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Report

Human spinal GABA neurons survive and mature in the injured nonhuman primate spinal cord

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SUMMARY

Spinal cord injury (SCI) leads to permanent neural dysfunction without effective therapies. We previously showed that human pluripotent stem cell (hPSC)-derived spinal GABA neurons can alleviate spasticity and promote locomotion in rats after SCI, but whether this strategy can be translated into the clinic remains elusive. Here, a nonhuman primate (NHP) model of SCI was established in rhesus macaques (*Macaca mulatta*) in which the T10 spinal cord was hemisected, resulting in neural conduction failure and neural dysfunction, including locomotion deficits, pain, and spasms. Grafted human spinal GABA neurons survived for up to 7.5 months in the injured monkey spinal cord and retained their intrinsic properties, becoming mature and growing axons and forming synapses. Importantly, they are functionally alive, as evidenced by designer receptors exclusively activated by designer drug (DREADD) activation. These findings represent a significant step toward the clinical translation of human spinal neuron transplantation for treating SCI.

INTRODUCTION

Spinal cord injury (SCI) leads to permanent deficits in neural function with no effective treatment currently (Ahuja et al., 2017). The nature underlying the failure of functional recovery is that dead and lost neurons cannot be reproduced, and severed axons cannot regenerate, leading to the fact that destroyed neural circuits cannot be restored (Sofroniew, 2018). Neural stem cells (NSCs), or neuronal progenitor cells (NPCs) that can generate neurons, have now become the focus for reconstructing neural circuits to recover function (Fischer et al., 2020). Recent studies have found that human pluripotent stem cell (hPSC)-derived spinal NSCs can induce robust axon regeneration and that human spinal neurons are extensively integrated into rat neural circuits, contributing to fast locomotion improvement (Kumamaru et al., 2018). However, these studies only focused on motor function through excitatory glutamatergic neurons to form relay circuits. Pain and spasticity develop in 90% and 65% of patients with SCI who are refractory to drugs, and many side effects occur due to systemic administration, which severely impacts quality of life (Ahuja et al., 2017). In the naive state, spinal dI4 GABA neurons contribute to the presynaptic inhibition of sensory terminals (Betley et al., 2009) and have pivotal roles in preventing abnormal sensation (Escalante and Klein, 2020), regulating muscle tone (Zhang et al., 2017), and securing the smoothness of forelimb grasping (Fink et al., 2014) and hindlimb locomotion (Koch et al., 2017). Loss of spinal GABA neurons after SCI (Gwak and Hulsebosch, 2011; Meisner et al., 2010) resulting in overexcitation of sensory transmission and excitatory interneurons could be the putative reason for pain and spasm, and studies have demonstrated the efficacy of rodent GABA neurons in reducing pain (Braz et al., 2012). However, no protocol exists that can generate human spinal GABA neurons until our previous studies (Gong et al., 2021; Xu et al., 2022). In rats, human spinal GABA neurons could mitigate hindlimb spasticity, improve locomotion (Gong et al., 2021), and alleviate pain (unpublished data), which makes them a good candidate for treating SCI. However, the efficacy demonstrated in rodent models cannot assure its success in patients because significant differences exist between rodents and humans (Courtine et al., 2007; Friedli et al., 2015). There has been a consensus that it is necessary to test the safety and efficacy of treatments in large animals or even nonhuman primates (NHPs) that closely resemble humans in neuroanatomy and neurophysiology before initiating clinical trials (Kwon et al., 2015), especially for cell
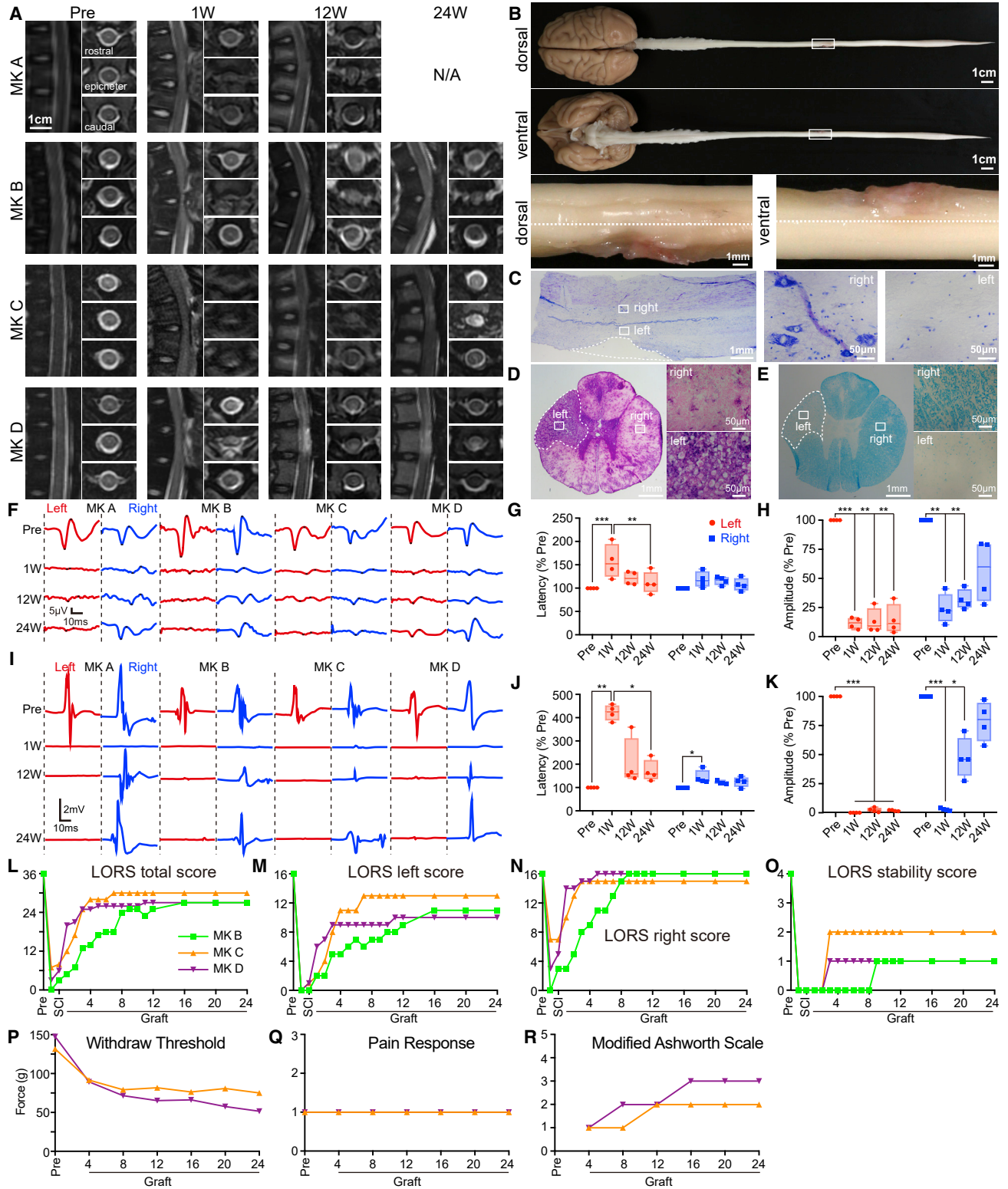
Figure 1. Establishing an NHP model of SCI

