Intranasal Wharton's Jelly-Derived Mesenchymal Stem Cell Therapy, Alone or in Conjunction With Therapeutic Hypothermia, Alleviates Neonatal Hypoxic-Ischemic Brain Injury in Mice

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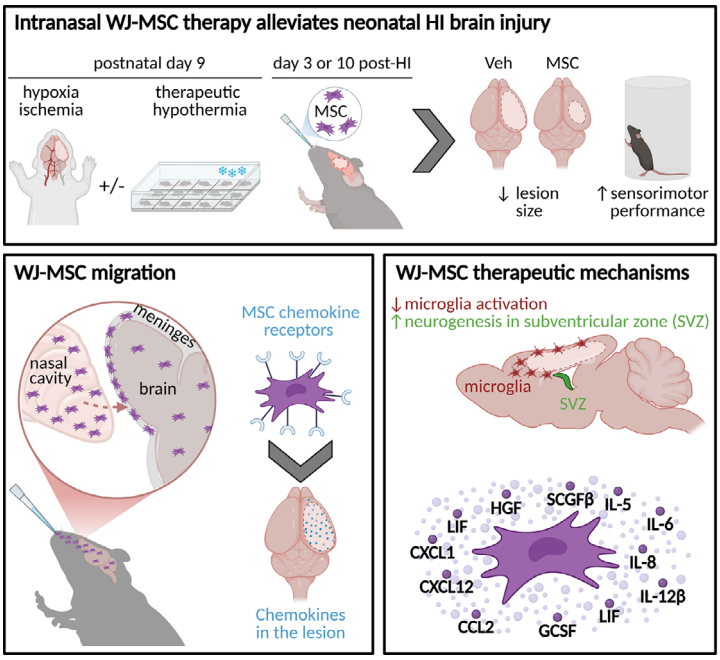

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This study demonstrates that intranasal Wharton's jelly-derived mesenchymal stem cell (WJ-MSC) administration at 3 or 10 days post-insult reduced the lesion size and sensorimotor impairment following neonatal hypoxic-ischemic (HI) brain injury in mice. WJ-MSCs expressed receptors for HI-upregulated chemokines and migrated from the nasal cavity into the meninges and brain parenchyma. WJ-MSCs inhibited neuroinflammation and boosted neuroregeneration via their secretome. Intranasal WJ-MSC therapy remains effective in conjunction with hypothermia, underscoring its translational potential as noninvasive adjunct to current clinical care. Created with BioRender.com.

Objective: Neonatal hypoxic-ischemic brain injury (HIBI) caused by perinatal asphyxia is a primary cause of long-term neurological morbidity. Hypothermia is the sole available clinical intervention despite its limited efficacy. Intranasal mesenchymal stem cells (MSCs) show promise for the treatment of HIBI. This study explores the efficacy, migration, treatment window, and therapeutic mechanisms of intranasally applied Wharton's jelly-derived MSCs (WJ-MSCs) in a neonatal HIBI mouse model, and assesses its therapeutic benefit in conjunction with hypothermia.
Methods: Nine-day-old C57BL/6 mice underwent hypoxia-ischemia (HI), with or without hypothermia, and were intranasally dosed with 0.1 to 2.0 $\times$ 10^6 WJ-MSCs, at 3 or 10 days post-HI. WJ-MSCs were traced using different techniques. Neurogenesis was examined 2 days post-treatment. Neuroinflammation, sensorimqotor outcome, and neuronal tissue loss was assessed 28 days post-HI. Additionally, anti-inflammatory and neuroregenerative properties of WJ-MSCs were investigated in non-contact co-cultures with microglia and neural stem cells, and by secretome profiling.
Results: Intranasally delivered WJ-MSCs reduced HI-induced lesion size and sensorimotor impairments. WJ-MSCs expressed multiple receptors for upregulated chemokines in the HI-lesion, and migrated from the nasal cavity into the meninges and brain parenchyma. WJ-MSCs secreted a broad spectrum of immunomodulatory and neuroregenerative proteins, and inhibited neuroinflammation and boosted neuroregeneration *in vivo* and *in vitro*. WJ-MSC potency was sustained across different donors. Importantly, intranasal WJ-MSCs augmented the therapeutic benefits of hypothermia following neonatal HIBI in mice.
Interpretation: This study provides new translational evidence that intranasal WJ-MSCs, either alone or in combination with hypothermia, mitigate the consequences of neonatal HIBI by resolving inflammation and boosting neurorepair through their secretome.

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Perinatal asphyxia is a major cause of neonatal hypoxic-ischemic (HI) brain injury that can lead to life-long neurological morbidity. Therapeutic hypothermia is currently the only clinically available treatment for HI and provides neuroprotection by reducing cerebral metabolism and mitigating oxidative stress shortly after injury.[1,2] However, because hypothermia reduces the risk of any adverse outcome with only 20% to 30%,[3–5] many children are left with major debilitating handicaps like cerebral palsy. Hence, the imperative to improve long-term outcomes following neonatal HI necessitates the development of novel therapies, ideally with additional benefits in conjunction with hypothermia. Strategies supporting neurorepair may be particularly promising.

Mesenchymal stem cell (MSC) application is implicated as a promising future therapy for neonatal HI brain injury.[6] Numerous studies have shown therapeutic benefits of MSCs in animal models, diminishing brain injury and subsequent neurodevelopmental impairments.[7] Intranasal application of MSCs offers potential advantages over intracerebral and intravenous administration, including noninvasive administration for targeted brain delivery.[8] Our group and others have previously demonstrated that intranasally administered bone marrow-derived MSCs (BM-MSCs) migrate to the HI-injured brain,[9–11] where they inhibit inflammation and promote endogenous regeneration of the neonatal brain, ultimately leading to long-term therapeutic benefits on anatomic and functional outcome without adverse safety issues.[12–14] Furthermore, intranasal BM-MSC therapy was shown to have a long therapeutic window of at least 10 days following HI.[9,13] Recently, a first-in-human phase I, open-label intervention study at the Neonatal Intensive Care Unit of the Wilhelmina Children's Hospital in Utrecht, The Netherlands, demonstrated feasibility and safety of intranasal BM-MSC administration in neonates with perinatal arterial ischemic stroke.[15]

For clinical use, MSCs derived from the umbilical cord provide practical benefits, as they are noninvasively obtained in large quantities from medical waste material. Umbilical cord-derived MSCs are isolated from umbilical cord blood or Wharton's jelly (WJ), the connective tissue surrounding the umbilical cord blood vessels. As recently reviewed, the therapeutic effects of WJ-MSCs in neonatal HI animal models have been examined using intraventricular, intraperitoneal, or intravenous administration.[7,16] However, the intranasal route remains understudied, with research limited to early administration within 24 hours post-HI.[7,16] Additionally, conflicting evidence on the benefits of MSC therapy in conjunction with therapeutic hypothermia requires further investigation to optimize MSC therapy for patients with perinatal asphyxia receiving hypothermia according to standard clinical care.[17] The current study is the first to assess the therapeutic potential

of intranasal administration of WJ-MSCs at late timepoints post-HI in a well-described mouse model for neonatal brain injury[18] and to map migration of WJ-MSCs to the brain. The anti-inflammatory and neuroregenerative mechanisms of WJ-MSCs were assessed *in vivo*, in non-contact co-cultures with microglia and neural stem cells *in vitro*, and by secretome profiling. Importantly, for translational applicability, efficacy of WJ-MSC therapy was tested also in conjunction with therapeutic hypothermia. We hypothesize that delayed intranasal WJ-MSC therapy following hypothermia provides therapeutic benefits, aiming to further improve neurodevelopmental outcome following neonatal HI brain injury.

Methods

Human WJ-MSC Culture

Human Wharton's jelly-derived MSCs were obtained from volunteers with written informed consent and isolated using a patented method (WO 2019/123407) by Chiesi Farmaceutici S.p.A. (Parma, Italy). The WJ-MSCs used in this study meet the release criteria set by the International Society for Cell and Gene Therapy (ie, more than 90% of the MSCs are CD73, CD90, and CD105 positive).[19] Furthermore, WJ-MSCs were tested to ensure sterility (ie, negative for bacteria, fungi, mycoplasma, and endotoxin). WJ-MSCs at passage 4 were seeded at a density of 0.15×10^5 viable cells/cm^2 in T75 flasks (Corning, New York, NY, USA) in a proprietary basal medium (Lonza, Walkersville, MD, USA), supplemented with 5% human platelet lysate (Lonza) and 2,000 U/L Heparin Sodium Injection (Sigma-Aldrich, St. Louis, MO, USA) and cultured for 48 hours prior to use. Alternatively, WJ-MSCs at passage 2 were seeded at 0.25×10^4 viable cells/cm^2 and cultured for 8 days with a passage at day 4. WJ-MSCs were harvested by 5 minutes trypsinization with 3 U/ml Recombinant Trypsin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and centrifugation (500 g for 10 minutes). WJ-MSCs were resuspended in cold DPBS (Gibco), filtered over a 40 μm cell strainer and counted (NC-200; Chemometec, Lillerød, Denmark). WJ-MSCs were centrifuged, resuspended in cold DPBS at the desired concentration, and kept on ice until *in vivo* use.

