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The cognition-enhancer nefiracetam inhibits both necrosis and apoptosis in retinal ischemic models *in vitro* and *in vivo*

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Running title:

Nefiracetam and retinal neuroprotection

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A list of abbreviations used in the paper:

LD: low-density, HD: high-density, LOG: low-oxygen glucose, PI: propidium iodide
BDNF: brain derived neurotrophic factor, TEM: transmission electron microscopy, PI
3-kinase: phosphoinositide 3-kinase, ERK: extracellular signal-regulated kinase, GCL:
ganglion cell layer, IPL inner plexiform layer, INL: inner nuclear layer, ONL: outer
nuclear layer, CBF: cerebral blood flow, ICMRgl: rate of local glucose metabolism

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Abstract

The retinal ischemic-reperfusion stress (130 mmHg, 45 min) caused neuronal damages throughout all cell layers and reduced the thickness of retinal layer by 30% at 7 days after the stress of mouse retina. The intravitreal injection of 100 pmol of nefiracetam, a cognition-enhancer, completely prevented the damages when it was given 30 min prior to and 3 h after the stress. Partial prevention was observed when it was given 24 h after the stress, or low dose (10 pmol) nefiracetam was given 30 min prior to the stress. However, aniracetam had no effect. In the retinal cell line N18-RE-105, the ischemic-reperfusion stress by 2 h culture under the serum-free condition with low oxygen (less than 0.4% O₂) and low glucose (1 mM), caused necrosis or apoptosis in the low-density (0.5x10⁴ cell/cm²) or high-density (5x10⁴ cell/cm²) culture, respectively. The necrosis showed membrane disruption, loss of electron density, and mitochondrial swelling, while apoptosis showed nuclear fragmentation and condensation in transmission electron microscopical analyses and in experiments using specific cell death markers. Nefiracetam inhibited both necrosis and apoptosis, while brain-derived neurotrophic factor (BDNF) inhibited only apoptosis. The cell-protective actions of nefiracetam were abolished by nifedipine and ω -conotoxin GVIA, L-type and N-type calcium channel blocker, but not by PD98059 or wortmannin, ERK1/2 or PI 3-kinase inhibitor, respectively, while those of BDNF were abolished by PD98059 and wortmannin, but not by nifedipine and ω -conotoxin GVIA. All these findings suggest that nefiracetam inhibit necrosis and apoptosis occurred in the ischemic/hypoxic neuronal injury through an increase in Ca²⁺-influx.

The retina is a part of the central nervous system that is well accessible for quantitative analysis of pathophysiological processes and experimental manipulation (Isenmann et al., 2003). Transient ischemia-induced damage of the retina, as well as the brain, results in a prolonged period of neuronal cell death. The mechanisms of ischemia-induced neuronal cell death include an early phase of excitotoxicity through glutamate, leading to necrosis and prolonged phase of apoptosis (Lombardi et al., 1994; Choi, 1996). Necrosis is characterized by the loss of membrane integrity, disruption of intracellular organelles, cellular swelling and lysis, while apoptosis involves reduction of intracellular volume, nuclear condensation and fragmentation (Kerr et al., 1977; Bredesen, 1995). Although the molecular mechanisms underlying apoptosis are now well characterized (Yuan and Yankner, 2000), and many therapeutic candidates have been proposed, little is known of mechanisms underlying necrosis and its therapeutic candidates. Since cell destructive features of necrosis induces secondary damages of surrounding cells, the inhibition of necrosis would be an important therapeutic target in the sense of minimization of ischemic neuronal injury.

Nefiracetam, N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide, is a member of oxopyrrolidine acetic acid derivatives such as aniracetam, piracetam and oxiracetam (Sakurai et al., 1989). Several pharmacological studies have shown that the anti-amnesic effect of nefiracetam is related to activation of cholinergic activity, GABAergic and/or monoaminergic transmitter systems (Fukatsu et al., 2002; Luthman et al., 1992). The electrophysiological studies revealed that nefiracetam activates voltage-dependent N-type and L-type calcium channels, thus promoting neural

transmission (Yoshii and Watabe, 1994; Yoshii et al., 1997). Recent studies demonstrate that nefiracetam has protective actions against memory dysfunction, electrophysiological and metabolic damages in the brain given ischemic stress (Takeo et al., 2003a; Takeo et al., 2003b; Jin et al., 2002). Although several neuromodulatory mechanisms underlying anti-amnesic actions of nefiracetam have been proposed in brain ischemia models, little is known of actions on survival activity of neurons. We firstly reported that nefiracetam protects apoptotic neuronal cell death under serum-free starvation stress condition (Fujita et al., 2001). We have also found that the neurons die by necrosis in the low-density culture under such serum-free condition, while they die by apoptosis in the high-density culture (Fujita et al., 2001; Fujita and Ueda 2003a). However, it remains to be determined whether nefiracetam could inhibit neuronal ischemia *in vivo*, and how it shows a neuroprotection in the culture system, particularly about the specificity for necrosis or apoptosis. Here we examined the neuroprotective actions of nefiracetam on the neuronal damages due to retinal ischemic-reperfusion stress in mice, and characterized its neuroprotective actions by use of mouse neuroblastoma x rat neural retina hybrid N18-RE-105 cells.

Materials and Methods

Materials

Nefiracetam and aniracetam were synthesized by Daiichi Pharmaceutical Co. Ltd. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan), and fetal bovine serum (FBS) was from Biosource int. (CA, USA). Propidium iodide (PI) and ω -conotoxin GVIA were purchased from Sigma (Tokyo, Japan). Nifedipine and wortmannin were purchased from Wako Pure Chemicals (Tokyo, Japan). PD98059 was purchased from Cell signaling (Tokyo, Japan). BDNF was a gift from Sumitomo Pharmaceutical (Osaka, Japan).

