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# Sonic hedgehog mediates BDNF-induced neuroprotection against mitochondrial inhibitor 3-nitropropionic acid

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ABSTRACT

Sonic hedgehog (SHH), a morphogen critical for embryogenesis, has also been shown to be neuroprotective. We have recently reported that pretreatment of rat cortical neurons for 8 h with brain-derived neurotrophic factor (BDNF; 100 ng/ml) affords protection against neurotoxicity of 3-nitropropionic acid (3-NP; 2.5 mM for 24 h), a mitochondrial complex II inhibitor. However, whether SHH is involved in BDNF-mediated neuroprotection remains unknown. Herein we tested whether BDNF induces SHH expression and if so, whether BDNF induction of SHH contributes to the observed neuroprotective effects. We found BDNF (100 ng/ml) increased SHH expression at both mRNA and protein levels. BDNF protection against 3-NP was abolished by cyclopamine (CPM; 5 μM), the SHH pathway inhibitor. Preconditioning of cortical neurons with N-terminal fragment of SHH (SHH-N; 0.1–1 ng/ml) was sufficient to confer resistance. These results indicate that BDNF induces SHH expression, which contributes to neuroprotection against 3-NP toxicity in rat cortical neurons.



Sonic hedgehog (SHH) is a mammalian member of hedgehog family known to regulate polarity of central nervous system (CNS) [1]. The biologically active N-terminal fragment of SHH (SHH-N), once secreted, exerts its actions through Patched [2,3], which is a 12-pass transmembrane protein that, in the absence of SHH-N, suppresses another 7-pass transmembrane protein called Smoothened [4]. In the presence of SHH-N, Smoothened may activate its downstream transcription factors of Gli family (for a recent review, please see [5]). Currently known target genes affected by SHH/Gli pathways include *N-Myc* [6], *Bcl-2* [7], and *Bmi1* [8] that participate, respectively, in the regulation of proliferation, survival and self-renewal.

Mitochondrial dysfunction has been implicated in the pathogenesis of Huntington's disease (HD), an autosomal dominant genetic disorder resulted from expansion of CAG repeats in the *huntingtin* gene [9]. 3-Nitropropionic acid (3-NP), a plant toxin that irreversibly inhibits succinate dehydrogenase in the complex II of mitochondrial electron transport chain, leads to depressed ATP levels and prolonged energy impairment [10]. In rats, systemic administration of 3-NP results in striatal lesions, dystonia and other abnormal motor behaviors secondary to a slowly progressive, excitotoxic striatal neuronal death [11,12]. Similar results were observed in nonhuman primates [13,14]. Thus, administration of 3-NP has been used as an approachable pharmacological model, both *in vivo* and *in vitro*, for the studies of HD.

Brain-derived neurotrophic factor (BDNF) is a pro-survival factor that belongs to neurotrophin family. Deficiency of the cortical BDNF has been proposed as one mechanism leading to neuronal demise in HD striatum due to insufficient neurotrophic support [15]. In addition to its critical roles in protecting striatal neurons, we have recently demonstrated that BDNF preconditioning may also protect cortical neurons against the neurotoxicity caused by 3-NP exposure [16]. However, the multi-faceted defensive mechanisms mediated by BDNF remain to be fully delineated. In the present study, we report that BDNF may induce the expression of SHH, which in turn results in neuroprotective effects against subsequent exposure to 3-NP in cultured rat cortical neurons.

## Materials and methods

*Rat cortical culture.* Primary neuronal cultures were prepared from cortices of Sprague–Dawley fetal rat brains at embryonic day 18 as previously described [17]. Cells were grown for 7–8 days *in vitro* to allow regeneration of dendrites before use. All the procedures for preparation of fetal rat cortical cultures were performed

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humanely in accordance with the guidelines described in the "User Manual of Laboratory Animal Center at National Yang-Ming University".