(A) MRI of the spinal cords of monkeys.

(B) Anatomical images of the spinal cord of monkeys. The white dotted line indicates the middle line.

transplantation (Kwon et al., 2013), which can substantially increase success in clinical trials while avoiding potential harmful events (Courtine et al., 2007). Here, we established an NHP model of SCI based on which the feasibility of transplantation of human spinal GABA neurons was tested.

RESULTS

Establishing an NHP model of SCI

All four monkeys (Table S1) underwent left hemi-section of the T10 spinal cord followed by intraspinal transplantation. The heart rate and mean arterial pressure were increased after SCI (Figure S1A), while all vital signs remained relatively stable during transplantation (Figure S1B). After two surgeries, monkeys had minimally lost body weight followed by progressive recovery (Figures S1C and S1D). The blood concentration of the immunosuppressant FK506 was kept in a range of 10–20 ng/mL (Figure S1E), which is safe and tolerable. The parameters of liver and kidney function were in the normal range (Table S2). Magnetic resonance imaging (MRI) revealed that the spinal cords were normal before SCI but were damaged 1 week after SCI; by 12 and 24 weeks later, the injury epicenter showed atrophy accompanied by high signal lesions (Figure 1A). Postmortem analysis confirmed that the injury was located in the left T10 segment and that the lesion extended to the midline (Figure 1B). Nissl staining revealed that neurons were preserved in the right gray matter but totally lost on the left side (Figure 1C). Caudal to the injury, hematoxylin and eosin (H&E) staining showed Wallerian degeneration and immune cell infiltration (Figure 1D), while Luxol fast blue (LFB) staining showed demyelination in the white matter on the left side (Figure 1E).