Mouse Model for Neonatal HI Brain Injury

All animal experiments were conducted in accordance with European guidelines (Directive 86/609, ETS 123, Annex II) and approved by the Dutch Central Authority for Scientific Procedures on Animals (project license AVD115002016751). C57Bl/6 mice (OlaHsa, Envigo, Horst, The Netherlands) were kept in individually ventilated cages with woodchip bedding, cardboard shelters, and tissues, provided on a 12 hour day/night cycle, in a temperature-controlled room (20–24°C and 45–65% humidity) with food and water ad libitum. Mice were bred in-house and dams were housed solitarily to give birth (ie, postnatal day [P]0). The HI was induced in P9 pups according to the Rice-Vannucci method, a well-validated and widely used preclinical model that replicates key pathological and functional features of neonatal HI brain injury.[18] Careful standardization of the surgical technique, hypoxic exposure, and temperature control has ensured experimental rigor and reproducibility at our laboratory for over 15 years.[13,20–22] Briefly, the right common carotid artery was permanently ligated under isoflurane anesthesia (3–4 vol%, for 5–7 minutes), followed by 75 minutes of recovery with their dam and 45 minutes of systemic hypoxia at 10% O_2 in a temperature-controlled humidified incubator (36.0–36.5°C). Sham-control animals were subjected to anesthesia and surgical incision only, without systemic hypoxia. Xylocaine and bupivacaine were applied to the wound for pre- and post-operative analgesia. Male and female pups were equally distributed and randomly assigned to the experimental groups (Supplementary Table S1).

Therapeutic Hypothermia

Based on the literature,[23] hypothermia was applied immediately following HI for 3 or 5 hours with a targeted rectal temperature of 32, 33.5, or 36.5°C (the latter as the normothermia group). Hypothermia for 5 hours at 33.5°C was chosen as the optimal protocol for later experiments. Following a subcutaneous injection with 50 μl saline to prevent dehydration, the mice were placed individually in separated cubicles in a Thermacage (Bioservices, Schaijk, The Netherlands) on a heating plate (UNO, Zevenaar, The Netherlands) controlled by rectal temperature of a sentinel pup, excluded from further analyses. Experimental pups returned to their dam immediately following hypothermia. Sham-control animals were not subjected to hypothermia, but remained with their dam to reduce maternal stress.

WJ-MSC Administration

The WJ-MSCs mice were intranasally administered at 3 or 10 days post-HI as previously described.[13] Briefly, 30 minutes after intranasal administration of hyaluronidase (2 droplets of 3 μl per nostril, 12 μl, 100 U; Sigma-Aldrich), the mice were treated with 0.1, 0.5 or 2.0×10^6 WJ-MSCs, or DPBS (vehicle) by administration of 3 droplets of 2 μl per nostril (12 μl in total) at 3 days post-HI or 2 droplets of 3 μl per nostril (12 μl in total) at 10 days post-HI. The intranasal dose of 2.0×10^6 WJ-MSCs was used for subsequent experiments. Administration at day 3 post-HI was used for all experiments except for the

extended time window experiment. WJ-MSCs were labeled with PKH26 or gold-nanoparticles for tracing as described in the Supplementary Data .

Sensorimotor Outcome

Unilateral motor impairment was measured in the cylinder rearing task at 28 days post-HI. Mice were placed in a transparent Plexiglas cylinder (ø 7.5 × 15 cm) and video-recorded for 5 minutes. Mice that did not perform at least 10 rearings were excluded for analysis (see Supplementary Table S1). The first weight-bearing forepaw contacting the cylinder wall during a full rear was scored by an experienced observer in a blinded manner as left (impaired), right (non-impaired), or both. Non-impaired forepaw preference was calculated as ((right–left)/(right + left + both)) × 100%.

Organ Collection for Immunohistochemistry

Mice were euthanized by an overdose of pentobarbital (Alfasan, Woerden, The Netherlands) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA; VWR, Radnor, PA, USA) to harvest organs for immunohistochemical stainings described in the Supplementary Data S1. Briefly, human-specific antibody against nuclear marker deleted in breast cancer gene 1 (DBC-1) was used to trace WJ-MSCs at 2 hours and 24 hours after intranasal dosing. Neurogenesis in the subventricular zone (SVZ) was assessed at 2 days post-MSCs using proliferation marker antigen Kiel67 (Ki67). Ipsilateral gray matter damage and neuroinflammation were assessed at 28 days post-HI by analyses of neuronal marker microtubule-associated protein 2 (MAP2) and microglia marker ionized calcium-binding adapter molecule 1 (IBA1), respectively. All immunohistochemical analyses were performed in a blinded manner.

WJ-MSC Non-contact co-Culture with Differentiating Neural Stem Cells

Mouse cortical neural stem cells (#NSC002; R&D System, Minneapolis, MN, USA) were cultured as neurospheres according to the manufacturer's protocol (0.2×10^6 cells/well) in 6-well plates in DMEM:F12 medium supplemented with 2% B27 (#0080085SA; Gibco) and 1% P/S (Gibco). Daily, 20 ng/ml recombinant Fibroblast Growth Factor-basic (bFGF, #100-47, Peprotech, Thermo Fisher Scientific) and 20 ng/ml recombinant heparin binding EGF-like growth factor (EGF; #100-18B; Peprotech) were supplied. Neural stem cells were passaged once and used at P3. One day before co-culture, 0.4×10^5 neural stem cells per well were cultured as a monolayer in poly-L-ornithine/laminin-coated 24-well plates. Meanwhile, 1 day before co-culture, WJ-MSCs or fibroblasts (CC-2512, Lonza, cultured according to the manufacturer's instructions) were embedded at a density of 0.8×10^5 WJ-MSCs per 100 µl HyStem hydrogel (Sigma-Aldrich) in hanging inserts (#MCHT24H48; Merck-Millipore, Burlington, MA, USA). Neural stem cell differentiation was initiated by adding medium without bFGF and EGF medium and non-contact co-culture was simultaneously started by adding inserts. Neurogenesis was assessed after 3 days by immunocytochemistry for βIII-tubulin as described in the Supplementary Data S1. Mean WJ-MSC viability in the gel was 81.8% at the end of the experiment.

WJ-MSC Non-Contact co-Culture with Primary Mouse Microglia

Primary cortical microglia were isolated from >30 P1 C57BL/6 mice, as described previously.[24] After 10 to 12 days *in vitro*, mixed glial culture flasks were shaken (20–22 hours, at 130–135 rpm, 37°C) and microglia were collected by centrifugation (120 rpm for 10 minutes), counted and seeded in poly-L-ornithine-coated 24-well plates at a density of 2.0×10^5 cells/well. Microglia from one isolation and 2 independent shakes were used. The same day, 0.8×10^5 WJ-MSCs were embedded in 75 µl of TrueGel hydrogel (Sigma) in hanging inserts. The next day, microglia were stimulated with 50 ng/ml lipopolysaccharide (LPS) (L4515; Sigma) and co-culture was started by adding inserts. After 48 hours of co-culture, inserts were removed (mean MSC viability: –LPS = 75.6% and +LPS = 82.6%). Microglia supernatant was collected and stored at −80°C for murine TNFα ELISA (Ucytech, Utrecht, The Netherlands) according to the manufacturer's protocol, and $IBA1^+$ area/cell was assessed by immunocytochemistry as described in the Supplementary Data S1.

Statistics

Data are presented as mean ± SEM when normally distributed and as median ± interquartile range (IQR) when not-normally distributed. Statistics were performed with Graphpad Prism version 10.1.2 (GraphPad Software, San Diego, CA, USA). Comparison of the 2 groups was tested using an unpaired *t* test, a Welch's *t* test for unequal variances, or a Mann–Whitney test for not-normally distributed data. When comparing >2 groups, 1-way analysis of variance (ANOVA) with Sidak multiple-comparison test, or a Kruskal-Wallis tests with Dunn's post hoc correction for unequal variances was used. $P < 0.05$ were considered statistically significant. Sample sizes are described in Supplementary Table S1.

Results

Intranasal WJ-MSC Administration Following Neonatal HI Improves Sensorimotor Outcome and Reduces Lesion Size

To evaluate the optimal dose of WJ-MSCs for treating neonatal HI brain injury, 0.1, 0.5, or 2.0×10^6 WJ-MSCs were intranasally administered at 3 days following HI (Fig 1A). Starting from an intranasal dose of 0.5×10^6 WJ-MSCs, HI-induced non-impaired forepaw preference in the cylinder rearing task was significantly reduced in comparison to vehicle treatment, suggesting that WJ-MSC treatment improved sensorimotor outcome at 28 days post-HI (Fig 1B). Furthermore, intranasal administration of 2.0×10^6 WJ-MSC significantly reduced gray matter damage in the ipsilateral hemisphere of HI-injured mice compared to vehicle treatment (Fig 1C, D). Therefore, the intranasal dose of 2.0×10^6 WJ-MSCs was used for all the following experiments.

