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Light delays synaptic deafferentation and potentiates the survival of axotomized retinal ganglion cells

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Abstract

Knowledge of the cellular mechanism underlying the therapeutic effect of stimulation and the optimal doses of such stimulation to maximize neuronal recovery is essential to guide clinical practice in neural rehabilitation. Using hamsters, we transected the optic nerve to demonstrate how light stimulation affects neuronal recovery. The c-fos protein was used as a neuronal connectivity marker. Here we show that: (a) in addition to cell death, a population of cells undergoes synaptic deafferentation and (b) light stimulation delays cell death and deafferentation. Among the three rearing conditions studied (6:18LD, 12:12LD, and 18:6LD), the 12:12LD condition appears to be the one achieving the optimal therapeutic effect. This study provides a solid base in the understanding of the neuroanatomical changes after traumatic brain injury and the need to establish an optimal level and timing for the environmental stimulation.

Keywords: Synaptic deafferentation; Optic nerve transection; Neuroprotection; Environmental stimulation; Cognitive rehabilitation

There have been a number of studies showing the positive effect of environmental stimulation on neural plasticity [19,29,30]. To increase the likelihood of producing an enriched environment favouring neuronal reorganization and recovery, a wide number of methods have been used, including functional electrical stimulation [16,23], psychostimulants such as amphetamines and methyphenidate [6,10], and injection of cyclic AMP (cAMP) or cAMP-elevating agents such as forskolin [7,9]. For the visual system, the most logical and common form of activity-dependent environmental stimulation is delivered via the manipulation of exposure to environmental light. Indeed, environmental light stimulation has been shown to enhance the survival and axonal regeneration of axotomized retinal ganglion cells (RGC) with peripheral nerve graft in adult cats [30]. Conversely, dark rearing, which provides no stimulation, has resulted in the death of axotomized RGCs irrespective of cell type. These observations signify that a light environment is essential for the survival of axotomized RGCs and their axonal regeneration. The optimal doses of stimulation to maximize neuronal recovery, however, is a question that has not been addressed yet.

Previous research has shown that environmental enrichment can inhibit spontaneous apoptosis, prevent seizures, and be neuroprotective. Some studies have reported improvement in the cognitive performance of neurologically damaged rodents if reared in an enriched environment [32]. Cognitive stimulation is also thought to increase the connectivity of neurons in certain brain areas [12]. On a microscopic level, laboratory animals housed in enriched cages have been reported to generate more dendritic spines, possess greater synaptic contact between cells, and show functionally stronger neuronal connections than animals housed in non-enriched cages [12,19]. However, the nature or mechanism that effects synaptic connections remains unanswered.

Synaptic deafferentation has long been speculated to be an important factor affecting neural cell death. The loss of synaptic inputs into the dendrites of axotomized RGCs in the visual system is thought to be a crucial factor in the shrinkage of receptive field centres in adult cats [26]. Furthermore, detachment of presynaptic terminals from postsynaptic membranes within a week after axotomy on motor neurons has also been reported

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in electron-microscopic studies [11]. Similar findings have been revealed for neurons of the Clarke's nucleus as well as for sympathetic ganglion cells [2,20]. It is probable that the loss of synaptic inputs into the axotomized RGCs leads to changes in presynaptic arrangements and a reduction in the membrane excitability of the axotomized RGCs [26]. On the other hand, long-term changes in a synapse's strength as a result of activity have been observed [21].

A total of 132 adult male Syrian hamsters (*Mesocricetus auratus*, 6–8 weeks old, weighing 60–80 g) were used in the present study. The number of animals per group was four ($n = 4$). Food and water was provided ad libitum. Animals were kept in the experimental light intensity environment with conventional light cycle (12-h light:12-h dark) to ensure that they were reared in a consistent light–dark environment before the commencement of the experiments. In order to determine if environmental stimulation serves to stabilize synaptic input and thus prevents or delays synaptic deafferentation, a connectivity marker needs to be identified. We adopted the proto-oncogene c-*fos* as an anatomic technique for metabolic mapping [24,25]. Sagar et al. [24] have reported that the *fos* protein was transiently expressed in neurons after synaptic stimulation. Since the activation of c-*fos* can be induced by a large variety of stimuli, and immunohistochemical Fos staining is localized in the cell nucleus [24], c-*fos* activation could serve as a functional tracer to map neuronal pathways with cellular resolution [22]. Previous studies have confirmed the feasibility of using the expression of c-*fos* as an indicator for metabolic mapping and functional markers in neuronal pathway tracing [8,22]. The expression of c-*fos* protein in immunohistochemically stained RGC thus indicates the intactness of the neural synaptic pathways [18], which is the focus of this program of studies. We used a Syrian hamster optic nerve transection (ONT) model and the c-*fos* protein as the connectivity marker to conduct three experiments to study the effect of environmental stimulation, ambient light, on the maintenance of synaptic input and to investigate the dose–response relationship between the extent of stimulation and its neuroprotective effect.

