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ORIGINAL RESEARCH

An In Vitro Model for Conditioning Lesion Effect

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Abstract
Axons of a peripheral nerve grow faster after an axotomy if it attains a prior injury a few days earlier. This is called conditioning lesion effect (CLE) and very much valued since it may provide new insights into neuron biology and axonal regeneration. There are established in vivo experimental paradigms to study CLE, however, there is a need to have an in vitro conditioning technique where CLE occurs in a maximally controlled environment. Mouse primary sensory neurons were isolated from lumbar 4–5 dorsal root ganglia and incubated at 37 °C on a silicon-coated watch glass that prevents cell attachment. After this conditioning period they were transferred to laminin coated culture dishes. Similar cultures were set up with freshly isolated neurons from control animals and from the animals that received a sciatic nerve cut 3 days earlier. All preparations were placed on a live cell imaging microscopy providing physiological conditions and photographed for 48 h. Axonal regeneration and neuronal survival was assessed. During the conditioning incubation period neurons remained in suspended aggregates and did not grow axons. The regeneration rate of the in vitro conditioned neurons was much higher than the in vivo conditioned and control preparations during the first day of normal incubation. However, higher regeneration rates were compromised by progressive substantial neuronal death in both types of conditioned cultures but not in the control preparations. By using neutralizing antibodies, we demonstrated that activity of endogenous leukemia inhibitory factor is essential for induction of CLE in this model.

Keywords Conditioning · In vitro · Neuron culture · Axon regeneration · Degeneration · Leukemia inhibitory factor · LIF

Introduction

We know for more than a century that unlike the central nervous system, regeneration of severed fibers is possible after an injury in the peripheral nerves. Interestingly, if a peripheral nerve receives two subsequent lesions, the first one strongly enhances regeneration after the second one. This phenomenon is called "conditioning lesion effect (CLE)" (Forman et al. 1980; McQuarrie et al. 1977; Thomas 1970). Understanding CLE has been highly valued since it may potentially contribute to develop strategies to encourage regeneration in the central nervous system, to enhance it in the periphery, and even to prevent neurodegeneration (Franz et al. 2009).

The most obvious result of CLE is the acceleration of axonal growth; which is probably due to increased protein synthesis (Jacob and McQuarrie 1993). Indeed, while regeneration after a standard injury can occur with existing protein pool, at least during the first few days, CLE requires transcription of new genes that takes place during conditioning period (Smith and Skene 1997). Two successive conditioning lesions had more stimulatory effect on axonal growth than a single one (Sjoberg and Kanje 1990a). Both central and peripheral axons of an in vivo conditioned neuron have enhanced regeneration suggesting that CLE does not only locally support the cut fibers but involves the whole cell, transforming it from transmission to growth mode (Mar et al. 2014).

Mechanism and mediators of CLE have been studied for decades, but the picture is still not complete. One important issue is the contributions of peripheral and central signals to CLE. This requires understanding the roles of inflammatory mediators, macrophages, lack of target derived factors and

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central and peripheral glial cells. When conditioning and test lesions were made at the same place, CLE was stronger, suggesting that lesion environment contributes to acceleration of axonal growth (Sjoberg and Kanje 1990a, b). Schwann cells from conditioned nerves support high regeneration rates in control axotomies (Torigoe et al. 1999) which may be related to high amount of laminin and fibronectin deposited in extracellular matrix during conditioning period (Ekstrom et al. 2003; Gardiner et al. 2007; Kwon et al. 2015). It was suggested that even chemical demyelination in a peripheral nerve is enough to induce a conditioning—like effect (Hollis et al. 2015). Although these findings suggest a peripheral induction and maintenance of CLE, central induction also seems possible. For example inducing inflammation in dorsal root ganglia (DRGs) produces a conditioning—like effect, which is thought to be mediated by satellite glial cells around the neurons (Lu and Richardson 1991).

High regeneration rates due to CLE are often attributed to acceleration of the axonal transport of structural proteins (Mar et al. 2014; McQuarrie 1986; Redshaw and Bisby 1987). Interestingly, a decrease in neurofilament expression following the test lesion was suggested to facilitate transport of tubulin by less interference (Tetzlaff et al. 1996).

cAMP is thought to play an essential role for CLE, especially in the spinal sensory fibers but not accountable for all of the improvements in regeneration (Blesch et al. 2012; Han et al. 2004; Knott et al. 2014; Qiu et al. 2002). It was reported that promotion of central fiber regeneration following a conditioning lesion to the peripheral branch requires IL-6 upregulation (Cafferty et al. 2004). Leukemia inhibitory factor (LIF) and neuropeptide galanin also play important roles in CLE (Cafferty et al. 2001; Sachs et al. 2007). Dibutyril cAMP injection into DRG produced a CLE-like effect by increasing LIF and IL-6 mRNA levels in the sensory neurons and in sympathetic nerves. CLE was shown to depend on LIF, IL-6, IL-11, and oncostatin M (gp130 cytokines) (Hyatt Sachs et al. 2010; Wu et al. 2007).