*Real-time RT-PCR.* Total RNA was isolated by RNeasy kit from QIAGEN Inc. (Valencia, CA, USA). Complementary DNA (cDNA) was produced from cellular RNA (2 μg) using the SuperScript II RNase H⁻ Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). After reverse transcription, the cDNA samples were diluted 200× in autoclaved dd$H_2O$. PCR primers were designed using PRIMER EXPRESS software (Version 2.0, Applied Biosystems, Foster City, CA, USA). The primers used are as follows: 5′-TGACTG AGGGCTGGGATGA-3′ (forward) and 5′-CCCTGTCAGACGTGGT GATG-3′ (reverse) for SHH; 5′-AGAGACAGCCGCATCTTCTTG-3′ (forward) and 5′-CGACCTTCACCATCTTGTCTATGA-3′ (reverse) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer pairs were blasted to avoid annealing to non-specific sequences during amplification. Reactions were performed in a 10-μl volume that contained 4 μl of diluted cDNA sample, 0.5 μl of each primer (final concentration at 200 nM), and 5 μl SYBR Green PCR Master mix (containing pre-mixed nucleotides, AmpliTaq Gold DNA polymerase, and optimized buffer components; Applied Biosystems) and assayed by Applied Biosystems Prism 7000 sequence detection system. Predicted cycle threshold ($C_T$) values were exported directly into EXCEL worksheets for analysis. After cycling, a melting curve was produced by slow denaturation of the PCR end-products to validate the specificity of amplification. GAPDH served as an internal reference for normalization of amplification efficiency.

*Western blotting.* Western analysis was performed as previously described [18,19]. The rabbit antibody against SHH (1:500; Cat. No. 2207, Cell Signaling Technology, Inc. Danvers,

