

Establishment of a human pluripotent stem cell-derived sensory neuron model for diabetic peripheral neuropathy and pharmacological evaluation

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Article

Keywords: Human pluripotent stem cells (hPSCs), Sensory neurons, Diabetic peripheral neuropathy (DPN), Axonal degeneration, Disease modeling, Drug screening

Posted Date: August 20th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10539421/v1>

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Additional Declarations: No competing interests reported.

Abstract

Objectives

Diabetic peripheral neuropathy (DPN) lacks effective disease-modifying therapies due to the shortage of standardized, human-relevant in vitro models. This study aimed to establish a human pluripotent stem cell (hPSC)-derived sensory neuron platform to model DPN pathogenesis and evaluate potential neuroprotective agents.

Methods

Human PSCs were directed into pure, mature sensory neurons (BRN3A⁺/ISL1⁺/Peripherin⁺/HB9⁻). A combinatorial DPN injury model was constructed using high glucose (HG, 45 mM) pre-treatment followed by acute hydrogen peroxide (H₂O₂)-induced oxidative stress, incorporating mannitol osmotic controls. The protective effects of N-acetylcysteine (NAC), minocycline, and metformin were evaluated via intracellular ROS levels, mitochondrial membrane potential, cellular viability, and 21-day neurite network integrity.

Results

High glucose specifically reduced neuronal viability via metabolic toxicity rather than hyperosmolarity. Pharmacological pre-treatments with NAC, minocycline, or metformin significantly suppressed HG/H₂O₂-induced ROS overproduction, prevented mitochondrial membrane potential collapse, and enhanced cellular viability. Crucially, long-term morphological analysis over 21 days demonstrated that early drug intervention effectively rescued neurite fragmentation and attenuated progressive axonal degeneration.

Discussion

This hPSC-derived sensory neuron platform faithfully recapitulates key metabolic and long-term morphological features of clinical DPN. By bridging acute mitochondrial dysfunction with chronic axonal degeneration, this human-relevant model offers a scalable and reliable in vitro tool for disease modeling and high-throughput neuroprotective drug screening.

1. Introduction

Diabetic peripheral neuropathy (DPN) is one of the most prevalent and debilitating complications of diabetes mellitus, affecting approximately half of all diabetic patients globally¹. Clinically, DPN primarily manifests as a sensory predominant neuropathy, initially impairing pain and temperature sensation in a “glove-and-stockings” distribution². The fundamental pathological hallmark of DPN is a “dying-back”

axonal degeneration of distal sensory nerve fibers, which eventually progresses to neuronal apoptosis³. Despite its high prevalence, current clinical management is largely limited to symptomatic pain relief and strict glycemic control, lacking effective disease-modifying therapies that can directly prevent or reverse sensory nerve degeneration⁴.

The development of targeted neuroprotective agents has been severely hindered by the lack of physiologically relevant human experimental models. Historically, DPN research has heavily relied on rodent models; however, substantial interspecies differences in metabolic rates, nerve conduction velocities, and regenerative capacities often lead to poor translational success in human clinical trials⁵. Furthermore, acquiring primary human sensory neurons from dorsal root ganglia (DRG) is constrained by ethical and logistical limitations⁶. In recent years, human pluripotent stem cells (hPSCs) have emerged as a powerful tool^{7,8}, offering a scalable and ethically viable source to generate specific human neuronal subtypes, thus providing an ideal platform for *in vitro* disease modeling and drug screening^{9,10}.

Pathologically, chronic hyperglycemia is recognized as the primary trigger of DPN, which subsequently induces severe intracellular oxidative stress and mitochondrial dysfunction in sensory neurons^{11,12}. Most existing *in vitro* models attempt to mimic this by simply exposing cells to high glucose concentrations. However, these simplified approaches often fail to replicate the complex acute-on-chronic metabolic stress seen in patients, and frequently overlook the critical confounding factor of hyperosmolarity¹³. Moreover, many *in vitro* pharmacological screenings rely primarily on short-term cellular viability readouts, failing to capture the long-term axonal morphological changes that are central to clinical DPN progression^{14–16}.

To address these critical gaps, the present study aimed to establish a highly reliable and standardized human *in vitro* model of DPN using hPSC-derived sensory neurons. We first directed hPSCs into highly pure mature sensory neurons, rigorously verified by the robust expression of typical sensory markers (TUBJ1, Peripherin, BRN3A, ISL1) and the absence of the motor neuron marker HB9. We then engineered a combinatorial injury model by integrating high glucose pre-treatment with acute H₂O₂-induced oxidative stress, strictly utilizing osmotic controls to rule out non-metabolic physical damage. Finally, using known neuroprotective and antioxidant agents (NAC, minocycline, and metformin), we validated the platform's capacity for drug evaluation by assessing both acute mitochondrial functional rescue (ROS levels and membrane potential) and long-term morphological protection against axonal degeneration. This study provides a robust, human-relevant platform for elucidating DPN mechanisms and accelerating neuroprotective drug discovery.

