



Application of two-photon microscopy to the study of cellular pharmacology of central neurons[☆]

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Abstract

Two-photon microscopy is an especially powerful tool for combining anatomical and physiological experiments in the central nervous system: the possibility of simultaneously studying physiological phenomena in well-defined anatomical compartments allows fluorescence imaging of neurons in deeper layers of the brain. In this review we summarize the most commonly used brain preparation techniques together with the methods of loading neurons with fluorescent indicators. We will focus primarily on issues of drug delivery specifically related to two-photon experiments highlighting the different ways of drug administration. Methods of chemical stimulation via caged neurotransmitters are also discussed. Finally a few specific areas of two-photon applications in drug research on neuronal tissue are highlighted.

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1. Introduction

The introduction of two-photon laser scanning microscopy (TPLSM) opened a new avenue which brings nerve cell imaging deeper in brain tissue while keeping the submicron spatial resolution of confocal microscopy at the millisecond time-scale of physiological responses. Cells are typically labeled by fluorescent probes loaded to the cells using various methods. The physical parameters of the exciting laser beam allow a reduction in the power thus diminish the damage of the tissue, while the use of infrared light has brought an important new aspect to neuroscience research by allowing the investigation structure in deeper layers of the tissue. Pioneers of the field revealed neural structure and function in an astonishing resolution in the brain tissue that formerly could not have been obtained in optical recordings, thus gaining recognition and attention for TPLSM used in neuroscience research [1–4].

2. Preparations of nervous tissue for TPLSM

2.1. Brain slice

The advances of TPLSM can perhaps best be exploited by using live brain slices. Usually 300–400 μm thick slices are cut from acutely isolated brain tissue of rodents, but hemisected whole amphibian forebrain with highly preserved interneuronal connections can also be used [5]. Although laser excitation is harmful to the tissue, the tissue damaging effect is relatively lower in case of two-photon excitation as compared to one-photon excitation, especially in the volumes above and below the focal plane compared to

multi-photon excitation. Transverse slices containing the hippocampus and the frontal cortex are dissected and placed in artificial cerebrospinal fluid (ACSF) and incubated for 30 min at 32 °C. Slices are typically left at room temperature for at least 30–45 min before use [6]. The experiments are performed at 36–37 °C and because of the vulnerability of brain tissue to oxygen/glucose supply, especially at this temperature, the speed of ACSF perfusion is recommended to set high (depending on the perfusion system, but typically higher than 3 ml/min). For electrical recordings and cell dialysis glass electrodes are filled with standard intracellular solution supplemented with various concentrations of a fluorescent indicator.

2.2. Organotypic slice culture

Imaging performed on organotypic slice culture offers a special opportunity for fluorescent labeling: the longer time scale of viability (several days) allows the development of fluorescent cells following transfection of the culture with fluorescent probes. There are a few advances of using organotypic cultures with fluorescent indicators. Gene transfer can be made only in neurons that are incubated for days. Neocortical or hippocampal slice cultures are prepared from postnatal tissue, and special neuron types, such as pyramidal neurons, can be identified based on their location and shape in the organotypic culture. Genetic probes (for example the enhanced green fluorescent protein (EGFP gene) can be introduced to the cells using biolistic gene transfer [7] and can be imaged a few days later [8]. Using this method, DNA of the fluorescent protein is “shot” into the culture by a gene gun. Although there is some damage due to the pressure shock, a few days later

the condition of the culture is normalized and the time window is sufficient for the expression of the marker. A recombinant vaccine virus or adenovirus vector can also be used to transfect organotypic neuronal slices [9,10]. Another possibility involves viral transfection. Sindbis vectors were successfully used to introduce the gene of EGFP into cells of an organotypic culture [11]. Organotypic cultures offer some further advances for drug application that becomes more effective compared to application in acute slices, as most tissue debris has been disappeared around the imaged neuron while determinant receptor profiles are maintained [12]. Thus the required drug concentration can be achieved more quickly and precisely to activate specific receptors in the appropriate localization. Another advantage is that the tissue has already adapted to the artificial oxygen supply that helps preserving viability of the slice in ACSF during imaging. The drop in the oxygen tension during imaging interferes less with the physiological responses compared to acute slice experiments when changing to the artificial environment occurs abruptly.

