Nerve growth factor increases electrical activity of neural cells derived from murine bone marrow stromal cells

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Abstract

OBJECTIVE: Nerve growth factor (NGF) triggers long-term neuronal excitability. We examined its effect on murine bone marrow stromal cells (BMSC)-derived neurons.

METHODS: With an optimal differentiation protocol, BMSCs were differentiated into neurons in culture. To confirm the probability of differentiation of BMSC into neuron, the expression of neuronal marker protein, neurofilament, was examined by immunocytochemistry. To examine the electrophysiological properties of BMSC-derived neurons, the field potentials were recorded either from nontreated (control) BMSC-derived neurons or from BMSC-derived neurons after the treatment with NGF by using extracellular recording techniques.

RESULTS: Most BMSC-derived neurons showed spontaneous discharges whose amplitudes were up to 2 mV. When NGF at a concentration of 100 ng/ml was applied to BMSC-derived neurons, the amplitudes of discrete field potentials were gradually enlarged within 1 min after NGF application and peaked 3 min later (20-fold the size of control). However, the enlargement of the amplitudes of field potentials almost disappeared 5 min after NGF application.

CONCLUSION: This finding indicates that neuronal cells derived from murine BMSCs generate discrete field potential activities spontaneously and that NGF has the effect of enlarging transient, but not sustained, electrical activity of BMSC-derived neurons.

ORIGINAL ARTICLE

INTRODUCTION

Bone marrow stromal cells (BMSCs) normally give rise to bone, cartilage, and mesenchymal cells. Recently, bone marrow cells have been shown to have the capacity to differentiate into neurons (Black & Woodbury 2001; Sanchez-Ramos 2002; Sanchez-Ramos *et al.* 2000; Woodbury at al. 2000), which raises the possibility of new therapeutic approaches in neural dysfunction. The use of BMSCs to generate neurons for transplantation offers a number of potential therapeutic advantages: no immunorejection by autologous transplantation, a safe and accessible source, and no need for genetic manipulation. BMSC-derived neurons express high levels of neuronal marker protein in immunocytochemical study, whereas it is not clear whether these neurons acquire electrical neuronal excitability.

Neurotrophins that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5 regulate the survival, growth, and differentiation of neurons in the central nervous system. Among these, NGF plays a prominent role in the regulation of sodium channel/current expression (Fjell *et al.* 1999). The biosynthesis and release of neurotrophins is sensitive to electrical activity and synaptic stimulation (Thoenen 1995). Experimental evidence indicates that brief exposure to NGF triggers long-term neuronal excitability through sodium channel gene induction in PC12 cells (Toledo-Aral *et al.* 1995). However, whether or not NGF is required for the acquisition of neuronal excitability in BMSC-derived neurons remains to be examined.

In the present study, to determine whether or not BMSC-derived neurons acquire electrical excitability, or whether or not exposure to NGF induces neuronal electrical excitability, we examine NGF's effect on BMSC-derived neurons using extracellular recording techniques.