2. Results

2.1. Directed differentiation and characterization of highly pure hPSC-derived sensory neurons

To establish an *in vitro* platform for DPN, we first adopted a small-molecule-driven stepwise protocol to direct the differentiation of human pluripotent stem cells (ESC-H9) into mature sensory neurons over a 35-day period (Fig. 1A). The hPSCs sequentially progressed through neural crest induction, sensory fate specification, and terminal maturation phases. Immunofluorescence staining at day 35 revealed a robust and dense neuronal network, characterized by the extensive expression of the pan-neuronal marker TUJ1 and the peripheral nervous system (PNS)-specific structural protein Peripherin (Fig. 1B, C). Furthermore, quantitative analysis demonstrated that over 80% of the cells expressed specific sensory neuron transcription factors, including BRN3A and ISL1 (Fig. 1E). Crucially, to confirm the lineage specificity of our differentiation paradigm, we assessed the expression of the motor neuron marker HB9. As expected, HB9 expression was virtually undetectable in our cultures (Fig. 1D, E), confirming that we successfully generated a highly pure population of human sensory neurons, free from motor neuron contamination, providing a reliable cellular foundation for subsequent DPN modeling.

2.2. Establishment of a combinatorial *in vitro* DPN model with strict osmotic control

DPN pathogenesis in patients involves chronic hyperglycemia coupled with subsequent oxidative stress. To faithfully recapitulate this complex microenvironment, we designed a combinatorial injury model consisting of a 24-hour high glucose (HG, 45 mM) pre-treatment followed by acute hydrogen peroxide (H_2O_2) exposure (Fig. 2A). We first investigated the isolated effect of high glucose. Compared to the culture medium control (25 mM glucose), exposure to 45 mM HG resulted in a modest but significant reduction in cell viability, as quantified by the ATP-based CTG luminescence assay (Fig. 2B, C). To rigorously exclude the possibility that this cytotoxicity was merely an artifact of hyperosmolarity¹⁷, we introduced an osmotic control group utilizing 20 mM mannitol supplemented into the basal medium (Fig. 2B). Importantly, the osmotic control showed no significant difference in cell viability compared to the normal control (Fig. 2D), unequivocally demonstrating that the observed neuronal damage was driven by glucose-induced metabolic stress rather than physical osmotic pressure. Furthermore, a dose-response evaluation of acute H_2O_2 treatment established an IC₅₀ of approximately 60 μ M (Fig. 2E), which was selected as the standard acute oxidative stressor for all downstream drug interventions.

2.3. Pharmacological attenuation of oxidative stress and mitochondrial dysfunction in the DPN model

Having established the HG/ H_2O_2 combinatorial injury model, we next evaluated its utility for drug screening by employing three well-documented neuroprotective/antioxidant agents: N-acetylcysteine (NAC)¹⁸, minocycline¹⁹, and metformin^{20,21} (Fig. 3A). H_2DCFDA staining revealed that the dual HG/ H_2O_2 insult provoked a massive, 7-fold accumulation of intracellular reactive oxygen species (ROS) compared to the control group (Fig. 3B, C). This severe oxidative stress was closely accompanied by mitochondrial membrane potential collapse, as indicated by a dramatic shift from JC-1 red (aggregates) to green

(monomers) fluorescence (Fig. 3D, E). Following a 2-hour pre-intervention with the selected drugs, we observed a remarkable phenotypic rescue. NAC, minocycline, and metformin all significantly suppressed ROS overproduction and restored the mitochondrial membrane potential, with the potent antioxidant NAC exhibiting the most pronounced restorative effect (Fig. 3B-E). Consistent with the restoration of mitochondrial function, all three agents significantly improved overall cellular viability against the HG/H₂O₂ challenge (Fig. 3F).

2.4. Long-term morphological evaluation reveals protection against DPN-associated axonal degeneration

While short-term viability and metabolic assays are informative, clinical DPN is predominantly characterized by progressive, long-term distal axonal degeneration. To determine whether our *in vitro* platform could model this crucial morphological hallmark, we extended the post-injury evaluation window to 21 days (Fig. 4A). Immunofluorescent tracking of the TUJ1-positive neural network revealed that the long-term HG/H₂O₂ insult induced severe neurite fragmentation and a drastic reduction in total axonal density, mimicking the “dying-back” neuropathy seen in DPN patients (Fig. 4B). Quantitative analysis confirmed a nearly 70% decrease in the relative TUJ1-positive area in the vehicle-treated model group (Fig. 4C). Strikingly, early intervention with NAC, minocycline, or metformin provided sustained neuroprotection, significantly preserving the integrity of the neurite network over the 3-week period (Fig. 4B, C). These long-term morphological results validate that our hPSC-derived sensory neuron platform can reliably recapitulate the core pathological features of DPN and serves as a highly predictive tool for screening therapies aimed at preventing axonal degeneration.

3. Discussion

Diabetic peripheral neuropathy (DPN) is a progressive and debilitating complication lacking effective disease-modifying treatments, largely due to the absence of reliable human-relevant *in vitro* models. In this study, we successfully engineered a robust, hPSC-derived sensory neuron platform to model DPN. By combining high glucose (HG) and acute oxidative stress (H₂O₂) with rigorous osmotic controls, our model accurately recapitulated key pathological features of DPN, ranging from acute mitochondrial dysfunction to long-term axonal degeneration. Furthermore, we validated the translational utility of this platform by demonstrating the neuroprotective efficacies of three distinct pharmacological agents.