2.3. Imaging brain areas *in vivo*

In vivo labeling is also a powerful method to produce labeled cells [13]. In this way intracortical injections of EGFP in a replication-deficient Sindbis expression vector can be pressure ejected at a depth of 300–500 μm in anesthetized rats through a small burr hole which has been drilled in the skull using stereotactic coordinates, to yield sufficient numbers of well separated neurons [14]. The skin is then closed and the animal is returned to its cage. After 24 h, brain tissue of the injected animals can be prepared as standard acute slices and ready for preparing cultures and subsequent imaging.

Following *in vivo* injection of a suspension of virus containing the gene for enhanced green fluorescent protein (described in 2.2.), tens to hundreds of neurons in virtually all cortical layers get infected [14]. The use of a replication defective virus to minimize its neuro-pathological effects is essential. A few days after infection the concentration of EGFP expressed in the cells is sufficiently high for TPLSM experiments. Animals are then anesthetized and their skull gets exposed. A custom-designed metal frame with a hole is attached to the skull with dental cement. Following craniotomy around the injection site the brain gets exposed. To reduce movement the surface is covered

with agarose and a cover slide. The frame is connected and fixed to the microscope to provide stability during imaging.

Another way of labeling neurons for *in vivo* TPLSM is to fill them with synthetic dyes introduced through intracellular recording electrodes [4,15].

Motility of small neural structures, such as spines, can be measured as a change in the length of individual protrusions as a function of time on the minute time-scale. To describe the structural dynamics for an individual protrusion, the average change of length per sampling interval can be used. These time-lapse imaging experiments were capable to reveal that spines and filopodia are highly motile *in vivo*: they can change their length and shape over tens of minutes which was even more pronounced in younger animals [13].

3. Loading fluorescence indicators into the cell

The acute introduction of the fluorescent dye into the cell to be imaged is an easy and often used method but it is less recognized since it raises several significant problems. Perhaps the fastest way of loading a cell is diffusing the dye from a pipette which is used for electrical recording the cell. However, in this case the loading characteristics depends on both the distance from the pipette and the time from the break-in. In proximal regions the intracellular concentration of the dye changes considerably at first, while at more distal sites the concentration of the dye may change during the whole experiment [16,17]. In order to use a relatively stable measure of ion dynamics visualized by the fluorescent indicator, meanwhile dye molecules are continuously diffusing into the cell and gradually increase the basal fluorescence, a normalized change in the fluorescence, the $\Delta F/F$ value is used by most investigators (Fig. 1). In detail, responses are expressed as relative fluorescence changes using this measure:

$$\left[\frac{\Delta F}{F} = \frac{(F_x - BG) - (F_0 - BG)}{F_0 - BG} = \frac{(F_x - F_0)}{F_0 - BG} \right]$$

where F_0 is the pre-stimulus fluorescence and BG is the background. However, a decrement in the $\Delta F/F$ value may occur as the indicator starts to significantly buffer the intracellular free Ca^{2+} (Fig. 1). This artificial decrease in the amplitude is accompanied by a prolonged

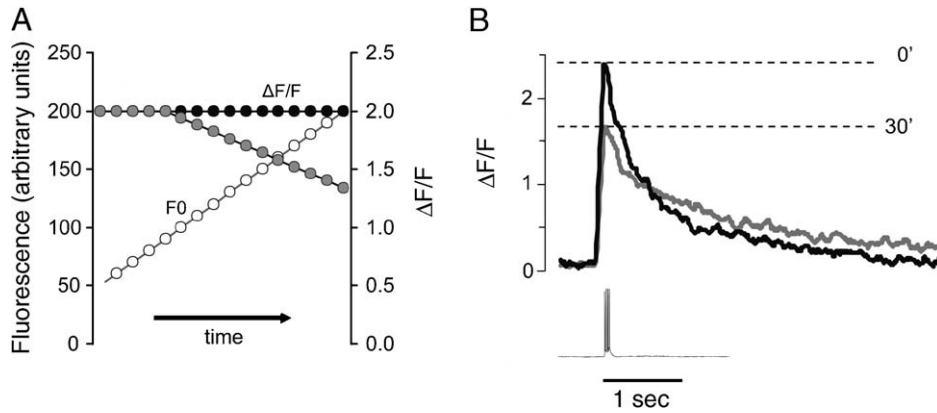


Fig. 1. Features of $\Delta F/F$ calculation in pipette-loaded cells. (A) A constant increase in the amount of intracellular indicator by 10 arbitrary units increase in the basal fluorescence (F_0) of the spine over time (open circles). The calculation of $\Delta F/F$ provides a stable parameter in spite of the changing indicator concentration (black circles). Significant buffering can cause a decrease in the peak amplitude of the evoked Ca^{2+} transient (gray circles). (B) Buffering of intracellular free Ca^{2+} causes decrement of spike train-evoked Ca^{2+} transients (22 Hz, 5 APs) in a neocortical pyramidal neuron within a 30-min time window. Note the prolonged decay of the transient at 30 min.

decay (Fig. 1B) because of the larger buffering of the indicator at higher concentration. The change in the decay time helps identify the buffering-induced decrease in the amplitude. The speed of amplitude decline must depend on the diffusion rate of the indicator (from the pipette to the cell).