MATERIALS AND METHOD

Cell cloning and culture

All experiments were performed under the housing care guidelines and experimental protocol approved by the Animal Care and Use Committee of the University of Tokushima School of Dentistry. Bone marrow was obtained from the dissected femora and tibiae of 6-week-old male C57B/6 mice. The epiphyses were removed, and marrow tissue was flushed from the shaft using 0.5 ml of α-MEM (Life Technologies, Grand Island, NY) expelled from a syringe through a 25-gauge needle. A single cell suspension was obtained by gently cells sequentially aspirating through 20- and 23- gauge needles, and finally a cell strainer (70 μm in sieve size, Becton Dickinson Labware, Frank1in Lakes, NJ) to exclude tissue debris. The cells were washed with α-MEM and seeded into T-75 flasks (Falcon Labware, Meylan Cedex, France) at a cell density of 4.0×10^5 nucleated cells/cm^2, and cultured in α-MEM containing 10% fetal bovine serum, which consisted of 10^{-8} M dexamethasone (Sigma Chemical, St. Louis, MO), 10^{-4} M L-ascorbic acid phosphate magnesium salt, 100 IU/ml penicillin, and 100 mg/ml streptomycin. After 1 week, the culture medium was changed for the first time. Thereafter, the medium was changed twice a week. After 2 weeks in culture, the adherent BMSCs were washed with Hanks'balanced salt solution, subcultured by treatment with trypsin-EDTA, and plated into 150 mm tissue culture dishes (Falcon Labware) at a cell density of 1.0×10^2 cells/cm^2. Only one-half of the culture medium was changed twice a week, until distinct colonies were identified. Thereafter, the medium was completely changed twice a week. Two to 3 weeks later, discrete colonies well separated from one another were cloned using cloning cylinders (Sigma). After cloning, the cells were expanded in number by passaging and plating at a density of 2×10^4 cells/cm^2 in successively larger tissue culture vessels. Based on their ability to proliferate continuously, five clones were selected and used in this study.

Neural differentiation

The cells were maintained beyond passage 30. To induce the neuronal differentiation, the cells at 60–70% confluence were washed with Dulbecco's modified Eagle's medium (DMEM) and thereafter cultured in DMEM containing 10% FBS, 100 ng/ml FGF-2, 10 ng/ml FGF-8, 10 ng/ml EGF, 10 ng/ml BDNF, and N-2 supplement (Invitrogen, Carlsbad, CA) for 2 to 7 more days. Neural differentiation was detected by the elongation of cellular processes.

Immunocytochemistry

The cells were seeded in two-well glass chamber slides. At 60–70% confluence, the cells were washed with DMEM and thereafter cultured in neural differentiation medium or control medium as mentioned. After 72 hours, the cells were fixed in 4% paraformaldehyde in PBS for 20 minutes. After 30-minutes incubation with 0.1% Triton X-100-PBS, the cells were blocked with Protein Block for 1 hours, incubated with rabbit anti-neurofilament 200 antibody in 1% BSA-containing PBS for overnight at 4 °C, and then washed 4 times with PBS and incubated with FITC-conjugated affinity-purified anti-rabbit IgG antibody (Sigma) for 1 hours at RT. The slides were mounted after nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma). The staining patterns were observed with fluorescence microscope (Nikon; Tokyo, Japan). The specificity of the immunoreaction was confirmed by incubation with rabbit IgG instead of the primary antibody.

Electrophysiology

Extracellular electrical activity was recorded in differentiated BMSCs using glass microelectrodes filled with 2 mol/l potassium acetate. The microelectrodes

were pulled from a 1.5 mm O.D. capillary glass on Brown-Flaming puller (model P-97; Sutter Instruments, Novato, CA) and selected to have resistance in the range between 20 and 40 MΩ. Extracellular field potentials were recorded with glass microelectrodes placed on neuronal cell bodies judged by visual placement of the recording pipette. Field potentials as well as membrane potential fluctuations were recorded and analyzed using a MEZ8301 amplifier (Nihon Koden, Tokyo, Japan) and SuperScope II data analyzing software (GW Instruments, Somerville, MA). NGF (Sigma) was applied to the cell by bath application at a concentration of 100 ng/ml.

Data analysis

Data are presented as mean ± standard deviation (SD). Differences among groups were analyzed using the two-way repeated-measures ANOVA. p-value <0.05 was considered as the borderline of statistic significance.

RESULTS

Within 2 days after neuronal differentiation, changes in the morphology of the BMSCs (Figure 1A) were apparent. Differentiated BMSCs progressively assumed neuronal morphological traits. Cytoplasm in the flat BMSCs retracted toward the nucleus, forming a contracted multipolar cell body, leaving membranous, process-like extensions peripherally. Cell bodies became increasingly spherical and refractile, exhibiting a typical neuronal perikaryal appearance. Neurite-like processes continued to elaborate, displaying primary and secondary branches, growth-cone-like terminal expansions and putative filopodial extensions (Figure 1B).