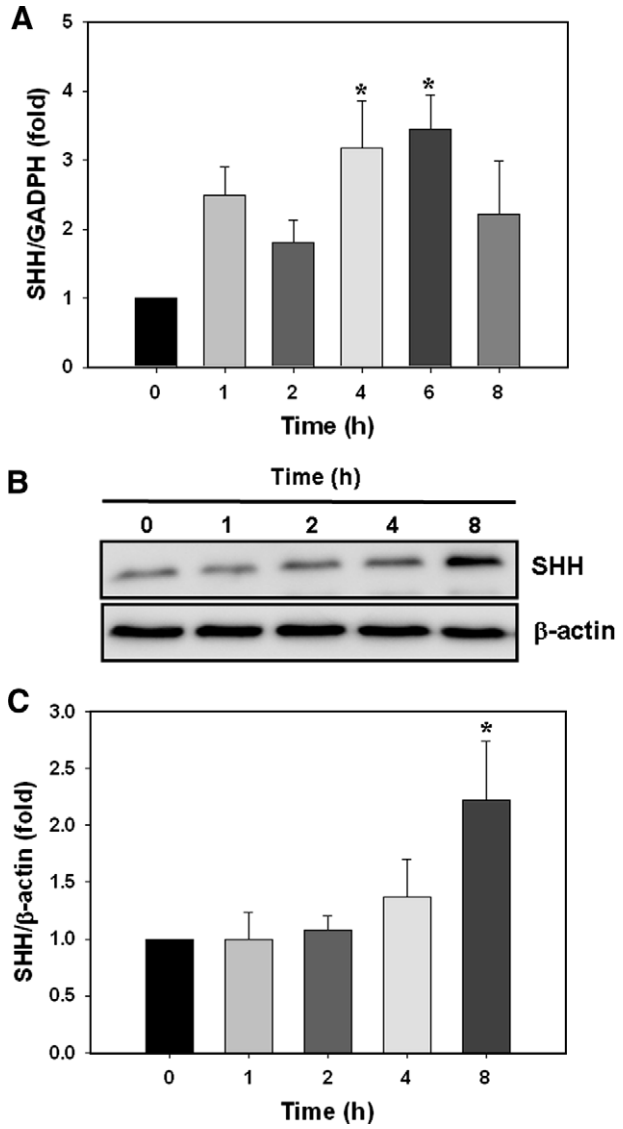


**Fig. 1.** Up-regulation of SHH by BDNF treatment in rat cortical neurons. (A) Real-time RT-PCR demonstrates BDNF-induced increase of SHH mRNA expression. Primary rat cortical cultures were incubated with 100 ng/ml BDNF at indicated times before total RNA isolation for amplification of SHH mRNA. GAPDH was also amplified as an internal standard for normalization. Combined results from 4 independent experiments using 4 different cultures with similar findings are shown. Mean ± SEM * denotes $P < 0.05$ as compared to the control cultures without BDNF treatment. (B) Western blots demonstrate an increased expression of SHH proteins upon BDNF exposure. Cultures were exposed to 100 ng/ml BDNF at indicated times before protein extraction for Western detection of SHH and β-actin, the latter served as a control for equal loading of proteins in each lane. Representative blots from 4 independent experiments using 4 different cultures are shown. (C) Quantitative results of 4 Western blots using 4 different cultures are shown. The experimental conditions were identical to (B). Mean ± SEM * denotes $P < 0.05$ as compared to the control cultures without BDNF treatment. (D) Confocal micrographs showing co-localization of SHH and MAP-2 immunostaining signals. Cortical cultures were treated with 100 ng/ml BDNF for 8 h before immunostaining with antibodies against MAP-2 (*red*) and SHH (*green*). Cultures were also stained with Hoechst 33258 to serve as counterstaining (*blue*). White arrows indicate the neurons expressing SHH. White bar is 10 μm.

MA, USA) was diluted in signal enhancer HIKARI solution 1 (Cat. No. NT08044-71R, Nacalai Tesque, Inc., Kyoto, Japan). The mouse antibody against β-actin (1:7000; Cat. No. MAB1501, CHEMICON International, Inc., Temecula, CA, USA) was diluted in blocking buffer (5% nonfat dry milk in TBST buffer containing 0.05% Tween 20, 137 mM NaCl, and 20 mM Tris–HCl, pH 7.5). The horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies were applied in fresh blocking buffer at 1:5000 to respectively detect the primary antibodies for SHH and β-actin. Filter membranes were pre-hybridized in blocking buffer for 1 h at room temperature. Hybridizations with the primary antibody of SHH and all the secondary antibodies were conducted at room temperature for 1 h. Hybridizations with the primary antibody of β-actin were performed at 4 °C overnight. Membranes were washed three times, each 10 min at room temperature, in TBST buffers between each hybridization and before detection. Immunoreactive signals were detected using ECL-Plus Western blotting detection reagents from Millipore Corp. (Boston, MA, USA). The blots were examined under Luminescence/Fluorescence Imaging System LAS-4000 (FUJIFILM, Tokyo, Japan). Quantification of protein expression was accomplished by using Multi Gauge analysis software (FUJIFILM).

*Immunocytochemistry by confocal microscope.* The following primary antibodies were used: the rabbit antibody for SHH (1:250; Cat. No. 2207, Cell Signaling Technology, Inc.), the mouse monoclonal antibody for microtubule-associated protein-2 (MAP-2, 1:100, Cat. No. MAB378, CHEMICON International, Inc.) and rabbit monoclonal antibody for glial fibrillary acidic protein (GFAP, Cat. No. Z0334, 1:600, DakoCytomation Denmark A/S, Denmark). The Texas Red-conjugated goat anti-mouse IgG secondary antibodies (1:150; Cat. No. T-6390; Molecular Probes, Eugene, OR, USA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:150; Cat. No. 02-15-06, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) were applied to respectively recognize the mouse IgG for MAP-2 and the rabbit IgGs for SHH or GFAP. The coverslips were examined under a laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan) equipped with filter sets to detect Texas Red (excitation/emission: 595-nm/615-nm) and FITC (excitation/emission: 494-nm/518-nm) fluorescence signal.

*Cell survival assays.* Hoechst staining and PI/Hoechst double staining to respectively assess cell survival and death were performed as described in details in our previous publication [16]. Note that unlike Hoechst staining alone where highly fluorescent, condensed nuclei indicative of cell death were excluded, in the PI/Hoechst double staining all the Hoechst-stained nuclei regardless of their morphology were included to serve as counterstaining. Counting of PI- or Hoechst-positive nuclei was performed by at least two individuals to prevent from personal bias. Each *N* represents data collected from a single coverslip. For each coverslip, at least 3 vision fields were randomly selected for counting to obtain the mean number of stained nuclei on this coverslip. Typically, approximately 100–120 Hoechst-positive nuclei with normal nuclear morphology were obtained from each vision field in the control cultures. In any given experiments, mean values were derived from combined results of at least three independent cultures with $N = 6–12$, as specified in figure legends for each experiment. For Hoechst staining, the "Cell Survival (%)" was defined as the mean numbers of surviving cells in experimental groups divided by those of control cultures in the same experiment and then multiplied by 100%. For PI/Hoechst double staining, the "Death Index" was defined as the number of PI-positive nuclei divided by that of Hoechst-positive nuclei in the same vision field.