Most of the in vivo conditioning lesion studies have been performed using sciatic nerve injury as an axotomy model (Savastano et al. 2014). Most current in vitro models also often start with a sciatic nerve injury; after a conditioning period L4 and L5 DRGs are removed, isolated primary sensory neurons are cultured and axonal outgrowth is quantified (Chen et al. 2016). While this method proved efficient to study consequences of CLE, it hardly allows to the study mechanism of conditioning as it develops under uncontrolled in vivo conditions. In this study, we developed a new technique that enables to model the conditioning with isolated neurons in a maximally controlled defined environment and demonstrated that LIF is required to induce CLE in this in vitro model.

Materials and Methods

All surgical procedures were conducted under sterile conditions and in conformity with institutional guidelines that are in compliance with European Economic Community Council Directive 86/609. All efforts were made to reduce the number of animals used. A local ethical committee approval was obtained for animal use in the experiments.

Graphical summary of the technique was presented in Fig. 1.

Neuron Culture

Adult Balb-c mice aged 6–12 weeks were used in all experiments. For in vivo conditioning experiments mice were anesthetized by an I.P. injection of ketamin (100 mg/kg, Ketalar, Pfizer, Istanbul, Turkey) and a unilateral sciatic nerve transection was done as a conditioning lesion 3 days before the cell culture as described earlier (Ozturk and Tonge 2001). Before surgically removing L4-L5 DRGs for cell culture under a stereomicroscope, ketamin (100 mg/kg, Ketalar, Pfizer) was administered intraperitoneally to anesthetize the mice, which were then sacrificed by cervical transection. Cultures of primary sensory neurons were set up as described before (Cengiz et al. 2012). Briefly, any attached nerves to DRGs were cut off with micro scissors and the ganglia were first digested with collagenase (Sigma) and then treated with trypsin (Sigma). The ganglia were then trituration for about 15 min by gently and repeatedly pipetting through the tips of narrowing bores and finally a fine injector needle. The cell suspension obtained was treated

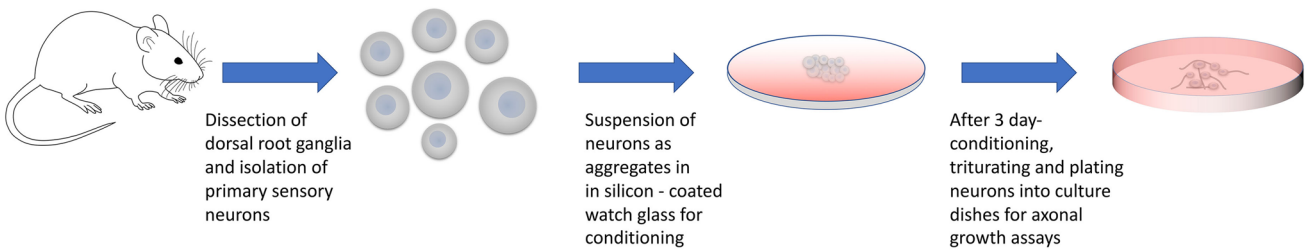


Fig. 1 Graphical summary of in vitro neuronal conditioning technique

with DNAse, spun and the pellet was re-suspended in Neurobasal A supplemented with B27 (Thermo Fisher), antibiotic solution, and glutamine (NBA-B27). To purify neurons, a three-layer percol (Sigma) gradient (60, 35, and 10% from bottom to top) was used and after spinning at 3000 g for 20 min. The neurons collected from 35% layer were washed with NBA-B27, spun once more and pellet was re-suspended in NBA-B27. The cell suspension was transferred on silicon (WPI-Dow Corning) coated watch glass that does not allow cell attachment. By this way neurons were incubated for 3 days (37 °C and 5% CO_2) as suspended aggregates and they were given time to get conditioned. At the end of the third day they were triturated and seeded on 35 mm diameter glass bottomed Petri dishes (WPI) coated with poly-L-lysine and laminin. In vivo conditioning and control group neurons were directly transferred to the culture dishes on the day of isolation. Neurons were incubated in NBA-B27.

Testing the Reliability of In Vitro Conditioning

In this part, we evaluated whether floating cells survived the in vitro incubation period and they extended neurites while in aggregates. That was because we aimed to inflict only one axotomy to the neurons, which inevitably occur during tissue dissociation. If neurons grew axons during in vitro conditioning, they would receive another axotomy during trituration before transferring to culture dishes. To confirm the identity of neurons in aggregates and define their morphological features, indirect immunofluorescence technique was used. Some aggregates were fixed in 4% paraformaldehyde at RT for 15 min and blocked and permeabilized for 30 min with phosphate buffered saline (PBS) containing 0.1% triton-x, 5% goat serum, and 0.1% Na-azide. Preparations were then washed with PBS and incubated with mouse anti-NFH IgG (Sigma, clone NE14) for 2 h. After washing with PBS, they were incubated with Alexa fluor 488 goat anti-mouse IgG (Thermo) for 2 h. Finally, preparations were given three more washes with PBS and imaged with a laser scanning confocal microscope (LSM510, Zeiss, Germany). To evaluate the viability of cells we added calcein AM (C-AM, 5 µm Calbiochem) and propidium iodide (PI, 7.5 µM Sigma), which stain live and dead cells respectively, to culture medium and incubated for 20 min at the end of the third day of in vitro conditioning. Then the cells were imaged with the laser scanning confocal microscope. Depending on the nuclear staining, percentage of viable neurons was calculated.

