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HIP/PAP prevents excitotoxic neuronal death and promotes plasticity

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Running head: HIP/PAP is neuroprotective in the developing brain
Abstract

Objectives: Excitotoxicity play a significant role in the pathogenesis of perinatal brain injuries. Among the consequences of excessive activation of the N-methyl-D-aspartate (NMDA)-type glutamate are oxidative stress caused by free radical release from damaged mitochondria, neuronal death and subsequent loss of connectivity. Drugs that could protect nervous tissue and support regeneration are attractive therapeutic options. Hepatocarcinoma Intestine Pancreas protein/Pancreatitis-Associated Protein I/Regenerating Gene 2 (HIP/PAP), which is approved for clinical testing for the protection and regeneration of the liver, is also upregulated in the central nervous system following injury or disease. Here we examined the neuroprotective/neuroregenerative potential of HIP/PAP following excitotoxic brain injury.

Methods: We studied the expression of HIP/PAP and two putative effector molecules in the neonatal brain, as well as the protective/regenerative effects of HIP/PAP in three paradigms of perinatal excitotoxicity: intracerebral injection of the NMDA agonist ibotenate in newborn pups, a pediatric model of traumatic brain injury, and cultured primary cortical neurons.

Results: HIP/PAP as well as two downstream molecules, cAMP-regulated phosphoprotein 19 (ARPP19) and growth-associated protein 43 (GAP-43), were expressed in the neonatal mouse brain. HIP/PAP prevented the formation of cortical and white matter lesions and reduced neuronal death and glial activation following excitotoxic insults in vivo. In vitro, HIP/PAP promoted neuronal survival, preserved neurite complexity and fasciculation and prevented reactive oxygen species production.

Interpretation: HIP/PAP has strong neuroprotective/neuroregenerative potential following excitotoxic injury to the developing brain, and could represent an interesting therapeutic strategy in perinatal brain injury.
Introduction

Excitotoxicity plays a key role both in acute insults to the brain such as perinatal brain damage\(^1,^2\), stroke\(^3,^4\) and traumatic brain injury (TBI)\(^5\), and in various neurodegenerative disorders\(^6,^7\), and is considered a major target for neuroprotection. During excitotoxicity, the excessive activation of glutamate receptors, particularly N-methyl-D-aspartate (NMDA) receptors, leads to massive calcium influx, damage to cellular membranes and leakage of reactive oxygen species (ROS) from mitochondria, inducing neural cell death and the loss of neurites and connectivity\(^8-10\). However, due to the important function of NMDA receptors, indiscriminate targeting induces localized neuronal death and behavioral deficits in adulthood\(^11,^12\) and massive and diffuse neuronal death in the developing brain\(^13,^14\). Preventing excitotoxic cell death or promoting neuritogenesis and neuronal connectivity without directly interfering with NMDA receptors is therefore an appealing therapeutic alternative, and several such candidate molecules and mechanisms have been tested in an attempt to achieve neuroprotection or neuroregeneration\(^15,^16\).

The Hepatocarcinoma Intestine Pancreas protein/Pancreatitis-Associated Protein I/Regenerating Gene 2 (HIP/PAP), a 16kDA acute-phase response protein belonging to the C-type lectin family, is expressed in the central and peripheral nervous systems\(^17\). HIP/PAP expression is increased following brain or peripheral-nerve injury in adult rodents and humans\(^18-20\). HIP/PAP protects cerebellar, cortical and hippocampal neurons against H\(_2\)O\(_2\)-induced cell death\(^19\), and both promotes Schwann cell proliferation during motoneuron regeneration\(^21\) and exerts neurotrophic effects on motoneurons themselves\(^22\). Its dual role - neuroprotection against oxidative stress and neurotrophism - suggest that it could be useful in preventing or reversing the effects of excitotoxicity in the developing brain. In addition, HIP/PAP has an advantage over other neuroprotective agents in that it has already been approved for clinical testing. A recombinant human HIP/PAP (rcHIP/PAP) has been found to be well tolerated in healthy volunteers in a phase 1 clinical trial\(^23\), and is currently being evaluated in a double-blind randomized multicenter phase 2 clinical trial for acute liver failure (NCT01318525).
HIP/PAP stimulates liver repair and regeneration by the PKA-dependent phosphorylation of cAMP-regulated phosphoprotein 19 (ARPP19), a protein phosphatase inhibitor that specifically inhibits protein phosphatase 2A. ARPP19 binds and stabilizes the 3'-untranslated region of the mRNA for Growth Associated Protein 43 (GAP-43), leading to an overall increase in GAP-43 protein. In the central nervous system, GAP-43 is essential for axon guidance, synaptic plasticity, and neuroregeneration, and we have previously demonstrated its key role in the plasticity induced by brain-derived neurotrophic factor (BDNF) after excitotoxic lesions of the perinatal mouse brain.