4. Drug delivery

There are two main routes for drug administration used in *in vitro* TPLSM experiments. One possibility is to take advantage of the rapid time-scale of TPLSM that calls for giving drugs at the temporal resolution of

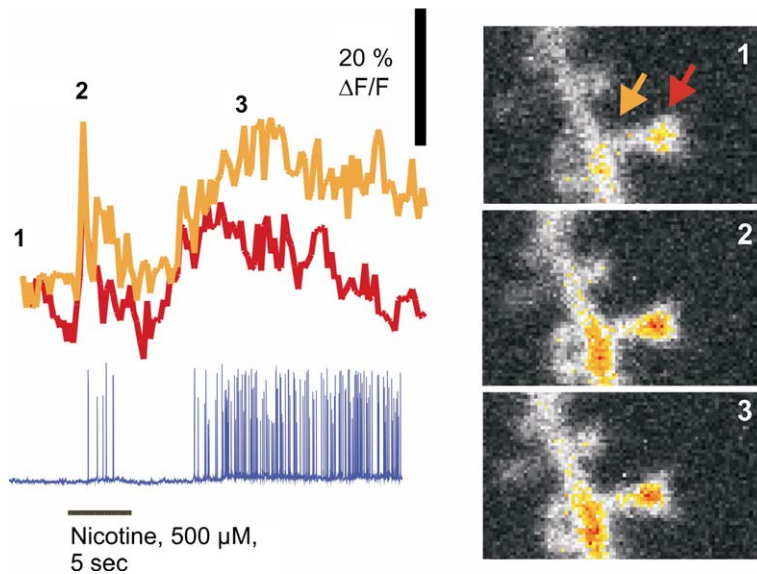


Fig. 2. Pressure ejection of drugs. Nicotine, dissolved in ACSF was ejected from a glass pipette positioned approximately $75 \mu\text{m}$ from the imaged area and caused firing of action potentials and elevations in the Ca^{2+} concentration of spines of CA1 pyramidal neurons indicated by the arrows and with corresponding colors in the right-hand image series. The numbers in the upper right corner of each image define periods in the trace when the corresponding image was taken.

imaging. The other method includes the application of drugs in the bath that produces a slowly rising drug concentration in the environment of the target cells. While the first method is perfect for evoking fast agonist responses, the second type of drug application is usually more appropriate for applying antagonists of receptors that knock out the receptor function between two functional tests of agonist response using either the first or second method. In *in vivo* experiments systemic administration is feasible.

4.1. Rapid drug application

The speed of a single line scan is typically in the range of 2–10 ms depending on the expected signal-to-noise ratio of fluorescence. Prolonging the line scan time the excitation gets stronger and therefore a larger fluorescence signal can be derived from a sample with lower indicator concentration. On the other hand, the stronger excitation causes larger photodamage especially in the vulnerable brain tissue. At an appropriate

