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Evaluation of Developmental Toxicity of Ultraviolet Absorber 2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-Chlorobenzotriazole in Rats

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2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as a UV absorber. In this study, the developmental toxicity of DBHCB was evaluated in rats. Pregnant rats were given DBHCB at 0, 62.5, 250, or 1000 mg kg^{-1} day^{-1} by gavage on days 5–19 of pregnancy. No deaths were observed in the pregnant rats of any group. No effect of DBHCB on the general conditions, body weight gain, or feed consumption was observed in the pregnant rats. There were no changes in the ovarian weight, gravid uterine weight, or necropsy findings in the maternal rats of the DBHCB-treated groups. No significant effects of DBHCB were found in the number of corpora lutea, implantations, live fetuses, resorptions or dead fetuses, incidence of pre- or postimplantation embryonic loss, viability of fetuses, fetal weight, or sex ratio of live fetuses. No significant difference in the incidence of fetuses with malformations or variations or degree of ossification was detected between the DBHCB-treated and control groups.

Keywords Benzotriazole, Developmental toxicity, Rat, UV absorber.

INTRODUCTION

2-(3',5'-Di-*tert*-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole (CAS no. 3864 99–1; DBHCB) is slightly yellowish powder, stable under ordinary conditions, and insoluble in water. Its melting point is 154–158°C, and its specific gravity

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is 1.26. This chemical provides effective light stabilization and prevents the yellowing and degradation of polymers such as polypropylene, high-density polyethylene, unsaturated polyester, styrene-based thermoplastics elastomer, polyamide and impact polystyrene and is used as a UV absorber (Chemical Land21, 2005). The finished polymers—which contain UV absorbers at levels not to exceed 0.5% by weight of polyethylene phthalate polymers, complying with 21 CFR 177.1630 (FDA, 2005a)—may be used in contact with some food types and used under certain conditions as described in 21 CFR 176.170 (FDA, 2000; 2005b). UV absorbers are used in food packages as plastic additives, their function being mainly to prevent polymer degradation and/or a change in the quality of the packed food due to UV rays.

It has caused some anxiety that humans have been exposed to these chemicals in occupational surroundings, from environmental contamination and from contamination in food migrated from packages. The possibility of these chemicals entering the biological system has aroused great concern about their toxic potential. Important information can be gained by studying the biological effects produced by environmental chemicals in laboratory animals, in order to investigate their possible influences on human health.

Recently, DBHCB was assessed for its estrogenic activity, using a recombinant yeast assay (Miller et al., 2001) and the yeast two-hybrid assay (Kawamura et al., 2003); it was reported that DBHCB was not estrogenic. Some information on toxicity is available (Everlight Chemical Industrial Corporation, 2002). The oral LD_{50} for DBHCB was greater than 5000 mg/kg in rats. DBHCB caused minimal irritation to the skin and slight irritation to the eyes in rabbits. A 90-day feeding study of DBHCB in rats, at 22–800 mg/kg, resulted in dose-dependent increases in liver weights and signs of liver toxicity. No effects were found at 3.7 mg/kg. However, no detailed information is available for the toxicity studies.

Although testing for reproductive and developmental toxicity has become an important part of the overall toxicology profile for chemicals, no information has yet been presented on the reproductive and developmental toxicity of DBHCB. Therefore, the current study was conducted to evaluate the developmental toxicity of DBHCB given orally to rats during pregnancy.

MATERIALS AND METHODS

This study was performed in compliance with the OECD Guideline 414 Prenatal Developmental Toxicity Study (OECD, 2001) in 2004 at the Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan).

Animals

International Genetic Standard [Crj: CD (SD) IGS] rats were used throughout this study. This strain was chosen because it is most commonly

used in reproductive and developmental toxicity studies, and historical control data are available. Males at 11 weeks of age and females at 10 weeks of age were purchased from Hino Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for 1 week prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared with a basal diet (CE-2; Clea Co., Ltd., Tokyo, Japan), water was provided *ad libitum*, and the animals were maintained in an air-conditioned room at 21.6–22.2°C, with a relative humidity of 45–58%, a 12-h light/dark cycle, and ventilation with 15 air changes/hour. Virgin female rats were mated overnight with male rats. The day when the sperm and/or vaginal plug was found to be day 0 of pregnancy. The copulated females, weighing 245–314 g, 11 weeks old, were distributed on a random basis into 4 groups of 20 rats each and housed individually. This experiment was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

Chemicals and Dosing

DBHCB was obtained from Musashino Geigy Co., Ltd. (Kitaibaraki, Japan). The DBHCB (lot no. 05004IX3) used in this study was 99.9% pure based on HPLC analysis, and it was kept in a dark place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before the study. Rats were treated once daily by gastric intubation with DBHCB at a dosage of 0 (control), 62.5, 250, or 1000 mg/kg on day 5 through day 19 of pregnancy. The dosage levels were determined based on the results of our dose-finding study in which a significantly increased liver weight was caused in males at 250 mg kg^{-1} day^{-1} and higher, but not in females even at 1000 mg kg^{-1} day^{-1}, after administration of DBHCB for 14 days in rats. DBHCB was suspended in 5% gum arabic solution. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body weight. The control rats were given only 5% gum arabic solution. The stability of the formulations in a dark and cool place under airtight conditions had been confirmed for up to 14 days. During use, the formulations were maintained under such conditions for no more than 7 days and were 97.3% to 100.1% of the target concentration.

Observations

All females were observed daily during the preadministration period and twice a day (before administration and 1 to 2 h after administration) during the administration period for clinical signs of toxicity. Maternal body weight was recorded on days 0, 5, 8, 11, 14, 17, 19, and 20 of pregnancy. Feed consumption was recorded on days 0–1, 5–6, 8–9, 11–12, 14–15, 17–18, and 19–20

of pregnancy. The pregnant rats were euthanized by exsanguination under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the uterus and ovaries were removed from the maternal body and weighed. The numbers of corpora lutea, implantation sites, and live and dead fetuses and resorptions were counted. The live fetuses were removed from the uterus and sexed, weighed, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected, fixed in alcohol, stained with alizarin red S (Dawson, 1926), and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to free-hand razor-blade sectioning (Wilson, 1973), and the thoracic areas were subjected to microdissecting (Nishimura, 1974) to reveal internal abnormalities.

Data Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. The initial body weight, body weight gain, and feed consumption of the pregnant rats, numbers of corpora lutea, implantations and live fetuses per litter, and fetal weight were analyzed with Bartlett's test (Snedecor and Cochran, 1974) for homogeneity of variance at the 5% level of significance. When the variance was homogeneous, Dunnett's test (Dunnett, 1996) was performed to compare the mean value in the control group with that in each DBHCB group. When the variance was heterogeneous, a Dunnett-type test (Miller, 1987) was performed to compare the mean value in the control group with that in each DBHCB group after rank conversion. The Dunnett-type test was used for the incidences of pre- and postimplantation embryonic loss and fetal anomalies and sex ratio of fetuses to compare the mean rank of groups treated with DBHCB and that of the control group. The incidence of dams with anomalous fetuses was analyzed with Fisher's exact test.

RESULTS

Table 1 shows the maternal findings in rats given DBHCB on days 5–19 of pregnancy. No deaths or clinical signs of toxicity were found in female rats of any group. There was no difference in the fertility rate between the control and DBHCB-treated groups. No effects of DBHCB on body weight gains on days 0–5, 5–14, 14–19, and 19–20 of pregnancy were observed. During the whole period of pregnancy, no effects of DBHCB were also detected in body weight gain. There was no difference in feed consumption during pregnancy between the control and DBHCB-treated groups. No effects of DBHCB on weights of the gravid uterus and ovaries were detected.