Induction of ischemic/reperfusion injury

We used a modified method of retinal ischemic-reperfusion injury in mice according to previously published methods in rats (Adachi et al., 1998). Briefly, male ICR mice (20-30g; Tagawa experimental animals, Nagasaki, Japan) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the pupil was fully dilated with 1% atropine sulfate drops. The anterior chamber was cannulated with a 33-gauge needle connected to a container of sterile intraocular irrigating solution (BSS PLUS dilution buffer, Alcon, Fort Worth, TX). Retinal ischemia was induced by elevating the intraocular pressure (IOP) to generate a hydrostatic pressure of 130 mmHg for 45 min by lifting the container. The retina was isolated 7 days after the ischemic-reperfusion treatment and used for evaluation of damage by Hematoxylin-Eosin (HE)-staining.

N18-RE-105 cell culture

Mouse neuroblastoma x rat retinal neuron hybrid N18-RE-105 cells were seeded onto 96-well culture plates, 8-well Lab-Tek™ chambers, or 3.5 and 9.0 cm culture dishes, which had been all coated with poly-DL-lysine and collagen, and cultured by DMEM containing 10% FBS at 37 °C in 5%-CO₂ atmosphere.

In vitro ischemic stress (Low-Oxygen and Low-Glucose; LOG stress)

N18-RE-105 cells were seeded in low-density (LD: 0.5 x10⁴ cells/cm²) or high-density (HD: 5 x10⁴ cells/cm²) 12-16 h before LOG stress. Cells were washed twice with balanced salt solution (BSS), added with de-aerated BSS containing 1 mM glucose and incubated for 2 h at 37 °C in 5% CO₂ and 95% N₂ (0.4% :O₂) (ischemia). After the ischemia stress, cultured medium was changed with fresh DMEM containing 10% FBS (reperfusion).

WST-8 assay

The viability of cells was assessed 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) reduction assay (Dojin Lab. Tokyo, Japan), according to the manufacturer's instructions. WST-8 was added to the culture for 1 h at 37 °C prior to the colorimetry. Percentage of WST-8 activity was represented as the ratio of activity at different time points to that in the beginning of reperfusion.

Hoechst 33342 and PI-staining assay

To assess the simultaneous observation of early phase of apoptotic and necrotic

features, cells on 8-well Lab-Tek™ chamber were treated with 10 µg/ml Hoechst 33342 (Molecular Probe, Eugene, OR), 10 µg/ml PI and RNase A 10 µg/ml for 15 min at 37 °C in the dark. RNaseA was added to reduce the non-selective PI binding to cytosolic RNAs. Cells were washed twice with ice-cold phosphate buffered saline (PBS), fixed with 4% paraformaldehyde (PFA) in PBS at 4 °C overnight and observed under a fluorescence microscope (BX50; Olympus; Tokyo, Japan).

Immunocytochemistry of caspase-3

Cells on 8-well Lab-Tek™ chamber were stained with PI and Hoechst 33342, fixed with 4% PFA in PBS for 30 min at 25 °C, and followed by permeabilization using 50% and 100% methanol for 5min, respectively. They were incubated in blocking buffer (2% low-fat milk powder, 2% bovine serum albumin, 0.1% Tween-20, in PBS, pH 7.4) for 1 h at 25 °C. Anti-cleaved caspase-3 antibody (1:100; Cell Signaling, Tokyo, Japan) was added to the cells. After 2 h incubation at 25 °C and washing, the cells were incubated with FITC-conjugated anti-rabbit immunoglobulin (IgG) (1:200; Cappel, Aurora) for 4 h at 25 °C. Immunolabeled cells were observed under a fluorescence microscope.

Transmission electron microscopy

Cultured cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 25 °C. The fixed cortical neurons were postfixated with 2% osmium tetroxide for 1 h at 25 °C, dehydrated in graded alcohol series, and embedded in Epon812. Ultrathin sections (80 nm thick) were cut with an Ultracut S (Leica, Austria), then stained with uranyl acetate and lead citrate for 30 and 5min, respectively. The stained sections were observed under an electron microscope (JEM-1200; JEOL, Tokyo, Japan).

Analysis of DNA fragmentation

Cells on 9-cm dishes were harvested and centrifuged at 350 x g for 5 min, and the pellet was used for DNA fragmentation analysis, as described previously (Fujita and Ueda 2003b). Briefly, the cells were lysed in 250 µl of lysis buffer (5 mM Tris-HCl, 20 mM EDTA and 0.5% Triton X-100, pH 8.1) with gentle shaking for 15 min at 4 °C. The lysates were centrifuged at 6,000 x g for 10 min at 4 °C to separate the fragmented DNA (supernatant) and intact chromatin DNA (pellet). The fragmented DNA was extracted with phenol/chloroform/isoamylalcohol, and the DNA in the aqueous phase was precipitated with 2 volume of ethanol following the addition of sodium acetate (final concentration, 0.3 M). The DNA was then collected by centrifugation (14,000 x g for 10 min at 4 °C) and dried. The samples were finally dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH8.1), incubated for 1 h at 37 °C with RNase A (10 µg/ml), and electrophoresed on a 2 % agarose gel. The gel was stained with ethidium bromide and then photographed on an ultraviolet illuminator.

Statistical Analysis

For the statistical analysis of data, Student's *t*-test following multiple comparisons of the analysis of variance (ANOVA) was used. The criterion of significance was set at $p < 0.05$.

Results

Neuronal protective actions of nefiracetam in retinal ischemia-reperfusion model

Retinal ischemic-reperfusion stress was performed by giving hydrostatic-pressure stress of 130 mmHg for 45 min to the anterior chamber of eye. The retina was then isolated 7 days after this ischemic stress, and used for HE-staining after the fixation with PFA and paraffin embedding. There was a reduction by approximately 70 % in thickness of retinal cell layers including ganglion cellular layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) as well as inner plexiform layer (IPL) after the ischemic-reperfusion stress, compared with sham control (Figs. 1A, 1B). The neuronal damages reached maximal 7 days after the ischemic-reperfusion stress. When nefiracetam was intravitreously given 30 min prior to the ischemic-stress at doses of 10 or 100 pmol in a volume of 1 μ l of phosphate buffered solution (pH 7.4), the damages throughout all layers were dose-dependently prevented (Figs. 1C, 1D). Moreover, when 100 pmol of nefiracetam was given 3 h after the ischemic stress complete prevention was observed, but no or if any weak prevention was observed when it was given 24 h after the stress (Figs. 1E, 1F). On the other hand, when aniracetam was intravitreously given 30 min prior to the ischemic-stress at doses of 100 pmol, did not protect ischemic-induced retinal cell death (Fig 1G). Moreover, quantification of retinal neurons revealed that 100 pmol of pre- or post-treated (3 h) nefiracetam significantly inhibited loss of neurons under the ischemia stress in GCL, INL and ONL, respectively (Figs. 1H-J).