To confirm the probability of differentiation of BMSC into neural cells, the expression of a neuron-specific intermediate filament, neurofilament, was examined by immunocytochemistry. Immunocytochemistry revealed that the expression of neurofilament was detected in all of differentiated BMSCs (Figure 2A–C). In control, undifferentiated BMSCs showed no expression of neurofilament in any cells (Figure 2D–L).

To examine the electrophysiological properties of BMSC-derived neurons, we recorded the field potentials extracellularly after 36–42 hours of differentiation. Under these conditions, most cells showed spontaneous discharges up to 2 mV. In subconfluent cultures where cell-cell contacts were noted, these BMSC-derived neurons also generated action-potential-like electrical activity spontaneously, which showed fast biphasic and discrete potentials (Figure 3A). No spontaneous discharges were recorded in undifferentiated BMSCs. Taken together, the results indicate that differentiated BMSCs are probably neurons and form synapses.

We have examined the effects of NGF on neural activity in BMSC-derived neurons. β-NGF was purified from mouse submaxillary glands and used at a final concentration of 100 ng/ml as described by Toledo-Aral *et al.* (1995). Amplitudes of field potentials increased gradually, as early as 1 min after the application of NGF, and peaked after 3 min (Figure 3B). At this point, the amplitudes of field potentials became about 20-fold of that before treatment (Figure 4). However, the enlargement of amplitudes did not persist for a long time. The amplitudes of field potentials diminished gradually and NGF's effects disappeared after 5 min (Figures 3B and 4). After NGF's effects disappeared, a concentration of 100 ng/ml was applied to the culture again. The amplitudes of field potentials, similar to the case in the first application, also increased up to 3 min and then diminished gradually, disappearing after about 5 min.

DISCUSSION

BMSCs acquired morphological features of neurons under specific conditions and BMSCs-derived neurons expressed high levels of a neuron-specific intermediate filament, neurofilament, in immunocytochemistrical study. However, it is necessary to demonstrate their functionality, because it is not clear whether these BMSCs-derived neurons acquire the electrical neuronal activity. Tondreau *et al.* (2004) found that bone marrow-derived mesenchymal stem cells already express specific neural proteins such as Nestin before differentiation. In the present study, BMSC-derived neurons generated action potential-like activities spontaneously although no spontaneous discharges were recorded in undifferentiated BMSCs, suggesting that these BMSC-derived neurons possess properties of functional neurons.

Neurotrophic factors are essential for modulating activity-dependent neuronal plasticity (Poo 2001). There is experimental evidence that neurotrophins elicit action potential and synaptic stimulation in central neurons. Blum *et al.* (2002) also showed that BDNF rapidly depolarizes neurons by activating a tetrodotoxin-insensitive sodium channel Nav1.9. Rapid neurotrophin-evoked excitation of neurons by TrkB-Nav1.9 causes prominent calcium transients through the opening of voltage-gated calcium channels in the plasma membrane. The rapid calcium signals lead to the activation of as-yet-unknown signal transduction pathways and ion channels such as transient receptor potential channel (Moran *et al.* 2004), and enhance concomitant activation of NMDA-receptor channels by synaptically released glutamate. Interestingly, NGF enlarged not the frequency but the amplitude of field potentials in the present study. Neurotrophins such as BDNF, in general, increase the frequency of action potentials by depolarizing neurons by activating sodium ion conductance (Kafitz 1999). Thus, electrical activity recorded from BMSC-derived neurons in the present study seems to show the response of synaptic transmission.