The animals were anesthetized with an intraperitoneal injection of Sodium Pentobarbitone (Nembutal, Rhone Merieux Australia Pty Ltd., Australia; 50 mg/kg) for all operations. The procedures were approved by the University of Hong Kong, Committee on the Use of Live Animals in Teaching and Research in accordance with the Department of Health, Hong Kong Special Administrative Region. Unilateral ONT (right eye) at 1.5 mm from the optic disc was performed. Retrograde labelling of surviving RGCs was carried out by placing a piece of Gelfoam (Upjohn, Kalamazoo, MI) soaked with 1 μl of 6% Fluoro-Gold (FG; Fluorochrome, Denver, CO) at the ocular stump immediately after operation. Quantification of surviving RGCs was done from 2 days post ONT onwards in order to allow time for the FG to reach the soma of RGCs from the stump.

The operated animals were reared under the conditions of either complete darkness (0:24LD) or conventional light with a 12 h light:12 h darkness cycle (12:12LD). All the light environments were maintained at an average light intensity of 1071 lx, while the light intensity ranged from 818 to 1217 lx within the animal cage. The animals were randomly assigned to be reared under one of the two environmental conditions right after ONT until the day of the animals were overdosed and sacrificed at 2, 3, 5, 7, or 14 days post axotomy (dpa). The right eyes (operated eyes) of the animals were enucleated and the retinae dissected in 4% paraformaldehyde (PFA). The slides were examined under fluorescence microscopy using an ultra-violet filter (excitation wavelength = 330–380 nm). The slides were coded, the codes of which were blind to the experimenter. Following a standardized procedure [3], labelled RGCs were counted along the median line of the four quadrants starting from the optic disc to the peripheral border of the retina at 500 μm intervals, under an eyepiece grid of 200 μm × 200 μm. The mean density of labelled RGCs in each retina was then computed. To ensure inter-rater reliability, a sample of the data was rated by a second rater. Any discrepancy was discussed until a 100% agreement was achieved before data collection continued.

Environmental stimulation was found to delay the degeneration of axotomized RGCs. RGC loss was observed beginning at 3 days post axotomy (dpa) in both the dark reared (0:24LD) and the normal lighting (12:12LD) groups (Fig. 1A) of animals. Both the density and the number of surviving RGCs were statistically different between the two groups at all times studied except at 2 dpa (Fig. 1A and B).

After confirming the positive effect of environmental stimulation on the survival of RGCs, we examined how environmental stimulation could enhance RGC survival or delay RGC degeneration. The operated animals were again reared under 0:24LD or 12:12LD, with light environments maintained as described earlier. Animals were assigned randomly to one of the two conditions, right after ONT until the day of sacrifice. In this part of the experiment, the animals were killed by vertebral dislocation since anesthesia such as pentobarbital reduces or even blocks c-*fos* expression [28]. Again, the animals were sacrificed at 2, 3, 5, 7, or 14 dpa. The numbers of FG-labelled RGCs and c-*fos*-antibody-labelled RGCs were counted through the whole length of the retinal section. The number of cells exhibiting co-localization of the two parameters was recorded. Counting was carried out in three retinal sections per animal to increase the reliability of the data. Since all the retinal section used in this study contained the optic nerve stump representing a section of the central retina, the lengths of all the retinal sections were comparable [4].

Yoshida et al. [31] have reported that under the condition of 12:12LD, hybridization of *c-fos* in the inner nuclear layer as well as in the ganglion cell layer is dependent on the onset of the light period. It is, therefore, necessary to determine the time of light exposure that would produce optimal *c-fos* expression in the experimental animals. We observed that the maximum c-*fos* protein expression in the retinal ganglion layer occurs 30 min after the onset of the light period. The animals in experiment that involved immunohistochemical staining of *c-fos* were therefore exposed to 30 min of ambient light before sacrifice 30 min after the onset of the light period in the 12:12LD or the other environmental light group, namely, 0:24LD. Immunohistochemical staining was carried out subsequently, followed by the quantification of RGCs and immunohistochemically stained RGCs.