Somatosensory evoked potentials (SSEPs) were readily elicited bilaterally before SCI but were hard to detect on both sides 1 week after SCI; 12 and 24 weeks later, SSEPs were still difficult to elicit on the left but showed reappearance on the right (Figures 1F–1H; Table S3). Similarly, motor evoked potentials (MEPs) were normally elicited bilaterally before SCI but were lost 1 week after SCI; by 12 and 24 weeks later, MEPs were still nearly absent on the

left but reappeared on the right (Figures 1I–1K; Table S3). The motor conduction velocity (MCV) test in peripheral nerves revealed that the velocity and amplitude remained unaltered on both sides (Figures S1F–S1H; Table S3), indicating that the conduction block was specific to spinal lesions. Evaluating quadrupedal locomotion revealed that monkeys scored 36 points before SCI (Figure 1L), with 16 points on both hindlimbs (Figures 1M and 1N) indicating normal stepping and 4 points on the trunk (Figure 1O) indicating consistent stability. One week after SCI, monkeys manifested severe locomotor disturbances, with full paralysis on the left but different extents of movement from full paralysis to weight-supported plantar stepping on the right, and no trunk stability existed. Thereafter, spontaneous partial recovery gradually reached a plateau (Figure 1L), with normal stepping on the right (Figure 1N) but only plantar stepping without stability on the left (Figure 1M), and no trunk stability recovered (Figure 1O). The withdrawal force threshold in the right hind paw gradually decreased (Figure 1P), suggesting the development of mechanical allodynia. However, the pain response always remained a spinal segment reaction with no exaggeration (Figure 1Q), indicating no development of hyperalgesia. The muscle tone of the left hindlimb progressively increased (Figure 1R), indicating the development of spasm. Taken together, these results demonstrated that an NHP model of SCI was successfully established in rhesus macaques.

Survival, maturation, axon growth, and synapse formation of human spinal GABA neurons

One monkey received intraspinal grafting of hPSC-derived spinal dI4 GABA NPCs and was euthanized 7.5 months later. By immunostaining with Stem121 and mCherry, human cells were identified in the spinal cord ranging from 6 mm rostral and 0.8 mm caudal to the lesion epicenter (Figures S2A and S2B). The two largest areas of graft were located 2 and 4.8 mm rostral to the epicenter (Figure S2C), and the total number of surviving human cells was approximately 41,140 (Figure S2D); thus, the survival rate was approximately 10.28%. The mean fluorescence intensity (MFI) of Stem121 and mCherry was relatively consistent (Figure S2E). By immunostaining with NeuN, human cells were identified in the left dorsal column out of the host

(C) Nissl staining of horizontal spinal cord sections through the lesion epicenter.

(D and E) H&E (D) and LFB staining (E) of coronal spinal cord sections caudal to the lesion epicenter.

(F–H) SSEP evaluation. Data are represented as the mean ± SEM.

(I–K) MEP evaluation. Data are represented as the mean ± SEM.

(L–O) Locomotion deficits after SCI.

(P and Q) Mechanical allodynia in the right foot after SCI.