Intranasally Administered WJ-MSC Migrate From the Nasal Cavity into the Meninges and into the Brain Parenchyma

To investigate the migratory capacity of WJ-MSCs, we first assessed which chemotactic signals for cell migration are expressed in the HI lesion. Multiple chemokine protein levels were increased in the ipsilateral hemisphere at 3 days post-HI compared to sham, including CCL2, 3, 5, 7, 12, and 17, and CXCL1, 10, and 16 (Fig 2A). Next, receptor profiling was conducted to investigate whether WJ-MSCs express receptors for the HI-induced chemokines. The microarray showed that receptors CCR1, 2, 3, and 5, CXCR3, and ACKR1 were expressed by WJ-MSCs, potentially accommodating cell migration toward the identified chemokines in the HI lesion (Fig 2B). Next, WJ-MSC migration to the brain was investigated by intranasal administration of 2.0×10^6 PKH26- or gold nanoparticle-labeled WJ-MSCs, or by staining for human-specific nuclear marker DBC-1 to detect WJ-MSCs in situ (Fig 2C). At 2 hours following intranasal administration, high numbers of WJ-MSCs were present in the nasal cavity, as observed by DBC-1^+ signal in 12 of 12 WJ-MSC-treated mice ($n = 6$ sham and $n = 6$ HI), whereas no DBC-1^+ signal was observed in vehicle-treated HI mice as negative controls (Fig 2D). WJ-MSCs were also detected in the lining of the olfactory bulb in 2 of 6 HI mice and 1 of 6 sham mice (Fig 2E), suggesting migration of WJ-MSCs into the meninges. To confirm this, meningeal tissue was isolated 24 hours after intranasal PKH26$^+$ WJ-MSC administration, and indeed PKH26$^+$ cells were present in the meningeal mounts in 10 of 10 mice (Fig 2F). Next, gold nanoparticle-labeled WJ-MSCs were intranasally administered for quantification purposes. At 24 hours following intranasal WJ-MSC administration, the number of WJ-MSCs was significantly increased in meningeal tissue compared to the negative control (vehicle administration; Fig 2G). Besides being present in the meninges, DBC-1^+ WJ-MSCs were occasionally traced at locations in the brain parenchyma in 3 of 6 MSC-treated HI mice at 24 hours following WJ-MSC administration (Fig 2H). In the other 3 of 6 WJ-MSC-treated HI mice, DBC-1^+ cell debris-like structures were observed, indicative for a prior presence of WJ-MSCs (Fig 2I). WJ-MSC tracing at 6 hours or 3 days following intranasal administration yielded similar numbers of WJ-MSCs in the brain parenchyma as at 24 hours (data not shown). Quantification of gold nanoparticle-labeled WJ-MSCs suggested that WJ-MSCs were distributed throughout the brain, because significant numbers of WJ-MSCs were detected in the ipsilateral hemisphere, contralateral hemisphere and cerebellum of HI mice at 24 hours post-MSC administration compared to the negative control (HI-vehicle; Fig 2J).

Intranasal WJ-MSC Administration Following Neonatal HI Boosts Neurogenesis

To investigate the neuroregenerative effects of intranasal WJ-MSC administration, Ki67$^+$ (proliferating) cells were quantified in the SVZ neurogenic niche at 2 days following WJ-MSC administration (Fig 3A). A significant increase in Ki67$^+$ cells was found in the ipsilateral SVZ of HI-injured mice treated with WJ-MSCs compared to vehicle treatment (Fig 3B, C). Furthermore, significantly more Ki67$^+$ cells were observed in the ipsilateral compared to the contralateral SVZ of WJ-MSC-treated mice, suggesting that WJ-MSCs specifically boost neurogenesis in the injured hemisphere. Next, the neurogenic effects of WJ-MSCs were tested *in vitro* by non-contact co-cultures with differentiating neural stem cells (Fig 3D). Exposure of differentiating neural stem cells to the WJ-MSC secretome significantly increased formation of βIII-tubulin$^+$ neurons in comparison to fibroblast secretome, which did not have neurogenic effects (Fig 3E, F). Additionally, co-culture with WJ-MSC secretome resulted in the formation of neurons with a greater morphological complexity, as measured by a significantly increased number of Sholl neurite intersections in comparison to co-culture with fibroblast secretome or medium (Fig 3G, H). Potential WJ-MSC donor variation was assessed by comparison of 3 different WJ-MSC donors in the neural stem cell differentiation assay. The secretome of all 3 WJ-MSC donors significantly increased the formation of βIII-tubulin$^+$ neurons to a similar extent (Fig 3I). Additionally, different WJ-MSC culture protocols, for either 2 or 8 days prior to co-culture with neural stem cells, also resulted in similar

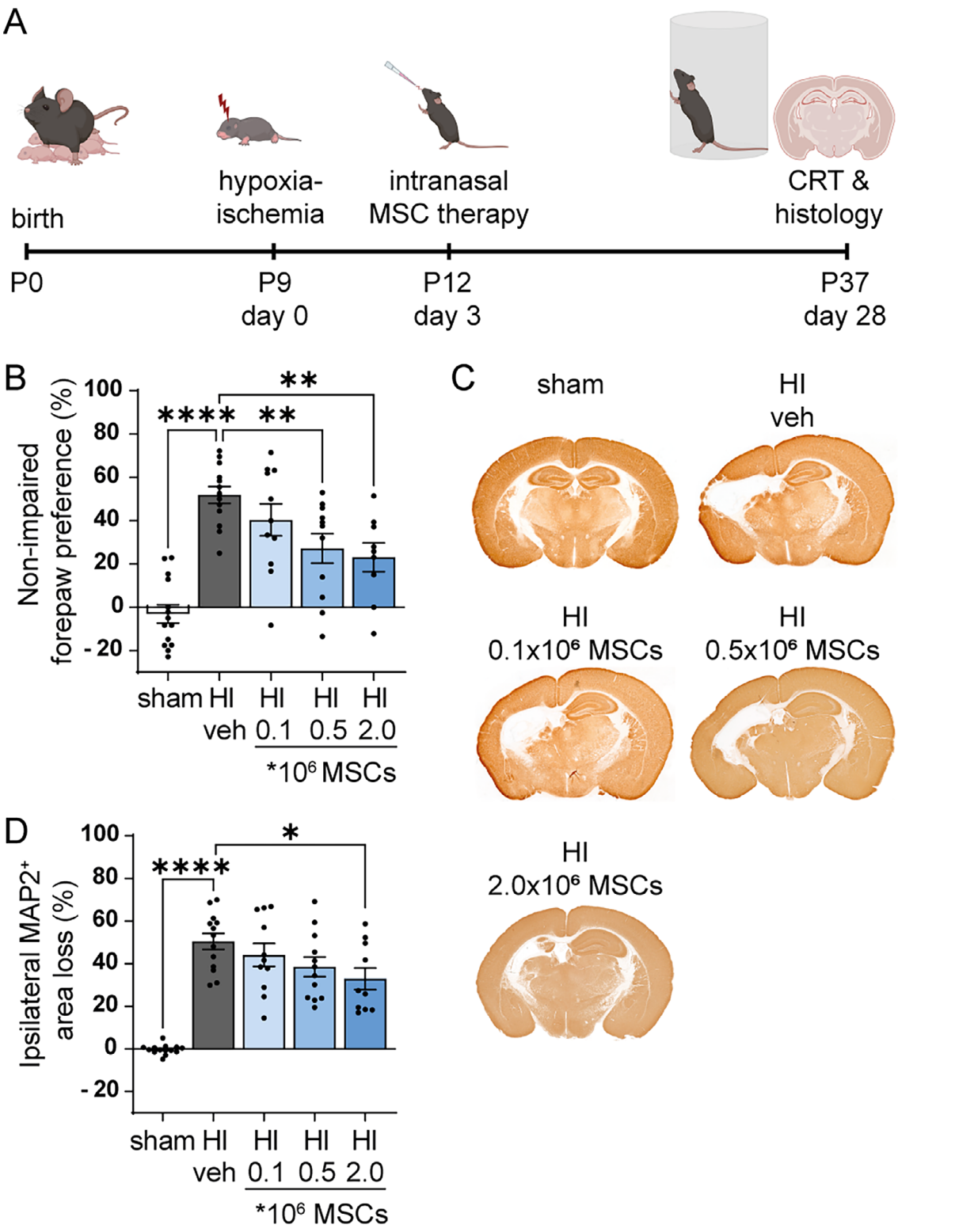

FIGURE 1: Therapeutic efficacy of intranasal WJ-MSCs in a mouse model for neonatal HI brain injury. (A) Schematic overview of the experimental set-up. (B) Starting from an intranasal dose of 0.5×10^6, WJ-MSCs improved sensorimotor impairment in HI-injured mice, compared to veh treatment. (C) Representative MAP2-stained coronal sections of sham-control mice or HI-injured mice treated intranasally with vehicle, 0.1, 0.5, or 2.0×10^6 WJ-MSCs. (D) Intranasal WJ-MSC therapy decreased ipsilateral $MAP2^+$ tissue loss in HI-injured mice starting from a dose of 2.0×10^6 WJ-MSCs. Data are presented as mean ± SEM. *$p < .05$, **$p < .01$, ****$p < .0001$. CRT = cylinder rearing task; HI = hypoxia–ischemia; MAP2 = microtubule-associated protein 2; P = postnatal day; veh = vehicle; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells. [Color figure can be viewed at www.annalsofneurology.org]