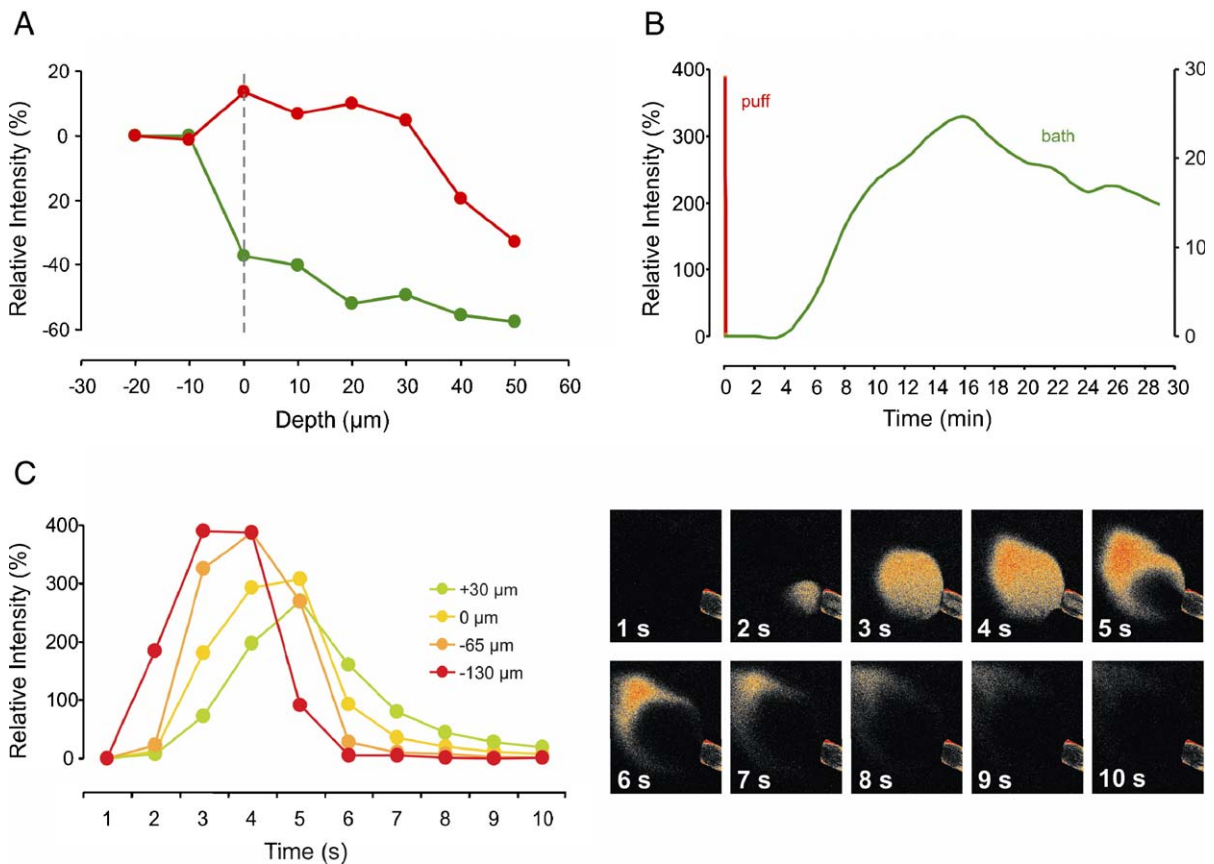


Fig. 3. Features of fast and slow drug delivery applications. The fluorescent dye Oregon Green BAPTA-1 (OGB-1) was added to the bath perfusion to model drug application. (A) The green line shows the vertical distribution of the dye. Measuring fluorescence from the surface down to the tissue (dashed line, 0 μm) a sharp reduction appears in the relative intensity as a consequence of volume exclusion from the cells. After this point relative intensity decreases only slightly with increasing depth. A thin glass tube filled with OGB-1 was used as a reference object: fluorescence intensity was stable (orange line) down to 30 μm where a decline was observed most likely referring to the absorption of emitted photons by the tissue. (B) Rapid drug application versus bath perfusion. Rapid drug delivery was made by a pressure ejection system. Pressure application caused an increase in the fluorescence intensity (red line) in the timescale of seconds. Bath perfusion (green line) allows much slower dynamics in the timescale of minutes. (C) Left: kinetics of rapid drug delivery using glass pipette with large tip in a multiple drug ejection device (DAD-12). Different colors correspond to different depths measured from the tip of pipette. Right: Two-photon images of a puff at -65 μm that corresponds to the orange line. To model the drug delivery we puffed fluorescent dye. Notice the rapid rise and fall in the concentration.

signal-to-noise ratio only a few points are required for getting a stable baseline and a clear response. Pressure ejection results in activation of receptors in seconds because of the drug diffusion into the tissue that occurs typically in the timescale of seconds. The applied pressure causes movement of the tissue in an unpredictable manner. Thus, time-lapse X – Y image series (frame imaging) is the most appropriate TPLSM mode for the observation of fluorescence responses to pressure ejection of drugs (Fig. 2). Pressure ejection can be accomplished using multibarrel glass pipettes with a large tip, which are typically used in multiple drug ejection devices. In this experimental design a relatively large area will be covered by the ejected drug and most receptors in the imaged cells will be activated by the drug (Fig. 3C). Using small tip size, such as the standard patch clamp pipette for pressure drug ejection delivers the drug to a smaller area (therefore the pipette must be much closer to the cell). The application of only a single drug may limit these investigations in some cases. Movement artifacts are not avoided by using this method. Movement artifacts can be minimized for all pressure ejection methods by avoiding the use of high pressure and by positioning the tip of the pipette close to the top of the slice without pushing it into the tissue.

