and its underlying survival signaling pathways would likely be compromised (Culmsee et al., 2005; Chen and Russo-Neustadt, 2007). Without N2, cell survival was compromised even further (Fig. 1), as was BDNF (Fig. 3), P-CREB (Fig. 4), and P-Akt (Fig. 5). Both BDNF and P-CREB were decreased to barely detectable levels, whereas P-Akt and P-MAPK were more selectively affected, depending on the inhibitor.

Because P-CREB and therefore, BDNF, is a possible convergence point from among several different pathways, the nature of their upstream inhibition may not be as selective. Inhibition of P-Akt and P-MAPK pathways, however, is potentially more revealing. In the presence of N2, LY294002, of course, inhibited P-Akt, compared to that of controls, but so did PD98059, ODQ, l-NAME, and CPTIO. Without N2, P-Akt levels were dramatically decreased in the presence of these inhibitors (Fig. 5). In the presence of N2, PD98059, of course, inhibited P-MAPK, but so did only l-NAME and CPTIO, suggesting NO involvement in this pathway (Lin et al., 2004). And without N2, neither ODQ nor LY294002 decreased P-MAPK levels below that of controls (vehicle (−N2)) (Fig. 6). The differential sensitivities of these pathways to various inhibitors are reminiscent of the pathway cross-talk we recently demonstrated (Chen et al., 2007, and references cited therein). Specifically, herein, we show that the MAPK inhibitor, PD98059, inhibited P-Akt immunoreactivity with respect to vehicle-treated cells (Fig. 5), but the PI-3K inhibitor, LY294002, did not inhibit P-MAPK immunoreactivity with respect to vehicle-treated cells (Fig. 6). And again, as in the case with P-Akt, neither l-NAME nor CPTIO by themselves had any effect on P-MAPK levels whether N2 was present or not, suggesting that the NOS inhibitor and scavenger were working maximally and the resultant lack of NO was more than adequate to suppress survival signaling via these two pathways, which the presence of N2 could not reverse. Further, when NO was completely removed from the system combined with either clonidine or norepinephrine, only BDNF (Fig. 3) and P-Akt (Fig. 5) decreased with respect to clonidine or norepinephrine alone, again indicating an essential role for NO on the Akt/PI-3K pathway in survival signaling (see Section 1). Conversely, when SNP is combined with either clonidine or norepinephrine, cell survival, BDNF, and P-CREB increased to comparable levels those elicited by either compound alone (Figs. 1, 3, 4). By itself, SNP also partially mimicked the effects of clonidine on all of these measures, perhaps through stimulating norepinephrine release from hippocampal neurons (Satoh et al., 1996; Stout and Woodward, 1995) via the stimulation of ATP-sensitive K channels through the MAPK pathway (Lin et al., 2004). Our results are consistent with the ability of l-NAME to block the stimulatory effect of BDNF on the differentiation of neural progenitor cells (Cheng et al., 2003) and decrease BDNF-treated motor neuron survival (Estevez et al., 1998).

The potent guanylate cyclase inhibitor, ODQ, inhibited cell survival, but did not significantly affect P-MAPK activation (Fig. 6B) and was only slightly, but not significantly increased when either clonidine or norepinephrine was included; ODQ, however, down-regulated P-Akt signaling (Chen and Russo-Neustadt, 2007; Ciani et al., 2002a,b; Ha et al., 2003; Kang et al., 2004), even in the presence of either clonidine or norepinephrine (Fig. 5B). These results are consistent with those of Zheng and Quirion (2004) who, in a hippocampal cell line undergoing media compromise, also found that the PI-3K, but not the MAPK, pathway was involved in the survival of serum-deprived neurons.

Because our experimental conditions were designed to model perhaps one aspect of a compromised cellular system *in vivo*, it should be noted that cells under such challenges might recruit more survival-promoting strategies. In the presence of N2, only four of the cell survival-promoting measures evaluated were correlated with each other (Table 1A); in the absence of N2, nine out of the ten cell survival measures were correlated with each other (Table 1B). This observation may represent a compensation to enhance survival in an environment that is proving to be a threat to homeostasis and, therefore, cell survival. Inconsistently, we show herein that cell survival (Fig. 1), P-CREB (Fig. 4), and P-MAPK immunoreactivity (Fig. 6) actually increase in response to clonidine + l-NAME + CPTIO. This utter depletion of NO, and therefore, posing a threat to cell survival, may have caused the neurons to marshall more resources to compensate, perhaps aided by clonidine, to increase cell survival and is reminiscent of the correlations displayed in Table 1, wherein 9 out of 10 survival correlations were statistically significant when N2 was lacking, as opposed to only 4 when N2 was present.

Alernatively, it is further probable that cGMP, PI-3K/Akt, and MAPK are by no means the only pathways through which NO mediates cell survival. There has been an increasing evidence that NO and also S-nitrosylates critical proteins, such as the NMDA receptor, caspases, p21ras (Kang et al., 2004) and the glutamate receptor 6 (Yu et al., 2008), protects against oxidative stress (Chieuh et al., 2000). Such posttranslational reactions are likely indispensible in supplementing the neuroprotective measures afforded by other signaling pathways.

The current study not only demonstrates that the noradrenergic system activation is neuroprotective under stress, but that these protective actions at least partly depend on NO signaling, as well as intact survival-promoting pathways. Our results support the idea that NO plays an important role in cell survival-promoting actions, in agreement with previous evidence that NO regulates neuronal survival, proliferation and differentiation (Cheng et al., 2003; Contestabile and Ciani, 2004). BDNF has been demonstrated to activate NOS expression and activity in cortical neurons (Cheng et al., 2003; Klöcker et al., 1999; Samdani et al., 1997; Xiong et al., 1999), whereas exposure of neurons to a NOS inhibitor has been shown to block BDNF-induced association of CREB with nNOS promoters (Riccio et al., 2006).

These results support our hypothesis that the norepinephrine-mediated cell survival signaling mechanism is NO-dependent. Future studies are needed to specifically investigate other points in this mechanism, and test the role of NO and other associated molecules in this pathway thought to promote cell survival.

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