In the present study, we used three experimental systems, i) excitotoxic perinatal brain injury by ibotenate injection, ii) a model of pediatric TBI that is predominantly mediated through excitotoxicity, and iii) primary cortical neuronal cultures exposed to NMDA, to investigate the protective effects of HIP/PAP on excitotoxic cell death and post-excitotoxic neuronal plasticity.
Materials and Methods

Experimental protocols were approved by institutional and local ethics committees, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health).

Animals and drugs

For in vivo experiments male and female Swiss pups (Iffa Credo, L’Abresle, France) and for in vivo experiments male and female C57Bl/6 mice were used. All animals were housed under the same temperature (25°C) and photoperiod (12h:12h light-dark cycle) conditions, and given free access to food and water.

Ibotenate and NMDA were purchased from Sigma (St-Louis, MO, USA). Ibotenate was diluted in phosphate buffered saline (PBS) containing 0.01% acetic acid. NMDA was diluted in PBS. The rcHIP/PAP protein is a recombinant human protein that corresponds to the addition of one amino-terminal methionine to the sequence of the secreted form (i.e. lacking the 26 amino acid signal sequence) of endogenous human HIP/PAP (NP_620355). It was produced in Escherichia Coli, purified to ≥99% and released in batches in compliance with the clinical grade manufacturing process, by PX’Therapeutics.

Quantitative RT-PCR analysis of HIP/PAP, ARPP19 and GAP-43

Experimental tissues came from the forebrain and hindbrain of embryonic day (E) 14 embryos (n=6), and from the neopallium and cerebellum of P0 (n=6), P5 (n=6) and P40 (n=5) mice. RNA extraction, cDNA synthesis and RT-PCR were performed and analyzed as previously described \(^2, 30\) and using RPLP0 as the housekeeping gene. Primer sequences are given in Table 1.

Neonatal excitotoxic brain lesions

We induced excitotoxic brain lesions by injecting ibotenate (10 µg) into the developing mouse neopallial parenchyma on postnatal day (P) 5 as previously described \(^2, 31, 32\). Pups
were sacrificed on P10 and following formalin fixation and paraffin embedding of the brain, 15-µm-thick serial coronal brain sections were cut, and every third section stained with cresyl violet to determine the maximal fronto-occipital extent of the lesion, used as an index of lesion severity. Lesion sizes were determined by an investigator blind to the treatment group.

**Pediatric TBI model**

P7 mouse pups were deeply anesthetized with isoflurane and subjected to a unilateral closed contusion head trauma as previously described. In brief, a 10 g weight falls from a height of 10 cm onto a circular footplate (2 mm diameter) resting on the animal's skull, through a skin incision made to expose the surface of the skull. The footplate is oriented parallel to the left parietal bone with the centre positioned 2 mm anterior and 1 mm lateral to lambda. The device was regulated to depress the skull surface by 1 mm. After surgery, animals were kept at 37°C to recover, and returned to their mothers 30 min later. On P28, whole brains were fixed in 4% formalin for 4-5 days at room temperature, processed for paraffin embedding and coronal sections, 16µm thick, cut and stained with cresyl violet. Brain atrophy induced by the degeneration of neurons after TBI was measured through the dilatation of the ipsilateral ventricle when compared to the contralateral ventricle. Measurements were made (NIH ImageJ) on 12 sections per subject, at six different levels (2 sections per level separated by by 320µm), and covering the entire extent of the ventricle. The 12 values were considered as dependant replicates.