4.2. Bath perfusion

The speed of drug delivery using bath perfusion is on the time scale of minutes and determined by the speed of the perfusion fluid (Fig. 3B). There is an optimal speed: slow perfusion rate does not provide optimal oxygen supply, while fast perfusion can lead to movement artifacts by blowing the tissue. The relatively slower rate of agonist delivery makes this drug application method unsuitable for two-photon imaging of the physiological changes themselves because the high temporal resolution of TPLSM cannot be fully exploited, while other systems, such as standard CCD cameras provide better cost/benefit ratio when the resolution of apical or basal dendrite is sufficient for making biological conclusions and single spine resolution is not required. However, antagonism by bath applied drugs is appropriate for TPLSM to study the inhibition a functional “fast kinetics” feature, such as synaptic activity or backpropagating spikes. In this scenario the rapid response of the cell, for example a

Ca^{2+} transient evoked by backpropagating spikes, can be blocked by a known concentration of an antagonist or channel blocker. In this case the well-defined concentration and uniform distribution of the drug after equilibration (Fig. 3A) has priority over the speed of delivery enabling us to correctly estimate the role of the given target molecule to be antagonized.

4.3. Uncaging

Uncaging of caged bioactive molecules by ultraviolet (UV) light offers a rapid and spatially confined drug application method for physiological experiments. Applying caged compounds (typically caged glutamate) and uncaging them locally by photolysis allows functional mapping of receptors on the cell [18,19]. Caged neurotransmitters are transmitter molecules inactivated by a photosensitive blocking group. When these caged molecules absorb UV light, the bond attaching the caging group is broken and active neurotransmitter gets released at the focal point of the excitation. However the usability of this method is limited, as the unfocused light in volumes above and below the focal plane also causes photolysis of caged neurotransmitter that limits depth resolution. This problem can be addressed either using optical uncaging with lasers used for two-photon imaging [20], or using high-precision chemical uncaging of double-caged molecules [18]. Both of these methods significantly limit uncaging effect to the focal plane thus improve spatial resolution allowing more precise investigation on functional receptors. Two-photon uncaging of receptor agonists/antagonists and simultaneous TPLSM will be an ultimate combination of methods to investigate neural function in the spatial context [21] but currently its use is limited by the lack of commercially available technology of preventing the interference between the two-photon excitation of the fluorescence indicator and the caged compound, and the relatively narrow spectrum of nontoxic and specific caged receptor ligands. The latter method has several advantages over the first as it uses the more stable double caged glutamate (two caging groups attached to one glutamate molecule) that requires absorption of two photons of UV light for conversion to active glutamate [18] thus providing a stable, high resolution and cost-effective method for rapid receptor investigation.

5. Fluorescence responses

TPLSM can be used to investigate neural structure and function, representing two main streams of two-photon studies. Taking advantage of the micrometer-scale spatial resolution, dendrites and spines can be imaged to study the change in their shape and size.

5.1. Functional anatomical studies

For time-lapse imaging dendritic segments are systematically sampled to collect image stacks in the minute time-scale. The morphology of neurons, especially in the critical period of sensory development is dynamically changing. To image the alteration and obtain detailed pictures with sharp contrasts, relatively stronger two-photon excitation is required during the experiments. It has been shown that spines are highly dynamic in the critical period as reflected by the change in motility [13]. The motility of spines could be modulated by drugs as well: vinpocetine, a clinically used brain enhancer molecule, could increase motility and induce small extrusions from existing spines [22]. Time-lapse imaging of the structure in the time-scale of minutes requires 3-dimensional image series because the imaged spine can move along the z axis that may appear as a change in the length or size of the spine at different focal planes. Fluorescence responses are different along the z axis of small anatomical structures (Fig. 4). In two-photon studies on faster fluorescence changes, such as bAP-evoked Ca^{2+} responses, axial movement may be negligible.