To determine whether the cells get damaged during trituration, Lucifer yellow (LY, 0.1% Sigma) or 4K fluorescein dextran (FD, 1 µg/ml BioChemica) was added into cell suspension before the in vitro conditioned cells were transferred to Petri dish. The cell suspension was then triturated; cells were seeded on culture dishes, incubated for 1 h and imaged with laser scanning confocal microscope. To prevent intake of these compounds by endocytosis the pH of cell suspension was reduced to 5.7 in some experiments (Davoust et al. 1987).

Comparison of Axon Regeneration from Control and Conditioned Neurons

The rates of axonal growth from in vivo or in vitro conditioned and freshly isolated DRG neurons were analyzed with a time lapse microscopy system (Cell observer, Zeiss) where multiple positions of preparations could be imaged at desired time intervals over long periods, while a physiological environment was created for the cells in an stage top incubator (Ozturk and Erdogan 2004). Images of individual neurons were digitally captured every 15 min for 48 h. The process was programmed and automatically executed using Axiovision 3.0 software. To monitor the viability of the neurons PI was added to the medium (7.5 µM).

Investigating Role of LIF in In Vitro Conditioning

In order to understand the role of LIF in in vitro conditioning, some preparations were treated with neutralizing anti-LIF antibodies (Sigma) at 1/1000 concentration during conditioning and culturing or conditioning only. To demonstrate existence of LIF and its receptor in the neurons, immunofluorescence staining was performed using goat anti-LIF (Sigma) and rabbit anti-LIF receptor beta (LIFRβ) (Santa Cruz) IgG's as primary and Alexa fluor 488 chicken anti-goat IgG and Alexa fluor 594 chicken anti-rabbit IgG (Thermo) as secondary antibodies. The staining protocol was similar to what was described above for neurofilament except that in blocking solution, chicken serum was used instead of goat. In some experiments, during conditioning incubation, protein transcription was inhibited with 50 µM 5.6 dicholorobenzimidazole riboside (DRB, Sigma) and translation with 1/1000 anisomycin (Sigma) to elucidate expression pattern of LIF.

Image and Statistical Analyses

For each experimental group, three replicates were performed using three different animals. Neurons from each animal were cultured in a single dish. About 50 neurons from a total of 12–15 microscopic fields in three culture dishes were tracked, analyzed, and the results were pooled for each group.

For image analyses, Image J software was used. The length of the first axon grown by each neuron was measured and regeneration rate (RR) was calculated for every 3-h intervals. Since not all axons continuously grow at every interval, a "regeneration activity index, (RAI)" was

produced by multiplying regeneration rates by the proportion of tracked neurons that grew axons in a given interval (RAI = RR × number of tracked neurons actively growing axons/number of all tracked neurons). Changes in LIF and LIFRβ immunoreactivities were assessed measuring the brightness of the signal in in vitro conditioned neurons with and without DRB and anisomycin. For the statistical evaluations, SPSS software package was used. For the comparison of means among groups one-way ANOVA and post-hoc LSD tests were used. To compare survival rates, Fisher's Exact test was conducted.

Results

DRG neurons clumped together and form aggregates during the in vitro conditioning. With NFH staining, large and round cells that make the main bulk of the aggregates were identified as neurons (Fig. 2). To test the viability of the neurons in these aggregates they were stained with C-AM and PI and then imaged with laser scanning confocal microscope. The strong C-AM staining with seldom PI-stained nuclei indicated high level of neuronal viability (96.4%), whereas there were numerous small red nuclei around the neurons, probably belonging to the satellite cells (Fig. 2). C-AM normally stains all extensions of a healthy neuron; we observed very few such stained extensions within the aggregates, indicating that in general, most of the neurons did not grow axons during conditioning period. To confirm this LY or FD was added to the culture medium just before the trituration at the end of the conditioning period, assuming that any potential unwanted axotomy would let these compounds into the neurons. Most of the neurons had slight fluorescent signal, which was probably due to endocytosis as this was prevented with low pH. Only few neurons (< 1%) stained brightly under neutral and low pH conditions. These were probably the neurons that extended axons while they were within the aggregates and received a second axotomy during trituration.

The percentages of neurons growing axons within first 24 h were 84.6 ± 2.5%, 82.4 ± 4% and 88.8 ± 1.8% in control, in vivo and in vitro conditioned groups respectively ($p > 0.05$ among the groups). Thus, more than 30 axon-growing neurons were imaged in each group.