(R) Muscle tone increase in the left ankle joint after SCI.
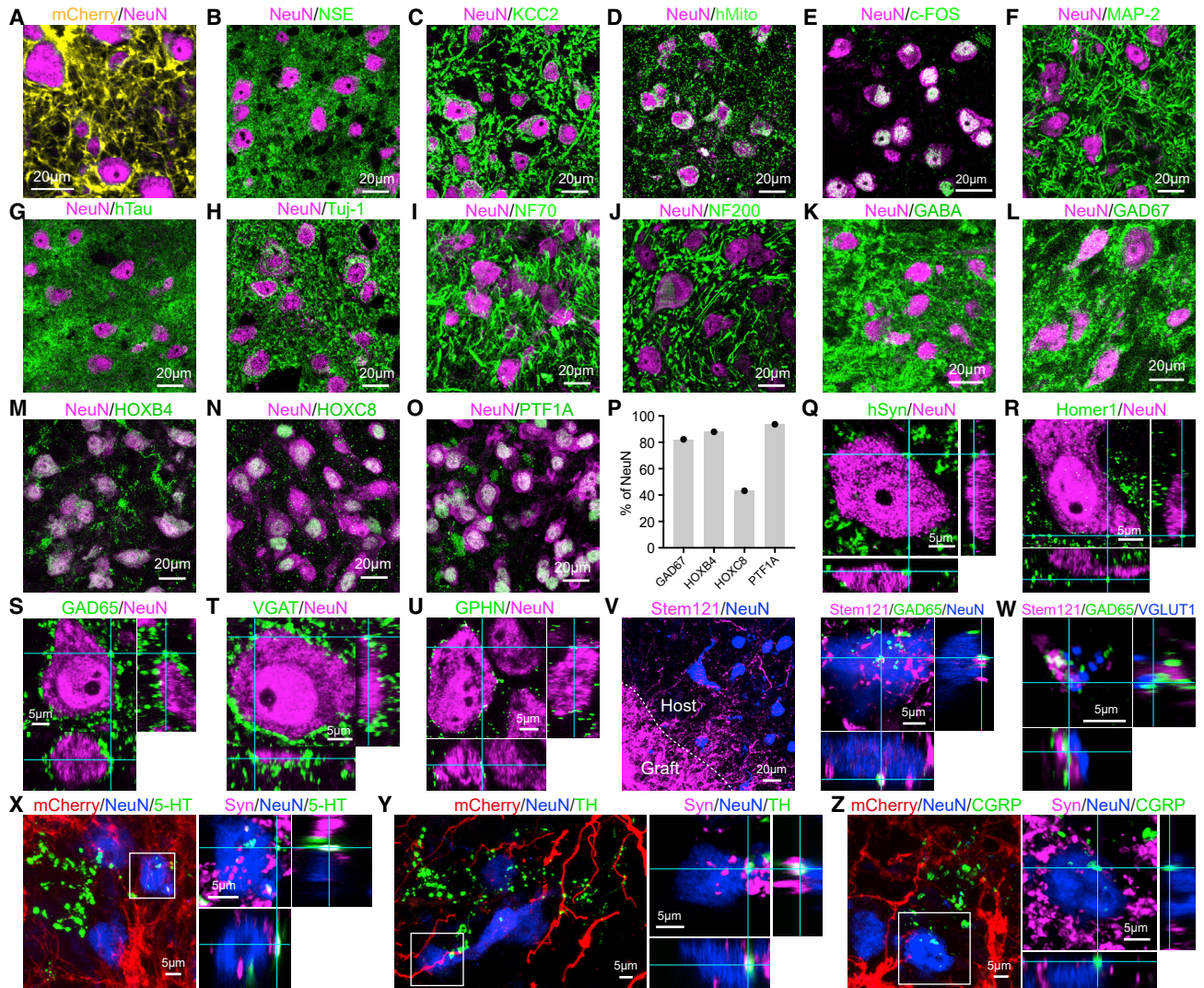
See also Figure S1.

Figure 2. Survival, maturation, axon growth, and synapse formation of human spinal GABA neurons

(A) Neuronal differentiation of grafted human cells.

(B–E) Functional survival of human neurons.

(F–J) Human neurons grew mature dendrites and axons.

(K–P) Characterization of human neurons.

(Q–U) Synapse formation on human neurons.

(V and W) Innervation of monkey neural circuits by human neurons.

(X–Z) Innervation of human neurons by monkey neural circuits.

See also Figures S2–S4.

monkey gray matter (Figure S3A). Within the graft, human neurons were present throughout the spinal cord sections (Figure S3B). The total number of human neurons was approximately 30,600 (Figure S3C); thus, the neuronal differentiation rate was approximately 74.38%. The diameter of human neurons was 19.1 ± 1.8 μm (Figure S3D). The MFI of human neurons was lower but closely approximated that of monkey neurons (Figure S3E). The expression of mCherry was observed around human neurons (Figure 2A),