5.2. Neurophysiological studies

Perhaps the most efficacious and simplest way of exploiting the advances of TPLSM in brain slices is recording the dendritic response to backpropagating action potentials (bAPs) [23]. Because of the large spatial resolution and the capability to optically section the tissue by TPLSM, it is possible to record from every spine filled with the fluorescence indicator. The low autofluorescence attributable to the higher wavelength excitation and the lack of fluorescent dye outside the neuron loaded through the patch pipette provides low background, which makes it possible to reliably measure Ca^{2+} transients evoked by action potentials (APs) or synaptic inputs even at the single spine resolution. The function of different spines at different distances from the soma can therefore be mapped to estimate the dendritic scaling of these responses. Furthermore, the good signal-to-noise ratio can be reached at a low laser power, which helps to prolong the duration of the imaging and makes it suitable to follow the effect of drugs in a longer time scale in pharmacological experiments. The main advantage of the bAP-evoked Ca^{2+} signaling is that it can be measured throughout the dendritic tree of a neuron to map the presence and size of the evoked Ca^{2+} responses indirectly showing the zone of the bAP spread even in small diameter dendrites, which are not accessible for dendritic patching. In most dendrites the amplitudes of Ca^{2+} responses are not equal in the entire length of the dendrite rather show an incremental [24] or decremental [25–27] scaling. The reasons for

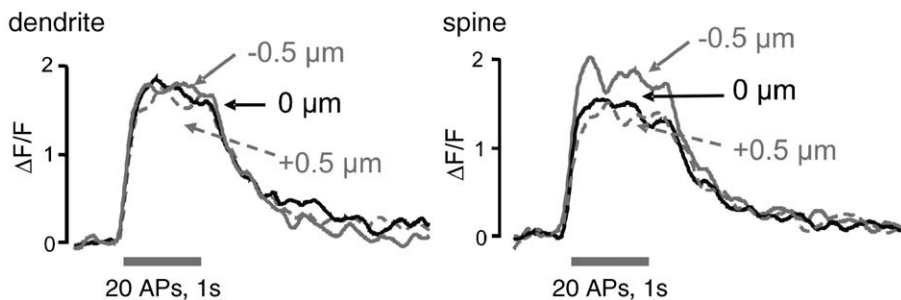


Fig. 4. Effect of small axial movements of line scans on the amplitude of Ca^{2+} transients. CA1 pyramidal neurons responded with large Ca^{2+} accumulation to a 20-AP, 22-Hz train. In the case of dendrites the change of z -position of the line scan in a 1 μm depth did not cause measurable difference in $\Delta F/F$. However, in smaller anatomical structures like in dendritic spines where F_0 is typically smaller by a factor of two, z -movement of the scan line may cause significant change in the amplitude of the evoked $\Delta F/F$ Ca^{2+} response. Although the noise of the fluorescence signal in spines is larger than in the dendrite (as it is composed of less image pixels), it is still appropriate for clear identification of the AP-evoked Ca^{2+} -transient.

the scaling derive from various sources: distribution of ion-channels, which produce the dendritic responses are skewed to one direction. Alternatively, factors (K^+ or HCN channels) that diminish the evoked transients are changing. The surface/volume ratio may also cause scaling effect moving from thicker to thinner dendrites. In stratum radiatum interneurons of the hippocampus the bAP-evoked Ca^{2+} transients exhibit an incremental scaling, i.e. the amplitude of the evoked responses increase with distance from the soma [24]. This is in sharp contrast with the distance-dependent scaling of pyramidal neurons where the amplitudes of single action potential-evoked Ca^{2+} responses decrease along the apical dendrites [26,28]. Attenuation of back-reporting signals occur parallel to the opposite scaling of synaptic potentials [25]. Investigations on bAP-evoked responses are of great importance because their interactions with synaptic inputs may underlie memory and learning at the cellular level: The interplay between current synaptic activity and traces of previous activities may be realized by the nonlinear summation of bAPs and synaptic responses [25–27].

From a pharmacological point of view the interesting question is whether the bAP-evoked Ca^{2+} signaling is subject to modulation by drugs. Indeed, it has been shown that the decrement of bAP-evoked dendritic calcium signals can be modified by cholinergic and GABAergic receptor activation [29–31].

6. Conclusions

To exploit the advances of TPLSM, drug delivery has to be fast enough to be able to observe the resulting changes in fluorescence intensity. Inhibition of receptor function can be achieved in the longer time-scale of drug application in the bath. The acute effects of receptor stimulation in TPLSM experiments can be studied using rapid drug application such as pressure ejection. However, some features of puffed drugs have to be taken into consideration: (1) a mechanical artifact by the pressure wave may modify the transient increase in the fluorescence intensity, (2) the efficiency of drug delivery declines with depth in planes where two-photon imaging is still possible. In the central nervous system, TPLSM offers special advances: neurons are complex three-dimensional structures receiving thousands of inputs. Thus, imaging the function in fine anatomical

structures of the nerve cells provides information that otherwise would not be available for electrophysiological or biochemical methods.

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