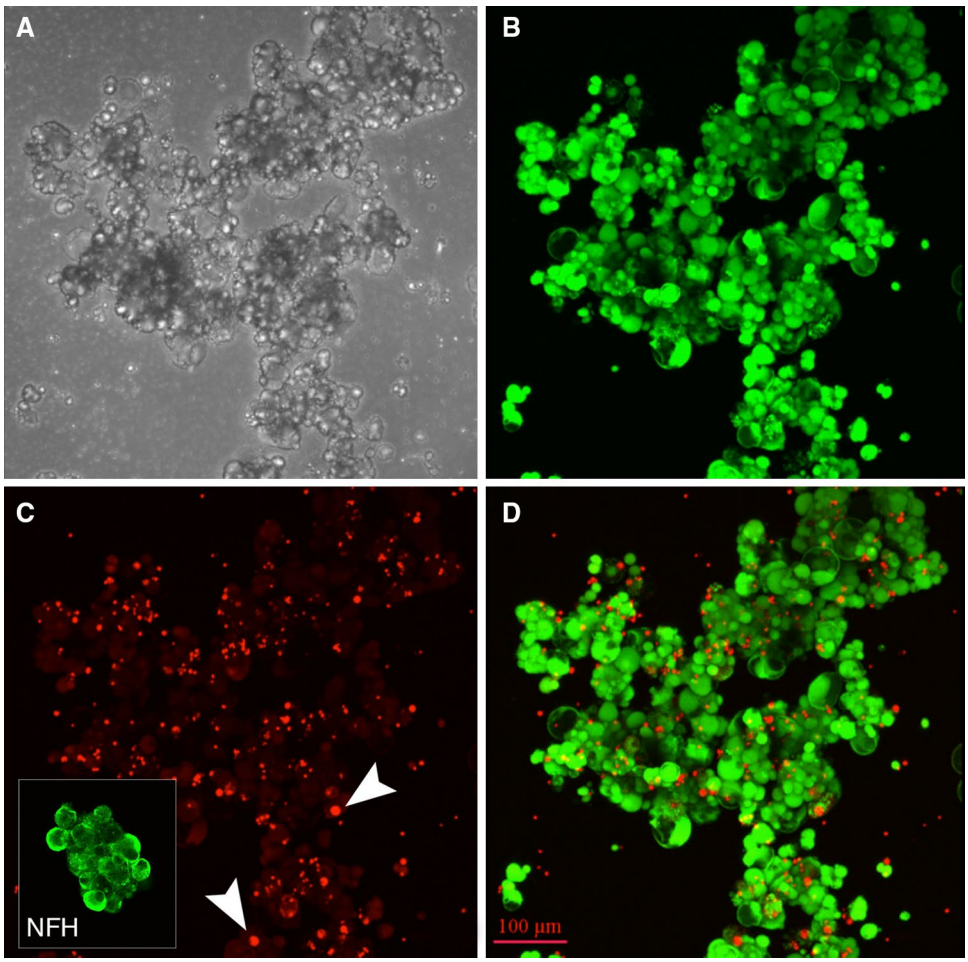


Fig. 2 Appearance of neuronal aggregates in suspension during conditioning incubation (**a**). Viability of neurons was high as assessed with calcein AM (**b**). Dead cell indicator propidium iodide stained many small nuclei around the neurons, probably belonging to satellite glial cells (**c, d**). Occasional PI-stained large nuclei probably belonged to dead neurons (arrow heads). The identity of the neurons was confirmed with NFH immunostaining (inlet)

Regeneration rate of axons growing out of control neurons almost steadily increased from about 5 µm/h to around 20 µm/h during 48 h of incubation (Figs. 3, 4). In vivo conditioned neurons started to grow axons almost twice as fast; the rate slightly increased during the course of incubation. The neurons conditioned in vitro, on the other hand, exhibited a reverse trend; a high rate over 20 µm/h during initial hours of incubation dropped to 5 µm/h at the end of 48th hours. While the maximum regeneration rate was more than five- and ten-fold higher in vivo and in vitro conditioned neurons respectively, the highest rate (162.3 µm/h) was scored by a neuron in control cultures during 31–33 h of incubation. RAI analyses showed that more neurons started to grow axons at the start of the incubation in conditioned preparations, but more neurons were active in control cultures after 24 h.

While all neurons survived in control preparations and only a few died in in vivo conditioned group, 30% died in conditioned cultures at 24th h of incubation (Fig. 4). However, at the end of second day while control cultures had only 15% loss, both in vivo and in vitro conditioned preparations lost about 60% of the neurons.

When the activity of endogenously expressed LIF was blocked by a neutralizing antibody during in vitro conditioning and culturing, conditioning was totally abolished and both average regeneration rate and RAI dropped even below control values (Fig. 5). When LIF inhibition was limited to the conditioning period, though regeneration rate was similar to control preparations, more neurons kept growing axons as reflected in a high RAI. Inhibition of transcription or translation decreased the amount of LIF and LIFRβ significantly.

