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Gold Nanoparticle-Mediated Laser Stimulation Causes a Complex Stress Signal in Neuronal Cells

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ABSTRACT

Gold nanoparticle mediated laser stimulation of neuronal cells allows for cell activation on a single-cell level. It could therefore be considered an alternative to classical electric neurostimulation. The physiological impact of this new approach has not been intensively studied so far. Here, we investigate the targeted cell's reaction to a laser stimulus based on its calcium response. A complex cellular reaction involving multiple sources has been revealed.

Keywords: gold nanoparticles, neurostimulation, laser-cell-interaction, neuroimplants, stress signaling

1. INTRODUCTION

Therapeutic use of electrical stimulation of the central and peripheral nervous system has proven to be efficient for various indications. Electric stimuli allow for the alteration of signal propagation, e.g. for the treatment of epilepsy or Parkinson's disease, or support of excitable tissues and organs, as in the case of pacemakers and sensory implants. However, due to the propagation of an electric field, these devices cannot reach the spatial resolution necessary to activate single cells. The resulting cross-activation can lead to adverse effects of the therapy. In the case of sensory devices like the cochlear implant, a blurred impression is the result.

To tackle these challenges, optical methods with superior spatiotemporal resolution have been developed. Gold nanoparticle mediated laser stimulation allows for the activation of a selected subset of cells. When irradiated at their plasmon resonance, the nanoparticles heat up rapidly, leading to the formation of bubbles that may rupture the cell membrane[1]. Furthermore, the rapid temperature transient increases the membrane capacitance, potentially evoking action potentials in neuronal cells[2]. The identification of this causal link entailed further efforts to prove this method's efficacy in neuronal activation[3,4].

Promising results have been obtained regarding optical neurostimulation. However, it is largely unknown to what extent nanoparticle mediated laser stimulation interferes with the cellular physiology. Here, we studied the immediate calcium response of a neuroblastoma cell line and primary murine cortex neurons by real-time fluorescence imaging. Several inhibitors were used to further characterize the nature of the calcium response. Calcium plays an important role in stress

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signaling in physiological as well as pathophysiological states. Analyzing a large set of single cell observations has revealed a multi-component process involving membrane perforation, instantaneous and stimulated calcium release from intracellular stores and possibly the involvement of Ca^{2+}-permeable membrane channels. Although the long-term effects have not been evaluated, it is unlikely that interference of this extent can be counteracted by a cell that is stimulated at moderately high frequencies, rendering this method not feasible for long-term *in vivo* applications.

2. MATERIALS AND METHODS

2.1 Cell culture

Cells of the neuroblastoma cell line N2A were cultured in MEM Eagle (PAN-Biotech, Germany) supplemented with 10% FCS and 1% Penicillin-Streptomycin (Merck, Germany). Before experiments, the cells were differentiated to exhibit a neuronal-like phenotype in medium without serum for two to three days. Primary mouse cortical neurons (MCNs) were obtained as a frozen aliquot and cultured according to the manufacturer's protocol (Thermo Fisher Scientific, USA). MCNs were used for stimulation experiments after 10-13 days in culture.

2.2 Laser stimulation

Cells were incubated with 0.5 µg/cm^2 200 nm gold nanoparticles (AuNP; Kisker Biotech, Germany) in culture medium for approx. 3 hours. Fluo 4 staining was performed according to the manufacturer's protocol (Thermo Fisher Scientific) at a final concentration of 5 µM (N2A) or 1 µM (MCN), respectively. Propidium iodide (PI) was added directly before stimulation at a final concentration of 2 µg/mL to visualize membrane perforation. The sample was placed onto an epifluorescence microscope and a 532 nm Nd:YAG laser with a pulse width of 850 ps was guided onto a single cell from above. Radiant exposures of 17, 25, 34, 42 or 51 mJ/cm^2 were used. The cell was irradiated for 40 ms and the fluorophore's signal was recorded simultaneously and up to 90 s after the laser pulse with an exposure time of 200 ms. The PI signal was recorded before and after irradiation.

2.3 Inhibitors

Laser stimulation was performed under the following conditions: culture medium, phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS), PBS with Ca^{2+} and Mg^{2+} (PBSC; both PAN-Biotech), medium + 2-Aminoethoxydiphenyl borate (2-APB, 75 µM), CGP37157 (CGP, 20 µM), lidocaine (Lid, 100 µM), ruthenium red (RR, 100 µM; all Cayman Chemical Company, USA) or ryanodine (Ry, 50 µM; Enzo Lifesciences, USA), respectively. Inhibitors were added to N2A cells after Fluo 4 staining, 10-15 min before laser stimulation.

