Influence of distant femtosecond laser pulses on growth cone fillopodia

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Abstract A 3 mW focused femtosecond laser spot at a distance ($>15\ \mu\text{m}$) has shown to attract the fillopodia from growth cones of primary neuronal cell cultures (mice E15). The phenomenological behavior of fillopodia is studied under short durations ($\sim 40\ \text{min}$) and different laser light conditions. The analysis of the fillopodia movement showed that they become significantly attracted towards the focused femtosecond laser light. In contrast, the use of continuous wave under the same conditions did not generate the same effect, the results of which were indistinguishable from when there was no laser light present (control condition). These results suggest the possible existence of an optically-induced signaling mechanism in growth cones.

Keywords Axon guidance · Growth cone · Fillopodia · Ultrashort pulses · Optical signalling · Light–cell interaction · Cell analysis

Introduction

Growing axons have a highly motile structure called the growth cone (Goldberg 2003; Jay 2000; Ramón y Cajal 1904). During normal development, the growth cone scans the extra-cellular matrix using the fillopodia, sensing guidance cues that ultimately guide axons towards the correct target. These guidance cues fall into several classes of biomolecules (Dickson 2002; Huber et al. 2003). Some of these biomolecules have been used externally to achieve control over neurite growth (Gallo and Letourneau 1998; Naka et al. 2004). These are, however, multifunctional and require specific receptors that activate complex intracellular signaling cascades in the growth cones (Tessier-Lavigne and Goodman 1996). As a result, precise and selective control on individual neurons can not be guaranteed. There have been significant efforts in finding alternative guidance cues that can manipulate and enhance the intrinsic natural growth process. Several methods have been suggested, including the use of electric fields (Patel and Poo 1982) and growth of neurons on micro-patterned structures (Fromherz et al. 1991; Kaehr et al. 2004; Prinz and Fromherz 2000).

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Biological cell–light interaction has been reported in a number of works. An example of this is the redox absorbance changes in living cells under near infrared (IR) laser irradiation (Karu et al. 2001). This irradiation causes, through an excitation of chromophores, an increase in the oxidative metabolism of transmembrane protein complexes in mitochondria. As a result, there is an enhancement of the mitochondria functionality and, therefore, of the cell metabolism. This is actually the basis for photobiomodulation therapy used in spinal cord nerve repair and wound healing (Desmet et al. 2006). Additionally, photoirradiation can generate significant biological effects such as cell proliferation, collagen synthesis, release of growth factors and the promotion of exocytosis (Henkel et al. 2006; Sommer et al. 2001). In other works, scattered IR light with 0.5–1 Hz modulation was shown to attract pseudopodia from 3T3 fibroblast cell lines (Albrecht-Buehler 1991).

One of the first uses of laser to indirectly guide axons was the laser photolysis of caged Ca^{2+} ions (Zheng 2000). In this work, an increase in the Ca^{2+} ion concentration in a localized region of the growth cone was shown to induce a tropic turning of the axon. The mechanical perspective of controlling the growth cone of different cell lines by continuous wave (CW) laser has also been explored. In these experiments a near IR CW laser beam was made to fall on the edge of the growth cone to optically drag the neuron towards a certain course (Ehrlicher et al. 2002). The main hypothesis in this work is that a tweezing-light like effect seems to gather chemical components at a certain location, enhancing local growth. With this idea in mind, axonal guidance was also achieved using an elliptical beam profile to better direct the optical forces in a chosen direction (Mohanty et al. 2005). In addition, the attraction efficiency while guiding axons with laser light has been tested for different wavelengths (Stevenson et al. 2006). Recently, neurite outgrowth of PC12 cell lines has been observed under LED illumination (Higuchi et al. 2007). However this outgrowth only occurred at a specific wavelength (479 nm). Remarkably, the underlying mechanism responsible for the light–cell interaction described above, has not been fully understood or is not known.