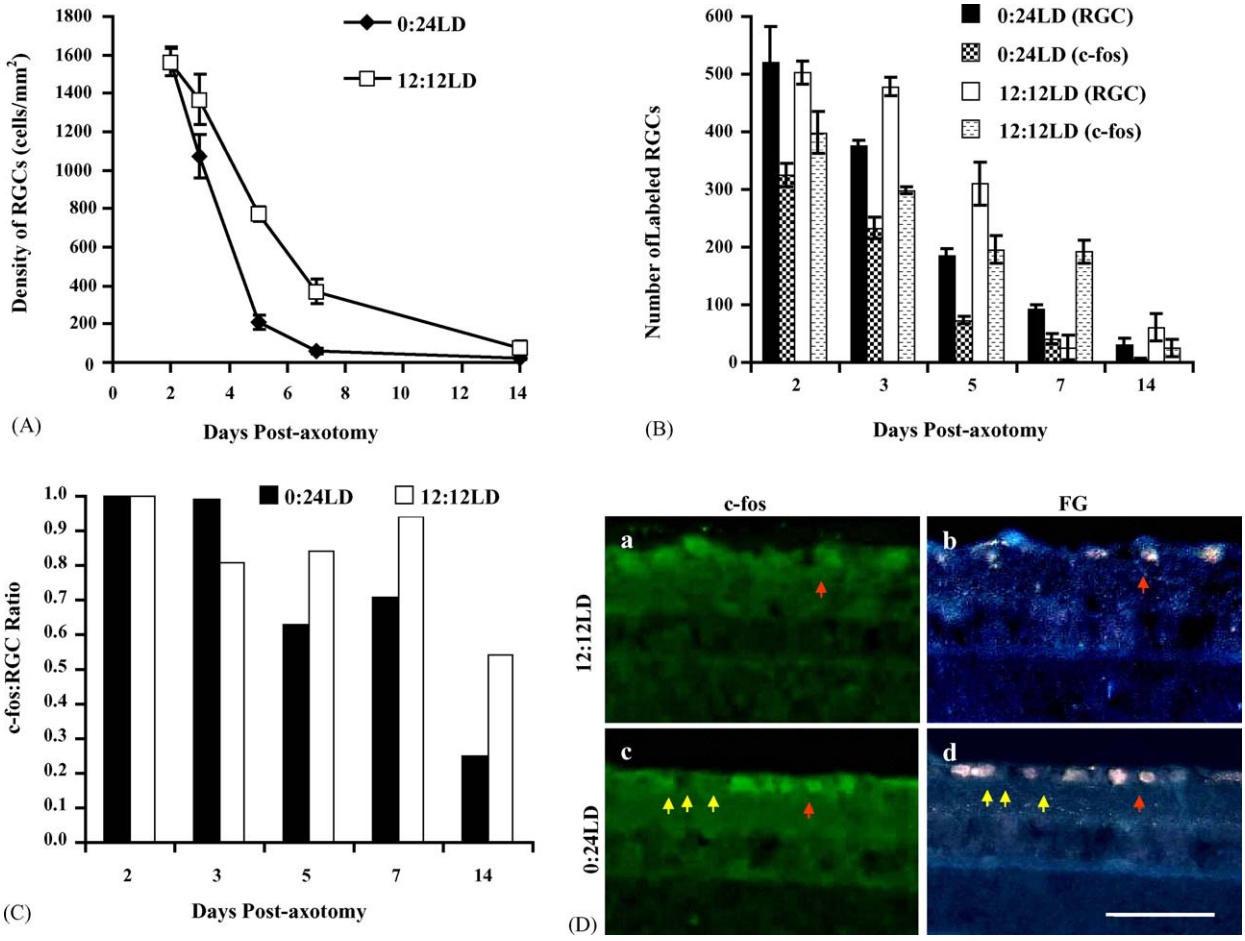

Fig. 1. Surviving RGCs in the retinas under complete light deprivation conditions following axotomy. (A) The density of the surviving RGCs of animals reared in complete darkness (0:24LD) and under conventional lighting (12:12LD) following 2–14 days of optic nerve transection. (B) The number of surviving RGCs and c-fos expressing RGCs in retinas of animals reared in completed darkness and conventional light at different days after axotomy. (C) The relative number of RGCs expressed as the ratio of c-fos reactive RGCs and FG-labeled RGCs in the dark reared (0:24LD—black bars) and in the normal lighting (12:12LD—white bars) groups of animals at 2–14 days following axotomy. Please note that there were more c-fos expressing RGCs in the 12:12LD group than that of the dark reared (0:24LD) at longer survival times. (D) Photomicrographs of retinal sections taken from 12:12LD animals 2 days post-axotomy (a and b) and from 0:24LD animals 3 days post-axotomy (c and d) showing c-fos expressing RGCs (a and c) and Floro-Gold (FG) labeled RGCs (b and d). Yellow arrows indicate surviving RGCs labeled by FG without c-fos reactivity (c, d) and red arrows indicate surviving RGCs with c-fos expression. Scale bar represents 100 μm.

We hypothesized that environmental stimulation that elevates cellular activity would prevent synaptic deafferentation and keep the efferent connectivity intact, thus preventing the injured neurons from dying (Fig. 2D). Therefore, as suggested by the results of our c-*fos* study, RGC survival is associated with synaptic connectivity. The numbers of both surviving RGCs as revealed by retrograde FG-labelled and c-*fos*-labelled RGCs in animals reared under 12:12LD were significantly