The reliability of any stem cell-based disease model fundamentally depends on the quality and specificity of the derived cellular population. Unlike typical protocols that yield heterogeneous neural mixtures, our directed differentiation paradigm generated a highly pure population of mature sensory neurons, evidenced by the uniform expression of sensory markers and the critical absence of motor neuron contaminants (HB9-negative). Moreover, when establishing the HG-induced injury, we utilized a mannitol-based osmotic control. This is a crucial methodological distinction, as many existing *in vitro* DPN models fail to differentiate between glucose-specific metabolic toxicity and non-specific

hyperosmotic shock. Our sensitive ATP-based CTG viability data clearly confirmed that the observed neuronal impairment was intrinsically driven by glucotoxicity.

A major limitation of traditional *in vitro* neurotoxicity screening is the over-reliance on acute cell death readouts, which do not fully reflect the chronic, "dying-back" neuropathy characteristic of clinical DPN. Our combinatorial model elegantly addresses this by bridging short-term metabolic responses with long-term morphological outcomes²². Acute H₂O₂ exposure triggered massive ROS accumulation and mitochondrial membrane potential collapse, which could be rapidly rescued by NAC, minocycline, and metformin. More importantly, by extending our observation window to 21 days, we captured the profound disruption of the neurite network. The ability of these pharmacological agents to prevent this long-term axonal degeneration highlights the robust predictive power of our platform for clinical efficacy.

While the current study successfully validates the phenotypic and morphological reliability of this hPSC-derived DPN model, we acknowledge certain limitations. Specifically, the detailed downstream molecular mechanisms underlying the neuroprotective effects of metformin (e.g., AMPK/mTOR pathway activation) or minocycline were not exhaustively explored²³. The primary focus of this work was to establish and validate a standardized, scalable human *in vitro* platform rather than to dissect individual signaling cascades²⁴. Future studies leveraging this validated system could incorporate transcriptomic (RNA-seq) or proteomic analyses to unearth the precise intracellular cascades driving human sensory axonal degeneration.

In conclusion, we present a highly characterized, hPSC-based *in vitro* platform that effectively mimics the metabolic and morphological hallmarks of clinical DPN. By bypassing interspecies differences and offering long-term readouts for axonal integrity, this model provides an invaluable tool for both elucidating human-specific DPN pathophysiology and accelerating the high-throughput screening of novel neuroprotective therapeutics.

4. Materials and Methods

4.1 Human Pluripotent Stem Cell Culture and Directed Differentiation

Human pluripotent stem cells (hPSCs, ESC-H9 cell line, RRID: CVCL_9773) were originally obtained from the WiCell Research Institute (Madison, WI) with no contamination. Briefly, hPSCs were maintained on Matrigel-coated plates (Corning) in mTeSR1 (Stemcell) medium at 37°C in a humidified incubator with 5% CO₂. Upon reaching 70–80% confluence, hPSCs were dissociated using ReLeSR (Stemcell) for passaging. The directed differentiation into sensory neurons was achieved through a precisely timed 35-day, small-molecule-driven protocol^{14,25}.

Phase 1 (Neural Crest Induction, Days 0–10)

hPSCs were seeded onto standard tissue culture plates and cultured in a DMEM/F12 medium (Gibco) supplemented with the dual SMAD inhibitor SB431542 (10 μ M, Sigma) and the GSK3 inhibitor CHIR99021 (3 μ M, Sigma) to efficiently generate rosette-like neural progenitors. Medium was refreshed every other day.

Phase 2 (Sensory Fate Specification, Days 10–20)

To drive the neural progenitors toward a sensory lineage, the medium was transitioned to incorporate BMP4 (10 ng/mL, Peprotech) and the Notch signaling inhibitor DAPT (10 μ M, Sigma), facilitating the emergence of nascent sensory neurons.

Phase 3 (Terminal Maturation, Days 20–35): For final structural and functional maturation, cells were cultured in a complete neuro-maturation medium (Neurobasal medium supplemented with B27) (Gibco) enriched with a cocktail of neurotrophic factors and signaling molecules: NGF (10 ng/mL, Peprotech), BDNF (10 ng/mL, Peprotech), GDNF (10 ng/mL, Peprotech), NT-3 (10 ng/mL, Peprotech), cAMP (1 mM, Sigma), and Ascorbic Acid (200 μ M, Sigma). Fully mature induced-sensory neurons obtained at day 35 were utilized for all downstream disease modeling experiments.

4.2 Establishment of the *In Vitro* DPN Model and Pharmacological Interventions

The combinatorial in vitro DPN injury model was constructed using Day 35 mature sensory neurons plated in 96-well or 24-well plates, depending on the downstream assay.