**Analysis of cell death, astrocytosis and microgliosis**

Antibodies used were cleaved caspase-3 (1/500; Cell Signaling Technology, Beverly, MA, USA), apoptosis-inducing factor (AIF, 1/500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), glial fibrillary acidic protein (GFAP, 1/500; DAKO, Carpinteria, CA, USA), and Iba1 (1/1000; Wako Chemicals, Richmond, VA, USA), and labeling was detected with avidin-biotin horseradish peroxidase kits (Vector). Adjacent sections were used for Fluoro-Jade B
(Histo-Chem Inc., Jefferson, AR, USA) staining. Five animals were included in each group and for each time point studied and analyses were carried out by two different investigators independently.

**Primary neuronal cultures**

On P0 primary neurons were cultured as previously described. Cells were plated at a density of $2 \times 10^4$ cells/cm$^2$ onto Poly-D-Lysine-coated Labtek chamber slides (Nunc, Roskilde, Denmark). After 24 h of incubation (DIV2) at 37°C in 5% CO$_2$, the serum-containing medium was removed. The cells were then cultured in serum-free medium for 12 hours after which they were treated with NMDA (300 µM) or H$_2$O$_2$ (30 or 100 µM) in the presence or absence of HIP (0.3 to 10 µg/ml) for the next 24 hours. Following treatment, the cells were rinsed with PBS and then fixed in 4% PFA.

**Oxidative stress measurement**

Untreated and NMDA-treated neurons were assayed for malondialdehyde (MDA), an end product of lipid peroxidation, using measured using the thiobarbituric acid (TBA) procedure as previously described. Results normalized using total protein content measured using a Bio-Rad Assay Kit.

**MTS assay and TUNEL assay**

Neuronal viability was quantified using the colorimetric CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche Applied Sciences, Indianapolis, IN, USA). Quantification of TUNEL-positive cells in cultures was performed by all counting TUNEL positive cells in a total of 10 random fields from two wells each.

**Analysis of neurite branching and length**
Cultured cells stained for beta-III tubulin (1:1000; Promega, Madison, WI, USA) at 4°C overnight, incubated with Alexa Fluor 488 anti-mouse secondary antibody (Molecular Probes, USA) for one hour at room temperature and mounted with Vectashield DAPI (Vector laboratories, Burlingame, CA, USA). The number of primary, secondary and tertiary neurites (labeled with beta-III tubulin) as well as the number of branch points were counted in captured images (Supplementary Figure 1). Neurite length was measured using Leica IM50 software.

**Statistical analysis**

All data are expressed as means ± S.E.M. Data were analyzed using a Student's t-test or a univariate ANOVA (GraphPad Prism version 4.01 for Windows, GraphPad Software). When ANOVA revealed significant differences between several experimental groups, multiple comparisons between experimental groups were performed using Dunnett’s or Bonferroni’s post-hoc test.
Results

HIP/PAP is expressed in the developing brain

Using quantitative RT-PCR, HIP/PAP, GAP-43 and ARPP19 expression levels were measured in the mouse cortex (or forebrain at E14) and cerebellum (or hindbrain at E14) from E14 to P40. HIP/PAP transcripts were barely detected in embryonic samples, but increased 31- and 150-fold (p<0.01) at P0 in the cortex and cerebellum, respectively (Figure 1). From P5 to P40, HIP/PAP transcripts decreased progressively to resume their original levels at P40 (Figure 1). GAP-43 was highly expressed in the cortex and cerebellum at all ages investigated. Its expression levels in the cerebellum were constant, but displayed a 6-fold increase at P5 in the cortex (p<0.0005) when compared with its original levels (Figure 1). ARPP19 was expressed at moderate but constant levels in the cortex and cerebellum at all ages (Figure 1).

rchIP/PAP protects the developing neopallium against excitotoxic injury

Overall mortality due to ibotenate excitotoxicity was low in the present study (< 3 % of the animals injected). No significant difference was observed in a test of contingency (the Fisher exact test) between the different ibotenate-treated groups. Epileptic manifestations including clonic or tonic seizures and apneas were observed in all ibotenate-treated animals. However, treatment with rchIP/PAP did not induce any detectable difference in the severity or frequency of seizures when compared to controls (data not shown).