Characterization of cell density-dependent death mode switch of neuroblastoma x retinal neuron hybrid cells after ischemic-reperfusion stress

To reproduce the cell density-dependent mode switch from necrosis to apoptosis, which has been originally observed in serum-free culture of cortical neurons (Fujita et al., 2001; Fujita and Ueda, 2003a), we attempted to characterize the cell death under various conditions of culture of neuroblastoma x retinal neuron hybrid cells. When the cell culture in the presence of DMEM containing 10 % FBS was given 2 h LOG (low oxygen and low glucose) ischemic stress (serum-free, 0.4 % O₂ and 1 mM glucose), followed by returning to the starting condition (reperfusion), they successfully showed a cell density-dependent survival activity and cell death mode switch. The cells in the low-density (LD) culture at 0.5 x 10⁴ cells/cm² died immediately after the reperfusion, as shown in Fig. 2. The survival activity measured by WST-8 became 55.4 % of control 1 h after the reperfusion, while attained to as low as 20 % at 3 h. However, the survival activity with WST-8 showed a reversal with the further culture at 18 h and later, possibly because of proliferation of residual cells. On the other hand, the cells in the high-density (HD) culture at 5 x 10⁴ cells/cm² after the reperfusion died more slowly than the case with LD culture. The recovery of WST-8 activity was also found 18 h after the reperfusion (Fig. 2)

We characterized cell death mode using propidium iodide (PI) staining and Hoechst 33342 under the LOG ischemic stress. The cells in the LD culture 1 h after the reperfusion showed a marked PI-staining (73.3%), but no nuclear fragmentation or condensation stained by Hoechst 33342 (6.2%), as shown in Fig. 3A. On the other hand,

the cells in the HD culture showed a marked nuclear fragmentation and condensation (60.7%), but not PI-staining (4.4%) 12 h after the reperfusion. The cells in the HD culture showed no significant change in PI (0.8%) or Hoechst 33342 (1.1%) staining 1 h after the reperfusion (data not shown).

The caspase 3 is a prosecutor molecule of apoptosis (Tewari et al., 1995; Nicholson et al., 1995). The immunoreactive caspase 3 was only observed in HD cells 12 h after the reperfusion (57.4%)(Fig. 3B), but not in LD cells 1 h after the reperfusion (3.1%) (Fig. 3B). DNA ladder formation which is also known as a representative indicator for apoptosis (Walker and Sikorska, 1994) mediated by activated caspase 3, was only observed in the HD cells (Fig. 3C).

We used TEM analysis in the present study to confirm in evaluation that PI-Hoechst 33342 or PI-caspase 3 stained showed the necrotic or apoptotic changes in the LD and HD culture. The cell immediately after the 2 h LOG stress did not show any change in the morphology in the TEM analysis, compared to the cell without LOG stress (Figs. 4A, 4B). However the cell in the LD culture 1 h after the reperfusion showed typical necrotic features, such as membrane destruction, loss of electron density and mitochondrial swelling accompanying loss of criste structures, but no significant change in the nuclei (Fig. 4C). On the other hand, the cell in the HD culture showed significant nuclear fragmentation, chromatin condensation and membrane blebbing, but no significant change in the mitochondria (Fig. 4D). All these findings strongly suggest that the retinal hybrid cells in the LD culture die by necrosis, while in the HD culture by apoptosis after the LOG stress.

Neuroprotective actions of nefiracetam on necrotic and apoptotic cell death

As shown in Figs. 5A and 5B, the rapid reduction of WST-8 survival activity of hybrid cells in the LD culture was markedly inhibited by 1 μ M nefiracetam which had been added to the culture before the start of LOG stress, and this inhibition was concentration-dependent between 1 nM to 1 μ M of this compound. However, BDNF at 0.1 - 100 ng/ml did not show any survival activity (Fig. 5C). On the other hand, both nefiracetam and BDNF significantly inhibited the slow decrease in the WST-8 activity of HD cells (Figs. 5D-5F). The concentration of nefiracetam required for the survival activity in the ischemia-reperfusion model was 100-times lower than the case with serum-free culture of cortical neurons (Fujita et al., 2001).

Nefiracetam significantly inhibited the PI staining (13.2%) (1 h after the reperfusion of the LD culture, while BDNF had no effect (75.4%) (Fig. 5G) Neither nefiracetam nor BDNF has effect on Hoechst 33342 staining (4.2% nor 6.0%). On the other hand, both nefiracetam and BDNF inhibited the nuclear fragmentation/condensation measured by Hoechst 33342 (5.1% and 3.9%) and caspase 3 activation (7.7% and 4.6%) under the HD culture (Fig. 5H).

In the TEM analyses, on the other hand, it was revealed that the necrotic features observed in the LD culture was inhibited by 1 μ M nefiracetam, but not by 100 ng/ml of BDNF (Figs. 6A-6C), while apoptotic ones in the HD culture was inhibited by both nefiracetam and BDNF (Figs. 6D-6F).

Distinct mechanisms for neuroprotective actions by nefiracetam and BDNF

The nefiracetam-induced increase in the survival activity of LD or HD culture with LOG stress was significantly inhibited by nifedipine and ω -conotoxin GVIA, L-type and N-type Ca^{2+} channel inhibitor, respectively (Figs. 7A, 7B). However, wortmannin, PI 3-kinase inhibitor, and PD98059, ERK1/2 inhibitor, did not affect the nefiracetam-induced increase in the survival activity in LD or HD culture with the LOG stress. On the other hand, BDNF-induced increase in the survival activity of HD culture was significantly inhibited by wortmannin or PD98059, but not by nifedipine or ω -conotoxin GVIA (Fig. 7B).