Metabolic Stress (High Glucose)

Cells were initially subjected to a 24-hour pre-treatment (Day 1–2) with high glucose (HG) medium (45 mM glucose in Neurobasal medium). A standard medium containing 25 mM glucose (Sigma) served as the control. To rigorously differentiate metabolic glucotoxicity from physical hyperosmolarity, an osmotic control group was established by supplementing the standard 25 mM glucose medium with 20 mM mannitol (Sigma)^{13,17}.

Acute Oxidative Stress and Drug Interventions

Following the 24-hour HG pre-treatment, cells were exposed to acute oxidative stress using hydrogen peroxide (H_2O_2 , Aladdin) for an additional 24 hours (Day 2–3). Based on a prior dose-response evaluation (ranging from 10 to 400 μ M), the IC₅₀ of H_2O_2 was determined to be approximately 60 μ M, which was strictly utilized for all subsequent injury models. For pharmacological rescue experiments, sensory neurons were pre-treated with N-acetylcysteine (NAC, 1mM, Sigma), minocycline (10 μ M, Sigma), or metformin (1mM, Sigma) for 2 hours immediately prior to the administration of H_2O_2 on Day 2.

4.3 CellTiter-Glo (CTG) Viability Assay

Cellular viability and metabolic activity were quantitatively evaluated using the luminescent ATP-based CellTiter-Glo Assay (Promega). After the 48-hour modeling and intervention period (Day 3), the culture plate was equilibrated to room temperature for 30 minutes. An equal volume of the prepared CTG reagent was added to each well containing the existing culture medium. The microplates were placed on an orbital shaker for 2 minutes to induce complete cell lysis, followed by a 10-minute incubation at room temperature to stabilize the luminescent signal. Luminescence, which is directly proportional to the amount of intracellular ATP and indicative of the number of viable cells, was recorded using a multimode microplate reader (Varioskan LUX, Thermo Fisher). Data were normalized and expressed as a percentage of the control group.

4.4 Assessment of Intracellular ROS

Intracellular reactive oxygen species (ROS) levels were assessed using the fluorogenic probe H₂DCFDA (Thermo Fisher). Following the respective 48-hour treatments, cells were washed gently with warm PBS and incubated with H₂DCFDA (5 μ M) for 30 minutes at 37°C in the dark. Cells were subsequently washed and counterstained with Hoechst 33342 (Abcam) to visualize cell nuclei. Green fluorescence images were acquired using a confocal microscopy (Leica). The relative ROS level was determined by calculating the average green fluorescence intensity per cell (normalized to Hoechst-positive nuclei) using ImageJ software (NIH), with values presented as the fold change relative to the control.

4.5 Assessment of Mitochondrial Membrane Potential

Changes in mitochondrial membrane potential were evaluated using the fluorescent probe JC-1 (MedChemExpress). Following the designated treatments, the culture medium was carefully aspirated, and the sensory neurons were gently washed once with warm PBS. Cells were then incubated with a 2 μ M JC-1 working solution for 30 minutes at 37°C in the dark. After incubation, the cells were washed twice with warm PBS to remove excess dye. Images were immediately acquired using a confocal microscopy (Leica). For quantitative analysis, the fluorescence intensities of JC-1 aggregates (red emission, ~ 590 nm) and JC-1 monomers (green emission, ~ 529 nm) were measured across multiple random fields per well using ImageJ software. The mitochondrial membrane potential was calculated and presented as the ratio of red to green fluorescence intensity.

4.6 Immunofluorescence Staining

Cultured neurons were gently washed with PBS (Gibco) and fixed with 4% paraformaldehyde (PFA, Biosharp) for 15–20 minutes at room temperature. After washing three times with PBS, cells were permeabilized with 0.1% Triton X-100 (HARVEYBIO) for 10 minutes and blocked with 5% normal goat serum (Jackson ImmunoResearch) in PBS for 1 hour at room temperature to prevent non-specific binding. Cells were then incubated overnight at 4°C with primary antibodies targeting specific markers: TUJ1 (Cell Signaling Technology), Peripherin (Abcam), BRN3A (Abcam), ISL1 (Abcam), and the motor neuron marker HB9 (Santa Cruz). The following day, cells were incubated with corresponding fluorophore-conjugated secondary antibodies (Alexa Fluor 488 or 555, Jackson ImmunoResearch) for 1 hour at room temperature in the dark. Nuclei were counterstained with DAPI (Abcam).

4.7 Long-term Axonal Degeneration Analysis

To evaluate long-term morphological changes and axonal degeneration, the DPN modeling and drug intervention timeline (HG + H₂O₂ + drugs) was strictly confined to the initial 48 hours, after which the cells were maintained in standard culture conditions without further stressors until the Day 21 endpoint. Cells were then fixed and immunostained for the pan-neuronal network marker TUJ1. To quantify the extent of neurite disruption, multiple random fields of view per well were imaged. The relative TUJ1-positive area was analyzed using ImageJ software by applying a consistent thresholding algorithm across all groups to isolate the fluorescent axonal signals from the background. Data were expressed as the fold change in TUJ1⁺ area relative to the control group.