NGF's effect was rapid, appearing within 1 min after it was applied, peaked at 3 min, and disappeared at 5

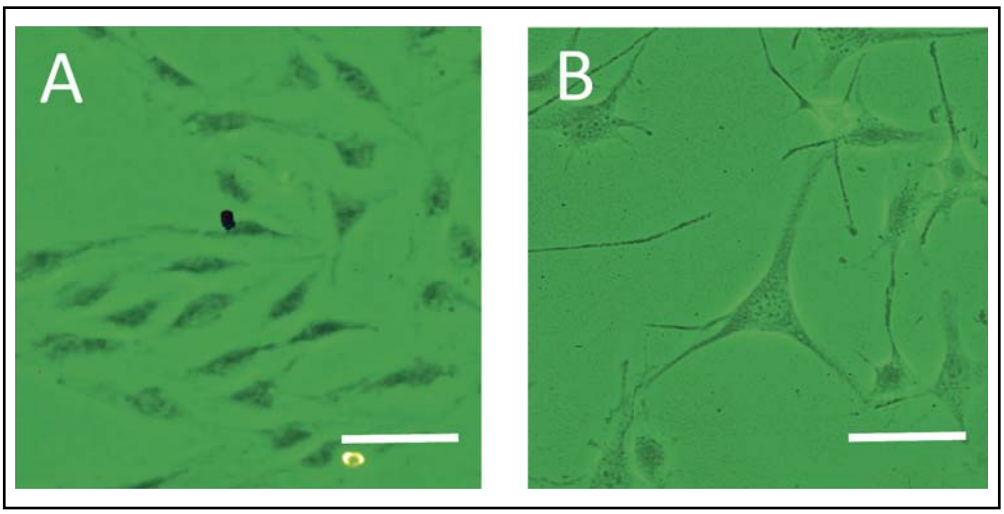

Fig. 1. Neuronal differentiation of BMSCs. (A) Undifferentiated BMSCs. (B) BMSC-derived neurons display condensed cell bodies and highly branched processes. Scale bar = 50 µm.

