

# Recovery from Paralysis in Adult Rats Using Embryonic Stem Cells

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**Objective:** We explored the potential of embryonic stem cell–derived motor neurons to functionally replace those cells destroyed in paralyzed adult rats.

**Methods:** We administered a phosphodiesterase type 4 inhibitor and dibutyl cyclic adenosine monophosphate to overcome myelin-mediated repulsion and provided glial cell–derived neurotrophic factor within the sciatic nerve to attract transplanted embryonic stem cell–derived axons toward skeletal muscle targets.

**Results:** We found that these strategies significantly increased the success of transplanted axons extending out of the spinal cord into ventral roots. Furthermore, transplant-derived axons reached muscle, formed neuromuscular junctions, were physiologically active, and mediated partial recovery from paralysis.

**Interpretation:** We conclude that restoration of functional motor units by embryonic stem cells is possible and represents a potential therapeutic strategy for patients with paralysis. To our knowledge, this is the first report of the anatomical and functional replacement of a motor neuron circuit within the adult mammalian host.

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The ability to specifically direct the differentiation of stem cells toward a particular mature cell lineage is a critical advance in stem cell biology because it allows researchers to generate an inexhaustible supply of relatively pure committed or fully differentiated mature cell types. These cells can then be used in biological studies *in vitro* or can be applied to the study of disease.

Spinal motor neurons can be generated efficiently by exposing mouse embryonic stem (ES) cells to retinoic acid (RA) and the developmental morphogen Sonic Hedgehog (Shh) or chemical agonists of Shh.<sup>1,2</sup> In this paradigm, RA serves both to neuralize and to establish a caudal positional identity for the ES cells, whereas Shh specifies a ventral positional identity. In response, many ES cells initiate a motor neuron–specific transcriptional pattern<sup>1</sup> and acquire immunohistochemical and electrophysiological features of mature motor neurons.<sup>3</sup> ES cell–derived motor neurons transplanted into embryonic chick spinal cord extend axons into the periphery and form neuromuscular junctions (NMJs).<sup>1</sup>

This work strongly suggests that ES cells can be induced to recapitulate normal developmental pathways for efficient generation of spinal motor neurons.

Several studies have examined the potential of stem cells to halt spinal motor neuron degeneration and restore function to animals with spinal cord injury or motor neuron disease.<sup>4–7</sup> However, none of these studies shows that transplanted cells can form functional neuronal circuits in the adult mammalian nervous system, in part because of the inhibitory nature of myelin for axonal growth.<sup>8</sup> Several pharmacological strategies have been developed that overcome these axonal repulsive cues, potentially enhancing the regenerative potential of transplanted stem cells in neurodegenerative disorders.<sup>9</sup>

Provision of attractive cues within the peripheral nervous system (PNS) or skeletal muscle may also be an important strategy in encouraging formation of stem cell/host NMJs. One candidate is glial cell–derived neurotrophic factor (GDNF), a neurotrophic factor that binds to GDNF family receptor  $\alpha$ -1 (GFR $\alpha$ -

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1), which is highly expressed on motor neurons and motor axons.<sup>10,11</sup> GDNF binding to GFR $\alpha$ -1 activates several signaling pathways<sup>12,13</sup> that result in trophic and tropic support of motor neurons.<sup>14,15</sup>

We have previously transplanted approximately 12,000 motor neuron-committed ES cells into the spinal cord of adult rats that had become paralyzed after infection with the selective ventral motor neuron-depleting virus Neuroadapted Sindbis virus.<sup>2,5</sup> We found that approximately 3,000 transplant-derived motor neurons survived until 3 months after transplantation.<sup>2</sup> We showed that by infusing dibutyryl cyclic adenosine monophosphate (dbcAMP) into the subarachnoid space to inhibit myelin-mediated axon repulsion, approximately 75 transplant-derived axons per animal extended into ventral roots (approximately 2.4% of surviving motor neurons). Transplant-derived axons did not reach skeletal muscle targets, and there was no electrophysiological or functional recovery of transplanted animals.

Based on these studies, we explored strategies of effectively inhibiting myelin-mediated axon repulsion and providing attractive cues within peripheral nerves to stimulate the formation of functional motor units composed of ES cell-derived motor axons and host skeletal muscle.

## Materials and Methods

### Antibodies

Antibodies and dilutions used in this study include: neurofilament (NF) 200KD (1:100, AB1982; Chemicon, Temecula, CA); synaptophysin (Syn; 1:100, AB9272; Chemicon); rat motor neuron-specific antibody (1:100, MO-1; Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA); synatobrevin-1 (1:1,000, 104 001; Synaptic Systems, Goettingen, Germany); anti-cholera toxin antibody (1:50–100, Sigma C3062; Sigma, St. Louis, MO); rabbit anti-GDNF (1:100, sc-9010; Santa Cruz, Santa Cruz, CA); VaChT (1:1,000, AB1578; Chemicon); mouse anti-green fluorescent protein (anti-GFP; 1:100, MAB3580; Chemicon); SV2 (1:100; DSHB); rabbit anti-GFP (1:100, AB3080; Chemicon); and mouse anti-HB9 (1:50, 81.5C10/MNR2; DSHB).

For the cell fate experiments, the following antibodies were used: M2 (1:10; DSHB),  $\beta$ 3-tubulin (1:500, MMS-435P; Covance, Princeton, NJ); choline acetyltransferase (1:500, Ab5851; Chemicon); GABA (1:1,000, Sigma A2052; Sigma); glutamate (1:5,000, G6642; Sigma); glycine (1:50, Chemicon Ab5020; Chemicon); Lim1 (1:500, Chemicon Ab14554; Chemicon); Lhx3 (1:4,000, Chemicon Ab14555; Chemicon); GFAP (1:50, Research Diagnostics RDI-PRO10555; Research Diagnostics, Minneapolis, MN); and Ki67 (1:25, Abcam Ab833).

The different primary antibodies were codetected by immunofluorescence, using goat anti-rabbit and anti-mouse IgG coupled to Alexa Fluor-488 (green) and Alexa Fluor-594 (red) (1:100, Molecular Probes).

### Reagents

Nissl red stain (1:100, N-21482; Molecular Probes, Eugene, OR), Nissl blue (1:100, N-21479; Molecular Probes) and tetramethylrhodamine  $\alpha$ -bungarotoxin (1:50, T-1175; Molecular Probes) were used following instructions on package insert. HhAg1.3 (Curis, Cambridge, MA) was made up as a 10mM stock in dimethylsulfoxide and was used at 1 $\mu$ M. All-*trans* retinoic acid (Sigma R-2625; Sigma) was made up as a 1mM stock in dimethylsulfoxide and used at 1 $\mu$ M. dbcAMP was obtained from Calbiochem (catalog #28745; San Diego, CA) and was used at 1 $\mu$ M. Rolipram was purchased from A.G. Scientific (R-1012-50MG; San Diego, CA) and was dissolved in 10% dimethylsulfoxide.

### Animal Care

All animals were cared for and procedures performed in accordance with the Johns Hopkins Animal Care and Use Committee guidelines. Five- to 7-week-old Lewis rats (Charles River, Wilmington, MA) were used. Paralysis was induced using Neuroadapted Sindbis virus as described previously.<sup>2,5</sup> Cyclosporine (CsA; Calbiochem) was given at 15mg/kg mixed in the food beginning on the day before surgery/transplantation, then every day after surgery.