indicating that the excitatory designer receptors exclusively activated by designer drug (DREADD) hM3Dq was still preserved in human neurons. Within the graft, extensive expression of neuronal-specific enolase (NSE), potassium-chloride cotransporter-2 (KCC2), and human mitochondria (hMito) (Figures 2B–2D) was identified. Upon activation with clozapine-N-oxide (CNO), an agonist of hM3Dq, human neurons expressed c-FOS (Figure 2E), suggesting that they were mature, functional, and alive.
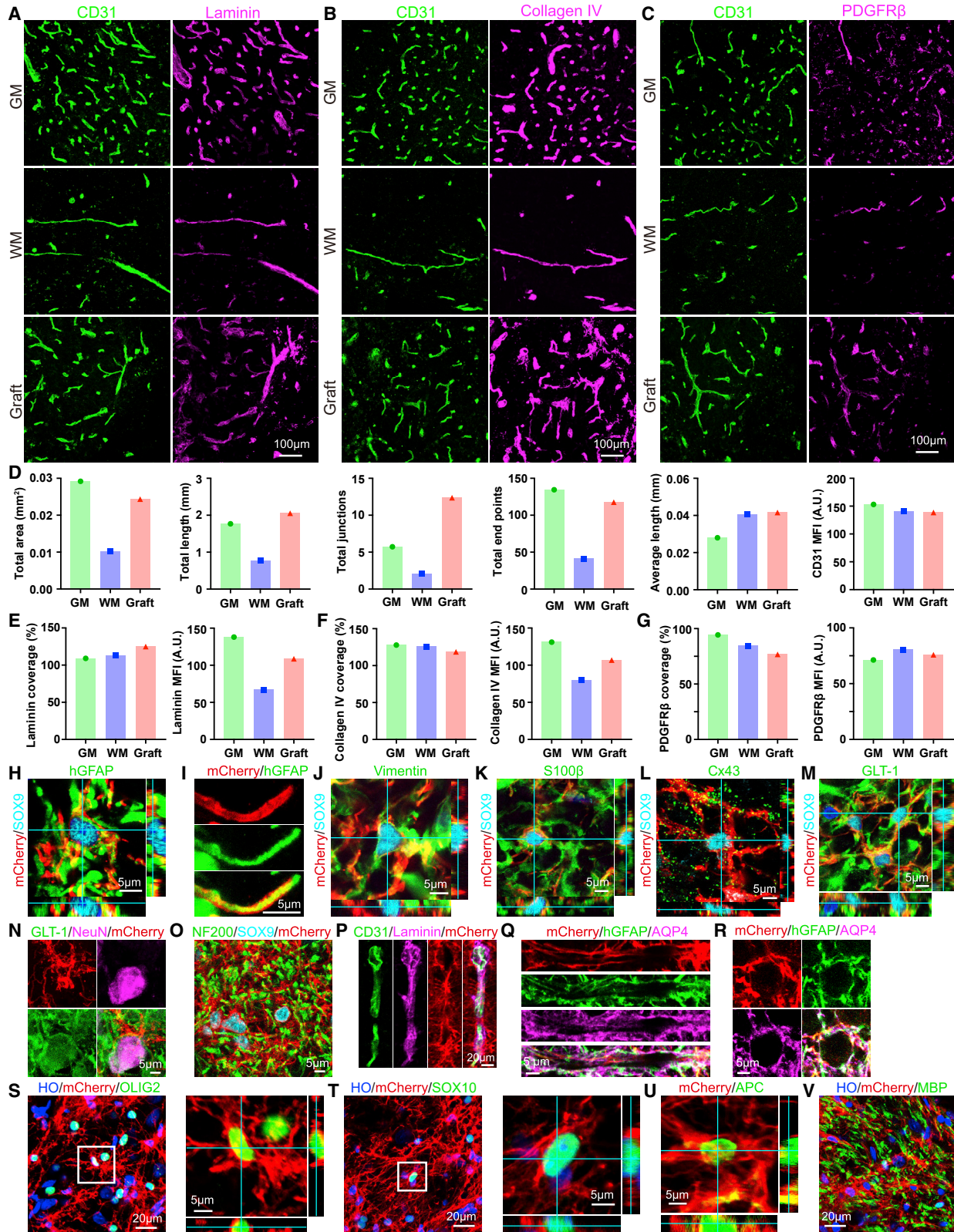
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Many dendrites, as verified by microtubule-associated protein 2 (MAP-2; Figure 2F), and axons, as confirmed by human tau (hTau), beta3-tubulin (Tuj-1), neurofilament 70 (NF70), and NF200 (Figures 2G–2J), were present within the graft. Moreover, Stem121 colocalized with MAP-2, NF70, and NF200 (Figures S4A and S4B), proving that these dendrites and axons were actually from human neurons, which, however, were not wrapped by the monkey myelin sheath (Figures S4C and S4D). A large amount of GABA was deposited around human neurons (Figure 2K). Approximately 82% of human neurons expressed glutamate decarboxylase 67 (GAD67), which is responsible for GABA synthesis; 88% and 43% expressed the spinal cord markers HOXB4 and HOXC8; and 94% expressed the dI4-specific marker PTF1A (Figures 2L–2P). Other subtypes of neurons were minor or even scarce (Figures S4E–S4S). Putative human synapses were formed on human neurons as verified by the pre- and postsynaptic markers human synaptophysin (hSyn) and Homer1 (Figures 2Q and 2R). Furthermore, these synapses were inhibitory as confirmed by GAD65, vesicular GABA transporter (VGAT), and gephyrin (GPHN) (Figures 2S–2U). There were also some excitatory synapses on human neurons (Figures S4T–S4V). Human axons had grown into the host dorsal horn and formed inhibitory synapses with monkey neurons and presynaptic inhibition structures with monkey sensory terminals (Figures 2V and 2W). Meanwhile, the monkey 5-HT$^+$ raphespinal tract, TH$^+$ cerulospinal tract, and CGRP$^+$ unmyelinated peptidergic nociceptive afferents grew into grafts and formed synapses with human neurons (Figures 2X–2Z).