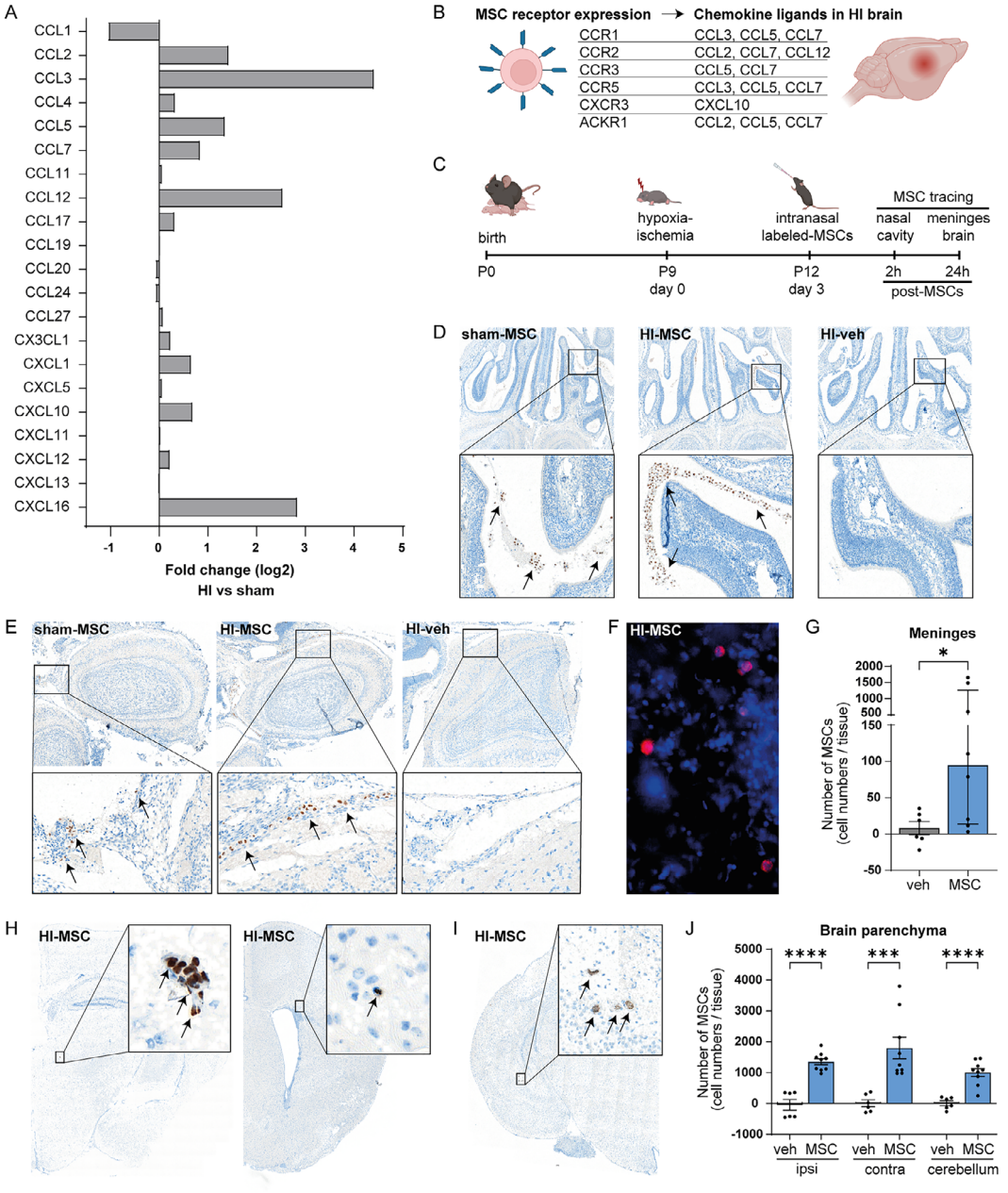

FIGURE 2: WJ-MSCs migrate from the nasal cavity to the meninges and brain parenchyma in a mouse model for neonatal HI. (A) Chemokine protein levels in the ipsilateral hemisphere at day 3 following HI compared to sham operation (measured in pooled homogenates of 23 sham and 40 HI ipsilateral hemispheres). (B) The mRNA expression of chemokine receptors on WJ-MSCs that can bind the HI-induced chemokines. Reported chemokine receptors were expressed in WJ-MSCs from 3 independent cultures. (C) Schematic overview of the experimental set-up. (D) Images of DBC-1^{+} WJ-MSCs present at high numbers in the nasal cavity at 2 hours following intranasal administration of 2.0 $\times$ 10^{6} WJ-MSCs in sham and HI mice, but not in vehicle-treated HI mice. (E) Images of DBC-1^{+} WJ-MSC migration into the lining of the olfactory bulb at 2 hours following WJ-MSC administration in HI and sham mice, but not in vehicle-treated control HI mice. (F) Image of PKH^{+} WJ-MSCs detected in isolated meningeal tissue of HI mice at 24 hours following intranasal administration of PKH-labeled WJ-MSCs. (G) Quantification of gold nanoparticle-labeled WJ-MSCs demonstrated a significant increase in numbers of WJ-MSCs in the isolated meninges of HI mice at 24 hours post-MSC administration compared to vehicle administration as a control. Data presented as median $\pm$ IQR. (H) Example images of DBC-1^{+} WJ-MSCs in the brain parenchyma of HI mice at 24 hours post-WJ-MSCs. (I) Image of DBC-1+ WJ-MSCs debris-like structures in the brain parenchyma of HI mice at 24 hours post-WJ-MSCs. (J) Quantification of gold nanoparticle-labeled WJ-MSCs demonstrated a significant increase in the number of WJ-MSCs in the ipsilateral and contralateral hemispheres and cerebellum of HI mice at 24 hours post-MSC administration compared to vehicle administration as a control. Data are presented as mean $\pm$ SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. ACKR = atypical chemokine receptor; CCL = chemokine (C-C motif) ligand; CX3CL = C-X3-C motif chemokine ligand; CXCL = chemokine (C-X-C motif) ligand; CCR = CC chemokine receptor; CXCR = CXC chemokine receptors; DBC1 = deleted in breast cancer gene 1; HI = hypoxia–ischemia; IQR = interquartile range; P = postnatal day veh = vehicle; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells. [Color figure can be viewed at www.annalsofneurology.org]