Table 1: Maternal findings in rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)			
	0 (control)	**62.5**	**250**	**1000**
No. of rats	20	20	20	20
No. of pregnant rats	17	18	17	18
No. of dead rats	0	0	0	0
Initial body weight	285 ± 11	280 ± 12	285 ± 18	288 ± 11
Body weight gain during pregnancy (g)[a]				
Days 0–5	30 ± 8	33 ± 5	31 ± 6	30 ± 6
Days 5–14	47 ± 7	44 ± 7	49 ± 5	43 ± 9
Days 14–19	71 ± 9	65 ± 10	67 ± 10	63 ± 12
Days 19–20	16 ± 6	17 ± 4	20 ± 5	18 ± 5
Days 0–20	163 ± 17	159 ± 19	167 ± 14	154 ± 20
Adjusted weight gain[b]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Feed consumption during pregnancy (g/day)[a]				
Days 0–1	24 ± 3	23 ± 3	23 ± 3	24 ± 4
Days 5–6	27 ± 3	27 ± 3	27 ± 3	27 ± 3
Days 8–9	28 ± 4	28 ± 3	28 ± 3	28 ± 2
Days 11–12	29 ± 4	29 ± 3	28 ± 2	29 ± 3
Days 14–15	28 ± 4	28 ± 3	28 ± 3	28 ± 3
Days 17–18	32 ± 4	30 ± 4	31 ± 3	31 ± 4
Days 19–20	29 ± 4	29 ± 3	31 ± 4	30 ± 3
Weight of gravid uterus (g)[a]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Weight of ovaries (mg)[a]	149 ± 21	137 ± 14	149 ± 19	139 ± 14

[a]Values are given as the mean ± SD.
[b]Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.

The reproductive findings in rats given DBHCB on days 5–19 of pregnancy are presented in Table 2. No totally resorbed litters were found in any group. No effects of DBHCB were observed on the number of corpora lutea or implantations, incidence of pre- or postimplantation loss, or the number of live fetuses or the sex ratio of live fetuses. There was no difference in the body weight of male and female fetuses between the control and DBHCB-treated groups. No abnormal findings were noted in the placentae of any group.

Morphological findings in the live fetuses of rats given DBHCB on days 5–19 of pregnancy are shown in Table 3. No fetuses with external malformations were observed in any group. Skeletal examination revealed no fetuses with skeletal malformations in any group. Fetuses with skeletal variations were observed in all groups including the control group. The incidence of fetuses with individual skeletal variations was not increased after the administration of DBHCB. The total number of fetuses with skeletal variations was also not increased in the DBHCB-treated groups. The degree of ossification, as evidenced by the numbers of sacral and caudal vertebrae and sternebrae in the DBHCB-treated groups, was not different from that in the control group. No fetuses with internal malformations were detected in any group. The fetuses with internal variations, such as thymic remnants in the neck, dilated renal

Table 2: Reproductive findings in rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)				
	0 (control)	**62.5**	**250**	**1000**	**Historical control values[d]**
No. of litters	17	18	17	18	652 (48 studies)
No. of litters totally resorbed	0	0	0	0	
No. of corpora lutea per litter[a]	16.9 ± 2.0	16.3 ± 1.1	17.1 ± 1.7	16.6 ± 1.9	13.8–17.5
No. of implantations per litter[a]	16.2 ± 1.4	15.8 ± 1.1	16.6 ± 1.6	15.1 ± 3.4	13.1–16.3
% Preimplantation loss per litter[b]	3.8	3.0	2.3	9.4	0.9–13.6
% Postimplantation loss per litter[c]	4.9	3.3	4.0	6.3	0–11.5
No. of live fetuses per litter[a]	15.4 ± 1.5	15.3 ± 1.3	16.0 ± 1.8	14.2 ± 3.6	12.4–15.5
Sex ratio of live fetuses (male/total)	0.51	0.47	0.48	0.48	0.38–0.59
Body weight of live fetuses (g)[a]					
Male	3.88 ± 0.22	3.87 ± 0.30	3.92 ± 0.19	4.00 ± 0.26	3.56–4.01
Female	3.68 ± 0.19	3.69 ± 0.31	3.70 ± 0.14	3.79 ± 0.29	3.33–3.81

[a]Values are given as the mean ± SD.
[b](No. of preimplantation embryonic loss/no. of corpora lutea) × 100.
[c](No. of resorptions and dead fetuses/no. implantations) × 100.
[d]Historical control values were obtained from the studies performed in SNBL during 1996–2004 using Crj: CD (SD) IGS rats.

Table 3: Morphological examinations in fetuses of rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)				
	0 (control)	**62.5**	**250**	**1000**	**Historical control values[b]**
External examination					
Total no. of fetuses (litters) examined	262 (17)	275 (18)	272 (17)	255 (18)	9178 (652): 48 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–0.8%
Skeletal examination					
Total no. of fetuses (litters) examined	136 (17)	141 (18)	141 (17)	132 (18)	3741 (516): 29 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–1.3%
Total no. of fetuses (litters) with variations	18 (7)	12 (10)	11 (8)	17 (11)	3.6–19.2%
Asymmetry of sternebrae	1	1	0	0	0–2.8%
Dumbbell ossification of thoracic centrum	1	3 (3)	2 (1)	2 (2)	0–5.5%
Splitting of thoracic centrum	0	0	0	1	0–3.0%
Full supernumerary ribs	0	0	1	0	0–4.4%
Short supernumerary ribs	16 (6)	8 (6)	9 (7)	14 (8)	0.3–17.1%
Short 13th ribs	0	0	0	1	0%
Degree of ossification[a]					
No. of sacral and caudal vertebrae	8.0 ± 0.4	8.0 ± 0.5	8.2 ± 0.4	8.1 ± 0.3	7.5–8.4
No. of sternebrae	5.4 ± 0.5	5.5 ± 0.6	5.7 ± 0.3	5.4 ± 0.5	4.7–5.7
Internal examination					
Total no. of fetuses (litters) examined	126 (17)	134 (18)	131 (17)	123 (18)	3459 (510): 30 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–0.8%
Total no. of fetuses (litters) with variations	2 (2)	5 (4)	8 (6)	10 (6)	0–22.4%
Thymic remnants in neck	1	2 (2)	2 (2)	3 (3)	0–10.0%
Dilated renal pelvis	0	0	3 (2)	3 (2)	0–14.2%
Dilated ureter	1	3 (2)	6 (4)	7 (4)	0–14.2%
Convoluted ureter	0	0	0	1	0–3.8%

[a]Values are given as the mean ± SD.
[b]Historical control values were obtained from the studies performed in SNBL during 1996–2004 using Crl: CD (SD) IGS rats.

pelvis, dilated ureter and/or convoluted ureter, were observed in all groups, including the control group. However, no significant differences in the incidences of the total number of fetuses with internal variations and individual internal variation were found between the control and DBHCB-treated groups.

DISCUSSION

The current study was conducted to determine the prenatal developmental toxicity of DBHCB. The data showed that the prenatal oral administration of DBHCB did not produce any adverse effects, including morphological anomalies in fetuses of rats.

DBHCB was given to pregnant rats during the time of implantation to the term of pregnancy, to characterize the effects of DBHCB on embryonic/fetal development. The number of implantations was slightly reduced, and incidence of preimplantation loss was slightly increased in the high-dosage group, a finding associated with the tendency for reduced maternal body weight gain during the administration period, with an increase in maternal body weight gain after completion of the administration period. These differences were probably associated with the variability in litter sizes in the high-dosage group and unrelated to the administration of the test chemical. No significant changes in any maternal parameters were noted, even at 1000 mg/kg. No significant changes in embryonic/fetal survival or growth parameters were found, even at 1000 mg/kg. These findings indicate that DBHCB is not toxic to maternal animals, embryonic/fetal survival, or fetal growth when administered during the time of implantation to the term of pregnancy.