Angiogenesis of monkey capillaries, differentiation, and integration of human astrocytes

Many CD31$^+$ capillaries were identified within the graft (Figures 3A–3C). The total area, length, junctions, and ending points (Figure 3D) were significantly higher than those in the white matter (WM) and approximate to those in the gray matter (GM). Furthermore, these capillaries were covered with basement membranes and pericytes as evidenced by laminin, collagen IV, and PDGFRβ staining (Figures 3A–3C). The coverage and MFI of the three markers in the graft were comparable to those in both the GM and WM (Figures 3E–3G). There was approximately 20.47%

astrocyte differentiation from human cells as verified by the expression of SOX9 and human glia fibrillary acidic protein (hGFAP), which colocalized with mCherry (Figures 3H and 3I). These human astrocytes also expressed vimentin, S100β, connexin 43 (CX43), and glutamate transporter 1 (GLT-1) (Figures 3J–3M). The processes of human astrocytes closely surrounded the neuron cell body (Figure 3N), supported the axons (Figure 3O), wrapped the capillaries (Figure 3P), and even expressed the water channel protein aquaporin 4 (AQP4; Figures 3Q and 3R). Approximately 2.58% of the OLIG2$^+$ oligodendroglia lineage differentiated from human cells, and a few of them had matured into SOX10$^+$ and APC$^+$ oligodendrocytes, which, however, did not form the myelin sheath (Figures 3S–3V). Finally, hPSCs were absent because of the lack of expression of the pluripotency markers OCT4, NANOG, and SOX2 (Figures 4A–4C). Some human NSCs existed as identified by SOX1 and human nestin, and they were proliferating (Figures 4D–4G). Human neurons had terminated migration and stopped axon growth as confirmed by negative expression of doublecortin (DCX) and growth-associated protein 43 (GAP43) (Figures 4H and 4I). Monkey CD3$^+$ lymphocytes were not found in the graft (Figure 4J), and apoptosis did not occur in human neurons, as evidenced by the absence of cleaved caspase-3 and TUNEL staining (Figures 4K and 4L).

DISCUSSION

In this study, an NHP model of SCI was established to test the feasibility of transplanting human cells. To the best of our knowledge, this is the first report where hPSC-derived spinal GABA NPCs engineered with DREADDs survived and matured into neurons, grew dendrites and axons, and formed putative synapses in the injured spinal cord of rhesus macaques. Thus, our study made a first step toward the demonstration of its long-term safety and efficacy in treating SCI.

The cells were grafted into the perilesion instead of the injury epicenter, as in our previous study in rats (Gong et al., 2021). Actually, around the lesion epicenter, there are spared, but reactive, neural circuits that undergo plasticity (O'Shea et al., 2017), which is responsible for spontaneous function improvement but also maladaptive

Figure 3. Angiogenesis of monkey capillaries, differentiation, and integration of human astrocytes
(A–C) Angiogenesis within human cell grafts.
(D) Quantification of the parameters of CD31$^+$ capillaries.
(E–G) Quantification of the coverage and MFI of laminin (E), collagen IV (F), and PDGFRβ (G) on CD31$^+$ capillaries.
(H–M) Differentiation and maturation of human astrocytes.
(N–R) Human astrocytes structurally integrate into the monkey spinal cord.
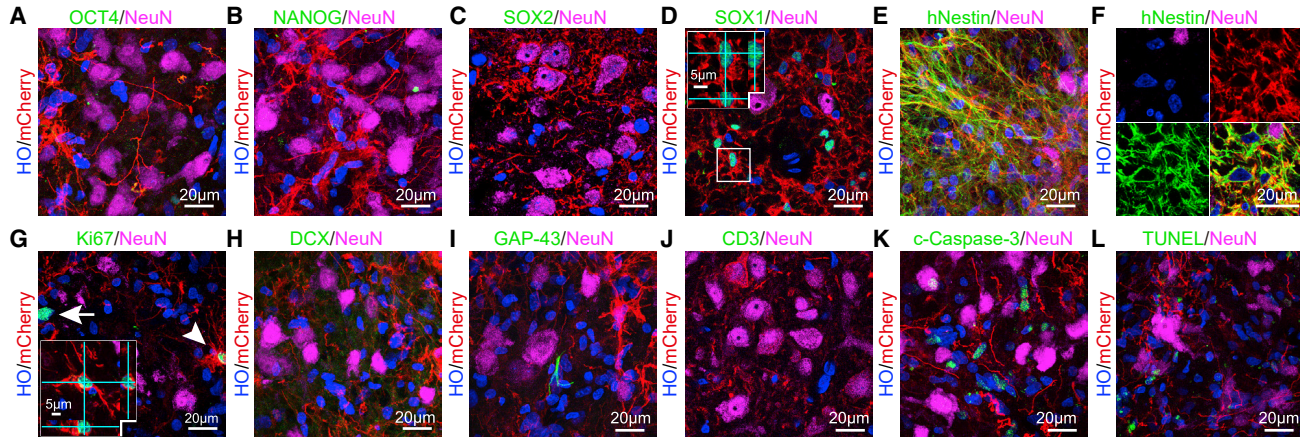(S–V) Differentiation of human oligodendrocytes.