larger than those in animals reared in complete darkness (0:24LD) at 3 dpa ($p < 0.05$), 5 dpa ($p < 0.05$), and 7 dpa ($p < 0.05$) (Fig. 1A and B). From 5 dpa onwards, the ratio of *c-fos* containing RGC to the number of surviving RGC was much higher in the 12:12LD than in 0:24LD groups (Fig. 1C). Some surviving RGCs (FG labelled) and *c-fos* expressing RGCs are shown in Fig. 1D.

In order to examine the dose response relationship between the extent of stimulation and its neuroprotective effect, we conducted the third experiment where the animals with transected ON were reared in four different light dark cycles (0:24LD, 6:18LD, 12:12LD, and 18:6LD) and the number of surviving RGC and number of *c-fos* expressing RGC were examined on 5 and 7 dpa. There was a significantly larger number of surviving RGCs among animals reared under the condition of 12:12LD than among animals reared under the conditions of 0:24LD ($p < 0.001$), 6:18LD ($p < 0.005$), and 18:6LD ($p < 0.05$) at 7 dpa. No significant differences in the surviving number of RGCs between the 0:24LD, 6:18LD, and 18:6LD groups at 5 dpa were observed (Fig. 2A). The number of c-*fos*-expressed RGCs was significantly larger in the 12:12LD group than in both the 6:18LD and 18:6LD groups at 5 dpa ($p < 0.05$ and $p < 0.001$) and at 7 dpa ($p < 0.005$ and $p < 0.001$) (Fig. 2B), while the 0:24LD group was having the smallest number of expressed RGCs. Correlation analysis of the number of surviving RGCs and c-*fos*-expressed RGCs showed that they were significantly correlated ($r = 0.972$, $p < 0.001$), while regression analysis revealed that c-*fos* expression in RGCs is a good predictor of the number of surviving axotomized RGCs ($R^2 = 0.918$, $p < 0.001$) (Fig. 2C).