Morphological examinations in the fetuses of exposed mothers revealed no fetuses with external malformations. However, some fetuses with skeletal and/or internal variations were found in all groups. The variations observed in the current study are of the types that occur spontaneously among the control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). A skeletal variation (i.e., full supernumerary ribs) has been described as a warning sign of possible teratogenicity and is known to occur in the presence of perturbation of maternal homeostatis. All other variations, short supernumerary ribs, sternebral variations, and bilobed centra of the vertebral column, are frequent variations, which were considered to be normal findings (Kimmel and Wilson, 1973). Although several types of skeletal variations, including full supernumerary ribs, were found in the control and DBHCB-treated groups, no consistent tendency was noted in the incidence of fetuses with these alterations. No significant differences between the control and DBHCB-treated groups were observed in the incidences of the total number of fetuses with skeletal variations or individual types of skeletal variation. Furthermore, these incidences were within the ranges of the background control data in the laboratory-performed current study. As for the internal variations, there was an increasing trend, according to the increasing doses, in the total number of

fetuses with internal variations and the number of fetuses with dilated renal pelvis or ureter. In the current study, the incidences of fetuses with internal variations, with dilated renal pelvis, and with dilated ureter at 1000 mg/kg were 7.5%, 2.1%, and 5.4%, respectively. In the background control data in the current study, these values were 0–22.4%, 0–14.2%, and 0–14.2% (Table 3). Because the incidences of fetuses with internal variations were within the range of the historical control data, and there were no statistically significant differences between the control and DBHCB-treated groups, these findings were considered unrelated to DBHCB and simply expression of the normal background incidence of such findings. Chahoud et al. (1999) noted that variations are unlikely to adversely affect the survival or health, and this might result from a delay in growth or morphogenesis that has otherwise followed a normal pattern of development. The alterations observed in the current study are not thought to be due to the administration of DBHCB, because they have occurred at a very low incidence and are of types that occur sporadically among control rat fetuses. Consideration of these findings together suggests that the morphological changes in fetuses observed in the current study do not indicate a teratogenic response and that DBHCB possesses no teratogenic potential in rats.

There was no available data for human exposure to this chemical. Actual human exposure to DBHCB may be estimated to be very low, because this chemical was not detected from polyethyleneterephthalate bottles in Brazil (Monteiro et al., 1998) and from polyethylene products in Japan (Kawamura et al., 1997). Consideration of these findings and the results of the current study together suggests that the risk of adverse effects of DBHCB on prenatal development of offspring is very low.

CONCLUSION

The current results showed that the administration of DBHCB to pregnant rats during the time of implantation to the term of pregnancy had no adverse effects on maternal rats and embryonic/fetal development, even at 1000 mg/kg no observed adverse effect levels. Based on these findings, it is concluded that the (NOAELs) of DBHCB for both dams and fetuses were 1000 mg kg^{-1} day^{-1} in rats.

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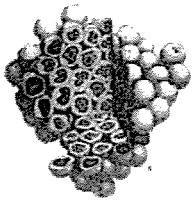

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Electrical Stimulation Modulates Fate Determination of Differentiating Embryonic Stem Cells

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ABSTRACT

A clear understanding of cell fate regulation during differentiation is key in successfully using stem cells for therapeutic applications. Here, we report that mild electrical stimulation strongly influences embryonic stem cells to assume a neuronal fate. Although the resulting neuronal cells showed no sign of specific terminal differentiation in culture, they showed potential to differentiate into various types of neurons in vivo, and, in adult mice, contributed to the injured spinal cord as neuronal cells. Induction of calcium ion influx is significant in this differentiation system. This phenomenon opens up possibilities for understanding novel mechanisms underlying cellular differentiation and early development, and, perhaps more importantly, suggests possibilities for treatments in medical contexts.
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INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells that can, in vivo and ex vivo, give rise to cells of different fates. Through induction, ES cells form embryoid bodies (EBs), and, in this way, ES cells are capable of differentiating into a variety of tissue types such as extraembryonic endoderm and neural and muscle tissue [1–3]. Many growth factors and signaling pathways initiate differentiation and modulate the course of cellular differentiation [4–7]. Several reports show that, with the application of certain growth factors or the alteration of culture conditions, ES cells can differentiate in a relatively efficient manner into specific neuronal cell types that are destined to become certain neuronal tissues [5, 8–11].

Another important factor that possibly influences developmental processes and is central for cellular homeostasis is transmembrane ion distribution. Unequal distributions of ions between the intra- and extracellular compartments yield electrical potential, which is crucial for neural transmission. Ions also play a role in shaping neuronal circuits during development via neural transmission; ions induce functional and structural refinement of synapses and neuronal networks by modulating activity-dependent gene transcription [12, 13]. Despite the important role of ionic density in development, very little information is available on the roles of intra- and extracellular ionic density in cell-fate determination. Here, we report that electrical stimulation can bias the fate of differentiating ES cells toward neuronal lineages. Growth factor-induced ES cells usually differentiate into rather restricted neuronal cell types. In contrast, electrically induced ES cells that ultimately differentiate into neurons are plastic in their capacity to differentiate into a wide variety of specific cell types. These ES cells are pluripotent, capable of differentiating into any neuronal lineage found within the various neuronal tissue types we examined.

MATERIALS AND METHODS

Fluorescent ES Cells

Venus-expressing ES cells were prepared by transfecting R1 ES cells with a construct containing Venus driven by a CAGGS promoter. R1 ES cells were a gift from Dr. Andreas Nagy. The Venus construct was made from plasmids provided by Dr. Jun-ichi Miyazaki [14, 15].

Differentiation Method in Cell Culture

The protocol for ES cell differentiation is schematized in Figure 1. Embryoid bodies (EBs) were made by culturing R1 ES cells with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) without leukemia-inhibitory factor (LIF) in noncoated bacterial petri dishes (Nunc, New York, http://www.nalgenunc.com). After 3 days of culture, electrical stimulation was applied to cells in a 4-mm gap cuvette under several voltage conditions (0, 5, 10, and 20 V; see supplemental data). One train of five pulses (950-millisecond interpulse interval) was delivered with an electroporater (CUY21E; Tokiwa Science, Tokyo, http://www.tokiwakagaku.jp). For cell culture experiments, stimulated EBs were maintained in DMEM with 10% FCS (GIBCO, Carslbad, CA,

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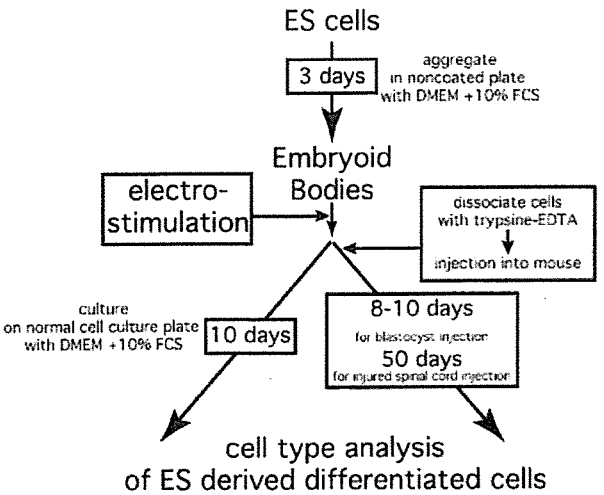

Figure 1. Experimental design. Embryoid bodies (EBs) were made by culturing embryonic stem (ES) cells with DMEM containing 10% FCS in noncoated bacterial petri dishes (Nunc). Electrical stimulation was applied to cells in a 4-mm gap cuvette under several voltage conditions (0, 5, 10, and 20 V; see supplemental data). For cell culture experiments, stimulated EBs were maintained in DMEM with 10% FCS on poly-D-Lysine-coated plates. For animal experiments, Venus-positive EBs were stimulated similarly (10 V, same 5-pulse train) and then dissociated with trypsin-EDTA for 3 minutes. Dissociated cells were injected into C57BL/6 blastocysts. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

http://www.invitrogen.com) on poly(D-lysine)-coated plates (BD, Franklin Lakes, NJ, http://www.bd.com). Ten days after stimulation, cells were fixed in paraformaldehyde in phosphate-buffered saline (pH 7.4) for immunocytochemical analyses. For animal experiments, Venus-positive EBs were stimulated similarly (10 V, same five-pulse train) and then dissociated with trypsin-EDTA for 3 minutes. Dissociated cells were injected into either mouse embryos or adult spinal cords.