All these previous works strongly suggest the existence of a signaling mechanism (direct or indirect) induced by light itself. It is very possible that

this mechanism depends not only on laser parameters such as the intensity or the wavelength, but also on the temporal regime (CW, CW-modulated or even pulsed), which can result in different light–biological matter interaction. In this work we have explored the effect of the temporal regime (ultrashort or CW) on the fillopodia response using primary cell cultures. Here we show that a stationary (static) laser beam of ultrashort pulses, focused at a certain distance from the growth cone of a neuron, can induce a tropic effect on the fillopodia. This attracting effect is characteristic of ultrashort pulses and was not observed using CW light. The underlying mechanism responsible for this attraction is unknown and requires extensive physical and biochemical investigations that are beyond the scope of this work which aims to report the observed phenomenological effect.

Methods

Cell culture

OF1 embryos (Iffa Credo, France) were used. The mating day was considered embryonic day 0 (E0). After anesthesia of the dams, embryos were dissected out and collected in 0.1 phosphate-buffered saline (PBS) and 0.6% D-glucose. Low-density dissociated cultures were established from the cerebral cortex of CD1 mouse embryos at 15 days gestation (E15). The dissected tissue was trypsinised (0.05% trypsin for 9 or 15 min, respectively at 37 °C) and dissociated by trituration. All neurons were plated on a poly-ornithine/laminin substratum in 35 mm diameter tissue culture petri dishes. The cerebral cortex neurons were grown on Neurobasal supplemented with 10% normal horse serum, L-glutamine, NaHCO_3 , D-glucose and supplement B27 (all from Gibco Life Technologies). Purified recombinant NGF (4 ng/mL) (Sigma) was added to the cultures at the time of plating. All neurons were grown for 18 h in a 5% CO_2 , 95% humidity incubator at 37 °C.

Experimental setup

The experimental setup was built around a commercial microscope (Nikon-Eclipse TE2000U) with a 60×, 1.4 NA oil immersion objective. The neurons were imaged using a CCD camera and the

conventional bright field illumination provided by the microscope. A chamber with an automatic heating system was built around the microscope to keep the neurons at 37 °C. A Kerr-lens mode locked Ti:sapphire laser (Mira 900f, Coherent) with a pulse duration of 150 fs, 76 MHz repetition rate and a central wavelength of 800 nm was used for the ultrashort pulsed (femtosecond case) experiments. For the CW case experiments the auxiliary cavity (without the pair of prisms) of the same laser was used to ensure a CW operation. The beam (either in the pulsed or CW regime) was coupled into the microscope through the back port. This was achieved using a pair of galvanometric mirrors, conventional relay optics and a 45° hot mirror to direct the beam through the microscope objective and towards the sample plane. The measured pulse duration at the sample plane was 240 fs, which implies a net group delay dispersion introduced by the microscope of $D = 2,100 \text{ fs}^2$. With the above parameters, the calculated spot size was $0.72 \mu\text{m}$.

The position of the focused beam was set by steering the beam to a fixed position in the XY plane before the start of each experiment. Fluorescence from a plane fluorescent sample was used to draw up a calibration curve between pixel coordinates in the CCD camera images and the galvanometric voltage which controlled the laser spot position. The cells were loaded after this calibration procedure. The laser spot could then be placed on the desired location by a simple mouse click on the image of the sample plane using a control software (programmed in Labview). The focal position of the laser beam spot was then adjusted by varying the height of the objective lens. Once the beam spot position was set, this remained unchanged (axially and transversally) through out the whole experiment. The program draws a coloured dot in the image indicating the position where the beam lies. During the experiment, the colour of this dot changes depending on whether the laser shutter is open or closed. Neutral density filters were used to attenuate the beam average power (in both pulsed and CW) down to 3 mW at the sample plane of the microscope. This yields 40 pJ energy per pulse and an irradiation of 2.4 mJ/cm^2 .

The control software was also used to capture the video-micrograph from the high resolution (pixel size of $4.5 \mu\text{m} \times 4.5 \mu\text{m}$) CCD camera at a rate of one frame each 5 s for the whole experiment. After the

experiment for each frame in each video we visually observed the image and manually marked the position of the tip of the fillopodium. This marking was done by loading the images into a Matlab program that captured the coordinates of the mouse pointer while clicking on the fillopodium tip position. These coordinates were then utilized to compute the distance of the fillopodium to the laser spot and for generating its trajectory.

