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Letter to Neuroscience

DISTANCE OF TARGET SEARCH OF ISOLATED RAT HIPPOCAMPAL NEURON IS ABOUT 150 μm

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Although the survival of neuronal cells is highly dependent on neural connections with afferents or targets,[10,14,15] little is known about the survival of immature neurons that have not yet encountered the partners. Herein, using cultures of isolated hippocampal neurons of rat embryos, we have attempted to elucidate the contribution of neurite outgrowth to neuron survival and found that neurons died at a certain degree of neurite length with apoptotic characteristics in cases of no contact with other neurons. The threshold was 143.4 μm, which was about five times as long as the cell body diameter. It was altered by depolarization or in the presence of basic fibroblast growth factor. Thus, neurons may be designed to kill themselves if they cannot find their targets after exploration within a particular area, the extent of which is variable due to cellular conditions. © 2000 IBRO. Published by Elsevier Science Ltd.

At the beginning of this study, we noticed that survival rate of isolated neurons strictly depended on culture density (Fig. 1, closed circle). When the density was higher than 10,000 cells/cm^2, the ratio of the number of surviving neurons at four days *in vitro* (DiV) to the number of initially-dispersed cells was constantly about 30%. However, lower culture densities (<5000 cells/cm^2) dramatically decreased the survivability. We computed the average interval of neurons, which was calculated by $10^4/\sqrt{\text{density}}$ μm (Fig. 1, broken line). The estimated neuron–neuron distance appears to correlate inversely with the survival rate; larger spacing between neurons caused less survivability. This suggests that the distance of target search of a developing neurite might be finite and restricted.

We next monitored neuron survival and neurite elongation in a low culture density so that neurons would not be able to make connections with others. Incidentally, under these conditions, neurons that succeeded in contacting other neurons had a better survival rate (37.5%, $n=56$) as compared with the neurons that failed to meet others (20.9%, $n=91$) ($\chi^2=5.103$, $P=0.024$), which again suggests that the survival is regulated at least in part by connections with other target neurons. Thus, the neurons that had not made connections with others were used in the following experiment. A representative morphological change of the neuron is shown in Fig. 2A. This neuron gradually extended neurites until three DiV and underwent degeneration at four DiV. The data in Fig. 2Ba show the survival rate at two, three and four DiV; the neurons were divided into three groups: Group 1, neurons that died by three DiV; Group 2, neurons that died by four DiV; and Group 3, neurons that survived up to and after four DiV. Changes in the neurite length are shown in Fig. 2Bb. Group 1 possessed initially long neurites but subsequently died. Group 2 possessed elongated long neurites and thereafter died. This is strongly suggestive of a threshold in neurite length for death of approximately 150 μm. In this context, the neurite length of Group 3 was always less than the putative threshold. Hence, we attempted to calculate the threshold more accurately.

Neurite lengths of 266 neurons, randomly selected at two, three and four DiV, are plotted as a cumulative frequency distribution (Fig. 2Bc), which closely conforms to a unimodal Gaussian distribution ($\mu=88.19$, $\sigma=55.23$). Thus, we defined the neurite threshold for death as the inflexion point of a decaying phase of the normal probability distribution because frequency in the distribution decreases at an accelerating rate at this point. For the inflexion point ρ in Gaussian distribution $\bar{P}$,

$$\bar{P}'' = \frac{1}{\sigma^3\sqrt{2\pi}}\,e^{-\frac{1}{2}\left(\frac{\rho-\mu}{\sigma}\right)^2}\left\{\left(\frac{\rho-\mu}{\sigma}\right)^2-1\right\}=0$$

in which case,

$$\int_{-\infty}^{\rho}\frac{1}{\sigma\sqrt{2\pi}}\,e^{-\frac{1}{2}\left(\frac{x-\mu}{\sigma}\right)^2}dx \cong 0.8414 \qquad (84.14.\%).$$

We then estimated ρ as the point 84.14% in the ordinate in Fig. 2Bc. The value of ρ was 143.4 μm, which is indicated by a dotted line; it is also superimposed as a dotted line onto Fig. 2Bb. It should be noted that the neurite did not grow beyond this threshold. The value is likewise superimposed onto Fig. 1 (indicated by a dotted line). At a neuron interval of 143.4 μm, the culture density was estimated to

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Abbreviations: bFGF; basic fibroblast growth factor; DiV, days *in vitro*; MAP2, microtubule-associated protein.