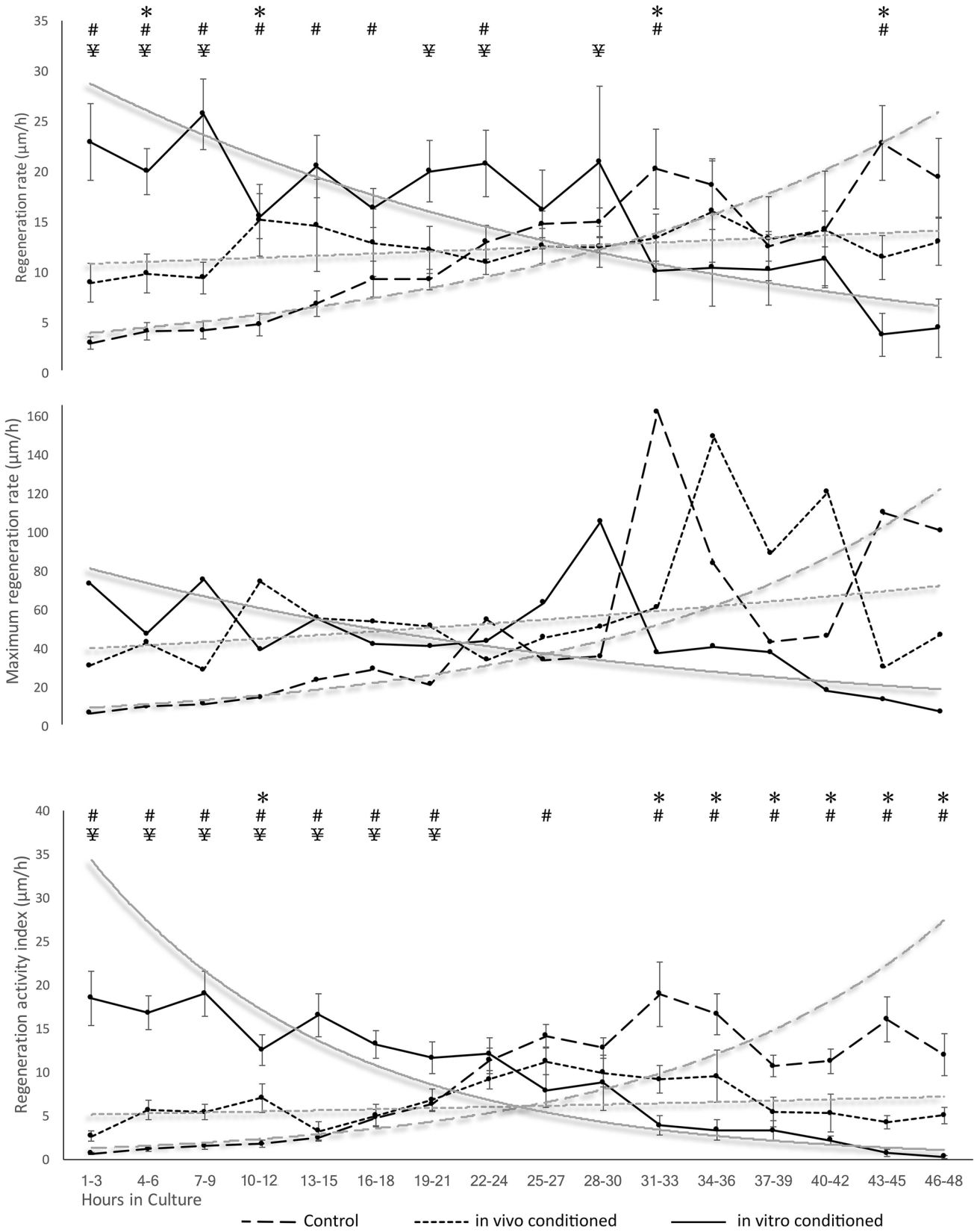
Discussion

In this study, we developed and showed reliability of an in vitro neuronal conditioning technique. During the dissociation for cell culture, the DRG neurons lose their axons due to enzymatic treatment and mechanical effects. In the current model, this was assumed to be the initial conditioning axotomy. The isolated neurons were kept in suspension to prevent axonal growth. This was a strategy to reduce energy expenditure and to enhance and preserve protein pool, which was presumed to create a conditioning effect. Three days was reported to be the shortest interval to observe an effective CLE (Jacob and McQuarrie 1993); consistent with this, we incubated DRG neurons in suspension for 3 days to condition. Fluorescent dye experiments with C-AM, LY, and FD confirmed that neurons survive the conditioning incubation period and only negligible number of neurons grew axons while in suspension, thus neurons were subjected to axotomy only once before plating. After plating, neurons in all groups started to extend axons at similar high percentages, which verifies their good health. There are some other in vitro conditioning techniques that involve detaching and replating cultured neurons (Frey et al. 2015; Saijilafu et al. 2013; Zou et al. 2009), which mimic the test injury. However, our model offers some advantages. First, as we do not let axonal growth during first incubation, the molecular products of conditioning process will be consumed less and be more abundant, which will make them easier to detect and their enhancing effect more pronounced after plating. Second, cell aggregates in suspension are anatomically more similar to ganglia, which is more suitable for any possible paracrine mechanism. Finally, conditioning the neurons in suspension is technically much easier and less costly than plating and detaching them.

According to in vitro and in vivo studies the first sign of CLE is early initiation of axonal growth. We use a very refined DRG neuron culture protocol where the axon growth starts within hours of incubation even in control preparations whereas others reported it only after 15–48 h (Kwon et al. 2015; Lankford et al. 1998; Saijilafu 2012). The initiation of axonal growth after a sciatic nerve cut in conditioned and unconditioned sciatic nerves was found to be 1.9 and 2.6 h (Sjoberg and Kanje 1990b) and in a film model 1 and 6 h respectively (Torigoe et al. 1999). Our model, in this respect, is consistent with in vivo situation (Figs. 3, 4).

The most obvious manifestation of CLE is reported to be increased axonal growth rate. In consistence with other reports in vivo conditioned neurons had longer axons than controls at 24th h. However, this was due to a significant acceleration of axonal growth at around 10–12th h of incubation and not because of a consistently faster regeneration. The in vitro conditioned neurons, on the other hand, scored much higher rates throughout first 24 h, sometimes five times faster than the control neurons (Figs. 3, 4) reaching up to 25 µm/h. This is the main evidence that the method we developed in this study successfully models CLE.

