differentiating into glial cells in our system (i.e., no 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 rtPCR 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 following 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 following 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) to the EB culture medium prior to 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, since 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 omega-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 rt-PCR analysis on EB after 3days 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 EYFP 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). 13 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 dpc embryos and 5 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 11dpc 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, while virtually none was observed 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 9 embryos at 11 days-post-coitum (dpc) and 4 embryos at 13 dpc. All these recovered embryos were morphologically normal. Upon closer examination in whole-mount preparations, fluorescence appeared to be primarily localized to dorsal structures (i.e., CNS) in 7 embryos (4 of the 11 dpc embryos and 3 of the 13 dpc embryos) with small pockets of fluorescence in other structures, including the PNS. In addition, 2 of the 11 dpc embryos, we observed minor fluorescence in the PNS. For 1 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 2 embryos recovered as 11 dpc and 1 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 Fig. 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 5 animals injected with stimulated EBs, one failed to survive due to injury

and all the others showed evidence of incorporation into the spinal cord as neuronal cells. Two of 5 animals injected with unstimulated EBs and 3 of 6 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, about 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, while unstimulated and stimulated ES cells, as well as unstimulated EBs, formed few neurons (Figure 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, co-localized 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 GABA-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 they are capable of differentiating into mature neurons 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 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: (i) destabilization of undifferentiated ES cells, (ii) modulation of cell fate direction, and (iii) differentiation to a mature, terminal cell state. Unlike other induction methods, electrical stimulation seems to work only during step 2. 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. In these reports, electrical stimulation alters their morphology, which probably correspond to step 3 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 dorso-ventral axes, since 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 towards cell types located in posterior structures [34].

A large proportion of the cells (about 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 and colleagues [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 indicated by expression of several markers including neurotransmitters. Importantly, stimulated EBs showed almost no proliferative activity 50 days after transplantation, unlike ES cells or unstimulated EBs, which showed proliferative and pathogenic features (Fig. 6). Because cells derived from stimulated EBs were extremely efficient in assuming a course of neuronal differentiation in an *in vivo* environment, they hold much promise for use in therapeutic applications.

Ca^{2+} is one of the most important signaling ions involved in various biological activities [19]. Ca^{2+} also appears to play an important role in the system we studied. We found that Ca^{2+} influx was necessary for neural fate determination of EBs. Our finding indicates that this influx did not result from cell membrane fissures; rather, it appears that Ca^{2+} channels may be responsible for Ca^{2+} uptake in our system for the three following reasons: (i) Passive influx via membrane fractures results in a much higher magnitude of intracellular fluorescence than what we observed in this study; (ii) Dye in

the culture medium failed to enter the cells upon electrical stimulation; and (iii) The original ES cells that did not take on a neuronal fate after electrical stimulation showed no evidence of Ca^{2+} influx (Fig. 3C). We attempted to identify these Ca^{2+} channels by culturing electrically stimulated EBs in media containing agents that block three major Ca^{2+} channels. Treatment with nifedepine, an L-type Ca^{2+} channel blocker, and ω-conotoxins, N-type and P/Q-type blockers, failed to block Ca^{2+} influx. Although the existence of these major Ca^{2+} channels in EBs could not be excluded, we conclude that EBs most likely express other minor Ca^{2+} transporters, and that these channels mediate the Ca^{2+} influx necessary for neuronal cell fate determination in this system. We conclude that EB formation is required for activation of these Ca^{2+} ion transporters, because unlike EBs, ES cells showed no change in intracellular Ca^{2+} density following electrical stimulation.

Ca^{2+} is also known to be involved in the noncanonical *Wnt* signaling pathway. In *Xenopus* and zebrafish, some Wnt ligands stimulate release of intracellular Ca^{2+} during development [36]. This Ca^{2+} release, however, induces differentiation into elements (e.g., non-neuronal cells) fated to become ventrally located structures [37]. This is in

stark contrast to our system in which Ca^{2+} induces differentiation into elements (e.g., neuronal cells) fated to become dorsally located structures.

In spite of our efforts, we do not fully understand the mechanisms for this differentiation system. Although we successfully demonstrated the Ca^{2+} flux is required for the induction (Fig. 3), this will not fully interpret the system. It is possible that other ions, such as potassium or sodium, can also be involved [38]. We are underway to further understand the mechanisms of neuronal induction ability of electrical stimulation on ES cells.

Signaling pathways that transmit information out of cells into the environment are usually activated by receptor-ligand recognition. However, in the present experiments, physical alteration of cell surface membranes may initiate signaling, even though the normal signaling molecule takes over later. This primitive signaling pathway may be a prototype that mediates environmental effects on cells. Receptor-ligand signaling systems may have evolved for stabilization and refinement of environmental cues impinging on cells. This simple system may possibly be invoked during early development and neuronal regeneration. In neuronal tissues, ionic currents are

continuously flowing, and these currents may instruct and ensure that undifferentiated cells assume the fate of neuronal progenitors. Ionic flux, therefore, may be a novel category of differentiation signals. Indeed, recent findings show that excitatory neural activity induces adult neural stem cells to adopt a neural fate [39,40]. In our study, we observed that cells affected by ionic currents were very close to being in an undifferentiated state. The effective differentiation step, therefore, corresponds to states that occur earlier than the neural stem cell state, since unstimulated cells could adopt various cell fates. Even though further studies are required to investigate the physiological role of ionic flow in early embryos, our observations suggest that transmembrane ion flow may play an integral role in early stages of development.

In summary, we described a simple and novel procedure for producing neural cells from ES cells. Further studies are needed to improve our procedure so that parameters are appropriate for therapeutic applications.

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