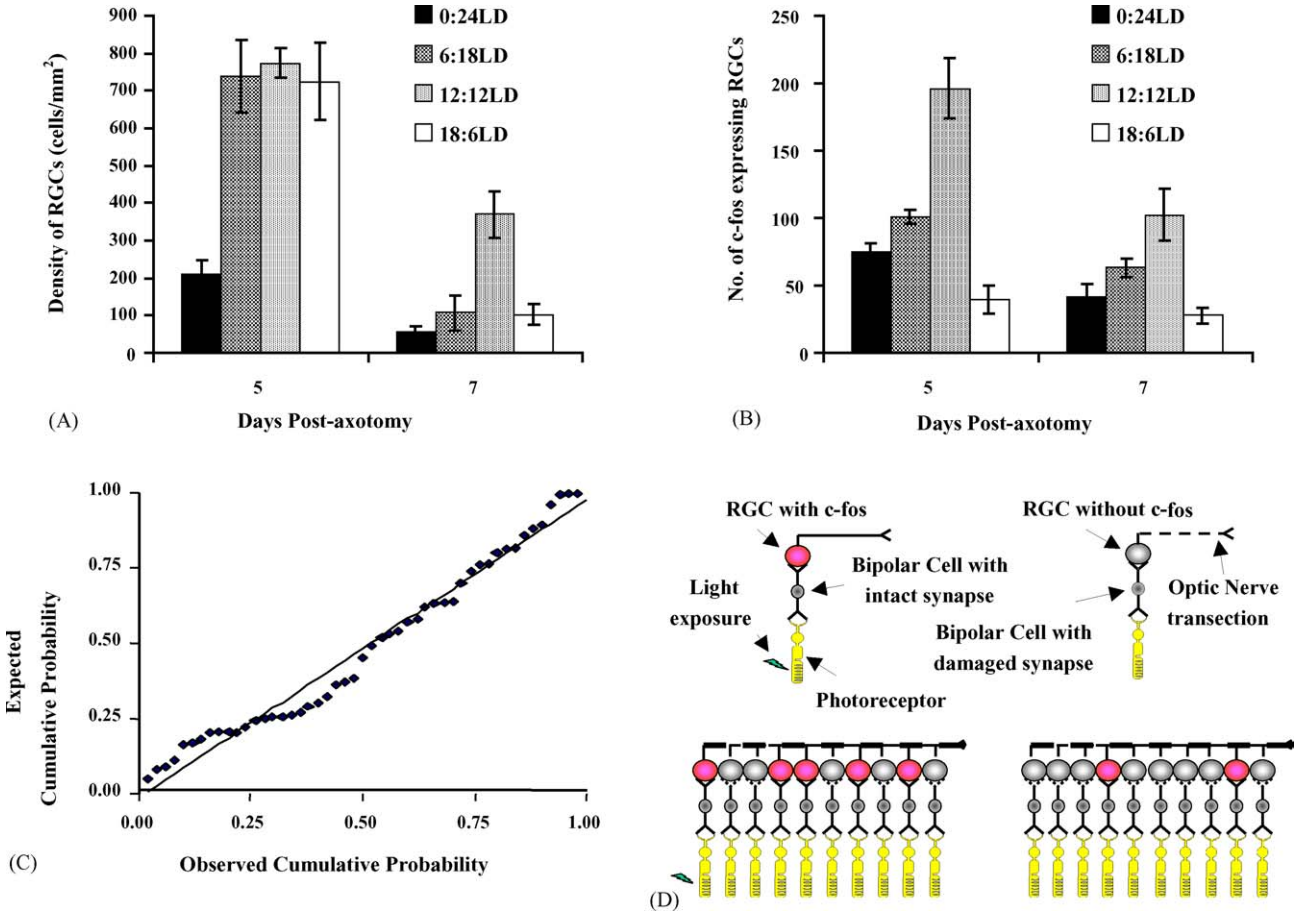

Fig. 2. Surviving RGCs in the retinas under various lighting conditions following axotomy. (A) The density of surviving RGCs reared under complete darkness (0:24LD), 6 h light and 18 h dark cycle (6:18LD), conventional light condition (12:12LD) and 18 h light and 6 h dark cycle (18:6LD) 5 and 7 days following optic transection. (B) The number of c-fos expressing RGCs under various lighting cycles 5 and 7 days post-axotomy. (C) Normal probability plot of regression showing the correlation between the observed number of c-fos expressing RGCs and the expected number of surviving RGCs. (D) Schematic diagram to illustrate c-fos as a functional marker to map synaptic connections or deafferentations in RGCs at two lighting conditions. The proportion of c-fos expressing RGCs shown refers to the results obtained from animals 5 days post-axotomy.

From our results, we found that the absence of light stimulation causes RGC death after axotomy at a faster rate than a conventional light cycle, especially at 3, 5, and 7 dpa. This finding clearly indicates the important role played by environmental stimulation in delaying the degeneration of injured RGCs. Furthermore, this therapeutic effect was observed to correlate with *c-fos* evidence of the synaptic connectivity of RGCs with bipolar and photoreceptor cells. Light appears to help preserve synaptic connection and hence maintain connectivity among cells. The possibility of widening the window of recovery in injured neurons by delaying their death, as suggested by our findings, has further clinical implications for it allows more time than usual for effective neuro-rehabilitation intervention to take place.