### Microscopy

Immunohistochemical studies were conducted by two-color confocal imaging with a Zeiss LSM510 microscope (Zeiss, Oberkochen, Germany). Images were acquired in both red and green emission channels by using an argon-krypton laser with single-channel, line-switching mode.

### Transplantation of Embryonic Stem Cells

Rolipram was administered at 0.5mg/kg/day subcutaneously beginning 2 days before ES cell transplantation and continuing for 30 days after transplantation. HB9-GFP ES cells were differentiated as described previously.<sup>1,2</sup> For differentiation of ES cells using RA alone, we used the 4–/4+ strategy described previously.<sup>16</sup> On the day of transplantation, 10ng/ml BDNF (Brain-Derived Neurotrophic Factor), 10ng/ml NT3 (Neurotrophic Factor 3), and 25ng/ml CNTF (Ciliary Neurotrophic Factor) (R&D Systems, Minneapolis, MN) were added to the culture medium. Embryoid bodies were then disaggregated with collagenase and dispase and resuspended in serum-free media. In all groups except Group 6, the cell suspension was supplemented with dbcAMP at 1mM. Transplantation was performed at 28 days after viral inoculation, as described previously.<sup>2</sup>

### C17.2-Glial Cell-Derived Neurotrophic Factor Cell Cultures and Preparation of Cells for Transplantation

Two types of C17.2 neural stem cells derived from day 8 cerebellar granule cells were used in this study: C17.2-Bleo and C17.2-GDNF.<sup>17,18</sup> All groups received 5 $\mu$ l injections of cells at 10<sup>5</sup> cells/ $\mu$ l into the sciatic nerve 4cm away from the spinal cord. Cholera toxin B (CTB) was injected in three muscles: the gluteal muscles (2.5cm from the transplantation site), quadriceps (6.5cm from the transplantation site), and the gastrocnemius complex (10.5cm from the injection site).

### *Electrophysiology Measurements In Vivo*

Motor unit number estimation (MUNE) was performed using previously described methods.<sup>19,20</sup> Animals were anesthetized with sodium pentobarbital (5mg/ml) injected intraperitoneally at a dose of 50mg/kg body weight. The abdomen and distal hind limbs were shaved, and animals were taped prone to a Styrofoam board. The stimulating electrodes were 0.7mm needles insulated with Teflon (Dantec sensory needle; Dantec, Skovlunde, Denmark). The cathode was placed close to the sciatic nerve at the proximal thigh, and the anode was placed subcutaneously 1cm proximally. Motor responses were recorded from a pregelled, self-adhesive surface recording strip (Nicolet Biomedical Inc., Madison, WI) cut to a length of 1.5cm and a width of about 0.5cm. This electrode was placed circumferentially around the animal's distal hind limb, and thus recorded activity in both flexor and extensor compartments. The reference electrode was a monopolar needle placed subcutaneously in the foot, 1.5 to 2cm distal to the recording electrode. Distance between stimulating and recording electrodes was 1.2 to 1.6cm. Both the right and left hind limbs were studied. Stimuli were 0.1-millisecond monophasic pulses of constant current delivered through a constant current stimulator (Medtronic Keypoint Electromyograph; Medtronic, Minneapolis, MN). Recordings were made through the same instrument. Filter settings were 300 and 3,000Hz. A maximum motor response from distal hind-limb muscles was recorded, representing the contributions of all viable motor units. Individual motor units were stimulated with submaximal stimuli slowly increased from subthreshold levels, to determine discrete response increments representing single motor units. The individual values were averaged to yield an estimate of average single motor unit action potential amplitude. This value was divided into the peak-to-peak amplitude of the maximum compound motor action potential to yield the MUNE.

### *Cell Count Estimations*

To estimate surviving mouse cells in the spinal cord and sciatic nerve, we generated 50 $\mu$ M tissue sections and conducted immunohistochemistry on every 10th (spinal cord) or 30th (sciatic nerve) section. We counted the total number of mouse-specific or neural subtype cells in which both the cytoplasm and nucleus could be identified using a Hoechst counterstain in all the sections. We then multiplied the counted number by 10 (spinal cord) or 30 (sciatic nerve) and subtracted 10% as a correction for the small number of cells that would be doubly counted to arrive at an estimate for cell survival.

### *Behavioral Assessment*

Functional recovery was assessed by evaluators blinded to treatment groups. Hind-limb grip strength was measured as described previously.<sup>5</sup> For the cohort given unilateral C17.2-GDNF cells, we defined an animal having recovered when it had proximal and distal movement of the leg. Two evaluators performed the analysis on live or videotaped animals in a blinded way. Scores were tabulated as the number of animals that had both proximal and distal limb movement ipsilateral and contralateral to the transplantation of C17.2-GDNF cells.

### *Statistical Analysis*

Reported values are means  $\pm$  standard error of the mean. Due to the nonparametric nature of the data, nonparametric equivalent tests of analysis of variance and repeated-measures analysis of variance were used to increase the robustness of the results. The Kruskal–Wallis test was performed to analyze differences between groups at each time point, and Friedman's nonparametric repeated-measures comparison was used to analyze differences across time within a group. Wilcoxon signed rank test was used for the comparison of two related samples. Significance was assessed at the 0.05 level.

## **Results**

### *New Strategies to Form Neuromuscular Junctions between Transplanted Embryonic Stem Cells and Host Muscle in the Adult Rat*

As before, we treated mouse ES cells with RA and a chemical agonist of Shh, HhAg1.3 (Curis), to induce differentiation of ES cells into motor neurons.<sup>1,2</sup> These ES cells are derived from a transgenic mouse that expresses GFP specifically in motor neurons, driven by the motor neuron-specific HB9 promoter. We disaggregated the differentiating cells 3.5 days after initiating the differentiation protocol and resuspended them in medium for transplantation. A total of 60,000 cells were transplanted into the ventral gray matter of the lumbar spinal cord of 5- to 7-week-old rats that had become paralyzed after Neuroadapted Sindbis virus infection. Approximately 12,000 transplanted cells were expressing GFP at this time and, therefore, were early motor neurons. We specifically tested three additional strategies in this study. First, in some animals, we resuspended the ES cells in a solution containing 1 $\mu$ M dbcAMP before transplantation to potentially increase their survival and ability to extend axons. Second, in some animals, we administered subcutaneously the phosphodiesterase type 4 inhibitor, rolipram, to potentially neutralize the inhibitory effects of myelin on axonal outgrowth. Third, in some animals, we delivered a motor axon tropic factor, GDNF, secreted by transplanted cells in the sciatic nerve, to potentially attract transplanted axons toward distal targets. We, therefore, defined eight groups of animals that would be treated singly or in combination (Table 1) and followed them after transplantation for immunohistochemical evidence of innervation of host skeletal muscle, electrophysiological evidence of functioning motor units, and functional recovery from hind-limb paralysis. Only Group 3 received the entire cocktail of intraspinal dbcAMP, subcutaneous rolipram, C17.2-GDNF cells into the sciatic nerves, and CsA to inhibit rejection of transplanted cells. Group 4 was the same except that animals were treated with C17.2-Bleo cells into the sciatic nerves instead of C17.2-GDNF. These cells express lower amounts of GDNF (1ng/10<sup>6</sup> cells/day vs

Table 1. Protocol for Transplantation of Embryonic Stem Cell-Derived Motor Neurons

Group No.	Animals at Beginning of Study, N	Cell Treatment		Pharmacological Rx		
		1	2	1	2	3
1	15	ES	C17.2-Bleo	dbcAMP	CsA	
2	15	ES	C17.2-GDNF	dbcAMP	CsA	
3	15	ES	C17.2-GDNF	dbcAMP	CsA	Rolipram
4	15	ES	C17.2-Bleo	dbcAMP	CsA	Rolipram
5	15		C17.2-GDNF	dbcAMP	CsA	Rolipram
6	15	ES	C17.2-GDNF		CsA	Rolipram
7	15	RA/ES	C17.2-GDNF	dbcAMP	CsA	Rolipram
8	15	ES	None	dbcAMP	CsA	

ES = embryonic stem; dbcAMP = dibutyryl cyclic adenosine monophosphate; (1 $\mu$ M); CsA = Cyclosporine (15 mg/kg/day); SQ = subcutaneously; GDNF = glial cell-derived neurotrophic factor; RA = retinoic acid; Rolipram (0.5 mg/kg/day SQ).