4.8 Statistical Analysis

All quantitative data represent the results from at least three independent biological replicates ($n \geq 3$) and are presented as the mean $\pm$ standard error of the mean (SEM) as indicated in the respective figure legends. Statistical analyses were performed using GraphPad Prism software (version 9.0). The normality of data distribution was assessed using the Shapiro-Wilk test. For comparisons between two experimental groups, a two-tailed unpaired Student's *t*-test was employed. For multiple group comparisons, a one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's *post hoc* test was utilized. A *p*-value of less than 0.05 ($p < 0.05$) was considered statistically significant, with specific significance levels denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Declarations

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Funding

This work was supported by the Youth Research Fund of Beijing Shunyi District Hospital (Grant No. ShunYi2025Q01).

Author Contribution

JL. Z. conceptualized and designed the study, performed the in vitro differentiation, disease modeling, and immunofluorescence staining. YF. Z., JQ. Z., ZB. N. assisted with data analysis and manuscript preparation. All authors read and approved the final manuscript.

Data Availability

The original contributions presented in the study are included in the article material. Further inquiries can be directed to the corresponding author upon reasonable request.

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Figures

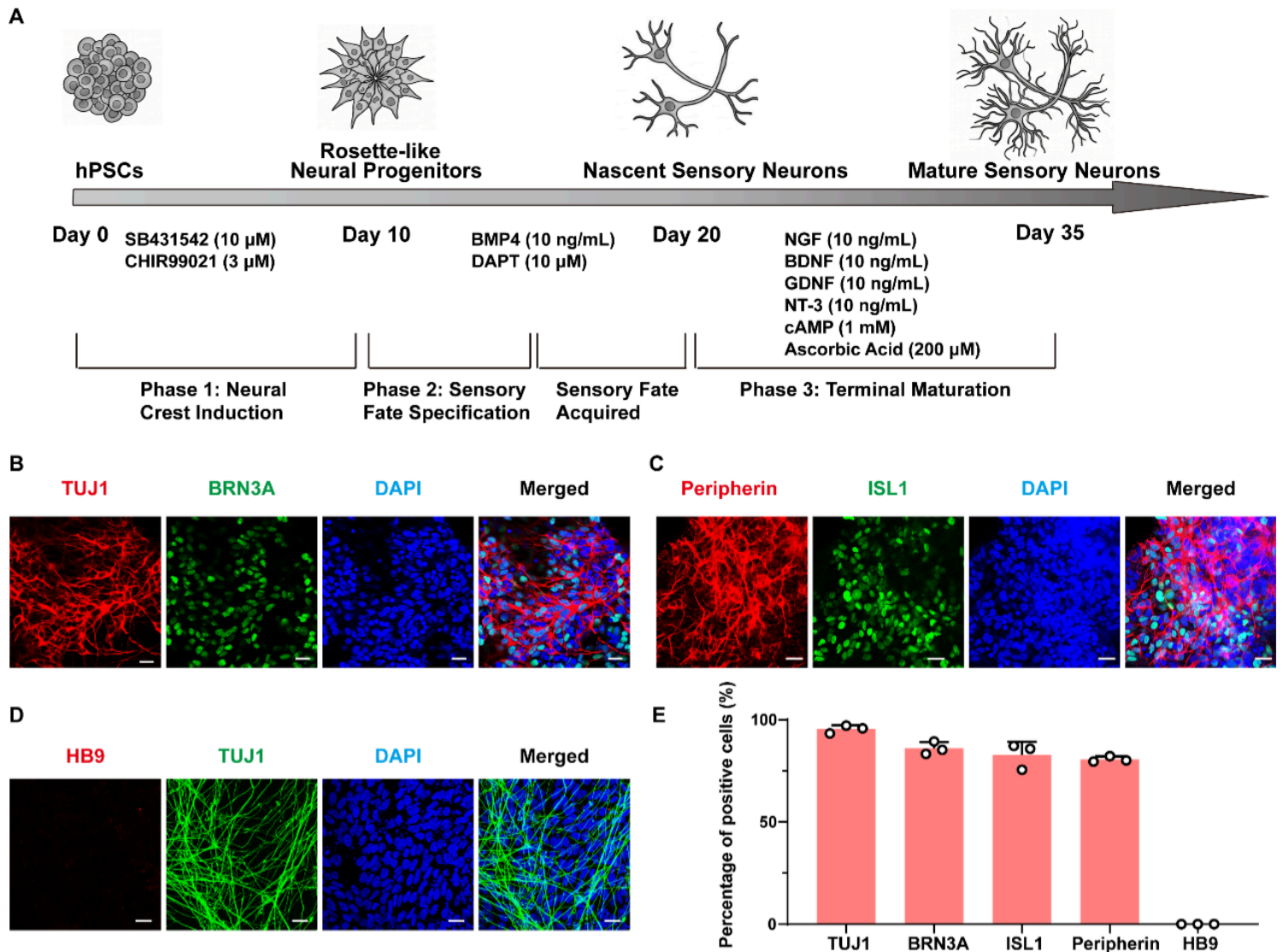


Figure 1

Directed differentiation and characterization of hPSC-derived mature sensory neurons.

(A) Schematic representation of the 35-day, step-wise differentiation protocol from hPSCs to mature sensory neurons, including neural crest induction, sensory fate specification, and terminal maturation phases.