Procedure

We use a focused beam at some distance ($>15 \mu\text{m}$ away from the closest fillopodium at the beginning of the experiment) and in front of the growth cone (within an angle of $\pm 90^\circ$) as Fig. 1a shows. The laser spot remained immobile in the same position during the whole experiment.

In order to perform the fillopodia analysis (described later) and determine the effects of the laser spot at a distance on the fillopodia, the following procedure was followed. Individual growth cones were recorded without any laser excitation for the first 20 min. This period was used as a reference for the fillopodium natural behaviour and is referred to as control or OFF period. After that, the laser shutter was opened for a second period of 20 min, referred to as laser period or ON period. For the ON period we used two different conditions: ultrashort pulses (femtosecond) and CW laser light. This procedure was applied to 40 neurons under the influence of ultrashort pulses and 36 neurons under the influence of CW light. The OFF-period can also be analyzed (following the statistical method) in order to determine the wild behavior of the fillopodia without laser illumination, this is referred to as control.

The 76 neurons were analyzed in 8 different sessions (4 for the CW regime and 4 for the femtosecond one) where only one fillopodium was analyzed per neuron. Each session was statistically treated as a different experiment to consider possible variations in the experimental conditions. None of the neurons were excited with more than one laser-period condition.

Fillopodium analysis

While observing the erratic-like fillopodia movement, it was evident that a formal analysis methodology

Fig. 1 **a** Snapshots of an axonal growth cone. The *green dot* represents the position of the focused beam. **b** Recorded trajectory of a filopodium during the control- (*dashed green trace*) and femtosecond laser- (*blue trace*) periods. P_f is the filopodium average position, m_f is the average displacement and σ_f the standard deviation (defined by the *circle*). S is the position of the static beam. $\vec{p}$ is the unitary polar vector and Δd_ρ the radial component for the extraordinary displacement vector $\Delta \vec{d}$

was required that obviates subjective decisions in determining if the laser beam exerts any influence on the behaviour (displacement) of the filopodia (i.e., a tendency to move towards or away the beam). A statistical approach was hence designed with the aim to distinguish between standard (normal) behaviour and changes induced by an external stimulus. In this framework, two types of movements (displacements) can be defined. *Ordinary* movements are those displacements of the filopodia that are comprised within the standard deviation of such displacement. Analogously, *extraordinary* movements are those displacements beyond this standard deviation.

Following this criteria, any external stimuli, including laser illumination, are assumed to be translated as an *increment* in the displacements beyond the standard deviation.

Taking as coordinate origin the beam position, the filopodium position $p_f(n)$ is obtained at each video frame n (that is related to time). This allowed us to calculate the filopodium average position $\bar{P}_f$, its displacement $p_f(n)$ with respect the average position resulting in a mean displacement m_f , and its standard deviation σ_f . These parameters were found considering the filopodium movements during both the OFF and ON periods. Figure 1b shows a typical example of the displacement followed by one filopodium, its average position and the region defined by the standard deviation (black circle). After that, our procedure is simply based on recording, as a function of the frame number n , the distance to the average position, P_f , and direction of these extraordinary displacements, i.e., those with $p_f(n) > \sigma_f$. These extraordinary displacements can be written in the vectorial form as $\Delta \vec{d}(n)$. This piece of information is then used to determine changes in activity and the influence of the laser illumination to induce a distal/proximal behaviour on a particular filopodium.

We start with the measurement of the filopodium *activity* before and during laser illumination. We can assume that a higher number of extraordinary displacements during laser illumination, implying an increase in the filopodium movements, means an increment in the activity. On the contrary, a decrease in the number of extraordinary displacements would result in a decrease in the activity. Therefore the *change in activity* can be measured by taking the ratio of the extraordinary displacements in the ON period with respect to the OFF period:

$$\Gamma = \frac{\sum_{n \in \text{ON}} |\Delta \vec{d}(n)|}{\sum_{n \in \text{OFF}} |\Delta \vec{d}(n)|}.$$

Note that zero change in the activity will give a coefficient with a value close to unity ($\Gamma \approx 1$). Within this framework, inactive neurons were those that had an activity one order of magnitude smaller ($\Gamma < 1$) than those with zero change in activity.