100ng/10<sup>6</sup> cells/day<sup>17,21</sup>), allowing us to determine the importance of high GDNF in peripheral nerves. In Group 2, we omitted only rolipram, and in Group 6, we omitted only dbcAMP, allowing us to determine the importance of these interventions in mediating axonal outgrowth. In Group 5, we omitted only the ES cell transplantation into the spinal cord, allowing us to define whether the other treatments (GDNF, dbcAMP, CsA, and rolipram) induced behavioral recovery independent of ES cell-derived motor neurons. In Group 7, we transplanted ES cells that were differentiated to neural cells using RA without an agonist of Shh so that these cells formed mature neurons but not motor neurons. Animals in this group also received the other potential modifiers of reinnervation, allowing us to determine the importance of motor neurons in the transplanted cells.

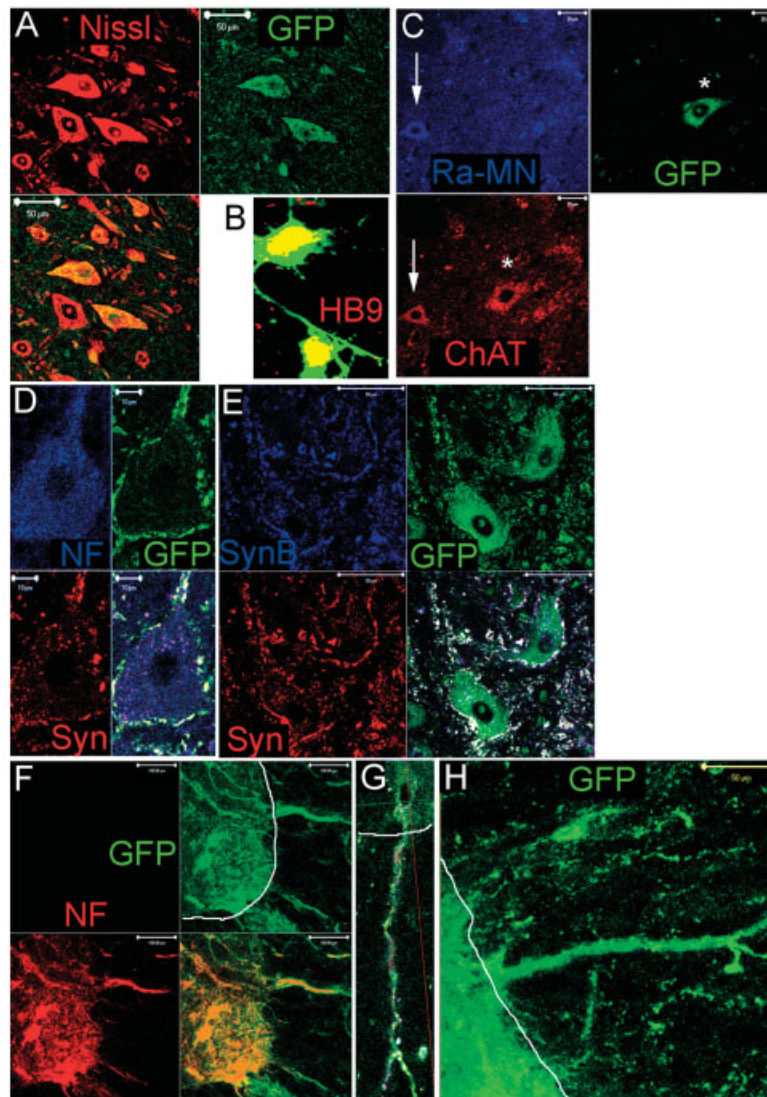
#### *Survival of Transplanted Embryonic Stem Cell-Derived Motor Neurons after Transplantation*

We first examined the survival and integration of transplanted motor neurons at 3 and 6 months after transplantation. At both time points, we saw surviving transplant-derived motor neurons (GFP<sup>+</sup>) within the gray matter of the spinal cord (Fig 1A). These transplant-derived motor neurons looked morphologically similar to host motor neurons and persistently expressed GFP, which served to distinguish them from remaining host motor neurons. Furthermore, transplant-derived (and not host-derived) motor neurons expressed HB9 (see Fig 1B) at 3 months after transplantation. To determine whether cellular fusion of transplant-derived cells with host motor neurons occurred, we looked for the presence of rat-specific immunoreactivity within the mouse-derived GFP<sup>+</sup> cells. A monoclonal antibody specific for rat motor neurons (MO-1)<sup>22,23</sup> failed to identify immunoreactivity on GFP<sup>+</sup> cells (see Fig 1C), strongly suggesting that cell fusion had not occurred. To determine whether surviv-

ing transplant-derived motor neurons integrate within a neural circuit, we defined the presence of axon-soma interactions between GFP<sup>+</sup> and GFP<sup>-</sup> cells (ie, non-motor neuron transplant-derived or host cells; see Figs 1D, E). We identified the presence of GFP<sup>+</sup> nerve terminals that also express the presynaptic marker synaptophysin (Syn<sup>+</sup>) (see Fig 1D) on GFP<sup>-</sup> neurons (NF<sup>+</sup>), confirming that transplant-derived collaterals formed synapses with other neurons in the spinal cord. Invariably, these other neurons were choline acetyltransferase-negative (data not shown), suggesting that the targets of these transplant-derived collaterals were not motor neurons and rather were sensory or interneurons. Similarly, we identified synaptic input from GFP<sup>-</sup> cells (Syn<sup>+</sup> and synaptobrevin<sup>+</sup>) onto transplant-derived motor neurons (see Fig 1E), confirming that these cells received afferent input from other neurons. As reported previously,<sup>2</sup> we found that few transplant-derived axons extended into the spinal white matter of animals not treated with inhibitors of myelin (data not shown). By contrast, in animals transplanted with ES cell-derived motor neurons and with both dbcAMP and rolipram (Groups 3–5), we saw many axons extending into surrounding white matter (see Figs 1F–H).