The nefiracetam-induced necrosis-inhibition (PI-staining) in the LD culture, and apoptosis-inhibition (Hoechst- or activated caspase 3-staining) in the HD culture were both significantly inhibited by nifedipine and ω -conotoxin GVIA, respectively (Fig. 7C and 7D). However, wortmannin and PD98059 did not affect the nefiracetam-induced necrosis-inhibition or apoptosis-inhibition. On the other hand, the apoptosis-inhibition by BDNF was significantly inhibited by PD98059, but not by ω -conotoxin GVIA (Fig. 7D).

Discussion

A number of studies revealed that nefiracetam has many desirable actions. The cognition-enhancing action has long been discussed in relation to the modulation of neurotransmission (Hiramatsu et al., 1997; Nisizaki et al., 1998; Oyaizu and Narahashi 1999; Nishizaki et al., 2000; Zhao et al., 2001). Nefiracetam also inhibits the development of morphine dependence and tolerance through acute morphine treated decreasing of intracellular cAMP levels (Itoh et al., 2000). Most recently, Rashid and Ueda (2002) reported that nefiracetam shows a potent analgesic action to the neuropathic pain, which is resistant to morphine (Rashid and Ueda 2002). In addition to such neuromodulatory actions, we have also reported that nefiracetam has a neuroprotective action through an activation of L- and N-type Ca^{2+} channels (Fujita et al., 2002).

In the clinical, retinal ischemic damages are observed in glaucoma and diabetic retinopathy, and in case of clinical detachment of a retina. In the present study we have examined the possibility of clinical advantage of nefiracetam against neuronal damages in the retinal ischemia-reperfusion stress model. The model has many advantages in evaluation of neuronal damages and their protection. The pressure stress causes reproducible, quantitative and time-dependent damages in GCL, IPL, INL and ONL. In addition, topical administration of drugs is available in clinic as well as in experiments. Under the present experimental condition, the degree of damage was maximal at 7 days after the stress, and it lasted at least for another week (data not shown). Recently neuronal stem cell was found in retinal ciliary corpus and ganglion

cell layer (Trobepe et al., 2000; Isenmann et al., 2003), however there regeneration did not enough recover damages from the ischemia-reperfusion stress. The intravitreal injection of nefiracetam 30 min before and 3 h after the stress completely prevented the retinal neuronal death (Figs. 1D, 1E). Partial prevention was observed when it was given 24 h after the stress, or low dose (10 pmol) nefiracetam was given 30 min prior to the stress. Moreover, although it remains whether the parenteral use of nefiracetam is useful for these damages, the topical use of this compound has potentially.

The next important issue in this manuscript is the characterization of the mode of neuroprotective actions of nefiracetam. For this purpose we used the retinal cell line neuroblastoma x retinal neuron hybrid N18-RE-105 cell. Our approaches are aimed to see effects of nefiracetam on either necrosis or apoptosis. In our previous experiments we firstly demonstrated that cortical neurons in the LD culture die by necrosis under the serum-free condition without any supplements, while they die by apoptosis in the HD culture (Fujita et al., 2001; Fujita and Ueda, 2003a). We have also found that nefiracetam had protective actions on the apoptotic cell death in the latter case (Fujita et al., 2002), but it remains to be determined whether this compound has anti-necrotic actions. In the present study we firstly attempted to create both necrotic and apoptotic cell death models, in the analogy of cell density-dependent cell death mode switch, observed in the primary culture of cortical neurons. As a result of various trials of stress model, we found that the serum-free culture with LOG stress condition, or low oxygen (0.4 %) and low glucose (1 mM) for 2 h (ischemia), followed by replacement with original 10 % FBS-containing medium (reperfusion) shows both necrosis and apoptosis

when it is performed in the LD and HD culture (Figs. 3, 4), respectively. Although glucose-deficient condition also shows necrosis in the LD culture, the cell death was so rapid that neuroprotective actions of nefiracetam was hard to be evaluated (data not shown). It was reported that cerebral blood flow (CBF) decreased to less than 20% of normal CBF in ischemic core region in brain (Iadecola C. and Walz W., 1999). Moreover, rate of local glucose metabolism (ICMRgl) in the core region decreased 20% of normal ICMRgl (Yao H. et al., 1995). Therefore, the LOG stress containing 1 mM glucose (cf. 5.5 mM glucose in DMEM) might have some validity for the model of ischemia.

The necrosis observed in the LD culture after the LOG stress was clearly demonstrated in the TEM analysis, showing membrane destruction, loss of electron density and mitochondrial swelling, and in experiments using PI staining (Figs. 3A, 4C). On the other hand, apoptosis observed in the HD culture after the LOG stress was also clearly demonstrated in the TEM analysis and in experiments using nuclear fragmentation and chromatin condensation by Hoechst 33342, immunocytochemistry of activated caspase 3 and DNA ladder formation (Figs. 3B-D, 4D). Nefiracetam prevented all these necrotic and apoptotic features, while BDNF prevented only apoptosis (Figs. 5, 6).

Previously we have observed that nefiracetam significantly inhibited the cell death due to apoptosis in cortical neurons at concentrations of 1 to 10 μ M (Fujita et al., 2002). However, the present study shows that this compound significantly inhibited the cell death due to either necrosis or apoptosis at a concentration of as low as 10 or 100

nM, respectively (Figs. 5B, 5E), while BDNF did not inhibit the necrotic cell death, but showed a significant inhibition of apoptosis in the present study (Figs. 5C, 5F), as previously reported (Fujita and Ueda., 2003b, Han and Holtzman, 2000; Klockner et al., 2000). This strongly indicates that the present experiment is very useful to evaluate selective neuroprotection against necrosis and apoptosis.