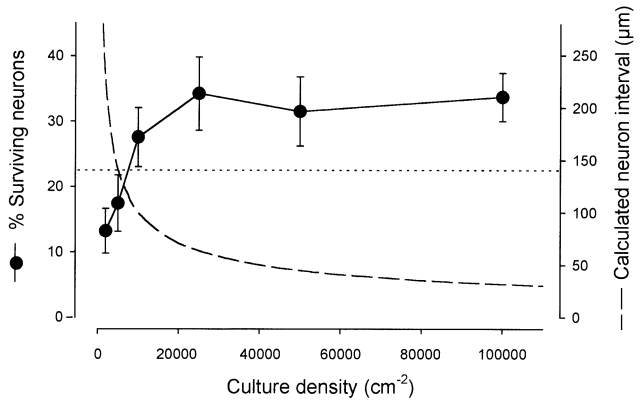

Fig. 1. Relationship between initial culture density of dispersed neurons and survival rate (closed circles). Estimated correlation of culture density with average neuron–neuron distance was superimposed as a broken line. A dotted line indicates the threshold of neurite length for degeneration (143.4 μm, see text). Note that there seems to be a strict correlation between neuron interval and survival ratio and that two relationship curves intersect almost at the threshold. Data represent mean ± S.E.M. of five to nine cases.

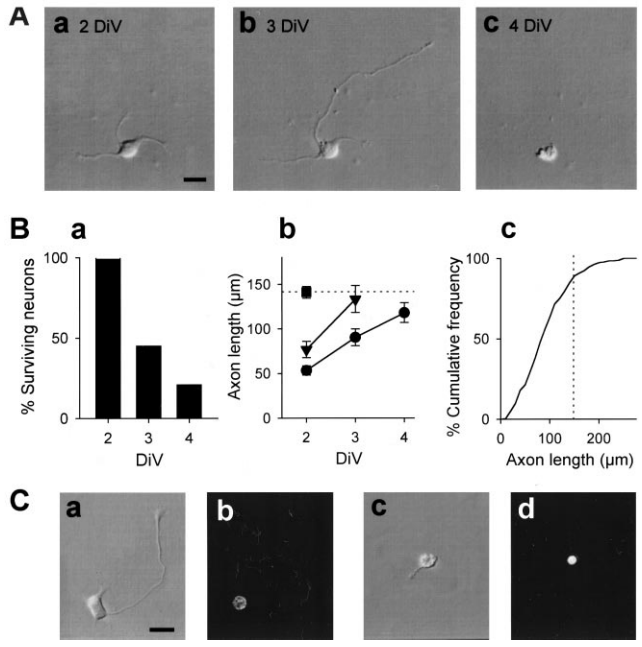

Fig. 2. (A) Hoffman modulation photomicrographs of one neuron were obtained at two (a), three (b) and four DiV (c). It gradually extended neurites until three DiV and died at four DiV. (Ba) Survival rate at two, three and four DiV is shown as a percentage of the value at two DiV ($n = 91$). (Bb) Neurons were divided into three groups, i.e. neurons that died by three DiV (closed squares), neurons that died by four DiV (closed triangle) and neurons that survived until four DiV (closed circles), and changes in the neurite length are shown. Data represent mean ± S.E.M. of 19–50 cases. (Bc) The distribution of neurite length for data obtained from 266 neurons. The cumulative frequency refers to the proportion of the total events. The threshold of neurite length for degeneration (143.4 μm) is indicated by a dotted line in (Ba) and (Bc) (see text). Note that neurites in any group did not cross over the threshold. (C) Hoffman modulation images (a,c) or acridine orange fluorescent images (b,d) of a live neuron (a,b) or a degenerated neuron (c,d) at four DiV. (b) and (d) were obtained from the same microscopic fields of view as (a) and (c), respectively. Note that the neuron with developing neurite displayed normal nuclear morphology and that the chromatin of the dead neuron was highly condensed. Scale bars represent 20 μm.