To quantify and define the fate and survival of transplanted ES cells within the host spinal cord, we conducted unbiased sampling of the lumbar spinal cord in three animals in each of Groups 3, 4, 6, and 7. We identified transplant-derived cells using a mouse-specific monoclonal antibody (M2) and used neural-specific polyclonal antibodies to determine neural fates within the grafted area. We found that there was no significant difference in the total number of surviving mouse cells at 3 months after transplantation in any of the groups: 11,742  $\pm$  640 in Group 3; 10,230  $\pm$  730 in Group 4; 11,975  $\pm$  780 in Group 6; and 10,654  $\pm$  541 in Group 7. Similarly, the percentage of M2<sup>+</sup> cells that coexpressed GFAP (astrocyte), CNPase (oligoden-





*Fig 1. Survival of embryonic stem (ES) cell-derived motor neurons in the spinal cord of paralyzed adult rats. (A) Three months after transplantation, rats were killed and spinal cords were isolated for immunohistochemical analysis. Lumbar spinal cord sections were probed with a green fluorescent protein (GFP) antibody to define transplant-derived motor neurons and counterstained with Nissl red to identify all motor neurons within the ventral gray matter. (B) Immunohistochemical analysis of transplanted animals at 3 months after transplantation was performed to identify whether transplant-derived motor neurons continued to express HB9 and GFP under control of the HB9 promoter. (C) The possibility that cellular fusion had occurred and accounted for the immunoreactivity was examined using a rat-specific motor neuron marker (MO-1). Asterisk denotes a transplant-derived motor neuron, whereas the arrow denotes a host motor neuron. (D) Transplant-derived axonal projections were synaptophysin-positive ( $Syn^+$ ) and terminated on GFP $^-$  neurons that were neurofilament-positive ( $NF^+$ ), but choline acetyltransferase-negative ( $ChAT^-$ ) (data not shown). (E) GFP $^-$  axonal projections that were both  $Syn^+$  and synaptobrevin-positive ( $SynB^+$ ) also terminated on transplant-derived GFP $^+$  motor neurons. (F–H) In the presence of inhibitors of myelin-mediated axonal repulsion, transplant-derived axons that were  $NF^+$  (F) exited the spinal gray matter into surrounding white matter.*

drocyte),  $\beta 3$ -tubulin (neuron), or no neural marker did not differ among groups (Table 2). We next defined subsets of neurons including motor neurons, GABA $^+$  neurons, glutamate $^+$  neurons, glycine $^+$  neurons, Lim1 $^+$  V0 interneurons, and Lhx3 $^+$  V2 interneurons. As expected, there was a small number of motor neurons in Group 7 animals (ES cells differentiated with

RA only), and the percentage of motor neurons in the other groups ranged from 35 to 39%. This corresponds to approximately  $4,110 \pm 450$  ( $\pm$  standard error of the mean) surviving motor neurons per animal in Group 3,  $2,553 \pm 720$  in Group 4,  $4,430 \pm 640$  in Group 6, and  $213 \pm 32$  in Group 7. Other neuron populations could also be identified in each group and

did not differ among groups with the exception that nonmotor neuron populations were more frequently identified in Group 7. The 3-month survival number is slightly higher than we reported previously,<sup>2</sup> perhaps due to more efficient differentiation of ES cells before transplantation or to enhanced survival of transplanted cells. At 6 months after transplantation,  $2,621 \pm 321$  surviving M2<sup>+</sup> motor neurons were identified in Group 3 animals (n = 3) and  $2,430 \pm 219$  in Group 6 animals (n = 3). We saw no M2<sup>+</sup>/Ki67<sup>+</sup> cells at either 3 or 6 months (n = 6), suggesting that these cells were not undergoing cell division at those times.

*Cotransplantation of Glial Cell-Derived Neurotrophic Factor-Secreting Cells into the Sciatic Nerve to Attract Embryonic Stem Cell-Derived Axons*

To further facilitate extension of ES cell-derived axons into the PNS, we cotransplanted GDNF-expressing cells into the PNS at the time of spinal transplantation. This strategy was warranted even though peripheral axon extension and muscle reinnervation can occur after acute injuries without exogenous support because our previous studies have shown that ES cell-derived axons that reach ventral roots of transplanted animals fail to innervate targets.<sup>2</sup> We reasoned that transplant-derived axons may respond to tropic cues, resulting in distal growth and innervation of skeletal muscle, and we chose GDNF as a candidate tropic molecule both because it is a potent stimulator of motor axonal growth<sup>24-26</sup> and because microarray data suggest that ES cell-derived motor neurons express high levels of the GDNF receptor, GFR $\alpha$ 1 (data not shown). We transplanted C17.2-Bleo (1ng GDNF/10<sup>6</sup> cells/day in vitro), as a control cell line, or C17.2-GDNF (100ng GDNF/10<sup>6</sup> cells/day in vitro) cells into one or both sciatic nerves of paralyzed animals that had been cotransplanted with ES cell-derived motor neurons in the spinal cord. At 6 months after transplantation, we

detected strong GDNF immunoreactivity only in the sciatic nerves of animals transplanted with C17.2-GDNF cell (Fig 2A) and not in the sciatic nerves of animals treated with C17.2-Bleo (see Fig 2B). Using dual-color confocal microscopy and antibodies to GDNF and the mouse-specific M2 antibody, we confirmed that GDNF within the sciatic nerve originated from transplanted cells (see Fig 2C). To define the survival and migration of C17.2 cells within the sciatic nerve, we conducted immunohistochemistry using the M2 antibody along the length of the sciatic nerve (see Fig 2D). We analyzed every 30th section of sciatic nerve from the spinal cord to below the knee (8.5cm from the spinal cord) in a subset of animals (n = 3 for each C17.2-Bleo and C17.2-GDNF). We found C17.2-Bleo or C17.2-GDNF cells in all sections of the sciatic nerve, though the highest density of cells was around the site of transplantation into the sciatic nerve (arrow denotes site of injection; see Fig 2E). There was no difference in the distribution of C17.2 cells between groups, and the estimated total number of surviving C17.2 cells in each group was similar ( $40,185 \pm 4,235$  in the C17.2-GDNF group;  $43,209 \pm 2,341$  in the C17.2-Bleo group).

We next examined whether any of the experimental modifications enhanced the outgrowth of transplant-derived axons as defined by the presence of GFP<sup>+</sup> axons within ventral roots (see Fig 2F). We identified all the ventral roots within 2 levels of the injection site (10 ventral roots, generated from 5 spinal levels; n = 5). We generated longitudinal frozen sections and counted the number of GFP<sup>+</sup> axons colocalizing with NF by immunohistochemistry. We found that animals in Group 3 exhibited more transplant-derived axons reaching the PNS than any other group ( $203 \pm 15$  vs  $123 \pm 3$  for Group 4 and  $76 \pm 11$  for Group 8;  $p < 0.02$ ). Because few transplant-derived axons were found in the ventral roots of Group 6 animals (all but dbcAMP), we conclude that local dbcAMP is critical