Mouse pups injected on P5 with intracerebral ibotenate developed typical cortical lesions and periventricular white matter cysts (Figure 2A). In P5 mice, rchIP/PAP administered i.c. immediately after ibotenate injection induced significant neuroprotection of the cortical plate and white matter when observed on P10 (Figure 2A-D). Similarly, rchIP/PAP administered i.p. immediately after ibotenate injection also induced significant and dose-dependent neuroprotection against ibotenate-induced cortical plate and white matter lesions (Figure 2C-D), an effect that was still seen at the highest dose of rchIP/PAP given 3h after ibotenate injection (Figure 2C-D). The protective effect of rchIP/PAP administered i.p. immediately
after ibotenate was accompanied by a significant reduction of cell death around the lesion site as measured by cleaved caspase-3, Fluoro-Jade B and AIF labeling one day post-ibotenate injection (Figure 3), a difference that was still observable at 5 days in the case of cleaved caspase-3. With respect to gliosis, rcHIP/PAP reduced microglial activation as measured by Iba-1 immunolabeling, and both prevented early astrocyte death (1 day post-ibotenate) and delayed astrogliosis (5 days post-ibotenate) as measured by GFAP immunolabeling (Figure 4).

rcHIP/PAP protects the developing brain against TBI

TBI induced a large increase in the cross-sectional area of the ipsilateral ventricle in saline-treated animals, an effect that reached significance 21 days post-TBI, i.e. at P28 (Figure 5). rcHIP/PAP administered i.p. immediately after TBI largely prevented this TBI-induced ipsilateral ventricular dilatation, but had no detectable effect on the contralateral ventricle (Figure 5). This protective effect was accompanied by a significant reduction of cell death at 1 day post-TBI as measured by cleaved caspase-3 and Fluoro-Jade B labeling (Figure 6), reduced astrogliosis at 5 and 21 days post-TBI as measured by GFAP immunolabeling (Figure 7A-C), and reduced microglial activation at 1 day post-TBI as measured by Iba-1 immunolabeling (Figure 7D-F).

rcHIP/PAP protects cultured cortical neurons against excitotoxic injury

Exposure of primary cortical neurons to NMDA induced significant cell death, as determined by MTS and TUNEL assays (Figure 8A-B). The addition of rcHIP/PAP completely abolished this NMDA-induced cytotoxicity (Figure 8A-B). As HIP/PAP is a ROS scavenger, its capacity to reduce oxidative damage to NMDA-treated cells was monitored next. The exposure of primary cortical neurons to NMDA induced a significant production of ROS, an effect that was completely blocked by co-treatment with rcHIP/PAP (Figure 8C). However, rcHIP/PAP under basal conditions did not significantly change the redox status of cells even after 5 hours. (Figure 8C). Further supporting a role for the antioxidant effect of rcHIP/PAP
in the protection of neurons against NMDA-induced damage, rcHIP/PAP significantly reduced neuronal cell death induced by H₂O₂ (Figure 8D).

rcHIP/PAP promotes post-excitotoxic plasticity of cultured cortical neurons

Exposure of primary cortical neurons to NMDA induced a significant reduction in the number of primary neurites per cell (Figures 9 and 10A), the proportion of cells with tertiary neurites (Figures 9 and 10B), the number of neurite branching points per cell (Figures 9 and 10C), and mean neurite length (Figures 9 and 10D). These deleterious effects of NMDA were largely counteracted by co-treatment with rcHIP/PAP (Figures 9 and 10).