The next step in our procedure was to determine if a particular filopodium had a proximal or distal behavior, i.e., a clear tendency to move in the

direction of the beam spot or away from it. Note that we took as coordinate origin the beam position. Therefore, in polar coordinates, the ρ component of an extraordinary displacement, i.e., Δd_ρ indicates any movement in the direction of the beam. In this way, $\Delta d_\rho < 0$ indicates displacements towards the beam and $\Delta d_\rho > 0$ displacements away from the beam.

Taking this into account, we can determine whether an individual filopodium has a net distal or proximal behavior during the ON or OFF period by defining a proximal parameter as:

$$\Omega_{\text{period}}^- = \sum_{n \in \text{period}} |\Delta \vec{d}_\rho(n)| \quad (\text{for } \Delta d_\rho < 0).$$

This parameter gives what is the total extraordinary displacement towards the beam. In the same way, the total displacement away from the beam is given by a distal parameter defined as:

$$\Omega_{\text{period}}^+ = \sum_{n \in \text{period}} \Delta d_\rho(n) \quad (\text{for } \Delta d_\rho > 0).$$

By comparing the proximal and distal parameters, it is possible to estimate the tendency of a filopodium to move in a particular direction. For example, $\Omega_{\text{OFF}}^-/\Omega_{\text{OFF}}^+ > 1$ indicates a clear tendency of the filopodium to move towards the beam position before the laser is switched on (during the OFF period). Similarly $\Omega_{\text{OFF}}^-/\Omega_{\text{OFF}}^+ < 1$ indicates a net distal tendency of the filopodium in the same period. Similarly, $\Omega_{\text{ON}}^-/\Omega_{\text{ON}}^+ > 1$ and $\Omega_{\text{ON}}^-/\Omega_{\text{ON}}^+ < 1$ would correspond respectively to a proximal or a distal behavior in the ON period. Taking this into account, an ideal example of *attraction* would be a filopodia with an initial distal behavior ($\Omega_{\text{OFF}}^-/\Omega_{\text{OFF}}^+ < 1$) that switches to proximal behavior during laser illumination ($\Omega_{\text{ON}}^-/\Omega_{\text{ON}}^+ > 1$).

Results

Each individual neuron was analyzed using the filopodia analysis described in the previous section. We first calculate the change in activity (Γ) for the control, CW and femtosecond cases. We found that the number of neurons of a given Γ was similar independently of the regime used. This can be seen in Fig. 2 where the frequency distribution of Γ is shown. This suggests that the presence of light (in either pulsed or CW regimes) at the employed

Fig. 2 Change of activity (Γ) of the different filopodia for the control (green), CW (red) and fs (blue) regimes

average intensities, seems not to negatively affect the amount of activity of the filopodia. The results also showed that in all the sessions, $<20\%$ of the neurons (see Table 1) exhibited a significant decrease in the activity ($\Gamma < 0.1$). This decreased measured activity might be attributed to internal neuron dynamics: neuron weakness, exhaustion of biological material needed for growth, or even the neuron undergoing apoptosis. Thus, in what follows, we have excluded from our analysis all those cases whose activity parameter is $\Gamma < 0.1$.

We now focus our attention on understanding the global tendency of the filopodia to move in the direction of the beam or away from it. This was done by calculating the percentage of filopodia showing a proximal behaviour, in both the OFF and ON periods (note that the distal behaviour is the complementary of the proximal). The results are summarized in Table 2. It should be noted that, in an ideal control

Table 1 Percentage of inactive neurons

	Number of neurons	Percentage of neurons with $\Gamma < 0.1$
Femtosecond	40	12.5
CW	36	19.4
Control	76	17.1

Table 2 Percentage of proximal behavior: $\Omega_{\text{period}}^-/\Omega_{\text{period}}^+ > 1$

Period	Control (%)	Cases CW (%)	Cases fs (%)
OFF	56	57	63
ON	61	65	88

situation, the population distribution should be equally spread (50%) between the distal and proximal behaviour (for both the OFF and ON periods as no laser is used). However, the number of cases with proximal behaviour is larger than the distal situation. This can be explained taking into account that the natural growth direction of the axon normally will occur within the semi-space in front of the growth cone, where the laser beam (in this case virtual) is located. This natural proximal behaviour offset is also observed during the OFF period for both the CW and femtosecond illumination. From these results we can calculate the probability of a filopodium to show a natural proximal behaviour in a $59 \pm 4\%$ of the cases. When using CW illumination, 65% of the filopodia showed a proximal behaviour. This falls very close to the calculated natural proximal probability, suggesting that the effect of CW illumination, if any, is very small.