Table 2. Fate of Transplanted Embryonic Stem Cells

Antigen	Group No. (mean % $\pm$ SEM)			
	3	4	6	7
GFAP (astrocyte)	22 $\pm$ 2	20 $\pm$ 4	19 $\pm$ 4	25 $\pm$ 5
CNPase (oligodendrocyte)	6 $\pm$ 3	8 $\pm$ 3	4 $\pm$ 1	9 $\pm$ 2
$\beta_3$ tubulin (neuron)	65 $\pm$ 13	59 $\pm$ 12	60 $\pm$ 13	51 $\pm$ 7
ChAT (motor neuron)	35 $\pm$ 7	39 $\pm$ 6	37 $\pm$ 9	2 $\pm$ 0
GABA (GABAergic neuron)	15 $\pm$ 2	12 $\pm$ 2	12 $\pm$ 3	23 $\pm$ 2
Glutamate (glutamatergic neuron)	7 $\pm$ 1	8 $\pm$ 1	5 $\pm$ 1	19 $\pm$ 6
Glycine (glycinergic neuron)	8 $\pm$ 2	2 $\pm$ 1	5 $\pm$ 1	9 $\pm$ 1
Lim1 (V0 interneuron)	10 $\pm$ 3	12 $\pm$ 3	7 $\pm$ 1	17 $\pm$ 1
Lhx3 (V2 interneuron)	13 $\pm$ 2	9 $\pm$ 1	14 $\pm$ 2	15 $\pm$ 5
Other	7 $\pm$ 3	13 $\pm$ 1	7 $\pm$ 1	15 $\pm$ 1

SEM = standard error of the mean; GFAP = glial fibrillary acidic protein; ChAT = choline acetyltransferase.

for extension of axons into the PNS, and that high levels of GDNF expression within the sciatic nerve enhance the ability of transplant-derived axons to reach the PNS. These and subsequent data regarding outcome measures are summarized for each group in Table 3.

#### Formation of Neuromuscular Junctions between Transplanted Motor Axons and Host Muscle

We examined the possibility that ES cell-derived axons reached skeletal muscle targets at three months after transplantation by conduction confocal microscopy on fixed-frozen muscle tissue from transplanted animals in each group. Exclusively in Group 3, we saw GFP<sup>+</sup> axons within quadriceps and gastrocnemius skeletal mus-

cle (Fig 3A). Transplant-derived axons often exhibited a branched morphology with apparent growth cones (see Fig 3A) and were strongly NF-positive (see Figs 3A, B). We also saw clustering of the acetylcholine receptor in apposition to advancing axons (defined by rhodamine-conjugated  $\alpha$ -bungarotoxin; see Figs 3C–E), though the morphology of these NMJs was simpler than that seen at 6 months (see later). We also saw colocalization of GFP<sup>+</sup> axons with the vesicular acetylcholine transporter, suggesting that these axons were active synaptically (see Fig 3F). To obtain further support for the observation that stem cell-derived axons reached skeletal muscle targets, we retrogradely labeled spinal motor neurons by injecting CTB into the quadriceps and gastrocnemius muscles of transplanted animals, then conducted immunohistochemistry. We identified transplant-derived motor neurons with GFP, all motor neurons with choline acetyltransferase, and retrogradely labeled motor neurons with CTB (see Figs 3G, H). As shown, we were able to retrogradely label both host (see asterisk in Fig 3G) and transplant-derived motor neurons (see arrowheads in Figs 3G, H). To determine the number of transplant-derived motor neurons capable of being retrogradely labeled from skeletal muscle, we conducted unbiased counting of GFP<sup>+</sup>/CTB<sup>+</sup> neurons in the spinal cord of five animals in each of Groups 1 through 5. We selected every fifth section from the spinal cord spanning the lumbar enlargement and conducted immunohistochemistry and blinded counting. We found that in each of Groups 1, 2, 4, 5, 6, 7, and 8, there were no dual-labeled motor neurons. In Group 3, however, there were  $123 \pm 47$  dual-labeled motor neurons per animal.

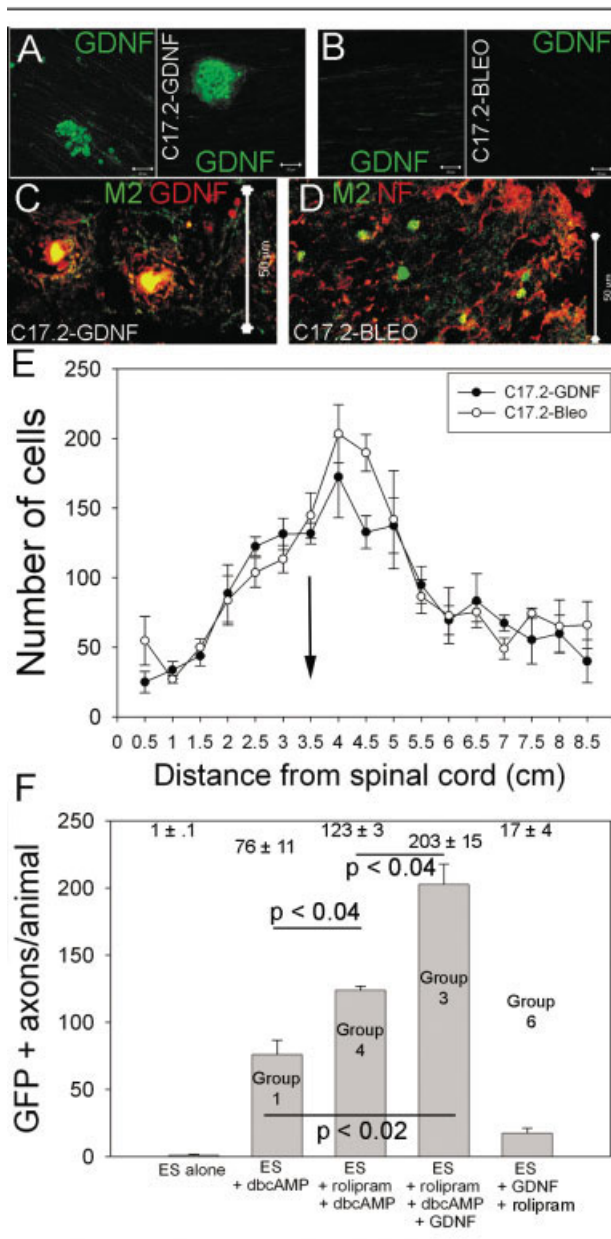


Fig 2. Glial cell-derived neurotrophic factor (GDNF)-secreting cells survive in the sciatic nerve of transplanted hosts for 6 months after transplantation. (A, B) At the time of embryonic stem (ES) cell transplantation, C17.2-GDNF or C17.2-Bleo cells were cotransplanted into the sciatic nerves of paralyzed rats. Immunohistochemical analysis at 6 months after transplantation showed clusters of GDNF-secreting cells only in animals transplanted with C17.2-GDNF cells. (C) Immunohistochemistry of sciatic nerve segments was performed using GDNF and mouse-specific (M2) antibodies confirming the source of GDNF after transplantation is the C17.2-GDNF cells. (D) C17.2-Bleo cells also could be identified using M2 immunoreactivity. (E) Distribution of C17.2-Bleo or C17.2-GDNF cells along the length of the sciatic nerve was assessed and quantitated by unbiased sampling of mouse-specific (M2) immunoreactivity. Arrow denotes the site of injection of cells into the sciatic nerve. (F) Ventral roots were harvested from transplanted animals and were subjected to confocal microscopy using neurofilament (NF) and green fluorescent protein (GFP) antibodies. The number of GFP<sup>+</sup> axons was scored for each animal ( $n = 5$ ), and the means and standard error of the means of each group are shown. dbcAMP = dibutyryl cyclic adenosine monophosphate.