Immunostaining

Cultured cells or histological sections were processed for immunostaining using the following antibodies: anti-TuJ1 mouse monoclonal antibody (mAb; 1:500; BAbCO, Berkeley, CA, http://www.babco.com), anti-GFP rat mAb (Nacalai, Kyoto, Japan, http://www.nacalai.co.jp), anti-Hu human polyclonal antibody (pAb; 1:1,000; a gift from Dr. Robert Darnell), anti-Ki67 rat mAb (DAKO, Glostrup, Denmark, http://www.dako.com), anti-MAP2 mouse mAb (1:200; Chemicon, Temecula, CA, http://www.chemicon.com), anti-ChAT rabbit pAb (1:200; Chemicon), anti-Islet1 mAb (1:400; Developmental Studies Hybridoma Bank [DSHB]), anti-Pax6 mAb (1:200; DSHB), anti-Pax7 mAb (1:400; DSHB), anti-MNR2 mAb (1:400; DSHB), and anti-Nkx2.2 mAb (1:400; DSHB). Histological sections were stained with the protocol described previously [16], and immunostained images were obtained with an LSM-510 confocal laser microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com/) by sequential scanning and analyzed with adjunctive software attached to the LSM-510. The thickness of the histological sections was less than 7 μm, and the z-axis sampling of the confocal images was less than 1 μm.

Measurement of Intracellular Ca^{2+} Concentration

ES cells or EBs were loaded with the Ca^{2+} fluorescence indicator fura-2 by incubating the cells in Hank's balanced salt solution (HBSS) containing 2 μM fura-2 AM (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) and 0.01% cremophor-EL (Sigma Chemicals, St. Louis, MO, http://www.sigmaaldrich.com/) at room temperature for 30 minutes. After loading, cells were washed in fresh HBSS and incubated an additional 15+ minutes before analysis of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was analyzed with an inverted fluorescent microscope (IX-70, Olympus, Tokyo, http://www.olympus-global.com) equipped with a filter exchanger (Lambda 10-2, Sutter Instruments, Novato, CA, http://www.sutter.com/index.html) and a cooled charge-coupled device camera (MicroMax, Roper Scientific, http://www.roperscientific.com/). One train of five pulses (950-millisecond interpulse interval) was delivered with an electroporater (CUY459G20; Nepa Gene, Chiba, Japan, http://www.nepagene.jp). The following optics were used: excitation filter, 340HT15, and 380HT15; dichroic mirror, 430DCLP; emission filter, 510WT40 (all from Omega Optics, Austin, TX, http://www.omegaoptics.com/); and objective lens, Uapo/340 20x/0.75 (Olympus). Metafluor 5.0 software (Universal Imaging) was used to control the system and analyze acquired images [17].

Spinal Cord Injury Model

Spinal cord injury was induced with a modified NYU impactor as described previously [18]. Briefly, female C57BL/6J mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After laminectomy at the T9 level, the dorsal surface of the dura matter was exposed. The vertebral column was stabilized with fine forceps and clamps at the T7 and T10 spinous processes and ligament, and then the animal's body was lifted. A 3-g weight (1.2-mm diameter tip) was allowed to drop from a height of 25 mm onto the dorsal surface of the dura matter. The muscles and the incision were then closed in layers, and the animals were placed in a temperature-controlled chamber until thermoregulation was reestablished. Manual bladder evacuation was performed twice per day until reflex bladder emptying was reestablished.

RESULTS

Cell Fate Determination of Electrically Stimulated Cells in Culture

We examined the influence of inter- and intracellular ionic balance on differentiation fate by application of weak electrical pulses. Embryoid bodies were stimulated at one of several intensities via an electrode (Fig. 1). The EBs were cultured for 10 days, and then fixed to assess differentiation fate (Fig. 1). Assessment for various markers (i.e., mainly for muscle and neural tissue) indicated that R1 ES cells showed no neuronal or myocytic differentiation, regardless of whether they were electrically stimulated (Fig. 2A, 2B, 2M, 2N). In contrast, EBs receiving electrical stimulation showed robust neuronal differentiation; control EBs (i.e., those receiving no stimulation) showed little differentiation (Fig. 2). Almost all colonies of EBs receiving 10-V stimulation contained cells immunoreactive for TuJ1, a marker for early committed neuronal cells, whereas less than 10% of control colonies contained TuJ1-positive cells derived from EBs receiving 0-V stimulation (Fig. 2A, 2C). We confirmed the neuronal identity of the cells from colonies that received 10-V stimulation: The majority of these cells were MAP2 immunoreactive (supplemental Fig. S1), whereas 20%–30% of cells showed immunoreactivity to MAP2 by retinoid treatment.

It is noteworthy that the neuronal cells in our system differentiated in a significantly shorter time than did those in most of other systems that use growth factors to initiate cell differentiation [5, 10, 11]. The differentiation efficiency decreased slightly in cells that received 20-V stimulation compared to that in cells that received milder stimulation. Although the morphology of these cells also clearly differed (e.g., thicker dendritic processes than in the ones receiving milder stimulation), all expressed TuJ1 (Fig. 2J). The size and number of colonies produced from cells stimulated with 20 V did not clearly differ from the size and number of colonies produced from the unstimulated cells. The size and number of colonies produced from