Although the details of mechanisms underlying nefiracetam-induced neuroprotection remain to be fully determined, it is evident that the anti-apoptotic action of nefiracetam is different from that of BDNF, as shown in Fig. 7 and Table 1. PD98059, a potent inhibitor of ERK1/2, and wortmannin, a potent inhibitor of PI-3 kinase, blocked the BDNF-induced anti-apoptosis action under the HD condition. These results are consistent with the previous findings that BDNF or other neurotrophic factors prevented apoptosis through either PI-3 kinase or ERK1/2 activation in downstream of Trk receptor (see review; Kaplan and Miller 2000). However, these inhibitors did not affect the nefiracetam's anti-apoptotic actions. On the other hand, nefiracetam-induced anti-apoptotic actions were inhibited by nifedipine, a potent inhibitor of L-type Ca^{2+} channel, and ω -conotoxin GVIA, a potent inhibitor of N-type Ca^{2+} , respectively, while BDNF-induced was not one (Fig 7 and Table 1). Moreover, nefiracetam also showed anti-necrotic action through an increase in Ca^{2+} -influx under the LD condition (Fig. 7C and Table 1). Thus, BDNF and nefiracetam have different mechanisms for neuroprotection. Recently, it has been reported that the ischemic preconditioning protects neurons from subsequently induced ischemic damages through an increase in Ca^{2+} -influx (Deng et al., 2003). Thus, it is interesting to consider the possibility that

JPET#061127

nefiracetam-induced neuroprotection is related to such precondition mechanisms.

The present study demonstrates that nefiracetam shows a potent neuroprotective action in the retinal ischemia-reperfusion model *in vivo* and in LOG ischemia-reperfusion stress model of neuroblastoma x retinal neuron hybrid cell *in vitro*. Nefiracetam inhibited both necrosis and apoptosis in a calcium channel inhibitor-reversible manner.

JPET#061127

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Footnotes

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Legends for Figures

Fig. 1. Neuronal protective actions of nefiracetam in retinal ischemia-reperfusion model. Cells were stained by hematoxylin and eosin. Light microscopical photographs of cross sections of the mouse retina 7 days after the ischemic-reperfusion. A, sham; B, ischemic vehicle-treated; C, ischemic and 100 pmol nefiracetam (nefi)-pretreated (30 min prior); D, ischemic and 100 pmol nefiracetam-posttreated (3 h after reperfusion); E, ischemic and 100 pmol nefiracetam-posttreated (24 h after reperfusion); G, ischemic and 100 pmol aniracetam (ani)-pretreated (30 min prior). H, I and J, Comparison of cell numbers in retinas following various treatments. Numbers of cells for these measurements taken in five adjacent areas (one area: 100 μ m) within 1 mm apart from the optic nerve were calculated. Data are the mean \pm SEM from 4-5 independent experiments. GCL; ganglion cell layer, IPL; inner plexiform layer, INL, inner nuclear layer and ONL; outer nuclear layer. * p <0.05, compared with sham vehicle, $^{\dagger}p$ <0.05, compared with ischemia vehicle.

Fig. 2. Time course of survival activity in LD or HD culture of N18-RE105 cells with LOG stress. Cells were cultured in DMEM containing 10% FBS under the low-density (LD: 0.5×10^4 cells/cm²) or high-density (HD: 5×10^4 cells/cm²), given 2 h LOG (low oxygen and low glucose) ischemic stress (serum-free, 0.4 % O₂ and 1 mM glucose) and followed by returning to the starting condition (reperfusion). The time course of survival activity after the reperfusion was measured by WST-8 assay. Data are the percent of control activity (0 time after the reperfusion) and expressed as the mean \pm SEM from 3 independent experiments.

Fig. 3. Cell density-dependent death mode switch after the LOG ischemic- reperfusion

stress. A: Results are representative photographs of double staining of PI and Hoechst 33342. The apoptotic cells were observed as intense signal after staining by Hoechst 33342 (HD 12 h after reperfusion). B: Representative photographs of activated caspase 3 staining. C: Ladder formation of DNA derived from cells harvested from control (0 h) or LOG stress-treated culture. Lane M; standard DNA markers, L; LD culture and H; HD culture, respectively. In each lane 10 µg DNA applied.

Fig. 4. TEM analysis of cell density-dependent death mode switch in the LOG ischemic-reperfusion stress. Representative photographs of cells cultures in the LD (A-C) or HD (D), without (A) and with ischemic- reperfusion stress (B-D). Inset figures show high-magnified photographs of mitochondria. Fragmented nuclei are indicated by arrows. Bar, 2 µm

Fig. 5. Neuroprotective actions of nefiracetam on necrotic and apoptotic cell death. A, D: Time course of nefiracetam (1 µM) or BDNF (100 ng/ml) induced survival activity after the LOG ischemic- reperfusion stress in LD (A) or HD (D) culture. Both compounds were added before the LOG stress till the end of experiments. B, C, E and F: Concentration-dependent survival activities of nefiracetam (B and E) and BDNF (C and F) 3 h or 12 h after reperfusion in LD (B and C) or HD (E and F), respectively. Survival activity was measured by WST-8 reduction activity. Data are the percent of control activity (0 time after the reperfusion) and expressed as the mean \pm SEM from 3 independent experiments. $*p<0.05$, compared with vehicle treatment. G: Double staining of PI and Hoechst 33342 1 h after reperfusion in the LD culture. H: Triple staining of PI, Hoechst 33342 and active caspase 3 (FITC labeling) 12 h after reperfusion in HD culture.

The apoptotic cells were observed as intense signal after staining by Hoechst 33342.

Fig. 6. TEM analyses of nefiracetam-induced inhibition of necrosis and apoptosis. Results are representative TEM photographs of the cell 1 h after the reperfusion under the vehicle (A), nefiracetam (1 μ M; B) and BDNF (100 ng/ml; C) in LD culture and 12 h after reperfusion under the vehicle (D), nefiracetam (1 μ M; E) and BDNF (100 ng/ml; F) in HD culture, respectively. Inset figures (A-C) show high-magnified photographs of mitochondria. Fragmented nuclei are indicated by arrows. Bar, 2 μ m

Fig. 7. Distinct mechanisms for neuroprotective actions by nefiracetam and BDNF. A and B: Effects of nifedipine (nife; 1 μ M), ω -conotoxin GVIA (ω -CTX; 1 μ M), wortmannin (wort; 1 μ M) and PD98059 (1 μ M). Inhibitors were added before the LOG stress till the end of experiments. Data are the percent of control activity (0 time after the reperfusion) and expressed as the mean \pm SEM from 3 independent experiments. * p <0.05, compared with vehicle treatment and $^{\dagger}p$ <0.05, compared with nefiracetam or BDNF treatment. C: Staining of PI 1 h after reperfusion in LD culture. D: Double staining of Hoechst 33342 and active caspase 3 (FITC labeling) 12 h after reperfusion in HD culture. The apoptotic cells were observed as intense signal after staining by Hoechst 33342.