Table 3. Summary of Outcome Data for Each Group

Group No.	Transplant Paradigm	Transplant-Derived Axons in PNS	Behavioral Recovery	Anatomic Reinnervations	Physiological Improvement
1	No Rolipram Low GDNF	±	N	N	N/A
2	No Rolipram	±	N	N	N/A
3	Complete paradigm	Y	Y	Y	Y
4	Low GDNF	Y	N	N	N
5	No ES Cells	N	N	N	N
6	No dbcAMP	±	N	N	N/A
7	Nonmotor neuron ES cells	N	N	N	N/A
8	No GDNF No Rolipram	±	N	N	N/A

PNS = peripheral nervous system; GDNF = glial cell–derived neurotrophic factor; ES = embryonic stem; N/A = not available.

Therefore, in Group 3, we have generated approximately 4,100 new motor neurons in the spinal cord, 200 new motor axons in the ventral roots, and 120 retrogradely labeled new motor neurons from skeletal muscle.

At 6 months after transplantation, we repeated and extended these studies using both proximal and distal limb muscles. Within the gastrocnemius muscle of transplanted animals (Group 3), we saw morphologically mature NMJs in close apposition to GFP<sup>+</sup> axons, suggesting that these axons were able to induce the organization of postsynaptic NMJ machinery (Fig 4A).

In a collapsed Z-stack image, GFP<sup>+</sup> axons can be seen in close apposition to clustered acetylcholine receptors, and single-layer, confocal, microscopic images confirm with orthogonal views that they form a single NMJ composed of transplant-derived axons and host-derived muscle (see Fig 4B). Furthermore, we found that transplant-derived axons within skeletal muscle were immunoreactive to synaptic vesicle proteins synaptobrevin (see Fig 4C), synaptophysin (see Figs 4D, E), and SV2 (see Fig 4E), suggesting that they had appropriately developed presynaptic vesicle machinery.

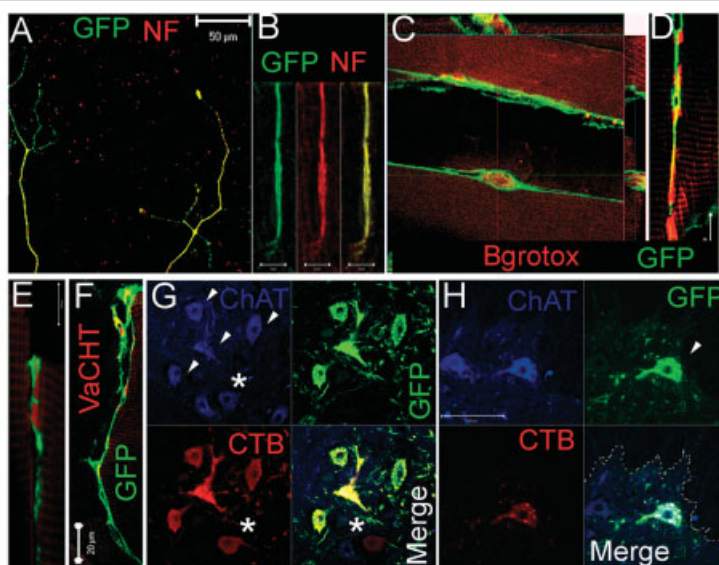


Fig 3. Embryonic stem (ES) cell–derived motor axons reach skeletal muscle targets at 3 months after transplantation. (A, B) Only in Group 3 animals were neurofilament–positive (NF<sup>+</sup>) and green fluorescent protein–positive (GFP<sup>+</sup>) axons identified within skeletal muscle, shown here in the gastrocnemius muscle. (C–E) GFP<sup>+</sup> axons reaching skeletal muscle often showed clustering of acetylcholine receptors as defined by staining of skeletal muscle with rhodamine–conjugated bungarotoxin (Bgrtox). (F) GFP<sup>+</sup> axons were examined for the presence of the vesicular acetylcholine transporter (VaCHT) as a marker of synaptic maturity. (G, H) Animals were injected with cholera toxin B (CTB) into the quadriceps and gastrocnemius muscles. Two days later, animals were killed and spinal cords were investigated for colocalization of GFP, choline acetyltransferase (ChAT), and the retrogradely transported CTB. Arrowheads denote transplant–derived motor neurons that were retrogradely labeled; asterisks denote a host motor neuron that was retrogradely labeled with CTB. (I) Dashed line denotes the gray/white matter junction.



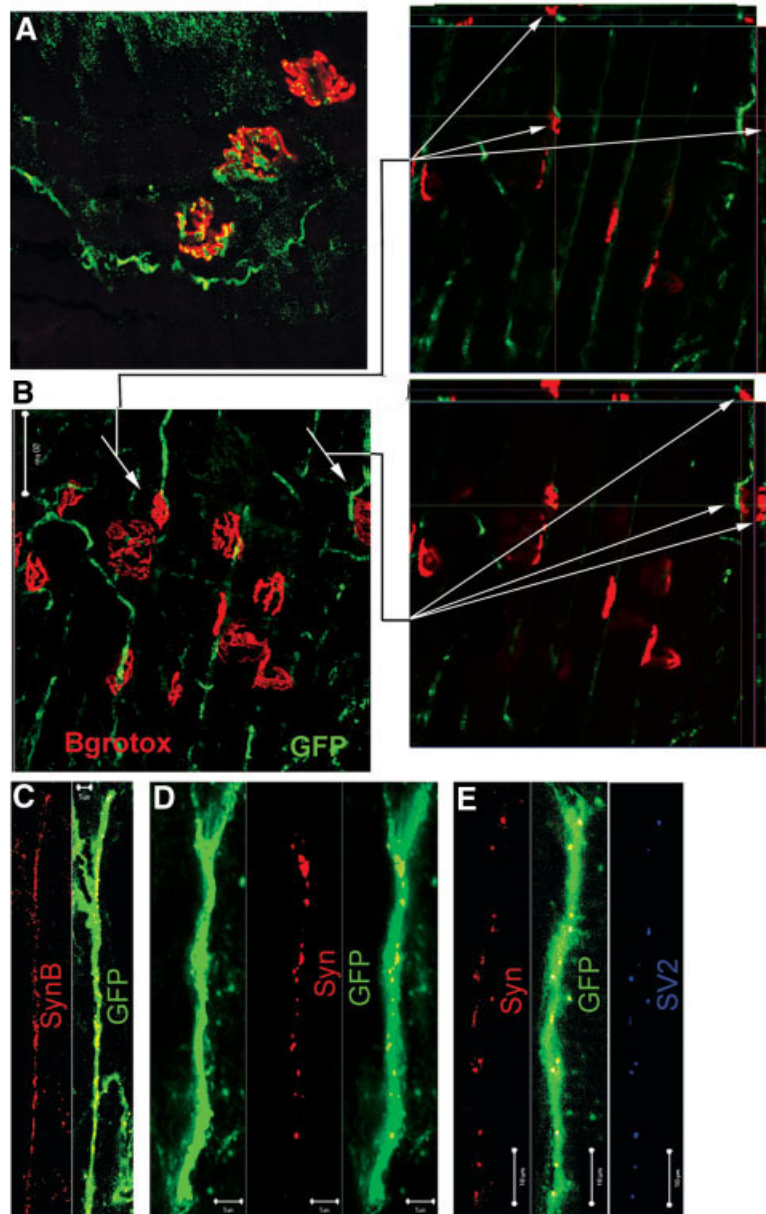


Fig 4. Formation of neuromuscular junctions (NMJs) composed of transplant-derived axons and host skeletal muscle at 6 months after transplantation. Proximal (A; quadriceps) and distal (B; gastrocnemius) skeletal muscle were examined for the presence of NMJs composed of transplant-derived axons and host skeletal muscle. (B, left) Collapsed Z-stack montage. (B, right) Single 1 $\mu$ M confocal images with orthogonal representations (y- and z-planes, arrows) confirming the apposition of transplant-derived motor axons and host-derived postsynaptic specializations. (C–E) Skeletal muscle was harvested from limb skeletal muscle (gastrocnemius) at 6 months after transplantation. GFP<sup>+</sup> axons colocalized with synaptobrevin (SynB). (D) GFP<sup>+</sup> axons colocalized with synaptophysin (Syn) (E) GFP<sup>+</sup> axons colocalized with Syn and SV2.