*Statistical analyses.* Results were presented as means ± SEM. Multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc Student–Newman–Keuls test. *P* values of less than 0.05 were considered significant.

## Results

### *BDNF induces SHH expression in rat cortical cultures*

To test the hypothesis that SHH is involved in the BDNF-dependent neuroprotective effects, we first determined whether BDNF may up-regulate expression of SHH in rat cortical neurons. Initial selection of BDNF dosage at 100 ng/ml was based on our previous report showing that BDNF at this concentration conferred neuronal resistance against 3-NP toxicity [16]. We found that BDNF (100 ng/ml) increased expression of SHH mRNA at 4–6 h, with the maximal induction at 6 h (3.45 ± 0.49-folds; Fig. 1A). Consistently, Western analyses revealed BDNF-induced up-regulation of SHH protein expression (Fig. 1B). Quantifications of Western blots from 4 independent experiments indicated that BDNF induction of SHH protein achieved the maximal extent at 8 h (2.23 ± 0.51-folds; Fig. 1C). The BDNF-induced increase of SHH protein levels lasted for at least additional 2 h after removal of BDNF (data not shown). To examine the cell type that expresses SHH proteins, we conducted confocal microscopy. Results revealed that immunostaining signal of SHH can be co-localized to that of MAP-2 (Fig. 1D), suggesting neuronal expression of BDNF-induced SHH proteins.

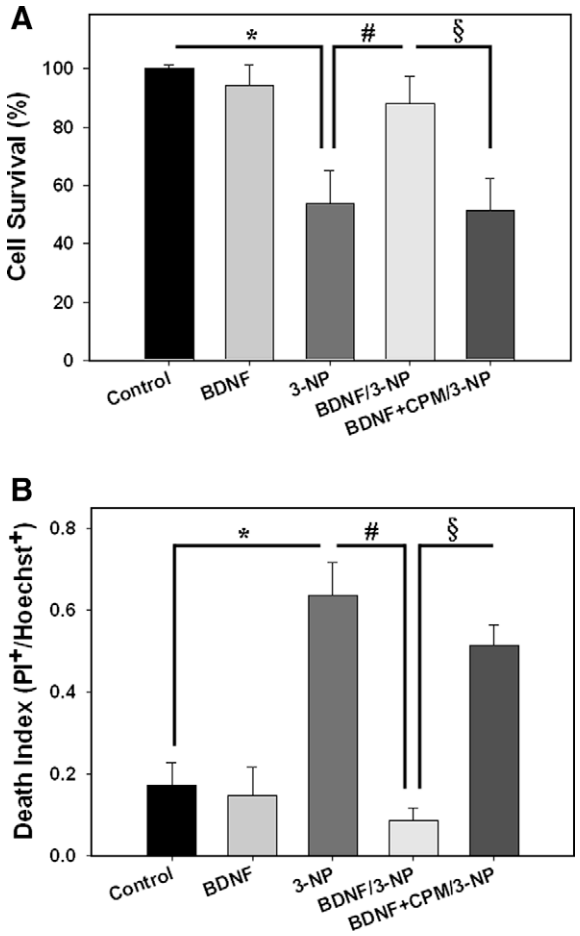


**Fig. 2.** Abolishment of BDNF-mediated neuroprotection by SHH inhibitor CPM. Cortical cultures were exposed for 8 h to culture medium alone or the medium containing BDNF (100 ng/ml) or BDNF (100 ng/ml) plus CPM (5 μM). This was followed by incubation with medium in the presence or absence of 3-NP (2.5 mM) for 24 h. Cells were then subjected to Hoechst staining (A) or PI/Hoechst double staining (B) to respectively determine the extent of cell survival and cell death. Mean ± SEM from $N = 6–9$ in (A) and $N = 6$ in (B). Combined results from 3 independent experiments using 3 different cultures were shown. *, #, and § all denote $P < 0.05$.