The sciatic nerve cut is the most common method to induce the conditioning in vivo (Savastano et al. 2014). Lumbar 4–6 DRGs that receive fibers from the sciatic nerve are removed and used for culture (Chen et al. 2016). However, in these DRGs, there are neurons that do not send axons to the sciatic nerve, thus not injured with the nerve cut (Rigaud et al. 2008). Presence of such unconditioned neurons in in vivo conditioned cultures may partially account for lower regeneration rates than in vitro conditioned neurons. However, in vivo and in vitro conditioning mechanisms may at least be partially different especially due to the absence of systemic influences in the in vitro model. Thus, while our technique in this study produces CLE it may not exactly mimic the in vivo conditioning; but it offers a model to investigate possible signaling pathways common and distinct in either condition. The second day of incubation for


Regeneration rate (µm/h)
Maximum regeneration rate (µm/h)
Regeneration activity index (µm/h)
1-3
4-6
7-9
10-12
13-15
16-18
19-21
22-24
25-27
28-30
31-33
34-36
37-39
40-42
43-45
46-48
Hours in Culture
Control
in vivo conditioned
in vitro conditioned

◂**Fig. 3** Mean and maximum regeneration rates and regeneration activity indices in conditioned and control neurons during 48 h incubation. Error bars represent standard error of means. Trend lines are superimposed on the chart. $^{*}p<0.05$ control versus in vivo conditioned, $^{\#}p<0.05$ control versus in vitro conditioned, $^{¥}p<0.05$ in vivo versus in vitro conditioned as calculated by one-way ANOVA and LSD post-hoc tests. Each time point represents means of about 30 neurons pooled from three replicate cultures

conditioned neurons witnessed a sharp decline in axonal growth. In this period control neurons were more successful and at the end of 48th h they had longer axons (Figs. 3, 4). Similar observations were done by others (Lankford et al. 1998). Probably this was, in most part, due to exhaustion of the neurons because of high metabolic state to support fast regeneration. Indeed, we observed axonal degeneration and substantial neuronal death among in vivo and in vitro conditioned neurons that exceeded 50% at the end of second day (Fig. 4). The high death rate in both types of conditioned preparations is probably due to rapid depletion of metabolic and energy resources because of accelerated axonal growth. Since we did not provide trophic support to neurons with growth factors or sera, this is not an unexpected observation. In this study we produced a regeneration activity index, which better represents the behavior of whole neuronal population in culture. The index shows a low start and an exponential rise for control, a medium start and almost steady progress in in vivo conditioning and a high start and an exponential decline for in vitro conditioning (Fig. 3). This casts doubts whether CLE in living organism may really contribute to recovery after nerve injury. There are functional studies where motor and sensory recovery was found improved significantly with CLE (Bisby 1985; Bontioti et al. 2003; Navarro and Kennedy 1990). This may suggest that in the animal, conditioned neurons are continuously supported by local and systemic factors that keep them growing axons and save them from death. In this sense, our model is very well suited to investigate such neurotrophic and neuroprotective mechanisms. Especially, the second day of incubation is an ideal period where preventing the neuronal death and decline in regeneration with potential interventions make good challenges.

Our model represents an intrinsic mechanism of conditioning, which makes it relatively easy to investigate the factors responsible for and test any factor on the neurons that may affect the conditioning process. For example, some studies suggest that after a peripheral conditioning lesion, macrophage activation is essential for CLE to promote regeneration in ascending sensory fibers of spinal cord as well in the conditioned peripheral axons (Kwon et al. 2013; Salegio et al. 2011). Chemokine CCL2 released from axotomized neurons trigger macrophage activation through CCR2 receptor and CLE abolished in CCL2 knock-out mice (Kwon et al. 2015, 2016; Niemi et al. 2016, 2013). However, according to our study, CLE does not seem to depend on the macrophage invasion since we eliminate them in neuronal cultures. Indeed, in a recent study, local application of ethidium bromide did not cause macrophage accumulation at the peripheral nerve but still induced CLE (Hollis et al. 2015).

We observed variability in survival and axonal regeneration capacity of neurons. This is something expected as the transcriptional response of DRG neurons to axotomy was shown to be highly heterogeneous (Hu et al. 2016). In fact, some suggest that CLE occurs only in a subset of DRG neurons (Kalous and Keast 2010).

LIF is a well-characterized survival factor for primary sensory neurons and its association with CLE induction in vivo has been documented (Cafferty et al. 2001; Murphy et al. 1991; Wu et al. 2007). Consistent with this, we demonstrated that LIF is essential for in vitro conditioning of neurons (Fig. 5). However, some of our findings need clarification. LIF is normally not expressed by primary sensory neurons but retrogradely transported from peripheral nerves where they are detected mostly in Schwann cells (Dowsing et al. 2001; Ito et al. 1998). A peripheral nerve injury increases the expression of LIF and its delivery to the primary sensory neurons (Banner and Patterson 1994; Curtis et al. 1994). LIF receptor, on the other hand, is constitutively expressed by these neurons (Gardiner et al. 2002; Scott et al. 2000). We detected immunoreactivity for both LIF and LIFRβ in cultured DRG neurons (Fig. 5). The source of LIF could be the satellite cells that surround the neurons (Banner and Patterson 1994). In fact, the massive loss of these cells during conditioning incubation may be due to their exhaustion as they upregulate LIF synthesis. However, we can still not rule out possibility of LIF expression by DRG neurons in culture since there is evidence that a similar cell type, olfactory sensory neuron can express it (Bauer et al. 2003). In either case, there should be extracellular LIF in order for the anti-LIF to interfere with its action since the antibodies can not penetrate the cell membrane. This could happen during its putative transfer from the satellite cell to the neuron. Additionally, since LIF has an autocrine effect (Dowsing et al. 1999), it may be continuously released by the neuron that accumulates it. When LIF inhibition was limited only to the conditioning incubation period, average regeneration rate decreased but RAI did not change; i.e., more neurons kept growing axons. On the other hand, application of anti-LIF during this culturing period completely abolished CLE and it even led to a regeneration rate lower than the control preparations during the second day of incubation. These may suggest that during conditioning incubation, excess amount of LIF may prepare the neuron for an enhanced regeneration and some of the factor is retained within the neuron, which would support an enhanced axonal regeneration at least during the first 24 h of incubation. Likewise, even smaller amounts of LIF, which could be acquired