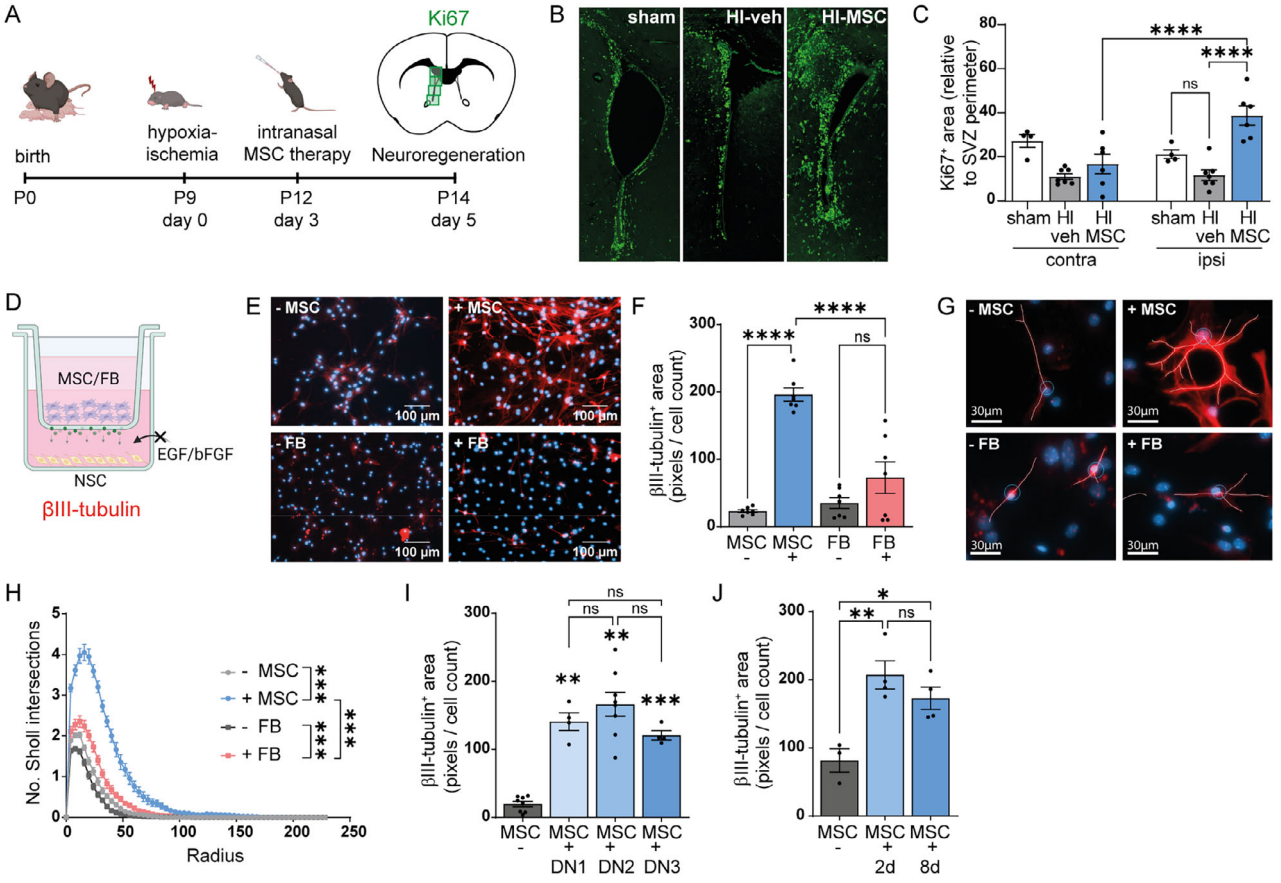

FIGURE 3: WJ-MSCs boost neurogenesis in a mouse model for neonatal HI brain injury and in an *in vitro* neural stem cell differentiation assay. (A) Schematic overview of the experimental set-up and schematic overview of image acquisition. (B) Representative images of Ki67$^+$ cells in the ipsilateral SVZ of sham-control or HI-injured mice followed by intranasal administration of 2.0 × 10^6 WJ-MSCs or veh. (C) Intranasal WJ-MSCs increased the number of proliferating cells in the ipsilateral SVZ of HI-injured animals compared to vehicle treatment. (D) Schematic representation of the non-contact co-culture of NSCs with WJ-MSCs or FBs (as controls) in the insert. Withdrawal of EGF and bFGF was used to induce NSC differentiation. (E) Representative images of βIII-tubulin$^+$ cells (ie, neurons) following NSC differentiation in absence or presence of FB or WJ-MSCs. In the MSC and FB conditions, inserts contain only MSC medium or FB medium, respectively. (F) Co-culture with WJ-MSCs, but not FBs, boosted the formation of βIII-tubulin$^+$ neurons from differentiating NSCs (all groups: n = 7 wells). (G) Representative images of Sholl analysis of βIII-tubulin$^+$ cells differentiated in absence or presence of FBs or WJ-MSCs. (H) Co-culture with WJ-MSCs increased neurite complexity of βIII-tubulin$^+$ cells in comparison to co-culture with FBs or only medium. (I) WJ-MSCs-induced increase in βIII-tubulin$^+$ cells from differentiating NSCs was confirmed to be comparable between 3 different WJ-MSC donors (– MSC: n = 8, DN1: n = 4, DN2: n = 8, DN3: n = 4 wells). (J) Comparable increases in the formation of βIII-tubulin$^+$ cells were observed when NSCs were co-cultured with WJ-MSCs that were either cultured for 2 or 8 days (– MSC: n = 3, 2 days: n = 4, 8 days: n = 4 wells). Data are presented as mean ± SEM. *p < .05, **p < .01, ***p < 0.001, **** p < .0001, ns = non-significant difference. FBs = fibroblasts; HI = hypoxia–ischemia; Ki67: Kiel 67; NSCs = neural stem cells; P = postnatal day; SVZ = subventricular zone; veh = vehicle; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells. [Color figure can be viewed at www.annalsofneurology.org]

neurogenic effects (Fig 3J). This implies a steady secretion of neurogenic proteins by WJ-MSCs between donors and over time. In addition, in the *in vivo* mouse model for neonatal HI brain injury, similar treatment effect sizes were observed on reduction of lesion size between different WJ-MSC donors (donor 1: $d_{Cohen} = -0.83$, donor 2: $d_{Cohen} = -0.78$) and between different WJ-MSC culture paradigms (2-days culture: $d_{Cohen} = -0.78$, for 8-days culture $d_{Cohen} = -0.76$, data not shown).

Intranasal WJ-MSC Administration Following Neonatal HI Inhibits Microglia Activation

The potential immunomodulatory effects of intranasal WJ-MSC administration was investigated at 28 days post-HI by assessing microglia activation (Fig 4A). Activated microglia, measured by a decrease in IBA1$^+$ cell perimeter and an increase in IBA1$^+$ cell circularity (both indicative for a more amoeboid shape), were observed in the ipsilateral cortex (Fig 4B–D) following HI and vehicle treatment. Intranasal WJ-MSC