(B, C) Representative immunofluorescence images of day 35 sensory neurons stained for the pan-neuronal marker TUJ1 (red) and sensory marker BRN3A (green) (B), as well as Peripherin (red) and ISL1 (green) (C). Nuclei were counterstained with DAPI (blue). Scale bars = 20 μ m.

(D) Immunofluorescence staining confirming the absence of the motor neuron marker HB9 (red) in the TUJ1-positive (green) sensory neuronal network. Scale bars = 20 μ m.

(E) Quantitative analysis of the percentage of cells expressing TUJ1, BRN3A, ISL1, Peripherin, and HB9.

Data are presented as mean $\pm$ SEM (n = 3 independent experiments).

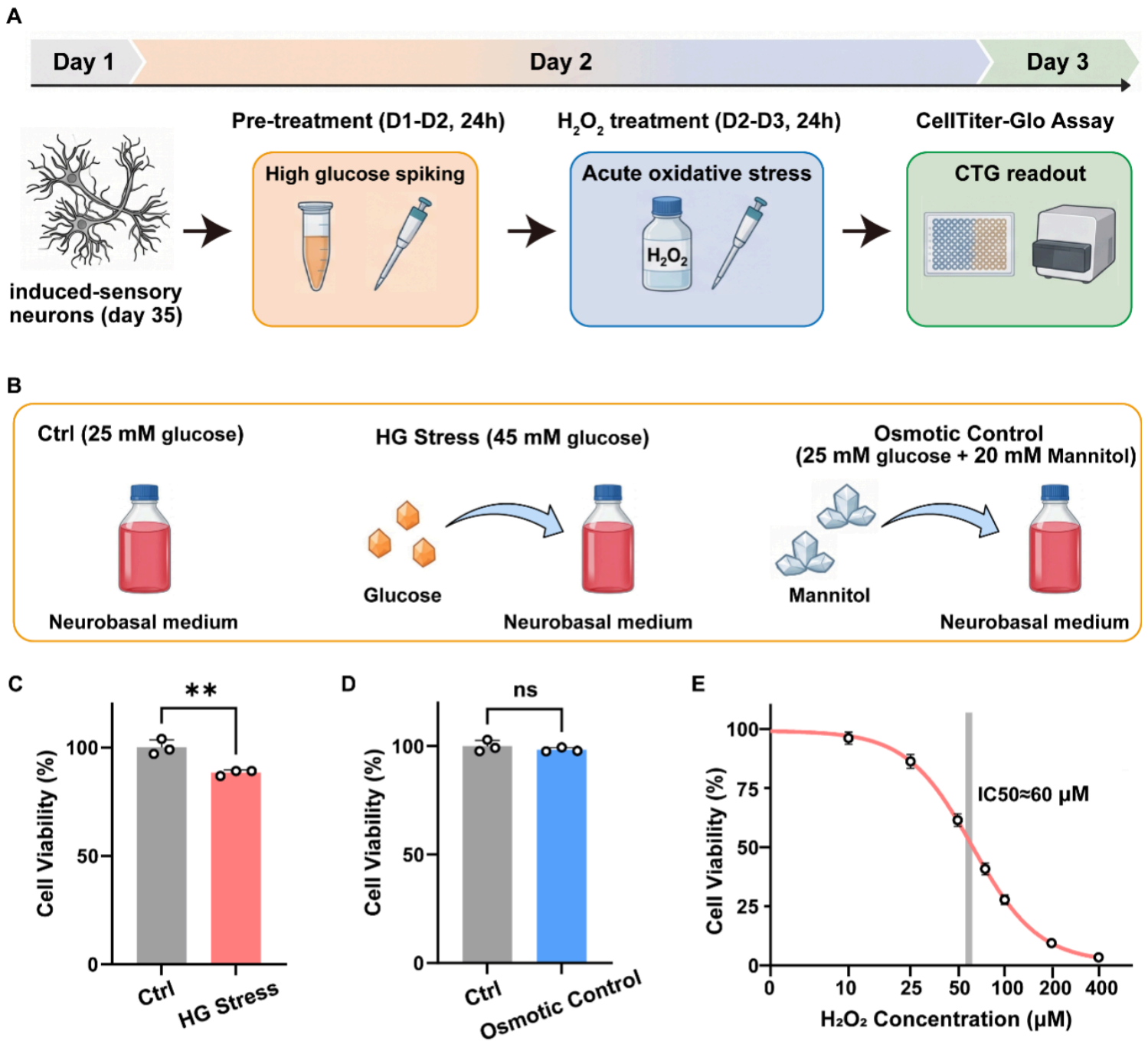


Figure 2

Establishment of the combinatorial *in vitro* DPN model and validation of metabolic glucotoxicity.

(A) Experimental timeline for the *in vitro* DPN modeling utilizing a 24-hour high glucose (HG) pre-treatment followed by acute H_2O_2 -induced oxidative stress, with cell viability evaluated via CTG readout.

(B) Schematic overview of the experimental groups: normal control (25 mM glucose), HG stress (45 mM glucose), and osmotic control (25 mM glucose + 20 mM mannitol).