Figure 4. Safety issues of grafted human cells

(A–C) Absence of hPSCs within the human cell graft.

(D–F) Preservation of undifferentiated NSCs within the graft.

(G) Proliferation of human cells.

(H) No migration of human neurons.

(I) Human neurons stopped axon growth.

(J) Lack of monkey lymphocytes within human cell grafts.

(K and L) Human neurons did not undergo apoptosis.

complications (Krupa et al., 2022). Modifying the excitation and inhibition balance of these neural circuits could produce significant functional improvement without bridging the lesion epicenter (Brommer et al., 2021; Chen et al., 2018). Thus, our cells are suitable for grafting into the perilesion part to form modulatory circuits, but this also leads to incomplete filling of the lesion cavity, which could become syringomyelia in the chronic phase. However, if cells were solely grafted into the cavity, they would be quickly rinsed by cerebral spinal fluid, resulting in no retention in the cavity but ectopic colonization in other sites of the CNS (Steward et al., 2014), raising safety concerns. Because our spinal GABA NPCs are mainly restricted to neuronal fate, which is postmitotic with low proliferative potential, in our successive work, the number of grafted cells might be scaled up to 1 to 20 million. More importantly, the fibrin matrix combined with a growth factor cocktail should be added into the graft (Rosenzweig et al., 2018) to retain the cells in the cavity and to support their survival and maturation. Alternatively, the cells could be seeded onto other biomaterials that have already shown efficacy in NHP of SCI (Rao et al., 2018; Slotkin et al., 2017) and then cotransplanted to better repair the lesion.

Nearly 80%–90% of human neurons are spinal dI4 GABAergic neurons, suggesting that they retain their intrinsic properties after grafting into the injured spinal cord, consistent with our previous rodent study (Gong et al., 2021). Human NF200 axons were present, consistent with our previous rat study (Gong et al., 2021) but contrary to the recent NHP study (Rosenzweig et al., 2018). This difference might be due to human cells being labeled with GFP, which was downregulated in mature neurons and axons, as in our previous study in mice (Chen et al., 2015), leading to the possibility that NF200 axons are GFP negative. Human dendrites are unmyelinated, which is a normal phenomenon. However, human axons were also unmyelinated, which is similar to a recent NHP study (Rosenzweig et al., 2018). The putative reason is that myelination is unnecessary for spinal interneurons that transmit information shortly and locally; another possibility is that the origin of axons and myelin is different in species since axons of grafted rat spinal neurons could be wrapped by host rat myelin (Hunt et al., 2017).

Putative GABAergic and glutamatergic synapses are formed on human neurons. Unfortunately, the hSyn antibody can recognize monkey synapses, making it uncertain whether these synapses authentically originate from human neurons, let alone quantification of the subtypes of human synapses. However, we believe these synapses are mainly human GABAergic because human GABA neurons dominated and were located in the dorsal column where monkey synapses were not present. However, the possibility that synapses from monkeys also formed on human neurons cannot be excluded. Indeed, monkey descending motor tracts and afferent sensory terminals were present in the graft and formed synapses with human neurons, resembling the normal circuit connections in the rodent spinal cord (Escalante and Klein, 2020). Furthermore, human neurons form inhibitory synapses with

monkey neurons and presynaptic inhibition structures with sensory terminals, which again mimics the circuits in rodent spinal cords (Betley et al., 2009). Thus, human spinal GABA neurons could find their appropriate targets to innervate and be innervated by proper host inputs when grafted into the injured spinal cord.