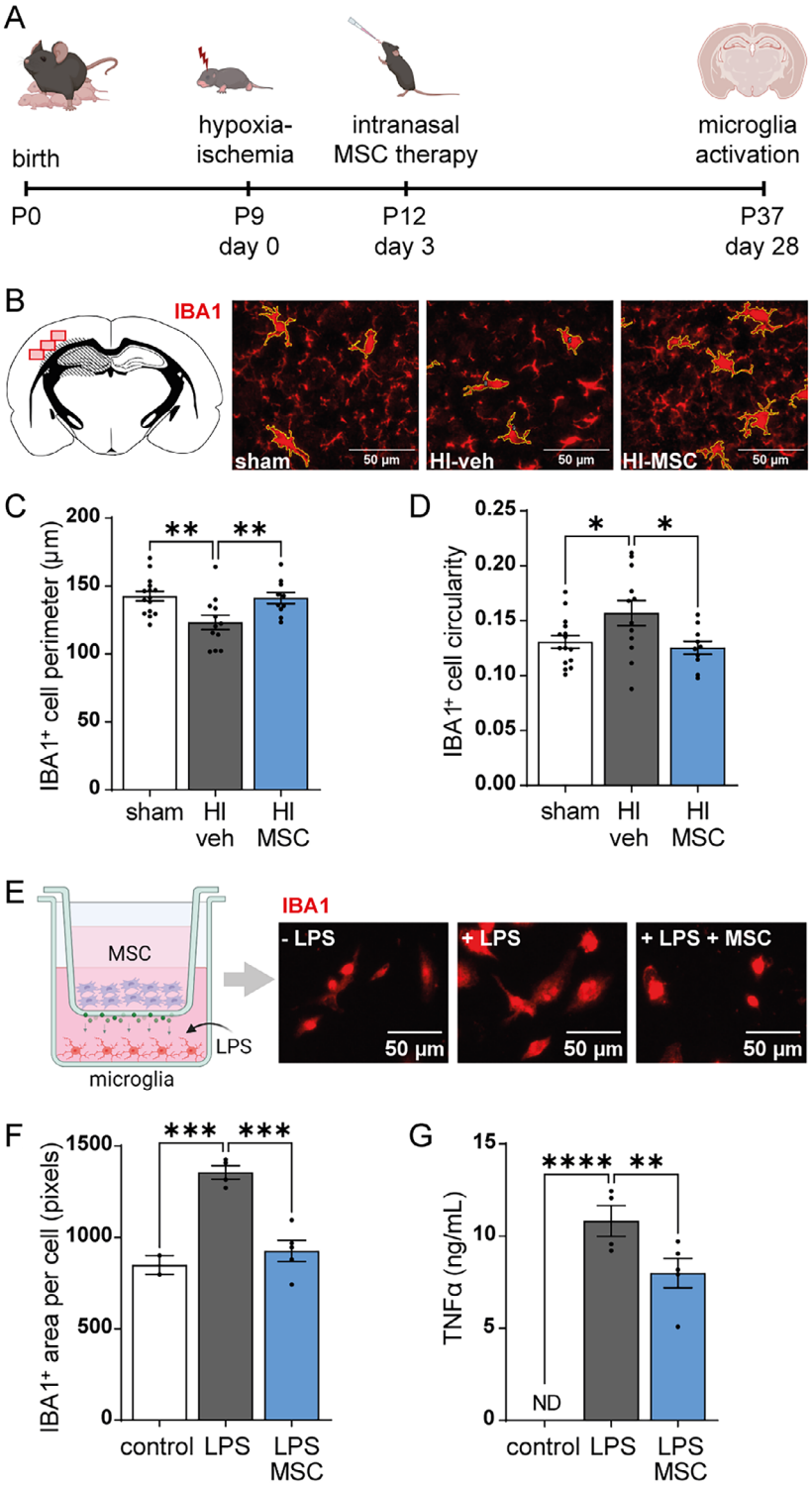

FIGURE 4: Intranasal WJ-MSC treatment dampens microglia activation in a mouse model for neonatal HI brain injury and in an *in vitro* microglia activation assay. (A) Schematic overview of the experimental set-up. (B) Schematic overview of image acquisition and representative images of IBA1$^+$ cells in the cortex of sham mice and HI mice intranasally treated with veh or 2.0 × 10^6 WJ-MSCs. (C) Intranasal WJ-MSCs restored the HI-induced reduction in IBA$^+$ cell perimeter. (D) Intranasal WJ-MSCs ameliorated the HI-induced increase in IBA1$^+$ cell circularity. (E) Schematic overview of the LPS-stimulated microglia in non-contact co-culture with WJ-MSCs. The right part shows representative images of cultured IBA1$^+$ microglia. (F) Co-culture with WJ-MSCs prevented the LPS-induced increase in IBA1$^+$ area per cell, as a morphological activation marker (control: n = 2, LPS: n = 4, LPS + MSCs: n = 5 wells). (G) Co-culture with WJ-MSCs also mitigated the LPS-induced secretion of TNFα by microglia. Data presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. HI = hypoxia-ischemia; IBA1 = ionized calcium-binding adaptor molecule 1, LPS = lipopolysaccharide; ND = non-detectable; P = postnatal day; veh = vehicle; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells. [Color figure can be viewed at www.annalsofneurology.org]

administration significantly ameliorated microglia activation in the ipsilateral cortex of HI-injured animals, as both IBA1$^+$ cell perimeter (see Fig 4C) and IBA1$^+$ cell circularity (see Fig 4D) were restored back to levels of sham-control mice. To confirm a direct effect of WJ-MSCs on microglia, LPS-activated primary-isolated mouse microglia were exposed to the WJ-MSC secretome in a non-contact co-culture assay (Fig 4E). The WJ-MSC secretome significantly reduced the LPS-induced increase in IBA1$^+$ area per cell (indicative for an active state; Fig 4F), and decreased LPS-induced microglial TNF-α secretion (Fig 4G), indicating that WJ-MSCs dampen microglia activity both on a morphological and functional level.

Because the WJ-MSC secretome directly affects neural stem cell differentiation and microglial inflammatory responses *in vitro*, the secretion of cytokines, chemokines, and growth factors by WJ-MSCs was determined (see the Table). Of the 48 proteins analyzed, WJ-MSCs secreted 33 proteins in low to very high amounts (1 pg/ml as the lower limit). The factors most abundantly secreted by WJ-MSCs included SCGFβ, HGF, IL-6, IL-8, CXCL1, and CCL2 (>1,000 pg/ml). The full panel of secreted factors can be found in the Table .

Treatment Efficacy of Intranasal WJ-MSC Therapy Persists When Treatment Is Postponed Until 10 Days Post-HI

To examine whether WJ-MSCs offer a late treatment window, the WJ-MSCs were administered intranasally at 10 days following HI (Fig 5A). The intranasal WJ-MSC doses that showed treatment efficacy at 3 days post-HI (see Fig 1B) remained highly effective when administration was delayed to 10 days post-HI. Both 0.5×10^6 and 2.0×10^6 WJ-MSCs significantly improved sensorimotor outcome (Fig 5B) and reduced gray matter damage compared to vehicle treatment (Fig 5C, D).

Intranasally Administered WJ-MSCs Provide Additional Therapeutic Benefits to Hypothermia Treatment Following Neonatal HI

Because therapeutic hypothermia is currently the standard clinical care for infants with moderate to severe HI encephalopathy, the effects of intranasal WJ-MSC therapy

TABLE. WJ-MSCs Secrete Immunomodulatory and Pro-Regenerative Factors

Very High Secretion (> 1,000 pg/ml in supernatant)		High Secretion (100–1.000 pg/ml in supernatant)		Medium Secretion (10–100 pg/ml in supernatant)		Low Secretion (1–10 pg/ml in supernatant)		No Secretion (< 1 pg/ml in supernatant)	
SCGFβ	203,484 ± 11,783	CXCL1	957 ± 16	GCSF	96 ± 3.6	CXCL10	7.9 ± 0.4	IL-13	0.7 ± 0.0
HGF	9,412 ± 2090	IL-6	692 ± 215	IL-5	66 ± 2.2	MCSF	7.8 ± 0.2	CCL3	0.6 ± 0.0
IL-8	4,130 ± 535	CCL4	625 ± 19	IL-1RA	65 ± 9.1	CXCL9	6.3 ± 0.3	IL-10	0.5 ± 0.1
CCL2	2,822 ± 303	CXCL12	478 ± 7	CCL7	60 ± 1.4	IL-9	3.8 ± 1.8	IL-3	0.5 ± 0.0
		IL-12β	319 ± 1	IFN-☒	56 ± 0.4	IL-2	3.3 ± 0.3	IL-12	0.5 ± 0.0
		LIF	179 ± 2	SCF	39 ± 1.6	IL-17	3.2 ± 0.1	IL-15	0 ± 0
		TRAIL	142 ± 6	FGFbasic	29 ± 0.3	IL-7	2.7 ± 0.3	IL-18	0 ± 0
				IL-1α	21 ± 0.3	IL-4	2.3 ± 0.1	CCL5	0 ± 0
				IFN-α2	12 ± 0.1	IL-1β	2.0 ± 0.0	CCL11	0 ± 0
				IL-2Rα	12 ± 0.1	GMCSF	1.8 ± 0.0	CCL27	0 ± 0
				TNFa	12 ± 1.0			β-NGF	0 ± 0
				IL-16	10 ± 0.3			MIF	0 ± 0
				TNF-β	10 ± 1.1			PDGFBB	0 ± 0
								VEGF	0 ± 0

A cytokine, chemokine and growth factor multiplex assay was performed on WJ-MSC supernatants under basal culture conditions. Protein concentration in pg/ml are expressed as mean ± SEM.
WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells.