Figure 1

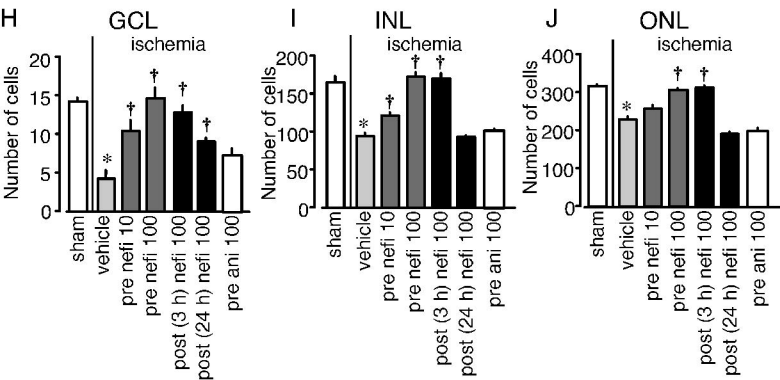
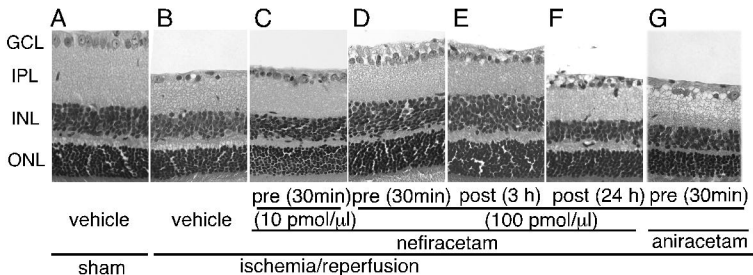


Figure 2

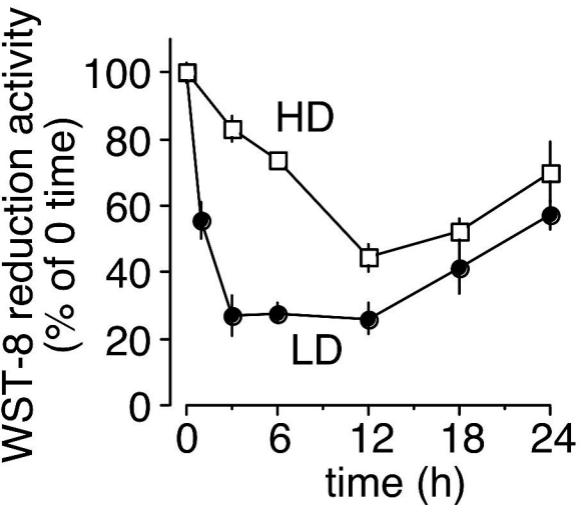


Figure 3

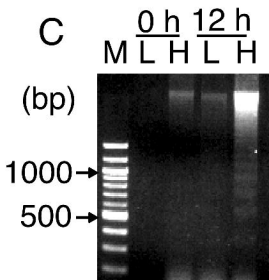
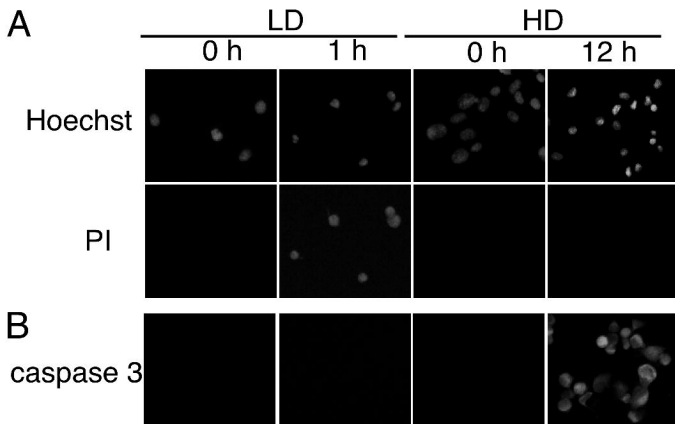
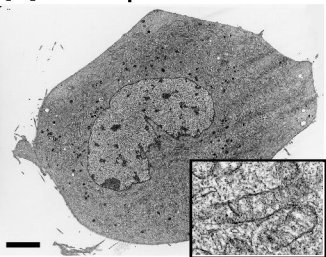
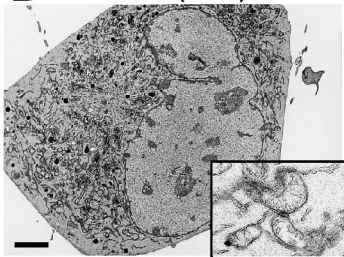


Figure 4

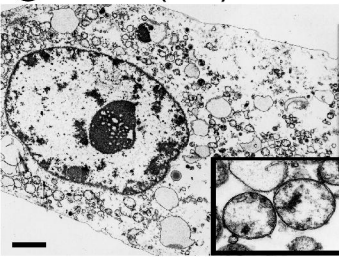
A LD preLOG



B LD (0 h)



C LD (1 h)



D HD (12 h)