(C) Cell viability of sensory neurons exposed to normal control versus HG stress conditions for 24 h.

(D) Cell viability comparison between the normal control and the osmotic control group, demonstrating no significant hyperosmolarity-induced cytotoxicity.

(E) Dose-response curve of sensory neurons treated with varying concentrations of H_2O_2 (0-400 μM) for 24 h. The calculated IC_{50} was approximately 60 μM .

Data are presented as mean $\pm$ SEM (n = 3 biological replicates). $**p < 0.01$; ns, not significant. Unpaired Student's *t*-test for (C) and (D).

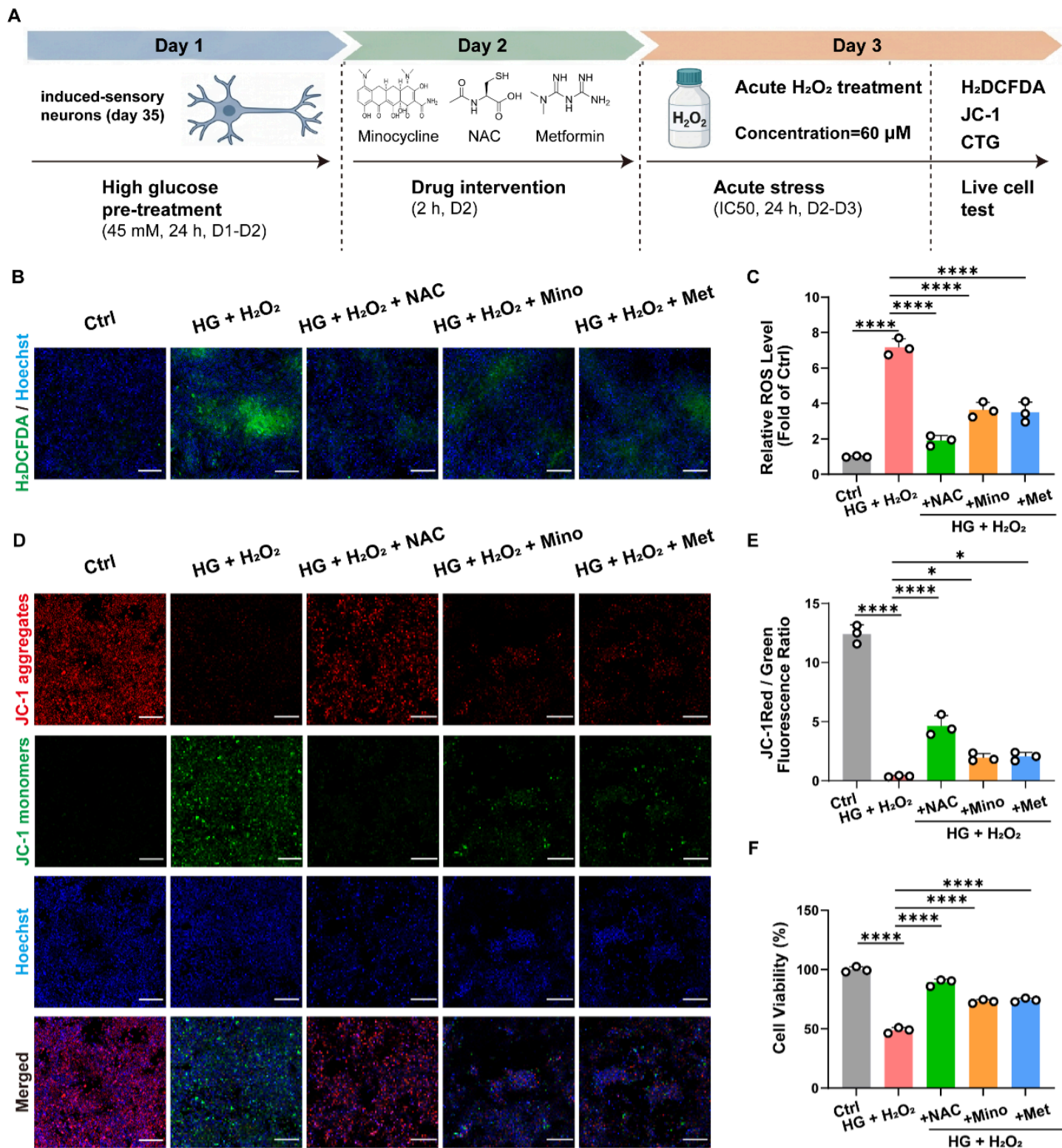


Figure 3

Pharmacological interventions rescue HG/ H_2O_2 -induced acute oxidative stress and mitochondrial dysfunction.

(A) Experimental design for the pharmacological screening of N-acetylcysteine (NAC), minocycline (Mino), and metformin (Met) in the established DPN model.

(B) Representative fluorescence images of intracellular ROS levels detected by the H₂DCFDA probe (green) across different treatment groups. Nuclei were stained with Hoechst 33342 (blue). Scale bars = 100 μ m.

(C) Quantification of relative ROS levels expressed as fold change relative to the control.