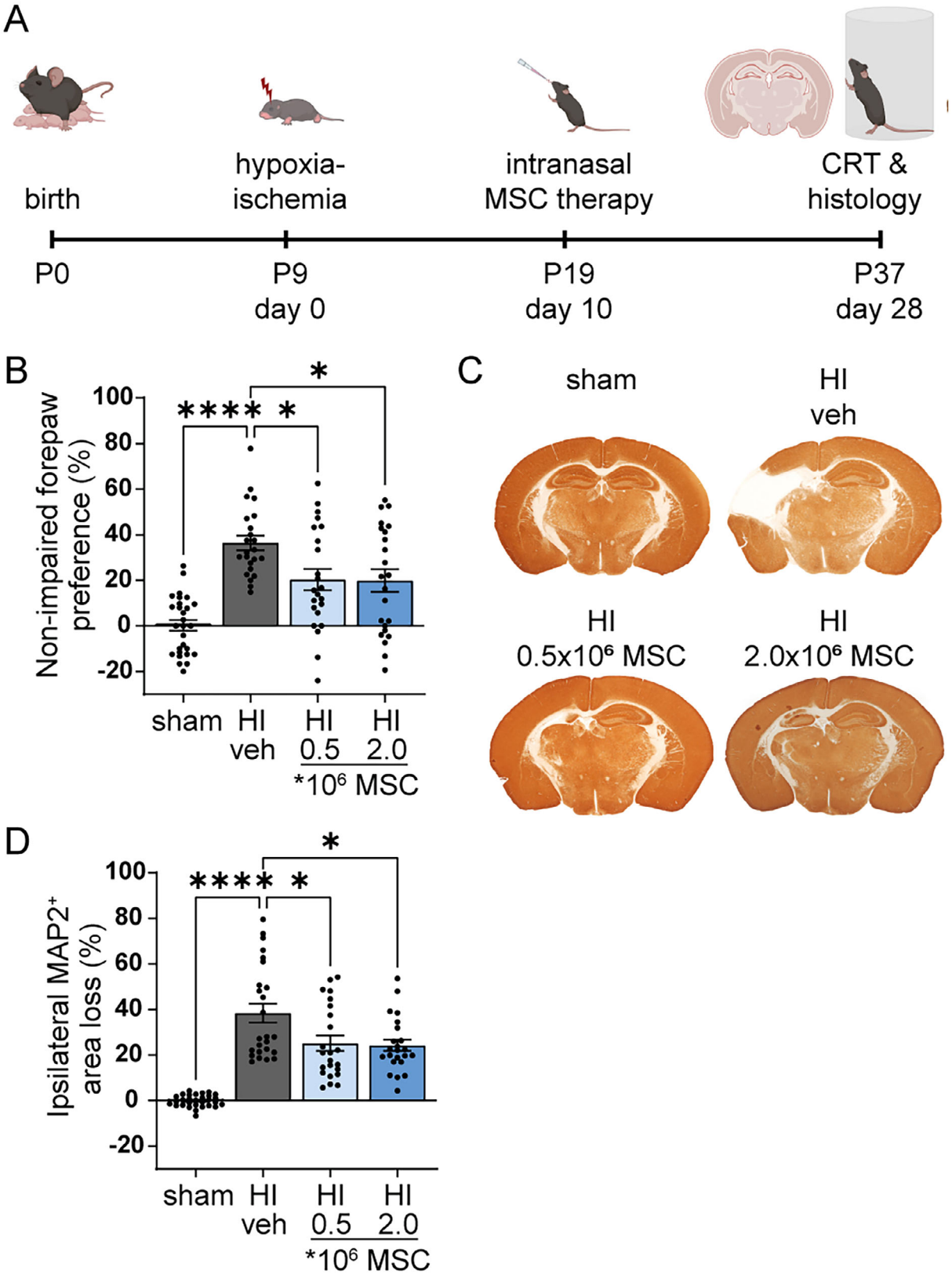

FIGURE 5: Intranasal WJ-MSC therapy remains effective for neonatal HI brain injury when administration was delayed until 10 days post-HI. (A) Schematic overview of the experimental set-up. (B) Intranasal WJ-MSCs improved sensorimotor impairment in HI-injured mice. (C) Representative MAP2-stained coronal sections of sham- and HI-injured mice treated intranasally with vehicle, 0.5 or 2.0 $\times$ 10^6 WJ-MSCs. (D) Intranasal WJ-MSCs ameliorated ipsilateral $MAP2^+$ area loss in HI-injured mice. Data are presented as mean $\pm$ SEM. *$p < 0.05$, ****$p < 0.0001$. CRT = cylinder rearing task; HI = hypoxia-ischemia; MAP2 = microtubule-associated protein 2; P = postnatal day; veh = vehicle; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells. [Color figure can be viewed at www.annalsofneurology.org]

were examined in HI mice subjected first to therapeutic hypothermia. To start, the optimal hypothermic paradigm was established by comparing the effects of normothermia (36.5°C) to deep hypothermia (32°C) or milder hypothermia (33.5°C) for 3 or 5 hours (Fig 6A). Both deep and milder hypothermia applied for either 3 or 5 hours effectively reduced MAP2$^+$ tissue loss at 10 days post-HI (Fig 6B). Hypothermia at 33.5°C for 5 hours provided the most significant neuroprotection, and was therefore used in subsequent experiments. Additionally, ipsilateral cerebral chemokine expression levels remained high in hypothermia-treated HI mice at day 3 post-HI when compared with sham-control mice, although levels of most chemokines were partially dampened compared to normothermia (Fig 6C). Because chemotactic signals CCL2, CCL3, CCL4, CSF1, CXCL4, and OPN were dampened at day 10 compared to day 3 post-HI + hypothermia (Fig 6D), the effect of WJ-MSC therapy in addition to hypothermia was subsequently tested by intranasal application at 3 days post-HI (Fig 6E). Therapeutic hypothermia following HI did not significantly improve sensorimotor outcome when compared to normothermic treatment at 28 days post-HI ($p = 0.26$; Fig 6F). However, intranasal WJ-MSC therapy did significantly improve sensorimotor outcome in hypothermia-treated HI animals, compared to hypothermia treatment alone. Hypothermia alone significantly improved gray matter damage compared to normothermia treatment in HI-injured animals at 28 days post-HI, indicating a long-lasting effect of hypothermia (Figs 6B and 6G). Importantly,

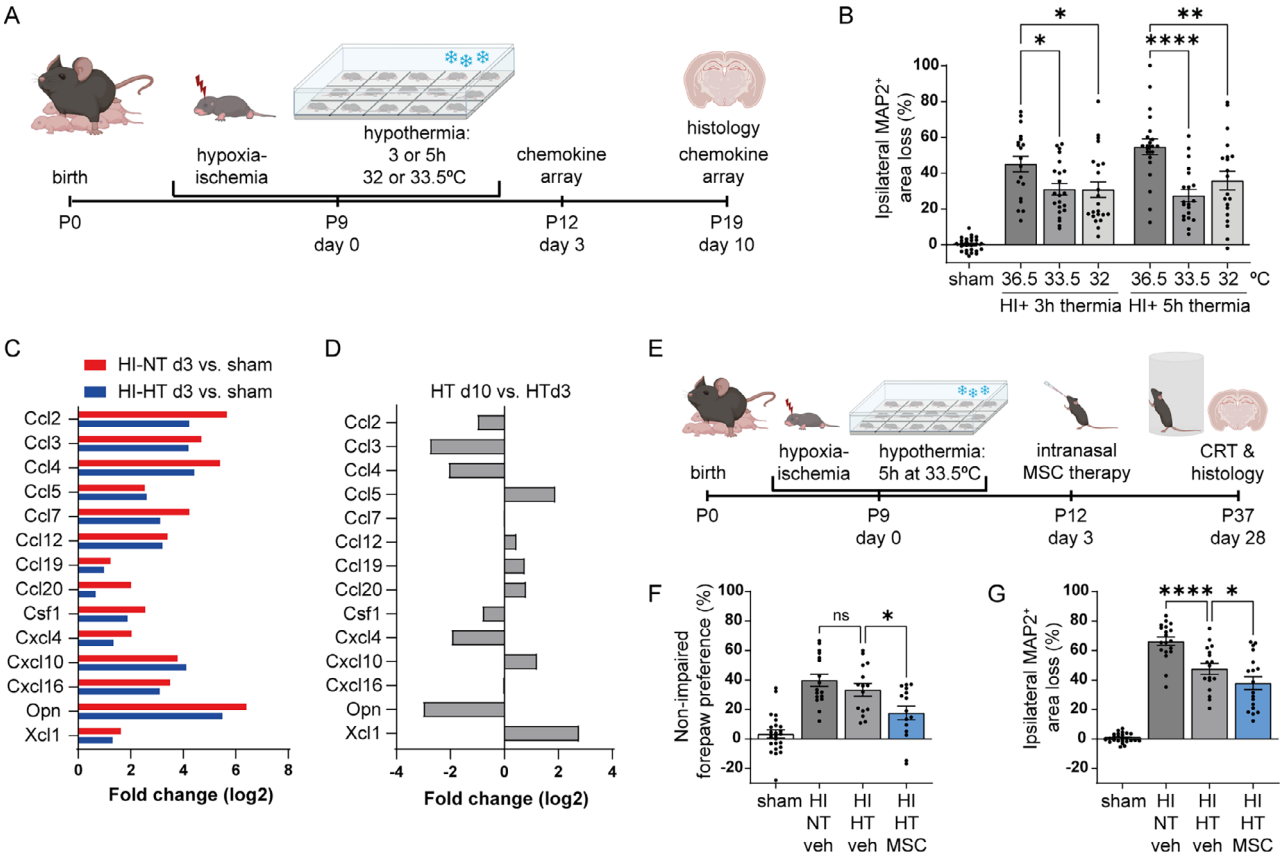

FIGURE 6: Intranasal WJ-MSC therapy provides additional therapeutic benefits in conjunction with early hypothermia treatment. (A) Schematic overview of the experiment for defining the most optimal hypothermic paradigm. (B) All therapeutic hypothermia protocols at 32°C or 33.5°C for 3 or 5 hours reduced ipsilateral MAP2$^+$ area loss at day 10 post-HI. Because hypothermia at 33.5°C for 5 hours gave the most significant reduction in brain injury this paradigm was used for the rest of the experiments. (C) Relative mRNA expression of chemotactic factors in the ipsilateral hemisphere at 3 days post-HI of mice that were subjected to normothermia or hypothermia (33.5°C for 5 hours) compared with sham brains. (D) Relative mRNA expression of chemokines in the ipsilateral hemisphere of HI mice subjected to hypothermia was compared between day 10 (d10) and day 3 (d3) post-HI. (E) Schematic overview of the experimental set-up combining early therapeutic hypothermia and intranasal WJ-MSC therapy at day 3 post-HI. (F) Intranasal WJ-MSCs improved sensorimotor impairment in HI-injured mice treated with hypothermia, compared to hypothermia treatment alone. (G) Intranasal WJ-MSCs ameliorated ipsilateral MAP2$^+$ area loss in HI-injured mice treated with hypothermia, compared to hypothermia treatment alone. Data presented are as mean ± SEM. *$p < 0.05$, **$p < 0.01$ ****$p < 0.0001$. CRT = cylinder rearing task; HI = hypoxia-ischemia; HT = hypothermia; MAP2 = microtubule-associated protein 2; NT = normothermia; P = postnatal day; veh = vehicle; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells. [Color figure can be viewed at www.annalsofneurology.org]

intranasal WJ-MSC administration at 3 days post-HI provided an additional benefit by further reducing gray matter damage in hypothermia-treated HI mice (Fig 6G).

