Supporting Information

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SI Text

NAS Derivative HIOC Displays Robust Neuroprotection Against Glutamate Excitotoxicity In Primary Neuronal Cell Cultures. To explore whether these NAS derivatives possess any of the neurotrophic effects, we pretreated the primary cortical neurons with various NAS derivatives for 30 min, followed by treatment with 100 μM glutamate for 20 h. The cell lysates were subjected to caspase-3 ELISA analysis. Both HIOC and NAS significantly blocked glutamate-triggered caspase-3 activity. As expected, BDNF also strongly inhibited caspase-3 activation by glutamate (Fig. S1*A*), underscoring that the NAS derivative HIOC possesses a prominent neuroprotective effect. To assess whether this caspase-3 suppressive activity by HIOC is through Trk receptors, we used K252a (100 nM), a small molecular Trk receptor inhibitor. As predicted, pretreatment of K252a substantially blocked HIOC and NAS's neuroprotective efficacy in primary neurons (Fig. S1*B Left*) as indicated by the cleavage of poly(ADP ribose) polymerase (PARP), a marker for apoptosis. K252a treatment also significantly blocked the suppression of glutamate-induced caspase-3 by HIOC and NAS (Fig. S1*B Right*), indicating that the neuroprotective effects of NAS and its derivative HIOC involve activating Trk receptors.

Toluidine Blue and Immunohistochemistry Staining. For toluidine blue staining, eyes were collected after the LIRD experiments and the morphology of the retina was analyzed. Briefly, eyes were removed and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 d. Eyes were subsequently washed three times in 0.1 M sodium cacodylate buffer, pH 7.4 (EMS), followed by removal of the lens. The eyecups were then embedded in an epoxy resin and processed for staining. Vertical sections of the retina were collected through the optic nerve head (ONH) followed by staining with toluidine blue using standard protocols. The thickness and density of the ONL were determined 2 mm superior and inferior to the ONH.

For immunohistochemistry, after extracted from perfused mice, eye and brain tissues were fixed in 4% paraformaldehyde overnight, then followed by paraffin embedding. Sections of 6 μm were cut. For immunohistochemical staining, paraffin sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 min and all slides were boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Phosphorylated TrkB (Y816) was detected using specific antibodies and Zymed Histo-SP AEC kit (Invitrogen). Slides were then counterstained with hematoxylin.

Pharmacokinetic Study of HIOC in Mice. Adult male Balb/C mice (2–3 mo old) were injected intraperitoneally with 40 mg/kg HIOC or 90 mg/kg NAS. At different time points (10, 30, 60, 120, 360, and 1,440 min after injection), the mice were killed and blood was collected retro-orbitally into tubes coated with lithium heparin immediately. The collected blood was centrifuged at 2,500 g for 15 min at 4 °C within 1 h after collection, and the plasma was harvested and kept frozen at −20 °C until use. One hundred μl of acetonitrile was added to 25 μl of plasma and mixed for 5 min on the plate shaker. The samples were centrifuged at 6,000 × *g* for 15 min at 4 °C; 90 μl of each supernatant transferred into a new tube and diluted with 100 μl of H_2O, centrifuged at 3,900 × *g* for 15 min. Twenty μl of the final sample was injected into the HPLC system.

Retinas were dissected and homogenized in 100 μl cold PBS using Sonic Dismembrator model 500 for 5 seconds and centrifuged at 10,000 × *g* for 10 min at 4 °C. Fifty μl of the supernatants were transferred into new tubes containing 100 μl of acetonitrile. Samples were mixed for 5 min on the plate shaker, centrifuged at 6000 g for 15 min at 4 °C, and 100 μl was transferred to a new tube. Samples were evaporated to dryness in Savant Speed-Vac, followed by reconstitution in 120 μl of acetonitrile: methanol: water (1:1:2). Forty μl of the final sample was injected into the HPLC system.

For the brain samples, the whole brain was removed immediately after collecting blood, and rinsed with cold saline (0.9% NaCl). Each brain was homogenized in 1.5 mL of cold PBS, pH 7.4, for 10 seconds on ice by an electric homogenizer (Ultra-turrax, T25 basic). Two hundred and fifty μl of chilled dextran (26%) was added to each tube containing 250 μl of the brain homogenate. Samples were centrifuged at 10,000 × *g* for 15 min at 4 °C and 100 μl of the supernatants were transferred into new tubes. Two hundred μl of acetonitrile was added to each sample, mixed, and centrifuged at 6,000 × *g* for 15 min at 4 °C. Two hundred μl of the subsequent supernatant were transferred into a new tube and evaporated to dryness in a Savant Speed-Vac. Samples were reconstituted in 125 μl of acetonitrile:methanol: water (1:1:2), and centrifuged at 3900 × *g* for 15 min. Forty μl of the supernatant was injected into the HPLC system.

The separation of the compounds was performed on an Ultrasphere ODS 250 × 4.6 mm column, 5 mm (Beckman Coulter) with 15: 0.1: 84.9 acetonitrile:acetic acid:water as the mobile phase. NAS or HIOC were detected by fluorescence with excitation and emission wavelengths set at 285 and 365 nm, respectively. External standards of NAS or HIOC with concentrations ranging from 80 pg to 80 ng/injection were used to build calibration curves.