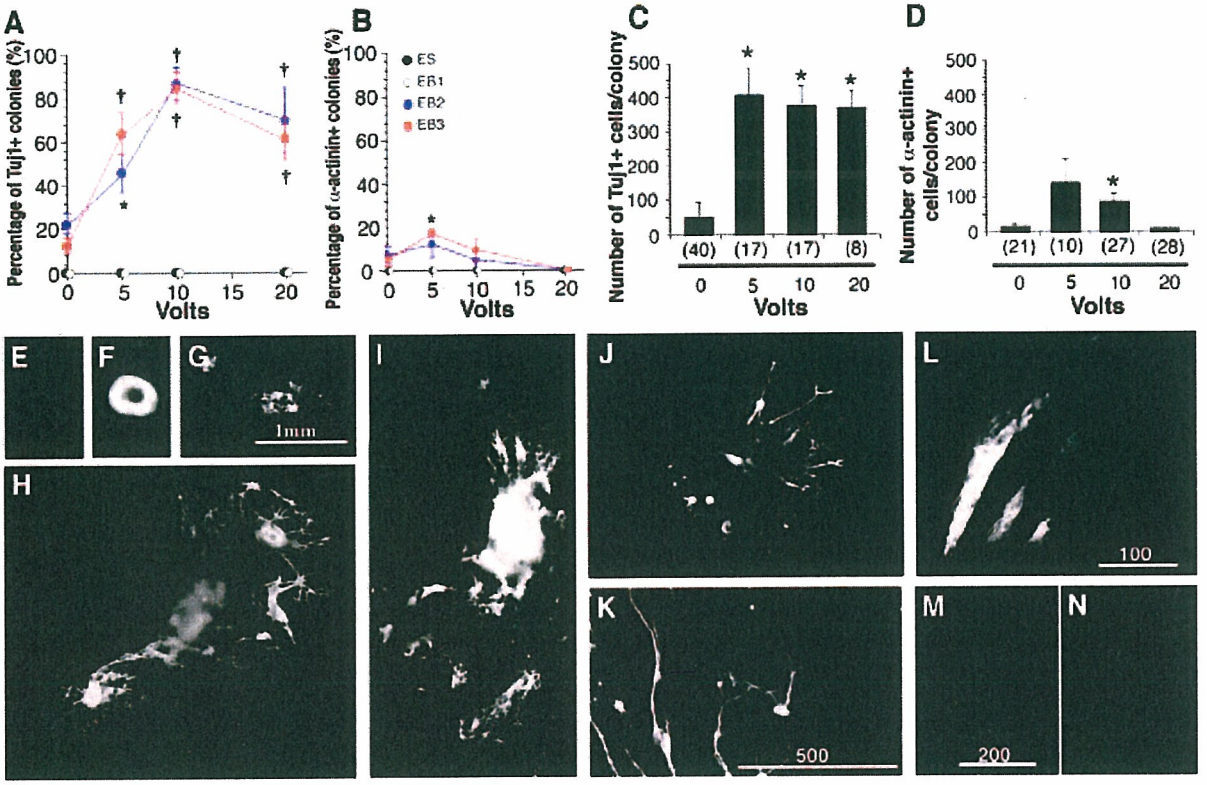

Figure 2. Effect of electrical stimulation on embryonic stem (ES) cell differentiation in culture. **(A–D):** Increasing, mild electrical stimulation disproportionately biases ES cell differentiation toward a neuronal fate. Percentage of colonies containing cells that express the neuronal marker TuJ1 **(A)**, and those that express the muscle marker α-actinin **(B)**: Black, filled circles, original ES cells; white open circles, ES cells cultured for 1 day to make embryoid bodies (EBs; before electrical stimulation); blue, filled circles, ES cells cultured for 2 days to make EBs (before electrical stimulation); and red, filled circles, ES cultured for 3 days to make EBs (before electrical stimulation). Number of TuJ1-positive cells per colony **(C)** and α-actinin-positive cells per colony **(D)**, both as a function of stimulation intensity. Daggers indicate $p < .001$ and asterisks indicate $p < .05$ compared to TuJ1-positive cells in zero-volt condition. Statistical differences between groups were assessed with Student's t test. A p value of at least $p < .05$ was considered significant. Numbers in parentheses in **(C)** and **(D)** indicate the number of colonies containing TuJ1-positive cells. **(E–G):** Appearance of unstimulated control EBs. Although the majority of colonies did not contain TuJ1-positive cells **(E)**, a few TuJ1-positive cells were present **(G)**. **(F):** Nuclear staining of EBs in **(E)** shows the density of cells. **(H–L):** Appearance of stimulated EBs. Anti-TuJ1 immunostaining of EBs subjected to either 5- **(H)**, 10- **(I)**, or 20-V **(J)** pulse stimulation. **(K):** Higher magnification of anti-TuJ1 immunostained EBs stimulated with 10 V. **(L):** Anti-α-actinin immunostained EBs stimulated with 10 V. **(M):** Nuclear staining demonstrates the existence of cells and lack of anti-TuJ1 immunostaining of ES cells. **(N):** Neuronal differentiation of ES cells was not induced. Nuclear stain was propidium iodide. Scale bars = 1 mm **(E–G)**, 100 μm **(H–J, L)**, 500 μm **(K)**, and 200 μm **(M, N)**.

cells stimulated with 5–15 V also did not differ. We note that the cell number and cell death did not show significant difference among EBs with or without electrical stimulation after outgrowth on the poly(D-lysine) plate (tunnel assay showed 11%–13% of cell death at 1 day and 3 days after outgrowth of EBs with electrical stimulation and without stimulation).

In general, we observed few muscle progenitors as a result of electrical stimulation, even though a slight but insignificant, increase in muscle progenitors was observed for EBs receiving 5-V stimulation (Fig. 2B, 2D). In addition, we did not observe cells differentiating into glial cells in our system (i.e., no glial fibrillary acidic protein [GFAP]-immunoreactive cells; supplemental Fig. S1). Electrical stimulation induced EBs to differentiate somewhat specifically into neuronal cells. However, because we failed to observe various differentiation markers (e.g., Islet1, Pax6, Pax7, MNR2, Nkx2.2, tyrosine hydroxylase, GAD65, Islet1) for specific neuronal cell types within 10 days of culture in immunocytochemical analysis, whereas we detected slight elevation of transcription of Pax6, NeuroD1, and some LIM-homeodomain genes by RT-PCR after 10 days of culture (data not shown), it is most likely that the TuJ1-positive cells of this ex vivo system did not reach the stage at which such neuronal markers are expressed.

Importance of Calcium Ion Influx for Modulation of Differentiation Fate

The mechanism that modulates fate determination in this system is unknown. To determine this mechanism, first, we examined the role of calcium by measuring intracellular Ca^{2+} concentration in ES cells and EBs before and after electrical stimulation (Fig. 3). It is well established that Ca^{2+} is an important signal transducer or modulator [19]. ES cells or EBs loaded with the fluorescent Ca^{2+} indicator fura-2 were electrically stimulated [17], and the resulting fluorescence was measured. ES cells showed no change in fluorescence emission after electrical stimulation, indicating that electrical stimulation did not induce release or uptake of Ca^{2+} in ES cells (Fig. 3B). In contrast, numerous cells within EBs showed small but significant changes in fluorescence after electrical stimulation, indicating that this stimulation induced an increase in Ca^{2+} concentration in EBs (Fig. 3A). Addition of the Ca^{2+} chelator EGTA (25 mM)

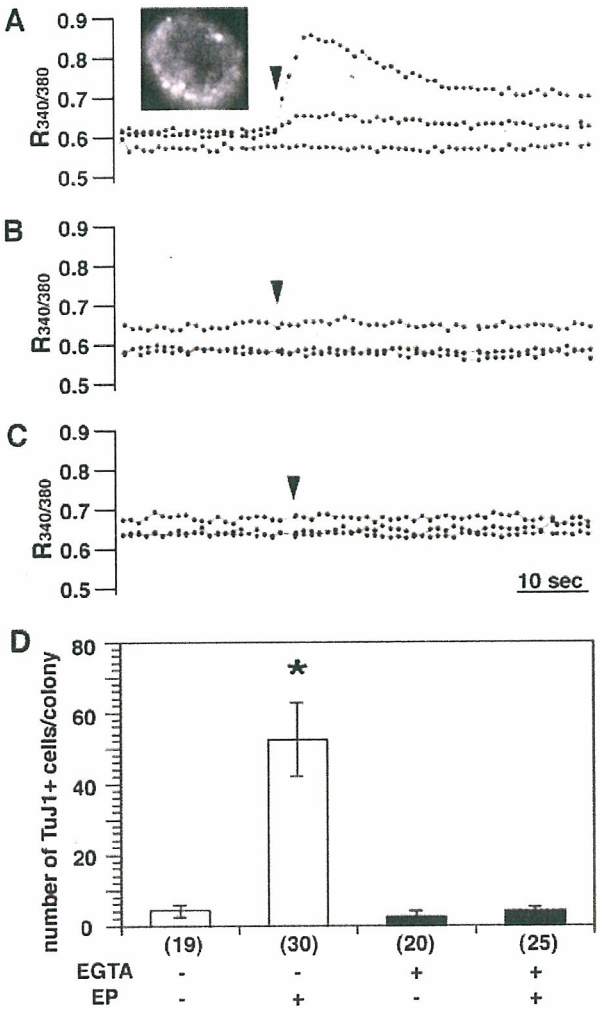

Figure 3. Electrical stimulation-induced calcium influx into cells. **(A–C):** Changes of $[Ca^{2+}]_i$ in embryoid bodies (EBs) **(A, B)** and in ES cells **(C)** after electrical stimulation. Ratio of fluorescence excitation intensities at 340 nm to 380 nm is plotted as a function of time. Filled arrowheads indicate time of electrical stimulation. Three typical patterns are displayed for each plot. Inset in **(A)** shows a fluorescent image of fura-2-loaded EBs excited at 380 nm. The culture medium contained either 2 mM Ca^{2+} **(A, C)** or 25 mM EGTA **(B)**. Cells were stimulated at 30 V (5–6 W) in this experiment. Due to the different buffer composition for this experiment, a higher voltage was required to produce comparable power (5–6 W) to that produced in the 10–15-V condition of the experiments presented in Figure 1. This stimulus intensity (one associated with 5–6 W) induces neuronal differentiation. **(D):** Mean number of neuronal cells counted per colony in the presence and/or absence of the calcium chelator EGTA and stimulation with electric pulses (EP). The number of neuronal cells decreased dramatically when Ca^{2+} was absent from the medium. The same stimulation condition that yielded TuJ1-positive cells failed to yield neuronal cells when 25 mM EGTA was added to the medium. Open bars show data for incubation without EGTA and closed bars with EGTA. Number of colonies counted is indicated in parentheses. Again, due to the difference in culture conditions in this experiment, cell growth was largely disturbed in comparison with the conditions used in the experiments of Figure 1. The absolute number of cells after culturing was approximately one-tenth of the number of cells in the experiments presented in Figure 1. Statistical differences between groups were assessed with the Student's *t* test. Error bars are SEM. $p = .00057$ for comparison (*) between stimulated EBs with EGTA present and stimulated EBs with EGTA absent.

to the EB culture medium before electrical stimulation prevented any measurable change in fura-2 fluorescence (Fig. 3C), indicating that the source of Ca^{2+} was extracellular rather than intracellular.