In vitro, control primary cortical neurons have a tendency to form aggregates, with neurites emerging from these aggregates to form fascicles (Figure 11). NMDA exposure induced a significant reduction in the number of neurons per aggregate and of the accompanying fasciculation of neurites (Figure 11). In contrast, rcHIP/PAP reversed this NMDA-induced disaggregation of neurons and increased the accompanying fasciculation of neurites emerging from these aggregates (Figure 11). Also, rcHIP/PAP alone increased neuronal aggregate formation and neurite fasciculation (Figure 11).
Discussion

We have shown in this study that HIP/PAP as well as two potential downstream effectors, GAP-43 and ARPP19, are expressed in the mouse cortex and cerebellum during the perinatal and early postnatal period. HIP/PAP protects the developing brain and its components against excitotoxic injury. In three different models of perinatal brain injury – ibotenate-induced lesions of the cortical plate and white matter, TBI of the cortex, and NMDA-mediated insult of cultured primary cortical neurons – HIP/PAP had significant neuroprotective effects, reducing cell death and gliosis in vivo, and improving survival, redox status and neuronal morphology in vitro.

The strength of the neuroprotective effects of HIP/PAP lies in its combined anti-excitotoxic and pro-regenerative capabilities. Work from our lab with the neurotherapeutic melatonin has shown similar properties. However, HIP/PAP is the only molecule tested so far to protect both the grey and white matter from excitotoxic lesion and to function with a delayed therapeutic window. These differences are of particular interest in the context of perinatal or pediatric TBI, for which few treatment options to protect the grey and white matter are available, especially those that could still be effective when given after a delay of several hours.

How are these neuroprotective effects of HIP/PAP mediated? In keeping with the involvement of excitotoxic processes both during perinatal brain injury and in the course of neurodegenerative disorders in adulthood, the paradigms we chose to study induced neuronal damage predominantly through an excitotoxic cascade involving the activation of the NMDA-type glutamate receptor. Following the injection of the NMDA agonist ibotenate, the brain of the newborn undergoes a series of cellular and molecular changes, ranging from inflammatory changes such as gliosis and cytokine production within a few hours of the insult, to neuronal loss within a few days, and the subsequent formation of a lesion demarcated by a glial scar and the remodeling of neuronal connections. In the case of
TBI also, there is an initial and rapid excitotoxic reaction mediated by glutamate receptor activation, which gives way to apoptotic degeneration over several days and scar formation. Since both these processes (ibotenate-induced excitotoxicity and TBI-induced neuronal death) involve mitochondrial damage and oxidative stress, it appears probable that HIP/PAP acts at least partly at this level to increase neuronal survival and reduce gliosis in these injured brains. Support for this hypothesis comes from our finding that the change in redox status (i.e. the increase in lipid peroxidation) of primary cortical neuronal cultures exposed to NMDA was blocked by the presence of HIP/PAP in the culture medium. This is in keeping with previous findings as to the mode of action of HIP/PAP in vivo in the liver and intestines, where it attenuates free radical damage induced by fas and dextran sodium sulfate, respectively, as well as in vitro in primary rat brain neuronal cultures, where it blocks peroxide-induced neurotoxicity.

In addition to, or perhaps in parallel with, these effects on oxidative damage triggered by neurons themselves, HIP/PAP could also act at the level of inflammatory processes, as indicated by the reduction of reactive gliosis (both astrocytes and microglia) in our animals. Findings from several groups also indicate that HIP/PAP reduces the injury-induced upregulation of inflammatory cytokines, such as TNFα and IL-6, in other tissues, and blocks the effect of TNFα on macrophage activation and inflammatory function. However, we have no direct evidence for an effect of HIP/PAP on glia in our models and any changes may reflect decreased cell death.