Incubation of HIOC With the Mouse Serum or Liver Microsomes. Twelve-week-old male Balb/C mice were euthanized with CO_2. The blood was collected from the heart and the whole liver was removed immediately and washed with ice-cold PBS for 3 times to remove the blood. The liver was put in a 15-mL tube, and 5 mL of homogenate buffer (0.154 M KCl, 0.25 M sucrose in 0.05 M phosphate buffer, pH 7.5) was added. The liver was cut into small pieces followed by homogenization for 10 seconds. The homogenate was centrifuged at 10,000 rpm for 22 min at 4 °C. The supernatants containing liver microsomes were collected. Then 20 μl of 1 mg/mL HIOC (10% DMSO in 0.9% saline) was added to 180 μl of liver homogenate. After mixing, 25 μl of aliquots were incubated for indicated time (0, 15, 30, 45, 60, and 120 min). At the end of incubation, ice was introduced to stop the reaction. In the blank control, only 25 μl of liver homogenate was added. At every time point, two concentrations were set: 100 μg/mL and 10 μg/mL. After incubation, 100 μl of acetonitrile: methanol (1:1, vol/vol) was added to each tube, mixed for 5 min on the plate shaker, and centrifuged at 6000g for 15 min at 4 °C. Ninety μl of each supernatant was transferred into a new tube, diluted with 100 μl of H_2O, and centrifuged at 3,900 × *g* for 15 min at 4 °C.

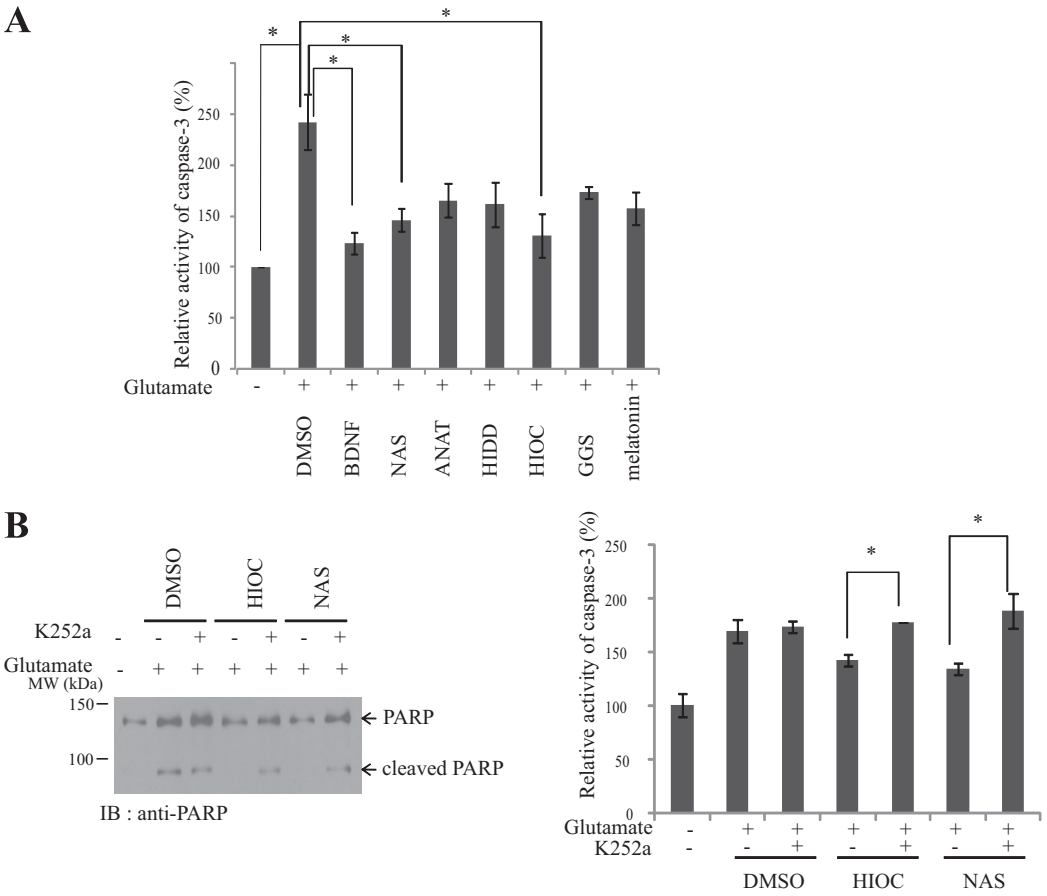

Fig. S1. HIOC possesses robust in vitro neuroprotective activity. (*A*) HIOC possesses neuroprotective activity. Primary rat cortical neurons from E18 embryos (12 DIV) were pretreated with 500 nM various drugs (or 100 ng/mL BDNF) for 30 min, followed by treatment with 100 μM glutamate for 20 h. The cell lysates were analyzed by active caspase-3 ELISA. (*B*) The neuroprotecive activity is Trk receptor-dependent. Primary rat cortical neurons were pretreated with DMSO or 100 nM K252a for 30 min, then added DMSO or 500 nM HIOC or NAS for 30 min, followed by 100 μM glutamate for 20 h. The cell lysates were conducted immunoblotting against PARP (*Left*), and also the cell lysates were analyzed by active caspase-3 ELISA (*Right*). The full-length PARP (116 kDa) and cleaved PARP (89 kDa) were indicated by arrows. The data were from two sets of replicated experiments ($n = 3$; mean ± SEM). (*$P < 0.05$, Student t test).