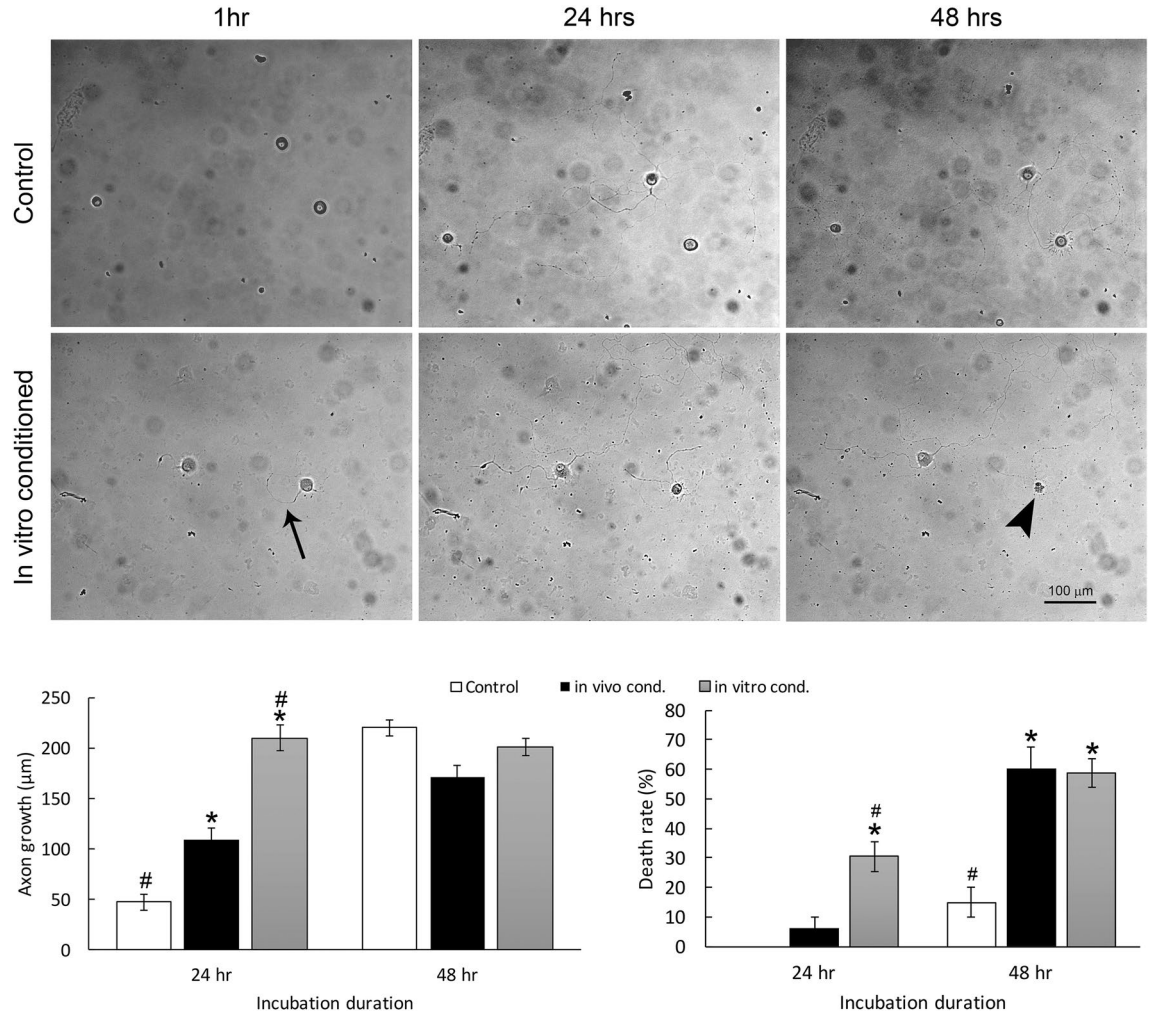


Fig. 4 Representative images of cultured control and in vitro conditioned neurons at different time points. Within first hour of incubation the conditioned neurons started to grow axons (arrow). At the end of 48 h, they had extensive axonal degeneration and death (arrow head). The mean lengths of the axons (below left) and death rates (below right) at the 24th and 48th h of incubation in conditioned and control neurons. Error bars represent standard error of means. $^{*}p<0.05$ versus control, $^{\#}p<0.05$ versus in vivo conditioned. In conditioned preparations, boosted axonal regeneration during the first day of incubation resulted in exhaustion and death of many neurons

even during dissection and cell dissociation, may be continuously released by the same neurons that have accumulated it. Decline in regeneration during the second day of incubation may be due to depletion of LIF. Experiments with DRB and anisomycin suggest that the upregulation of LIF and LIFRβ expression occurs by translation of existing mRNAs and also transcribing new mRNAs.