Angiogenesis with a normal blood-spinal cord barrier (BSCB) occurs in the graft to supply metabolism to human neurons while preventing harmful molecule infiltration. Furthermore, mature human astrocytes are present around neurons, participating in functions including glutamate clearance, supporting axon growth and forming the BSCB, consistent with our and other previous studies in rodents (Chen et al., 2015; Lien et al., 2019). By virtue of angiogenesis and human astrocytes, human neurons could be anticipated to survive for a more protracted time. Indeed, human neurons did not undergo apoptosis and were functionally alive, as verified by DREADD activation. No hPSCs existed, excluding the safety issue of teratoma formation. However, there were some NSCs in proliferation, probably due to insufficient time to complete differentiation; another explanation is that they are destined to be preserved, as in CNS development, where NSCs are preserved during adulthood (Johansson et al., 1999). Once stimulated, they can differentiate into neurons and glia.

Finally, a recent study revealed that autologously grafted porcine induced pluripotent stem cell (iPSC)-derived NPCs could survive and mature in the spinal cord of minipigs in the long term without immunosuppression (Strnadel et al., 2018). This strategy holds great promise in translation, where patients with SCI could receive syngeneic grafts while avoiding side effects due to immunosuppression and tumorigenicity. In addition, a recent first-in-human phase I study (Curtis et al., 2018) demonstrated the feasibility and safety of human fetal spinal cord-derived NSC transplantation for chronic SCI, which directly indicates the promise of human NSC transplantation and implies the need to test human embryonic stem cell (ESC)- or iPSC-derived spinal cord NSCs (Kumamaru et al., 2018) in patients with SCI; however, much work still should be done in more NHP models. Only based on more future translational studies could human NSC transplantation be employed to treat SCI successfully.

EXPERIMENTAL PROCEDURES

Resource availability

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Materials availability
This study did not generate new unique reagents.

Data and code availability
No large datasets were generated in this study. No original code was generated in this study.

Cell differentiation
Human spinal dI4 GABA NPCs were generated from hM3Dq-expressing hESC lines (line WA09) under our step-by step protocol (Xu et al., 2022). The details are presented in the supplemental information.

SCI and cell transplantation
Adult male rhesus macaques were subjected to left T10 spinal cord hemisection, followed by intraspinal transplantation of cells or medium 2 weeks after SCI. Immunosuppression was employed until sacrifice. The details are presented in the supplemental information.

Behavioral assessment
Locomotion was evaluated by the locomotor observational rating score (LORS). Pain was evaluated by mechanical allodynia. Spam was evaluated by the modified Ashworth scale. The details are presented in the supplemental information.

Electromyography
SSEP was performed to evaluate the ascending somatosensory function of the dorsal column. MEPs were utilized to assess descending motor function in the corticospinal tract(CST). Nerve conduction velocity (NCV) was performed to evaluate the function of peripheral nerves. The details are presented in the supplemental information.

MRI
Sagittal and axial T2WI images were acquired with clinical 3T scanners. The details are presented in the supplemental information.

Histology
The monkeys were euthanized 6 to 7.5 months after grafting. The grafted monkey received a CNO injection to induce c-FOS expression before sacrifice. The spinal cord segment centered on the lesion was dissected and cut into coronal or horizontal sections. H&E, LFB, and Nissl staining were used to characterize the lesions. Immunofluorescence was employed to characterize the grafted cells. Fluorescence images were acquired with laser scanning confocal microscopy and further analyzed with FIJI and AngioTool. The details are presented in the supplemental information.

Statistics
No statistics were used to determine strategies for randomization, sample size estimation, and inclusion and exclusion of any data. All statistical analyses were performed using GraphPad Prism 8 software. The Kolmogorov-Smirnov test was used to determine data normality. The results are shown with individual data points. For statistical analyses of EMG, two-way repeated ANOVA followed by Tukey's test was employed for both SSEP and MEP, while mixed-effects models followed by Tukey's test were utilized for NCV.

Differences were considered significant for p <0.05 (*p < 0.05, **p < 0.01, and ***p < 0.001).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.12.016.

AUTHOR CONTRIBUTIONS

W.W, H.C., D.P., and S.-C.Z. conceptualized the study. X.Z., W.W., H.C., and S.-C.Z. designed the experiments. H.C. performed cell differentiation. B.Z. and X.Z. performed SCI and transplantation. J.X., Y.H., and X.Z. performed EMG. D.L., W.Z., and X.Z. performed MRI. X.Z. performed behavioral tests, histology, and immunofluorescence and recorded and analyzed the data. F.G. and Y.D. performed animal care under the guidance of D.C., administered immunosuppressants, and assisted in behavioral tests. Z.L. performed some of the immunofluorescence analyses. X.Z. drafted and revised the paper. All of the authors read, edited, and approved the final version of the manuscript.

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CONFLICT OF INTERESTS

The authors declare no competing interests.

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