2.4 Data analysis

The mean gray value for each cell in each frame was normalized to its gray value before the laser pulse using Fiji[5] (ImageJ v.2.35). The change in fluorescence (% $\Delta F/F_0$) over time was depicted and the maximum value as well as the time to peak were recorded. Correlational analysis and data visualization was done with Tableau (v.10.2).

3. RESULTS AND DISCUSSION

Laser irradiation of both N2A cells (culture medium) and MCNs (PBSC) with different radiant exposures reliably evoked a calcium response in the presence of AuNP. Fluorescence increased by about 150-400 % in N2A cells and 20-60 % in MCNs within five to ten seconds. Probability of activation was 80-100 % for N2A cells and 60-100 % for MCNs at radiant exposures of at least 25 mJ/cm^2. No dependence of activation on radiant exposure was observed for higher values. Furthermore, the maximum change in fluorescence does not depend on the radiant exposure. However, there is a negative correlation between time to peak and radiant exposure in N2A ($R^2 = 0.57$), but not in MCNs. The overall characteristics of the laser-induced calcium response appeared to be very reproducible, although atypical signatures with particularly large peak or time to peak values have been observed as well. A negative correlation between time to peak and radiant exposure could hint at the involvement of membrane pores: The higher the energy, the more membrane defects would be expected to occur through which Ca^{2+} could enter the cell. However, the peak fluorescence values would be expected to increase as well if more calcium enters through membrane pores.

The calcium signature does not match that observed before by activation of voltage gated calcium channels by action potentials[7]. To investigate the source of the observed calcium trace, N2A cells were tested under different conditions (see methods). For these experiments, radiant exposure was held constant at 25 mJ/cm^2. Removing extracellular calcium (PBS) had the strongest effect on peak fluorescence and strongly reduced peak values compared to samples in medium or PBSC (Fig. 1). CGP, 2-APB and Ry and are inhibitors of intracellular calcium pathways[8,9]. All three components reduce peak values, however not to the level observed in PBS. Lidocaine, which inhibits Na^+-influx as would occur during an action potential[10], does not affect the calcium response of N2A cells. This is consistent with the above conclusion that electrophysiological activity is not likely to be the cause of the observed signals. MCNs kept in PBS also exhibited a marked reduction in $\Delta F/F_0$ (data not shown). The kinetics and general characteristics of the observed calcium transients in cells in PBS do not generally differ from cells in the reference medium, i.e. time to peak and the signature are similar.

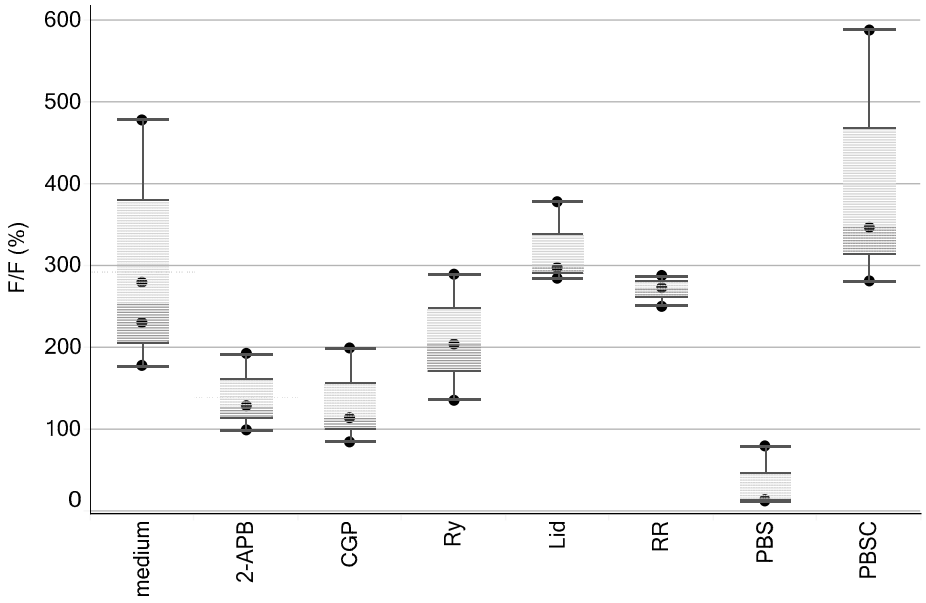

Figure 1: Maximum fluorescence values of N2A cells divided by experimental condition. Black circles are mean values for one experiment. Dashed lines are arithmetic means for the respective conditions. Inhibitors of intracellular pathways (2-APB, CGP, Ry) reduce $\Delta F/F_0$ values. Absence of extracellular Ca^{2+} (PBS) does not completely suppress the calcium signal.