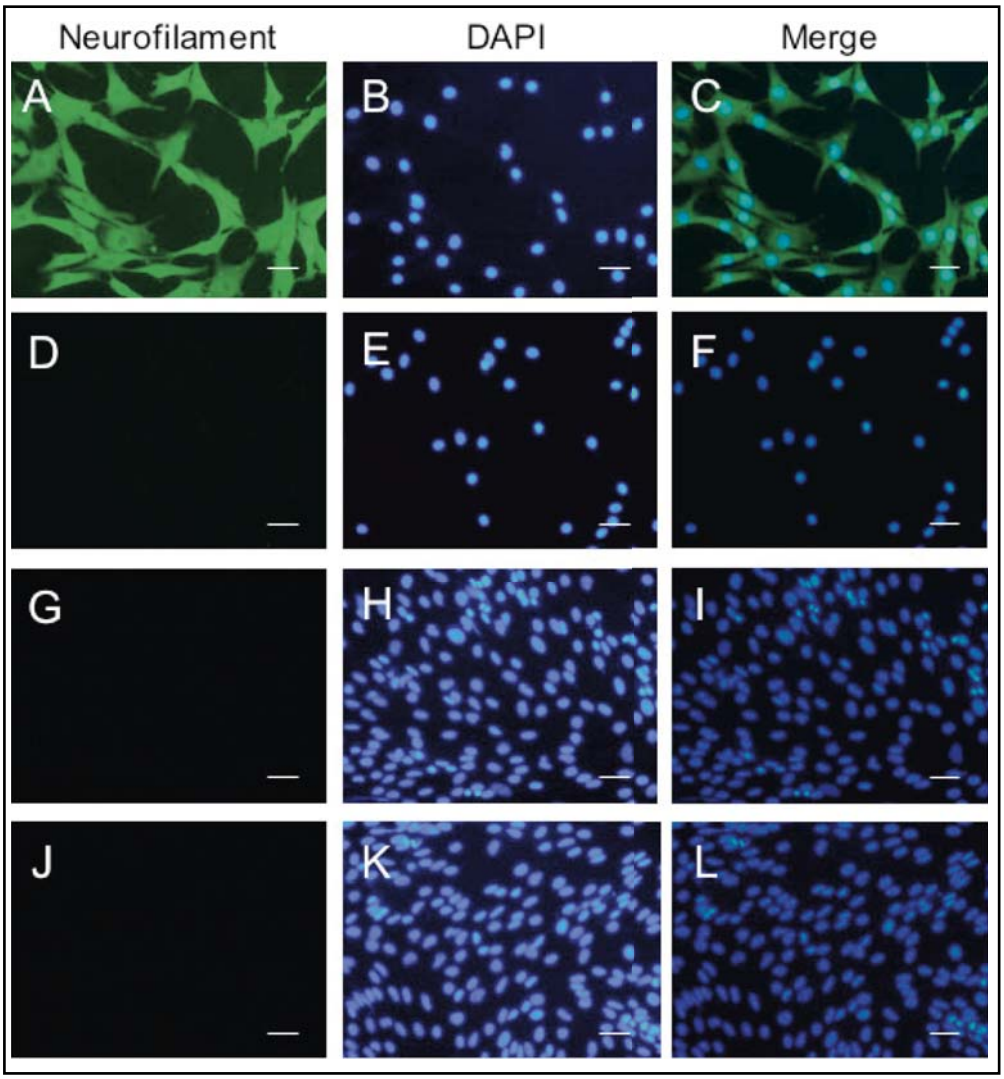

Fig. 2. Expression of neurofilament and DAPI by BMSC-derived neural cells or control BMSC cells. A–F: BMSC-derived neurons. G–L: Undifferentiated BMSCs. A–C, G–I: Neurofilament. D–F, J–L: normal rabbit IgG. BMSC-derived neurons (A–C) showed expression of neurofilament in all cells. Besides, in control cells (D–F) and undifferentiated BMSCs (G–L), no expression of neurofilament was observed. All of cells were easily seen by DAPI staining. Scale bar = 50 µm.