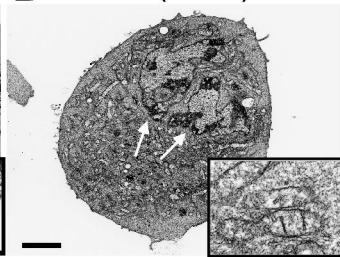


Figure 5

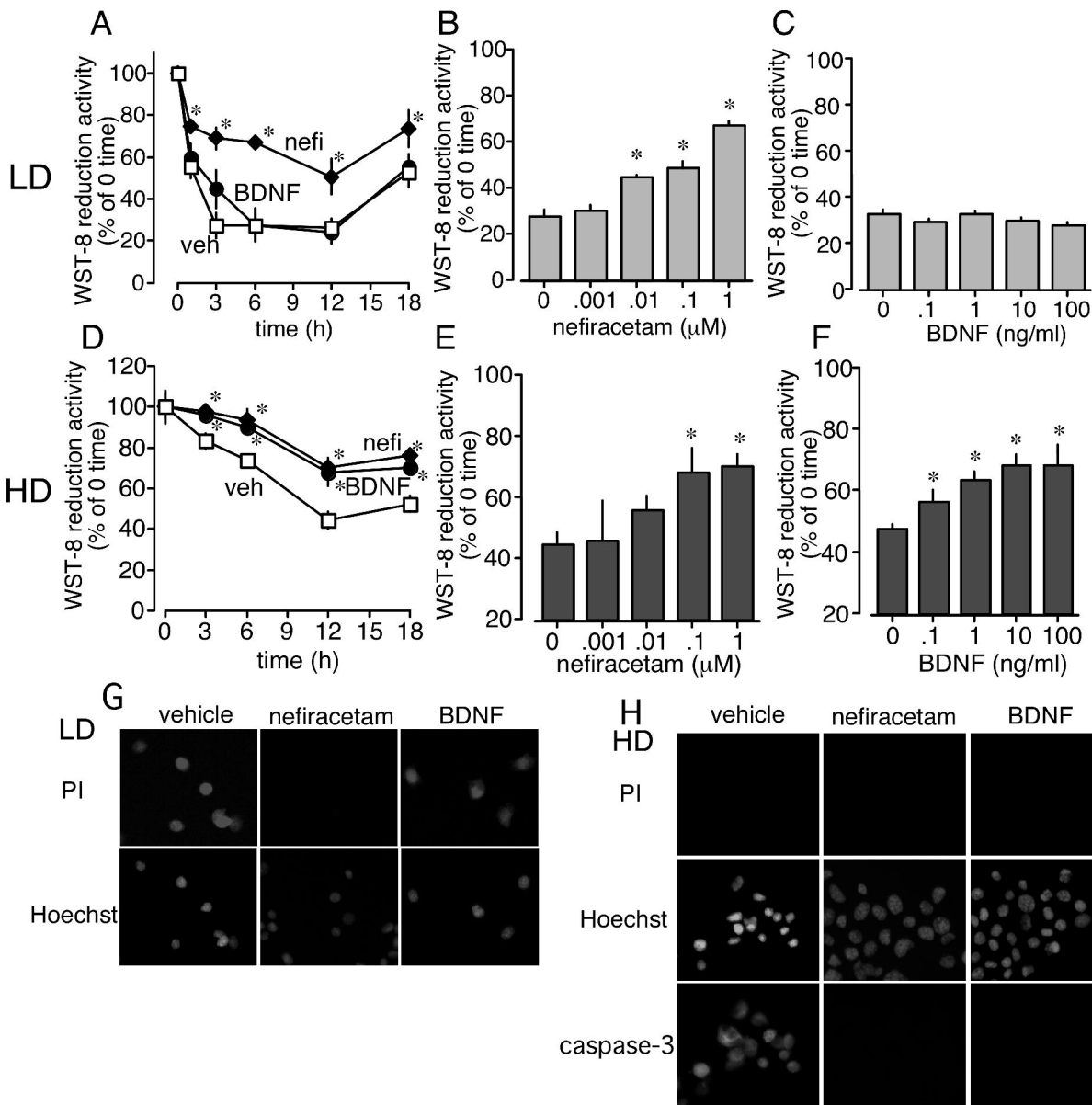


Figure 6

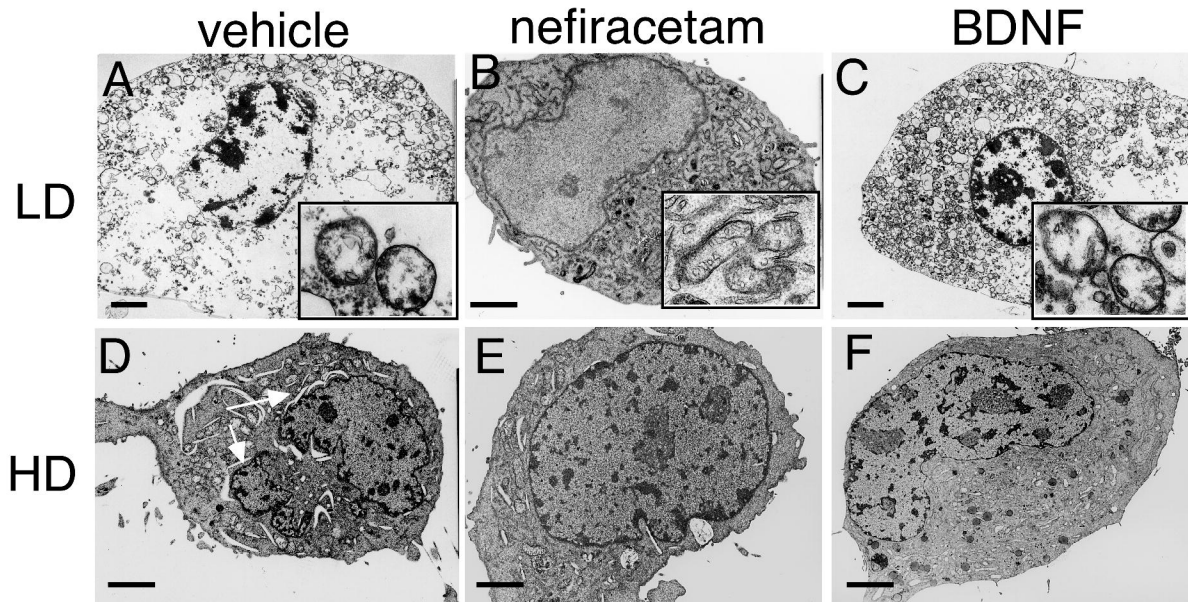


Figure 7

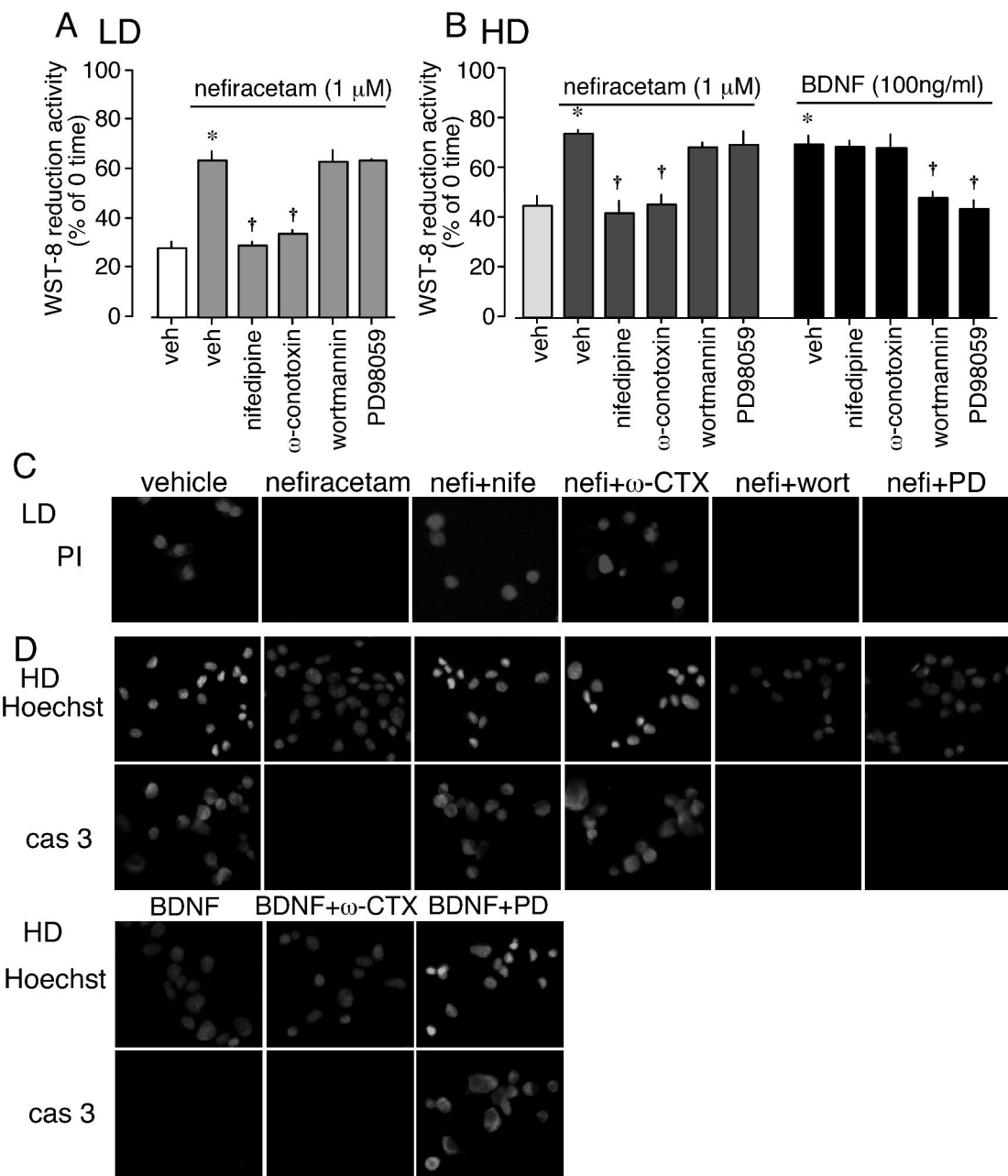


Table 1 Distinct actions of various inhibitors on the neuroprotection by nefiracetam and BDNF

Sample	LD			HD		
	PI	Hoechst	caspase-3	PI	Hoechst	caspase-3
Vehicle	73.3±8.14	6.2±3.24	3.1±2.98	4.4±4.09	60.7±10.22	57.4±5.42
Nefiracetam (1 µM)						
+ Vehicle	13.2±3.88*	4.2±5.56	2.9±2.74	1.5±0.07	5.1±1.56*	7.7±2.77*
+ Nifedipine	70.8±4.82 [†]	3.1±4.98	2.2±1.78	0.9±0.13	58.3±9.91 [†]	50.3±3.13 [†]
+ ω-CTX	66.4±6.11 [†]	5.1±3.11	4.9±2.21	1.0±0.47	60.2±2.68 [†]	55.5±11.4 [†]
+ Wort	12.4±3.29	3.8±1.98	2.7±1.73	1.5±0.11	4.9±1.54	3.4±3.11