The situation drastically changes when using femtosecond illumination. Here the proximal behaviour increased up to 88% of the cases, clearly outside the calculated natural proximal probability. This

shows that the femtosecond laser illumination is inducing a clear attraction effect on the filopodia.

The significance obtained by computing the binomial test between the pulsed case and the control or the CW cases is $\alpha = 0.01$. This clearly shows that there is a statistical difference between the presence of the femtosecond laser spot and the use of a CW spot.

To confirm this result and check the feasibility of the statistical method used, we averaged the filopodia displacement $p_i(n)$ over all the analyzed neurons under femtosecond and CW laser light conditions. Figure 3a, c shows the obtained results. The vertical dividing line (at 20 min) in figures shows the transition from the OFF to the ON-periods. To obtain an even better understanding we also computed the displacement frequency distribution (see Fig. 3b, d) corresponding to Fig. 3a and c, respectively. It is important to observe that, for the pulsed femtosecond regime (Fig. 3a), the average position of the filopodia is clearly displaced towards the beam. This appears as a downward shift of the blue curve indicating the frequency distribution for femtosecond laser period when compared to the OFF period

Fig. 3 a, c The displacement in the beam direction averaged over all the filopodia during the laser and control periods for pulsed and CW cases, respectively. b, d The displacement frequency distribution of the average displacement of figures a and c, respectively. In the pulse regime, a strong bias towards the beam can be observed. This behaviour is not evident for the CW case at the same average power. Control (green), pulsed (blue), CW (red)

indicated by the green curve. During the OFF period the mean distance of the fillopodia to the laser spot was 15 μm with a standard deviation of 0.8 μm . Analogously, the mean distance of the fillopodia to the beam during the femtosecond laser-period was 12 μm with a similar standard deviation (0.9 μm). This analysis confirms that the pulsed laser has induced a measurable fillopodia attraction towards the beam. In contrast, the results with the CW case in Fig. 3c do not show such average displacement of fillopodia during the CW laser-period. Here, in both OFF and ON- periods, the fillopodia mean position remains close to 15 μm with identical standard deviation (0.8 μm), resulting in an overlapped area in the displacement frequency distribution shown in Fig. 3d and an almost identical mean position. This clearly demonstrates that the population keeps behaving similarly in both periods and CW light does not induce any measurable effect on the fillopodia behavior.

Discussion and conclusion

In this paper, our aim was to demonstrate the capability of ultrashort pulsed lasers to induce a measurable effect on fillopodia of a single axon from primary cell cultures. Our methodology is based on placing the beam spot at some distance from the axonal growth cone and on using average powers of 3 mW to avoid any tweezing or heating effects. Importantly, the non contact characteristic of our technique (as opposed to the use of CW under an optical tweezer-like effect) can be used simultaneously in several fillopodia (and thus axons). It is important to note that the results here presented were obtained using primary cell cultures. This demonstrates the viability of the method for biomedical applications. This is in contrast to previous studies, performed on cell lines, in which a CW beam was touching the cell and the employed average powers were 1–2 orders of magnitude higher (Ehrlicher et al. 2002; Mohanty et al. 2005; Stevenson et al. 2006).