To determine the significance of Ca^{2+} influx for electrically induced neuronal differentiation, EBs were electrically stimulated in the presence or absence of EGTA, and then these were cultured in the absence of EGTA and monitored for signs of differentiation fate. EBs electrically stimulated in the presence of EGTA failed to assume a neuronal fate, whereas those stimulated in the absence of EGTA assumed a neuronal fate (Fig. 3D). These results indicate that Ca^{2+} influx is, at the very least, necessary for neuronal cell fate determination in this system.

It is possible that this Ca^{2+} influx was not mediated by ionic channels, but instead, simply by physical disruption of the cell membranes caused by electrical stimulation. We believe that the latter is unlikely, because the emission ratio for fura-2 was significantly lower in electrically stimulated cells compared to that in cells with membrane fractures (Fig. 3A), suggesting that the increased Ca^{2+} influx did not result from passive influx caused by membrane fractures. In addition, we observed no blue staining in the cells when we applied trypan blue to the culture medium at the time of stimulation with parameters that successfully induced neuronal differentiation. However, we did observe staining in cells stimulated with higher voltage pulses (data not shown). The lack of trypan blue staining with milder stimulation parameters indicates that our parameters were sufficiently weak to avoid membrane fractures. Thus, the increased Ca^{2+} influx we observed was most likely mediated by Ca^{2+} ion channels, which probably play a key role in fate determination in our model system. However, we could not determine the type of Ca^{2+} channel at work in this system by applying inhibitors such as nifedepine or ω-conotoxin MVIIC. In addition, we could not detect significant expression of subunits from either of Ca^{2+} channels L, N, and P/Q types, by reverse transcriptase polymerase chain reaction (RT-PCR) analysis on EB after 3 days of aggregation (data not shown).

Incorporation of Electrically Stimulated ES Cells into Neural Tissues of Embryos

To determine whether electrically stimulated EBs were capable of differentiating into mature neurons in vivo, stimulated or unstimulated EBs were injected into mouse embryos, and the fate of these cells was traced during the course of embryonic development. In this set of experiments, we used an ES cell line ubiquitously expressing Venus, an enhanced yellow fluorescent protein derivative [14]. When injected into mouse blastocysts, line LCVL10 ES cells incorporated equally into the bodies of embryos and neonatal mice. As before, ES cells expressing Venus were induced to make EBs, which were then either stimulated with 10-V pulses or were unstimulated, and then injected into mouse blastocysts (supplemental Fig. S2). Thirteen embryos and 15 embryos were recovered from the 10-V and unstimulated conditions, respectively.

Unstimulated EBs failed to incorporate into specific tissues in most embryos tested (10 of the 11-days-postcoitum [dpc] embryos and five of the 13-dpc embryos; supplemental Fig. S2). We observed fluorescence nonspecifically across the embryo and in extraembryonic structures, such as in the yolk sac of two embryos (one from 11-dpc and the other from 13-dpc embryos; supplemental Fig. S2; supplemental Table S2). The 11-dpc embryo that ES cells heavily contributed showed abnormality (supplemental Fig. S2). In two other 11-dpc embryos, strong fluorescence was observed only in the yolk sac, whereas virtually none was observed