+ PD98059	13.8±2.22	4.5±0.18	4.4±3.07	1.8±1.01	3.2±2.17	4.4±1.90
BDNF (100 ng/ml)						
+ Vehicle	75.4±7.42	6.0±3.0	2.1±1.17	3.3±1.13	3.9±1.13*	4.6±2.44*
+ Nifedipine	76.6±6.56	5.3±3.16	4.8±2.05	2.7±0.14	2.9±0.18	2.4±1.63
+ ω-CTX	74.6±3.19	3.2±3.18	1.9±4.11	3.7±2.18	2.8±0.22	1.6±1.98
+ Wort	77.4±9.91	4.3±2.08	5.5±0.14	3.8±1.24	61.5±2.13 [†]	61.9±2.34 [†]
+ PD98059	70.2±2.30	8.1±3.25	6.5±4.40	4.2±0.24	58.4±7.93 [†]	50.2±7.79 [†]

Data are the percent of total cells (150-300 cells in each group are counted), and expressed as the mean ± SEM from 3 independent experiments. **p*<0.05 (vs vehicle) and [†]*p* <0.05 (vs nefiracetam + vehicle or BDNF + vehicle). All inhibitors was used at 1 µM.

ω-CTX: ω-conotoxin GVIA, Wort: wortmannin