Discussion

This study demonstrates the dosing, timing, and efficacy of intranasal therapy using WJ-MSCs in mitigating brain injury and sensorimotor impairments following neonatal HI. Whereas previous research has extensively explored the therapeutic potential of intranasally administered MSCs from alternative sources in models for neonatal HI brain injury,[7] the effects of intranasally applied WJ-derived MSCs remain relatively understudied. The neuroprotective potential of nasal WJ-MSCs has been described when applied at early timepoints after hypoxia-ischemia (ie, within 24 hours after the insult), resulting in reduced brain injury and improved functional outcome via the inhibition of apoptosis.[25–27] The current study applied for the first time an extended treatment window of 3 or 10 days post-HI and revealed that intranasal WJ-MSCs exhibit therapeutic effects by attenuating neuroinflammation and promoting neuroregeneration at relative later phases post-HI. These findings align with observations from preclinical studies utilizing MSCs from alternative sources, including bone marrow.[13] Because WJ-MSCs isolated from the umbilical cord can be noninvasively obtained in larger quantities and exhibit higher self-renewal capacity than other MSCs,[28] they may be preferred for clinical use. MSCs from different sources may exhibit distinct properties that can influence their therapeutic potential, such as differential chemokine receptor expression and secretome profiles.[29,30] Comprehensive direct comparison studies of MSCs from different sources to treat neonatal HI brain injury are currently lacking, necessitating further research to determine the most efficacious MSC source for clinical application.

The existing literature on the combination of MSC therapy with therapeutic hypothermia reports conflicting results, potentially due to variations in hypothermia protocols and MSC source, and administration timing and route.[17] Benefits of MSC therapy in conjunction with hypothermia have been tested mostly using intracerebral injection of cord blood-derived MSCs, whereas intranasal administration remains understudied.[17] Herz et al. conducted a study relatively similar to ours, intranasally dosing BM-MSCs at 3 days following HI in P9 mice, and observed unexpected exacerbation of brain injury on some outcome parameters, potentially due to a pro-inflammatory response of BM-MSCs to the HI-hypothermia brain milieu.[31] In the contrary, a follow-up study demonstrated additional treatment benefits in conjunction with hypothermia when using intranasal extracellular vesicles from clonally expanded immortalized MSCs, indicating that the brain remains permissive to the MSC secretome after hypothermia.[32] In a newborn piglet model of neonatal HI brain injury, intranasally administered WJ-MSCs demonstrated therapeutic effects s in conjunction with hypothermia, highlighting their potential for clinical translation.[25] In the current study, we confirmed the presence of remaining chemotactic signals in the HI-lesioned hemisphere post-hypothermia and demonstrated that WJ-MSC therapy at 3 days post-HI improved anatomic and functional outcome in conjunction with hypothermia. Additionally, we showed lower expression of multiple chemotactic signals (eg, Opn, Ccl3, and Cxcl4) in the brain at 10 days compared to 3 days post-hypothermia, suggesting a potential implication for MSC migration. However, other chemokines were not affected or upregulated, including Cxcl10, which we have recently identified as a crucial chemokine for attracting MSCs to the brain after intranasal delivery.[22] Future studies should address whether hypothermia affects the therapeutic window of MSC therapy at timepoints later than 3 days, which is critical information for designing clinical trials. Although we have previously shown that multiple dosing at day 3 and day 10 post-HI did not improve MSC efficacy compared to a single dose,[13] the effects of multiple dosing regimens following hypothermia should also be assessed.

We hypothesized that upon intranasal administration, WJ-MSCs migrate from the nasal cavity into the brain using chemotactic signals. Indeed, WJ-MSCs express multiple receptors for HI-induced chemokine expression in the brain parenchyma. WJ-MSCs were traced in the nasal cavity and the lining of the olfactory bulb at 2 hours following administration, and entered the brain parenchyma at 24 hours. The distribution of WJ-MSCs was comparable across the ipsilateral hemisphere, contralateral hemisphere, and cerebellum, with relatively low percentage of intranasally administered WJ-MSCs reaching the brain parenchyma. Because WJ-MSC tracing at 6 hours or 3 days post-administration resulted in similar numbers of WJ-MSCs in the brain parenchyma (data not shown) and MSC debris was observed at 24 hours post-administration, the peak of WJ-MSC migration probably takes place between 6 and 24 hours. This study is the first that traced intranasally administered MSCs in the meninges after neonatal HI brain injury. Future studies should examine whether WJ-MSC migration into the brain parenchyma is essential, or that WJ-MSCs could partially exert their beneficial effects remotely, for example, from within the meninges. To get a complete overview of the destination of MSCs following intranasal administration,

biodistribution analyses should be performed, including CSF and draining lymph nodes, but also peripheral organs to assess whether WJ-MSCs have been inhaled, swallowed, or taken up systemically. Given the significant differences between murine and human nasal cavities, effective migration should be validated in a large animal model with nasal cavity and brain anatomy more closely resembling that of human neonates.

The non-contact co-culture systems used in this study with primary microglia and neural stem cells proved that the anti-inflammatory and pro-regenerative effects of WJ-MSCs are, at least in a large part, driven by the WJ-MSC secretome. WJ-MSCs secreted factors with pleiotropic effects in the brain, including anti-inflammatory factors, such as HGF, GCSF, and IL-1RA[33–35] and known pro-regenerative factors, such as GCSF, LIF, and FGF-basic.[36,37] Future studies investigating the effect of individual MSC-secreted factors on inflammatory responses by microglia and on differentiation of neural stem cells in the described *in vitro* assays will provide better understanding of the therapeutic cocktail of the MSC secretome. A limitation of our study is that the multiplex assay to study the WJ-MSC secretome was limited to 48 proteins, so potentially other important protein targets in the secretome could have been missed. In addition, we analyzed the secretome under naïve culture conditions. Because MSCs can adapt their secretory profile to environmental cues, for instance, MSCs secrete pro-angiogenic proteins in response to hypoxia and immunosuppressive proteins in response to inflammation, it should be taken into account that the secretome of MSCs may adapt upon *in vivo* HI conditions.[38] Therefore, future proteomic analysis of the WJ-MSC secretome under normal, hypoxic, or inflammatory conditions would provide a full assessment of secreted proteins. Identification of the crucial factors responsible for the anti-inflammatory and pro-regenerative effects of WJ-MSCs would facilitate the development of optimization strategies to further enhance the efficacy of MSC therapy.

MSC-based therapies for neonatal HI brain injury are steadily advancing toward clinical application. In the first-in-human intranasal safety trial, MSC dose was based on the relative brain surface area between rodents and humans due to the targeted delivery route, and treatment timing was based on days following insult due to the temporal dynamics of chemotactic signaling. A key goal moving forward is the development of a high-quality, off-the-shelf MSC product, characterized and tested in validated potency assays. In this study, we used a co-culture with differentiating neural stem cells to demonstrate consistent neuroregenerative potency of WJ-MSCs across donors and culture protocols, implying stable secretion of neurogenic factors. The near-future validation of potency assays will be crucial to compare MSC source, donor, and batch variability in future clinical application, and to assess optimization strategies to enhance MSC efficacy, such as preconditioning. In addition, optimization of MSC administration may involve refining dose and frequency, or tailoring optimized MSCs to specific neonatal populations (eg, preterm vs term infants). Ultimately, human efficacy trials are essential to confirm the therapeutic benefit of intranasal MSC therapy in neonates with HI brain injury, with the overarching aim to improve the quality of life for patients and their families.

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Author Contributions

Conception and design of the study: C.T., C.N., F.B., M.B., K.D., G.V., and D.A. Acquisition and analysis of data: C.T., C.N., S.D.P., J.V., S.R., K.D., G.V., and D.A. Drafting a significant portion of the manuscript or figures: C.T., S.D.P., and C.N.

Potential Conflicts of Interest

K.D., G.V., and D.A. are employed by Chiesi Farmaceutici S.p.A., the company which own the patents rights to the WJ-MSCs tested in this study. M.B. received consultancy fees from Chiesi Farmaceutici S.p.A. that were not related to nor did it influence any aspects of the current study. The other authors declare no conflicts of interest.

Data Availability

The array and multiplex datasets supporting the findings of this study are deposited in DataverseNL and accessible under doi:10.34894/PW1JFT. Full datasets of this study are available from the corresponding author upon reasonable request.

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