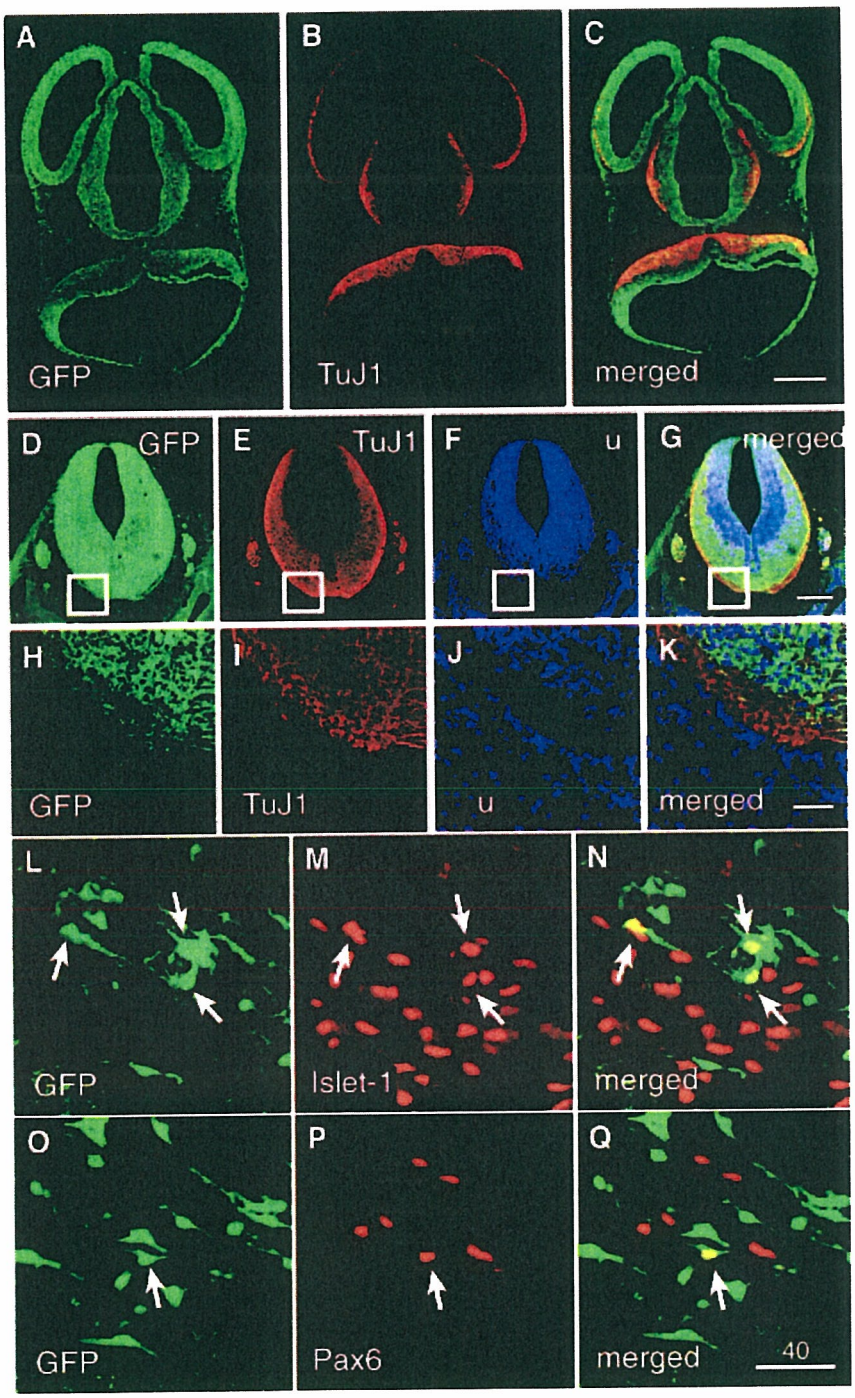

Figure 4. Distribution of electrically stimulated embryonic stem (ES) cells implanted in mouse embryos. An embryo at 11 days postcoitum (dpc) was sectioned to examine cell-type specificity of incorporated fluorescent ES cells in the central nervous system (CNS). Incorporation of ES cells into brain **(A–C)** and spinal cord **(D–Q)**, shown in transverse sections. Green fluorescent puncta are anti-GFP-positive cells expressing Venus **(A, C, D, G, H, K)**, red are TuJ1-positive cells, indicating differentiated neurons **(B, E, G, I, J)**, and blue shows nuclear staining with TO-PRO3 (Molecular Probes) **(F, G, J, K)**. Small boxes in **D–G** show areas of high magnification presented in **H–K,** respectively. **(L–Q)** High magnification images of a 13-dpc embryo showing that ES cells differentiated into a variety of neuron types, including motor neurons, interneurons, or their precursors. Green signals represent GFP, and red signals, Islet1 **(L–N)**, Pax6 **(O–Q)**. Scale bars = 500 μm **(A–C)**, 250 μm **(D–G)**, 50 μm **(H–K)**, and 40 μm **(L–Q)**. Abbreviation: GFP, green fluorescent protein.

in the embryo proper (data not shown). In the remaining 11 embryos, EBs did not incorporate (data not shown).

Cells arising from the stimulated EBs (EPs) tended to incorporate into neural tissue (supplemental Fig. S2; supplemental Table S2). We recovered nine embryos at 11 dpc and four embryos at 13 dpc. All of these recovered embryos were morphologically normal. Upon closer examination in whole-mount preparations, fluorescence appeared to be primarily localized to dorsal structures (i.e., the central nervous system [CNS]) in seven embryos (four of the 11-dpc embryos and three of the 13-dpc embryos) with small pockets of fluorescence in other structures, including the peripheral nervous system (PNS). In addition, in two of the 11-dpc embryos, we observed minor fluorescence in the PNS. For one embryo acquired at 13 dpc, the heart emitted strong fluorescence, and the PNS emitted weak fluorescence (supplemental Table S2). In summary, we observed that ES-derived Venus positive cells contributed primarily to CNS in 7 embryos among 13 recovered embryos when electrical stimulation is applied.

To determine whether incorporated EBs differentiated into proper neurons, we assessed several neuronal markers immunohistochemically. Transverse sections of two embryos recovered

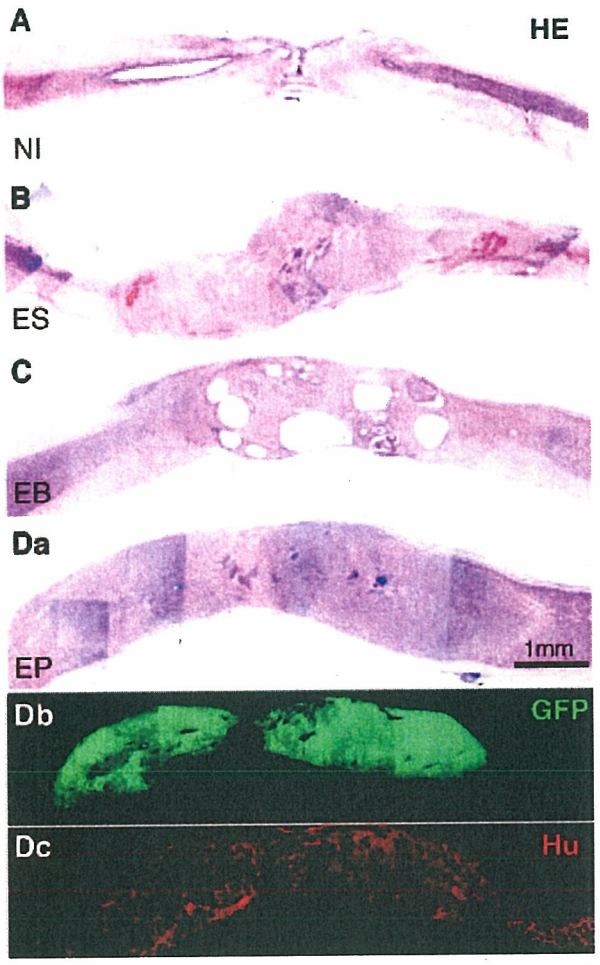

Figure 5. Longitudinal sections of injured, adult mouse spinal cords injected with electrically stimulated or nonstimulated cells. **(A–D):** Hematoxylin-eosin staining. Injured spinal cords 57 days after trauma was induced without injection of ES cells (ES; control) **(A)**. **(B–D):** Untreated or treated ES cells were injected 7 days after injury. Histological analysis was performed 50 days after injection with unstimulated ES cells **(B)**, with nonstimulated EBs **(C)**, or stimulated EBs (EP) **(D)**. Scale bar = 1 mm. **(D):** Incorporation of electrically stimulated EBs into injured adult spinal cord. **(Db):** Cells derived from stimulated EBs (EP) are positive for GFP. **(Dc):** ES cells from stimulated EBs differentiated into Hu-positive neuronal cells. Abbreviations: EB, embryoid body; EP, electropulsed; ES, embryonic stem; GFP, green fluorescent protein.

as 11 dpc and one from 13-dpc embryo were prepared and double immunostained for GFP and one of the following neuronal makers: TuJ1, Islet1, Pax6 (Fig. 4), LIM3, Pax7, or MNR2 [20]. All three preparations of CNS structures clearly contained numerous double-immunostained cells, as shown in Figure 4, for Islet1 and Pax6 (i.e., cells that were immunoreactive for both GFP and one of the above-mentioned neuronal markers), suggesting that, in vivo, cells from electrically stimulated EBs can differentiate into proper functional neurons. Although electrically stimulated cells tended to incorporate into ventral spinal cord to form motor neurons and interneurons, they can, in principle, incorporate into various neural structures across the dorsoventral axis to form different types of mature neurons (Fig. 4). With respect to the anteroposterior axis, we also found GFP-labeled cells in forebrain, hindbrain, brain stem, and spinal cord (Fig. 4).

Together, our data demonstrate that electrically stimulated EBs differentiate primarily into early committed neuronal cells. These cells are plastic and can differentiate into a variety of specific neuronal cell types in accordance with the environment.

Electrically Stimulated EBs Contribute to Injured Adult Spinal Cords

Our embryo experiments prompted us to examine whether electrically stimulated EBs are capable of differentiating into neurons when grafted into the injured spinal cords of adult mice. Stimulated EBs produced from Venus-expressing ES cells were injected into spinal cords of mice 7 days after injury, and their survival and phenotype within the spinal cord was assessed 50 days after transplantation (Fig. 5D). As control experiments, we also grafted unstimulated EBs or unstimulated ES cells into the spinal cords of adult mice (Fig. 5A–5C). In each animal, 10^6 cells were injected in a volume of 5 μl. Of the five animals injected with stimulated EBs, one failed to survive due to injury, and all of the others showed evidence of incorporation into the spinal cord as neuronal cells. Two of five animals injected with unstimulated EBs and three of six animals injected with unstimulated ES cells died within 50 days. All of the survivors from both of these control experiments showed tumorigenic or pathogenic features (Fig. 5B, 5C).