Under the condition of 12:12LD, we observed that the surviving RGCs started to decrease in number only from 3 dpa onwards. Cheung et al. [3] showed that this 3-day delay in RGC death is caused by the activation of an endogenous neuroprotective pathway. In fact, Akt and phosphor-Akt (pAkt) can be sustained for 3 days, after which RGC death will commence. However, in the 0:24LD (dark reared) group, a significant decrease in surviving RGC numbers was observed at 3 dpa in comparison with 2 dpa, indicating that RGC death has already been initiated before 3 dpa. One possible explanation is that a different endogenous neuroprotective pathway or a different duration of endogenous neuroprotection was recruited in these two groups of hamsters, which received different environmental light stimulation.

The expression of c-*fos* was shown to correlate with the number of surviving RGCs and provides a good predictor of the survival of axotomized RGCs after environmental enrichment. In the present study, we used the expression of c-*fos* as a marker of the intactness of synaptic connections and tested the hypothesis that synaptic connectivity correlated with neuron survival within the visual pathway. In this study, we observed significantly more c-*fos* expression and surviving RGCs in the group of hamsters reared under the condition of 12:12LD than in the other three groups. We conclude that environmental stimulation in the form of 12:12LD light cycle can maintain neural synaptic connectivity integrity, which correlates with a higher survival rate of axotomized neurons. This is important because of its implications for the understanding of, and for future research on, the neural mechanism of the survival of injured neurons.

A peak level of axotomized RGC survival occurred when the animals were reared under the condition of 12:12LD at 7 dpa, suggesting an optimum rather than a dose-dependent positive effect of environmental stimulation on injured neurons. Stimulation of 12:12LD is therefore considered to be the optimum dose for maximizing neuronal survival. There were fewer surviving RGCs in the 6:18LD group than in the 12:12LD group. This could be explained by the insufficient stimulation that limited the prevention of synaptic deafferentation, thus leading to more RGC deaths. This speculation is further supported by the lower expression of c-*fos* in the 6:18 group than in the 12:12LD group. On the other hand, an unexpected low RGC survival rate was observed in the 18:6LD group. A higher dose of environmental stimulation did not yield a higher survival rate among neurons. An alternative explanation would be that the content and patterning of the light offered in the 12:12LD condition enables it to achieve optimal therapeutic effect. These speculations await verification in future research.

Taking all these observations together, we conclude that there exists a non-linear function of stimulation that has a neuroprotective effect, and that there is an optimum dose of stimulation, which is species and/or injury specific, that maximizes its effect. This observation is significant both to neural recovery research and to clinical practice as it suggests the importance of identifying and then implementing this optimum dose of stimulation to facilitate maximum neural recovery. Following this line of thought, we speculate that phototoxicity might play a role in the unexpectedly low RGC survival rate in the 18:6LD group. Indeed Jin et al. [13] reported that any exposure to light with an intensity of more than 100 lx can cause light damage to the retina, while constant exposure to bright light has frequently been shown to induce photoreceptor degeneration [1,17]. With up to 3 days constant exposure to ambient bright light (2500 lx), rats with intact visual system would have the total number of photoreceptor cells decreased. Other cell types in the retina also exhibit different extent of apoptotic cell death [5]. c-*fos* expression was found to be much lower in the 18:6LD group than in the 12:12LD group at both 5 and 7 dpa ($p = <0.001$). This result correlates well with the speculation that photoreceptor cell death prevents the transmission of activity signals to target RGCs and prevents expression of the c-*fos* proto-oncogene.

To conclude, this study has verified two important concepts: (1) environmental stimulation that elevates neural cell activity is essential to delay neural cell degeneration via mechanisms that prevent synaptic deafferentation, and (2) there exists a non-linear relationship between environmental stimulation and its therapeutic effect. The existence of a critical period and an optimal dose for maximum therapeutic effect is indicated. In other words, therapeutic intervention at an optimal dose delivered during the critical period should generate favorable outcomes of neuro-rehabilitation. Furthermore, given that synaptic connection is one of the means of preventing the death of injured cells, further methods of reconnecting the neural pathway or preventing synaptic deafferentation within the expanded window should be considered. Scientists have tried to design neurocognitive programs for improving functional outcomes after various neurological conditions (e.g. [14–15,27]). Generalizing our findings to human subjects, we now understand one of the avenues by which environmental stimulation promotes neural recovery in patients with brain impairments. We have also learned that the treatment effect does not necessary increase with an increasing dose of stimulation. Rather, it is important to administer the optimum dose of stimulation to maximize the treatment effect. The next research step should be to identify the range of optimum doses of stimulation for different kinds of neuronal damage caused by different neurological conditions.

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