HIP/PAP could also affect neuroregeneration or plasticity following injury as indicated by its effects on neurite growth, branching and fasciculation in this study. In addition, ARPP19, which promotes neurite outgrowth, is expressed in the brain over the course of normal development in our study, and HIP/PAP acts via the PKA-dependent phosphorylation of ARPP19 to induce regeneration in the gut. Regeneration is of particular importance as dying neurons undergo neurite degeneration and axonopathy is a hallmark of brain damage in preterm infants and those with TBI.
This study achieved its aim of describing the neuroprotective effects of HIP/PAP in models of damage to the immature brain. Given the current paucity of neurotherapeutic strategies for excitotoxic injuries in the perinatal and pediatric population, these data suggest that HIP/PAP might have clinical potential to reduce the burden of neurological damage associated with these injuries. Further work to elucidate the precise molecular effectors of HIP/PAP in the brain may strengthen this potential and enhance our ability to effectively use this drug.
Acknowledgements

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References

Figure Legends

Figure 1. Ontogenic expression of HIP/PAP, GAP-43, and ARPP19 mRNA in murine cerebral cortex and cerebellum, as determined by quantitative RT-PCR. Data are presented as mean gene of interest / GAPDH ratios ± s.e.m.
Figure 2. A-B. Cresyl violet-stained sections showing brain lesions induced by ibotenate injected at P5 and studied at age P10. Brains from pups treated with i.c. ibotenate alone (A) or treated with i.c. ibotenate and rcHIP/PAP (B), showing typical neuronal loss in layers II-VI (arrow) and the white matter lesions (*). LV, lateral ventricle. Bar: 40 µm. C-D. Effect of i.c. and i.p. rcHIP/PAP on ibotenate-induced lesions. Pups were injected on P5 and sacrificed on P10. Drugs indicated on the X axis were injected immediately (T0h) or three hours (T3h) after ibotenate. Values represent the mean length of the lesions ± s.e.m. Asterisks indicate statistically significant difference from the appropriate controls (black bars); *: p< 0.05, **: p< 0.01, ***: p< 0.001 by ANOVA with Dunnett's multiple comparison test.
**Figure 3.** Effect of rcHIP/PAP on ibotenate-induced cell death determined by cleaved caspase-3 immunohistochemistry (A), Fluoro-Jade B staining (B), and AIF immunohistochemistry (C). Pups were injected with ibotenate on P5 and sacrificed 1 or 5 days later. PBS or rcHIP/PAP (0.75 µg) were injected i.p. immediately after ibotenate. Values represent mean density of positive cells ± s.e.m. Asterisks indicate statistically significant difference from controls (black bars); *: $p< 0.05$, **: $p< 0.01$, ANOVA with Dunnett's multiple comparison test.
Figure 4. Effect of rcHIP/PAP on ibotenate-induced gliosis determined by immunohistochemistry for GFAP (A) and Iba1 (B). Pups were injected with ibotenate on P5 and sacrificed 1 or 5 days later. PBS or rcHIP/PAP (0.75 µg) was injected i.p. immediately after ibotenate. Values represent mean density of positive cells ± s.e.m. Asterisks indicate statistically significant difference from controls (black bars); *: p< 0.05, **: p< 0.01, ANOVA with Dunnett's multiple comparison test.
**Figure 5. A-C.** Cresyl violet-stained sections showing ventricular enlargement (red asterisk) induced by TBI performed on P7 and studied 21 days later. Ipsilateral brain hemispheres from a control animal (no TBI) (A), from an animal with TBI treated with PBS i.p. (B), and from an animal with TBI and treated with rcHIP/PAP (0.75 µg) i.p. (C). Bar: 80 µm. **D-F.** Effect of rcHIP/PAP on TBI-induced ventricular enlargement. Pups underwent TBI on P7 and were sacrificed 1 (D), 5 (E) or 21 (F) days later. PBS or rcHIP/PAP was injected i.p. immediately after the TBI. Values represent ventricular area ± s.e.m. Asterisks indicate statistically significant difference from the contralateral hemisphere of PBS-treated animals (light blue bar); § indicates a statistically significant difference from the ipsilateral hemisphere of PBS-treated animals (dark blue bar); **§§**: p< 0.01, ANOVA with Dunnett's multiple comparison test.