Immunostaining revealed that a large part of electrically stimulated EBs were capable of differentiating into Hu-positive neuronal cell types (Fig. 5D; Fig. 6A–6D). We detected no cells that differentiated into GFAP-positive astrocytes or NG2-positive oligodendrocytes (data not shown). In these experiments, approximately 80% of grafted cells assumed a neuronal lineage, clearly expressing neural markers including Hu (Fig. 6A–6D, 6M). Furthermore, Venus-positive cells also immunostained for MAP2, indicating that the exogenous cell population differentiated into neurons (Fig. 7A–7C). On the contrary, even surviving animals possessed few Hu-positive neuron-like cells derived from unstimulated EBs or ES cells (Fig. 6E–6M). Notably, in all the survivors, we observed pathology within the grafts (e.g., infiltration of inflammatory cells, such as macrophages, or ectopically formed tubular structures surrounded by epithelia-like cells; Fig. 5B, 5C). In both cases, many of the injected cells were reactive for phosphorylated histone H3 (phospho-H3) or Ki67 antibodies, suggesting that the grafted cells maintained proliferative activity and thus can be tumorigenic (Fig. 6E–6L, 6N). On the other hand, stimulated EBs showed essentially no phospho-H3 and Ki67 immunoreactivity (Fig. 6A–6D, 6N).

In summary, stimulated EBs contributed robustly to form neurons within the spinal cord, whereas unstimulated and stimulated ES cells, as well as unstimulated EBs, formed few neurons (Fig. 5). In addition, grafts appeared to display pathological features (Fig. 5).

To analyze in more detail the fate of these neural cells, we performed additional immunohistochemical analyses using antibodies against several neuronal markers. ChAT and Islet1, markers found in spinal motor neurons, colocalized within Venus-expressing cells (Fig. 7D–7F; data not shown) [21], indicating that grafted cells have the potential to differentiate into motor neurons. We detected a few parvalbumin- and γ-aminobutyric acid-positive cells among the Venus-expressing cells that displayed typical neuron-like morphology (Fig. 7G–7I; data not shown). Stimulated EBs incorporated quite well into spinal cord tissues, differentiating into a variety of neuronal cell types and mixing with the recipients' own cells (Fig. 7).

These experiments with injured spinal cord established that stimulated EBs are capable of differentiating into mature neu-

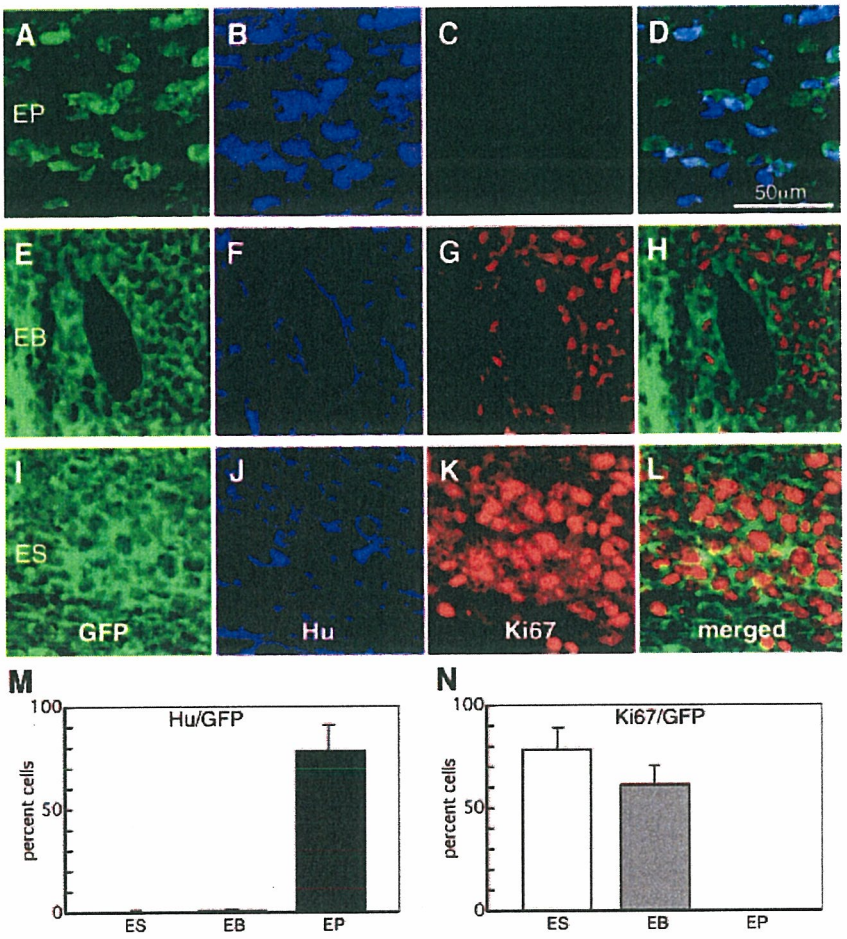

Figure 6. Stimulated EBs frequently adopted the appearance of neuronal cells when injected into spinal cord. **(A–D):** Almost all the cells derived from stimulated EBs (EP) displayed Hu immunoreactivity but not Ki67 immunoreactivity, whereas cells derived from unstimulated EBs (EB; **E–H**) or ES cells **(I–L)** displayed Ki67 immunoreactivity but not Hu immunoreactivity. **(M):** Graph showing the percentage of cells that coexpress both GFP and Hu. **(N):** Graph showing the percentage of cells that coexpress both GFP and Ki67. Percentages indicated were average of percentages of Hu- or Ki67-positive cell counts obtained by tallying the GFP-positive cells observed in more than 10 different focal planes of each EP, EB, and ES examined. Green, blue, and red signals indicate GFP, Hu, and Ki67 immunoreactivity, respectively **(A–L)**. Scale bar = 50 μm. Abbreviations: EB, embryoid body; EP, electropulsed; ES, embryonic stem; GFP, green fluorescent protein.

rons when inserted into an in vivo environment. It is possible that injury to the spinal cord provided environmental cues necessary to direct differentiation. This hypothesis is supported by findings that adult neuronal stem cells differentiate into glial cells in response to spinal cord injury [22–24]. Growth factors and cytokines are released, and these factors, in turn, can modulate proliferation and differentiation of neuronal stem cells [25–28].

DISCUSSION

ES cells that have initiated differentiation steps will preferentially assume a neuronal fate when electrically stimulated. In comparison with other systems that produce neural cells from ES cells, the neuronal cells in our system differentiated in a significantly shorter period than did those produced by other methods [5, 10, 11]. Moreover, these stimulated ES cells could be easily transplanted into animal tissues. According to our observations, the differentiation process can be divided into three steps: (a) destabilization of undifferentiated ES cells, (b) modulation of cell fate direction, and (c) differentiation to a mature, terminal cell state. Unlike other induction methods, electrical stimulation seems to work only during the second step. There are several reports on electrical stimulation-induced neurite extension and growth cone guidance cue of PC12 cells or some neural cells [29–33], which are mostly destined to neural fate, but not pluripotent, as ES cells are. In these reports, electrical stimulation alters their morphology, which probably corresponds to the third or even later phase of developmental process in this scheme.

These cells possess terminal differentiation plasticity, requiring further steps to determine a neuronal destiny. They failed to express any specific markers indicative of mature neurons ex vivo, and were flexible or plastic; thus, the differentiation course of these multipotent cells can be further molded by the environmental context and can differentiate into cells suitable for the region in which they have been transplanted. Indeed, the cells derived from treated EBs showed varied neural specificities, both when injected into blastocysts and when implanted into adult spinal cords. Electrically stimulated EBs seem to incorporate into the CNS with no specific preference for anterior-posterior or dorsoventral axes, as we observed that the injected cells contributed to the entire CNS in our blastocyst injection experiments. Some induction protocols may specifically direct cells to preferentially contribute to certain tissues. For example, the protocol that uses retinoids directs cells to differentiate toward cell types located in posterior structures [34].

A large proportion of the cells (approximately 80%) derived from stimulated EBs displayed neuronal identities when injected into injured spinal cord, an environment that is non-neurogenic (Fig. 5). This amount of neural differentiation is extremely high in comparison with that observed by Ogawa et al. [35], who adopted a similar experimental design with in vitro expanded neural stem/progenitor cells derived from fetal spinal cord. In the present study, the cells we grafted differentiated into various types of neurons, as