In conclusion, the in vitro neuronal conditioning model developed in this study proved a strong experimental paradigm to study the mechanism of conditioning and to learn more about neuronal response to injury. Isolated environment of the model that successfully produces CLE without added serum or growth factors can allow activation or inhibition of signaling pathways, addition or inhibition of growth factors, and co-culturing neurons with other cells like macrophages and glial cells, whereby any potential process leading to CLE can be investigated. In this respect, we have demonstrated that LIF is essential for induction of CLE in this study.

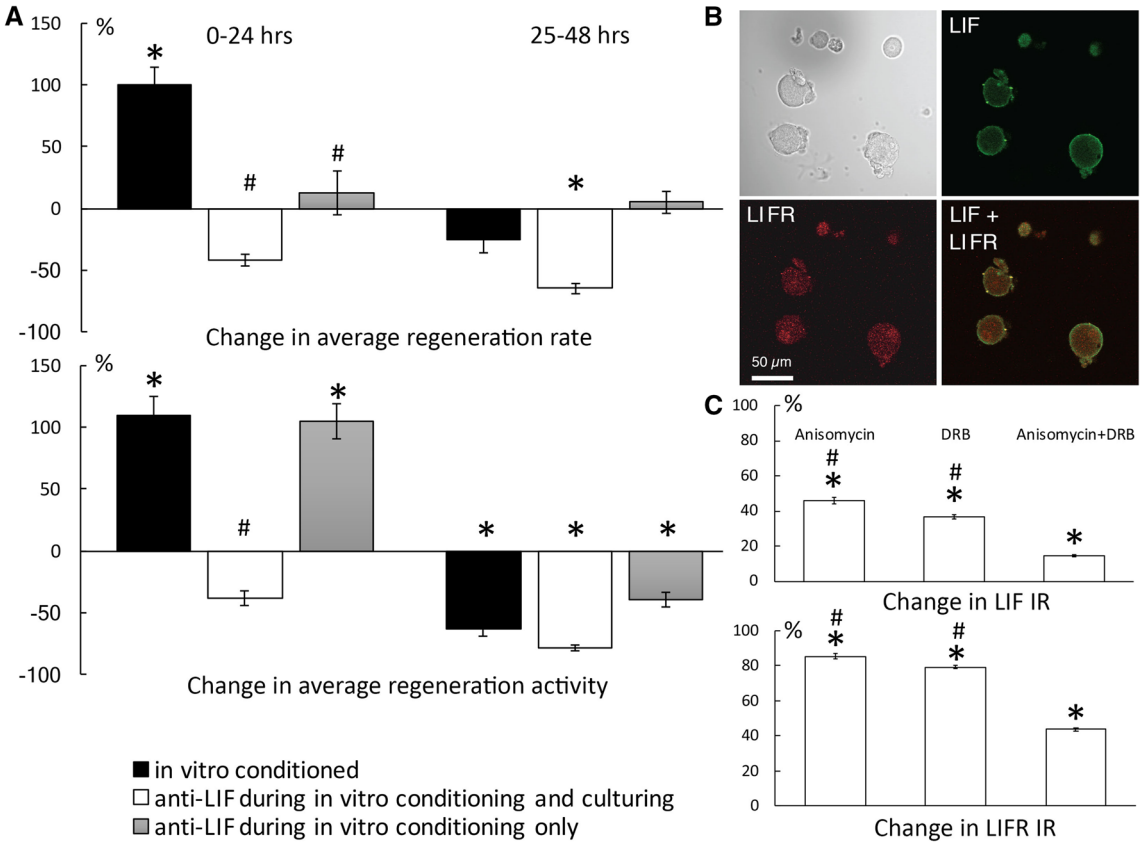


Fig. 5 Effect of prevention of activity of endogenous LIF in the induction of CLE (**a**). Data are presented as percentage changes relative to control preparations. Blocking LIF abolished the increase in axonal regeneration due to conditioning. $^{*}p<0.05$ versus control, $^{\#}p<0.05$ versus in vitro conditioned. Representative immunofluorescence images of cultured DRG neurons stained with antibodies against LIF and LIFRβ (**b**). Inhibition of translation and transcription during conditioning significantly decreased the immunoreactivity for LIF and LIFRβ (**c**). The data are presented as percentage changes in average pixel brightness relative to in vitro conditioned preparations. $^{*}p<0.05$ versus in vitro conditioned, $^{\#}p<0.05$ versus anisomycin + DRB

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Author Contributions Elif Kaval Oğuz: Conducting experiments, image analysis, drafting the manuscript. Gürkan Öztürk: Designing the experiments, statistics, revision of the manuscript. Both authors have reviewed the final version of the manuscript and approved it for publication.

Compliance with Ethical Standards

Conflict of interest Authors declare that they have adhered to ethical standards in this study and that they have no conflict of interest to disclose.

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