Figure 6. Effect of rcHIP/PAP on TBI-induced cell death determined by cleaved caspase-3 immunohistochemistry (A-B), Fluoro-Jade B staining (C-D), and AIF immunohistochemistry (E-F). Pups underwent TBI on P7 and were sacrificed 1 or 5 days later. PBS or rcHIP/PAP (0.75 µg) was injected i.p. immediately after the TBI. Values represent mean density of positive cells ± s.e.m. Asterisks indicate statistically significant difference from the contralateral hemisphere of PBS-treated animals (light blue bar); § indicates statistically significant difference from the ipsilateral hemisphere of PBS-treated animals (dark blue bar); **§§: p < 0.01, **§§§: p < 0.001, ANOVA with Dunnett's multiple comparison test.
Figure 7. Effect of rcHIP/PAP on TBI-induced gliosis, as determined by immunohistochemistry for GFAP (A-C) and Iba1 (D-F). Pups underwent TBI on P7 and were sacrificed 1, 5 or 27 days later. PBS or rcHIP/PAP (0.75 µg) was injected i.p. immediately after the TBI. Values represent mean density of positive cells ± s.e.m. Asterisks indicate statistically significant difference from the contralateral hemisphere of PBS-treated animals (light blue bar); § indicates statistically significant difference from the ipsilateral hemisphere of PBS-treated animals (dark blue bar); §: p< 0.05, §§: p< 0.01, ***: p< 0.001, ANOVA with Dunnett's multiple comparison test.
Figure 8. A-B Effect of rcHIP/PAP on NMDA-induced cell death of cultured primary cortical neurons, as determined by MTS (A) and TUNEL (B) techniques. C. Effect of rcHIP/PAP on NMDA-induced oxidative stress in primary cortical neurons, as determined by quantification of lipid peroxidation (malondialdehyde or MDA assays). D. Effects of rcHIP/PAP on H_2O_2-induced cell death of primary cortical neurons determined by the MTS technique. Values represent means ± s.e.m. Asterisks indicate statistically significant difference from the black bar; § indicates statistically significant difference from the hatched bar; *: p< 0.05, **: p< 0.01, ***: p< 0.001, ANOVA with Dunnett's multiple comparison test.
Figure 9. Effects of rcHIP/PAP on neurite extension and branching. **A-B.** Cultured primary cortical neurons treated with vehicle. **C-D.** Primary cortical neurons treated with NMDA + vehicle. **E-F.** Primary cortical neurons treated with rcHIP/PAP. **G-H.** Primary cortical neurons treated with NMDA + rcHIP/PAP. Bar: 16 µm (A, C, E, G) or 8 µm (B, D, F, H).
**Figure 10.** Effects of rcHIP/PAP on the number of primary neurites (A), the percentage of cells with tertiary neurites (B), the number of branching points (C) and neurite length (D) in primary cortical neurons. Values represent means ± s.e.m. Asterisks indicate statistically significant difference from light blue bar; §§ indicates statistically significant difference from dark blue bar; *, §: p < 0.05, **: p < 0.01, ***: §§§: p < 0.001, ANOVA with Dunnett's multiple comparison test.
Figure 11. Effects of rcHIP/PAP on neuronal aggregation. A. Primary cortical neurons treated with vehicle. B. Primary cortical neurons treated with rcHIP/PAP. C. Primary cortical neurons treated with NMDA + vehicle. D. Primary cortical neurons treated with NMDA + rcHIP/PAP. Bar: 16 µm (A-D). E. Values represent mean percentage of aggregates containing a given number of neurons, ± s.e.m. Asterisks indicate statistically significant difference from light blue bar; § indicate statistically significant difference from dark blue bar; ***, §§§: p < 0.001 in ANOVA with Dunnet's multiple comparison test.
Supplementary Figure 1. Elements taken into account for the quantitative analyses performed on cultured neurons to assess neurite branching.
Table 1. Sequences of primers used for real-time PCR.

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<td>5'-GTTCTTGCCCATCACGCACC-3'OH</td>
</tr>
</tbody>
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