*SHH induction is required for BDNF neuroprotection*

We then investigated whether SHH activity is required for BDNF-dependent neuroprotection against 3-NP toxicity. Cyclopamine (CPM) is a steroid–alkaloid that inhibits the hedgehog signaling through interaction with the hedgehog receptor Smoothened [20]. To test if inhibition of SHH activity may abolish BDNF neuroprotection, rat cortical cultures were pretreated for 8 h with BDNF (100 ng/ml) in the presence or absence of CPM (5 μM) before subsequent exposure to 3-NP (2.5 mM for 24 h). Results derived from Hoechst staining shown in Fig. 2A indicated that BDNF-dependent neuroprotection (percent cell survival, 84.77 ± 6.29% versus 49.88 ± 7.70%; BDNF/3-NP versus 3-NP) was completely abolished by co-incubation with CPM during preconditioning phase (percent cell survival, 46.29 ± 7.60% versus 84.77 ± 6.29%; BDNF+CPM/3-NP versus BDNF/3-NP). Similarly, PI/Hoechst double staining shown in Fig. 2B revealed that BDNF-mediated reduction of "Death Index" (0.09 ± 0.03 versus 0.64 ± 0.08; BDNF/3-NP versus 3-NP) was also reversed by 5 μM CPM (0.51 ± 0.05 versus 0.09 ± 0.03; BDNF+CPM/3-NP versus BDNF/3-NP). CPM at this dosage did not cause cell death (data not shown). Together these results establish the notion that SHH is required for BDNF-dependent neuroprotection.

*Exogenous SHH-N is sufficient to neutralize 3-NP toxicity*

In addition to confirming that SHH is required for BDNF-dependent neuroprotection, we also tested whether an increase of SHH activity alone is sufficient to neutralize 3-NP toxicity in cultured rat cortical neurons. As revealed in Fig. 3A, preconditioning of rat cortical cultures with the biologically active N-terminal fragment of SHH (SHH-N) for 8 h conferred neuronal resistance, with the maximal efficacy observed between 0.1 ng/ml (percent cell survival, 70.70 ± 3.53% versus 46.43 ± 6.31%; SHH-N/3-NP versus 3-NP) and 1 ng/ml (percent cell survival, 71.37 ± 4.72% versus 46.43 ± 6.31%; SHH-N/3-NP versus 3-NP). Initial selection of 8-h was based on our earlier report showing that BDNF neuroprotection requires an 8-h pretreatment time [16]. To further optimize the conditions for SHH-N preconditioning, cortical cultures were incubated with 1 ng/ml SHH-N for various times prior to 3-NP challenge. Results shown in Fig. 3B indicated that SHH-N at this