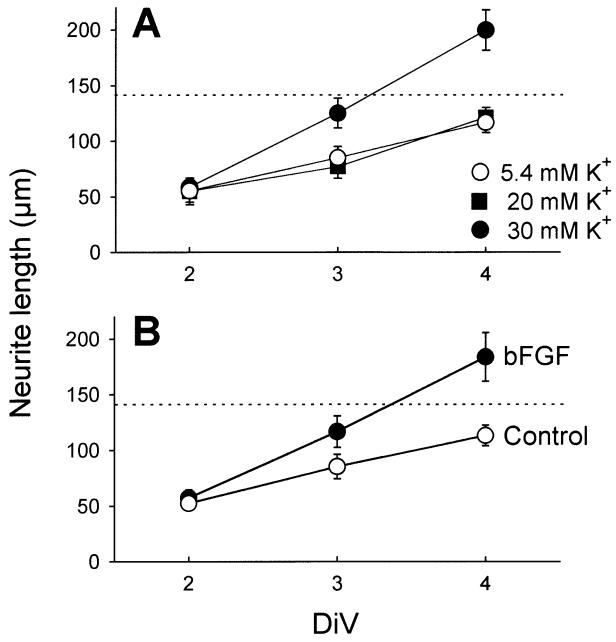

Fig. 3. Facilitatory effect of K^+ depolarization (A) or bFGF (B) on neurite outgrowth. Neurons were cultivated in high K^+- or bFGF-containing medium from two DiV to four DiV. (A) Neurite elongation in the presence of 30 mM K^+ was significantly facilitated as compared to that in normal K^+ concentration (5.4 mM) [$F(2,99) = 15.07$, $P < 0.001$: two-way analysis of variance; $Q(3,99) = 6.84$, $P < 0.01$: Tukey's multiple range test]. (B) Neurite outgrowth of the neuron treated with bFGF (1 ng/ml) was also extensively accelerated ($F(1,66) = 13.51$, $P < 0.001$, $Q(3,99) = 5.20$, $P < 0.01$). A dotted line on each panel indicates the threshold of neurite length for degeneration (143.4 μm). Note that the neurite length after the exposure to 30 mM K^+ or bFGF crossed over the putative threshold at four DiV. Data represent mean ± S.E.M. of 10–13 cases

be 4862 cells/cm^2, which almost coincides with the critical density that severely aggravated neuron survivability. Therefore, the ρ value possibly serves as the threshold for neuronal death.

The naturally-occurring degeneration of developing neurons often exhibits apoptosis.[4,15] Because the nuclear pyknosis of dying or dead cells has apoptotic characteristics,[6,17] we elucidated the nuclear morphology of neurons which had extended neurites near or longer than the threshold, using acridine orange staining.[8] Living neurons with developing neurites displayed normal nuclear morphology (Fig. 2Ca,b). However, the condensed chromatin was observed in degenerated neurons (Fig. 2Cc,d). The same results were obtained in all tested cases ($n = 27$).

If the threshold of neurite length was categorical and unprescribable, neurons would be unable to encounter their targets located further apart than the threshold. However, the hippocampal neurons *in vivo* can find the partners even at distances of >150 μm.[1–3] This predicts that the threshold fluctuates dynamically in response to environmental or surrounding conditions. Numerous reports indicate that the rate of neuron survival is improved by neuron activity (i.e. depolarization)[7,11,18] or diverse neurotrophic and growth factors.[5,9,12] Therefore, we evaluated the effect of K^+ depolarization or basic fibroblast growth factor (bFGF) on neurite outgrowth. Neurite outgrowth was significantly driven in the presence of 30 mM K^+, and the neurite length at four DiV crossed over the threshold, while the neurite elongation of neurons cultivated in medium containing 20 mM K^+ did not differ from that in normal K^+ concentration (Fig. 3A). Chronic treatment

with bFGF (1 ng/ml) also promoted neurite extension, and the final reach of the neurite exceeded the threshold. These results suggest that cellular conditions and environmental stimuli shift the threshold.

As a novel concept, the threshold in neurite length for death has been proposed and actually determined by the behaviors of hippocampal neurons. Furthermore, we have also shown that the neurons that reached the threshold underwent apoptosis-like cell death. Thus, neurons might be forced to disappear if they failed to acquire their targets within certain restricted space, because such neurons would disturb normal development of the nervous system. From this point of view, the variable threshold may play a role in regulating survivability according to circumstances and also manipulating the behaviors of neurons individually, i.e. excluding undesired neurons with mistimed neurites, allowing neurons with particular neurites to survive, determining the extent of neurites for a subset of neurons or even canalizing neurites. Further investigations following the present study would help to explain the complicated and controversial mechanisms underlying developmental processes of the nervous system.

EXPERIMENTAL PROCEDURES

Hippocampal neurons were prepared from 18-day-old embryos of Wistar rats, as described previously,[13,16] and were cultivated in serum-free Eagle's medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with N2 hormone. The immunostaining for microtubule-associated protein indicated that >99% of cells were neurons under these conditions. For measurement of neurite length, cells were dispersed at a density of 2500 cells/cm^2. Neurons were randomly selected at two DiV and photographed using an inverted microscope. Immediately after the recording, the culture medium was changed to high K^+- or bFGF-containing medium if necessary. The medium containing 20 or 30 mM K^+ was prepared by iso-osmotic replacement of Na^+. Basic fibroblast growth factor was cotreated with 5 μM nicardipine. The same neurons were repeatedly photographed after 24 or 48 h, and their longest process was measured as neurite length.

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