#### Electrophysiological Analyses of Transplanted Rats Show Increased Motor Unit Nerve Numbers in Hind Limbs

To determine whether these hybrid NMJs are functionally active, we conducted electrophysiological analyses of live animals, examining in a blinded way whether the number of functioning motor units changed over time after transplantation. We generated

data regarding MUNE (a measure of the number of functioning motor units) and single motor unit action potential (a measure of the amplitude of individual motor units) using a preselected cohort of five to six transplanted animals in each of Groups 3 to 5. Each animal was studied in a blinded manner at 12 days after transplantation and again at 120 days after transplantation. Mean MUNE counts at baseline were sim-

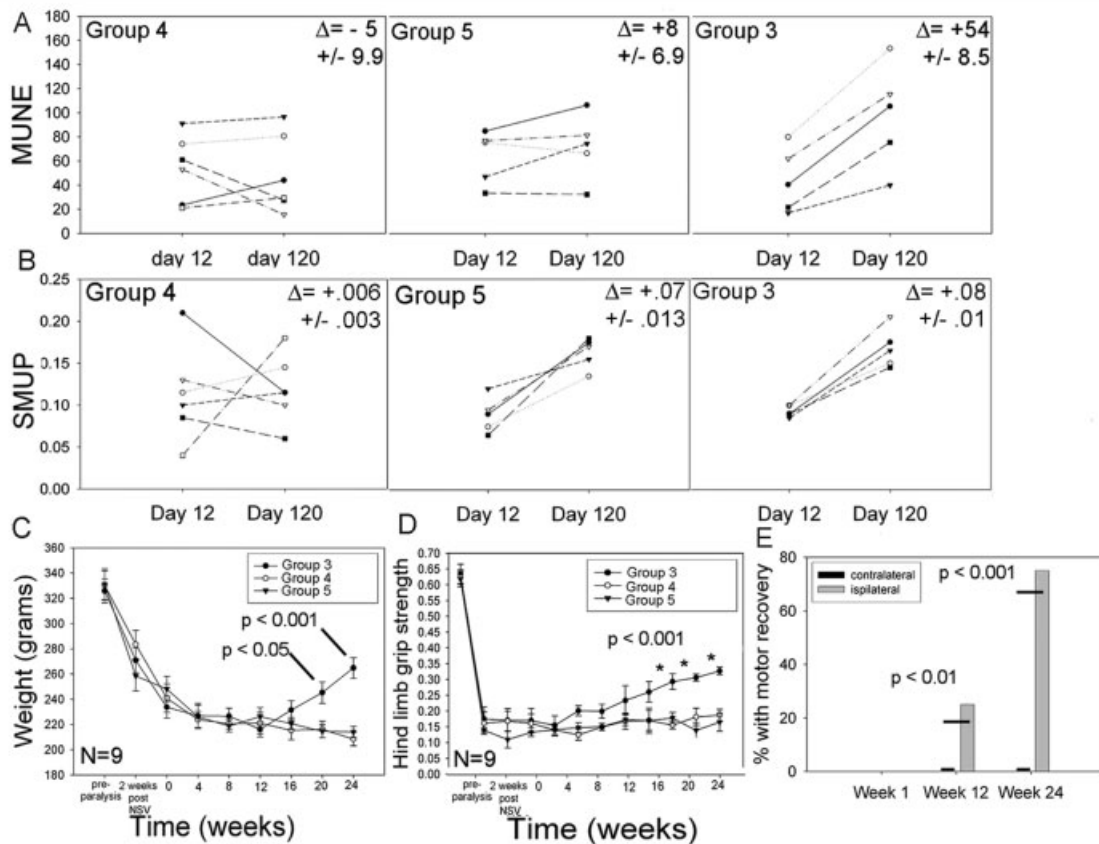


Fig 5. Electrophysiological and behavioral analysis of transplanted animals at 6 months after transplantation shows electrophysiological and functional recovery. (A) Motor unit number estimation (MUNE) of animals in Groups 4 (left), 5 (middle), and 3 (right) at 6 months after transplantation. Five to six animals in each group were examined electrophysiologically at 12 days and again at 120 days after transplantation. Each line represents the change of a single animal over that time period and the  $\Delta$  score for each group is presented at the top right of each graph. (B) Single motor unit action potential (SMUP) was defined for the same animals in the same groups. (C) Animals in Groups 3 to 5 were weighed weekly after transplantation, and the group means were plotted for each group. (D) Nine animals each from Groups 3 to 5 were scored blindly for functional recovery, defined by improvement in hind-limb grip strength. (E) A cohort of animals was transplanted with embryonic stem (ES) cell-derived motor neurons in the spinal cord bilaterally and C17.2-GDNF cells unilaterally. Animals were followed for 24 weeks and were assessed blindly for functional recovery as defined by the ability to flex the proximal leg under the animal and to push off with the foot.

ilar between the groups (not statistically significant). Animals in Groups 4 (Fig 5A, left panel;  $\Delta = -5 \pm 9.9$ ) and 5 (see Fig 5A, middle panel;  $\Delta = +8 \pm 6.9$ ) had no significant change in MUNE. However, in Group 3, we saw a significant increase in the MUNE at 120 days after transplantation compared with 12 days (see Fig 5A, right panel;  $\Delta = +54 \pm 8.5$ ;  $p < 0.04$  for intragroup change;  $p < 0.01$  for intergroup difference at 120 days). The simplest interpretation of these findings is that ES cell-derived motor neurons have the capability to establish electrically active motor units when transplanted with dbcAMP, systemically administered rolipram, and GDNF in the PNS.

Single motor unit action potential increases were observed in Groups 3 and 5 between days 12 and 120 ( $p < 0.05$ ), suggesting that GDNF transplantation results in enhanced axonal sprouting of motor axons (see

Fig 5B), and that this increase is independent of ES cell transplantation. However, it is clear that axonal sprouting mediated by GDNF was not sufficient to mediate functional recovery in the absence of transplanted ES cell-derived motor neurons (see later).

#### Functional Recovery of Transplanted Animals

We followed a cohort of animals for functional recovery by blindly assessing weight (see Fig 5C) and hind-limb grip strength (see Fig 5D) for up to 6 months after transplantation. The code of animal grouping was broken at 6 months after transplantation, and there was a significant improvement in animal weights only in Group 3 beginning at 20 weeks after translation ( $p < 0.05$  at 20 weeks;  $p < 0.001$  at 24 weeks). This suggested that these animals had become more mobile

in the cage and were better able to obtain food than their littermates in other groups. Indeed, hind-limb grip strength improved only in Group 3 with statistical distinction from other groups achieved at 18, 22, and 24 weeks ( $p < 0.001$ ). Two representative videos of a single Group 3 rat at the time of transplantation and 24 weeks later are provided in the supplementary material (see Supplementary Videos 1 and 2).