(D) Representative images of JC-1 staining evaluating mitochondrial membrane potential. Red fluorescence indicates JC-1 aggregates (healthy mitochondria), whereas green fluorescence indicates JC-1 monomers (depolarized mitochondria). Scale bars = 100 μ m.

(E) Quantitative analysis of the JC-1 red/green fluorescence ratio.

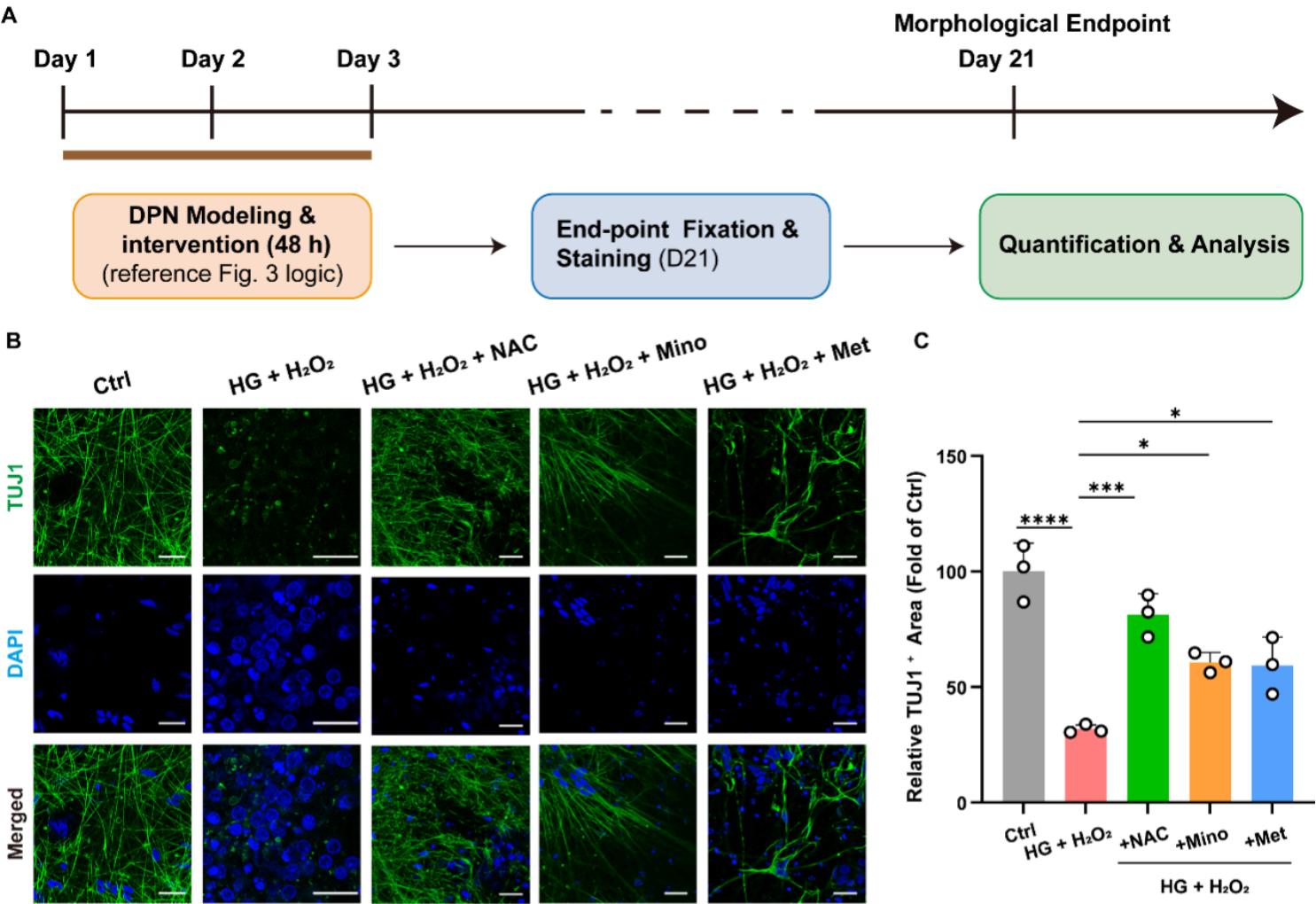


Figure 4

Long-term morphological evaluation reveals neuroprotective effects against DPN-associated axonal degeneration.

(A) Schematic timeline of the long-term experimental paradigm. The DPN modeling and drug intervention were restricted to the initial 48 h, followed by standard culture until the morphological endpoint at day 21.

(B) Representative immunofluorescence images of the TUJ1-positive (green) neurite network at day 21 across all experimental groups, illustrating HG/H₂O₂-induced axonal fragmentation and the protective effects of NAC, Mino, and Met. Nuclei were counterstained with DAPI (blue). Scale bars = 20 µm.

(C) Quantification of the relative TUJ1-positive area, normalized as fold change of the control group. Data are presented as mean ± SEM (n = 3 independent experiments). **p* < 0.05, ****p* < 0.001, *****p* < 0.0001, One-way ANOVA followed by Tukey's *post hoc* test for (C).