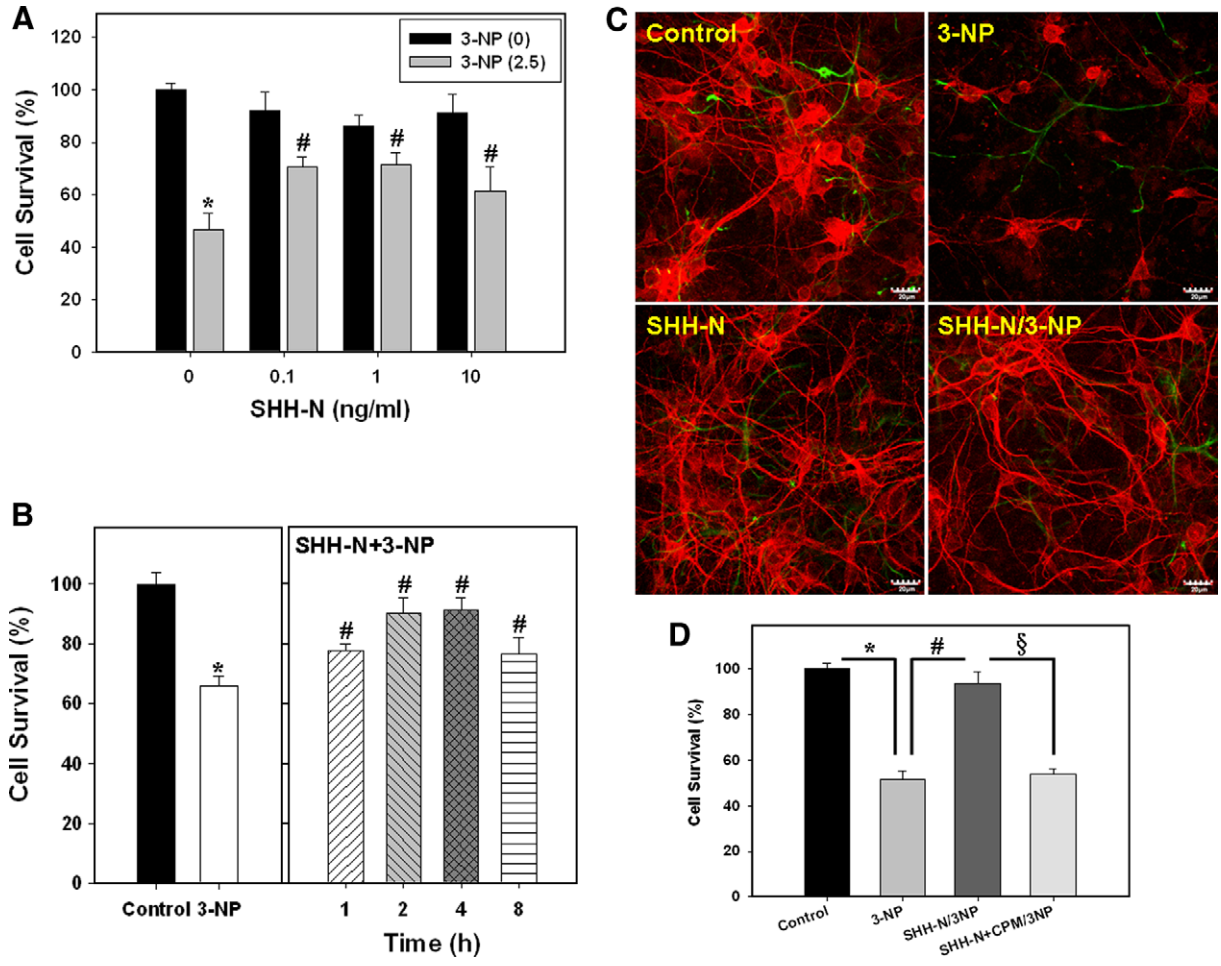


**Fig. 3.** SHH-N-mediated neuronal resistance to 3-NP toxicity. Cortical cells were incubated with SHH-N at indicated concentrations for 8 h (A) or with 1 ng/ml SHH for indicated times (B) before exposure to 2.5 mM 3-NP for additional 24 h. Cell survival was assessed by Hoechst staining. Mean ± SEM from $N = 10$ in (A) and $N = 9$–12 in (B). Combined results from 3 independent experiments using 3 different cultures were shown. * denotes $P < 0.05$ as compared to the corresponding control cells. # denotes $P < 0.05$ as compared to the cells challenged with 3-NP alone without SHH-N preconditioning. (C) Primary fetal cortical cultures were incubated with 2.5 mM 3-NP for 24 h with or without prior exposure to SHH-N at 1 ng/ml for 8 h. The cells were then immunostained with antibodies against MAP-2 (*red*) and GFAP (*green*) before confocal microscopy. White scale bar in the lower right corner of each micrograph is 20 μm. Note restoration of neuronal morphology by SHH-N preconditioning (*SHH-N/3-NP*) as compared to the culture treated with 3-NP alone (*3-NP*). (D) Cortical cultures were exposed for 8 h to culture medium alone or the medium containing SHH-N (1 ng/ml) or SHH-N (1 ng/ml) plus CPM (5 μM). This was followed by incubation with medium in the presence or absence of 3-NP (2.5 mM) for 24 h. Mean ± SEM from $N = 9$. Combined results from 3 independent experiments using 3 different cultures were shown. *, #, and § all denote $P < 0.05$. Note complete abolishment of SHH-N-dependent protective effects by CPM.