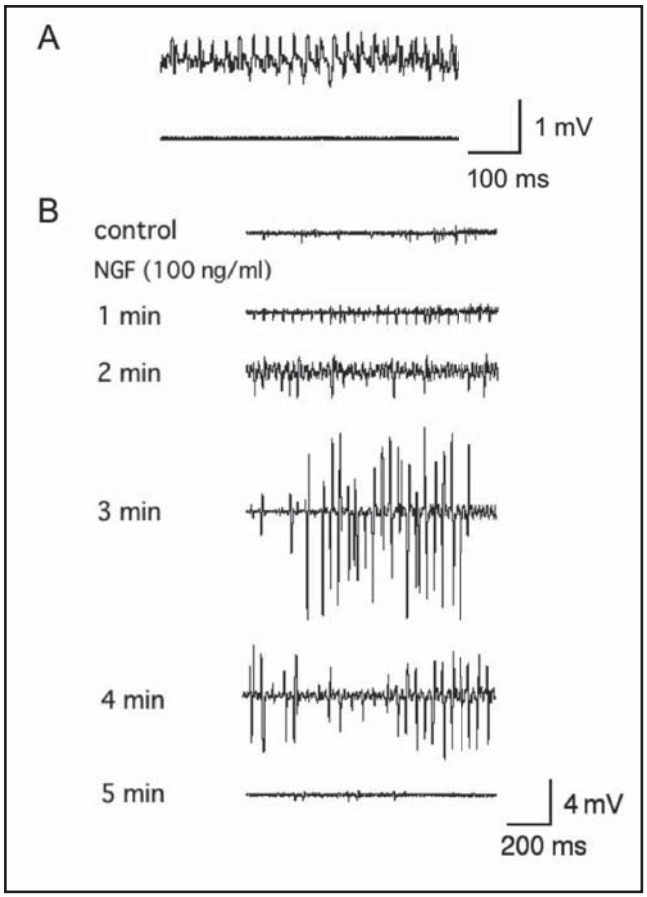

Fig. 3. NGF's effects on neuronal electrical activity in BMSC-derived neurons. (A) Action potential-like electrical activity acquired from BMSC-derived neurons. The top trace shows an example of spontaneous action-potential-like discharges recorded from BMSC-derived neurons. These potentials show fast biphasic and discrete field potentials. The bottom trace shows the potential out of cell touch. Scale bars, 1 mV and 100 ms. (B) Shown is an example of electrical recordings before (control) and after bath application of NGF in BMSC-derived neuron. As soon as NGF was applied at a final concentration of 100 ng/ml, the amplitudes of field potentials increased gradually, peaking after 3 min. Then, the amplitudes of field potentials diminished gradually and NGF's effect disappeared after 5 min. Scale bars, 4 mV and 200 ms.

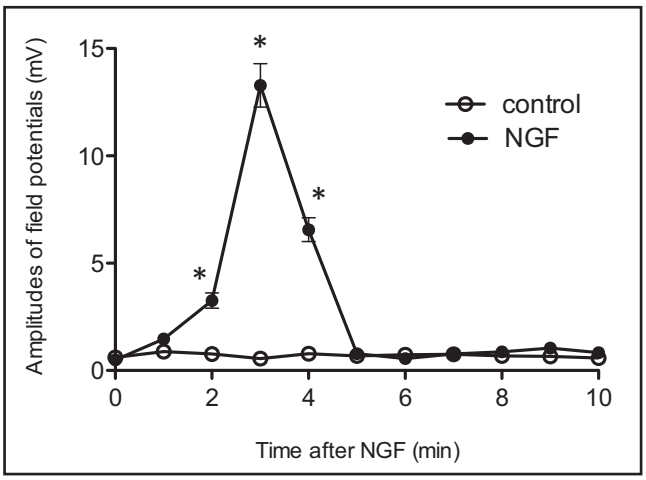

Fig. 4. Amlitudes of the field potentials in differentiated BMSCs measured at the indicated times after the treatment with NGF. The closed circles indicate NGF-treated cells, and the open circles indicate nontreated differentiated BMSCs (control). The values shown are the mean ± SD (n = 23). The differences between control and cells treated with NGF are significant (asterisk, $p<0.05$) according to the two-way repeated-measures ANOVA.

min in the present study. Although BDNF causes very rapid membrane depolarization in the low-millisecond range (Kafitz 1999), NGF slowly elicits neuronal excitability through the sodium channel from 3 hr to 24 hr after 1 min of NGF treatment (Toledo-Aral *et al.* 1995). However, Carter *et al.* (1995) demonstrated that the addition of NGF in cultured sympathetic neurons increased the formation of Ras-GTP rapidly, peaking at 2 min, followed by a quick decline to the same level as in nonstimulated cells. The most rapid biochemically detectable tyrosine phosphorylation of Trk receptors after neurotrophin administration is in the range of 30 seconds (Kahle *et al.* 1994). Rose *et al.* (2004) also showed that rapid neurotrophin-evoked neuronal activity may regulate neurotrophin-evoked intracellular signaling cascades that occur on the time scale of seconds to minutes. In addition, NGF acutely increases the amplitudes of voltage-sensitive calcium currents within 3–5 min, and this increase is partly mediated by protein kinase Cα phosphorylation (Jia *et al.* 1999). Taken together, these findings indicate that NGF's rapid action in the present study may result from the enhancement of glutamatergic synaptic transmission by the increase of intracellular Ca^{2+} via the stimulation of an as-yet-unidentified signal transduction pathway through TrkA tyrosine kinase or the activation of a voltage-sensitive calcium channel.

In conclusion, the data presented here suggest that neuronal cells derived from murine bone marrow stromal cells generated action-potential-like electrical activities spontaneously and that the neurotrophin NGF induces a rapid and transient increase in the electrical activity of BMSC-derived neurons.

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