Our results show that fillopodia activity can be effectively assessed by analyzing its random displacement, showing first that laser light does not induce any negative effect on our samples. We showed this for three situations with different stimuli (CW, femtosecond and control conditions). After

rejecting inactive neurons, we also showed that fillopodia are in fact attracted by distant stationary focused ultrashort laser pulses. Under the same conditions, the CW light produced no clear effect on the fillopodia showing a similar behavior compared to the control situation. These results were obtained using a methodology based on fillopodia movements and by analyzing those displacements beyond the standard deviation. These results were further confirmed by analyzing the average displacement behavior of all the fillopodia. There are many parameters (wavelength, focused spot size, intensity, pulse duration, repetition rate, culture medium, age of neurons, etc.) that can be varied and that could enhance the robustness of this effect. However, the preliminary results here presented are unambiguous and represent an interesting discovery that could result in a new optical signaling mechanism.

When guessing the physical mechanism that triggers this fillopodia attraction, the low average powers used in the experiment discard any thermal effect as origin. It has been reported that a 15 mW CW laser at 780 nm produced negligible heating effects at a distance of 5 μm away from the focal spot (Stevenson et al. 2006). In our work the 3 mW pulsed femtosecond laser at a distance 15 μm should then not result in any heat accumulation on the growth cone. Alternatively, the pulse nature of the source of light and the high intensities involved also suggest that shockwaves, induced by focused femtosecond light, could be one of the mechanisms responsible for the effect (Doukas and Flotte 1996; Lin et al. 2003). At lower intensities, pressure waves generated by the periodical nature of the pulsed beam or by piezoelectric effects induced in the media could result in similar effects. Other mechanisms, suggested by recent works, indicate that the electric fields involved in laser light may play a significant role as light can induce a wave-like alternating electric field in a medium that is able to interact with polar structures and produce dipole transitions at different cellular and biochemical levels (Amat et al. 2006). Additionally, the large electric field of the ultrashort pulses could be manifested on the distant cells in the form of direct scattering or in the high order fringes originated after focusing the beam (Patel and Poo 1982). On the same line, it has been reported that magnetic fields can affect free radical recombination and other chemical reactions (Harkins and Grissom 1994;

Timmel et al. 1998). In particular, extremely low electromagnetic fields (ELEMf) have been shown to increase the expression and function of voltage-gated Ca^{2+} channels and that Ca^{2+} influx through Ca_v1 channels plays a key role in promoting neuronal differentiation of neural stem/progenitor cells (Piacentini et al. 2008). Furthermore the naturally occurring photoreceptors (opsins) in the brain could play a role in sensing the light spot (Henkel et al. 2006) at a distance. Several nonretinal/nonpineal opsins like RGR, peropsin, melanopsin, encephalopsin, and neuropsin are expressed in mammalian brain (Kumbalasiria and Provencio 2005). Finally, the laser may also be affecting the nutrients/microglial cell distribution, influencing thus filopodia activity.

In the future, the intracellular mechanisms implicated in growth cone guidance promoted by light will be evaluated. In this sense and looking all the data generated on chemoattractant molecules different types of cytoskeletal proteins, proteins regulating addition of cell membrane, Rho family of small GTPases, kinases, calcium and cyclic nucleotides have been shown to be intracellular targets of guidance signals (Wen and Zheng 2006; Tessier-Lavigne and Goodman 1996). In order to clarify the mechanisms implicated in this promoting effect observed by laser light, experiments to clarify the possible molecular identity of the receptors and the signal transduction mechanisms implicated will be performed. To address this issue the use of both a genetic approach in the nematode *Caenorhabditis elegans* and a functional biochemical approach on in vitro neurite outgrowth assays stimulating with femtosecond laser light will allow initially to elucidate these mechanisms and to test if light acts in a similar pattern like the well known chemical chemoattractants and chemorepellents.

In summary, we have shown the ability of pulsed femtosecond laser light to induce a measurable effect on filopodia of axons from primary cell cultures. This has been observed following a simple methodology that involves the use of a conventional microscope to record the trajectory of filopodia under the influence of focused (ultrashort) pulsed lasers at some distance from the axon. Our preliminary results represent what we believe is an interesting discovery but with lots of room for improvement. There are many parameters (intensity thresholds, wavelength dependence, focused spot

size, intensity, pulse duration, culture medium, age of neurons etc.) that can be varied and that could enhance the robustness of our method. Nevertheless, upon identification of the molecular mechanisms involved, this technique could unveil new possibilities in the field of neuronal guidance.

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