dosage was most effective when applied 2 h (percent cell survival, 90.06 ± 5.39% versus 65.95 ± 3.14%; SHH-N/3-NP versus 3-NP) to 4 h (percent cell survival, 91.10 ± 4.07% versus 65.95 ± 3.14%; SHH-N/3-NP versus 3-NP) before 3-NP exposure. In addition to Hoechst staining, immunocytochemical studies with antibodies against MAP-2 and GFAP, the respective marker protein for neurons and glia, were also conducted to visualize the neuroprotective action of SHH-N against 3-NP. Results shown in Fig. 3C clearly reveal that SHH-N (1 ng/ml for 8 h) restored neuronal morphology with recovery of extensive dendritic structures that were damaged by 3-NP (2.5 mM for 24 h). Furthermore, this effect was specific because Fig. 3D shows that SHH-N-mediated neuroprotection against 3-NP (percent cell survival, 93.70 ± 5.13% versus 51.74 ± 3.70%; SHH-N/3-NP versus 3-NP) was abrogated by CPM (percent cell survival, 53.80 ± 2.46% versus 93.70 ± 5.13%; SHH-N+CPM/3-NP versus SHH-N/3-NP). Overall, these data indicate that an increase of SHH activity alone by preconditioning is sufficient to neutralize 3-NP toxicity in cultured rat cortical neurons.

## Discussion

BDNF belongs to the neurotrophin family that may affect neuronal survival and differentiation. SHH is a morphogen important for the embryonic development. Potential correlation between BDNF and SHH is, however, less well studied. One recent study has shown that SHH expression was up-regulated prior to the induction of BDNF mRNA in Schwann cells adjacent to the injured site in an animal model of sciatic nerve injury [21]. Consistent with a causative relationship between the induction of SHH and BDNF, continuous administration of hedgehog inhibitor CPM to the injured site suppressed the increase of BDNF expression and, notably, deteriorated the survival of motor neurons in lumbar spinal cord [21]. In our study, we demonstrated BDNF-induced up-regulation of SHH at both mRNA and protein levels; further, up-regulation of SHH mRNA at 4–6 h precedes the heightened expression of SHH proteins at 8 h following exposure to BDNF (Fig. 1), suggesting involvement of a transcriptional mechanism, at least in part. Expression of SHH induced by BDNF can be localized to the cells that also express MAP-2 (Fig. 1D), indicating that neuronal populations contribute to the expression of SHH. SHH induction mediates the neuroprotective effects of BDNF because the SHH signaling inhibitor CPM was capable of abolishing BDNF protection (Fig. 2). Further, exogenous SHH-N alone mimicked BDNF action that was sufficient to attenuate 3-NP toxicity towards cortical neurons in a dose- and time-dependent fashion (Fig. 3). These results thus establish a "BDNF → SHH → 3-NP resistance" pathway in rat cortical cultures.

The molecular mechanisms underlying SHH-mediated protective effects against 3-NP toxicity remain to be fully delineated. Recently, a protective action exerted by SHH has been revealed in several animal models of ischemia/reperfusion. Thus, SHH may augment blood flow recovery and limb salvage following operatively induced hind-limb ischemia in aged mice [22]. Pretreatment of SHH has been shown to protect cardiomyocytes against hydrogen peroxide-induced cytotoxicity *in vitro* [23]. SHH gene transfer also reduced the number of cardiomyocytes positively stained with activated caspase-3 after myocardial infarction in mice [23]. Notably, SHH is able to activate the promoter of anti-apoptotic *Bcl-2* gene [7]. Thus, SHH may afford both anti-oxidative and anti-apoptotic actions under appropriate circumstances. In addition to BDNF-induced SHH as shown in current study, we have previously proposed another potential signaling pathway of "BDNF → NOS/NO → PKG → thioredoxin → Bcl-2 → 3-NP resistance" that may have contributed to the observed BDNF effects [16]. Whether SHH induction leads to activation of this signaling cascade is currently under investigation.

Metabolic stress induced by compromised mitochondria has been implicated in both acute and chronic neurodegenerative disorders such as ischemic stroke, Alzheimer's disease, Parkinson's disease (PD), and HD. It has also been shown that SHH reduces behavioral deficits induced by intrastriatal 6-hydroxydopamine (6-OHDA) lesion and suggests that SHH may be useful in the treatment of disorders that affect the nigrostriatal system, such as PD [24]. Results derived from the present report thus pave a foundation for future *in vivo* studies in the application of BDNF and/or SHH-N for therapeutic intervention of HD or other neurodegenerative disorders involving mitochondrial dysfunction.

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