Two distinct possibilities exist to explain the effects of GDNF in this regard. Perhaps the GDNF is secreted systemically, creating a general permissive environment for axonal growth. Alternatively, the GDNF may act as a focal attractive source attracting axons distally. To distinguish between these possibilities, we transplanted C17.2-GDNF cells unilaterally into the sciatic nerve and ES cell-derived motor neurons bilaterally into the spinal cord and assessed for functional recovery (see Fig 5E) as described earlier; all animals received rolipram and CsA. We reasoned that if GDNF is critical within a focal region of the PNS to act as an attractive cue, then we should see asymmetric recovery ipsilateral to the transplanted C17-GDNF cells. If, however, GDNF secretion by transplanted C17.2 cells acts diffusely, then we would see growth both ipsilateral and contralateral to the C17.2 side. We initially attempted to use hind-limb grip strength as the readout for motor recovery as described earlier. However, preliminary studies showed that several animals exhibited asymmetric (ipsilateral) recovery, and we found that unilateral grip strength measurements were unreliable in this setting. Animals were followed for 24 weeks and were assessed blindly for functional recovery as defined by the ability to flex the proximal leg under the animal and to push off with the foot. We scored the percentage of animals that could do both at 0, 12, and 24 weeks after transplantation both ipsilateral and contralateral to the C17.2 GDNF transplantation. Raters were not aware which sciatic nerve had been transplanted with C17.2-GDNF cells. Although none of the animals regained the ability to bear weight and step contralaterally to the C17.2-GDNF cells, 25 and 75% of animals regained the ability to bear weight and step ipsilateral to the C17.2-GDNF cells at 12 and 24 weeks after transplantation ( $p < 0.001$ ). Two representative videotapes of one rat used in this analysis are provided in the supplementary material (see Supplementary Videos 3 and 4). In these videos, the transplanted rat recovers the ability to ambulate with the right hind limb (ipsilateral), and yet does not recover any hind-limb movement in the left hind limb (contralateral). We conclude from these studies that GDNF acts as a focal attractive cue for ES cell-derived motor axons, and that when coadministered with dbcAMP and rolipram, facilitates the establishment of NMJs between transplant and host, resulting in physiological and behavioral recovery.

## Discussion

We have defined a protocol that allows for the functional restoration of motor units in paralyzed adult rats using mouse ES cells. The critical features of this protocol are first, the directed differentiation of pluripotent stem cells into committed motor neuron progenitors using RA and a chemical agonist of Shh. As shown by others<sup>27,28</sup> to stimulate axon regeneration, the inhibitory effects of myelin need to be inhibited by intraspinal infusion of dbcAMP and systemic administration of a phosphodiesterase 4 inhibitor. Because it is clear that modulation of intracellular cAMP levels also alters or switches the response of neurons to axon guidance cues,<sup>29,30</sup> it is also possible that dbcAMP and rolipram treatments rendered the transplanted cells responsive to these cues. In addition, we applied a focal attractant, GDNF, within the PNS to directionally attract transplant-derived axons distally, resulting in the formation of host/transplant NMJs.

In this context, we reasoned that if transplant-derived axons could reach the PNS, then attractive cues could be used to result in the formation of transplant/host NMJs. It has been shown that GDNF acts as a powerful stimulator of motor axonal growth.<sup>14,31,32</sup> Therefore, we administered C17.2-Bleo or C17.2-GDNF cells into the PNS of paralyzed animals that had been cotransplanted with ES cell-derived motor neurons in the spinal cord. Consistent with other reports suggesting that GDNF is a critical motor axon tropic factor, only the C17.2-GDNF transplanted rats improved, strongly supporting the conclusion that GDNF mediated this recovery.

Most significantly, these strategies resulted in the formation of anatomically, physiologically, and functionally active motor units between transplanted axons and host muscle. Function of the NMJ is closely tied to the function of several proteins associated with presynaptic vesicles. Vesicular acetylcholine transporter is responsible for the packaging of acetylcholine into secretory vesicles and has been found to accumulate in the terminals of regenerating axons.<sup>33</sup> Synaptobrevin and synaptophysin reside on exocytotic vesicles and mediate fusion to the plasma membrane,<sup>34,35</sup> whereas SV2 is required to prepare vesicles for a fusion event.<sup>36</sup> Animal models involving modulation of these vesicular proteins have shown that they are necessary for junction survival.<sup>36,37</sup>

The most definitive proof of the functional reestablishment of motor units is the physiologically enhanced MUNE only in animals treated with all of the above interventions. Electrophysiological examination of nerve-muscle interaction provides a quantitative measure of reinnervation of skeletal muscle, and MUNE analysis has been found to reliably track changes in function over time.<sup>19</sup> Studies in humans, as well as in animal models of amyotrophic lateral sclerosis, illus-



trate the sensitivity of MUNE not only to early detection of abnormalities, but also to gain of function as a result of therapeutics.<sup>20</sup> MUNE studies have also suggested that the total number of functioning motor units in a human limb muscle range from 65 to 479.<sup>38</sup> It has also been reported that patients with progressive motor neuron diseases are asymptomatic until 70 to 80% of motor units are lost.<sup>39</sup> By extension, using the highest MUNE for the large muscles of the lower extremities (ie, 479), it can be inferred that muscles that have a MUNE of approximately 100 are likely to be near normal in strength. In our transplantation paradigm in rats, we generated an increase of approximately 50 in the MUNE to the distal lower extremity. Animals with this number of functioning motor units would not be expected to be normal, and indeed, the hind-limb grip strength improved to approximately 50% of the preparalysis strength.

One report has previously generated cholinergic neurons from human ES cells and has shown that these cells, transplanted into the rat spinal cord, are capable of extending axons to and occasionally forming NMJs with host muscle.<sup>4</sup> However, although this study showed the potential of reconstituting motor circuits *in vivo*, it did not show that the transplanted cells formed functional connections with host muscle, or that these connections were required for the observed improvement in gait. In this study, we defined that animals transplanted with inhibitors of myelin and GDNF within the PNS formed approximately 125 new connections with host skeletal muscle, 50 of which were active electrically in the distal hind limb. We also provide several lines of evidence that strongly suggest that this functional innervation was required for the behavioral recovery observed. First, transplanted animals who had any of the steps omitted (dbcAMP, rolipram, GDNF) had the same number of surviving, transplant-derived motor neurons, and yet had no innervation of host muscle and recovery. Second, transplantation of ES cells fully capable of differentiating into neurons, but not into motor neurons, did not result in functional recovery. These nonmotor neurons may be expected to provide as much support to the host environment, and yet this is clearly insufficient. Third, application of all the tested modalities (dbcAMP, rolipram, CsA, and GDNF) that might be expected to provide trophic and tropic support to host motor neurons did not result in functional recovery unless motor neurons were transplanted into the spinal cord. Fourth, there was a strong temporal association between the formation of first anatomic reinnervation at 3 months, functional innervation at 4 months, and behavioral recovery beginning at 4 months. By contrast, in our previous studies in which stem cells were used to provide tropic and trophic support to host motor neurons in this same model of paralysis, recovery was much more

rapid, being nearly complete by 3 months after transplantation.<sup>5</sup>

We conclude that in adult paralyzed rats, functional restoration of motor units with ES cell-derived motor neurons is possible, and ES cells represent a potential therapeutic intervention for humans with paralysis.

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