Since comparable stimulation parameters that were used here have been shown to enable delivery of molecules by transient cell perforation[1], it is reasonable to assume that calcium inflow from the surrounding medium through laser-induced defects in the cell membrane might be the main source of increased intracellular calcium. Indeed, the strong decrease of $\Delta F/F$ values in cells in PBS shows that extracellular calcium plays an important role, although it is not the only source. PI was used as an indicator of membrane perforation, since the kinetics of its uptake after laser irradiation has been described before[1]. The hydrodynamic radius of the PI-cation is considerably larger than that of Ca^{2+} ion[11]. However, strong interactions between the smaller cation and the negatively charged headgroups of the cell membrane largely impair fast ion flux through small pores[12], despite of the large electrochemical gradient. Therefore, Ca^{2+} inflow through a damaged membrane is possible in the absence of a PI-signal, but pores large enough to allow for PI-influx should also markedly facilitate Ca^{2+}-influx. Data from all N2A cells except for cells kept in PBS was pooled to compare peak and time to peak values of PI-positive vs. PI-negative cells. The mean peak value for PI-positive cells is 301.6 ± 204.9 % $\Delta F/F_0$ vs. 194.5 ± 129.7 % $\Delta F/F_0$ for PI-negative cells. The mean time to peak values are 4.3 ± 3.1 s vs. 6.3 ± 4 s, respectively. The large variations kept in mind, there appears to be a trend that cells with large membrane defects

experience more and faster Ca^{2+} inflow. However, a number of observations contradict the hypothesis that the observed calcium signal is primarily caused by a damaged membrane:

- Small pores, as might be present in PI-negative cells, constitute a large energy barrier for cations that impairs fast flow into the cell[12].
- Apart from the aforementioned weak trend, there is no evidence for different kinetics of the calcium response in PI-positive and negative cells. The overall signature does not change; some variation might have been expected and attributed to varying pore size and number.
- The proportion of PI-positive cells increases with radiant exposure (data not shown), but the peak fluorescence values do not.
- Several studies have suggested that while the rate of ion inflow is highest during the first ~10s after laser irradiation, pores can remain open for minutes[1,13]. Assuming that the large Ca^{2+} gradient across the membrane cannot be balanced immediately (due to the energy barrier within the pore), a proportion of cells should be expected to show fast influx during the first seconds that would then be slowed down when the first pores close, and continue to approximate the extracellular calcium concentration. Almost all cells that did show a second, slower rate of fluorescence increase after the initial fast rise were kept in PBS. Extracellular calcium can be excluded as a source in those cases.

We therefore propose a mechanism relying on one or more intracellular calcium sources, triggered by cytosolic accumulation of Ca^{2+}. Enough calcium is only accumulated if extracellular ions flow into the cell – this possibility is excluded in the case of a calcium-free buffer (PBS), and the respective pathways are not activated. Inhibitors of intracellular calcium channels tend to reduce peak calcium levels, but none suppresses the cellular reaction completely. Multiple sources, both extra- and intracellular, are likely to be involved, highlighting the severity of the interference with cellular homeostasis. High cytosolic calcium levels by themselves impose stress on a cell[14] and, to a certain extent, trigger further accumulation by calcium-induced calcium release (CICR). If the stressor posed by nanoparticle-mediated cell stimulation is strong enough, it could activate irreversible and potentially lethal pathways.

Considering our observations, gold nanoparticle-mediated stimulation with a weakly focused laser and a pulse duration of 40 ms constitutes a stressor large enough to potentially induce cell death. Regardless of the exact mechanisms involved, profound changes in cellular physiology, disruption of cellular homeostasis and membrane damage have been observed that make this approach very problematic for long-term *in vivo* applications. It could however prove a useful tool in research: A metabolic cue can be given at high spatiotemporal resolution, enabling the study of the long-term impact of stressors affecting only single cells in a network, priming cells for stress resistance or neurons for memory formation or studying the complex interplay of physiological and pathophysiological pathways.

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